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PROCEEDINGS OF THE

26TH WORKSHOP ON THE DEVELOPMENTS IN THE ITALIAN PHD RESEARCH ON FOOD SCIENCE TECHNOLOGY AND BIOTECHNOLOGY

A S T I

19TH - 20TH - 21ST
SEPT 2022

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Proceedings of the 26th Workshop on the Developments in the Italian PhD Research on Food Science, Technology and Biotechnology

Università degli Studi di Torino – Asti (Italy), 19th-21st September 2022

Abstract: This book collects the conference proceedings of the *26th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology*, held at the *UniASTISS Polo Universitario Asti Studi Superiori "Rita Levi Montalcini"* from 19th to 21st September 2022. The goal of the conference is to gather PhD students from all Italian universities of whom projects deal with food-related topics to define the state of the art of the Italian academic research in this area of study.

Keywords: Food science, Food technology, Microbiology, Biotechnology, Italian PhD Research, PhDFood 2022.

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26th PhD Workshop Program

Workshop venue: **UniASTISS**
Polo Universitario Di Asti Studi Superiori *"Rita Levi Montalcini"*
Piazzale Fabrizio De André, Asti, Italy

Monday, September 19, 2022

Registration opens at 10:00 time

13:30 – 14:00 **Workshop opening ceremony and welcome note (Aula Magna 1, in parallel streaming Aula Magna 2)**

Maurizio Rasero, Sindaco Città di Asti
Mario Sacco, Presidente Polo Universitario UniASTISS
Stefano Geuna, Magnifico Rettore Università di Torino
Rosalba Lanciotti, Presidente SIMTREA
Ernestina Casiraghi, Presidente SISTAL

14:00 – 14:45 **Plenary lecture (Aula Magna 1, in parallel streaming Aula Magna 2)**
"The 12 pleasures of science"
Marcel Zwietering, Wageningen University, The Netherlands

14:45 – 16:15 **Parallel session 1: Microbial fermentation (Aula Magna 1)**
Session Chairs: **Monica Gatti, Gianluigi Mauriello**

Marilisa Giavalisco, SAFE, University of Basilicata
"Use of *Lactiplantibacillus* strains and yeasts for the production of fermented table olives and extra virgin olive oil"

Anđela Martinović, DeFENS, University of Milano
"The ability of *Streptococcus thermophilus* ST870 to modulate urease activity in healthy subjects' fecal samples depends on the biomass production process"

Samantha Rossi, DISTAL, University of Bologna
"Biotechnological approaches to valorise alternative protein source, waste and by-products of food industries."

14:45 – 16:15 **Parallel session 2: Bakery products (Aula Magna 2)**
Session Chairs: **Maria Cristina Messia, Monica Laureati**

Veronica Oliviero, Department of Agriculture, University of Napoli Federico II
"Design and validation of healthy leavened bakery products: Focus on chemical-physical and sensory properties"

Martina Moretton, DI4A, University of Udine
"Formulation and processing strategies for obtaining bakery products tailored to the elderly's needs"

Alice Costantini, Faculty of Science and Technology, Free University of Bozen-Bolzano
"Leavened baked goods for improving the functionality"

Oumayma Toumi, Department of Agricultural Sciences, University of Sassari
"Use of response surface methodology to investigate the effect of partial substitution of sodium chloride with *Salicornia ramosissima* powder in wheat dough and bread"

16:15 — ***6th What For Award*, Federalimentare**
Video presentation of the selected final proposals (Aula Magna 1 and Aula Magna 2)

16:15 – 17:00 — **Coffee break**
Poster viewing (PhD I year Aula 6 - ground floor; PhD II year Aula seminari – first floor)

17:00 – 18:30 — **Parallel session 3: Food analysis (Aula Magna 2)**
Session Chairs: **Ernestina Casiraghi, Angelita Gambuti**

Silvio Iacovino, DiAAA, University of Molise
"Flour rheological properties assessed through empirical and fundamental methods"

Anna Luparelli, Department of Chemistry, University of Bari
"Development of innovative methods for the multiple analysis of allergens in processed foods"

Luca Menegoz Ursol, DI4A, University of Udine
"Optimization of rapid analytical protocols for monitoring the contamination with hydrocarbons of petrogenic origin in the olive oil supply chain"

Giacomo Bedini, DIBAF, University of Tuscia
"Use of non-destructive analysis techniques for the technological and chemical-physical characterization of fruit and vegetables and for monitoring of the drying process"

17:00 – 18:30 — **Parallel session 4: Microbial characterization (Aula Magna 1)**
Session Chairs: **Alessio Giacomini, Marisa Manzano**

Cecilia Crippa, Department of Agricultural and Food Sciences, University of Bologna
"Application of Next Generation Sequencing for the characterization of microbial hazard in Italian dairy and meat food productions realized in small-scale plants"

Federica Barbieri, Department of Agricultural and Food Sciences, University of Bologna
"Characterization of new bio-protective and functional lactic acid bacteria isolated from spontaneously European fermented sausages"

Luca Bettera, Food and Drug Department, University of Parma
"Non-starter Lactic Acid Bacteria: origin and characterization for a potential targeted use in cheesemaking"

Rossella Filardi, DeFENS, University of Milano
"Isolation and characterization of new isolates of *Akkermansia muciniphila* with a focus on antibiotic-resistance phenotypic and genotypic traits"

Tuesday, September 20, 2022

08:30 – 09:15 **Plenary Lecture (Aula Magna 1, in parallel streaming Aula Magna 2)**
"From idea to business start-up - developing entrepreneurial skills for life"
Jonathan Tait, EIT Food, Belgium

09:15 – 10:45 **Parallel session 5: Food sustainability (Aula Magna 1)**
Session Chairs: **Monica Anese, Massimiliano Rinaldi**

Mirella Noviello, DiSSPA, University of Bari
"Sustainable approaches to winemaking and wine aging"

Marika Valentino, Department of Agricultural Sciences, University of Napoli Federico II
"Biopolymer active coating to extend the shelf-life of minimally processed fruits and vegetables"

Angela Michela Immacolata Montone, DIIN, University of Salerno
"Development of edible coating functionalized with hydroxyapatite, complexed with bioactive compounds for the shelf-life extension of food products"

Valeria Frigerio, DeFENS, University of Milano
"Shelf-life estimation as a strategic tool for the eco-design of a sustainable food packaging"

09:15 – 10:45 **Parallel session 6: Food biotechnology (Aula Magna 2)**
Session Chairs: **Prospero Di Pierro, Angela Capece**

Serena Malabusini, DeFENS, University of Milano
"Bioetholgy of a promising parasitoid associated with fig pests"

Chiara Purgatorio, Food Sciences, University of Teramo
"Alternative antimicrobial strategies for the replacement of traditional preservatives and evaluation of the impact on stability and safety of food products"

Francesca Melini, DIBAF, University of Tuscia
"Application of metabolites secreted by plant growth-promoting bacteria to selected crops and evaluation of nutritional quality thereof"

Francesco Salini, DI4A, University of Udine
"Heterologous expression of two novel antimicrobial peptides and investigation of their dedicated protease"

10:45 ***6th What For Award*, Federalimentare**
Video presentation of the selected final proposals (Aula Magna 1 and Aula Magna 2)

10:45 – 11:30 **Coffee break**
Poster viewing (PhD I year Aula 6 - ground floor; PhD II year Aula seminari – first floor)

11:30 – 13:30 **Parallel session 7: Food processing (Aula Magna 2)**
Session Chairs: **Maria Cristina Nicoli, Giuseppe Gambacorta**

Nazarena Cela, SAFE, University of Basilicata
"Optimization of microbrewing process for high quality gluten free beer production"

Aniello Falciano, Department of Agricultural Sciences, University of Napoli Federico II
"Processing and innovation in the Neapolitan Pizza manufacturing"

Fosca Vezzulli, DiSTAS, Università Cattolica del Sacro Cuore
"Multifactorial traceability and characterization of green and roasted coffee"

Davide Emide, DeFENS, University of Milano
"The complexity of protein network in foods: insight in the protein structure in cereal products"

Andrea Bresciani, DeFENS, University of Milano
"Effects of processing on pulses and related products"

11:30 – 13:30 **Parallel session 8: Probiotics, prebiotics and nutraceutics (Aula Magna 1)**
Session Chairs: **Carlo Rizzello, Fabio Minervini**

Stefan Klettenhammer, Faculty of Science and Technology, Free University of Bozen-Bolzano
"Innovative techniques to encapsulate food-grade bioactives"

Claudia Cappello, Food engineering and Biotechnology, Free University of Bozen-Bolzano
"A novel functional herbal tea containing probiotic *Bacillus coagulans* GanedenBC^{30}: an *in vitro* study using the Simulator of the Human Intestinal Microbial Ecosystem (SHIME)"

Margherita D'Alessandro, Department of Agricultural and Food Sciences, University of Bologna
"Development and investigation of functional foods conceived for specific categories of consumers and produced with selected strains isolated from healthy vaginal environment and human breast milk"

Annalisa Porrelli, DiSSPA, University of Bari
"Functional food and *per os* microbial delivery system with a potential role in the prevention of diseases related to the human intestinal microbiota"

Giovanni Turchetti, DIBAF, University of Tuscia
"Identification and characterization of bioactive plant extracts and evaluation of viable use in the food industry"

13:30 – 14:30 **Lunch**
Poster viewing (PhD I year Aula 6 - ground floor; PhD II year Aula seminari – first floor)

14:30 – 15:15 **Plenary lecture (Aula Magna 1, in parallel streaming Aula Magna 2)**
"The future of wine-making… the role of the scientific research"
Luigi Moio, University of Napoli Federico II and President of the OIV, Italy

15:15 – 16:45 **Parallel session 9: Food circularity (Aula Magna 1)**
Session Chairs: **Matteo Mario Scampicchio, Rossella Di Monaco**

Patricia Dahdah, Department of Agricultural Sciences, University of Sassari
"Valorization of olive oil extraction by-products through functional bread making"

Marco Montemurro, DiSSPA, University of Bari
"Exploitation of unconventional plant matrices and agri-food waste through biotechnological processes"

Marica Troilo, DiSSPA, University of Bari
"Grape pomace as an innovative flour for the formulation of bakery products: how nutritional, textural and sensorial properties were affected?"

15:15 – 16:45 **Parallel session 10: Targeted nutrition (Aula Magna 2)**
Session Chairs: **Ilario Ferrocino, Francesca De Filippis**

Massimiliano Tucci, DeFENS, University of Milano
"Definition and validation of a healthy and sustainable dietary pattern, enriched with

plant-based foods rich in bioactives compounds, in the context of the MIND FoodS Hub project"

Flavia Casciano, DISTAL, University of Bologna
"*In vitro* study of short-term effect on gut microbiota of foods and ingredients for specific consumer categories"

Cinzia Franchini, Department of Food and Drug, University of Parma
"Promotion of nutrition knowledge and sustainability of dietary behaviors in different student populations"

Veronica D'Antonio, Bioscience and Agro-Food and Envr. Technology, University of Teramo
"Role of typical foods from Abruzzo region in reducing oxidative, inflammatory and metabolic stress in frail elderly people and/or affected by degenerative diseases"

16:45 – 17:00 **Awarding Ceremony (Aula Magna 1, in parallel streaming Aula Magna 2)**
Fondazione Prof. Roberto Massini – ETS

17:00 – 18:30 **Poster viewing and discussion**
(PhD I year Aula 6 - ground floor; PhD II year Aula seminari – first floor)

17:30 – 19:00 **PhD Coordinators meeting (Aula 5)**

19:00 **Bus departure from the Workshop venue (Piazzale Fabrizio De André, Asti) to the Gala Dinner**

20:00 **Gala Dinner (Foro Boario Pio Corsi, Piazza Garibaldi, Nizza Monferrato, AT)**

23:30 (est.) **Bus departure from the Gala Dinner venue back to Asti and Torino**

Wednesday, September 21, 2022

09:00 – 09:45 **Plenary lecture (Aula Magna 1, in parallel streaming Aula Magna 2)**
"Sustainable nutrition: the new challenge for the Italian food system"
Mauro Fontana, President of the Italian AgriFood Cluster (CLAN), Italy

09:45 – 11:45 **Parallel session 11: Innovation in the food system (Aula Magna 1)**
Session Chairs: **Marco Dalla Rosa, Fernanda Galgano**

Federico Basso, DI4A, University of Udine
"Hyperbaric storage: An innovative and sustainable technology to extend stability and improve functionality of food"

Giulia D'Alessio, Bioscience and Agro-Food and Envr. Technology, University of Teramo
"Impact of high dynamic pressure treatments on the physicochemical properties and technological functionality of pea proteins"

Giulia Romano, DI4A, University of Udine
"Optimization of cooking for food service: matching quality and nutritional requirement as drivers for development of innovative tools"

Mehmet Onur Oral, DAFNE, University of Foggia
"Adding unprecedent economic and social values to the side- and by-products of Mediterranean fruit and vegetables by reshaping them in novel source of nutrients and tailored food products mediated by 3D printing technology"

Vincenzo Valentino, Department of Agricultural Sciences, University of Napoli Federico II
"Validation of Microbiome Mapping Strategies for the Food Industry"

9:45 – 11:45 **Parallel session 12: Wine quality and analysis (Aula Magna 2)**
Session Chairs: **Fabio Mencarelli, Daniela Fracassetti**

Francesco Maioli, DAGRI, University of Firenze
"Monitoring and management of chemical and physical wine parameters by using different tank materials into the winemaking process"

Giulia Scalzini, DISAFA, University of Torino
"What is the best time to harvest red grapes cv. Nebbiolo destined to withering? A three-years study"

Sabrina Voce, DI4A, University of Udine
"Yeast strain and processing technology affect the composition of yeast autolysates: characterization and potential effects on wine evolution"

Paola Bambina, SAAF, University of Palermo
"^{1}H-NMR-based metabolomics to assess the impact of the soil on the chemical composition of Nero d'Avola red wines"

Rolla El Harati, Department of Agricultural Sciences, University of Sassari
"Utilization of citral as antimicrobial on the mutants of Σ1278b library of *Saccharomyces cerevisiae*"

11:45 – 12:30 **Awarding ceremony (Aula Magna 1, in parallel streaming Aula Magna 2)**
***6th What For Award*, Federalimentare**

12:30 – 13:00 **Closing ceremony (Aula Magna 1, in parallel streaming Aula Magna 2)**

13:00 – 14:30 **Lunch and farewell**

14:00 ***Post-event:* COSTAL meeting (Aula 5)**

List of contributions

1st year PhD Students: **PhD Dissertation Projects & Miniposters**

2nd year PhD Students: **Contributions & Poster Communications**

3rd year PhD Students: **Contributions & Oral Communications**

Workshop contributions

1st year: PhD Dissertation Projects

Low-cost non-destructive sensors for monitoring polyphenols, volatile compounds and quality parameters in grapes, musts and wines

Gianmarco Alfieri (gian.alfieri@unitus.it)
Dept. DIBAF, University of Tuscia, Viterbo, Italy
Tutor: Prof. Andrea Bellincontro

In the context of improving the quality and lowering the costs chemical analyses in the viticulture and oenology sector, the main goal of this PhD project is to realise a collection of low-cost and non-destructive sensors for monitoring polyphenols and volatile compounds in grapes, musts and wines. Moreover, the data obtained from the different non-destructive technologies (i.e., VIS-NIR spectrophotometry, surface acoustic waves (SAW)) will be handled and shared throughout IoT technologies (clouding) to obtain a shared data library available for different companies and research institutions.

Sensoristica non distruttiva low cost per il monitoraggio dei polifenoli, composti volatili e parametri di qualità in uve, mosti e vini.

Nell'ottica del miglioramento del prodotto e dell'abbattimento dei costi dell'analitica per il settore viticolo-enologico questo progetto di dottorato mira a realizzare diversi sensori non distruttivi e a basso costo per il monitoraggio dei polifenoli e dei composti volatili in uve, mosti e vini. I dati raccolti dai diversi approcci sensoristici (dalla spettrofotometria VIS-NIR alle onde acustiche di superfice (SAW)) verranno elaborati e stoccati utilizzando le moderne tecnologie IoT di connettività (clouding) con lo scopo di creare una libreria di informazioni condivisa tra le diverse aziende e enti di ricerca.

1. State-of-the-Art

In wine sector, the quality is easier to detect than define and it has always been at the centre of major discussions. This is mainly due to quality being primarily subjective, and strongly influenced by different extrinsic factors. Hence, the chemical composition of wine is determined by numerous factors, such as grape variety and maturity, environmental condition during the development stage (climate, soil, health status), and vinification technology, as well as fermentation and ageing conditions. Polyphenols profile can be considered as one of the most interesting chemical parameters to define the quality of grape, must and wine. From a detailed polyphenols characterization is possible to identify grape variety, grape ripening stages, grape geographical origin and much more. Moreover, polyphenols evaluation can be a useful tool to monitor wine evolution during vinification and ageing (Garrido et al., 2013). For polyphenols monitoring the most commonly used methods are destructive analytics (i.e. Folin-Ciocalteau method, HPLC-DAD/MS and NMR), which are expensive analytical techniques which requires expensive instruments, highly specialised personnel and chemical reagents. In recent years, low-cost, non-destructive analytical methods have been proposed as eco-friendly and cheap alternative to the classical analytical methods and, among them, one of the most efficient approaches is based on the use of sensors, such as surface acoustic wave (SAW) sensors. The detection of a substance deposited on a vibrating element (resonator) by means of acoustic devices dates back to Sauerbrey in 1959. To date, acoustic transducers find application in the production of a large number of sensors, such as quartz crystal microbalances (QCM), Rayleigh wave sensors, horizontal surface acoustic wave (SAW) and shear wave sensors, and Love wave sensors. Very recently, Gagliardi et al., (2022) by functionalising quartz crystal microbalances (QCM-D) with peptides and proteins were able to discriminate and quantify different tannin families. The QCM-based approach uses a piezoelectric resonator to generate bulk acoustic waves (BAW) that propagate within the resonator and their frequency changes when an event occurs, such as the adsorption of a molecule onto the sensor surface or a chemical reaction involving the analyte (Jang et al., 2021). Another non-destructive approach widely tested in the wine industry for determining the chemical and physical properties of wine is VIS-IR spectroscopy (Cozzolino et al., 2006). Recently, the interest in non-destructive technology is shifting to the development of low-cost sensors and efficient chemometric techniques to create predictive models based on indirect sensor measurements (Chandra et al., 2017). For instance, Pampuri et al. (2021) developed a low-cost spectrophotometer prototype UV-VIS with an LED light source for monitoring grape ripening parameters, creating fairly robust predictive models (PLS) for berry sugars measurements and models (to be improved) for polyphenol measurements. For the measurement of volatile compaunds destructive and expensive techniques such as GC-MS/FID and distillations are routinely used. In recent years, the use of e-noses and volatile sensors as been largely tested and improved. E-nose is defined as "an instrument, which comprises an array of electronic chemical sensors with partial specificity and appropriate pattern-recognition system, capable of recognising simple or complex odours." (Rock et al., 2008). Common volatile compounds sensors instruments can be based on doped metal oxides (MOX) and MOSFET, conducting polymers (CP) and optical sensor arrays, quartz microbalance (QMB) and surface acoustic wave SAW sensors (Rodríguez-Méndez et al., 2016).

In the context of cost reduction, simplification and lower environmental impact, this PhD project aims to test different low-cost non-destructive approaches (specifically SAW, VIS-NIR spectroscopy and sensors volatile compaunds) to be applied in the wine sector, mainly focusing on polyphenols and volatile compounds profile.

2. PhD Thesis Objectives and Milestones

The PhD project will be organized into the following activities and according to the Gantt diagram given in Table 1:

A1) **Data acquisition for SAW wave lab-on-chip sensor calibration.** Specifically polyphenols-enriched wine samples will be analysed (A1.1) with classical analytical approach **(**HPLC-DAD) to quantify polyphenols content. Quantification will be performed by using specific calibration curve built with polyphenol standards (A1.2). The same sample will be then mesaured with the Lab-on-a-Chip biosensors to perform tests and calibrations [Collaboration with UNIPI and CNR-Nano Pisa].

A2) **Spectral acquisitions with low-cost prototype spectrophotometers.** Wines and grapes samples with a known chemical composition and polyphenols content will be analysed by using nature 4.0 VIS prototype (A2.1) and nature 4.0 VIS-NIR prototype (A2.2). [Collaboration with UNIPI]

A3) **Measurements with low-cost sensors volatile compounds.** The volatile compounds sensors will be tested for sulphur dioxide and volatile parameters monitoring in wine (A3.1). The wine will be also analysed with the destructive classical approaches (GC-MS) to compare the aromatic profile with the sensors response (A3.2). [Collaboration with UNIPI, Group sensori e Microsistemi Roma Tor vergata].

A4) **Realisation of multivariate predictive models**. The data from the different non-destructive approaches (i.e. lab-on-chip (A4.1), spectral data (A4.2), volatile sensors measurements (A4.3)) and traditional destructive analyses will be analysed by using chemometrics approaches with the final goal to built robust predictive models for polyphenols and volatile parameters discrimination in wine matrices [Collaboration with UNIPI]

A5) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

Table 1 *Gantt diagram for this PhD thesis project.*

Activity	Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	***Sensors lab-on-chip SAW***																								
	1)HPLC-DAD polyphenols standard																								
	2)HPLC-DAD polyphenols enriched wines																								
A2)	***VIS-NIR spectrophotometer***																								
	1) Nature 4.0 UV-VIS																								
	2)Nature 4.0 VIS-NIR																								
A3)	***Sensors volatile compounds***																								
	1) Monitoring SO2 e volatile acidity																								
	2) Destructive correlation analyses																								
A4)	***Development of predictive models***																								
	1)Lab-on-chip																								
	2) *VIS-NIR spectrophotometer*																								
	3) Sensors volatile compounds																								
A5)	***Thesis and Paper Preparation***																								

3. Selected References

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Development of a multifunctional cooking appliance: evaluation of food quality indexes and cooking functions

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Department of Food, Environmental and Nutrition Science (DeFENS), University of Milan, Italy
Tutor: Prof. Ernestina Casiraghi

This PhD research project aimed at contributing to the development of a multifunctional cooking device that embeds different cooking functions to be optimized versus target foods preparation. The cooking functions that will be considered, individually and together, in the appliance include air frying, high-temperature pizza oven, medium-temperature steam cooking and *sous vide* cooking, besides conventional oven functions. The performance of the multifunctional appliance will be tested developing, and subsequently applying, methods able to evaluate target foods attributes and cooking-induced modification, using new instrumental-sensorial (E-sensing technology) and nutritional approaches. Each cooking function will be optimized and cooking programs will be implemented on the multifunctional device.

Sviluppo di un apparecchio di cottura multifunzionale: valutazione di indici di qualità degli alimenti e di programmi di cottura

Questo progetto di ricerca contribuisce allo sviluppo di un dispositivo di cottura multifunzionale che incorpora diverse funzioni di cottura da ottimizzare rispetto alla preparazione degli alimenti target. Le funzioni di cottura che verranno considerate, singolarmente e insieme, nell'apparecchio multifunzionale includono la frittura ad aria, un forno per pizza ad alta temperatura, la cottura a vapore a media temperatura e la cottura sottovuoto, oltre alle tradizionali funzioni del forno. Le prestazioni dell'apparecchio multifunzionale saranno testate sviluppando, e successivamente applicando, metodi in grado di valutare le caratteristiche degli alimenti target e le modificazioni indotte dalla cottura, utilizzando nuovi approcci strumentali-sensoriali (tecnologia E-sensing) e nutrizionali. Ogni funzione di cottura sarà ottimizzata e i programmi di cottura saranno automatizzati.

1. State-of-the-Art

According to the aim of developing a multifunctional cooking system, it's necessary to study methods suitable for evaluating each system "cooking function-target food", using instrumental sensory analysis and nutritional indices to assess the desired quality for the considered cooking function. The cooking functions that will be implemented in the multifunctional cooking device are:

1.1 Air frying

Air frying is a technique alternative to deep-frying in oil; it preserves the characteristic taste and texture of deep-fried foods, avoiding over browning the food, undesirable flavours and odours that unpaired the food sensorial and nutritional value. Air frying induces food dehydration with hot air in a frying chamber; achieving typically crust fried food with low fat content. The reduced oil content has a positive impact on the nutritional quality and eliminates the problem of used oil disposal. Gouyo et al. (2020) have reported that texture is one of the important quality aspects, and crispy crust is a relevant sensory property and one of the most important consumers' quality parameters of fried products.

1.2 Pizza

The crust browning and the cheese melting on top of pizza can be monitored by means of image analysis, as shown by Sun and Brosnan (2003). Gökmen and Mogol (2010) have examined the potential application of an algorithm, for potato crisps and cookies, to predict acrylamide concentration. It could be possible to develop a color segmentation algorithm for the calculation of a browning ratio and cheese melting effect and to correlate it with acrylamide and 5-hydroxymethylfurfural level.

1.3 Steam

Steam cooking have effects on the nutritional value and tenderness of beef. Gerber et al. (2009) reported significant differences produced by different cooking methods (grilling, braising and boiling) on the FA composition and mineral content in beef. However, there is still a shortage of reports concerning mineral contents, tenderness and eating quality of steam-cooked beef combine with others cooking treatments (i.e. microwaves).

1.4 *Sous vide*

Sous vide cooking differs from traditional cooking methods in two fundamental ways: the raw food is vacuum-sealed in heat-stable, food-grade plastic pouches and the food is cooked using precisely controlled heating. Vacuum-sealing has several benefits: it allows heat to be efficiently transferred from the water (or steam) to the food; it increases the food's shelf-life by eliminating the risk of recontamination during storage; it inhibits off-

flavors from oxidation and prevents evaporative losses of flavor volatiles and moisture during cooking (Church and Parsons, 2000).

2. PhD Thesis Objectives and Milestones

Consumers are increasingly aware of the relationship among proper nutrition, health and environment protection. They are more and more avoiding high temperature cooked foods (e.g. fried, roasted or grilled food), which are highly tasty but potentially harmful to health. Along with the technological development and lifestyle changes, the consumers want to replace small appliances and maximize space. Besides, they want to cook everything with a good control and, finally, they want to optimize time. So, the appliance industry is focused on specific cooking techniques that can answer these consumer demands. In particular, a multifunction cooking system will be developed. This PhD thesis project is organized into work packages (WP). Each work package investigates and analyzes a different cooking function: high temperature (HT), such as air frying and pizza cooking; medium temperature (MT), like steam cooking; low temperature (LT), such as *sous vide*. For each function the consumer expectations need to be translate into food quality attributes, i.e. sensory characteristics instrumentally measurable and nutritional characteristics. These food attributes need to be identified and measured by reproducible and discriminant methods. This will be one of the focuses of this PhD project. Another one will be the optimization of each cooking function by appropriate time-temperature binomial and/or sensor system, based on desired food quality attributes.
The following Gantt chart (*Table 1*) shows the activities that will be performed for each work package:
WP1) **Study of air frying cooking function;**
WP2) **Study of pizza cooking function;**
WP3) **Study of steam cooking function**;
WP4) **Study of *sous vide* cooking function**.
Meanwhile, **writing and editing** of the PhD thesis, scientific papers and oral and/or poster communications will proceed.

Table 1 Gantt diagram for this PhD thesis project.

		Activity \ Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
HT	WP1	***Air Frying***																								
		1) crispness method evaluation																								
		2) physical-chimical analysis																								
		3) sensory analysis																								
	WP2	***Pizza***																								
		1) recipe development																								
		2) texture method evaluation																								
		3) browning by IA																								
		4) thermal damage products																								
MT	WP3	***Steam***																								
		1) nutritional indexes evaluation																								
		2) texture and IA methods																								
LT	WP4	***Sous Vide***																								
		1) nutritional indexes evaluation																								
		2) texture and IA methods																								
		Thesis and Paper Preparation																								

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4. Acknowledgements

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Using consumer science to improve healthy and sustainable eating behavior

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This Ph.D. research project is aimed at setting up strategies to promote healthier and more sustainable eating choices across the population to protect both human health and the environment.

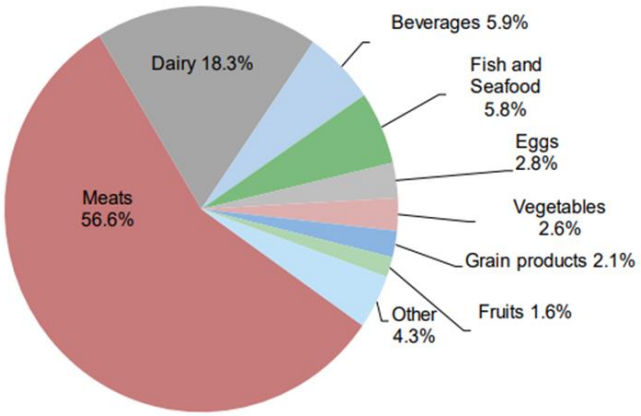
Utilizzare la consumer science per promuovere abitudini alimentari salutari e sostenibili

Questo progetto di dottorato mira a studiare ed analizzare strategie che possano supportare i consumatori nel fare scelte alimentari che siano salutari e sostenibili, per proteggere sia il proprio stato di salute che l'ambiente.

1. State-of-the-Art

Unhealthy diets and lack of physical activity are among the global risks to health leading to many chronic non-communicable diseases, such as heart disease, diabetes, and cancer. Thus, healthy diets could prevent diseases, promote health, and prolong life among the population.
At the same time, food choices don't influence only human well-being but also the planet. Food production is a major source of greenhouse gas (GHG) emissions, showing the huge impact consumers have on climate change. Furthermore, animal-based foods produce roughly twice the emissions of plant-based products. Nevertheless, despite a vegetarian diet greatly reduces an individual's carbon footprint, switching to less carbon-intensive meats can have a major impact as well. As a matter of fact, beef's GHG emissions per kilogram are 7.2 times greater than those of chicken.
As meat, especially red meat, has an impact on both human health and the environment, one possible solution to tackle both dimensions is the promotion of alternative protein sources and the literature research shows that the literature on alternative proteins is developing rapidly. In addition, overconsumption of meat and animal-based products contributes to the preventable suffering and slaughter of approximately 500 to 12,000 animals over the lifetime of each human.
Therefore, developing interventions to reduce the consumption of meat and animal-based foods could carry widespread societal benefits. During the last years, many studies have focused on fostering plant-based diets and reducing the consumption of animal and animal-based products; several interventions have been tested and consumers' perceptions of protein alternatives investigated. Among the main focal factors of behavioral change addressed by interventions, personal, socio-cultural, and external factors are the most frequently investigated. Geographically, the majority of studies were conducted across North America and Europe, and only a few research explored cross-national or cross-cultural dimensions. Numerous studies compared the acceptance of alternative proteins, such as pulses, seaweed, insects, and cultured meat, to that of traditional animal products. Specifically, healthiness, environmental concerns, taste, convenience, and appearance are the most frequent drivers towards protein alternatives, while food neophobia and disgust are among the main barriers.

Figure 1. Greenhouse gases' contribution by food type on average.



Center for Sustainable Systems, U. of M. | "Carbon F. Factsheet." | Pub. No. C.-05. (2021). Sustainability Indicators For Complete Set of Factsheets visit. www.nature.org/greenliving/carboncalculator/

As of now, most studies focused on understanding drivers for acceptance and barriers more than on testing the

effectiveness of behavioral interventions. Future research is needed to further explore how to best support consumers in making healthier and more sustainable eating choices and support a transition towards the reduction of animal and animal-based products.

Thus, this Ph.D. project aims at contributing to the above-presented field by studying and implementing strategies to promote the reduction of animal and animal-based products. The project foresees a cross-national comparison between Italy and the US to further evaluate cultural differences and dimensions.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above, this PhD project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) **Definition of the strategy:** to promote the reduction of animal and animal-based products as a possible intervention will be tested. Its definition will take place after the Summer Schools and conferences attended on this topic, allowing a deep discussion with experts in the field. Thus, the target group and the method selection will be defined.
A2) **Pilot phase:** A pilot phase will take place to test the approach and explore its possible limitations.
A3) **Data collection in the US:** data will be collected in the US.
A4) **Data collection in Italy:** data will be collected in Italy.
A5) **Data analysis and dissemination:** after the data are collected in both countries, the analysis will take place and a cross-national evaluation will be performed. Throughout this phase, dissemination activities are foreseen to share preliminary results and discuss them with experts.
A6) **Writing and Editing** of the PhD thesis, scientific papers, and oral and/or poster communications.

Table 1. Gantt diagram for this PhD project.

Activity	Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	**Definition of the strategy**																								
A2)	**Pilot phase**																								
A3)	**Data collection US**																								
A4)	**Data collection Italy**																								
A5)	**Data analysis and dissemination**																								
	1) Data analysis																								
	2) Dissemination																								
A6)	**Writing and Editing**																								

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Sustainable approach to obtain innovative fresh cheeses by increasing shelf-life and nutritional features

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The research activity of this PhD project aims to evaluate the effect of the addition of functional molecules, obtained by upcycling agri-food by-products, in the production of innovative fresh cheeses, thus using a sustainable approach. The influence of the added molecules on the nutritional, sensory, textural, and shelf-life characteristics of the obtained products will be evaluated. Moreover, the research project will focus on the possibility of reusing UHT milk close to expiry in the cheese-like formulation, thus limiting food losses.

Approccio sostenibile per ottenere formaggi freschi innovativi aumentando la shelf-life e le caratteristiche nutrizionali

L'attività di ricerca del presente progetto di dottorato si propone di valutare l'effetto dell'aggiunta di molecole funzionali recuperate da sottoprodotti agro-alimentari, nella produzione di formaggi freschi innovativi, utilizzando un approccio sostenibile. Sarà valutata l'influenza delle molecole aggiunte sulle caratteristiche sensoriali, nutrizionali, strutturali nonché sulla shelf-life dei prodotti ottenuti. Inoltre, il progetto di ricerca verterà sulla possibilità di reimpiego di latte UHT prossimo alla scadenza nella formulazione di simil-formaggi, limitando così gli sprechi alimentari.

1. State-of-the-Art

Sustainable food production means shared responsibility for the production, supply, and consumption of safe and nutritious food. Global food production systems must change their actions to minimize their environmental impact and allow the world to produce food in the future. Furthermore, this would lead to an improvement in the quality of life now and in the future (Anastasiadis *et al.*, 2021). One of the strategies for a sustainable approach is the reformulation of foods to extend their shelf-life and ameliorate the nutritional aspect using food waste and by-products (Iriondo-DeHond *et al.*, 2018).

The reuse of by-products is known in the literature, both for the extraction of bioactive compounds, of which they are often rich and for the possibility of giving them economic value (Campos *et al.*, 2020). In the context of the dairy sector, there is a strong interest in extending the shelf-life of fresh cheeses to allow them to be marketed in wider markets and a longer time to use, without forgetting the possibility of obtaining nutritional benefits from the new foods (Zappia *et al.*, 2020). For the production of fresh cheeses, extracts rich in polyphenols obtained from vegetation waters and solid residues generated by the transformation of olives into oil were used. These bioactive compounds allowed increasing the shelf-life of the product thanks to their antioxidant activity (Roila *et al.*, 2019). The technological and nutritional potential of pomegranate and grape skins also allowed their incorporation in the formulation of different cheeses. Their addition determined an antimicrobial activity and a slowing down of lipid oxidation, which was positively reflected in the shelf-life of the product (Frühbauerová *et al.*, 2021; Mahajan *et al.*, 2015). Other by-products such as pomace and grape seeds, artichokes, broccoli stems and leaves (Lucera *et al.*, 2018), mango skin and kernels (Posseti *et al.*, 2020) and wheat germ (Çetinkaya *et al.*, 2020) are rich in numerous bioactive compounds like polyphenols, fatty acids, and fibers. These molecules have a positive effect on human health, such as a greater supply of essential nutrients and the prebiotic effect that promotes the proper functioning of the intestinal microbiota. The addition of agri-food by-products in cheeses also showed a positive effect on composition, texture, and sensory characteristics. These advantages encourage and justify the interest in their application in the dairy sector.

Another approach to limit food losses is reusing raw materials of animal origin. It is possible to use UHT milk near the deadline for the recovery of the functional molecules, like casein, present in it. These compounds could be used for the production of new cheese formulations, improving their nutritional and technological characteristics.

Based on these considerations, the main objective of this research project is to produce innovative fresh cheeses with increased nutritional characteristics, shelf-life, and sustainability by upcycling agri-food chain by-products and by reducing food loss. To this aim, minor milk, less involved in cheese making (such as goat) will be used, thus exploiting the nutritional and technological properties they provide.

2. PhD Thesis Objectives and Milestones

Taking into account the objectives mentioned above, the activities of this doctoral thesis project can be divided according to the Gantt diagram (Table 1).

A0) **Bibliographic search**

A1) **Production of spreadable cheeses based on minor milk with the addition of functional molecules extracted from waste/by-products**

A1.1) Extraction of functional molecules like xylooligosaccharides from vine shoots, polyphenols from the vegetable agri-food chain, and related chemical characterization.

A1.2) Development of the technology for the production of goat milk-based spreadable cheeses added of functional molecules and subjected to different heat treatments and fermentation parameters. The product will be characterized by nutritional, textural, rheological, and sensory characteristics.

A2) **Study the shelf-life of dairy products by adding vegetable extracts to the preserving liquid**

A2.1) Addition of different concentrations of extract in preserving liquid of various dairy products.

A2.2) Characterization of the preserving liquid and the related dairy product. Analyses will be carried out to assay the quality of the dairy product and the shelf-life by analyzing composition, microbiological parameters, color, texture, volatile compounds, and sensory profile.

A3) **Production of cheese-like products reducing food loss (use of UHT milk close to expiry)**

A3.1) Development of an extraction method from UHT milk that selectively extracts the casein molecules.

A3.2) Production tests of casein-based cheeses with the possibility of mixing with agri-food by-products to obtain a functional product. Various formulations will be tested. Characterization of the product and possible improvements regarding the sensory and texture features.

A4) **Data processing, writing, and editing of the scientific papers and the PhD thesis**

Table 1. Gantt diagram for this PhD thesis project.

Activity \ Months		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A0)	***Bibliographic search***																								
A1)	***Spreadable functional cheeses***																								
	1) Study of functional molecules																								
	2) Cheesemaking and characterization																								
A2)	***Study of dairy product shelf-life***																								
	1) Extract application in dairy product																								
	2) Dairy products characterization																								
A3)	***Cheese-like reducing food loss***																								
	1) Tests of casein extraction																								
	2) Production of casein-based cheeses																								
A4)	***Thesis and paper preparation***																								

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Inclusion of fresh forage in lactating ruminant diet. Evaluation of sustainability and effect on the chemical and sensory properties of milk and dairy products

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This PhD thesis research project aims to evaluate the chemical and sensory properties of milk and dairy products derived from lactating ruminants fed with fresh fodder-based diets to improve nutritional quality. The investigations will be carried out on both small and large ruminants to find the most appropriate dietary strategy in relation to environmental and economic efficiency.

Inclusione di foraggio fresco nella dieta dei ruminanti da latte. Valutazione della sostenibilità e dell'effetto sulle proprietà chimiche e sensoriali di latte e prodotti caseari

Questo progetto di tesi di dottorato si propone di valutare le proprietà chimiche e sensoriali del latte e dei prodotti caseari derivati da ruminanti in lattazione alimentati con diete a base di foraggi freschi per migliorare la qualità nutrizionale. Le indagini saranno effettuate sia su piccoli che grandi ruminanti per trovare la strategia dietetica più appropriata in relazione all'efficienza ambientale ed economica.

1. State-of-the-Art

The dairy-based food market is broadly segmented and continues to differentiate according to new consumer needs. For reasons related to human health and environmental sustainability, dairy products made with milk obtained from pasture-fed ruminants are gaining interest among consumers. Although several factors affect the composition of milk, it is widely documented in scientific literature that the nutritional and sensory properties of milk and dairy products are closely related to animal feeding (Cabiddu et al., 2019). Through appropriate dietary management regimes which include the supply of fresh fodder to replace the conserved ones, it is possible to naturally emphasize important healthy components in milk, and hence in milk products, such as omega-3 polyunsaturated fatty acids (improving the omega 6 / omega 3 ratio), vaccenic acid, conjugated linoleic acids (CLA), carotenoids, phenolic compounds, other antioxidant compounds, etc. (Table 1). The phenological stage and the type of forage crops also contribute to influencing the chemical and sensory composition of milk. The different chemical composition of milk affects the flavour and some physical properties of dairy-based products (Cabiddu et al., 2019; Sant'Ana et al., 2019). These chemical and sensory differences could allow a traceability and control of the authentic quality of dairy products (Cabiddu et al., 2019). By electronic nose analysis, Falchero et al. (2009) quickly discriminated the milk from cows that grazed two different Alpine vegetation types.

Table 1 Variation of the chemical and sensory properties of dairy products from animals fed with fresh forages

Feeding system	Sample	Main results	References
Native pasture-fed in replacement of confinement diet with conserved forage	Goat's milk and cheese	Higher MUFA, PUFA and trans-FAs content and lower medium chain FAs. exclusive identification of α-terpineol and β-caryophyllene. Higher herbaceous flavour intensity and lower butter flavour intensity	Sant'Ana et al. (2019)
Replacement of silage forage with fresh one	Buffalo milk and mozzarella cheese	Lower level of butanoic acid, 2,3-pentanedione and propyl acetate; Higher content of alcohols and esters (sweet and fruity notes)	Sacchi et al. (2020)

CLA: Conjugated linoleic acids; FA: Fatty acid; MUFA: Monounsaturated FAs; PUFA: Polyunsaturated FAs.

In Italy, forage maize crop is the main forage crop used in intensive dairy farming. However, such a forage system is highly demanding for water and non-renewable sources. The economic convenience of forage maize production is also decreasing due to the increasing costs of the production inputs and price volatility on international markets (Tabacco et al., 2018). Optimising livestock husbandry and feeding techniques can help meet animal welfare and environmental sustainability requirements. Grazing ruminants can express their natural behaviours, and it is an efficient system that has low competition for the direct production of human-edible crops (Knaus, 2016). Proper pasture management contributes to the maintenance of plant and animal biodiversity, especially in the breeding of local small ruminants. However, due to some constraints related to the availability of space, climatic conditions or the need for a high production yield, an extensive system is not always feasible or not practicable all year round. Some new technologies could help integrate fresh fodder into farms where grazing is inaccessible. In a such

context, a role may be played by hydroponically cultivated forages. Hydroponics is a soilless cultivation technique used in place of traditional agricultural systems, especially in areas characterised by large fluctuations of rainfall and temperature, and restricted water and arable land availability (Agius et al., 2019). Automated hydroponic forage systems can produce large quantity of green fodder of constant quality, in any place and season and in a short time, to be fed daily to livestock. Hydroponic forage could be a valid alternative in intensive dairy farming of large ruminants, like buffalo breeding in Campania.

2. PhD Thesis Objectives and Milestones

The PhD research project includes two experimental plans.

Noble goat's milk and cheese
Milk and cheese samples will be collected in the "I Moresani" farm located in Casal Velino (SA). Twenty pluriparous Saanen goats, homogeneous for age, body weight, parity and milk yield will be divided in two groups. Control group will be fed hay ad libitum and concentrate (400 g/head/day) composed by corn meal, barley, faba bean and peas bean. Pasture group will be fed on permanent pasture with the supplementation of concentrate in the same quantity of Control group, according to the disciplinary of the Noble Method.

Buffalo milk and mozzarella cheese
Milk and mozzarella cheese samples will be collected in the "Cerase" farm and "La Baronia" dairy factory located in Pontelatone (CE). Fifteen lactating buffaloes, homogeneous for calving order, days of lactation, body condition score, and milk yield will be divided in two groups. The Control group will be fed a diet based on the maize silage produced on the farm which, in the Experimental group, will be replaced by 50% (trial 1) and 100% (trial 2) hydroponic forage. The diets will be isonitrogenous and isoenergetic and will meet the buffalos' needs.

The research objectives will be achieved by carrying out the following analyses for both experimental plans according to the Gantt diagram given in Table 1:
A1) **Authenticity control** by electronic nose on milk samples.
A2) **Sensory profile** by descriptive sensory profile (QDA test) and discrimination test (triangle test).
A3) **Chemical and nutritional profiles** phenolic compounds by Folin-Ciocâlteu and HPLC-DAD (A3.1), antioxidant activity by ABTS method (A3.2), fatty acids by GC (A3.3), VOCs by SPME-GC/MS (A3.4).
A4) **Energy and economic efficiency** by evaluation of costs and profitability indicators, dependence on non-renewable energy sources and energy efficiency indices.
A5) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

Table 1 Gantt diagram for this PhD thesis project.

Activity \ Months		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	***Authenticity control***																								
A2)	***Sensory Profile***																								
A3)	***Chemical and nutritional profiles***																								
	1) Phenolic compounds																								
	2) Antioxidant activity																								
	3) Fatty acids																								
	4) Volatile organic compounds																								
A4)	***Energy and economic efficiency***																								
A5)	***Thesis and Paper Preparation***																								

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Use of deep learning in combination with FT-NIR spectroscopy for the analysis of extra virgin olive oil

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The aim of the present thesis is to use Fourier Transform near-infrared spectroscopy (FT-NIR) in combination with deep learning algorithms to develop predictive models for i) determining the chemical composition of extra virgin olive oil (EVOO) and ii) identifying its possible adulteration.

Fattibilità di impiego dell'apprendimento profondo in combinazione alla spettroscopia FT-NIR per l'analisi dell'olio extra vergine di oliva

La presente tesi ha lo scopo di utilizzare la spettroscopia nel vicino infrarosso in trasformata di Fourier (FT-NIR) in abbinamento ad algoritmi di apprendimento profondo (deep learning) per sviluppare modelli predittivi per i) determinare la composizione chimica dell'olio extravergine di oliva (EVOO) e ii) identificarne possibili adulterazioni.

1. State-of-the-Art

The importance of extra virgin olive oil (EVOO) in the globalised market is increasing economically and qualitatively. Quality aspects depend on environmental, agronomic, genetic and technological factors. Traditional laboratory analyses, also named wet chemistry, are widely used to assess olive oil quality. However, the methodologies used are characterised by complex procedures that require specific laboratory knowledge and do not allow timely answers. Such analyses can be also expensive and not eco-friendly because they produce laboratory waste that has a significant environmental impact.
Nowadays, new analytical techniques have also been employed to substitute traditional assays for olive oil. Among these, NIR spectroscopy is interesting because it is a quick and easy to use, economically accessible, non-destructive and multi-analytical technique, as multiple constituents can be determined from a single measurement (Massantini et al., 1997). The possibility for simple and timely determination of chemical composition and sensory characteristics of olive oil is the basis for product optimisation efforts. NIR spectroscopy combined with chemometrics is successfully used to assess the EVOO quality in terms of free acidity, peroxide number, UV spectrophotometric indices and oxidative stability, as well as chemical constituents (e.g., phenols, tocopherols, volatile compounds) (Stella E. et al., 2015). In addition, NIR spectroscopy can be used to identify any possible adulteration (Wójcicki et al., 2014), as well as to verify the genuineness and origin of the product.
Difficulty in using the NIR spectroscopy occurs prior to the end-user use and basically involves the researcher deputed to the computation of spectral data and the development of the mathematical models used to predict the analyte from the spectral profile of the product. The researcher must have specific knowledge of chemometrics and quantum mechanics to perform feature engineering (i.e., extract information from raw spectral data) and achieve high-performance prediction models. Consequently, NIR-based model development is subject to the empirical choices of the researcher with related subjectivity issues in processing of complex spectral data (Acquarelli et al., 2017), with the risk to affect the robustness and resilience of the predictive models.
Research in the field of NIR spectroscopy currently undergoes a paradigm change from classical chemometrics to deep chemometrics, which is based on the use of deep neural networks (DNNs), with the goal of assigning the feature engineering task to artificial intelligence. Preliminary studies demonstrate the efficiency of DNNs for classification and regression on spectral data (Nallan Chakravartula et al., 2022), with comparable, or even better, performance than predictive models developed using classical chemometrics. In fact, there are not studies on the use of DNNs in analysis of EVOO quality through NIR spectroscopy. Only few studies have been conducted to quantitatively identify vegetable oils (Wu X. et al., 2022).
The project aims to develop accurate and precise prediction models for the quality assessment of EVOOs by combining the use of Fourier-Transform NIR spectroscopy with deep learning algorithms.

2. PhD Thesis Objectives, milestones and deliverables

In order to achieve the objectives of the dissertation project, programmed activities will be carried out according to the GANTT diagram shown in Table 1.

A1) **Development of the research strategy (A1)**. Acquisition of knowledge and skills for the project development through the following tasks: A1.1 - the study of the state of the art in the use of NIR spectroscopy for the qualitative-quantitative analysis of EVOOs (Q1-Q3); A1.2 - selection and testing of classical analytical methods for quali-quantitative assessments in EVOOs (Q4); and A1.3 - selection and testing of deep learning

algorithms to be combined with NIR spectral analysis of EVOOs (Q4). *Milestones*: M1, the current knowledge for the development of DNN models for the analysis of EVOOs through NIRS is acquired.

A2) **Chemical-profile and shelf-life prediction models (A2).** The activity consists in the development of FT-NIR-based DNN models for the assessment of the chemical profile and shelf-life of EVOOs: A2.1 - shelf-life tests on EVOOs at controlled storage conditions (Q5-Q8); A2.2 - FT-NIR spectral scan and chemical analysis of EVOO samples (Q5-Q8); A2.3 - data acquired during the Q5-Q7 period are used to calibrate models using both classical and deep chemometrics; A2.4 – predictive models are validated using the external dataset acquired during the Q8 period, and models are evaluated for their performances. *Milestones*: M2, chemical-profile and shelf-life prediction models are calibrated. *Deliverables*: D1, validated chemical-profile and shelf-life prediction models based on DNN algorithms.

A3) **Adulteration identification models (A3).** The activity is focused on the development of FT-NIR-based DNN models for the identification of adulteration in EVOOs: A4.1 – adulterated EVOO samples are prepared and scanned using FT-NIR spectroscopy (Q9-Q12); A4.2 – calibration of both classical and deep chemometrics models using data acquired during the Q9-Q11 period; A4.3 - models are validated with new oil blends (i.e., a real external dataset) and evaluated for their performances (Q11-Q12). *Milestones*: M3, adulteration identification models are calibrated. *Deliverables*: D2, validated adulteration identification models based on DNN algorithms.

A4) **Exploitation and dissemination (A4).** The resulting activities will be used to take part to conferences, fairs, workshops and similar events (A4.1; Q3-Q12), to supervise master's theses and submit scientific articles (A4.2; Q3-Q12) and to finalise the PhD thesis (A4.3; Q9-Q12). *Deliverables*: D3, attendance to the workshops, conferences, etc. is achieved; D4, student theses are supervised, and scientific papers are published; D5, the PhD thesis is finalized.

Table 1 GANTT diagram for the PhD thesis project.

	Activity	Q1	Q2	Q3	Q4	Q5	Q6	Q7	Q8	Q9	Q10	Q11	Q12
A1	***Development of the research strategy***				M1								
	1. State of the art of EVOO quality assays												
	2. Selection and test of analytical methods												
	3. Test of deep chemometrics methods												
A2	***Chemical-profile and shelf-life pred. models***												
	1. Shelf-life tests of selected EVOOs												
	2. Spectral and chemical data acquisition												
	3. Calibration of deep chemometrics models							M2					
	4. Validation of models and performance tests								D1				
A3	***Adulteration identification models***												
	1. Sample adulteration and FT-NIR scans												
	2. Calibration of deep chemometrics models											M3	
	3. Validation of models and performance tests												D2
A4	***Exploitation and dissemination of results***												
	1. Conference, workshops, fairs, etc.												D3
	2. Student theses and scientific articles												D4
	3. PhD thesis development												D5

M, milestones; D, deliverables; Q, quarter

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Insights on the mechanisms underlying the colour expression and stability of Italian rosé wines

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This PhD project aims to investigate the mechanisms of formation, evolution and stabilisation of colour in rosé wines. It also aims to evaluate the effectiveness of different winery strategies, able to allow the management of this component of the quality of wines produced and its maintenance during shelf-life.

Studio sui meccanismi alla base dell'espressione cromatica e della stabilità del colore dei vini rosati italiani

Questo progetto di dottorato mira ad indagare i meccanismi di formazione, evoluzione e stabilizzazione del colore nei vini rosati. Si intende inoltre valutare l'efficacia di diverse strategie di cantina, in grado di consentire la gestione di questa componente della qualità dei vini prodotti e il suo mantenimento durante la shelf-life.

1. State of the art

Over the last decade, the consumption of rosé wines in the world has seen steadily growing statistics. In the 2018 campaign (latest available data), the total volume marketed was 25.6 million hectolitres (+31% compared to 2002), with France (28% of the total), the USA (17%) and Spain (15%) leading the sector (Roseè Wine World, 2020). Italian rosé wines represent 10% of world production with excellent export performance (+13%) and average export prices in constant growth (+35% since 2008). Italian rosé is therefore popular abroad but is struggling to find space in Italy. Producers could seize the opportunity offered by the positive trend in international preferences, improving communication and attractiveness of rosé wine on the domestic market. Colour is one of the main visual features of wines and plays a key role in defining the perceived quality of the product. Probably even more than for white and red wines, the colour of rosé wines is a strong quality factor since it can influence purchasing choices. The phenolic class of anthocyanins and the pigments coming from them are primarily responsible for the colour of red and rosé wines. Pigments are localised in the epidermal cells of red grapes and are extracted during the short maceration phases implemented for rosé wines (He et al., 2012). During storage, however, anthocyanins undergo a variety of reactions and condensations, often linked to oxidoreductive kinetics, leading to the formation of anthocyanin derivatives (the latter crucial for colour stability) (Hernandez et al., 2011). The types of reactions involved include self-association and co-pigmentation (for young wines), condensation with catechins and procyanidins to form polymer molecules or, finally, the formation of new pigments such as pyranoanthocyanins and their polymers (typical of developed wines) (He et al., 2012). The colour and stability of these derivatives vary from compound to compound but in general, it is a common opinion that condensation products between anthocyanins and flavan-3-ols, mediated or not by the presence of acetaldehyde, are the most suitable for stabilising the colour of wines (Hernandez et al., 2011). The compositional specificity of rosé wines allows that the expression and stability of colour strongly depend on I) the grape variety, II) the number of anthocyanins extracted during short macerations, III) the molar ratio between anthocyanins and tannins (extracted from the berry or added during vinification), IV) the oxidation-reduction state of the wine and V) the extent of the reactions involving these latter classes of compounds during fermentation and ageing (He et al., 2012). Excessive amounts of anthocyanins can lead to drastic colour losses as a result of oxidation. Indeed, in rosé wines, the oxidation of flavanols (catechins and procyanidins) triggers the phenomenon of non-enzymatic browning (Li, Guo, & Wang, 2008). An excess of polyphenols can also negatively affect the aroma of wines due to the possible formation of quinones that can irreversibly react with varietal aromas such as thiols, giving rise to aromatic losses (Gil et al, 2019).

2. PhD Thesis Objectives and Milestones

The PhD project can be divided into the following activities, visible also in the Gantt diagram shown in Table 1:

A1) Bibliographic research on the different distinct and established analytical methodologies for the determination of colour, anthocyanins and tannins in rosé wines.
A2) Evaluation of analytical techniques for colour and tannins that are easy to apply in the production environment, based on spectrophotometric determinations, including: a) acquisition of UV/Vis spectra in absorbance and reflectance; b) vanillin-reactive phenols; c) p-DAC-reactive phenols; d) gelatine-reactive tannin index (astringent power); e) Bate-Smith method; f) methylcellulose method
A3) Anthocyanins and tannins interactions in a model matrix to model the phenomena under study; it is important to work under laboratory conditions, using synthetic matrices or white and red musts that have been previously characterised and subsequently tested at different combinations to vary the ratio of pigments, tannins and phenolic acids.
A4) Development of a colour and tannic profile map through the execution of an analytical-sensorial screening of Italian rosé wines, bought on the market and coming from national territories historically vocated to these products (Bardolino, Valtenesi, Abruzzo, Apulia), but also emerging districts (Tuscany, Sicily) or dedicated to sparkling rosé wines (Lambrusco base in Emilia, Pinot noir base in Franciacorta or Trento).
A5) Evaluation of the influence of maceration time and the presence of oenological tannins of different origins on the evolution of colour over time (spectrophotometry, CIELab profile) and on the phenolic (HPLC-DAD-TOF) and volatile (GC-MS) component of laboratory vinified rosé wines.
A6) Meso-scale vinification in at least one of the two vintages following the first year, to study two different oenological techniques applied to rosé wines: sparkling and macro/micro-oxygenation.
A7) Writing and publication of PhD thesis, posters, scientific articles and oral presentations.

Table 1 - Gantt diagram for this PhD thesis project.

Activities	Month	2	4	6	8	10	12	14	16	18	20	22	24	26	28	30	32	34	36
A1)	***Bibliographic research***	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	
A2)	***Evaluation of analytical techniques for colour and tannicity***		■	■	■														
A3)	***Anthocyanin and tannin interaction in a model matrix***				■	■	■												
A4)	***Development of a colour and tannin profile map***			■	■	■	■												
A5)	***Evaluation of the influence of maceration time and presence of tannins***							■	■	■	■	■	■						
	1) colour, phenol, volatile analysis of wines							■	■	■	■	■	■						
	2) Study shelf-life/evolution of the above parameters										■	■	■						
A6)	***Vinification on a meso scale***												■	■	■	■	■		
	1) sparkling wine and macro/micro-oxygenation study													■	■	■			
	2) colour, phenol and volatile analysis of wines and their evolution														■	■	■		
A7)	***Publications - communications and final thesis writing***						■	■	■	■	■	■	■	■	■	■	■	■	■

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Novel plant protein sources for the beverage sector: technological functionality, nutritional properties, sensory characteristics, and consumer acceptability

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This Ph.D. research, supported by PON Ricerca e Innovazione 2014-2020 (DM1061/2021) and Lavazza S.p.A, aims at identifying unconventional plant proteins suitable for the beverage sector by filling the knowledge gap about their technological, nutritional and sensory performance. More specifically, the main objectives are: (i) to set up a database about plant protein sources; (ii) to develop novel technological strategies for the production of protein-rich plant ingredients with improved technological, nutritional and sensory properties; (iii) to develop novel protein-rich plant beverages.

Nuove fonti proteiche vegetali per il settore delle bevande: funzionalità tecnologiche, proprietà nutrizionali, caratteristiche sensoriali ed accettabilità del consumatore

Questo percorso di dottorato, finanziato da PON Ricerca e Innovazione 2014-2020 (DM1061/2021) e Lavazza S.p.A, ha come obiettivo quello di identificare proteine vegetali non convenzionali da impiegare nel settore delle bevande, colmando l'attuale carenza di informazioni relative alle loro proprietà tecnologiche, nutrizionali e sensoriali. Più specificamente, gli obiettivi principali sono (i) sviluppare un database delle fonti vegetali per l'ottenimento di proteine, (ii) sviluppare nuovi processi tecnologici per la produzione di ingredienti vegetali ricchi di proteine con migliorate proprietà tecnologiche, nutrizionali e sensoriali; (iii) sviluppare bevande vegetali ricche di proteine.

1. State-of-the-art

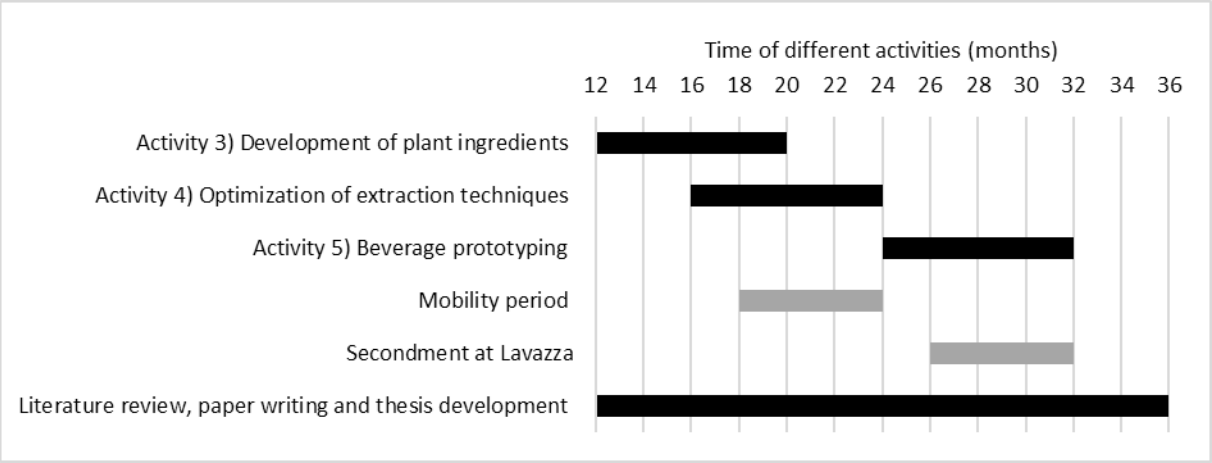
In the context of a constantly growing beverage sector, there has been a surge in the consumer demand for novel products. Such an increase is due to the typical advantages of beverages, being easy to use/easy to be consumed/easy to be digested, which make them suitable to all consumer categories. The growing interest in health and wellbeing is heralding the expansion of functional beverages. Among these, protein beverages are predicted to emerge as the fastest-growing subsector (Jiménez-Munoz et al., 2021; Penha et al., 2021). Based on amino acids composition, digestibility, and bioavailability, dairy proteins are considered optimal ingredients for the preparation of protein-rich beverages. Nevertheless, they present some major issues related to negative environmental impacts, enhanced risk of developing cancer and cardiovascular diseases and possible consumer aversion due to ethical issues (e.g., vegan philosophy) (Kumar et al., 2021).
Plant proteins present much lower environmental and social criticisms than dairy ones. They can be isolated from several plant matrices as well as from by-products and waste (e.g., soy okara, potato juice) of plant processes, with the additional advantage of increasing the circularity of food production (Fasolin et al., 2019; Prandi et al., 2019). However, the extensive use of these ingredients is hindered by the impaired techno-functional properties and unpleasant sensory attributes, besides the poor essential amino acids profile and lower digestibility/bioavailability (Kumar et al., 2022). Although thermal-assisted protein extraction is able to enhance the techno-functional properties of the ingredients, it might reduce protein's nutritional and sensory profile (Kumar et al., 2021,2022). Further advantages could derive from applying innovative non-thermal treatments, such as those based on high pressure, electric fields and mechanic waves (Akharume et al., 2021).
In addition, the preparation of dried ingredients integrally retaining the original plant materials dry matter could represent an alternative technological strategy to the preparation of protein concentrates and isolates. The latter are often associated with low production yields and not negligible plant residues, negatively contributing to the environmental impact (Mondor & Hernández-Álvarez, 2022). The integral use of the plant materials could not only reduce the impact of traditional extraction steps, but also avoid the loss of valuable nutritional plant components (e.g., dietary fiber and phenolic compounds).

Based on these considerations, the aim of this Ph.D. project is to develop novel protein-rich plant ingredients for the beverage sector.
To this aim, the research activity will be organized as follows (Fig.1):
Activity 1. Development of a database of literature data about possible plant protein sources and their characteristics. The database will comprehensively organize data relevant to protein content, nutritional characteristics, technological and sensory properties, and consumer/market/regulatory aspects of plant sources

having different TRL from laboratory level to availability on the market. To this aim, cereals, pulses, oilseeds and their by-products will be considered as possible protein sources.
Activity 2. Selection of the most interesting plant protein sources based on clustering of the different sources reported in the database (Activity 1) according to the analysis of their pros and cons.
Activity 3. Development of protein-rich plant ingredients by integral use of the plant materials. To this aim, different size-reduction operations (dry and wet grinding, ultrasounds, high-pressure homogenization), assisting technologies (pulsed electric fields, ultrasounds) and drying procedures (air, freeze and supercritical drying) will be investigated. Optimal processing parameters will be selected based on protein content, technological, nutritional, sensory properties, and economic/environmental sustainability of the obtained protein-rich plant ingredients.
Activity 4. Optimization of extraction techniques (wet and dry extraction), with or without the assistance of pulsed electric fields and ultrasounds, when the integral use of the plant source will be not feasible. This could be the case of plant sources providing low protein content of the ingredients obtained in activity 2 or having non-adequate hygienic properties (e.g., by-products and waste materials).
Activity 5. Beverage prototyping, including formulation, production process, and packaging to allow product quality, safety and stability as well as easy use by target consumers. The beverage aminoacid profile will be optimized by studying the combination of different protein-rich plant ingredients. Beverages will be finally tested for consumer acceptability. Activity 4 will be performed within a 6-month secondment at Lavazza S.p.A.

The first year of my Ph.D. course has been devoted to the development of a database collecting literature data about the extraction yields, processing parameters, technological properties, nutritional value, sensory properties, novel food status, EFSA opinion, allergenicity, market availability and cost of 73 plant protein sources from all over the World (Activity 1). Based on this data set, some pulses sources have been selected and processed according to Activity 2. Ingredients obtained by grinding and supercritical CO_2 drying of soy okara and pea by-products resulted particularly interesting since colorless and free of the typical off-flavors of the original plant matrix.

Figure 1. Timeline of expected activities



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Development of sustainable and innovative strategies for the valorization of by-products of the brewing industry

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Beer is one of the most consumed alcoholic beverages in the world. The brewing industry, as well as the whole agri-food chain, is responsible for the production of a high number of several by-products (spent grain, spent hop, and spent yeast). In a broad context, this PhD thesis research project is aimed to implement sustainability, resilience, and circularity strategies within the brewing chain, through biorefining and valorization of the brewery by-products. In particular, this project will focus on the reuse and valorization of brewer's spent grain, the main by-product of the brewing industry.

Sviluppo di strategie sostenibili e innovative per la valorizzazione dei sottoprodotti dell'industria birraria

La birra è tra le bevande alcoliche più consumate al mondo. L'industria birraria, come l'intera filiera agro-alimentare, è responsabile della produzione di un'elevata quantità di sottoprodotti (trebbie, trub e lievito esausto). In un contesto più ampio, questo progetto di tesi di dottorato ha lo scopo di migliorare la sostenibilità, la resilienza e le strategie circolari all'interno dell'industria birraria attraverso la bioraffinazione e la valorizzazione dei suoi sottoprodotti. In particolare, il presente progetto sarà focalizzato sull'utilizzo e la valorizzazione delle trebbie, principale sottoprodotto dell'industria birraria.

1. State-of-the-Art

In recent years, the topic of food loss is gaining in importance due to serious environmental issues caused by the increase in food waste and the costs of their disposal. From a sustainable point of view, the circular economy aims to reuse and recycle waste, source of valuable compounds, to produce new added-value products. In 2014, the Food and Agricultural Organization of the United Nation (FAO) defined "food loss" as the "decrease in quantity or quality of food caused by the functioning of the food production and supply system or its institutional and legal framework". Furthermore, FAO defined "food waste" as "a part of food loss" caused by unintentional or intentional disposal of edible food mass at any stage of the supply chain.

Beer is one of the most common alcoholic beverages in the world. The brewing process involves several stages of production and at the end of some of these, brewery by-products are obtained. Brewer's spent grain is obtained by lautering at the end of the mashing process. Brewer's spent hop is obtained by separation in the whirlpool at the end of the boiling stage. Brewer's spent yeast is obtained at the end of the fermentation stage (Cimini et al., 2021). Given the large global annual production of beer and the large production of this waste that will have to be disposed, the brewing industry contributes to the increase of the environmental negative impact. However, it is very important to consider that the high volume of by-products from breweries represents a feedstock to produce valuable product streams in the perspective of a circular economy (Kerby et al., 2017).

Brewer's spent grain represents the main by-product of the brewing industry (about 20 kg of spent grain for hl of beer produced), accounting for about 85% of the total by-products. The global annual production of spent grain is ~39 million tons. In the European Union, the production is ~3,4 million tons, ~219 thousand tons of which are produced in Italy according to the Assobirra Report 2020. Brewer's spent grain consists of coating layers of seeds, pericarp, and husk of barley grain. It represents variable heterogeneous biomass depending on the type of barley grain, harvest time, location, the conditions of malting and mashing, the quality and type of adjuvants used during the production of beer processes. The major challenges associated with using of spent grain as a feedstock include the high moisture content, ~70-80%, which makes it susceptible to microbial growth and spoilage within a short period. Brewer's spent grain is composed by fiber (30-50%, w/w), proteins (19-30%, w/w), lipid (10%, w/w) and ash (2-5%, w/w). The fibrous fraction is divided in cellulose (12-25%, w/w), hemicellulose (20-25%, w/w) and lignin (12-28%, w/w) (Lynch et al. 2016).

Currently, brewer's spent grain is mainly sold to farmers as animal feed, with a low market value of ~35 Euro/ton or landfilled. Finding alternatives, higher-value uses for spent grain is therefore particularly attractive from the point of view of brewery economics (Buffington, 2014). In addition, the components of brewer's spent grain are also considered as precursors for fine chemicals or as energy sources in microbial fermentations. The brewer's spent grain can be used in food production (e.g., bakery) due to the rich composition in protein and fiber with health benefits (Bravi et al. 2021). Furthermore, brewer's spent grain can be used in the production of bioethanol, building materials (bricks), biogas, and bioplastics (food packaging) (Karlović et al, 2020; Chetrariu et al., 2020; Jackowski et al., 2020).

This PhD research project has the overall objective to implement sustainability, resilience, and circularity strategies within the brewing chain. According to the concepts discussed in this section, the purpose of this PhD thesis project is:

- evaluate the employ of by-products of the brewing industry for different applications;
- achieve an integral fractionation technique of brewer's spent grain, mainly by-product, for the recovery of protein and fibrous components;
- optimize strategies for the valorization of the protein component of brewer's spent grain;
- develop sustainable strategies for the conversion of fibrous components into innovative and biodegradable materials for food packaging.

2. PhD Thesis Objectives and Milestones

Within the overall objectives mentioned above, this PhD thesis project can be subdivided into the following activities, according to the Gantt diagram in Table 1:

A1) Knowhow of the brewing process and by-products. Study of the state of the art of the reuse and recycle of by-products (spent grain, spent hop, and spent yeast). Characterization of by-products of the brewing industry (e.g., chemical-physical and thermal) employing official and instrumental methods.

A2) Development and optimization of fractionation process of the brewer's spent grain. The aim of this activity is the separation of all the main components (proteins, cellulose, hemicellulose, and lignin), starting from the data in the literature. Analysis of the several fractions recovered by brewer's spent grain in terms of molecular structure, morphological and thermal characteristics, employing instrumental techniques (e.g., ATR-FTIR spectroscopy, thermogravimetric analysis, etc.). The purpose of this monitoring is the identification of optimal protocols mainly in terms of sustainability, yield of the processes, and quality of the components.

A3) Valorization of brewer's spent grain. Employ brewer's spent grain or its individual components in formulations to produce bioplastics. For example, the use of these as functional fillers for the improvement of the mechanical, antimicrobial, barrier, and antioxidant properties of the polymeric films for packaging used on the market, and non-renewable, will be evaluated. Furthermore, the possibility of employing the protein component of brewer's spent grain in the formulation of innovative ingredients will be evaluated.

A4) Literature, writing and editing of the PhD thesis, publication of scientific papers and presentation to National and International meetings.

Table 1 Gantt diagram for this PhD thesis project.

Activity	Months	1	2	3	4	5	6	7	8	9	10	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
A1)	**Characterization of brewing by-products**																																			
	1) Study of the state of the art of the reuse of by-products																																			
	2) Characterization of brewer's spent grain, spent hop and spent yeast																																			
A2)	**Development and optimization of fractionation process of the brewer's spent grain**																																			
	1) Fractionation of all the main components																																			
	2) Analysis of the several fractions (cellulose, hemicellulose, lignin and protein)																																			
	3) Optimization of fractionation process to obtain optimal protocols																																			
A3)	**Valorization of brewer's spent grain**																																			
	1) Realization of formulations for the production of bioplastics																																			
	2) Evaluation of mechanical, antimicrobial, barrier, and antioxidant properties																																			
	3) Evaluation of the protein component in the formulation of innovative ingredients																																			
A4)	**Literature, writing and editing**																																			

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ECO-sustainable packaging materials for the food industry (ECOPACKMAT)

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The overall objective of this PhD thesis research project is to develop a new class of paper-based materials, characterized by a low environmental impact, intended to feed the food packaging industry. Specifically, the PhD program is focused on scouting and valorizing disparate cellulosic industrial wastes, which nowadays do not find efficient reuse. At first, such wastes will undergo an extractive process to obtain cellulose with high purity degree from which, through a top-down approach, microfibrils (MFCs) and nanocrystals (CNCs) are manufactured. Secondly, MFCs and CNCs will be added to the cellulosic pulp both "in bulk" and as a thin layer on the substrate, thus potentially leading to the differentiation of available materials for packing specific categories of food products.

Materiali per packaging ecosostenibili per l'industria alimentare (ECOPACKMAT)

Questo progetto di tesi di dottorato si prefigge come obiettivo principale la creazione di una nuova classe di materiali cartacei, caratterizzata da un basso impatto ambientale, da destinare all'industria dell'imballaggio alimentare. Nella fattispecie, il percorso è incentrato sulla ricerca e successiva valorizzazione di differenti prodotti di scarto industriale a base cellulosica, i quali non trovano attualmente un riutilizzo efficiente. In primo luogo, tali sottoprodotti verranno sottoposti ad un processo estrattivo con il fine ultimo di ottenere cellulosa con un elevato grado di purezza da cui, tramite approccio top-down, produrre microfibrille (MFCs) e nanocristalli (CNCs). Successivamente, MFCs e CNCs verranno adoperati sia come riempitivo della matrice cellulosica che depositati in strato sottile (coating) sulla superficie del substrato, con lo scopo di garantire una potenziale differenziazione dei materiali a disposizione per l'imballaggio di specifiche categorie di prodotti alimentari.

1. State of the Art

The increasing attention towards "green" packaging materials, together with the combination of current legislation constraints, has prompted dramatic changes in the design and development of new materials that can replace conventional (oil-derived) solutions. Among others, cellulose and its derivates, such as micro-fibrillated cellulose (MFCs) and cellulose nanocrystals (CNCs), are sought after with outstanding interest because of their inherent biodegradable features (Motaung et al; 2018). Cellulose is the main constituent of higher plant tissues, and it is also largely abundant in several waste biomasses, namely industrial by-products, recycling streams, and rubbish (Rampazzo et al; 2017).

Table 1: Main features of biomasses to be investigated during this PhD project.

	Arundo Donax	**Posidonia Oceanica**	**Coffee Silverskin**
Amount of waste product	Present in large amount as invasive plant throughout the Italian territory	There is no data, but an articles report that on the beach of Algero, Sardinia, 905 metric tonnes/year are deposited	In Italy, the industrial processing of coffee generates 7500 tons of waste per year
Amount of cellulose [w/w]	39.6 %	≈ 35 %	≈ 20-30%
Current applications	Cellulose extraction, phytodepuration, land stabilization, biogas production	Dietary supplement for animal feed, composting	Cosmetics, cellulose extraction

Within this research project, we selected three different matrices as promising candidates for cellulose extraction and functionalization purposes (Table 1), namely *Arundo Donax*, an invasive and perennial plant, *Posidonia Oceanica*, a common aquatic plant, and *Coffee Silverskin*, a coffee production waste (Collazo-Bigliardi et al; 2018). This choice accounts for the scarce availability of data in the current literature, thus gaining new knowledge as well as setting up a platform for the proper valorization of heterogeneous substrates of cellulosic origin. Within this project proposal, the MFCs and CNCs will be added 'in bulk' in the cellulosic pulp manufactured at the

industrial plant. Within this activity, different formulations will be tested depending on the different characteristics and concentrations of MFCs and CNCs. The final specimens will be finally characterized in terms of surface wettability and roughness, mechanical strength, barrier properties to gases, and resistance to grease. Besides using MFCs and CNCs within the cellulosic network, I will investigate the effect arising from the deposition of MFCs and CNCs as a thin layer on the main cellulosic substrate on the overall performance of the final material. According to this scenario, it is somehow obvious that cellulosic materials (paper and paperboard) are looked with renewed interest because they may represent the starting point for a new class of biodegradable and/or compostable materials for food packaging applications, with no use of plastic or metal layers, using solely water-based solutions (no organic solvents). Accordingly, the success of this project may represent a relevant competitive advantage at industrial level.

2. PhD thesis Objectives and Milestones

The Gantt diagram (Table 2) provides an overview of all activities foreseen to be run during this PhD project. More specifically, the whole work is distributed within four main Work Packages (WPs) according to the following schematic description:

WP1: Cellulose recovery and MFCs/CNCs preparation
T1: Extraction of cellulose from residues and/or waste generated by the agri-food industry/industrial partner;
T2: Production of MFCs and CNCs using top-down approaches;
T3: MFCs and CNCs production from by-products;
T4: Dimensional, yield, and structural characterization of MFCs and CNCs.

WP2: Bulk insertion of MFCs/CNCs into paper-based materials
T1: Addition of MFCs/CNCs within the cellulosic substrate at different concentrations;
T2: Assessment of surface roughness of achieved samples;
T3: Scale-up to pilot plant.

WP3: Production of MFCs/CNCs-based coatings
T1: Coating deposition on paper-based materials;
T2: Physicochemical characterization of the final products;
T3: Evaluation of mechanical properties (tensile, and puncturing resistance);
T4: Physicochemical characterization of packaged foods.

WP4: Dissemination
T1: Publication in peer-reviewed journals;
T2: Communications at conferences/meetings;
T3: Report activity toward the industrial partner
T4: Writing the final thesis

Table 2*: Gantt diagram for this PhD thesis project.*

		Years											
		1				2				3			
		Months											
WPs	Task	3	6	9	12	15	18	21	24	27	30	33	36
1	1												
	2												
	3												
	4												
2	1												
	2												
	3												
3	1												
	2												
	3												
	4												
4	1												

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Development of innovative tools for nutrition education: a promising strategy to tackle and prevent malnutrition and to reduce the environmental impact of food through the adoption of healthier and more sustainable diets

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This PhD thesis research project aims to create innovative digital tools for the promotion of healthy and safe nutrition, and conscious and sustainable lifestyles, to simultaneously tackle the environmental impact of food and the double burden of malnutrition in a simple, immediate, and effective way. More sustainable diets and healthier lifestyles must be sought, for the reduction of non-communicable diseases (NCDs) that make innovative nutritional solutions not only healthy, but also environmentally sustainable and customised for different consumer groups increasingly necessary.

Sviluppo di strumenti innovativi per l'educazione alimentare: una strategia promettente per combattere e prevenire la malnutrizione e per ridurre l'impatto ambientale del cibo attraverso l'adozione di diete più sane e sostenibili.

Questo progetto di tesi di dottorato mira a creare strumenti digitali innovativi per la promozione di una alimentazione sana e sicura e di stili di vita consapevoli e sostenibili, per affrontare contemporaneamente l'impatto ambientale legato all'alimentazione e la malnutrizione per difetto e per eccesso, in modo semplice, immediato ed efficace. La necessità di una maggiore sostenibilità alimentare e di stili di vita più salutari finalizzati alla riduzione delle malattie non trasmissibili (NCD) rendono sempre più necessarie soluzioni nutrizionali innovative non solo salutari, ma anche sostenibili e personalizzate per diversi gruppi mirati di consumatori.

1. State-of-the-Art

The food system is constantly evolving as consumer preferences, production methods and policies change over time. Public health systems are also under increasing pressure as malnutrition-related NCDs, including obesity, diabetes, and some cancers, pose a threat to long-term health. At the same time, food systems contribute substantially to climate change, loss of biodiversity and depletion of natural resources (1). Changes in food systems are needed not only to address the increase in food-related NCDs, but also to promote a shift towards an environmentally sustainable future. This is not an easy task, nor it can be accomplished by any one actor or action (1). The Mediterranean Diet (MD) is a model of eating based on the traditional foods and drinks of the countries surrounding the Mediterranean Sea. Over the last few decades, it has been promoted worldwide as one of the healthiest dietary patterns and has been reported to be consistently beneficial with respect to chronic diseases and longevity (2). Indeed, as scientific evidence has shown, to date the MD represents a model of healthy and sustainable diet, which is a determining factor in prevention, counteracting the risk of the onset of major chronic diseases (3). At the same time, the DM represents a sustainable diet model with its positive effects in the environmental and economic context. In fact, agricultural and agri-food productions, together with the culinary tradition, on the one hand ensure quality from an organoleptic point of view and, on the other, guarantee compliance with ethical and environmental criteria (3). MD is characterized by a balanced lipid profile (low in saturated fats (SFAs) and high in monounsaturated fats (MUFAs), with olive oil as the main source of fat), a high intake of low glycemic index carbohydrates, fiber, antioxidants, cereals (preferably as whole grain), legumes, fruit, vegetables, and nuts, and a moderate consumption of fish and shellfish, white meat, eggs, and dairy products (4-6). Recently, the environmental impact of MD has been addressed and considered in an updated version of the MD Pyramid, which emphasizes a lower consumption of red meat and bovine dairy products, and a higher consumption of legumes and locally grown eco-friendly plant foods (7,8). In this context nutrition education, based on the principles of the Mediterranean lifestyle, is the first and most effective tool to protect health and the environment, both as action and prevention (9). In particular, educating to a MD-based diet from school age onwards is a way to make the younger generations understand the role that its adoption can play in both health protection and environmental sustainability (10). Innovative technological educational tools are extremely important for adults, but even more so for the younger generation as they enable them to apply 'learning through play'. Indeed, games and activities improve understanding of any nutritional topic (9,11). All the mentioned objectives can be achieved through 'smart' technological supports, services, digital innovation, new technologies, tools, also based on the analysis of drivers influencing behavior, communication and education, digital technologies and labelling, and considering the role of collective catering (12).

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) **Market survey and co-creation:** characterisation, with the synergy of MEDEGUS S.r.l., of existing tools to reduce malnutrition and the environmental impact of diets, trying to identify gaps and strengths. Representative samples of the target population are interviewed and tested using different tools and different educational content to verify the levels of learning and usability. By following a co-creative strategy with user interviews, it is possible to build digital tools based on the real needs of the target population.

A2) **Development of interactive digital tools based on the results of the co-creation:** design and development of interactive digital tools specifically addressed to different target populations suffering from malnutrition (different age groups and physiological conditions) to tackle malnutrition through nutrition education. Tips, educational content, healthy and sustainable recipes and menus are examples of the services available through these innovative tools.

A3) **Implementation and evaluation:** the innovative educational tools are tested on a representative sample of subjects of different ages and with different malnutrition problems, to verify the acquisition of the contents in terms of adopting healthier and more sustainable eating habits. The functionality and effectiveness of the innovative tools are evaluated according to specific objectives defined a priori.

A4) **Industrial transferability/use:** a prototype of the innovative digital tool will be tested on a large scale in collaboration with the company partner (MADEGUS S.r.l) to demonstrate its functionality and the feasibility and effectiveness of the project.

A5) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications. This activity also includes a series of actions to share the results of the project with the aim of raising awareness and contributing to knowledge building and the advancement of innovation. The dissemination plan is planned both through social networks for the general population and through scientific publications and conference proceedings.

Table 1 Gantt diagram for this PhD thesis project.

Months		1	2	3	4	5	6	7	8	9	10	11	**12**	13	14	15	16	17	18	19	20	21	22	23	**24**	25	26	27	28	29	30	31	32	33	34	35	**36**
Activity																																					
A1	Market survey and co-creation																																				
A2	Development of interactive digital tools																																				
A3	Implementation and evaluation																																				
A4	Industrial transferability/use																																				
A5	Writing and Editing																																				

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Valorization of milk and dairy products in healthy and sustainable dietary patterns

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This PhD thesis research project is aimed at: i) elaborating and optimizing sustainable and healthy Mediterranean-based dietary patterns including milk and dairy products; ii) comparing their nutrient adequacy and environmental impact with those of the dietary patterns replacing dairy foods with plant-based alternatives; iii) investigating the impact of the optimized dietary patterns on nutritional, metabolic and physiological status in target groups of the population.

Valorizzazione del latte e dei prodotti lattiero caseari in modelli dietetici sani e sostenibili

Questo progetto di tesi di dottorato mira a: 1) elaborare e ottimizzare modelli dietetici salutari e sostenibili ispirati al modello Mediterraneo che includano il latte e i suoi derivati; 2) confrontare la loro adeguatezza nutrizionale e il loro impatto ambientale con quelli di modelli dietetici nei quali i prodotti lattiero-caseari sono sostituiti con prodotti di origine vegetale; 3) valutare l'impatto dei modelli alimentari ottimizzati sullo stato nutrizionale, metabolico e fisiologico di specifici gruppi di popolazione.

1. State-of-the-Art

Our times are characterized by several food challenges, including several forms of malnutrition, depletion of the environmental resources and the population growth. Some of sustainable development goals, in particular goals number 2, 3, 12 and 13, underline the need to find a solution to these challenges to promote prosperity while protecting the planet (Morton *et al.*, 2017). In this scenario, there is the urgency to encourage the transition towards dietary patterns with a low environmental impact, hence characterized by a high consumption of plant-based foods and a low intake of animal products (Lindgren *et al.*, 2018). However, animal products could be crucial for specific targets of population since they are an excellent source of essential aminoacids, and highly bioavailable micronutrients (e.g., calcium, phosphorous, B2 and B12 vitamins) (Seves *et al.*, 2017). As a consequence, it is necessary to optimize dietary models in order to guarantee an adequate intake of energy and nutrients while limiting the environmental impact of the whole dietary pattern.
In this context, within Mediterranean-based dietary pattern (MD), milk and dairy products could play a key role in this challenge as they have a lower environmental impact than the other animal products (Barilla Center for Food & Nutrition, 2014). Due to their important nutritional role, all guidelines for a healthy diet recommend daily consumption of milk/yogurt; however, as shown in Figure 1, in Italy, we are facing a progressive decrease in milk consumption. This reduction has been observed especially among individuals between 6 and 24 years, range of age in which the consumption of milk may be crucial to reach the peak bone mass (Istat, 2022).
Besides ethical and healthy reasons (e.g., allergy), one of the main reasons of this reduction could be the unfavorable communication against these products, often not supported by scientific evidence.

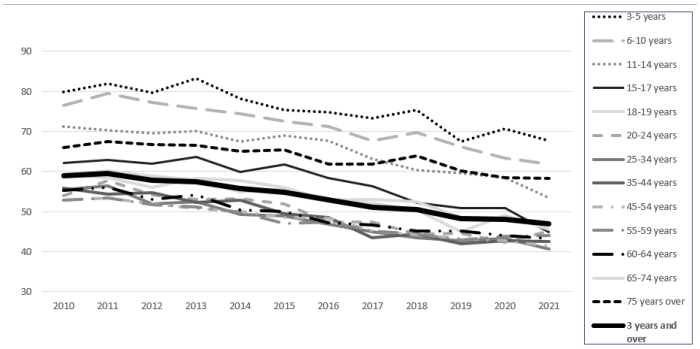

Figure 1 *Percentage of people aged 3 years and over consuming milk at least once a day from 2010 to 2021 in Italy.*

Thus, this PhD thesis project will be directed to develop sustainable and healthy dietary patterns taking into consideration the role of milk and dairy products on human health. In addition, we will investigate the nutritional and environmental impact of these products to implement knowledge about the role of dairy category which may represent an additional tool to fight against fake news.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above, this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) **Development and optimization of Mediterranean-based dietary patterns including milk and dairy products.** Indications of weekly frequencies and serving sizes of the different food groups will be developed and will be translated into a dietary plan considering traditional dishes of the MD (A1.1). All the analysis will be performed by using a specific software for nutritional assessment to simulate dietary patterns. The environmental impact will be evaluated with the use of data available in literature, such as SU-EATABLE LIFE (SEL) database (Petersson *et al.*, 2021) (A1.2).

A2) **Simulation of replacement of milk and dairy products with dairy analogues** on nutritional (A2.1) and environmental impacts (A2.2) using methods described above**.**

A3) **The comparison of these dietary patterns** will be performed in terms of: i) nutrient adequacy for the general populations and for specific target groups by using dietary reference values of Nutrients and Energy for Italian population (LARN, 2014) (A3.1); ii) environmental impact, by using data available in literature (A3.2).

A4) **Research and development of innovative dairy products and their optimization for target groups**; the results from the activities described above could be useful to identify the need of developing new dairy products specifically target for selected target groups of the populations.

A5) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

A6) **Dissemination of results will be performed** through different communication campaigns and with the use of several means of communication, to promote a correct knowledge about the role of milk and dairy products on human health and their inclusion in sustainable healthy dietary patterns.

Table 1 *Gantt diagram for this PhD thesis project.*

Activity / Months		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	***Dietary patterns with milk and dairy products***																								
	1)Development of the MD-based dietary patterns																								
	2) Evaluation of nutritional profile and environmental impact																								
A2)	***Dietary patterns with dairy analogues***																								
	1) Development of the MD-based dietary patterns																								
	2) Evaluation of nutritional profile and environmental impact																								
A3)	***Comparison of dietary patterns***																								
	1) Nutrient adequacy and health impact																								
	2) Environmental impact																								
A4)	***Innovative dairy products***																								
	1) Analysis of critical issues																								
	2) Optimization of new dairy products																								
A5)	***Thesis and Paper Preparation***																								
A6)	***Dissemination of the results***																								

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Antioxidant activity of Maillard reaction products by multiresponse kinetic models

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The Maillard reaction is one of the most important reactions in foods with large implications for color, taste, flavor, and nutritional value. Moreover, during the reaction between reducing sugars and amino acids, the antioxidants activity typically increases. However, the mechanism that control such changes remains unclear. Thus, this PhD project aims to develop a multiresponse kinetic model that could predict the antioxidant formation during cooking of baked and brewed products. Through the understanding of the kinetic evolution of antioxidants in Maillard reaction, it will be possible to design new foods with the highest antioxidant functionalities.

Attività antiossidante dei prodotti della reazione di Maillard mediante modelli cinetici a risposta multipla

La reazione di Maillard è una delle reazioni più importanti negli alimenti, con grandi implicazioni per il colore, il gusto, il sapore e il valore nutrizionale. Inoltre, durante la reazione tra zuccheri riducenti e amminoacidi, l'attività antiossidante tipicamente aumenta. Tuttavia, il meccanismo che controlla tali cambiamenti rimane poco chiaro. Pertanto, questo progetto di dottorato mira a sviluppare un modello cinetico a risposta multipla che possa prevedere la formazione di antiossidanti durante la cottura di prodotti da forno e la produzione di birra. Attraverso la comprensione della cinetica di formazione di antiossidanti sarà possibile progettare nuovi alimenti con la massima funzionalità antiossidante.

1. State of the Art

Maillard reaction is a complex mix of chemical reactions that occurs between reducing sugars and amino acids or proteins. This reaction is one of the most studied in foods as it is responsible for their aroma, flavours and colour. Typical examples include the browning and flavours formation of cooked meat, backed products, soy sauces, brewing and toasting of coffee.

Although the reaction is known since 1912, when it was discovered by the French chemist Louis Camille Maillard, only in the 50s with John Hodge (USDA), that a mechanism for the chemistry of non-enzymic browning was proposed. However, since that time, the kinetic mechanism could not be completely revealed because of the concomitant influences of too many factors, such as pH, water activity, temperature, and the content and type of lipids, sugars and proteins. Such complexity is well illustrated by the thousands of known reaction products and intermediates. (Martins & Van Boekel, 2005)

Despite such complexity, the essential skeletal of a Maillard Reaction mechanism consists of the initial condensation reaction between the aldehyde or ketone group of a reducing sugar and the free amino group of amino acids. This forms Schiff base products (or glycosylamines). Such intermediates quickly undergo Amadori rearrangement to form amino-deoxy-ketoses (or ketosamine). The Amadori products degrade in pathways that depend, as said before, on several factors (pH, T, Aw, etc.). However, during the Maillard Reaction, amino groups are released, forming new sugar fragments (deoxy-osones), that may further act as oxidizer of amino groups via Strecker degradation, leading to the evolution of flavors (volatile aldehydes and ketones) and melanoidines (brown pigments). (Feng et al., 2022)

Maillard Reaction Products (MRP) are of high interest in food science not only for their organoleptic properties, but also for their bioactivities, in particular their antioxidant and antimicrobial properties.

The antioxidant properties of some compounds produced during the progress of the Maillard reaction, i.e. Amadori compounds, premelanoidins, and melanoidins, have been reported in processed foods. The antioxidative activity of MRPs in model and food systems is due to different antioxidant mechanisms, including chelation of metal ions, radical chains breaking, breakdown of hydrogen peroxide, and scavenging of reactive oxygen species. (Nooshkam et al., 2019)

Their antioxidant activity is widely studied in literature since the antioxidant properties are fundamental for food maintenance.

For example, many studies have been done on model solutions of amino-acids and mono or di-saccharides.

MRPs produced by model solutions of specific protein or non-protein amines have also been studied for their antioxidant properties. For example, someone worked on MRPs obtained from casein and glucose solution, or studied Maillard Reaction between porcine plasma proteins and sugars or on stingray non-protein nitrogenous fraction and sugar model system Maillard Reaction derived Products.

Some studies on MRPs antioxidant activity have also been conducted on more complex systems, like chitooligomer, ovalbumin and d-aldohexoses. (Feng et al., 2022)

Despite this, just a few works have been done on food matrixes.

Since MRPs can have both positive and negative bioactivities as here described, would be interesting to understand at which stage of the reaction the MRPs have a positive biological activity and when, instead, they start becoming dangerous for human health and food.

In particular, the aim of my PhD project is to develop a multiresponse kinetic model that could predict the antioxidant formation during cooking of baked and brewed products. Through the understanding of the kinetic evolution of antioxidants in Maillard reaction, it will be possible to design new foods with the highest antioxidant functionalities.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

M1- Literature review

M2- Studies on Maillard Reaction model solution: studies on kinetic of Antioxidant Compounds production during heating phase of Glucose-Glycine solution;

M3- Studies on more complex systems containing proteins and complex sugars;

M4- Studies on real food matrixes like cookie dough and beer;

M5- Period abroad probably at Wageningen University under the supervision of Professor van Boekel, working of Reaction Kinetics;

M6- Writing and Editing of PhD thesis, papers and/or poster communication.

Table 1 Gantt diagram for this PhD thesis project.

Milestones	**1st year**				**2nd year**				**3rd year**			
Terms	I	II	III	IV	I	II	III	IV	I	II	III	IV
M1 Literature review												
M2 Studies on model solutions												
M3 Studies on complex model solutions												
M4 Studies on food matrix												
M5 Period abroad												
M6 Writing												

3. References

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Strategies for developing functional food products by creating a polyphenol-phytosterols complex

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The aim of this PhD project is to develop functional foods by studying the binding capacity of specific polyphenolic classes with phytosterols (complex forms), estimating both their effects on the human organism and the bioaccessibility of the complex by developing at least an analytical validated method for analysing and monitoring the oxidation products of phytosterols.

Strategie per lo sviluppo di alimenti funzionali attraverso la formazione di un complesso polifenolo-fitosterolo

Lo scopo del presente progetto di dottorato consiste nello sviluppo di alimenti funzionali attraverso lo studio della capacità di legame tra specifiche classi di polifenoli e fitosteroli stimandone, quindi, il loro effetto sull'organismo umano e la loro bioaccessibilità attraverso lo sviluppo di almeno un metodo analitico validato per l'analisi e il monitoraggio dei prodotti di ossidazione dei fitosteroli.

1. State-of-the-Art

Plant sterols and stanols, also known as phytosterols, are bioactive compounds found in foods of plant origin belonging to the triterpene family (Trautwein *et al.*, 2018; Gachumi *et al.*, 2021). Recently, they have received particular attention due to their ability to reduce the intestinal absorption of cholesterol, thereby reducing the occurrence of both cardiovascular diseases and colon cancer (Cuevas-Tena, Alegría and Lagarda, 2018). Since phytosterols are structurally similar to cholesterol, when consumed, they compete with latter absorption from the gastrointestinal track reducing low-density lipoprotein (LDL-C) levels by competing with cholesterol in the enterocytes (Gachumi *et al.*, 2021). Phytosterols intake from natural sources ranges between 200 and 400 mg/day with habitual diets and up to 600 mg/day under vegan- or vegetarian-type diets. However, that intake is not sufficient to achieve the cholesterol-lowering effect; in fact, the hiring of 2 to 3 g of phytosterols per day is essential to significantly reduce the total cholesterol and LDL-C levels in the blood by 10 % (Cabral and Klein, 2017; Trautwein *et al.*, 2018). Due to the well-established cholesterol lowering effect of phytosterols, commercial food products such as margarine and dairy products, with added phytosterols either in their free or esterified form, have been marketed for decades (Lin, Knol and Trautwein, 2016). However, the phytosterols added into food products is challenging because of their water insolubility and susceptibility to oxidation. The current techniques used for integrating phytosterols into foods are various (e.g. microemulsion, micelles or microcapsule) but critical points are highlighted, such as low stability, high cost reflected in the final price of the product or low protection against oxidation (Tolve *et al.*, 2020; Cercaci *et al.*, 2007). Consequently, the high concentration of phytosterols in foods could lead to a high presence of oxidised phytosterols; in fact, as it has been reported for cholesterol, also phytosterols are prone to be oxidized generating several isomers, which are collectively known as Phytosterol Oxidation Products (POPs) (O'Callaghan, McCarthy and O'Brien, 2014; Tolve *et al.*, 2020). The actual literature is conflicting for what concern the effects of POPs on human health. Several studies report an association between the intake of POPs and the onset of various diseases and negative health implications but, at the same time, anti-diabetic and anti-cancer activities are also highligthed (Scholz, Guth, *et al.*, 2015; Wang and Lu, 2018; Gachumi *et al.*, 2021). In addition, another point to be considered is the bioavailability and bioaccessibility of POPs. Again, the literature is poor about that; Grandgirard et al., (1999) reported an intestinal absorption rate of the 7-keto- and epoxy-isomers of sitosterol and campesterol about 1.4 % and 4.7 %, respectively, which were higher than sitosterol (1.2 %) and lower than campesterol (7.0 %). Others, reported an absorption of (oxy)-campesterols (15.9%) and (oxy)-β-sitosterols (9.12%) higher than campesterol (5.47%) and β-sitosterol (2.16%) (Wang and Lu, 2018; Grandgirard *et al.*, 1999). Thus, it is required to limit the oxidation of phytosterols and to properly monitor them, in order to limit the possible negative effects on human health given the limited knowledge. One solution to counteract POPs formation is the use of antioxidant compounds, such as polyphenols, tannins or tocopherols (Gachumi *et al.*, 2021), potentially exploiting the ability of them (such as gallic acid) to bind sterol structures by modifying their solubility (Zeng *et al.*, 2020). Finally, since commercial reference standards of oxidised phytosterols as well as a validated analytical method are not available, the accurate determination of POPs is often complex. The oxidation products of phytosterols are usually present in trace amounts and purification techniques may result in sample loss or degradation of compounds or also formation of artefacts. These analytical obstacles result not only in a limited quantitative dataset on the occurrence and formation of phytosterol oxidation products in selected enriched foods, but also in the comparison of existing data (Scholz, Wocheslander, *et al.*, 2015).

2. PhD Thesis Objectives and Milestones

The PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) **Development of the analytical methods for POPs determination.** Formation of standard mixes of the oxidized phytosterols and development of, at least, one method of analysing these products and validated.

A2) **Study of the interaction between polyphenols and phytosterols.** Selected polyphenols will be tested and their complexing activity with different phytosterols (β-sitosterol, campesterol, stigmasterol, brassicasterol among the most common) will be studied in a model system at different temperatures and pH.

A3) **Study of the selected phytocomplex in food products** based on the results obtained from the model systems. Therefore, foods tested with the complex will be monitored during their shelf-life.

A4) **Bioaccessibility studies** through the official product protocol; different analysis will be performed on the samples before the digestive process and at the end of each phase, in order to monitor the fate of the complex.

A5) **Writing and Editing** PhD thesis, scientific papers and oral and/or poster communications.

Table 1 Gantt diagram for this PhD thesis project.

		MONTHS																																		
	ACTIVITY	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
A1)	**Development of the analytical methods for POPs determination**																																				
	1) Development of pure phytosterol oxidation products																																				
	2) Validation of an analytical method for POPs determination																																				
A2)	**Study of the interaction between polyphenols-phytosterols**																																				
	1) Selection of polyphenols able to bind phytosterol																																				
	2) Assessment of the complex stabilty in model systems																																				
A3)	**Study of the selected phytocomplex in food products**																																				
	1) Test in different food matrix																																				
	2) Evaluation of phytocomplex shelf-life																																				
A4)	**Bioaccessibility studies**																																				
A5)	**Thesis and paper preparation**																																				

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Biobased approaches at modulating the interaction between legume biopolymers and bioactives: a perspective for the production of gluten-free baked goods

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This Ph.D. thesis project aims to evaluate the effect, through a biomolecular approach, of innovative technological processes on bean seeds (*Vigna unguiculata*), for the production of bakery products with and without gluten with higher health value. In particular, the application of the sprouting process, a reliable, economic, and sustainable process, was evaluated in order to improve the technological, nutritional, and biomolecular properties of beans. Once the best germination conditions have been determined, the development of bakery products (bread) with sprouted and non-sprouted bean flours (control), and the subsequent technological, chemical and biomolecular characterization will be conducted.

Approcci biomolecolari per lo studio delle interazioni tra biopolimeri e bioattivi nei legumi: una prospettiva futura per la produzione di prodotti da forno gluten-free

Lo scopo del progetto della tesi di dottorato è valutare l'effetto, attraverso un approccio biomolecolare, di processi tecnologici innovativi sui semi di fagiolo (*Vigna unguiculata*), per la produzione di prodotti da forno con e senza glutine a maggiore valore salutistico. In particolare, al fine di migliorare le proprietà tecnologiche, nutrizionali e biomolecolari dei fagioli si è valutato il processo di germinazione, un processo affidabile, economico e sostenibile. Determinate le migliori condizioni di germinazione, si procederà allo sviluppo dei prodotti da forno (pane) con le farine di fagioli germinati e non (controllo) e della successiva caratterizzazione tecnologica, chimica e biomolecolare.

1. State-of-the-Art

Legumes are gluten-free plants with good climate resilience, low production cost, and a well-balanced nutritional profile made of a good amount of proteins, dietary fiber, resistant starch, vitamins, minerals, and unsaturated fatty acids (Gomes Los et al., 2018). Among legumes, Cowpea beans (*Vigna unguiculata*) are an important and versatile crop with high protein content and technological features (de Souza Rocha et al., 2014). Cowpea proteins include globulins (44-55%), albumins (20-35%), glutelins (~22%) and prolamins (1-3%). The main cowpea storage protein is vicilin, a 7S globulin characterized as a trimer of 150 to 170 kDa formed by similar subunits of 40-70 kDa with no disulfide exchange. Another important cowpea protein is lectin, with a molecular weight of approximately 55 kDa. Unlucky, beans are characterized by a low protein digestibility and nutrient bioavailability due to trypsin inhibitors such as lectin (de Souza Rocha T. et al., 2014), and phytates, which require the application of technological and biological processes to increase their nutritional value.
During the first twelve months of this Ph.D. project, we investigated the effect of sprouting on cowpea seeds by assessing the residual levels of phytates and trypsin inhibitors, as well as the extent of protein hydrolysis in germinated seeds. Sprouting was performed on previously soaked seeds (weight ratio 1:2, 8 hours). After keeping the soaked seeds at room temperature and 90% relative humidity for 24, 48, and 72 hours, samples of germinated seeds were collected. Phytates content and trypsin inhibitors in aqueous extracts from individual meals were determined using a commercial kit and the EN ISO 14902 standard, respectively, after drying and grinding. SDS-PAGE and the OPA assay for free amino groups were used to assess protein hydrolysis. The results show that sprouting decreased trypsin inhibitor activity and phytates content while increasing protein hydrolyses in a time-dependent manner. At 48 hours of germination, the maximum effect had already been reached. Although more research is needed to better define the effect of germination on protein levels, these preliminary findings highlight the importance of this bioprocess for increasing the nutritional value of legumes for use in the formulation of bakery products with a higher health impact.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this Ph.D. thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) **Literature research**: research, reading, and comprehension of the most recent publications related to the PhD project.

A2) **Application and characterization of innovative technological processes:** application of sprouting condition in Cowpea bean seeds (A2.1) and assessment of the best technological conditions by biochemical assay (SDS-PAGE, OPA assay, phytates content, and trypsin inhibitory activity) (A2.2).

A3) **Product development:** development of bakery products (bread) with sprouted and non-sprouted bean flours (control) (A3.1).

A4) **Characterization of prototype breads**: The prototype breads will be characterized from a technological (A4.1) and biochemical (A4.2) point of view. Subsequently, they will be subjected to in vitro digestion to evaluate the bioaccessibility of nutrients and bioactive compounds (A4.3).

A5) **Evaluation of antioxidant and anti-inflammatory properties of digestive selected breads in cell culture:** the antioxidant (A5.1) and anti-inflammatory (A5.2) properties of the digested selected breads will be evaluated in cultured intestinal cells.

A6) **Data dissemination** of the obtained results in scientific papers and conferences communications **and Ph.D. thesis writing.**

Table 1 Gantt diagram of the Ph.D. thesis project.

ACTIVITY			DURATION (months)																																		
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
A1)	***Literature research***																																					
A2)	***Application and characterization of innovative technological processes***																																					
	1)	Application of innovative technological processes																																				
	2)	Biochemical characterization of innovative technological processes																																				
A3)	***Product development***																																					
A4)	***Characterization of prototype breads***																																					
	1)	Evaluation of technological properties																																				
	2)	Biochemical characterization																																				
	3)	In vitro bio-accessibility																																				
A5)	***evaluation of antioxidant and anti-inflammatory properties in cell culture***																																					
	1)	Evaluation of anti-oxidative properties																																				
	2)	Evaluation of anti-inflammatory properties																																				
A6)	***Data dissemination and PhD thesis writing***																																					

3. Selected References

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Assessment of the stability and efficacy of a newly developed probiotic blend in the context of IBS through a pilot multicentre study

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It is recently emerged the central role of the gut microbiota in irritable bowel syndrome (IBS) and the beneficial use of several probiotic products. The main objective of this PhD project is to develop a bacterial blend based on selected and *in vitro* characterized SOFAR's strains. The effects of this multi-strains supplement will be assessed *in vivo* through a pilot multicentre study with IBS patients. Since an important technological limitation in clinical trials is the actual amount of microbial cells delivered, the analysis of formulation's stability during the shelf-life and a microbiological characterization by flow cytometer will be also performed.

Valutazione della stabilità e dell'efficacia di un nuovo prodotto probiotico multi-ceppo nel contesto dell'IBS tramite uno studio pilota multicentrico

Recentemente è emerso il ruolo centrale del microbiota intestinale nel contesto della sindrome dell'intestino irritabile (IBS) e dei benefici derivanti dall'utilizzo di prodotti probiotici. L'obiettivo principale di questo progetto di dottorato è lo sviluppo di una nuova miscela composta da ceppi SOFAR selezionati e caratterizzati *in vitro*. Gli effetti di tale integratore multi-ceppo saranno valutati *in vivo* mediante l'allestimento di uno studio pilota multicentrico in pazienti IBS. Poiché un importante limite tecnologico in tali studi clinici riguarda il quantitativo di cellule microbiche somministrate, verrà inoltre condotta un'analisi di stabilità della miscela durante la *shelf-life* e una caratterizzazione microbiologica tramite citofluorimetro.

1. State-of-the-Art

Irritable bowel syndrome (IBS) is a common enigmatic functional gastrointestinal disorder characterized by heterogeneous symptoms – such as abdominal pain, bloating, diarrhoea, constipation, nausea – and parallels with other somatic comorbidities and psychiatric conditions like anxiety and depression. These features may explain why it is also associated with a decrease in patients' quality of life (QoL). This multi-factorial condition is diagnosed through the Rome IV criteria and can be divided into 4 different IBS subgroups, which are patients with diarrhoea predominance (IBS-D), predominant constipation (IBS-C), mixed or alternating bowel habits (IBS-M), or un-subtyped IBS (IBS-U).

Due to the global prevalence of around 10%, IBS causes such a significant economic burden for health systems that there is an increasing need to develop protocols to manage this issue. What has recently emerged is the pathogenetic contribution of the intestinal microbiota in IBS, further supported by several reported beneficial effects of the use of probiotics in improving the symptoms of this syndrome. Systematic reviews of the literature and meta-analysis indicate that probiotics have significant therapeutic effect on IBS manifestations, though their efficacy resulted to be heterogeneous according to the IBS subgroup and the kind of product considered, and depending on the strain administered (Li *et al.*, 2020).

Moreover, the understanding about the mechanism of action by which probiotics exert their beneficial actions in humans is limited because these aspects were evaluated only in a small number of clinical trials. For instance, the ability of *Bifidobacterium bifidum* MIMBb75 to well adhere to intestinal cells may play a pivotal role in increasing the intestinal barrier, proving to effectively alleviate global IBS symptoms with a concurrent improvement of QoL (Guglielmetti *et al.*, 2011). On the other hand, in a study on healthy volunteers, the intake of *Lacticaseibacillus paracasei* CNCM I-1572 significantly modulated faecal Eubacteriales (formerly Clostridiales) bacteria and butyrate levels, potentially conferring a health benefit to the host. Coherently, as the same strain proved to be able to modulate both gut microbiota structure and function and to reduce immune activation in IBS, this immunostimulation may be the consequence of perturbation of gut microbiota (Cremon *et al.*, 2018).

In this context it has emerged that the administration of suitable dose combined of lactobacilli and bifidobacteria can have prepotent effect compared with single species, suggesting the existence of a possible complementary or synergistic interaction between different probiotic strains which improves their effectiveness (Liang *et al.*, 2019). Indeed, a product consisted of a mixture of 5 strains of lactic acid bacteria and bifidobacterial was texted in a prospective, double-blind, randomized placebo-controlled study conducted by Francavilla et al. (2019) to assess its capability to decrease the severity of IBS-type symptoms in patients with Celiac Disease (CD) despite a gluten-free diet (GFD). The authors demonstrated that this probiotic combination is superior to placebo to alleviate the IBS symptomatology, speculating that part of this effect might be secondary to a positive modification of gut microbiota shown by the steady and persistent increase of the bifidobacteria count in faecal samples of CD patients, detectable 6 weeks after discontinuation of probiotics.

These findings may explain why, although it has not been scientifically demonstrated if a combination of strains is more efficacious than a single strain, the number of commercially available multispecies probiotic formulation is increasing (Taverniti *et al.*, 2019). Thus, this project aims to develop a microbiological blend consisting of some SOFAR's proprietary strains – each one chosen on the basis of the clinical studies included in the reported literature – which could potentially aptly perform in IBS context.

2. PhD Thesis Objectives and Milestones

According to the abovementioned objective, this PhD project can be divided into 3 interconnected Work Packages (WPs), which include the activities detailed below as Tasks, as shown into the Gantt diagram reported in Table 1:

WP1) **Definition and microbiological characterization of the probiotic blend**, both by an *in vitro* characterization of strains' probiotic properties from the functional point of view (Task 1.1) and through strains' genome sequencing and analysis (Task 1.2), in order to identify one or more unique regions to develop primers for the preparation of a strain-specific qPCR protocol to be used in a clinical study.

WP2) **Stability and microbiological characterization of the probiotic blend**, since an important limitation in clinical trials with probiotic products consists in the actual amount of microbial cells delivered. The stability during the shelf-life of the multi-strains supplement will be evaluated at defined time-points by viable count (Task 2.1), and the probiotic formulation will be also characterized by cytofluorimetric analysis (Task 2.2).

WP3) ***In vivo* clinical trial** to assess the effect of the probiotic multi-strains supplement in subjects with IBS. Taxonomic profiling of bacterial DNA and other more specific analysis in both faecal and blood samples (i.e. SCFAs and some biomarkers respectively) will be conducted (Task 3.1), and the final results of these endpoints will be elaborated (Task 3.2).

These WPs will end with the **Writing and Editing** of the PhD thesis and scientific papers.

Table 1 Gantt diagram for this PhD thesis project.

		Months																						
	Activities	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**
WP1)	***Definition of the Probiotic Blend***	■	■	■	■	■	■	■	■	■															
Task 1.1.	*In vitro* Strains' Probiotic Properties	▒	▒	▒	▒	▒	▒																		
Task 1.2.	Genome Sequencing and Analysis						▒	▒	▒	▒															
WP2)	***Stability of the Probiotic Blend***									■	■	■	■	■	■	■	■								
Task 2.1.	Stability by Viable Count									▒	▒	▒	▒	▒	▒	▒	▒								
Task 2.2.	Characterization by Flow Cytometry											▒	▒	▒	▒	▒	▒								
WP3)	**In vivo *Clinical Trial***									■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
Task 3.1.	Endpoints Evaluation									▒	▒	▒	▒	▒	▒	▒	▒	▒	▒	▒	▒				
Task 3.2.	Elaboration of results																	▒	▒	▒	▒	▒	▒	▒	
	Thesis and Paper Preparation																					■	■	■	■

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Valorisation of artichoke by-product as ingredients for functional dairy foods

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Tutor: Prof. Alessandra Del Caro, Co-tutor: Prof. Pietro Paolo Urgeghe

This PhD thesis research project aims to study the valorization of artichoke by-products (leafs, stems and bracts) as ingredients for the production of functional dairy foods. This research includes the chemical-physical characterization of freeze-dried artichoke leafs, stems and bracts. The goal is the production and optimization of a fortified dairy food like the yogurt, with potential benefits to human health, evaluation of its rheological characteristics, sensory profile, fiber content and antioxidant activity

Valorizzazione dei sottoprodotti del carciofo come ingredienti per la produzione di alimenti lattiero caseari funzionali

Questo progetto di tesi di dottorato si propone di studiare la valorizzazione dei sottoprodotti del carciofo (foglie, steli e brattee) come ingredienti per la produzione di alimenti lattiero-caseari funzionali. La ricerca comprende la caratterizzazione delle foglie, degli steli e delle brattee attraverso analisi chimico-fisiche. L'obiettivo finale è la produzione e ottimizzazione di un alimento lattiero-caseario fortificato, lo yogurt, con potenziali benefici per la salute umana, attraverso la valutazione delle sue caratteristiche reologiche, del profilo sensoriale, del suo contenuto in fibre e delle sue proprietà antiossidanti.

1. State-of-the-Art

The artichoke (*Cynara cardunculus* subsp. *scolymus*) is a natural source of minerals, fiber, inulin, and polyphenols (Rizzo *et al*., 2019). The artichoke crop in the world contributes significantly to the Mediterranean agricultural economy, where more than 60% of the world's production of this vegetable and generates an high quantity of by-products. In 2017, 1.505.328 t of artichoke where harvested worldwide. Overall, artichoke by-products from the canning industry (leaves, outer bracts and stems) represent a high amount of waste material, about 80% of the plant's total biomass, generating 1.204.262 t/year of by-products.

In recent years there has been a growing demand for the use of waste products both to reduce environmental pollution, and to produce fortified foods that are beneficial for human health.

Many studies have focused on the use artichoke waste as an alternative feed for dairy production with a potentially improved nutritional profile (Monllor *et al*., 2020). Dairy is the first Italian food sector and represents more 12% of the total turnover of national food. This sector shows a strong innovative capacity, in fact, most of the recent research is based on the direct addition of agricultural wastes into dairy products (Soleimanian et al., 2021; Iriondo-DeHond, et al., 2018).

Furthermore, proteases represent the most important and commercialized class of enzymes in the world due to their coagulating capacity in milk. The coagulation of milk by enzymatic action is an essential step in the production of dairy products. The food industry can search for these enzymes not only from animal and bacterial sources but also from plant sources, such as the artichoke, by-products.

Recent studies have evaluated the feasibility of fortifying Greek yogurt with a new ingredient consisting of food waste powder of various plant origin. The yogurts were analyzed using rheological and chemical-physical analysis, including whey release, water retention capacity, firmness, flow and viscoelastic properties, protein content, available lysine, and antioxidant activity (Osorio-Arias *et al.*, 2020).

Several studies have also highlighted the ability of inulin, present in artichoke by-products, to increase the consistency and compactness of the yogurt without affecting the gelling process (Wang *et al.*, 2019).

Thus, this PhD thesis project will be based on the use of artichoke waste to fortify a dairy product, the yogurt. At present the preliminary chemical characterization and the study of the proteolytic activity of leaves, stems, and bracts, (Silva, *et al.*, 2021) has been performed. Moreover, the fiber content of three artichoke by-product has been calculated (Total Dietary Fiber Assay Kit, Megazyme®) and the phenolic content and antioxidant activity is going to be obtained.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) **Bibliographic research**

A2) **Characterization of freeze-dried artichoke (leaves, stems, bracts) by physicochemical analysis**: total protein, fiber content, proteolytic activity, polyphenol oxidase activity, phenolic content characterization.

A3) **Development of a yogurt from whole ovine milk fortified with artichoke by-products:** choice of the fortification level and optimization of the process variables.

A4) **Characterization of the fortified yogurts:** chemical-physical and sensory analysis will be performed both for validating optimisation steps and to characterise these functional foods. The analyses will include the evaluation of syneresis and water holding capacity; proteolysis; rheological and textural properties, color evaluation and sensory characteristics; volatile compounds analysis

A5) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

Table 1 Gantt diagram for this PhD thesis project.

Activity \ Months		2	4	6	8	10	12	14	16	18	20	22	24	25	26	27	28	29	30	31	32	33	34	35	36
A1)	***Bibliographic research***																								
A2)	***Characterization of freeze-dried artichoke***																								
A3)	***Development of a yogurt from whole ovine milk fortified with artichoke by-products***																								
A4)	***Characterization of the fortified yogurts***																								
A5)	***Thesis and Paper Preparation***																								

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Eco-design tool development for sustainability optimization in food production systems and food waste reduction

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This PhD research project aims at developing an eco-design tool based on Life Cycle Assessment methodology to allow food systems have a reliable resource when approaching to sustainability. The project wants to identify the most appropriate model to evaluate accurately the eco profile of selected food productions and provide a user friendly and strategic decision-making tool for businesses.

Sviluppo di strumenti di eco-design per l'ottimizzazione della sostenibilità nei sistemi di produzione alimentare e la riduzione degli sprechi alimentari

Questo progetto di ricerca di dottorato mira a sviluppare uno strumento di eco-design basato sulla metodologia di valutazione del ciclo di vita per consentire ai sistemi alimentari di disporre di una risorsa affidabile in ambito di sostenibilità. Il progetto vuole identificare il modello più appropriato per valutare con precisione il profilo ecologico di produzioni alimentari selezionate e fornire uno strumento decisionale di facile utilizzo e strategico per le aziende.

1. State-of-the-Art

The food supply chain is one of the main contributors to several pressing environmental problems such as climate change, eutrophication, and biodiversity loss (EEA, 2018), aiming to a sustainable food system is therefore a priority.
To reach a sustainable food system, is fundamental the cooperation of all the actors involved in the supply chain, from the cultivation step, through production and transformation ones also including the use and consumption phase, and not neglecting the waste management processes.
To monitor and then reduce environmental impact along the food supply chain it is fundamental to use recognized and validated methodologies. In this context, the Life Cycle Assessment (LCA) can provide the response representing a scientific method internationally recognised (ISO, 2021a and ISO, 2021b) that allows to assess environmental impacts through a rigid methodology and can help to design and increase sustainability in the food supply chain (Colley et al., 2020).
The LCA methodology is identified as a means of achieve a more sustainable food system, but at the same time, significant issues concerning its use emerge.
On the one hand, food supply chain stakeholders argue that the rules, the models, the descriptions of the inputs required for a LCA, and the interpretation of the results are too difficult to define, identify and understand. As a result, they only hire professional analysts to assist them in understanding the system and applying LCAs to identify all potential sources of uncertainty in the model's output and to use the LCA model effectively and responsibly in any decision-making process (Cucurachi et al., 2022). On the other hand, LCA tools are increasingly being used to provide decision makers with quantitative evaluations of the decisions they make throughout the lifecycle of their products, systems, or services. However, the current generation of tools or is (i) aimed for specialists or users with extensive experience in industrial and environmental operations (Borrion et al., 2019) or (ii) represent well-publicized open-source solutions lacking scientific foundations that can only undermine the system and do not serve as a support tool for environmental management.
The preliminary results of an ongoing bibliographic and commercial review project revealed that there are currently more than 100 tools available that can be used in the food chain, with 35% of them completely free, 43% require registration to access the tool, and the remaining 20% require a subscription. Solely 91 percent of these tools analyse only farm or field activities (with only 26 percent analysing the meat and dairy industry), while only 9 percent present a solution that analyses the entire chain from farm to consumer. More than 70% of the tools only provide results related to the carbon footprint, whereas less than 30% of the tools provide a comprehensive and complete study of the system's environmental impacts.
It is also evident that there is a substantial heterogeneity among the LCA tools proposed by the market in fact, even if they offer a solution to monitor agricultural and field operations, they do not respect common LCA methodologies and some of these are obsolete or do not offer a complete picture of the resulting environmental impacts from the activities analysed. To interface with the largest number of operators in the agri-food chain, it is essential to work in the direction of simplifying the LCA analysis, moving from methodologies that may seem complex to tools that reduce the possibility of making mistakes and guide the user throughout the process. of analysis. however, it is essential not to stop only at field / breeding operations, even if these lead to the evaluation

of the environmental impacts of most of the food raw materials, one should go beyond what are the current boundaries analysing the processes of transformation, packaging and quantifying the potential food waste along the supply chain to create a new generation of LCA tools.
This PhD program aims at offering a concrete solution to the problems listed above, to create a set of tools that are based on scientific criteria and are reliable and recognized throughout the supply chain, capable to be used by the greater number of operators in the food chain starting from the grower / breeder up to the distributor.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) **Critical analysis of LCA tools to identify strengths and weaknesses** in terms of (i) application field, (ii) food systems evaluated, and (iii) methodological approaches underlying the different tools (A 1.1). Moreover, the same analysis will help in the identification of food production chains whit high necessity to monitor the system.
Milestones: the ranking of the tools and the identification of two food chains to be analysed.

A2) **Development of eco-design tool for two production chains.** From the results of activities A1 strengths and weaknesses points of the different tools identified and analysed will be considered. Guidelines and recognised procedures will be used to give to the tool developed a robust structure for both mathematical and statistical aspects. The activity will require different steps: execution of the full LCA study of the system identified (A 2.1), simplification of the assessment procedure (A 2.2) and the creation of a tool (A 2.3).
Milestone: the final version of the (beta version) eco-design tool.

A3) **Validation of the tool developed** via statistical analysis to obtain reliable and recognised tool. In this activity the tool will be stressed using different data to check the output. Moreover, the tool can be statistically compared with the reference LCA study to identify its compliance, variability factors, and criticisms. Finally, once the tool has been validated, it can be compared with some of the tools identified and analysed (A1) to quantify the variability range among the available tools.
Milestone: the validation of the created tool.

A4) **PhD project management activities.** PhD thesis, scientific papers, and oral and/or poster communications preparation.

Table 1. *Gantt diagram*

	Activities	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1	**Critical analysis of LCA tools to identify strengths and weaknesses**																								
	1. Identification of methodological strengths and weaknesses of the different tools																								
A2	**Development of eco-design tool for two production chains**																								
	1. Execution of the full LCA study																								
	2. Simplification of the assessment procedure																								
	3. Eco-design tool development																								
A3	**Validation of the tool developed via statistical approach**																								
	1. Test using different data of different origin																								
	2. Comparison with with full LCA studies and other tools																								
A4	**PhD project management activities**																								

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Bio-sustainable technologies to produce raw materials and food products with improved physico-chemical, nutritional and safety properties

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The objective of this PhD project is the application of bio-sustainable treatments, such as germination and fermentation, to improve the quality of cereals and legumes. The nutritional, physico-chemical and safety properties of cereals and legumes could be improved by the enzymatic modifications brought about by these treatments. This would favour their application in the production of leavened breads of high nutritional and functional quality.

Trattamenti biosostenibili per produrre prodotti lavorati e semilavorati dal migliorato valore qualitativo

Lo scopo di questo progetto di dottorato è l'applicazione di trattamenti bio-sostenibili, come la germinazione e la fermentazione, per migliorare la qualità di cereali e legumi. Le proprietà nutrizionali, chimico-fisiche e di sicurezza di cereali e legumi potrebbero essere migliorate grazie alle modifiche enzimatiche apportate da questi trattamenti. Ciò favorirebbe la loro applicazione nella produzione di pani lievitati arricchiti dall'elevata qualità nutrizionale e funzionale.

1. State-of-the-Art

Sprouting and fermentation treatments can potentially improve the nutritional and functional profile of cereals and legumes (Nkhata et al., 2018). They are treatments well perceived by consumers demanding healthy and bio-processed foods (Diez-Ozaeta and Astiazaran, 2022; Lemmens et al., 2019). Indeed, the trend of eating foods containing sprouted and fermented cereals has increased in recent years (Olaerts and Courtin, 2018). Worldwide, cereals and legumes are essential dietary ingredients and important sources of macronutrients, micronutrients, phytochemicals, as well as anti-nutritional factors (ANFs) (Nkhata et al., 2018). Fermentation and sprouting are biochemical processes mediated by exogenous microbial and endogenous enzymes, respectively. The changes brought about by these treatments usually involve the breakdown of structural matrices and the hydrolysis of some of their components. Sprouting and fermentation make nutrients and phytochemicals freer and more accessible to digestive enzymes. As a consequence, starch and proteins improve their digestibility, the minerals and bioactive compounds are released and ANFs are reduced (Lemmens et al., 2019). Moreover, sprouting and fermentation can also increase sensory quality and, in the case of fermentation, ensure safety and extend the shelf life of food products (Nkhata et al., 2018).

The use of sprouted and/or fermented cereals and legumes in leavened bread formulations could be an effective strategy to increase the nutritional value of a worldwide consumed food. However, an excessive enzymatic activity after sprouting has sometimes been found to negatively affect the physico-chemical properties of the doughs and the resultant baking performance. This make the use of sprouted grains for bakery products more challenging (Olaerts and Courtin, 2018). On the other hand, fermentation has sometimes shown positive effects on the technological performances of fermented grains, showing an improvement in flours baking capacity, especially if the fermented flours were added as sourdoughs (Garrido-Galand et al., 2021).

Nevertheless, the technological quality of flours should not be limited to the sensory aspect of the fresh product, but also to its quality over time. In fact, during shelf-life, the quality losses due to bread staling are the cause of considerable product waste worldwide. The major contributors to bread staling are amylopectin retrogradation, starch-gluten interactions and water redistribution at different structural levels within bread phases and bread polymers (Fadda et al., 2014). Sprouting and fermentation could affect the bread staling process. In fact, some studies have reported a positive effect of the use of sourdough starters and the activities of α-amylase and other enzymes, as they can modify the structure of starch and proteins and thus influence the rate of amylopectin retrogradation and water redistribution. In addition, hydrocolloids of microbial origin could stabilise the dough, influence changes in moisture content during storage and crust structure (Fadda et al., 2014). However, detailed studies on the shelf-life of enriched breads and the effect of fermentation and germination on bread ripening are not available, although assessing bread staling is a fundamental issue when high-quality products are pursued.

Therefore, the aim of this PhD project is to study the quality of breads enriched with fermented and sprouted sorghum (*Sorghum bicolor* (L.) Moench) and chickpea (*Cicer arietinum* L.) flours, as well as the staling mechanisms characterizing their products. The applicability of sprouted and fermented sorghum and chickpea flours in bread formulation will be evaluated by physico-chemical, structural, rheological, and thermal analyses of flours, doughs, and breads, in order to define the best conditions for producing breads with higher nutritional and functional properties.

2. PhD Thesis Objectives and Milestones

According to the objectives mentioned above, this PhD project can be divided into different activities as shown in the Gantt chart in Table 1:

A1) **Cereals and legumes fermentation.** Fermentation of cereals/legumes by highly functional LAB strains (high proteolytic activity, EPS production, aromas production) (A1.1) and characterization of the functional properties of the fermented flours (A1.2).

A2) **Development of high-quality bread enriched with fermented cereals/legumes.** Assessment of the best way to add fermented flours to the bread formulation (e.g., dry flour, sourdough) (A2.1) and evaluation of the effect of fermented grains on bread staling (7 days of storage) (A2.2).

A3) **Cereals and legumes sprouting**. Evaluation of different sprouting conditions on the functional (A3.1) and nutritional (A3.2) properties of legumes/cereals flours.

A4) **Development of high-quality bread enriched with sprouted flours.** Assessment of the effect of the percentage of addition (10-20-30%) of sprouted grains on bread quality and staling (7 days of storage) (A4.1) and evaluation of the effect of flours different particle size on bread sensory quality (A4.2).

A5) **Writing and Editing** of the PhD thesis, and publication of scientific papers.

Table 1 Gantt chart about this PhD project.

Activity	**Months**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**
A1)	*Cereals and legumes fermentation*																								
	1) LAB strains fermentation																								
	2) Functional properties																								
A2)	*Development of high-quality bread*																								
	1) Different addition methods																								
	2) Bread staling study																								
A3)	*Cereal and legumes sprouting*																								
	1) Nutritional properties																								
	2) Functional properties																								
A4)	*Development of high-quality bread*																								
	1) Percentage of addition																								
	2) Effect of flour particle size																								
A5)	*Thesis and papers preparation*																								

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Non-conventional Inactivated Yeasts and Tannins: evaluation of their impact on the chemical, physical and sensorial characteristics of wines

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This PhD thesis research project is aimed at characterizing and evaluating non-conventional inactivated yeasts and tannins, as novel biotechnological tools for the production of low chemical input yet stable wines.

Lieviti inattivati non-convenzionali e tannini alternativi: valutazione del loro impatto sulle caratteristiche chimico-fisiche e sensoriali dei vini

Questo progetto di tesi di dottorato mira a caratterizzare e valutare lieviti non convenzionali inattivati e tannini alternativi come strumenti innovativi per la produzione di vini a basso input chimico ma stabili.

1. State-of-the-Art

Microbiological and physical-chemical stability of wine is normally obtained through enological processing aids and additives. Sulfur dioxide, due to its antioxidant and antimicrobial properties, is the most commonly utilized additive, and it is added at different stages of the production process. However, because its well documented toxic effect on sensitive individuals and the greater attention of consumers towards minimally processed wine and "natural "additives, its replacement with harmless compounds is of great interest for the wine industry.

An oenological practice that allows to protect wine from oxidation and to improve its stability is the aging on lees. However, permanence on yeast lees is time consuming and may be risky, since it may facilitate the production of sulfur compounds and wine spoilage. Thus, the utilization of inactivated dry yeast (IDY), that mimic the effect of yeast lees, seems therefore much more convenient. Indeed, IDY are characterized by cell components, such as glycoproteins, that may positively impact on a range of wine features such as the protein and tartaric stability, reduction of astringency and bitterness, increase in viscosity and stability of the foam of sparkling wines. Other components are glutathione and small sulfur-cointaining compounds able to protect wine from oxidation. While all that has been clearly demonstrated for yeast derivatives obtained from *Saccharomyces cerevisiae*, much less is known on the inactive non-*Saccharomyces* yeasts and their utilization in winemaking. On the other hand, non-*Saccharomyces* yeasts are currently proposed as biotechnological tools for the achievement of specific oenological objectives and commercialized in pure or mixed cultures with *S. cerevisiae* due to their biocontrol activity in pre-fermentative stages and during wine conservation, the release of polysaccharides able to protect wine from protein haze and their general impact on wine quality.

Besides IDY, tannins represent other interesting and "greener" enological coadjuvants, able to promote wine stability mainly because of their antioxidant properties. In this context, tannins extracted from pomegranate peels have been recently shown to have interesting antioxidant properties. However, the only types of tannin allowed in oenology are at the moment those extracted from grape skins, grape seeds, chestnut and oak wood, quebracho and galls. Tannins obtained from alternative botanical sources such as fruit/agri-food waste are not allowed (RESOLUTION OIV-OENO 613-2019).

2. PhD Thesis Objectives and Milestones

Based on the observation above reported, with this PhD thesis project we aim to characterize and to evaluate inactivated non-*Saccharomyces* yeasts and pomegranate peels tannins on the wine stability at different stages of winemaking.

The activities can be subdivided according to the following Gantt diagram (Table 1):

A1)

1. **Selection of non-*Saccharomyces* yeasts** based on specific capacities (i.e. colloidal stability, antioxidant activity)
2. **Production of inactivated non-*Saccharomyces* yeasts**
3. **Evaluation of their effect on wine stability,** with particular attention on protein stability and antioxidant properties

A2)

1. **Extraction process optimization of tannins from pomegranate peels**
2. **Evaluation of their effect on wine stability,** with particular attention on antioxidant activity and protection of the colour;

A3) **Evaluation of the combined action of non-*Saccharomyces* yeast derivatives and pomegranate** tannins on the stability of wines at different stages of winemaking;

A4) **Optimisation of the processes** to determine the main parameters necessary for an industrial scale.

A5) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

Table 1 Gantt diagram for this PhD thesis project.

Activity \ Months		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**
A1)	***Non-conventional yeasts***																								
	1) Screening																								
	2) Optimization biomass production inactivation processes																								
	3) Test on model wine and real wine																								
A2)	***Alternative Tannins***																								
	1) Extraction process optimization																								
	2) Test on model wine and real wine																								
A3)	***Evaluation of the interaction between selected IDY and alternative tannins***																								
A4)	***Process Optimisation for industrial application***																								
A5)	***Thesis and Paper Preparation***																								

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Strategies to increase the sustainability of plant-based proteins

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The main aim of this PhD project is to develop agri-food chains for the production of plant-based proteins more sustainable compared to the products currently available on the market. The aim will be persecuted following different strategies including the selection of raw materials, the application of different agricultural practices, the optimisation of production processes and the valorisation of the generated by-products. Proteins will be mainly extracted from conventional alternative protein sources, such as soy and pea seeds, but also from non-conventional sources, such as corn germ and oil seed cakes.

Strategie per aumentare la sostenibilità delle proteine di origine vegetale

L'obiettivo principale di questo progetto di dottorato è sviluppare filiere agroalimentari per la produzione di proteine di origine vegetale più sostenibili rispetto ai prodotti attualmente disponibili sul mercato. L'obiettivo sarà perseguito seguendo diverse strategie che includono la selezione delle materie prime, l'applicazione di diverse pratiche agricole, l'ottimizzazione dei processi produttivi e la valorizzazione dei sottoprodotti generati. Le proteine saranno estratte principalmente da fonti proteiche alternative convenzionali, come soia e pisello, ma anche da fonti non convenzionali, come germe di mais e panelli di semi oleosi.

1. State-of-the-Art

In the past decade, the number of people following a vegan/vegetarian lifestyle has increased significantly. This is due to the fact that more people are becoming aware of the environmental impact associated to a diet rich in animal products.

The livestock sector's need for natural resources, such as land, water and energy, is increasing and this sector has a severe environmental impact on air, water and soil1. These impacts arise from various emissions into the environment as well as from the consumption of resources associated with production processes (Djekic I,2015 ; Lopez-Ridaura *et al*, 2009).

Even though enormously developed in the last years, the plant-based protein industry is still in its infancy and destined to continuously grow in the next decades. The United Nations have indicated that food is a key contributor to climate change and global warming, and moving more toward plant-based proteins can significantly reduce an individual's carbon footprint.

Soy protein from soybeans, historically identified in the Asian region, is considered an important food source to meet protein demand for the human body (Rizzo *et al.*, 2018). Soybean flour can contain up to more than 40% protein and protein concentrates or isolates (with typically more than 70 and 90% protein content, respectively) can be obtained through appropriate extraction processes.

Pea protein is relatively similar to soy protein in terms of its production methods but with protein quality, structure, and functionality can be highly different and peas are naturally low in fat but rich in starch, thus a defatting step is not necessary but high amounts of starchy residues are generated (Gwiazda *et al.*, 1979).

Pea flour is typically 20-30 percent protein, depending on the starting protein content of the whole peas (Tian *et al.*, 1999; Tulbek *et al.*, 2016). Pea protein concentrate can potentially be produced also just through dry milling but hardly achieving more than 60 % protein, while production of pea protein isolate (more than 90% protein content) requires a specific extraction process, similar to what done for soy proteins (Arntfield and Maskus, 2011; Pelgrom, 2015).

Sustainability of plant-based proteins still needs to be carefully assessed, especially when the production of meat analogues is considered. In fact, as mentioned above, proteins need to be extracted using conventional extraction processes that require a large use of energy, water and sometimes also chemicals. The environmental impact of such processes needs to be improved working on selection of high protein varieties, of low environmental impact agricultural practices, evaluation and implementation of innovative more eco-friendly extraction processes and a full valorisation of the fibre, fatty or starchy residues that are generated from the extraction processes.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above, the following research activities are planned:

1) Selection of different pea and soy varieties characterised by a high protein content and characterisation for overall chemical composition.
2) Selection of specific pea and/or soy varieties and evaluation of the influence of the year and/or agricultural practice (conservative agriculture versus conventional one) on protein content and quality.
3) Application of an improved conventional extraction process (with an alkaline extraction step followed by an isoelectric precipitation step) in terms of reduced use of water and chemicals and valorisation of solid and liquid residues for agricultural, feed and food applications.
4) Application of alternative extraction processes based on the use of pulsed-electric-field pre-treatments.
5) Evaluation of the influence of points 1, 2 and 4 on the extraction yield and on the technological functionality of the extracted proteins (for example gelling and emulsifying properties).
6) Evaluation of LCA for some of the protein products / processes developed.
7) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

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Shelf-life modelling as a strategic tool to validate sustainable food/packaging solutions: the case of coffee

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This PhD thesis research project is aimed at creating a shelf-life predictive model that can supply the necessity of finding the right trade-off between the environmental sustainability and the physical-technical performances of new and sustainable packaging materials for a specific food product. Shelf-life models based on gas permeability (O_2/CO_2) and water vapour transmission through flexible packaging will be developed and validated with coffee powder as a case study. The relationships between the sensitivity of coffee to gas and vapour, the packaging characteristics and the environmental conditions will be examined to provide useful insights in eco-design process of sustainable packaging solutions. The ultimate purpose is to implement shelf-life estimation in LCA studies, thus including both direct (production, processing, and end-of-life) and indirect (food loss and waste) packaging environmental impacts. In this way, a scientifically validated decision-making tool will be made available to food companies that are facing the green revolution and the ecological transition.

La modellazione della shelf-life come strumento strategico per validare nuove soluzioni di packaging sostenibili: il caso del caffè

Questo progetto di ricerca di tesi di dottorato mira a creare un modello predittivo di shelf-life in grado di trovare il giusto compromesso tra la sostenibilità ambientale e le prestazioni fisiche di materiali di imballaggio nuovi e sostenibili per un prodotto alimentare specifico. I modelli di shelf-life basati sulla permeabilità ai gas (O_2/CO_2) e sulla trasmissione del vapore acqueo attraverso imballaggi flessibili saranno sviluppati e convalidati usando caffè in polvere come caso studio. Verranno esaminate le relazioni tra la sensibilità del caffè a gas e vapori, le caratteristiche dell'imballaggio e le condizioni ambientali per fornire utili approfondimenti nel processo di eco-progettazione di soluzioni di packaging sostenibili. Lo scopo finale è quello di implementare la stima della shelf-life negli studi di LCA, includendo sia gli impatti ambientali diretti (produzione, lavorazione e fine vita) che indiretti (perdita di cibo e rifiuti). In questo modo, uno strumento decisionale scientificamente convalidato sarà messo a disposizione delle aziende alimentari che stanno affrontando la rivoluzione verde e la transizione ecologica.

1. State-of-the-Art

The challenge that the food packaging industry has faced in the last few years is the transition from traditional, performing but not recyclable materials to new and more sustainable packaging solutions. Replacing one material with another requires considerable effort, including verifying that the new material preserves the quality of the food during the primary and secondary shelf life. For coffee, be it in beans or ground, the traditionally used packaging are multi-layer solutions with high barrier properties composed by PET/Aluminum foil/PE or PP /Aluminum foil/PE, for example. The element that mainly limits the recyclability of these multilayer structures is the presence of materials difficult to separate and recycle. Its elimination must necessarily be compensated by the adoption of solutions capable of avoiding major physical-chemical changes that may compromise the quality and acceptability of the coffee. Aroma volatilization, carbon dioxide release, surface oil migration, water vapor absorption, oxidation reactions are only a few of the decay reactions that coffee undergoes during its storage, with consequent sensory variations that limit its acceptability (Manzocco and Lagazio, 2009). Therefore, a complete shelf-life evaluation model for packed coffee should be an interesting decision-making tool that can allow the prediction of the overall quality according to the specific characteristics of the packaging (i.e. shape and size), the materials (i.e. barrier performance) and the environment (i.e. oxygen, humidity and temperature). This kind of model may ideally integrate, one global quality parameter evolution, impact of headspace gases composition on this global quality evolution and consumer acceptability (Coffigniez et al., 2021). In the case of coffee, such full shelf-life models integrating the three components are poor in the literature due to the complexity of their implementation and the difficulties in their validation (Anese *et al.*, 2006). From the environmental point of view, a packaging development team in the food industry has also to consider the environmental impact that the new packaging solution has from production until the end-of-life. There are three main aspects to achieve the sustainability of food packaging: reducing CO_2 emission, choosing energy-efficient processes, and improving the waste management level keeping the attention on the food quality and the shelf-life extension to reduce food waste (Peelman et al., 2013). As the food packaging is a wide field with multiple environmental aspects to consider, conducting an LCA is the best way to calculate the overall environmental impact of a food product and its packaging solution (Molina-Besch et al., 2019).

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:
A1) **Characterization of new packaging solutions** in terms of mechanical, geometrical and barrier properties (Tasks 1.1 and 1.2). An in depth analysis of the aroma scalping and permeation properties of new recycle materials will be also carried out (Task 1.3).
A2) **Development and validation of a shelf-life (SL) model for packed roasted coffee:** based on the state-of the-art and literature research on shelf-life models done in the previous months, experiments devoted to the definition and/or validation of critical quality indicators and acceptability limits of coffee will be arranged (Task 2.1). Accelerated SL tests will be organized to model the temperature dependence of both coffee and materials' permeability (Task 2.2). A shelf-life model that integrates the global quality parameter evolution, the gas exchanges on the global quality, the packaging and materials features, the consumer acceptability, will be developed and validated (Tasks 2.3 and 2.4).
A3) **Evaluation of the environmental assessment of the packed coffee chain** to carry out complete LCA studies including both direct and indirect impacts of the packaging solutions, organizing interviews and questionnaires both for consumers and companies to validate the new decision-making tool (Task 3.1).
A4) **Scientific Coordination** between the research group, the Company and the research supervisor through meetings and updates**.**
A5) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

Table 1 Gantt diagram for this PhD thesis project (considering the last 24 months).

Activity	Month	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	***Characterization of new packaging solutions***																								
	1.1) New recyclable packaging materials characterization																								
	1.2) Study of packaging materials barrier features as function of T and RH																								
	1.3) Study of aroma scalping and permeation of new materials																								
A2)	***Shelf-Life Modelling***																								
	2.1) Definition of critical attributes of coffee and their acceptability limits																								
	2.2) Setting-up of accelerated SL tests as function of temperature																								
	2.3) Development of a SL model based on packaging-food interactions																								
	2.4) Validation tests																								
A3)	***Evaluation of the environmental assessment***																								
	3.1) LCA studies implementing direct and indirect effects of packaging																								
	3.2) Validation of the decision-making tool																								
A4)	***Scientific coordination***																								
A5)	***Thesis and Paper Preparation***																								

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Engineering of bioaerogels as key ingredients in the development of functional foods to deliver health through diet

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This Ph.D. research project aims at exploiting the peculiar characteristics of innovative porous food-grade materials, called bioaerogels, in the development of foods with tailored health-related functionalities. In particular, bioaerogels will be used to develop: (i) delivery systems able to protect bioactives through the gastrointestinal tract; (ii) fat replacers rich in unsaturated fatty acids, able to mimic the technological functions of traditional saturated hard fats; (iii) lighting ingredients, able to incorporate air in food formulations, thus leading to caloric density reduction.

Ingegnerizzazione di bioaerogels come ingredienti chiave nello sviluppo di alimenti funzionali per migliorare la salute attraverso la dieta

Questo progetto di dottorato di ricerca prevede di sfruttare le peculiari caratteristiche di innovativi materiali porosi per uso alimentare, chiamati bioaerogels, nello sviluppo di alimenti con funzionalità specifiche per la salute. In particolare, i bioaerogels saranno utilizzati per sviluppare (i) sistemi di veicolazione per proteggere composti bioattivi attraverso il tratto gastrointestinale; (ii) sostituti di grassi ricchi in acidi grassi polinsaturi capaci di mimare le funzionalità tecnologiche dei tradizionali grassi saturi concreti; (iii) ingredienti sfruttabili nell'alleggerimento degli alimenti, capaci di incorporare aria nelle formulazioni alimentari, riducendo la densità calorica.

1. State-of-the-Art

The development of "functional foods", *i.e.*, foods that, beyond basic nutrition, provide documented health benefits allowing the improvement of the population's well-being and the prevention/management of chronic diseases, has attracted large attention in the last years. The strategies for the development of functional foods can be classified as follows: (i) enrichment, *i.e.*, the addition of foods with healthy compounds which are delivered and protected through the gastrointestinal tract (*e.g.*, vitamins, minerals, antioxidants); (ii) replacement, referring to the substitution of unhealthy components (*e.g.*, saturated fatty acids) with healthier ones (*e.g.*, unsaturated fatty acids); (iii) clearing or lighting, based on caloric density reduction and/or removal or reduction of unhealthy components (*e.g.*, salt, sugar, fats). An ideal functional ingredient should be thus not only efficient in increasing food healthy profile, but also versatile in terms of both technological functionalities and applicability in different matrices, environmentally sustainable, and cheap. In this regard, bioaerogels could represent optimal highly innovative candidates for the development of functional ingredients.

Bioaerogels are food-grade materials characterized by high porosity (70.0-99.8%), low density (0.0003-0.5 g/cm^3), and high surface area (50-1200 m^2/g), produced by removing a solvent from a biopolymeric gel with a proper technique (most commonly freeze-drying and supercritical CO_2 drying) (García-González *et al*., 2019). By using different molds and gelation conditions bioaerogels of countless formats, shapes, sizes, and textures can be obtained. Up to now the research on food applications of bioaerogels is still pioneering and very limited information on the performances of aerogels in real food matrices is reported. Nevertheless, the results reported in the literature support the promising role of bioaerogels as functional food ingredients. In particular, the high absorptive internal surface of bioaerogels has been exploited to load high amounts of bioactives, which were effectively protected during processing, storage, and digestion by the aerogel polymeric network (Ubeyitogullari & Ciftci, 2019). Bioaerogels have been also exploited to prepare fat-replacers as they can absorb large amounts of liquid oil begetting semi-solid systems with structural properties comparable to those of traditional saturated fats (Plazzotta *et al*., 2021). Finally, bioaerogels might be added to foods to incorporate air with a remarkable energy intake reduction. Air incorporation has also been demonstrated to be an efficient strategy to emphasize the perception of aromas and tastes such as salty and sweet. Although the potential role of bioaerogels in reducing food salt and sugar content, no studies on these aspects have been reported to date.

Bioaerogels can be made of polysaccharides, proteins, or their combination. In the case of proteins, animal ones (gelatin, collagen, diary, and egg white proteins) are the most used for aerogel preparation, while the use of plant proteins is limited to a few studies relevant to potato proteins (patatins) and zein (Andlinger *et al*., 2021; Santos-Rosales *et al*., 2019). This is due to the poor gelling capacity of vegetable proteins as compared to animal ones, which hinders the possibility of obtaining a solid network able to withstand the conversion from hydrogel to aerogel. However, the possibility of exploiting vegetable proteins in the preparation of bioaerogels would offer a range of advantages: (i) plant proteins are more sustainable than animal ones in terms of greenhouse gas emissions and water consumption, very attractive in the context of the expected "plant-protein transition"; (ii) plant proteins

are increasingly demanded by consumers that follow specific dietary regimes (*e.g.*, vegans, vegetarians); (iii) plant proteins are of great nutritional interest as a source of bioactive peptides.

Based on these considerations, the present Ph.D. project aims at filling the knowledge gaps in food applications of bioaerogels to enable their application as functional food ingredients delivering health through diet. To this aim, based on the literature research conducted during the first months, the activities have been organized in two main research themes, which will be articulated over the 2 years PhD programme as shown in Table 1:

1. Development of bioaerogels based on plant proteins. To this aim, the activities will be characterized by:
   - Identification of possible sources of vegetable proteins, including the waste streams of vegetable processing industries. In this regard, samples will be directly collected from selected food industries.
   - Assessment of the gelling capacity of the selected proteins under different conditions in terms of pH, antisolvents, temperature, ionic strength, and their combination. In this step, rheological measurement and differential scanning calorimetry will be crucial techniques to study the gelling properties of the samples,
   - Conversion of selected plant gels based on their rheological properties, into aerogels using freeze-drying and supercritical-CO_2 drying and characterization of the physical properties (*e.g.*, mechanical properties, BET, SEM, capacity of entrapping air or absorbing food liquids) of the obtained aerogels.
2. Use of bioaerogels as functional ingredients in real food matrices. To this aim, the research activities will be focused on:
   - Evaluation of bioaerogels compatibility with model food systems. In particular, the effect of solvents with different polarities on bioaerogel structure and integrity will be evaluated.
   - Identification of strategies to control surface porosity with the aim of protecting loaded bioactives during food processing, storage, and digestion, and increasing air incorporation.
   - Development of functional food prototypes by using bioaerogels as functional ingredients to deliver specific compounds. In this phase, the technological performances of bioaerogels will be evaluated by comparing the physical and sensory properties of reformulated foods with those of standard control samples.
   - The nutritional effect of reformulation will be evaluated in-depth. To this aim, proper methodologies will be used to assess food nutritional functionality, including *in vitro* digestibility assays and cell culture models.

Table 1. Gantt diagram for this Ph.D. thesis project.

Activity \ Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1. Development of bioaerogels based on plant proteins																								
a. Identification of protein sources	■	■																						
c. Assessment of protein gelling capacity		■	■	■	■																			
d. Aerogel production and characterization					■	■	■																	
2. Use of bioaerogels as functional ingredients in real food matrices																								
b. Evaluation of bioaerogels compatibility with food								■	■	■														
c. Development of strategies to control aerogel porosity											■	■	■											
c. Prototyping foods containing bioaerogels													■	■	■	■								
d. Assessment of nutritional functionality																	■	■	■	■	■	■		
Mobility period																	■	■	■	■	■	■		
Bibliographic research	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
Paper preparation	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
Thesis development															■	■	■	■	■	■	■	■	■	■

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Enzyme biotechnology to recovery byproducts from agricultural and food wastes

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This PhD thesis research project is configured as an industrial doctorate funded by the National Operational Programme (NOP) on Research and Innovation 2014-2020 "Action IV.5 - "Doctoral Programmes on Green topics" and aims to develop and standardize extraction processes by using assisted enzymatic extraction for the recovery of byproducts from waste generated in agri-food production and processing. In particular, enzymatic processes and the functionalities of the different byproducts obtained through the disassembling-reassembling strategy will be tested on a laboratory scale and then transferred on a pilot scale to the company (Euronut Spa) involved in the project.

Biotecnologie enzimatiche nel recupero di byproducts da scarti agroalimentari

Questo progetto di tesi di dottorato si configura come un dottorato industriale finanziato nell'ambito della misura PON "Ricerca e Innovazione" 2014-2020" Azione IV.5 – "Dottorati di ricerca su tematiche Green" e mira a sviluppare e standardizzare processi di estrazione utilizzando l'estrazione enzimatica assistita per il recupero di byproducts da scarti delle produzioni e trasformazioni agroalimentari. In particolare, processi enzimatici e le funzionalità dei diversi byproducts ottenuti attraverso la strategia del disassemblaggio-riassemblaggio verranno testati in scala di laboratorio per poi essere trasferiti su scala pilota nell'azienda (Euronut Spa) coinvolta nel progetto.

1. State-of-the-Art

The impending environmental crisis has made us participants in the greatest global battle: the one for our planet. The challenge in which each of us is called to take part affects all sectors of the economy in a transversal manner, which goes from linear to circular but it does not mean that the currency, the market or consumer goods have changed, rather that every substance previously considered "waste" now takes on a new value, re-entering a new production cycle as a by-product.
Circular economy criteria have wide application in the agro-food sector and, thanks to the macro and microscopic characterization of by-products that come from processing waste of the supply chain, it is possible to highlight the presence of any nutraceutical substance in them in order to offer to the consumer a new functional commercial product. The nutritional aspect, however, is not the only one to take into consideration; in fact, through these applications, it is possible to insert the resulting material in the formulation of biofuels, to extract molecules, which could be used in cosmetics or as ingredient in nutraceutical food, in new packaging methods, all thanks to biotechnologies. Among them, the one that goes out the best result is the applied enzymology that perfectly meets the green criteria as demonstrated by Ara *et al.* (2013) who found useful glycoside hydrolases (GHs) to extract flavonoids, an important class of antioxidants. Also Rodriguez-Colina *et al.* (2013) found several advantages in using an enzymatic approach to obtain GOS (galactooligosaccharides) which promote the formation of a bifidus microbiota in the intestine of milk-fed babies. In the field of vegetal matrices and extraction of substances from them, Milosevic *et al.* (2022) compared the conventional and the enzymatic strategy to extract pectin from butternut squash, actually there were several advantages in pectin extraction with enzymatic strategy: higher solubility of the fiber, bigger phenolic acid content, better antioxidant capacity.
In this way it is therefore possible to reduce waste and improve the approach to consumption, creating a true and environmental awareness in the consumer, who becomes a key player in the production and recovery processes.
Enzymatic biotechnologies are an important tool for pursuing, through an interdisciplinary approach, the objectives of sustainable and eco-compatible development by the isolation of the principal macro elements: polysaccharides, lipids, proteins (as said by OECD -Organization for Economic Cooperation and Development).

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) **Individuation of agri-food wastes** proximate composition analysis (moisture, ash, lipid, protein and carbohydrate contents) of samples (A1.1), choice of the best residues through the evaluation of the best cost-benefit ratio (A1.2).

A2) **Choice of the best enzymatic strategy** to disassembling the selected wastes by testing different hydrolases (protease, carbohydrase, lipase, etc) and standardize the main operating variables (pH, T, liquid-solid ratio,

enzyme substrate ratio) to obtain the best byproduct yields (A2.1). The obtained byproducts will be characterized for their physico-chemical properties (z-potential, size, solubility, hydrolysis index, hydrophobic index) (A2.2) and technological properties (solubility, water and oil holding capacity, foaming and emulsion ability) (A2.3).

A3) **Scaling- up of enzymatic process** to obtain byproducts in the pilot plant scale to validate the processes parameter identified during activity A2

A4) **Use of byproducts** to assess their use as food ingredient (A4.1) food additives, (A4.2) and feed formulation (A4.3).

A5) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

Table 1 Gantt diagram for this PhD thesis project.

Activity		Months																							
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	***Individuation of agri-food wastes***																								
	1) Proximate composition analysis																								
	2) Choice of the best residues																								
A2)	***Choice of the best enzymatic strategy***																								
	1) Enzymatic assay																								
	2) Physico-chemical properties																								
	3) Technological properties																								
A3)	***Scaling-up of enzymatic process***																								
A4)	***Use of Byproducts***																								
	1) Food ingredient																								
	2) Food additives																								
	3) Feed improvement																								
A5)	***Thesis and Paper Preparation***																								

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Impact of different filtration processes on genetic traceability of monovarietal wines

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Nowadays wine production is changing all the agri-food sector in an economic and environmental way. In fact, the development of new methodologies and new techniques to reduce the environmental impact of some oenological phases and also to improve traceability in this field is becoming more and more important. One of the aims of this PhD thesis is to evaluate how different filtration techniques and filtration's adjuvants, could impact on wine genetic traceability.

L'impatto di diversi processi di filtrazione sulla tracciabilità genetica dei vini monovarietali

Oggigiorno la produzione del vino sta cambiando l'intero settore agroalimentare, sia da un punto di vista economico che ambientale. Lo sviluppo di nuove metodologie e nuove tecniche diventa così sempre più importante per ridurre l'impatto a livello ambientale del processo enologico e per migliorare la tracciabilità di filiera. Uno degli obbiettivi del dottorato è quello di valutare come diverse tecniche di filtrazione ed alcuni coadiuvanti possono impattare sulla tracciabilità genetica dei vini.

1. State-Of-The-Art

The wine production has an increasing impact on the world's economy. This makes wine subject to fraud and manipulations, that can occur in many forms, to increase quality and to modify some characteristics (Versari et al., 2014).

The quality of wine depends from different factors: winemaking process, agricultural practices, and the area, but also is very important the grape variety. Grapevine varieties can influence not only wine quality parameters, but it has an impact on the price and on the market placement, especially for the Designation of Origin (DO) wines. For this reason, the knowledge of the chemical, aromatic and phenolic profile of a grapevine variety and the ability to track the entire winemaking process is fundamental to ensure the quality, the authenticity and the typicity of the final product (Pereira et al., 2011).

Legislative guidelines and instrumental techniques are used to avoid frauds and to trace the product, particularly linking wine's composition to the grape's characteristics through the study of chemical, physical and sensorial parameters, like anthocyanin and sensory profile. This kind of analysis are usually expensive both under an economic and time point of view. Moreover, those parameters are too much influenced by agricultural and cellar practises and they can result inaccurate (Pereira et al., 2012; Villano et al., 2017). For this reason, in the last few years the attention is moving to a genetic approach, based on DNA analysis. Today grapevine's DNA can be extract from every part of a plant, but this process is more complex when it is related to musts and wines. Indeed, these two products undergo some oenological operations like clarify, adjuvant addition or filtration processes that can reduce DNA availability (Boccacci et al., 2020; Gambino et al., 2022).

With the aims of improve the sustainability of the wine making process, understand the impact of genetic traceability in the supply chain and have a varietal characterization of some grapevine varieties, one of the purposes is to evaluate how different filtration methods and filtration's adjuvants, usually used in the wine making activities, could impact on DNA's traceability of monovarietal wines.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) **Investigation on the genetic traceability** and on the impact of some filtration methods and adjuvants on some monovarietal wine's DNA, performing in a laboratory scale different filtration technique (A1.1), sampling for analytical analysis and for DNA extraction (A1.2).

A2) **Improve sustainability in the wine making process** through the reduction of use of some adjuvants and coadjuvants such as sulphur dioxide (A2.1) and the application of new technologies to improve wine chain sustainability (A2.2).

A3) **Varietal characterization** of some grapevine varieties, particularly the ones used for monovarietal D.O. wines production, to understand their characters and tipicity through experimental microvinifications (A3.1) and the study of phenolic and aromatic profile, with chemical characterizations, analytical determinations (A3.2) and sensorial activities (A3.3).

A4) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

***Table 1**. Gantt diagram for this PhD thesis project*

Period	Year 1												Year 2												Year 3											
Activity	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12
A1. Genetic traceability																																				
1. Filtration techniques																																				
2. Sampling & DNA analysis																																				
A2. New metodologies for improve sustainability																																				
1. Reduction of SO_2																																				
2. Application new technologies																																				
A3. Varietal characterization																																				
1. Microvinifications																																				
2. Analytical determinations																																				
3. Sensorial activities																																				
A4. Writing and editing																																				

3. Selected References

Boccacci P., Chitarra W., Schneider A., Rolle L., Gambino G. (2020). Single-nucleotide polymorphism (SNP) genotyping assays for the varietal authentication of 'Nebbiolo' musts and wines. *Food Chemistry*, 312, 126100.

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Health-promoting whey protein-derived peptides in functional foodstuff

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Tutor: Prof.ssa Nadia Innocente

This PhD thesis research project is aimed to develop enzymatic hydrolysis processes of whey proteins to obtain bioactive peptides with tailored functionalities for application in functional foodstuff. The characterization of whey protein hydrolysates and the enrichment of the bioactive peptides with enhanced functionalities will be assessed. The final objective is therefore to design a functional food added in whey-derived bioactive peptides and to determine the preservation of bioactivity and bioaccessibility throughout food formulation, processing, storage and, ultimately, *in vitro* digestion.

Impiego di peptidi bioattivi ottenuti dall'idrolisi delle proteine del siero in alimenti funzionali

Il presente progetto di ricerca si propone di sviluppare un processo di idrolisi enzimatica delle proteine del siero per l'ottenimento di peptidi bioattivi con funzionalità personalizzate per l'applicazione in alimenti funzionali. Gli idrolizzati di proteine del siero verranno caratterizzati e sarà valutato l'arricchimento di peptidi bioattivi con valorizzate proprietà funzionali. L'obiettivo finale sarà quindi la progettazione di un alimento funzionale con l'aggiunta di peptidi bioattivi da proteine del siero e la determinazione del mantenimento della bioattività e bioaccessibilità durante la formulazione, la trasformazione, lo stoccaggio ed infine la digestione *in vitro.*

1. State-of-the-Art

In the last decades, consumer demand for functional foods has increased, because of their health-promoting beneficial activities and the risk reduction for both curable and chronic diseases. In this context, food and nutraceutical industries have focused the attention on bioactive peptides. These molecules are fragments of proteins consisting of 2 to 20 amino acids residues that are encrypted in the parent protein in an inactive form. Bioactive peptides are involved in many regulatory and modulatory processes in the human body associated with anti-hypertensive, antioxidant, immunomodulatory, antimicrobial and many other activities. To perform their specific and independent functions, bioactive peptides need to be released from the native protein sequences through hydrolysis, microbial fermentation, or enzymatic digestion (Pihlanto-Leppälä, 2000).

Among these possible strategies, enzymatic hydrolysis is the most common adopted technique to produce bioactive peptides. Furthermore, targeted bioactive peptides can be obtained by adequately designing and optimizing the hydrolysis process parameters, such as choice of the enzyme, enzyme/substrate ratio and reaction time, temperature, and pH (Hinnenkamp and Ismail, 2021). Also, pre-treatment of proteins, before their exposure to enzymatic hydrolysis, (e.g. heat, high pressure, pulsed electric field) enhances the refolding of secondary structure conformation, thus leading to an improved endoprotease access and hydrolysis. This could have an impact on both technological and bioactive properties (Kumar *et al.*, 2018). However, further studies are necessary to better understand the effect of different hydrolysis parameters and pre-treatments on the functional and technological properties of bioactive peptides.

Potentially, all type of proteins found in nature could be sources of bioactive peptides. Among all, whey proteins are already known in the food industry sector for their high nutritional value and extremely versatile technological properties. Whey is a high-value by-product of the dairy manufacturing sector and whey derived bioactive peptides represent a sustainable approach for its revalorization. Moreover, whey-derived bioactive peptides are known to exert different physiological effects (Dullius *et al.*, 2018). In this respect, the acknowledge antimicrobial activity of these peptides appears to be particularly interesting in the perspective of their use in foods aiming at a reduction of food risks.

Compared to the research interest in the development of bioactive peptides-based foods, the application of whey bioactive peptides in food formulation is still delayed. This is because of a lack of information about chemical reactions that could occur during food processing and food storage. Additionally, during food formulation, micro and macro nutrients could easily interact with the numerous reactive and susceptible groups that bioactive peptides possess, leading to changes in the peptide structure and thus facilitating a change or even depletion of bioactive properties. However, the mechanisms through which processing, formulation and storage can act on peptide structure are still not well established (Chakrabarti *et al.*, 2018).

Furthermore, possible changes in peptide conformation could occur as an effect of reactions between gut proteases and peptides during the digestive process. A major challenge when using whey-derived bioactive peptides as functional food components is the possible loss of peptide biostability during the gastrointestinal transit. For this reason, designing delivery systems to encapsulate, protect, deliver bioactives in a controlled and improved bioaccessible form at the target site is a promising solution (Sun and Udenigwe, 2020).

In conclusion, future studies should focus on the characterization of the interactions between bioactive peptides and food components, food processing and storage conditions and biological processes in humans.

2. PhD Thesis Objectives and Milestones

The main objective of this research program is the development of whey protein hydrolysates to be used as functional food ingredients. To this aim, bioactive peptides with target functionalities will be produced through a scalable and efficient hydrolysis process and then characterized in terms of biological and functional properties. Within this purpose the main steps of the research will be:

1) Set up of different methods to produce whey protein hydrolysates enriched with bioactive peptides.
2) Characterization of whey protein hydrolysates in terms of degree of hydrolysis, molecular weight profile, and targeted functional and biological activities as a function of the hydrolysis process.
3) Development of functional foods containing whey protein hydrolysates.

Within the overall objective mentioned above, this PhD thesis project can be subdivided into the following activities according to the Gantt diagram (Table 1):

A1) **Characterization of enzyme cleavage patterns** to determine the degree of hydrolysis (A1.1), the peptides profile by RP-HPLC and SDS-PAGE (A1.2) and to identify peptides sequences by mass spectrometry (A1.3).
A2) **Assessment of whey protein hydrolysates' properties** in terms of functional properties (e.g. solubility, gelation, emulsifying and foaming capacity, viscosity) (A2.1) and *in vitro* possible bioactivities (e.g. anti-hypertensive property, antimicrobial and antioxidant activities, immunomodulatory effects, …) (A2.2).
A3) **Bioactive peptides enrichment** through set-up of molecular weight cut-off membrane filtration process.
A4) **Functional foods design** trough the selection of possible food targets (yogurt, fermented milk, plant-based beverages) (A4.1), the identification of a suitable delivery systems (e.g. liposome, nanoemulsion, microemulsion) (A4.2), the evaluation of physicochemical and sensory characteristics of whey protein hydrolysates-added food (A4.3) and the determination of peptides bioactivity preservation during processing, shelf-life and *in vitro* digestion of functional foods (A4.4).
A5) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

Table 1 Gantt diagram for this PhD thesis project.

Activity / Months		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	***Characterization of whey protein hydrolysates***																								
	1) Assessment of hydrolysis degree																								
	2) Identification of peptides profile																								
	3) Mass spectrometry analysis																								
A2)	***Assessment of whey protein hydrolysates' properties***																								
	1) Evaluation of functional properties																								
	2) *in vitro* bioactivities assessment																								
A3)	***Bioactive peptides enrichment***																								
A4)	***Functional foods design***																								
	1) Selection of food target																								
	2) Selection of delivery systems																								
	3) Development of peptides-added foods																								
	4) Assessment of peptides bioactivity preservation in foods																								
A5)	***Thesis and Paper Preparation***																								

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Use of vegetable by-products for the formulation and study of foods with high health value and to prolong their shelf-life

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Tutor: Prof.ssa la Gatta

This PhD thesis research project is aimed at using vegetable by-products as ingredient in the formulation of foods with high health value and at evaluating their shelf-life, according to a sustainable and circular economy for the agro-alimentary production chain.

Utilizzo di sottoprodotti vegetali per la formulazione e lo studio di alimenti ad elevato valore salutistico e per il prolungamento della shelf-life

Il progetto di questa tesi di dottorato mira all'utilizzo di sottoprodotti vegetali da aggiungere, come ingredienti, nella formulazione di alimenti al fine di incrementarne il valore salutistico e condurre studi al fine di valutarne l'impatto sulla shelf-life, nell'ottica di un'economia circolare e sostenibile per la filiera agro-alimentare.

1. State-of-the-Art

Over the years, the amount of food waste has increased, with constant losses through the supply chain and losses of water, lands and energy. The management of this situation has become a concern for governments due to their impact on economy and environment. Generally, the food waste is brought to landfills and incinerated, used as animal feed or used for biofuels, biogas or biofertilizers. As per the FAO (Food and Agriculture Organization of the United Nations) vegetable wastes have created a significant higher "carbon footprint"; it estimated that one third of food products are either wasted or lost and total value of the waste is up to US$1 trillion (Ben-Othman, 2020).

Food by-products are generated by industries and includes dairy products, cereal, fish processing, meat processing and fruit and vegetable by-products. These last types of waste are around 40-50% of total discards, but they have a huge potential of being recycled because they are sources of bioactive compounds that include fiber, pectin, phenolic acid, carotenoids, enzymes, pigments, vitamins and aromatic compounds. The waste from fruit and vegetable can be in form of peel, pulp, seeds, crop, leaf, stem, root or tubers, depending on raw materials and industrial process. They can be easily used to add foods additional properties, like the antioxidant or antimicrobial activity, enhancing their functional and nutritional properties (Dilucia, 2020; Ganesh, 2022; Ben-Othman, 2020; Melini, 2020).

In this project, the stabilization of vegetable by-products will be headed through a new physical, non-thermal, under patenting process that permit to obtain new ingredients with high health value without the utilization of chemical solvents. This kind of process will allow a reduction of energetical and environmental costs and the obtaining of nutraceutical, more stable resources from vegetable, even when they are not available due to the seasonality problem.

Thus, this PhD project will be directed to demonstrate the feasibility of vegetable by-products as ingredients or food preservatives, reducing food waste in agro-food industrial chain. Although, following the "Green curriculum" of the PhD, the purpose of the project is to use development strategies in the food supply chain according to a sustainable and circular economy for the agro-alimentary industries. Moreover, the PhD project will be aimed to understand consumer behavior to buy new foods enriched with the vegetable by-products rich in biocomponents and/or nutraceutical compounds because during COVID-19 pandemic, the consumer attitude has changed, showing more attention to healthy and sustainable foods.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) **Stabilization and Characterization of vegetable by-products powders,** with the new non-thermal, under patenting process and selection of vegetable richen in bioactive compound, through chemical and sensorial analysis.

A2) **Formulation and characterization of bakery products,** using the powder obtained as new ingredients.

A3) **Industrial Scale-up of the bakery products** with vegetable by-product, in the pilot plant, as mentioned in activity A2

A4) **Bioavailability and immunoreactivity study of the obtained products**, to the Manchester Institute of Biotecnology.

A5) **Writing and Editing** of the PhD thesis, scientific papers, research project, interdisciplinary works and oral and/or poster communications.

Table 1 *Gantt diagram for this PhD thesis project*

Activity	Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	**Stabilization and Characterization of powders**																								
	1) Under patenting process																								
	2) Chemical and sensorial analysis																								
A2)	**Formulation of bakery product**																								
A3)	**Industrial Scale Up**																								
	1) Chemical and sensorial analysis																								
A4)	**Immunoreactivity study**																								
A5)	**Thesis and Papers Preparation**																								

3. Selected References

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Sustainable technologies and valorisation of food by-products by the recovery of bioactive compounds

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This PhD project aims to apply green technologies in recovering bioactive compounds from food by-products in order to develop functional food, and ingredients. In addition, the combined impact of green technologies and natural compounds to increase the quality and sustainability of food products will be studied.

Tecnologie sostenibili e valorizzazione dei sottoprodotti della filiera alimentare attraverso il recupero di composti bioattivi

In questo progetto di dottorato verranno utilizzate tecnologie verdi per il recupero di composti bioattivi da sottoprodotti della filiera alimentare, con l'obiettivo di sviluppare alimenti funzionali ed ingredienti. Inoltre, sarà studiato l'impatto combinato delle tecnologie verdi e dei composti naturali per migliorare la qualità e la sostenibilità dei prodotti alimentari.

1. State-of-the-Art

Food by-products are a considerable amount of the entire food production, and this share is rising annually. Despite the drawbacks arising from these by-products, they could be considered low-cost, renewable, and prevalent sources for extracting natural bioactive compounds (e.g., antioxidants, vitamins, proteins, etc.). The recovery of these compounds could reduce the economic and environmental influence caused by food waste. Nevertheless, it is challenging to find an efficient method to fulfil this aim. Since conventional extraction methods may lessen these high-sensitive components, nonthermal technologies, such as microwave, ultrasound, cold plasma, pulsed electric field, pressurized liquid, and ohmic heating technology, are more engaging as alternatives. As the temperature is not the main factor in mild technologies, they require lower energy.
Moreover, they have a lower effect on the loss of sensitive compounds, resulting in better recovery of bioactive constituents. Several research projects have previously reported that these sustainable methods could cause high bio-accessibility and stability of these compounds (Ebrahimi & Lante, 2022). Therefore, it could be an excellent circular economic approach to use the extracts obtained by mild technologies to formulate functional food products (figure 1) with high nutritional value and quality (Laganà et al., 2022; Tinello & Lante, 2019). Indeed, adding bioactive compounds to food products could establish a connection between food and health (Difonzo et al., 2020). Moreover, the combined usage of some green technologies and natural ingredients in producing functional food is another potential procedure that can increase their quality and safety (Bouzid et al., 2021; Cropotova et al., 2021; Martín-García et al., 2020).

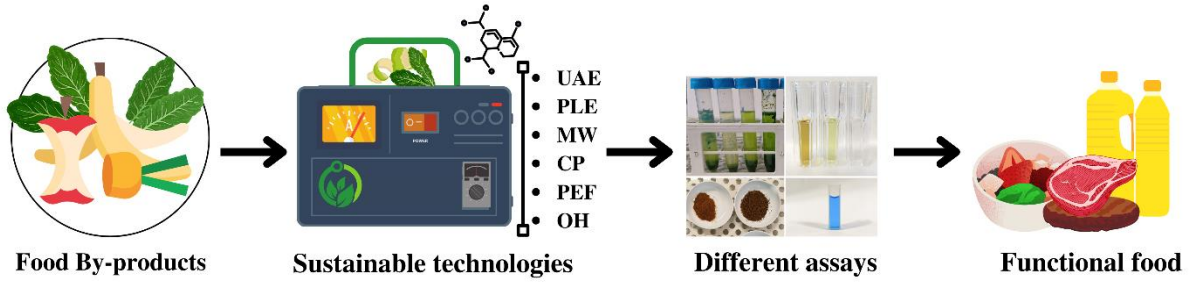

Figure 1 Schematic of Bioactive compounds' recovery and their usage in functional food
UAE: Ultrasound-Assisted Extraction; PLE: Pressurized Liquid Extraction; MW: Microwave; CP: Cold Plasma; PEF: Pulsed Electric Field; OH: Ohmic Heating.

In addition, the control of polyphenol oxidase (PPO) during the extraction of phenolic compounds is another crucial issue for maintaining their nutritional attributes since the reaction between PPO and polyphenols gives rise to their oxidation (Ebrahimi & Lante, 2021). The mild technologies are able to decrease the activity of enzymes and inhibit the enzymatic browning and degradation of polyphenols (Wang et al., 2021).
In this respect, it is pivotal to research the potential strategies to maximize the recovery and exploitation of bioactive compounds at low or moderate temperatures and find the best solutions to produce functional food products with added safety and quality. Accordingly, this PhD thesis will investigate the impact of sustainable technologies on PPO and recovering bioactive compounds for the valorisation of food by-products.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above, this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:
A1) **Bibliographic research on the state of the art and the main analysis techniques**: An overview of the literature on the topic will be done.
A2) **Implementing the trials and optimization of the extraction parameters** to reach the sustainability of the process.
A3) **Designing functional food** with high quality, safety, and nutritional value.
A4) **Writing and Editing** the PhD thesis, scientific papers, and oral and/or poster communication.

Table 1 Gantt diagram for this PhD thesis project.

Activity \ Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1) ***Bibliographic research***																								
A2) ***Implementing the trials***																								
A3) ***Designing functional food***																								
1) Finding the suitable food products																								
2) Testing the different quality parameters																								
A4) ***Writing Thesis and Papers***																								

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Authentication of hay milk and its dairy products by nuclear magnetic resonance spectroscopy

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Hay milk is a high quality product that its authenticity needs to be investigated with an innovative approach to protect both producers and consumers against possible counterfeits. This research project aims to determine the authenticity of hay milk with two analytical approaches: (1) targeted cyclopropane fatty acids biomarker detection and quantification and (2) non-targeted fingerprinting in hay milk and milk from cows fed either with maize or grass silage employing high resolution liquid state ^{1}H-NMR (600 MHz) spectroscopy. Furthermore, the ^{1}H-NMR approach for hay milk authenticity will be applied on other dairy products such as cheese, butter, and yoghurt.

Autenticità di Latte Fieno e suoi derivati con spettroscopia di risonanza magnetica nucleare ad alta risoluzione

Il latte fieno è un prodotto di alta qualità la cui l'autenticità deve essere indagata con un approccio innovativo per tutelare sia produttori che consumatori da possibili contrafazzioni. Lo scopo di questa ricerca di dottorato è di determinare l'autenticità del latte fieno sia con un approccio di fingerprinting non-target, che target sull'identificazione e quantificazione dell'acido grasso ciclopropanico del latte fieno e latte di mucche alimentate con insilato di mais e di erba con la spettroscopia ^{1}H-NMR (600 MHz). Successivamente, l'approccio del NMR sarà applicato per determinare l'autenticità dei prodotti di latticini derivati dal latte fieno come formaggio, yoghurt e burro.

1. State-of-the-Art

Hay milk is produced in South Tyrol, Italy, following traditional alpine practices is protected under the EU Traditional Specialty Guaranteed quality scheme since 2016. The hay milk comes from the cows that have been grazed in the alpine pastures and were fed exclusively with unfermented hay and herbs that silage feeding is strictly prohibited (EU Regulation 2016/ 304). Recent studies showed that hay milk has high nutritional values, higher concentration of essential fatty acids such as linoleic acid, alfa-linolenic acid, lysine, and putrescine (Van den Oever et al., 2021).

Cyclopropane fatty acids (CPFAs) have been recently detected in milk and dairy products as a consequence of silage feeding of the cows that they are released by the bacteria through silage fermentation process (Caligiani et al., 2016). Therefore, the presence of CPFAs in milk and dairy products have been demonstrated to be a valid marker for the authentication of Protected Designation of Origin (PDO) cheeses, like Parmigiano Reggiano, (Caligiani et al., 2016).as well as hay milk (EU Regulation 2016/ 304) where the use of silage in cow feeding is not allowed.

NMR spectroscopy can be considered as an effective alternative to other conventional analytical techniques such as chromatography and mass spectroscopy, because it requests minimal sample preparation and short data acquisition time and hence, it has non-destructive characteristics. Moreover, NMR has considerable advantages both as a non-targeted technique for screening due to its structure sensitive response of the sample at molecular level (Le Gall and Colquhoun, 2003) and simultaneous detection of high number of compounds in complex matrices like milk (Lamanna et al., 2011). Therefore, NMR spectroscopy can be applied on hay milk authentication with the detection and quantification of the CPFAs as a biomarker for hay milk and its dairy products (Lolli et al., 2018) and hence a molecular fingerprinting approach for hay milk authentication.

2. Ph.D. Thesis Objectives and Milestones

The objectives of the present PhD thesis project are following:

- **Identification of CPFAs (dihydrosterculic and lactobacillic acid) with ^{1}H-NMR spectroscopy as a bio-marker for hay milk authentication**

According to the 250 NMR spectra of hay and non-hay milk samples analyzed so far, ^{1}H-NMR technique provides very precise chemical information about the cyclopropane ring of the CPFAs, especially the signals of the cis H atoms of cyclopropane ring with the chemical shifts at between-0.30 and -0.35 ppm, where there is no other overlapping signal.

• Non-targeted spectroscopic fingerprinting approach as a rapid and powerful tool for hay milk authentication

An effective and representative hay milk fingerprinting model is to be established to investigate the conformity of concerned milk sample as hay milk.

• The assessment of reliability of ^{1}H-NMR method in hay milk authentication with compared to gas chromatography- mass spectroscopy method

The results obtained by ^{1}H-NMR analysis will be compared with the results which were previously obtained at Laimburg Research Centre, collaborator within the HEUMILCH project, by GC-MS method to investigate the reliability of the NMR technique on hay milk authentication.

• Application of ^{1}H-NMR hay milk authentication method on South Tyrolean dairy products

The ^{1}H-NMR fingerprinting will be applied for quality and authenticity assessment of hay milk derived dairy products such as cheese, butter, and yoghurt which are produced in South Tyrol.

Table 1. *Gantt diagram of the present PhD thesis project activities*

Activity	Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	**^{1}H-NMR method development for hay milk authenticity**																								
	Literature review																								
	Preliminary experiments																								
A2)	***NMR Analysis***																								
	Milk sample lyophilization																								
	2) NMR analysis																								
A3)	***Data Analysis***																								
	1) Spectral data analysis																								
	2) Multivariate data analysis																								
A4)	***Assessment of the reliability of* ^{1}H-NMR method**																								
	1) Comparison of the result with GS-MS method																								
A5)	**Application of ^{1}H-NMR method to other dairy products**																								
A6)	***Thesis and Paper Preparation***																								

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Ozone technology for sanitization and product quality in the dairy supply chain

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This PhD thesis research project aims to develop the use of ozone technology within the dairy supply chain to control mainly the microbiological proliferation and/or development of parasites and insects, such as mites and moulds, without altering the normal process of the production of fresh and mature cheeses. The ozone technology will be use in multiple sectors of the dairy supply chain, as for the sanitization of process water (reuse), following "green" lines of social, environmental and economic attention. Evaluation of dairy products is expected for the maintenance of product quality.

La tecnologia dell'ozono per la sanitizzazione e la qualità di prodotto nella filiera di produzione casearia

Questo progetto di tesi di dottorato mira a mettere a punto l'uso della tecnologia dell'ozono all'interno della filiera lattiero – casearia per controllare principalmente la proliferazione microbiologica e/o lo sviluppo di parassiti ed insetti, quali acari e muffe indesiderate, senza alterare i normali processi di produzione di formaggi freschi e stagionati. Tale tecnologia sarà usata in più comparti della filiera lattiero - casearia, come per la sanitizzazione delle acque di processo (riuso), seguendo linee "green", di attenzione sociale, ambientale ed economica. Si prevede una valutazione dei prodotti per il mantenimento della qualità di prodotto.

1. State-of-the-Art

In the dairy supply chain, there has been the introduction of innovative techniques over the years, with an improvement in the art of dairy at instrumental and performance level to meet the challenges of an increasingly competitive market. Wood is not easy to replace, because the wood essences are important for sensory nuances typical of each cheese, but it is an ideal environment for microbial colonization and it is at the ripening stage that the coexistence of traditional and innovative methods is declined in the use of efficient cleaning and sanitizing materials (Piscopo, 2016). Alternative sanitization technologies include the use of ozone. Ozone (O_3) is triatomic oxygen. It has antimicrobial ability because of its oxidation potential (Genecya *et al.*, 2020). It is in 1996 that, in Italy, the Ministry of Health recognizes the use of ozone in the treatment of air and water and, in 2010, the National Committee for Food Safety gives a favorable opinion on the ozonation of cheese maturing chambers (CNSA, 2010). The treatment of wastewater with ozone allows to obtain effluents that can be discharged in natural aquatic systems (Martins and Quinta-Ferreira, 2010). The use of ozone treatment can result in significant reductions in counts of microorganisms (Afonso *et al.*, 2022). Mozzarella cheeses cooled with pre-treated water were characterized by a low microbial load and an increased shelf life (Segat *et al.*, 2013). The shelf life of perishable dairy products like yoghurt and cheese depends on several factors, such as the degree of sanitation during packaging. However, in a study with ozone, some sensorial alterations were observed (Alexopoulos *et al.*, 2017). The implementation of a standardized sensory language seems imperative (Khattab *et al.*, 2019). Recently, it was studied the effectiveness of air disinfection by ozonation or hydrogen peroxide aerosolization in dairy environments for food safety and product shelf life (Masotti *et al.*, 2019). The advantages of using ozone in dairy processing plants include the low environmental impact (Panebianco *et al.*, 2022). We intend to experiment the use of ozone technology on fresh, semi-aged or aged cheeses, on process water and in other sectors of the dairy supply chain with a product quality evaluation.

2. PhD Thesis Objectives and Milestones

This PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) **Determination of the optimal doses of ozone, contact times and other treatment variables** through the study of the existing bibliography (A1.1) and through experiments and replicas (A1.2).

A2) **Use of ozone for sanitization in several sectors of the dairy supply chain** with on-site application (company internship) (A2.1) and its monitoring (A2.2).

A3) **Analysis of the product quality**, microbiological, qualitative (lipid content, dry matter content, moisture and ash, nitrogen and total protein content, peroxides), technological evaluation (weight loss, texture, colour) (A3.1) and sensory analysis (A3.2). All data will be statistically evaluated (A3.3).

A4) **Study of shelf life** of ozonated products;

A5) **Sustainability assessment** with environmental impact (A5.1) and cost analysis (A5.2)
A6) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

Table 1 *Gantt diagram for this PhD thesis project.*

		Months																																			
	Activity	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**	**31**	**32**	**33**	**34**	**35**	**36**
A1)	***Treatment variables***																																				
	1) Bibliography																																				
	2) Experiments/replicas																																				
A2)	***Use of ozone***																																				
	1) On-site application																																				
	2) Monitoring																																				
A3)	***Product quality***																																				
	1) Quality evaluation																																				
	2) Sensory analysis																																				
	3) Statistical analysis																																				
A4)	***Study of shelf life***																																				
A5)	***Sustainability assessment***																																				
	1)Environmental analysis																																				
	2) Cost analysis																																				
A6)	***Writing and Editing***																																				

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Selection of Next-Generation Probiotics (NGPs) from the gut microbiome of subjects with different dietary patterns

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The aim of this PhD project is to create a collection of microbial strains isolated from the human gut of individuals with different diets, promising as Next Generation Probiotics (NGPs). The term Next-Generation Probiotics has been developed to refer to microbial strains that can have a positive effect on human health, but do not belong to common probiotic species (e.g., lactic acid bacteria). NGPs are promising for the treatment and prevention of chronic and degenerative non-communicable diseases (e.g., diabetes). Strains will be characterized at phenotypic and genomic level and tested for the production of beneficial metabolites.

Selezione di probiotici di nuova generazione dal microbioma umano di individui con abitudini alimentari diverse

L'obiettivo di questo progetto di dottorato è la creazione e caratterizzazione di una collezione di ceppi microbici isolati dall'intestino di individui con diete diverse, potenzialmente utilizzabili come probiotici di nuova generazione (NGPs). Il termine Next-Generation Probiotics è stato coniato per indicare ceppi microbici che possono esplicare un'azione positiva sulla salute umana, ma che non appartengono a specie comunemente utilizzate (ad esempio, batteri lattici). Gli NGPs sono promettenti per il trattamento e la prevenzione delle malattie cronico degenerative. I ceppi saranno caratterizzati a livello fenotipico e genomico e testati per la produzione di metaboliti benefici.

1. State-of-the-Art

In the last decade, thanks to the development of novel knowledge on the human gut microbiome and its influence on health, it has been realized that it constitutes an unexplored source of microorganisms with a potential beneficial role. Therefore, the term Next-Generation Probiotics (NGPs) has been developed to refer to microbial strains that can have a positive effect on human health, but do not belong to common probiotic species (e.g., lactic acid bacteria, LAB). NGPs are promising for the treatment and prevention of chronic degenerative diseases. Probiotics currently available on the market generally belong to a narrow range of microbial species (mostly LAB). However, recent studies about the importance of the gut microbial commensals on human health highlighted that several different taxa (e.g., species within *Faecalibacterium*, *Akkermansia*, *Bacteroides* genera) may exert a positive role on human health (Chang et al., 2019). For this reason, academic and industrial research is focused on identifying and testing novel microbial strains of gut origin for the development of next-generation probiotics (NGPs). Next-generation probiotics are microbial taxa that conform to the traditional definition of probiotics, but do not have an history of use for health promotion. They also fit well within the US Food and Drug Administration (FDA) definition of a LBP: "a biological product that: (1) contains live organisms, such as bacteria; (2) is applicable to the prevention, treatment, or cure of a disease or condition of human beings; and (3) is not a vaccine" (O'Toole et al., 2017). One of the main issues in NGP isolation and culturing is the identification of the best growth medium.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD project can be divided into the following activities, according to the Gantt diagram given in Table 1:

A1) **Literature screening** for the identification of most promising taxa
A2) **Selection of suitable culture media** for the isolation of microbial strains from human gut.
A3**) Isolation of strains from the human gut** of individuals with different diets, potentially promising as NGPs.
A4) **Characterization of strains at phenotypic and genomic level** and testing for the production of beneficial metabolites.
A5) **Evaluation of the possibility of industrial scale production** (e.g., evaluation of the best storage methods to preserve strains viability).
A6) **Evaluation of the most promising strains in a mouse model** to verify their effect on health and the ability to modulate the gut microbiome.
A7) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

Table 1 *Gantt diagram of the PhD project*

Activity	Months	2	4	6	8	10	12	14	16	18	20	22	24	26	28	30	32	34	36
A1)	**Literature screening**																		
	1) Identification of the most promising taxa and isolation methods																		
A2)	**Selection of suitable culture media for the isolation of strains from the human gut**																		
	1) test of culture media with different formulations in terms of vitamins, minerals and fatty acids																		
A3)	**Isolation of strains from the human gut**																		
A4)	**Characterization of strains**																		
	1) Phenotypic characterization 2) Genomic analysis 3) Beneficial metabolites production (e.g., short-chain fatty acids, gamma-aminobutyric acid)																		
A5)	**Evaluation of the possibility of industrial scale production**																		
	1) Evaluation of storage methods to preserve strains viability																		
A6)	**Mouse model**																		
A7)	**Thesis and Papers Preparation**																		

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Development of strategies for the adaptation of the livestock sector to the new climate regime with machine learning and artificial intelligence methods

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Tutor: Prof. Giovanni Chillemi

The objectives of this PhD program are aimed at identifying potentially useful strategies to reduce the environmental impact of the livestock sector. Using innovative technologies available in the field of precision livestock farming, aimed at improving nutrition management. And also, to identify food strategies (e.g. use of additives) that can reduce enteric emissions of CH_4 from animals.

Sviluppo di strategie per l'adattamento del settore zootecnico al nuovo regime climatico con metodiche di machine learning ed intelligenza artificiale

Gli obiettivi del presente percorso di dottorato mirano ad individuare delle strategie potenzialmente utili per ridurre l'impatto ambientale del settore zootecnico. Tramite l'utilizzo in campo di tecnologie innovative disponibili, nell'ambito della zootecnia di precisione, volte a migliorare la gestione dell'alimentazione. Ed inoltre individuare delle strategie alimentari (es. utilizzo di additivi) che possano ridurre emissioni enteriche di CH_4 dagli animali.

1. State-of-the-Art

The livestock sector is directly responsible for climate change as it must change the composition of gases in the atmosphere through GreenHouse Gas emission (GHG), such as methane (CH_4), carbon dioxide (CO_2), nitrous oxide (N_2O) and ammonia (NH_3). GHG emissions from the livestock sector can take place both directly (fermentation and manure) and speed up (with processing, fertilization, food production, etc.) (Cassandro & Finocchiaro, 2021). Of the GHGs produced by ruminants CH_4 is the most impacting because the effect on global warming is approximately 25 times that of CO_2 (Cassandro et al., 2013). The quantity of CH_4 produced by animals is influenced by many factors: species, production level, feed composition, the type of carbohydrates present, the level of ingestion, the degree of saturation of the lipids presents in the feed, some environmental factors, such as temperature (McAllister et al., 1996), and genetic factors, such as feed conversion efficiency. The enteric CH_4 emission from the livestock sector is equal to 39% of the CH_4 emissions attributable to animal production, and the livestock sector is responsible for about 14.5% of the total anthropogenic GHG emissions (Grossi et al., 2019). Al present, this impact is already important, but is further amplifying in the consequent demand of the growing world-wide for products of animal origin. The desirable reduction of CH_4 emissions would not only be beneficial for the environment, with the mitigation of the impact, but would lead to more efficient, less impacting, and therefore more sustainable animals. There is a direct link between the intensity of GHG emissions and the animal efficiency: the more productive the animal, the lower the environmental impact (per unit of product). Both the quality of management and the expression of the full genetic potential are necessary to increase the efficiency of production. Currently, CH_4 measurement techniques can be divided into direct and indirect (Okpara, 2018). The direct techniques are based on the use of sulfur hexafluoride (SF6), laser tracer, GreenFeed system and respiratory chamber, the latter considered the "gold standard". These techniques are able to measure emissions directly on animals using special equipment but have various disadvantages such as invasiveness on the animal routine and the need for animal training (e.g. respiratory chambers), and are also not very applicable on a large scale, for which are currently useful for research purposes. Indirect quantification methods include prediction equations based on animal phenotypes (e.g. milk fatty acids, body weight, milk production, dry matter ingestion) (Ellis et al., 2010; van Engelen et al., 2015). Compared to the direct ones, the latter can be applied on a large scale without interfering with the animal's routine and without being invasive. Furthermore, thanks to the advent of Precision Livestock Farming (PLF), today there is an enormous amount of data available on different phenotypic traits of the animal at an individual level, even if the costs are, for some types, still high. The use of direct methods on a large scale is inapplicable. Several studies suggest the usefulness of indirect methods for predicting methane emission, including milk fatty acid profiles and the evaluation of some components of the ration, such as NDF, ADF and lignin (Ellis et al., 2007; Knapp et al., 2014; Castro-Montoya et al., 2017). With PLF, it is now possible to know directly in the company the composition of the ration distributed using infrared spectroscopy. It has the advantage of being a multi-analytical technique that allows to predict multiple parameters at the same time, it is fast, non-destructive, precise, and economical, compared to other laboratory techniques (Yakubu et al., 2020). This technique could be adopted at a company level and facilitate self-control analysis, increasing the quality and food safety of animal products, if the costs of the equipment were even lower and the use of the tools were automatic.
In recent years, infrared spectroscopy is assuming more and more potential because the fields of application on

which it can be used are expanding. Currently, in the field of precision feeding (PF), portable NIRS instruments capable of analysing in real time the chemical-physical composition of the unifeed diet intended for animals are widespread. Knowing these aspects, in real time, allows you to promptly make changes, based on the real dry matter, to the distributed diet and, consequently, improve food efficiency without compromising the health and well-being of the animals. Improving food efficiency would lead to a reduction in the intensity of CH_4 (e.g. CH_4 / kg of milk produced), simply because fewer animals are needed to produce the same amount of milk. According to Knapp et al. (2014) practices aimed at increasing the quality of the diet showed a potential reduction of enteric methane of about 5% per unit of corrected milk.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) **Study of the art and framing of the problem**: how this sector becomes an actor in the production of GHG, in particular methane (CH_4).

A2) **Identification of a narrower range of wavelengths in the near infrared** and apply them in the field analyses. Firstly, this analysis will be conducted on already available data, applying ML (Machine Learning) methods to identify the most important wavelengths, to identify the fibrous fractions in the animal feed in a precise way (NDF, neutral clean fiber; ADF, clean acid fiber; ADL, clean acid lignin). After, a portable instrument will be applied in the field, and compared with laboratory analyses.

A3) **In vivo experimental tests** for the evaluation of additives (such as: essential oils, tannins, fats, marine algae, 3-nitroxypropanol (3NOP)) that can be useful to reduce enteric emissions of CH_4.

A4) **Training abroad**. Period abroad for the PhD program.

A5) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

Table 1 Gantt diagram for this PhD thesis project.

Activity / Months		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	***Study of the Art***																								
A2)	***Study of wavelengths in the near infrared for the determination of the components of animal feed***																								
A3)	***In vivo experimental tests***																								
A4)	***Training abroad***																								
A5)	***Thesis and paper preparation***																								

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Development of a food-waste derived bioink for sensing applications

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This PhD thesis research project is aimed at formulating the right mixture of natural polymers extracted from food waste in order to produce a printable hydrogel that can be used as substrate for growing plant cells that can be make it conductive and attached to a sensor to monitor the plants´ health status. In this way it is possible to have a controlled agriculture and therefore limit food waste.

Sviluppo di un bioinchiostro ottenuto a partire da scarti alimentari per applicazioni sensoristiche

Questo progetto di tesi di dottorato mira a mettere a punto una formulazione ottimale di un bioinchiostro composto da polimeri naturali ottenuti a partire da scarti alimentari in modo da ottenere un hydrogel stampabile che possa essere usato come substrato di crescita per cellule vegetali e che possa essere reso conduttivo per essere poi collegato ad un sensore in grado di monitorare lo stato di salute della pianta. In questo modo è possibile aver un tipo di agricoltura controllata e di conseguenza utile per limitare lo spreco alimentare.

1. State-of-the-Art

A new emerging approach to reach the "green" goal in the electronic field is the biological printing. The bioprinting, in fact, allows to deposit cells in a viscous biomaterial in a precise space thanks to the use of a computer-aided printer (Jovic et al. 2019). What a bioprinter need is a "bioink" which is composed of a viscous fluid, an hydrogel or a polymeric solution that can be loaded with cells (animal or plant) because mimics the ideal environment for cell culture (Montero et al. 2019). Other than polymers, which constitutes the supportive scaffold, they can contain additives like growth factors or signaling molecules, depending on their function (2). The majority of these materials can be plant-derived and consequently they can also be extracted from some of the food industry by-products. They have a great advantage due to their enhanced bioactivity, biocompatibility, biodegradability and mechanical stability (Yegappan et al. 2018). The main candidates are extracted both from land plants, such as cellulose, starch, rubber and pectin, but also from marine algae, including alginate, fucoidan, agarose and carrageenan (Jovic et al. 2019). In addition, these bioinks could be conductive, thanks to the use of nanoparticles that can be added to the ink´s formulation and this allows to incorporate these materials in sensors. Consequently, conductive hydrogels can respond to various elements such as temperature, pH, enzymes and also to the presence of pathogens (Wang et al. 2022). Depending on the type of nanoparticles present in the polymeric network of hydrogels (also called nanocomposite hydrogels),they can give different properties to the scaffold. For example, they can became electroconductive, thermally conductive, optically active and mechanically strong (Chakraborty et al. 2021). Manufacturing hydrogel-based sensors implies more demanding requirements in terms of mechanical performance, structure, chemical and thermal stability as well as other features because these kind of sensors must have an increased selectivity and functionality (Sun et al. 2021). One limit that can be found in this new technology, is given by the poor mechanical strength and biodegradation that can be a restraint for some application (Utech, Boccaccini, 2016), although, biodegradability is favorable if it is meant for agri-food application.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) **Literature review**: research of most recent literature in order to get to the right direction of studies. Start working in the laboratory: first developing and testing the right bioink formulation and its printability.
A2) **Assessment and printability test of the bioink:** this test will be performed using a 3D bioprinter with different nozzles in order to set the right printing parameters.
A3) **Testing plant growth in a conductive bioink:** at this step semiconductive nanoparticles will be added to the ink formulation and once the substrate is printed, plant cells will be added in order to see if it´s suitable for their growth.
A4) **Development and testing of a sensor attached to the hydrogel:** the sensor will be useful to monitor the plant growth and specifically the loss of nutrients.
A5) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

Table 1 Gantt diagram for this PhD thesis project.

Activity \ Months		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	***Literature review***																								
A2)	***Printability test***																								
	1) Rheological characterization																								
A3)	***Plant growth evaluation***																								
	1) Conductive bioink																								
	2) Plant seedling																								
A4)	***Sensor development***																								
	1) Sensor set up																								
	2) Growth monitoring																								
A5)	***Thesis and Paper Preparation***																								

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Applications of Cold Atmospheric Plasma as Green Technology for Food Shelf-life Extension

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This PhD thesis research project is aimed to investigate the applications of cold plasma technology on food preservation and modification and to increase the knowledge of its effects on different food matrices during production and processing.

Applicazioni del plasma freddo come trattamento *green* per il prolungamento della shelf-life degli alimenti

Questo progetto di ricerca di dottorato ha lo scopo di investigare le applicazioni della tecnologia al plasma freddo sulla conservazione e la stabilità di differenti alimenti, e di aumentare la conoscenza dei suoi effetti su diverse matrici alimentari durante la loro trasformazione e produzione.

1. State-of-the-Art

Many chemical, biological, thermal and non-thermal food processing have been introduced and applied in the food industry for preservation and decontamination (Zhang *et al*., 2019). However most of them are characterized by various criticality related to food safety and quality: some are not validated and approved by regulatory agencies, and have issues related to economic feasibility and environmental impact (Picart-Palmade *et al*., 2019). To address the existing issues green and non-thermal technologies which is cold plasma (in gaseous form)(Capelli *et al*., 2021) and plasma activated water (PAW) are being explored (Laurita *et al*., 2021).

Plasma (gaseous) is often referred to as the fourth state of matter, after solid, liquid and gas. It is generated by applying any kind of energy to the gas and that contains wide range of reactive species that can play a key role in various food applications. Promising results have been obtained in relation to the decontamination/detoxification of microorganisms, pesticides, food allergens, mycotoxins, and to the functionalization of food components and shelf-life extension (Luo *et al*., 2020). However, contrasting results have also been observed in terms of colour and lipid oxidation (Foligni *et al.*, 2022). Since plasma is mainly considered a very superficial treatment with low penetration depth, and given the encouraging results obtained on different products, a deeper understanding of drying efficiency, safety and food property modification is certainly needed. Inconsistencies in the obtained findings in the literature often result from the lack of process optimization and the use of different types of plasma devices, application modes, etc., which makes very difficult to compare data. Finally, exposure of the different products to the reactive species induced by cold plasma can potentially lead to the formation of toxic components. Currently, there are very few studies evaluating these aspects, which is absolutely necessary for the approval of cold plasma by governmental agencies. Further research in this direction is strongly recommended.

PAW is generated by exposing water to a plasma discharge that contains reactive species, creating an acidic environment that leads to changes in the redox potential and conductivity. Its application in the food sector showed promising results in relation to decontamination and detoxification purposes, and technological and functional modifications of foods. Moreover, the treatment showed good uniformity and environmental friendliness, proving to be a valid alternative to the conventional use of chemical and hydrothermal techniques. However, there are some issues to be addressed such PAW storability, safety of PAW treated food, scale-up and, because of the complexity of chemistry and process parameters and treatment tailoring in terms of its optimization and validation for different food products. Therefore, this PhD thesis project will be focused on the deepening the knowledge and clarifying various aspects of the effect of cold plasma aimed at food preservation and functionalization.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above, this PhD research project can be subdivided into the following activities, according to the Gantt diagram given in Table 1:
A1) **Literature review** of previous studies on the application of cold plasma in food (A1.1), properties and types of plasma (configuration, diagnostic methods and characterisation of plasma) (A1.2);
A2) **Application of PAW to modify food starches considering different starch types** (waxy maize, normal maize and potato) and to evaluate the effects of PAW on rheological and pasting (A2.1), structural and thermal properties (A2.2);
A3) **Application of cold plasma for fruit drying** with the aim to improve heat and mass transfer, investigating drying kinetics (A3.1) and measuring quality parameters (A3.2);
A5) **Application of cold plasma to extend the shelf-life of foods**, with objective the main investigating the ability of cold plasma to preserve foods from spoilage and quality degradation;
A4**) Inactivation of microorganisms from food by cold plasma,** with the aim of increasing thier biological safety
A6) **Evaluate the safety/toxicity, scalability and environmental impact of cold plasma,** contributing to the approval and acceptance of cold plasma for commercial purposes
A7) Writing and editing of PhD thesis, posters, scientific papers and and oral and/or poster communications

Table 1 *Gantt diagram for this PhD thesis project.*

Activity	Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	26
A1)	***Literature Review***																									
	1) Application of Cold Plasma																									
	2) Types and Diagnostic of Plasma																									
A2)	***Application of PAW for Starch Modification***																									
	1) Rhelogical and Pasting Properties																									
	2) Thermal and Functional Properties																									
A3)	***Application of Cold Plasma for fruit Drying***																									
	1) Drying Kinetics and Efficiency																									
	2) Quality Evaluations																									
A4)	***Application of Cold Plasma to Extend Shelf-life of Foods***																									
A5)	***Inactivation of Microorganisms Using Cold Plasma***																									
A6)	***Evaluation of Safety, Scale-up and Environmental Impact of Cold Plasma***																									
A7)	***Thesis and Paper Preparation***																									

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Integrated green strategies for the management, recovery and recycling of waste in a dairy factory

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Currently, unsold pasteurized milk cannot re-enter the food chain and is mostly destined to animal feeding, even though this causes a great depreciation of its value. Among milk components, proteins have unique properties from both the technological and nutritional point of view. This PhD project aims at developing novel non-food uses of casein recovered from unsold milk. In particular, it will focus on the development of a casein-based biomaterial suitable for different applications by modifying casein properties through chemical and enzymatic treatments. Different approaches will be adopted to obtain a biomaterial having targeted mechanical and thermal properties as well as good vapor-barrier properties necessary to satisfy minimal standards for real applications.

Strategie sostenibili integrate per la gestione, il recupero e il riciclo dei sottoprodotti in un'industria lattiero-casearia

Allo stato attuale, il latte invenduto non può essere reintrodotto nella supply-chain alimentare e viene per la maggior parte destinato all'alimentazione animale, anche se questo causa una forte svalutazione del prodotto. Tra i componenti del latte, le proteine possiedono proprietà uniche sia dal punto di vista tecnologico sia dal punto di vista nutrizionale. Questo progetto di dottorato mira allo sviluppo di nuovi usi non-food della caseina, recuperata dal latte invenduto. In particolare, si focalizza sulla produzione di un biomateriale casein-based con diverse applicazioni attraverso la modifica delle proprietà della caseina mediante trattamenti chimici ed enzimatici. Verranno adottati numerosi approcci per ottenere un biomateriale con mirate proprietà meccaniche e termiche, come anche proprietà di barriera al vapore, necessarie per soddisfare gli standard minimi per applicazioni reali.

1. State-of-the-Art

Both the increasing consumer awareness of environmental impacts of crop and food production as well as the current energy crisis have led the food industry to devise more sustainable strategies in manufacturing processes, packaging technologies and delivering steps of their products. Environmental problems involve many food sectors, including dairy, which is not exempt from losses and waste. Waste deriving from dairy industries is estimated to range from 4 to 11 million tons per year and, due to the high organic content, it represents a real threat for the environment (Ahmad *et al.*, 2019). Most of pasteurized milk is sold through large-scale retail channels and the amount that remains unsold is downgraded to "Animal by-products: Category 3 materials" (Reg. CE 1069/2009), no longer suitable for human consumption. This implies a great depreciation of a product that already caused a high environmental impact due to the production, processing and distribution.

Among milk components, proteins have unique technological properties, due to the open random coil structure of casein molecules and their ability to form intermolecular interactions. Therefore, the use of milk proteins in non-food areas is an innovative topic covered by the recent literature (Rebouillant & Ortega-Requena, 2015). Casein represents the main fraction (80%) of milk protein and it can be recovered with low-impact approaches, like isoelectric precipitation or rennet coagulation. The former allows to obtain insoluble casein which needs neutralization with alkali (e.g. NaOH) to obtain soluble caseinates.

These recovery methods allow to use milk casein for biomaterial production. In particular, Sodium caseinate has been used for film production with advantages and disadvantages. On the one hand it provides a barrier for gases, good tensile strength and good elongation attributes, but on the other hand it shows a high-water vapor permeability and does not prevent water diffusion (Daniloski *et al.*, 2021). Chen *et al.*, (2019) reported, among several advantages, a high level of biodegradability, with reduced environmental impact if compared to the use of synthetic polymers. It has been demonstrated that casein concentration adopted in film production process affect yield, thickness, and tensile strength. Data showed a positive correlation of the three parameters when increasing the concentration of casein up to 9.5% (Sabil *et al.*, 2021).

To improve film properties, some substances may be added. Plasticizers, such as glycerol or polyethylene glycol, have proven to improve flexibility of the protein network, whereas hydrophobic constituents, like beeswax or oils, improve water permeability. Different approaches can be used to produce films with Sodium caseinate (Belyamani *et al.*, 2014). Blown extrusion seems to be a highly efficient process with potential uses at large-scale level. However, at present, film casting is the most used process in lab-scale studies (Kandasamy *et al.*, 2021). Film casting involves solubilization of caseinate in water, addition of plasticizers, followed by casting and drying.

Based on this background, the aim of this PhD project will be the definition of modifications and additives necessary to obtain a casein-based material with suitable performances and setting a green protocol to obtain a film with reproducible features. As mentioned above, this PhD project is intended to find green, sustainable uses

for expired milk; since this product is classified as "Animal by-products", it will not be possible to use the derived casein-based film directly in contact with food. However, the chemical, mechanical and thermal characterizations of the modified casein will allow to identify suitable applications for the developed biofilm.

2. PhD Thesis Objectives and Milestones

The activities of this PhD project include five sections corresponding to the relative milestones as shown below and in the Gantt chart (Table 1):

A1) **Casein recovery and modification**: casein will be recovered from unsold milk by testing different approaches. After the recovery phase, a lyophilization process will be adopted to reach the correct amount of moisture and to produce a homogenous powder that will be used as the main ingredient in the formula of the biomaterial (A1.1). In order to obtain a matrix with suitable mechanical and physical properties, different crosslinking approaches will be evaluated, considering physico-chemical and enzymatical ones (A1.2). In the first case, casein will be exposed to heat, extreme pH values, oxygen or light, while in the latter two different types of microbial Trans-Glutaminase that catalyze the formation of covalent isopeptide bonds between glutamine and lysine residues will be evaluated (Raak & Corredig, 2022). A combination of the two approaches would be also considered.

A2) **Characterization of powders**: the powders produced in A1 will be characterized to define the gross composition (A2.1) and to validate the modifications carried out to promote crosslinking (A2.2).

A3) **Developing the biomaterial**: different additives will be tested in the formulation to create a biomaterial with good filming properties.

A4) **Production and characterization of casein-based film**: selected casein-based biomaterial with desired characteristics will be used to produce a film that will be characterized for their structural, mechanical and thermal properties to define the best application.

A5) **Writing and Editing**: scientific papers, poster or oral communications, and PhD thesis.

Table 1: Gantt chart for the PhD project.

Activities	*months*	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	***Casein recovery and modification***																								
A1.1	Casein recovery and lyophilisation																								
A1.2	Crosslinking with different approaches																								
A2)	***Characterisation of powders***																								
A2.1	Powder characterisation																								
A2.2	Validating of different crosslinking																								
A3)	***Developing the biomaterial***																								
	Additives behaviour in the biomaterial																								
A4)	***Production & characterization of the film***																								
	Properties investigation																								
A5)	***Writing and Editing***																								

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Fermentation of plant by-products by using probiotic microorganisms to obtain functional foods with potential health benefits

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This PhD thesis research project aims to assess the *in vitro* effect of fermentation on agri-food by-products, which can lead to the improvement of functionality and bioactivity, therefore enhancing their biological value to obtain functional foods. The valorization of by-products represents an interesting and cheaper source of potentially functional ingredients, while promoting sustainability and circular economy concept.

Fermentazione di sottoprodotti vegetali tramite l'utilizzo di microrganismi probiotici per l'ottenimento di alimenti funzionali con potenziali benefici sulla salute

Questo progetto di tesi di dottorato mira a valutare l'effetto *in vitro* della fermentazione di sottoprodotti vegetali, che può portare ad un miglioramento del valore biologico per ottenere alimenti funzionali. La valorizzazione dei sottoprodotti rappresenta una risorsa interessante e poco costosa di ingredienti potenzialmente funzionali, promuovendo al contempo il concetto di sostenibilità ed economia circolare.

1. State-of-the-Art

Over the last years, a growing consumption of fermented foods has been observed in Western countries, thanks to their positive health benefits. Currently, numerous fermented foods and beverages are present on the market, obtained by using a wide range of different raw materials, microorganisms, and procedures. In particular, the growing number of people with lactose intolerance, the unfavorable cholesterol content of fermented dairy products and the increasing popular trends such as veganism and vegetarianism, have pushed towards the development of novel plant-based fermented products. The non-consumed parts of plants are generally originated from primary agri-food production processes or are discarded by consumers due to their low acceptability. A recent report from FAO (FAO, 2019) estimated that one-third of edible parts of food is lost or wasted every year. The agri-food by-products represent an interesting and unexpensive source of added value compounds, such as peptides, carotenoids, dietary fiber and phenolic compounds; hence it is important to look for valid strategies to exploit such materials. Therefore, in a context of circular economy, the lactic acid fermentation can be a valid strategy to exploit and manage by-products as a potential source of low-cost substrates. Lactic acid fermentation of fruits and vegetable is the oldest method of food bio-preservation. The microorganisms employed in lactic acid fermentation are lactic acid bacteria (LAB), such as species of the genus *Lactobacillus*, *Leuconostoc*, *Weisella*, *Pediococcus*, *Streptococcus* and *Enterococcus*, which possess the Generally Recognized As Safe (GRAS) status and probiotic potential. During food fermentation, the metabolic activity of LAB leads to numerous and significant changes of the nutritional and functional properties of food, due to transformation of substrates and formation of bioactive and bioavailable end-products which can have beneficial effects on human health (Cunningham et al., 2021). These fermented foods, composed of bioactive compounds with demonstrated physiological health benefits beyond nutritive functions, are defined as functional foods. For example, bioactive peptides formed by LAB exhibit functional properties such as immunomodulatory, antithrombic and antihypertensive activities. Moreover, food fermentation enhances the microbial safety and extends the shelf life due to the synthesis of antimicrobial molecules, such as organic acids, diacetyl, ethanol and bacteriocins. It has been reported that fermented foods have antioxidant properties, providing protective action against oxidative damage which is involved in chronic diseases. Functional properties of fermented foods include bioactive peptide production, fibrinolytic activity, degradation of anti-nutritive compounds, antithrombic and antihypertensive activities, immunomodulation capacity, and anticarcinogenic ability (Tamang et al., 2016).

2. PhD Thesis Objectives and Milestones

According to the overall objective mentioned above, this PhD project can be subdivided into the following activities, according to the Gantt diagram given in Table 1:

A1) **Screening and determination of suitable plant by-products** to be used as substrate for lactic acid fermentation testing different combination of LAB strains and substrates (A1.2). Evaluation of kinetic of bacterial growth using different technique (A1.2) and optimization of parameters as temperature and time to enhance fermentation process (A1.3).

A2) **Characterization of fermentation using different methods** to detect and analyse nutritional changes (A2.1) and bioactive compounds produced during fermentation by LAB strains (A2.2).

A3) **Evaluation of physiological benefits** with a demonstrated impact on human health using *in vitro* (A3.1) and (possibly) *in vivo* approaches (A3.2).
A4) **Production and characterization of novel functional foods** and sensorial analysis; evaluation of rheology properties and consumers acceptance (A4.1).
A5) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

Table 1 Gantt diagram for this PhD thesis project.

Activity \ Months		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	***Sample Collection and Screening***																								
	1)Strain/substrate combination																								
	2) Bacterial growth																								
	3)Time/temperature optimization																								
A2)	***Characterization of Fermentation***																								
	1)Physicochemical evaluation																								
	2)Bioactive compounds																								
A3)	***Functional Health Benefits***																								
	1)Antimicrobial activity																								
	2)*In vivo* functional properties																								
A4)	***Final Product Evaluation***																								
	1)Sensorial analysis																								
A5)	***Thesis and Paper Preparation***																								

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Morphological and genetic characterization of *Phaseolus vulgaris* and *P. coccineus* landraces grown in the Aniene's Valley and evaluation of agronomic and nutritional performances of *Helianhtus tuberosus* accessions of different geographical origin cultivated in marginal environments

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This Ph.D. thesis research project aimed to valorise the agro-biodiversity and the typical agri-food production of Aniene's valley through the characterization and conservation of autochthonous bean landraces, which are now faced with the danger of extinction. Furthermore, to strengthen the local agricultural system, the present project aimed to introduce into cultivation in the Aniene' valley varieties/clones of Jerusalem artichoke (Heliantus tuberosus), a non-traditional species in this area, but that is often adapted to marginal environments. This species is a multi-purpose crop used for human food consumption, pharmaceutical applications, and biomass and bioenergy production.

Caratterizzazione morfologica e genetica delle varietà autoctone di *Phaseolus vulgaris* e *P. coccineus* tipiche della Valle dell'Aniene e valutazione delle "performances" agronomiche e nutrizionali di accessioni di diversa origine geografica di *Helianthus tuberosus* coltivate in aree marginali

Questo progetto di tesi di dottorato mira a valorizzare la biodiversità e le produzioni agro-alimentari tipiche della Valle dell'Aniene attraverso il recupero, caratterizzazione e conservazione delle varietà autoctone di fagioli, che ora sono a rischio di estinzione. Inoltre, per rafforzare il sistema agricolo locale, il presente progetto ha l'obiettivo di introdurre in coltivazione nella Valle dell'Aniene varietà/cloni di *Helianthus tuberosus*, una specie non tradizionale di questa area, ma che spesso si adatta ad ambienti marginali. Questa specie è considerata una coltura polivalente utilizzata per il consumo alimentare umano, nel settore farmaceutico e per la produzione di biomassa e bioenergia.

1. State-of-the-Art

The Aniene Valley, due to its highly natural aspects, orographic conditions and the sustainable management of the agricultural practices represents a territory particularly suitable for the development and application of activities for the conservation, characterization and valorisation of the natural and agricultural biodiversity. These activities are supported by the presence of a protected area, such as the Natural Regional Park of Monti Simbruini. In this context, the "Casa delle sementi della Valle dell'Aniene" project (www.arsial.it) is particularly interesting because aimed, on one hand to develop *in situ*/on farm and *ex-situ* conservation strategies of autochthonous genetic resources of agricultural interest, and on the other hand to valorise the typical agri-food productions, with the involvement of local farmers. Among the various plant species of agricultural interest, bean landraces play a significant role in the agricultural ecosystem of the Aniene's Valley due to their economic, socio-cultural and landscape-environmental values (Piergiovanni et al., 2006; Alimonti, 2010). In the Regional Voluntary Register are listed six *P. vulgaris* and one *P. coccineus* landraces, still cultivated in the Aniene's Valley, at high risk of genetic erosion (www.arsial.it). Although bean landraces are important component of agro-biodiversity in Italy, few studies have been focused to understand how genetic diversity is structured within a given landrace and how the natural processes such as selection, genetic drift and migration and the agriculture practices interact to shape and maintain within- and among-landrace population diversity (Tiranti and Negri, 2007).
In recent years, Jerusalem artichoke (*Helianthus tuberosus*) has been indicated as suitable and sustainable crop in marginal areas due to its high adaptability to diverse environments, good freezing and draught tolerance, and high resistance to pests and plant diseases (Liava et al., 2021). Jerusalem artichoke is a multi-purpose crop used mainly for human food consumption and diverse pharmaceutical applications, as both above- and underground parts of the plant contain various chemical constituents such as proteins, glucose, fructose, sucrose and inulin (Liava et al., 2021; Sawicka et al., 2020). Moreover, this species is considered an emerging energy crop for bioethanol and biogas production due to its high biomass yield (Rossini et al., 2019). The tubers are rich in inulin, a fructose polymer, which is used as a prebiotic, fat replacer, sugar replacer, and for the development of functional foods due to its beneficial role in gastric health (Yang et al., 2015). Tubers, leaves and stems contain several bioactive compounds, including polyphenols and flavonoids, which confer to their extracts high antioxidant and antimicrobial activities (Chen et al., 2013; Sawicka et al., 2020).

2. Ph.D. Thesis Objectives and Milestones

Within the overall objectives mentioned above this Ph.D. thesis project can be subdivided into the following activities according to the Gantt diagram given below.

A1) **Farm census** in the Aniene's Valley where are grown the autochthonous bean landraces and collection of the seeds (A1.1) to integrate also the activities planned within the "Casa delle sementi" project.

A2) **Seed morphological analysis** for each collected accession (farm) of the bean landraces according to the International Board for Plant Genetic Resources (IBPGR) descriptors (A2.1).

A3) The **biochemical characterization** will concern the analysis of the main seed proteins, phaseolins and phytohemagglutinins (A3.1), while the **genetic characterization** will be carried out by using SSR molecular markers (A3.2).

A4) The **agronomic evaluation** of *H. tuberosus* accessions will be performed in two different sites: 1) a mountain area (Vallepietra, RM), in the Natural Regional Park of Monti Simbruini, in which Jerusalem artichoke has never been cultivated before; 2) a hillside area where it is noticed that this crop growth well (A4.1). **The chemical and nutraceutical characterization** (A4.2) of the tubers and aerial parts of the different accessions growth in the two different sites will be performed by determining the main bioactive compounds and the antioxidant and antimicrobial activities of their extracts.

A5) **Writing and Editing** of the Ph.D. thesis, scientific papers and oral and/or poster communications.

Table 1 *Gantt diagram for this PhD thesis project*

Activity	Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	***Farm census***																								
	1) Seeds collection																								
A2)	***Morphological analysis***																								
	1) Quali-quantitative seed descriptors according to IBPGR																								
A3)	***Biochemical and genetic characterization***																								
	1) Seeds protein analysis																								
	2) Molecular markers analysis (SSR)																								
A4)	***Agronomical, chemical, and nutraceutical evaluation of different Helianthus tuberosus accessions***																								
	1) Growth of *Helianthus tuberosus* accessions in different sites																								
	2) Chemical analysis and antioxidant activity of tubers and aboveground (scrap)																								
A5)	***Thesis and Paper Preparation***																								

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Analysis and characterization of Sicilian cereals landraces to destined at malting and brewing industry

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This Ph.D research project aims to provide a comprehensive view of the production of malt from old landraces of cereals, identifying the varieties that have the best aptitude for malting and evaluating their application in brewing. The projects aim also to study and to characterize malting conditions that are characterized by high quality, new sensory profiles, and strong link with the territory.

Analisi e caratterizzazione delle varietà autoctone siciliane di grano e orzo da indirizzare all'industria del malto e della birra

Questo progetto di ricerca di dottorato si propone di fornire un quadro chiaro e completo della produzione di malto da antiche varietà autoctone di cereali, individuando pertanto le varietà che hanno la migliore attitudine alla maltazione e valutandone l'applicazione nella produzione della birra. Il progetto si propone inoltre, lo studio e la caratterizzazione di nuovi processi di maltazione al fine di ottenere tipologie di malto che si contraddistinguono per l'alta qualità, nuovi profili sensoriali e un forte legame con il territorio.

1. State-of-the Art

In the last two decades, in Italy, the constant development of the craft beer sector and the spread of craft breweries has led to a growing interest in the study of the use of old landraces of cereals for the production of malt and beer. Despite the fact that Italian beer is known for its great quality, most Italian breweries and microbreweries currently use foreign ingredients, highlighting that the beer ingredient's self-sufficiency is the main problem that affects the Italian beer supply chain.

Therefore, the analysis and characterization of old cereals landraces to be used for malt and beer productions is the key element to guiding the sector towards sustainable growth that ensure the production of raw materials within national borders and products innovation. Moreover, the inclusion of old landraces in the beer production process achieves the European sustainable goals which aims to promote the short supply chain, protect biodiversity, preserve the soil, reduce transport and emissions with the purpose to increasing the well-being and improving the health of current and future generations.

Recently there has been a growing interest in the use of unconventional cereals for beer production and some studies have been carried out on Italian varieties of cereals to evaluate their malting and brewing performance (Alfeo et al., 2018, 2021; Blšáková et al., 2021; Faltermaier et al., 2014; Mascia et al., 2014). Many old landraces of wheat showed great aptitude for malt production, furthermore they are all characterized for a high rusticity and adaptability to the territory which make these cereals the ideal raw materials for the development of organic and sustainable productions ensuring the reductions of fertilizer and herbicides. Besides the use of these varieties for the production of malt, there is also a new trend for using some of the old landraces in beer production as an unmalted supplement to improve sensory and healthy characteristics in beer. About this issue, Mascia et al., 2014 made a comparison between craft beer brewed with the unmalted Italian wheat cultivar Senatore Cappelli and two other industrial wheat beers, which the results showed a higher polyphenol content and more balanced taste in the first case.

The Southern Italy, and in particular the Sicily, can be considered a remarkable part in terms of old landraces of wheat, barley and spelt. The high biodiversity of the Sicilian cereals was ensured during the centuries by various climatic, altimetric and pedologic conditions, which are the characteristics of the Sicily Island.

In a recent study, Alfeo et al., 2021 investigated the physical and chemical properties of malt obtained from eight old Sicilian wheat varieties in order to evaluate their suitability in the production of beer. Considering the most important parameters commonly used to define malt quality, some varieties of common and durum wheat such as Romano, Maiorca, Bufala nera corta and Bufala lunga corta showed excellent characteristics.

Among the most important parameter for malters and brewers to evaluate the malt quality, these cultivars showed high value of extract even above 80% db. The malt extract is one of the most important quality parameters for understanding malt suitability in beer production; it measures the amount of carbohydrates released from malt during mashing that is related to the grain modification and enzyme activity. Malt extract is also a general indicator of brewhouse yield and performance. Also the protein content of the majority of these varieties enters in an optimal range for beer cereals. The protein contents influence the choice of malt in beer production and it is known that also influence several quality attributes of wort and beer. In particular, the protein content, type and dimensions direclty influence the filtration during wort production, the wort fermentability, the foam stability and haze in beer and wort (Faltermaier et al, 2014). Many other quality parameters such as diastatic power, alpha and beta amylase

content, beta glucan content, Kolbach index, fermentability have values close to the optimal ones for beer cereals. The quality attributes of malt and beer depend on several morpho-physiological grain parameters like kernel size, shape, grain hardness, moisture, dormancy, and microbial contamination but also on biochemicals aspect as protein content, β-glucan, starch (Rani et al., 2021); therefore, understanding the correlation between these aspects is important in the selection of new malt varieties.

2. PhD Thesis Objectives and Milestones

To achieve the objectives defined by the Ph.D research project, it is planned to carry out the following activities according to the Gantt diagram given in Table 1:

A1) **Analysis of state of the art**.

A2) **Preliminary evaluation of unmalted wheat and barley landraces** will be performed to identify the cultivar with greater attributes for malting; therefore will be evaluate the morpho-physiological and biochemical aspects of unmalted grains identifying thus that have a greater attribute for malt production.

A3) **Pilot-scale malting trials** will be performed in an automatic micro-malting system at the University of Palermo to better understand each variety's response to the different parameters adopted in the steeping, germination and kilning phases, thus identifying the better malting program related to the cultivar.

A4) **Physico-chemical evaluation of malts and wort** through the analysis of the most important quality index for malters and brewers as the content of extract, total starch, total protein content, soluble protein content, FAN, Kolbach Index, β-glucan content, diastatic power, α-amylase and β-amylase content, β-glucanase content. The analysis of malt and wort samples will be performed according to the methods of Analytica European Brewery Convention (EBC).

A5) **Pilot-scale brewing, physico-chemical and sensory evaluation of beer**. The malt with the highest quality attributes will be tested in beer production that will occur in a microbrewery pilot plant at the University of Palermo. The physico-chemical aspects of beers will be evaluated and compared with sensory evaluation performed by trained panelists.

A6) **Participation in conferences, preparation of scientific articles and thesis**.

Table 1. *Gantt diagram for this PhD project.*

Activity / Months		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
A1)	**Analysis of state of the art**	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
A2)	**Preliminary evaluation of wheat and barley landraces**			■	■	■	■	■	■	■	■	■																									
A3)	**Pilot-scale malting trials**										■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■										
A4)	**Physico-chemical evaluation of malts**													■	■	■	■	■	■	■	■	■	■	■	■	■	■										
A5)	**Pilot-scale brewing, physico-chemical and sensory evaluation of beer**																							■	■	■	■	■	■	■	■	■	■				
A6)	**Participation in conferences, preparation of scientific articles and thesis**						■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■

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Use of Wild Edible Plants as Environmental Indicators and as Ingredients for the Creation of new Functional and Enriched Products

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This PhD thesis research project aims at identifing edible wild plants to be used both as additional ingredients for the production of new functional and/or enriched food products and as environmental indicators to assess the level and type of contamination in a given area.

Uso delle piante selvatiche commestibili come indicatori ambientali e come ingredienti per la creazione di nuovi prodotti funzionali e arricchiti

Il seguente progetto di ricerca per la tesi di dottorato mira a identificare piante selvatiche commestibili da utilizzare sia come ingredienti aggiuntivi per la produzione di nuovi prodotti alimentari funzionali e/o arricchiti, sia come indicatori ambientali per valutare il livello e il tipo di contaminazione presente in una determinata area territoriale.

1. State-of-the-Art

Wild edible plants (WEPs) suitable for human consumption are non-cultivated plants that grow wild in nature. They play an important role in the prevention and treatment of many diseases. This is possible thanks to the presence of beneficial molecules, in particular vitamin E, vitamin C, carotenes, xanthophylls, phenolic compounds, organic acids and minerals, which reduce the risk of developing diseases such as diabetes, cancer, coronary heart disease and ageing (Petropoulos S.A et al., 2019) . Vitamin E comprises two different classes of molecules; tocopherols and tocotrienols. Tocopherols exist in nature in multiple stereoisomer forms and in particular as: α-tocopherol (α-T), β-tocopherol (β-T), γ-tocopherol (γ-T) and δ-tocopherol (δ-T). Tocotrienols also exist in different stereoisomer forms, the most common being: α-tocotrienol (α-T3), β-tocotrienol (β-T3), γ-tocotrienol (γ-T3) and δ-tocotrienol (δ-T3). Vitamin C, or ascorbic acid, is a water-soluble vitamin with antioxidant properties. Many works in the literature show that edible wild plants have higher amounts of vitamin C than conventional vegetables. The main carotenoids present in WEPs are α-carotene, β-carotene, lutein and β-cryptoxanthin. α-Carotene and β-carotene are precursors of vitamin A, also known as retinol. In this respect, the scientific community has ascertained the function of β-carotene as a precursor to retinol and its role in achieving the recommended total intake of vitamin A. Xanthophylls act as antioxidant molecules by protecting WEPs from excessive exposure to sunlight. Finally, polyphenols are a class of compounds with a high antioxidant power and are divided into four classes: phenolic acids, flavonoids, stilbenoids and lignans. WEPs, therefore, can be regarded as important sources of bioactive and high-nutrient compounds that are easily accessible and cheap (Luta G *et al*., 2020). The addition of WEPs in new food formulations can enable the production of functional and/or enriched foods (Panfili G *et al*., 2020). Moreover, the addition of WEPs in regularly consumed food products, such as pasta, biscuits and juices, can be a healthy and cost-effective strategy to improve the intake of nutrients and bioactive compounds. Fortification of foods with wild edible plants has not received much attention from the scientific community. Therefore, it is interesting to study new formulations that allow the production of enriched foods by assessing their nutritional quality, together with technological and sensory aspects. In recent times, the intensification of agriculture has led to the widespread use of multiple chemical preparations. Examples are pesticides, herbicides and other industrial products. These substances are very useful in controlling pests and diseases, but on the other hand have negative effects on the environment and human health. Classes of molecules such as chlorinated hydrocarbons, polychlorinated biphenyls, dioxins, furans and heavy metals present in formulations used in agriculture can be released into the environment resulting in soil and water contamination (Rimayi C *et al*., 2022). These molecules known as persistent organic pollutants (POPs) are included in the list of the most toxic agents called "the dirty dozen" by the Stockholm Convention on POPs. To these contaminants can be added polycyclic aromatic hydrocarbons (PAHs), which are released into the atmosphere from natural or anthropogenic sources and can be deposited, carried by atmospheric particles, on soil and plants. Since POPs are highly stable molecules with an apolar character, they take a long time to decompose and consequently accumulate in the environment. Despite recent bans on the use of these substances in agriculture, residues of these molecules are still found in plants and animal tissues, which are the main sources of human exposure. As a result, WEPs can present very different types of contaminants and contamination levels depending on the area and the type of emission sources present. Some authors have used wild edible plants to assess the pollution of a given area and consequently also of the food in that area (Fismes J *et al*., 2004). Plants can absorb multiple contaminants from the soil through their roots, including heavy metals, dioxins and polychlorinated biphenyls. Not only soil, but also outdoor air is a potential source of contamination for wild plants. In particular, for broad-leaved plants and vegetables, which have large broad leaves, the latter act as a 'filter' for the air passing through them, resulting in the accumulation of high-

diameter particulate matter (Shi J *et al*., 2017) .It is therefore possible to use WEPs as environmental indicators of environmental pollution for a given geographic area.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) **Bibliographic research**: work planning and international paper analysis.
A2) **Identification and classification of the WEPs under study**.
A3) **Choice and characterization of sampling areas**: choice of different sampling arrays to compare relative contamination levels.
A4) **Development of analytical methods**: a) Development of analytical methods suitable for the analysis of contaminant molecules (chlorinated hydrocarbons, polychlorinated biphenyls, dioxins, furans and PAHs) and heavy metals in plants. b) Development of analytical methods suitable for the analysis of bioactive molecules (such as tocols and vitamin C, polyphenols, carotenes and xanthophylls) in plants.
A5) **Sampling activities**: sampling of soil and WEPs species in different territorial areas.
A6) **Analytical activities**:a) analyses for the quantification of bioactive molecules b) analyses for the determination and quantification of different contaminants.
A7) **Study of new food formulations**: a) Determination of the quantities and physical state of the WEPs to be added in the formulation. b) Realization of the new products through the use of pilot plants.
A8) **Characterization of the new products**: study of compositional, nutritional and sensorial aspects.
A9) **Development of the obtained data**.
A10) **Reports, thesis and paper preparation**: throughout the research period, reports on the obtained results will be made and scientific paper will be prepared.

Table 1 Gantt diagram for this PhD thesis project.

Activity	Months																	
	2	4	6	8	10	12	14	16	18	20	22	24	26	28	30	32	34	36
A1) Bibliographic research																		
A2) Identification and classification of the WEPs under study																		
A3) Choice and characterization of sampling areas																		
A4) Development of analytical methods																		
A5) Sampling activities																		
A6) Analytical activities																		
A7) Study of new food formulations																		
A8) Characterization of the new products																		
A9) Development of the obtained data																		
A10) Reports, thesis and paper preparation																		

3. Selected References

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Plant health monitoring of crops of high territorial relevance, identification with advanced diagnostic tools and design of sustainable prevention systems

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The research will be organized in order to monitor the phytosanitary status of the three relevant crop species in Italy (*Solanum tuberosum*, *Solanum lycopersicum*, *Triticum durum*) and to suppress the plant pathogens linked to the cultivated species, while improving the quality of the soil by applying sustainable agricultural practices. The work will be carried out in different farms in the area of Tuscia, Province of Viterbo, depending on crop species that are going to be examined. The research will be held: a) In field; b) In laboratory (controlled conditions).

Strategia sperimentale per monitorare la salute delle colture fortemente rappresentate sul territorio italiano

La ricerca sarà organizzata al fine di monitorare lo stato fitosanitario delle tre specie vegetali rilevanti in Italia (*Solanum tuberosum*, *Solanum lycopersicum*, *Triticum durum*) e per sopprimere i patogeni legati alle specie coltivate, migliorando al tempo stesso la qualità del suolo applicando le pratiche agricole sostenibili. I lavori verranno eseguiti in diverse aziende agricole della Tuscia, Provincia di Viterbo, a seconda della coltura specie che verranno esaminate. La ricerca si terrà: a) In campo; b) In laboratorio (condizioni controllate).

1. State-of-the-Art

Many conventional agricultural practices are putting pressure on the environment causing soil degradation, water shortages, pollution and loss of natural habitats and biodiversity. Basing agriculture on chemical fertilizers and pesticides has serious consequences on public health and environment and plant health as well as climate changes. Innovative agricultural plans and technologies are crucial for a development of sustainable agricultural system, which implies environmental, social and economic sustainability. The New European Green Deal represents new efforts and actions of EU in order to reflect an increased level of ambition to reduce significantly the use and risk of chemical pesticides, as well as the use of fertilizers and antibiotics. As for the purpose of this project three agricultural crop species previously mentioned will be examined, the following challenges arise: Investigation of effects of compost and alternative agricultural practices on disease suppression in potato, tomato, wheat and caused by major soil-borne pathogens. The importance of these crops is reflected by the amount of annual production in Italy. The statistics of annual production are provided by the National Institute of Statistics (ISTAT). Late blight of potato and tomato is a common disease caused by *Phytophthora infestans* which is considered as a highly aggressive and destructive pathogen. Additionally, foot rot of wheat is a disease spread in all Italian cereal areas and it is caused by various fungi species: *Microdochium nivale*, *Bipolaris sokokiniana*, *Fusarium spp*. Some of alternative agricultural strategies will be examined in order to supress plant pathogens and soil-borne. Their impact on soil conditions and mentioned crop species as well as their effectiveness in disease suppression will be studied in detail for the purpose of the project.

2. PhD Thesis Objectives and Milestones

Taking into account the aim of the PhD project mentioned above we can outline main objectives:

A1) Territorial survey on some diseases of horticultural species, in this case potato (*Solanum tuberosum*), and wheat (*Triticum durum*) in the area of Tuscia, Province of Viterbo and sampling of these crop species through different phenological phases.
The sampling of potato (*Solanum tuberosum*) will be carried out once in a year in the phase of flowering or maturation of tubers, while the sampling of wheat (*Triticum durum*) will be done three times in a year following all the phenological phases: 1. Tilling; 2. Rising; 3. Ripening. The sampling of both crop species will be done in order to analyze the microbiota surrounding the rhizosphere. The plots are divided according to the treatments used (organic compost and mineral fertilizer).

A2) Territorial survey on some diseases of tomato (*Solanum lycopersicum*) in the coastal area of Lazio, sampling of these crop species taking into account disease symptoms present on leaf area.
The sampling will be carried out in field selecting the plants showing disease symptoms and collecting soil samples in order to determine its conditions.

A3) Isolation and characterization of microbiota associated to crops mentioned above by using NGS technique and bioinformatics and metagenomic tools

The microbiome analyses will be done from fine soil particles collected around the root system and soil samples collected from at the depth of 10-20cm. The NGS reactions are going to be performed followed with

sequencing and bioinformatic analyses by using Nucleotide blast program and MEGAN Community Edition (version 6.22.2.) in order to determine the sequences and taxonomy of pathogens isolated from our samples.

A4) Analysis of diffusion of *Phytophthora infestans* propagules by captaspore in tomato cultivation

The captaspore (Lanzoni VPPS 2000 sampler), supplied by the DIBAF for flight analysis of the spores in our case of *Phytophtora infestans* will be placed in the vicinity of the field trials in June and in early July. Every week the sampling tape will be taken and changed to a new one for sampling the next week.

A5) Management of pathogens in sustainable way by decreasing the utilization of pesticides and their residues using alternatives

Investigation of compost-based sustainable alternatives to improve the soil quality and effectiveness in suppression of plant pathogens. Several experiments are going to be carried out in order to examine the right management strategies of pathogens in sustainable way by using alternatives. The formulations and proportion of the alternative strategies are still to be defined.

A6) Writing and Editing of the PhD thesis, scientific papers and oral and/or poster communications

Table 1: *Gantt diagram for this PhD thesis project.*

	Activity Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	30
A1)	**Territorial survey and sampling**																									
	1) *Solanum tuberosum*																									
	2) *Triticum durum*																									
A2)	***Territorial survey and sampling of Solanum lycopersicum***																									
A3)	***Characterization of microbiota***																									
	1) NGS analyses																									
	2) Bioinformatic and metagenomic analyses																									
A4)	**Analysis of diffusion of *Phytophthora infestans* propagules by captaspore in tomato cultivation**																									
A5)	***Management of pathogens***																									
A6)	***Writing and editing of PhD thesis, papers***																									

The expected findings will be convenient to point out that along the agricultural production chain, the crops pass through critical phases from the point of view of the healthiness and the quality of the product, which is influenced in a great part by the management and agricultural practices used in food production. The supposed results will be also functional to underline the correlation between soil health and health of the plant as well as importance of soil conditions and application of innovative and sustainable agricultural practices in suppression of soil-borne pathogens.

3. Selected References

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Development of methods and technologies for the specific inhibition of enzymes relevant for low colour stability of fruit juices and nectars

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This PhD thesis research project is aimed to develop strategies and test new technologies for the inhibition of enzymes relevant for colour stability in fruit juices and nectars. In particular, red fruits will be used for producing nectars and juices as well as thermal and non-thermal technologies will be tested.

Sviluppo di metodi e tecnologie per l'inibizione specifica di enzimi rilevanti per il colore di succhi e nettari di frutta

Questo progetto di tesi di dottorato mira a sviluppare strategie e testare nuove tecnologie per l'inibizione di enzimi rilevanti per la stabilità del colore in succhi e nettari di frutta. In particolare, i succhi e i nettari saranno ottenuti da frutti rossi e sa ranno testate tecnologie termiche e non termiche sui prodotti ottenuti.

1. State-of-the-Art

The fruit juice industry is interested in increasing stability, since a longer shelf life would reduce production, transportation, and storage costs significantly. New technologies which would prevent the need for freezing could offer annual potential savings of almost 10 Mio € (AIJN 2016). In addition, this would also take a significant load from the environment. Moreover, the colour stability of red fruits is an important quality marker which can be affected depending on the method of preservation or lack of. This project aims therefore at elucidating the causality of the low colour stabilities associated with red fruit juices, in particular the role played by enzymes in the change of colour during storage. A second major focus is placed on preservation technology. HiStabJuice has a strong focus on endogenous enzymes (polyphenol oxidases peroxidases, ascorbic acid oxidases, ß-glucosidases) that are the presumed key players for colour degradation as many of them seem to survive current preservation methods that are optimized rather for the inactivation of microorganisms than of enzymes (Cao et al., 2010). HiStabJuice aims at the identification and characterization of endogenous enzymes, which show (at least partial) resistance to heat treatments or other preservation methods and thereby contribute to colour degradation, at the biochemical and molecular level in juices and nectars of color stable and colour unstable fruits.

2. PhD Thesis Objectives and Milestones

Figure 1 Sample preparation and processing

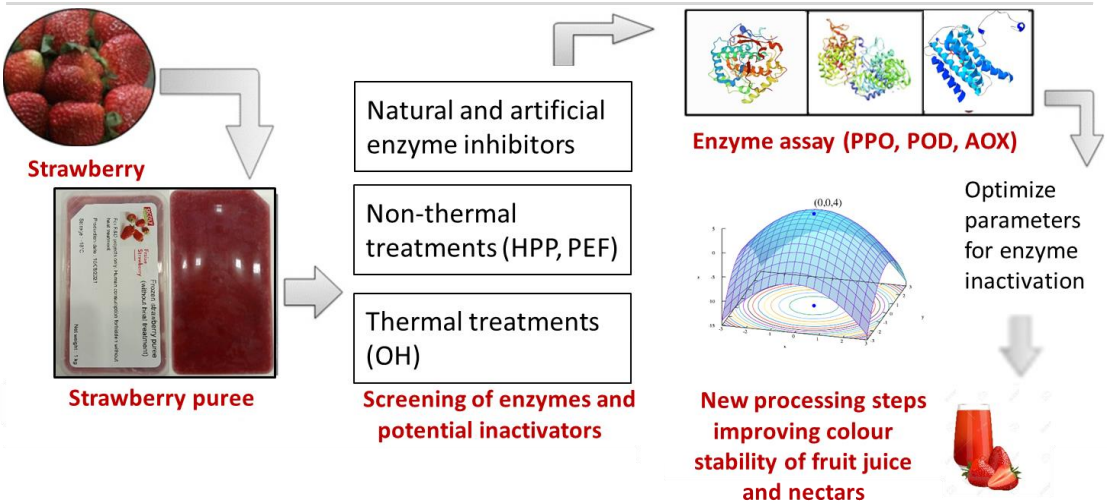
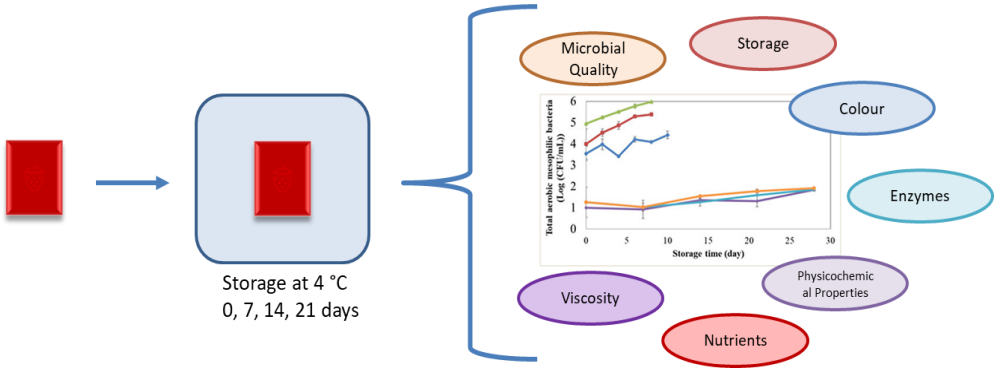


Figure 2 Shelf-life and evaluations



1. To establish methods for the inactivation of enzymes (PPO, POD, AOX, beta-Glucosidase) via non-thermal and thermal treatments (OH, HPP, PEF)
2. To improve shelf life of juices and nectars by optimized technology (proteases, glucose oxidases, exclusion of oxygen),
3. To produce juices and nectars and to evaluate storage stability,
4. To test natural and artificial enzyme inhibitors
5. To optimize assays for the determination of the enzyme activities

Expected Results

1. Optimized process parameters of conventional and not conventional treatments for appropriate inactivation of endogenous enzymes relevant for colour instability,
2. Optimized processing steps for increased shelf-life of fruit juices and nectars

Table 1 Gantt diagram for this PhD thesis project

Activity / Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
Training at UP																																				
List of most promising inhibitors providing colour stability (Ranking of best 10)																																				
Kinetics of at least 3 enzymes relevant for colour loss – exchange in SSICA																																				
Kinetics of at least 3 enzymes relevant for colour loss																																				
Optimized parameters for enzyme inactivation to increase colour stability																																				
Test methods for the inactivation of enzymes via thermal treatments (OH) exchange in CTCPA																																				
Test methods for the inactivation of enzymes via thermal treatments (PEF) exchange in ELEA																																				
Test methods for the inactivation of enzymes via non-thermal treatments (HPP)																																				
New processing steps improving colour stability of fruit juice and nectars																																				

Results: 2 Abstracts submitted for international conferences (oral presentation), Participated in PEF summer school 2022

3. Selected References

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Cell cultures of Mediterranean Plant Species Involved in the Production of Novel Food

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This Ph.D. thesis research project aims to develop an experimental protocol that allows the use of cell cultures Mediterranean plant species, for the analysis and the extraction of secondary metabolites in order to employ them as novel food with beneficial properties on human health.

L'uso di colture cellulari di specie di piante mediterranee coinvolte nella produzione di novel food

Questo progetto di tesi di dottorato mira a mettere appunto un protocollo sperimentale che permetta l'uso di colture cellulari di specie di piante mediterranee per l'analisi e l'estrazione di metaboliti secondari da poter impiegare come "novel food" che possano avere proprietà benefiche per la salute umana.

1. State-of-the-Art

In the last years, the demand for plant-based food has increased as it is considered healthier and more sustainable than animal-based food. Nevertheless, crop farming for human consumption accounts for almost one-third of the impact that food loss and waste have on climate change. Furthermore, recent trends claim that the number of people on earth is set to increase from 7 to 10 billion by 2050 and therefore it will be challenging to supply the world population with a sufficient amount of high-quality food. Here, plant cell culture (PPC) may represent a new approach to consider for plant-based food production as this method can be used to produce food additives and healthy ingredients. Indeed, there has already been an extensive adoption of plant cell culture for the cosmetic industry whereas the idea of using PPCs for the food industry has only recently attracted attention. This will require a full comprehension of the effects that these advanced methodologies can have on human well-being (Gubser et al., 2020). The commercial products derived from PPCs are mainly secondary metabolites (SM) which are crucial for several biological activities concerning plants and humans. SMs mostly involved in plant-based food and particularly abundant in Mediterranean fruit trees' species, such as apple, pear, lemons, are polyphenols and terpenes. Polyphenols can be distinguished into three sub-classes: phenolic acids, flavonoids, and tannins. These metabolites are widely distributed in different parts of the plant such as seeds, leaves, roots, and stems and they have been often proposed as food supplements as they are characterized by anti-inflammatory, anticancer, and antihypertensive properties that are essential for the human diet. Terpenes constitute a group of natural compounds largely diversified in their structures and functions. These compounds are very well-known to have a peculiar fragrance that suits them perfectly as food additives. Moreover, they have been discovered to have numerous positive effects that make them appropriate as treatments for respiratory, nervous, and cardiovascular disorders (Chiocco et al., 2021). It has also been demonstrated that PPCs can be engaged not only for the extraction of SMs but also as "novel food". Eibl et al. (2018), demonstrated that it is possible to produce 70% dark chocolate bars from *in vitro* cocoa powder obtained by freeze-dried cell cultures. Generally, PPCs products are based on plant suspension cells grown from two types of callus cultures, as shown in Figure 1.

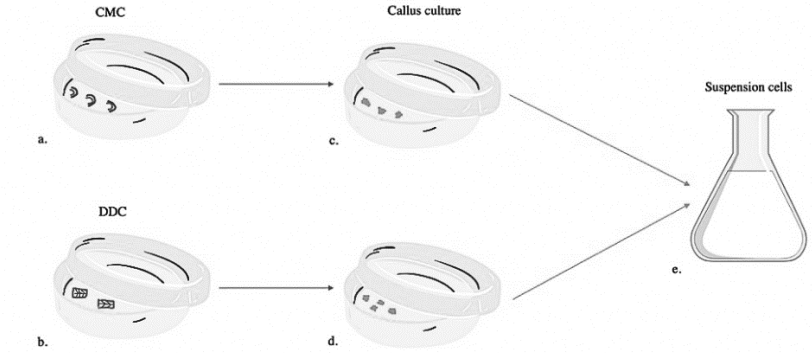

Figure 1 a. Cambium meristematic cell-derived callus culture (CMC); b. Dedifferentiated cell-derived callus culture (DDC); c., d. Callus culture; e. Suspension cells.

PPCs are considered a suitable alternative to crop farming since there is no seasonal dependence on *in vitro* production of SMs whose production will be much less time-consuming. Moreover, plant cells are mostly cultivated in media consisting of very few and simple ingredients such as salts, sugar, and some low concentrations of vitamins and phytohormones. Therefore, the water needed is reduced and fertilizers, as well as pesticides, are not required preventing a negative impact on the ecosystem. In this scenario, it is also worth to highlight that plants' secondary metabolites can be obtained by food industry wastes and by-products boosting the shift from a linear economy model to a circular economy one. Indeed, the greatest amount of SMs have been identified in components of plants and fruit that are usually discarded, such as leaves, seeds, peels, and pomace. Furthermore, the adoption of certain substances, called elicitors, within PPCs induces a higher delivery of healthy compounds reducing or even suppressing the presence of those that are harmful to the consumer. Nonetheless, the reduction of dangerous substances is also due to the replacement of the synthetic SMs which help to avoid the high content of extraction solvents that can be toxic to human health (Eibl et al. 2018). Although plant cell cultures offer an attractive option for plant-based food backgrounds, they must align with human health safety limits imposed by the EU Regulation 2015/2238 on novel food. Given this, one of the most important risks to consider for consumers is the use of synthetic growth regulators within plant cell growth media. Until now it has not been clear which amount of the added growth regulators are still present in the harvested biomass at the end of the cells' cultivation. However, the omission of hormones led to a significant decrease in biomass accumulation after a few subcultures. Therefore, it is necessary to evaluate the acute toxicity of plant cell cultures. In addition, taste, odor, and consistency need to be tested for consumer acceptance (Hakkinen et al., 2020).

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:
A1) Determination of *in vitro* plant cell culture protocols; understanding of which part of plant needs to be used to induce the callogenesis. Analysis of the type of sterilization to perform, and of sugars, vitamins, and phytohormones to use as components of growth cell culture media.
A2) Assessment of the effects of different elicitors on *in vitro* plant cell culture to identify the most suitable elicitor to increase the final production of secondary metabolites.
A3) Extraction and analysis of secondary metabolites in PPCs at the end of cell cultivation cycle, to assess the type of healthy compounds present within the cell cultures.
A4) Evaluation of the extracted compounds as food additive in order to determine the plant cell culture as possible novel food
A5) Writing and Editing of the PhD thesis, scientific papers and oral and/or poster communications.

Table 1 Gantt diagram for this PhD thesis project.

Activity	Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	***In vitro plant cell culture protocols***																								
	1) Sterilization and callogenesis																								
	2) Growth culture media																								
A2)	***Use of different elicitors***																								
	1) Test of different elicitors																								
A3)	***Analysis of secondary metabolites***																								
	1) Extraction																								
	2) HPLC for SMs' analysis																								
	3) Metabolomic analysis																								
A4)	***PCCs as novel food***																								
	1) Assessment of SMs' toxicity																								
	2) Analysis of SMs as food additive																								
A5)	***Thesis and Paper Preparation***																								

3. Selected References

Chiocco I, Mandrone M, Tomasi P, Marinchic L, Poli F (2021) Plant Secondary Metabolites: An Opportunity for Circular Economy. Molecules 26, 495

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Food and food bioactives fighting chronic inflammation

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Co-tutor: Ph.D. Marilisa Alongi

The aim of this project is to evaluate the influence of bioactive compounds contained in raw and processed plant-based foods, and their industrial processing by-products on the immune landscape and homeostasis of the gut affected by chronic inflammation.

Alimenti e composti bioattivi nel trattamento dell'infiammazione cronica

L'obiettivo di questo progetto è valutare l'influenza che i composti bioattivi contenuti in alimenti di origine vegetale trasformati e non, e dei relativi sottoprodotti derivanti dalla lavorazione industriale possono avere sul contesto immunitario e sull'omeostasi dell'intestino affetto da infiammazione cronica.

1. State-of-the-Art

Inflammation can be defined as the response that the immune system establishes upon the onset of harmful stimuli, such as pathogens, damaged cells, toxic compounds, or irradiation, and acts by removing injurious stimuli and initiating the healing process. Inflammation is therefore a defence mechanism that is vital to health (Chen *et al.*, 2018). Normally, acute inflammation is characterized by cellular and molecular events and interactions which efficiently remove the noxious stimulus and, in the end, lead to the restoration of tissue homeostasis and resolution of the acute phase. However, uncontrolled acute inflammation may become chronic, contributing to the onset of a variety of disorders. Chronic inflammation has been linked to many degenerative conditions such as cancer, autoimmune, cardiovascular, neurodegenerative diseases, and metabolic syndrome (Furman *et al.*, 2019). Inflammatory bowel disease (IBD) is a clear example of a condition generated by the establishment of chronic inflammation in the intestine (Zhang and Li, 2014). IBD has been a world- wide health-care problem with increasing incidence. Scientific investigations have found that IBD is characterized by an aberrant and continuing immune response directed to the microbes in the gut. The true aetiology although remains largely unknown. Current therapeutic strategies include corticosteroids and immunosuppressive drugs but also biological treatments. Various reports have also linked the role of the diet in the onset and progression of IBD (Marion-Letellier, Savoye and Ghosh, 2016; Roda *et al.*, 2020). Dietary interventions can become a standard approach to improve chemical therapies, due to bioactive components naturally ingested with daily food. Indeed, there is a plethora of scientific evidence which proves that food-derived bioactive compounds (e.g., Vitamin A and D, resveratrol, quercetin) have a potential role in modulating inflammation through the regulation of the immune response (Veldhoen and Brucklacher-Waldert, 2012; Malavolta *et al.*, 2014; Gurău *et al.*, 2018). Therefore, combined therapies with diets rich in bioactive molecules may help to reduce drug dosage, the side-effects and contribute to the prevention of chronic inflammation-linked diseases. Raw and processed plant-based foods, as well as their industrial processing by-products are known to be rich in bioactive compounds although the latter are often considered as waste. In the last few years, based on the idea of the existence of a linear cause-effect relation, studies conducted on dietary bioactive compounds, however, have often been carried out considering single components. Since no beneficial and sometimes paradoxical effects have been detected in vivo, the emerging hypothesis is that this approach represents a gross simplification. It is likely that, when contained in the food matrix, bioactives act collectively, often in synergy, exerting single or multiple physiological effects. This nonlinear relationship can be reasonably attributed to the chemical interactions among the huge variety of bioactives and bioactive families present in the food matrix together with the effect of food structure. The latter can greatly affect bioactive release and fate in the gastrointestinal tract, thus affecting their bioavailability (Connolly, Tuohy and Lovegrove, 2012). In order to overcome above-mentioned criticality in understanding the role of food bioactives on gut inflammation, a bottom-up approach has been pursued: food model systems exerting increasing structure complexity and containing selected bioactives will be designed. In particular, samples mimicking different degrees of cellular deconstruction will be prepared and the fate of the contained bioactives on the gastrointestinal tract and on in vitro model of gut chronic inflammation will be studied. This Ph.D. research is supported by PON Ricerca e Innovazione 2014-2020 (DM1061/2021) and with collaboration of BIOFARMA GROUP.

2. PhD Thesis Objectives and Milestones

The first 6 months of this PhD research activity were focused on the selection of bioactives to be studied. Based on literature review and analysis of the widely consumed fruit and vegetables derivatives, some polyphenols were selected. In the next months, the following research steps will be pursued:

A1) Literature review;
A2) Preliminary tests: setting up of analytical methodologies (antioxidant activity, lactase activity, in vitro digestion):
A3) Design of bioactive containing model system exerting increasing level of cellular deconstruction mimicking vegetables derivatives such as juice puree, homogenate;
A4) In vitro digestion;
A5) Analysis of the bioaccessibility and bioavailability of bioactive compounds in the digest by using different food model systems and evaluation of the mechanism of absorption (e.g. lactase activity against glycosilated polyphenols);
A6) Development of an experimental model of gut chronic inflammation;
A7) Analysis of the influence that the products obtained in step A4 can have on gut epithelial homeostasis and on immune cells populations in the experimental model:
A7.1) Treatment of the experimental model with the product obtained in step A4;
A7.2) Analysis of the immune landscape (regulatory immune cells populations by flow cytometry, cytokine expression profiles by ELISA, epigenetic regulation of cytokine production).
A8) Company period;
A9) Paper writing and thesis development.

Table 1 Gantt diagram for this PhD thesis project.

Activities	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1) Literature review	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
A2) Preliminary tests	■	■	■																					
A3) Design of bioactive containing model system				■	■	■	■																	
A4) *In vitro* digestion								■	■	■	■													
A5) Analysis of the bioaccessibility and bioavailability of bioactive compounds										■	■	■	■											
A6) Experimental model development														■	■	■								
A7) Immunologic analysis																	■	■	■	■				
A8) Company period																			■	■	■	■	■	■
A9) Paper writing and thesis development	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■

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Production and analytical characterization of new and traditional foods: focus on sustainability

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The PhD research project pursues the main objective to enhance the diversity of food production through the application of sustainable technologies for developing new food products based on local ingredients and raw materials. Strategies for the food by-products and waste valorization to recover high added value bioactive compounds are applied on a lab scale with the opportunity to be transferred to pilot and industrial scale in a circular economy approach.

Produzione e caratterizzazione analitica di alimenti tradizionali ed innovativi: focus sulla sostenibilità

Questo progetto di tesi di dottorato ha il principale scopo di promuovere la diversificazione nella produzione di alimenti. Ciò si può ottenere attraverso l'applicazione di tecnologie sostenibili per lo sviluppo di nuovi alimenti impiegando materie prime ed ingredienti locali. Sono impiegate anche strategie per la valorizzazione di sottoprodotti e sprechi alimentari per l'estrazione di importanti composti bioattivi, utilizzando tecnologie su scala di laboratorio. I risultati così ottenuti potrebbero consentire uno scale-up delle metodologie impiegate su scala industriale e semi-industriale, in un contesto di economia circolare.

1. State of the Art

Malnutrition is a matter of major concern globally, and it is also addressed by the United Nations in its Agenda 2030 within the Sustainable Development Goals (SDG) 2 through the promotion of practices voted to eradicate all forms of malnutrition (FAO, 2020).
It is well known that around 1.3 billion tonnes of food is lost or wasted every year globally, nearly one third being edible parts, mostly from fruits, vegetables and cereals. As a matter of fact to increase the affordability of healthy diets, the costs of nutritious foods must come down, as their accessibility has to rise (FAO, 2020). One of the strategy to reach this goal is reducing pre-harvest and post-harvest losses and wastes, both in terms of quantity and quality and in each food supply chain, through the valorization of by-products, with a resulting increase in sustainability and circularity of the whole food sector (FAO, 2020). According to Garn and Leonard (1989), more than 7'000 crop species have been cultivated and domesticated, but no more than 150 species are intensively cultivated for commercial purpose and just three main crops provide 60 % of world's food energy intake (Garn & Leonard 1989). Moreover, the use of local and traditional species can increase agricultural sustainability by reducing the need for external inputs, such as pesticides and fertilizers, and, depending on their species, can also improve soil fertility and the resilience of the entire system against climate change. Food insecurity is severe especially in developing countries, but it is gradually improved due to the increased agricultural export and growing needs demands from consumers. In fact, consumers are more and more attracted by healthy foods; markets are following this trend by highlighting this kind of products and adding functional ingredients to regular foods (Meyerding et al. 2018).
In this framework, the enrichment of local foods with bioactive compounds would meet the market demand for healthy products, and a proper labelling, with indication of the geographical area of provenience and possibly the health claim can be also a driver of economic growth for developing countries by exporting new food products (Bradley et al., 2011).
It is particularly important to choose the appropriate technological approaches for supplemented food preparation in such a way new foods, produced from traditional and local ingredients or raw materials and added with extracted compounds with beneficial health effects, can have a place in global market (Hedhili et al., 2021). One of the major causes of food insecurity is climate change: the increase in the severity of natural disaster events in such a way it has become fundamental to discover strategies to cope with that. Agroecology is an integrated approach that applies ecological and social concepts for the management of the food sector and the agricultural system, optimizing the interactions between plants, animals, humans and the environment taking into consideration the social aspects for a sustainable production (FAO, 2020). The agroecology approach is coherent with the use of by-products for the formulation of new enriched food and the promotion of knowledge on local biodiversity and raw material; in addition to that the improvement of food production processing techniques is another driver towards a more efficient and sustainable food making.
Sustainable food technologies play a very relevant role in determining the global sustainability of the food systems;

some of them work without the direct application of thermal energy and use of chemicals such as the cold pressing for the production of unconventional and specialty edible oils, which recently have gained a lot of attention due to their useful and beneficial properties (Vladic et al., 2020). Besides the use of non-thermal technologies, another example of sustainable extraction method is vacuum distillation which is used for the separation of mixtures with thermolable compounds. It allows to operate at low temperatures, avoiding the degradation of several compounds in the extract. This technique can be applied to obtain natural products (e.g. essential oils). Different techniques could be applied for the separation of oil fraction from the seeds such as the more sophisticated supercritical fluid extraction and the simpler cold pressing (Vladic et al., 2020).

2. PhD Thesis Objectives and Milestones

The PhD project focuses on sustainable technologies for the production as well as analytical characterization, both sensory and instrumental, of new food products and ingredients. The research project is implemented according to the activities reported in the Gantt chart here below.

A1) **Bibliographic research** regarding the analytical characterization of food products obtained from different local raw materials. Research in the literature is also voted to the study of innovative and sustainable technologies for the valorization of food wastes and losses to recover valuable compounds.

A2) **Sustainable food technologies:** food raw materials, by-products and new food products are prepared using local raw materials, ingredients and treated food by-products and waste. The bioactive compounds, to be incorporated within the new formulation, are extracted using sustainable technologies. In addition, the raw materials, ingredients and by-products are also treated with sustainable technologies (e.g. co-milling, vacuum distillation, air-driying,…). For example, flavored olive oils have been prepared by co-milling using by-products as flavoring agents; also essential oils have been extracted using vacuum distillation allowing to use lower temperatures and using water as solvent.

A3) **Analytical characterization of food products:** the instrumental and sensory evaluation of the new food products are carried out to characterize them (raw materials, by-products and new food products).

A4) **Labelling** of new and traditional food products.

A5) **Writing and editing** of the PhD thesis and research papers.

Table 1 Gantt diagram for this PhD thesis project.

Activity	Months	2	4	6	8	10	12	14	16	18	20	22	24	26	28	30	32	34	36
A1)	***Bibliographic research***																		
A2)	***Sustainable food technologies***																		
	1) Co-milling																		
	2) Vacuum distillation																		
	3) Non-thermal drying																		
	4) Other technologies																		
A3)	***Analytical characterization of food products***																		
	1) Characterization of raw materials																		
	2) Characterization of by-products																		
	3) Characterization of new food products																		
A4)	***Labelling***																		
A5)	***Writing of the thesis and research papers***																		

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"Green" Technologies in the supply chain of agri-food company

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This PhD thesis research project is aimed to change, in the context of globalization, the industrial approach through the development of sustainable processes, devices and approaches, thus improving competitiveness and implementing green and digital. For these reasons, my research project is based on the implementation of green technologies and digitalization in a kiwifruit company, with the aim of improving the effectiveness and efficiency of the agri-food chain.

Implementazione di tecnologie "green" nella filiera di un'azienda ortofrutticola

Questa proposta di tesi di dottorato ha l'obiettivo di modificare, nel contesto della globalizzazione, lo scenario industriale attraverso lo sviluppo di processi, dispositivi e approcci sostenibili, migliorando così la competitività e implementando le tematiche del verde e del digitale. Per questi motivi, questo progetto di ricerca ha l'obiettivo di implementare le tecnologie green e la digitalizzazione in un'azienda di kiwi, con il fine di migliorare l'efficacia e l'efficienza della filiera agroalimentare.

1. State-of-the-Art

Kiwifruit is appreciated by consumers for its high bioactive compound content with health-beneficial effects (Goffi, Magri et al. 2020) and for this reason, the consumption of fresh kiwifruits is booming (Meena, Nirmal Kumar, et al. 2018). Yellow-fleshed kiwifruit possesses many interesting agronomic features (Goffi, Magri, et al. 2020) but it is not suitable for long-term storage, as demonstrated by several studies (Goffi, Modesti et al. 2018). The lack of proper maturity harvest indices is a major drawback in postharvest management because many pre- and postharvest factors are involved in the deterioration of fresh fruit quality and storage life. Therefore, the development of novel techniques to maintain the quality and shelf life of fruits after harvesting is a major challenge (Gwanpua SG, Jabbar A et al. 2018). It was also observed that the ineffective water use increases the cost of crop production and that water stress can induce changes in fruit yield and quality (Pinto, R., Valin, et al. 2021). In other studies it has been shown that fuit size is generally reduced by water deficit as opposed to the quality of the fruits, such as the increasing of sugar content or improved color, which is enhanced by lower irrigation. The technical approach to optimize irrigation program is based on monitoring soil water moisture content and plant water status (Steduto, P., Hsiao, et al. 2012) and for this reason it is necessary to carry out analysis of the soil and the humidity of the stem and the speed of the sap. To carry out these analyzes, the PNRR proposes the use of new multifunctional devices, and an exemple in our experiment is the use of a new 4.0 Tree Talker technology, based on IoT systems, that can be used for the real-time observation of trees physical and biological parameters applicable to the monitoring of forests, agro forestry systems and urban green infrastructures. This device allows monitoring such parameters as stem temperature and moisture, sap flow, and canopy light transmission. Data are transmitted via wireless LoRa (long-range) connection to the router (TT-Cloud) with an hourly frequency (Valentini, R., Marchesini, et al. 2019). In conclusion, the goal is implement green technologies and digitalization of the agri-food chain, increase effectiveness and efficiency in food traceability processes, general benefits for the supply chain, increase in sales and exploitation of data as a wealth of knowledge for businesses. (www.osservatori.net)

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above, this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) **Bibliographic research**: Literature research of the most promising digital sensors in the agricultural field, study of kiwis and their qualitative and conservative characteristics

A2) **Selection of experimental plants**: Visit to the company and selection of the kiwi plants that will be subjected to experimental tests.

A3) **Implementation of three different experimental irrigations**: A first sample will be sprayed with a "standard" volume and two sample tests will be irrigated, at the same times established, with volumes corresponding to 80% and 60% of the standard volume used, in order to analyze any stress of the plant.

A4) **Plant water stress assessment:** Kiwifruit plants will be analyzed to evaluate the water stress of the plants through the variations of the chlorophyll pigment on the leaves, the sap flow and the soil humidity for the three different irrigations during the six month period of the experimental test, These analyzes will be carried out through the use of Tree Talker sensors in the field

A5) **Sensors device management by software:** These sensors will have to be managed by software that allow to receive field data in real time on various media managed by a single computer platform

A6) **Study of harvest fruit set and storage:** Experiments will be carried out, with the aim of evaluating the better fruit harvest periods of differently irrigated horticultural products, and this quality parameters will be checked at 15-20 day intervals

A7) **Statistical analysis:** analysis about qualitative and conservation characteristics of kiwi fruits (A6), data analysis obtained by Tree Talker (A5) and analysis about water stress (A3) will be statistically evaluated.

A8) **Stage in the company:** 12 months will be spent in the company in which field activities will be carried out.

A9) **Writing and Editing:** PhD thesis, scientific papers and oral and/or poster communications will be carried on during the PhD period.

Table 1 *Gantt diagram for this PhD thesis project.*

		Months																												
Activity		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
A1	***Bibliographic research***																														
A2	***Selection of experimental plants***																														
A3	***Implementation of three different experimental irrigations***																														
A4	***Plant water stress assessment***																														
A5	***Sensor management by software***																														
A6	***Study fruit ripening***																														
A7	***Statistical analysis***																														
A8	***Stage in the company***																														
A9	***Writing and Editing***																														

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www.osservatori.net

Application of an eco-sustainable technology: use of direct and photodynamic UV light for the microbial decontamination on food industries

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This PhD thesis research project is aimed to investigate the inactivation capacity of planktonic cells and microbial biofilms by UV light and blue light on surfaces and food products. These technologies will be coupled with photoactive materials. The obtained results will be optimized to adapt the use of this technology on company machineries.

Applicazione di una tecnologia ecosostenibile: utilizzo della luce UV LED diretta o fotodinamica per processi di decontaminazione microbica nell'industria alimentare

Questo progetto di dottorato mira allo studio delle capacità di inattivazione di cellule planctoniche e biofilm della luce UV e blu emessa da dispositivi LED su superfici e alimenti. Tali tecnologie saranno inoltre combinate con materiali foto-attivabili. I risultati ottenuti verranno quindi usati per ottimizzare l'uso della tecnologia in macchinari prodotti da una azienda.

1. State of the art

Thermal and chemical inactivation techniques have been traditionally used for microbial decontamination. Recently, UV light has been well established for water treatment, air disinfection, and surface decontamination, according to the FDA and EFSA guidelines (Koutchma, 2008). Traditionally UV lamps contain mercury, but in recent years light-emitting diodes (LEDs), mercury-free, have been released as a new UV light source, having a long lifetime, low heat emission, and versatility of use (Hinds et al., 2019).
The antimicrobial effect is brought mainly by the UV-C portion (200-280 nm) of the electromagnetic spectrum which has a limited penetration capacity, so the germicidal effect is obtained only by applying UV-C light directly on the target and not in shade, in pores, or in orifices (Guerrero-Beltrán & Barbosa-Cánovas, 2004). Recently, it was noted that wavelengths from 400 to 480 nm (blue light) can be used for microbial inactivation. Although blue light is less germicidal than UV light, it is less harmful to host tissue, and microorganisms are then selectively inactivated (Halstead et al., 2016).
The germicidal effect of LED lamps can be amplified if coupled with photocatalytic materials able to produce reactive oxygen species (ROS) by irradiation. These materials can include inorganic nanomaterials (i.e., TiO_2, ZnO) or organic molecules (i.e., porphyrins, phthalocyanines, etc)(Comuzzi et al., 2020), and can be designed to kill both Gram-negative and Gram-positive bacteria, and fungi.
Among the microorganisms isolated from the food environment, there are pathogens such as Listeria, Salmonella, Staphylococcus, Escherichia coli, etc. or spoilage microorganisms such as Pseudomonas spp. which can undergo a different germicidal effect both in relation to the species, the strain, and the physiological condition in which they are found (planktonic or biofilm form). The presence or absence of a biofilm can strongly affect the amount of light irradiation required to inactivate microorganisms (Gora et al., 2019); a substantially higher amount of light dose may be required to inactivate microorganisms protected by biofilms than those not protected by a biofilm. Currently, there are only few studies that investigate the efficacy of visible light against biofilms, but it is necessary to consider it since biofilm is commonly formed in environments. It is therefore important to obtain a high level of hygiene for surfaces and equipment intended for contact with food.
Although light irradiation can be exploited for disinfection purposes of surfaces and the environment, these types of treatment may induce the chemical transformation of lipids, bioactive compounds, and proteins present in foods (Hinds et al., 2019). LEDs lamps could be easily used for decontamination of surfaces for food preparation or incorporated into equipment devoted to food preservation, so it could be successfully applied to guarantee a hygienic level of environment and a prolongation of shelf-life of the products.
This project aims to develop a suitable innovative disinfection strategy for surfaces and environment addressed to food contact using irradiation eventually coupled with photoactive materials. The application of these technologies should guarantee antimicrobial action in the environment as well as in foods. The tests will provide the results necessary to optimize conditions of the germicidal effect of these technologies for use in devices built by the company.

2. PhD Thesis Objectives and Milestones

This PhD project could be divided in the following activities according to the Gantt diagram reported in Table 1:
A1) **Assessment of the germicidal effect on planktonic cells and biofilm**: to optimize the efficacy of microbial inactivation the parameters that influence the process (exposition time, dose, LED lamps, distance) will be considered. The efficacy of the treatment will be assessed by counting viable cells before and after irradiation (A1.1). Also, adhesion and formation kinetics of premature and mature biofilms will be studied on different materials (A1.2).
A2) **Tests with photoactive materials**: already existing photoactive organic materials (e.g., porphyrins) will be exploited for their antimicrobial activity. Organisms will be making grow on the materials to verify the germicidal effect (A2.1). Some tests will be done to evaluate the amount of ROS released and how they adhere to the surface (A2.2). It will be considered the synthesis of new photoactive materials (A2.3).
A3) **Analysis of food product**: to assess the quality of the treated food products, pH, total titratable acidity, colour, firmness, lipid oxidation, phenolic content will be analysed (A3.1). According to the obtained results, for each selected condition, a specific protocol will be developed to apply the antimicrobial treatment (A3.2).
A4) **Laboratory prototype**: after all the tests, a prototype will be designed to be used in the company's laboratory. The efficacy of light irradiation and photoactive materials will be evaluated in the company's appliances (A4.1). Also, microscopy (SEM, CLSM) will be used to verify if the treatments have caused any damage to the microbial cell and to observe the biofilms' structures (A4.2).

Table 1. *Gantt diagram for this thesis project*

Activities/months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
Literature research																																				
UV lamps, materials, and microorganisms selection																																				
Strain's characterization (planktonic/ biofilm form)																																				
Test for germicidal effect with UV and light blue LED																																				
Test for germicidal effect on photoactive materials																																				
Evaluation of the prototype in the company																																				
Test on food																																				
Workshops, elaboration of the results, manuscript elaboration																																				

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Bioactive Compounds From Hop Leaves: A Green Opportunity

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In order to make hop cultivation sustainability-oriented and with a bioeconomic approach, this Ph.D project aims to value the hop plant vegetative biomass to obtain bioactive compounds, potentially useful for food and non-food industry. To reach this objective, several hop genotypes, grown in field and i*n vitro*, will be screened, to characterize their bioactive compound composition; moreover, since the bioactive compound synthesis in plants could be stress induced, the same hop plants will be subjected to stresses to stimulate their secondary metabolism and to diversify the bioactive compound profile and their concentration.

Composti Bioattivi da Foglie di Luppolo: Un'Opportunità Green

Al fine di rendere più sostenibile la coltivazione del luppolo e conferirle un approccio bio-economico, questo progetto di dottorato mira a valorizzare la biomassa vegetativa di piante di luppolo, utilizzandola come matrice per ottenere composti bioattivi che saranno successivamente caratterizzati al fine di valutare la loro potenziale applicazione nell'industria alimentare e non. Per raggiungere questo obiettivo, diversi genotipi di luppolo, coltivati in pieno campo ed *in vitro*, verranno sottoposti ad uno screening per caratterizzare il loro contenuto in composti bioattivi. Inoltre, poiché la sintesi dei composti bioattivi nelle piante è considerata anche una risposta allo stress, le stesse piante di luppolo, saranno sottoposte a stress per stimolare il loro metabolismo secondario e diversificare il profilo dei composti bioattivi e la loro concentrazione.

1. State of the Art

Sustainable Development Goals (Agenda 2030 ONU) emphasizes the importance of sustainable conservation and rational utilization of biodiversity, thus, investigating the wealth in bioactive compounds of agricultural species must be a priority (Ofosu et al. 2020). Hop, *Humulus lupulus* L., a widely known cultivated plant, whose different parts are rich in bioactive compounds, with antioxidant, antimicrobial and antiviral activity (Astray et al. 2020), is drawing the attention of several food and non-food enterprises, interested in producing natural extracts. In detail, hop plant produces important bioactive secondary metabolites, such as terpenoids, phenolic compounds, alkaloids and bitter acids (Bocquet et al.2018). Even if hop leaves are as reach in these compounds as fruits, stems and roots (Muzykiewicz et al. 2019) they are considered waste and used, mainly, for compost production (Abram et al., 2015). European Commission strongly underlines that agricultural waste recycling is an important step for environment protection, agricultural development and circular economy purposes (Diacono et al. 2019), therefore, valuing hop vegetative biomass, so rich in bioactive compounds, can be a precious resource for food, pharmaceutical and nutraceutical industries, but can also represent a second income for hop growers (Nionelli et al. 2018). Unfortunately, the supply of hop biomass to industries is not continuous, being hop a deciduous plant. To overcome this problem FAO recognized Plant Tissue Culture as an important tool for the large-scale production of bioactive compounds (Dias et al. 2016), since plants synthetize *in vitro* the same bioactive compounds present in their corresponding in nature (Espinosa- Leal et al. 2018). Other than by the genetics, the synthesis of bioactive compounds is highly influenced by the exposition of the whole plant, or some of its part, to biotic or abiotic stress (Cheynier et al. 2015), with the advantage that their effect is much more under control in *in vitro* conditions (Chandran et al. 2020). Few are the research exploring the influence of any kind of stress on bioactive compound biosynthesis in the vegetative biomass of field-grown hop plants (Ceh et al. 2007), but none regarding *vitro*-derived hop plantlets; instead, several are the research studying the influence of cultural conditions on bioactive compound synthesis in other plant species of interest (Smetanska 2018).

2. PhD Thesis Objectives and Milestones

The general activities and related objectives of this Ph.D project are:
A1) **Screening for vegetative biomass total bioactive compound content of hop open field grown plants**. This activity will foresee the selection of a set of hop genotypes, within those grown in the collection field of UNIPR, in order to characterize their leaves, in terms of total polyphenol content and antioxidant activity.
A2) **Regulation of bioactive compound synthesis in open field plant vegetative biomass induced by abiotic stress.** This activity will include the individuation of a valid combination genotype/plant treatment to induce a diversification in the bioactive compound profile and in the antioxidant activity of leaves from open field cultivated hop plants.
A3) **Evaluation of total bioactive compound content in *vitro*-derived hop plantlets.** This activity will be focused, firstly, on the *in vitro* establishment of a hop collection, then on the characterization of *vitro*-derived

plantlets in terms of their total polyphenol content and their antioxidant activity.
A4) **Regulation of bioactive compounds synthesis through *in vitro* culture techniques.** This activity will be carried out to set up the *in vitro* cultural conditions that better regulate the synthesis of bioactive compounds in *in vitro* cultured plantlets.
A5) **Chemical characterization of hop leaf bioactive compound**s. In this activity, that will be carried out in collaboration with the partner company Packtin s.r.l., extracts obtained in A 1-4 will be characterized, to evaluate their polyphenol profile.
A6) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.
Timetables and milestones of proposed activities are summarized in the Gantt charts given in Table 1.

Table 1. Gantt diagram for this Ph.D thesis project.

Activities	Months																																		
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
A1) Screening for vegetative biomass total bioactive compound content of hop open field grown plants.												M1						M1																	
A2) Regulation of bioactive compound synthesis in open field plant vegetative biomass induced by abiotic stress.																			M2											M2						
A3) Evaluation of total bioactive compound content in vitro*-derived hop plantlets.*						M3							M4											M4											
A4) Regulation of bioactive compounds synthesis through in vitro *culture techniques*																			M5						M5							M5			
A5) Chemical characterization of hop leaf bioactive compounds										M6				M7						M8			M6					M9				M10			
A6) Writing and Editing												M11						M12						M13											M14

Milestones M1: Identification of the most promising hop genotypes in terms of polyphenol content and antioxidant activity. **M2**: Evaluation of the influence of stress treatments on vegeto-productive parameters. **M3**: *In vitro* collection of hop genotypes. **M4**: Identification of the most promising *in vitro* cultured genotypes. **M5**: identification of the best *in vitro* cultural conditions. **M6**: Intership at the "Packtin" company. **M7**: Chemical characterization of open field leaves. **M8**: Chemical characterization of *vitro*-derived leaves. **M9**: Chemical characterization of open field derived leaves stress treated. **M10**: Chemical characterization of *vitro*-derived leaves treated with elicitors. **M11**: Presentation of first year results. **M12**: Research at foreign universities. **M13**: Presentation of second year results. **M14**: Presentation of final results.

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Application of innovative, non-thermal technology and edible coating to extend the *shelf life* of fresh cut fruits

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This industrial PhD project has been founded by MIUR through the Research and Innovative National Operative Program (PON) and developed in collaboration whit Universidad Politécnica de Valencia and Campo D'Oro S.r.l. company. The main aim of the PhD project is to extend the shelf life of fresh-cut products through the application of innovative, non-thermal technologies, such as UV, ultrasound or cold plasma, and / or the use of edible film/coating. The effectiveness of the different treatments will be assessed through chemical, physical and microbiological analyses with the aim of establishing the most suitable treatment for making a fresh-cut product that is safe and organoleptically acceptable by the consumer.

Applicazione di tecnologie innovative, non termiche e coating edibili per prolungare la *shelf life* dei prodotti di IV gamma

Il progetto di dottorato industriale è stato finanziato dal MIUR attraverso il Programma Operativo Nazionale di Ricerca e Innovazione (PON) e sviluppato in collaborazione con Università Politecnica di Valencia e la società Campo D'Oro S.r.l. L'obiettivo principale del progetto di dottorato è prolungare la durata di conservazione dei prodotti di IV gamma grazie all'applicazione di tecnologie innovative, non termiche, come UV, ultrasuoni o plasma freddo, e/o l'uso di film/coating edibili. L'efficacia dei diversi trattamenti sarà valutata attraverso analisi chimiche, fisiche e microbiologiche con il fine di stabilire il trattamento più adatto a realizzare un prodotto di IV gamma sicuro e accettabile dal consumatore.

1. State of the Art

The fruit and vegetable processing and supply chains represent an important pillar of the food industry. Consumption of fresh fruits and vegetables is deemed healthful for the high nutritional value of these products. However, the fresh-cut product can also be a source of foodborne illness. The high perishability of the product is due to endogenous reasons, related to enzymatic activity, and exogenous, such as, for example, the high risk of microbial contamination before and during the production stages. The traditional method currently in use involves a pre-treatment based on hypochlorite or chlorine with a concentration ranging between 50 and 200 ppm, or the use of trisodium phosphate for the prevention of pathogenic fungi. These pre-treatments, in addition to having an environmental impact, are not appreciated, and considered safe by consumer who prefers alternative methods to chemical. Currently, to reduce the biochemical and physiological changes and prevent the microbiological degeneration, there are different physical and chemical approaches. Among the physical methods are worthy of mention the use of UV-C rays at 257 nm, the use of low-frequency ultrasound, non- thermal, non-toxic, and safe technology, which integrated with other biological and physical procedures have given good results in terms of prevention or microbial inactivation. Finally, the pulsed light, non-thermal technology, replacing UV-C rays and cold plasma, have been investigated too. To the methods described above to preserve freshly cut fruit can be added edible coating or film based on polysaccharides, lipids and proteins obtained from the processing of industrial and non-industrial waste that act as a barrier to water vapor and oxygen. The films are sometimes integrated with chemical or natural additives, such as essential oils or plant extract. However, the immediate inclusion of essential oils in the films can reduce the mechanical characteristics, load capacity, and increase the risk of an "oil effect". Otherwise, use of nano-emulsions, oil/water emulsions with an average diameter between 100 and 500 nm give greater transparency, stability, and resistance and greater antimicrobial efficacy. This PhD thesis project will be directed to evaluate the most promising single or combined innovative treatment to extend the shelf life of fresh cut products.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A.1 Literature research and experimental planning

A.2 Effect of ultrasound treatment on texture, colour and microbiology, enzymatic activity, and Aw.
A.2.1 Investigation of the effect of the ultrasonic bath with or without additives (ascorbic acid, calcium chloride). Evaluation of the effectiveness of the treatment. It depends on the intrinsic characteristics of the product or on the treatment parameters (frequency, time, power (W) and temperature).
A.2.2 Study on the effect of ultrasonic cutting on fresh-cut products on colour, pH and sensory attributes.

A.3 Study on the effects of cold plasma on fresh-cut products. Cold plasma is an emerging antimicrobial technology for the sanitation of various food products. Plasma is composed of gas molecules, in a dissociated state thanks to an energy input 24. The goal is to evaluate the shelf life of the final product in relation to gas, flow rates, power (W) and treatment time.

A.4 Realization and study of the effects of edible active film or coating with controlled release of natural antimicrobials and antioxidants.

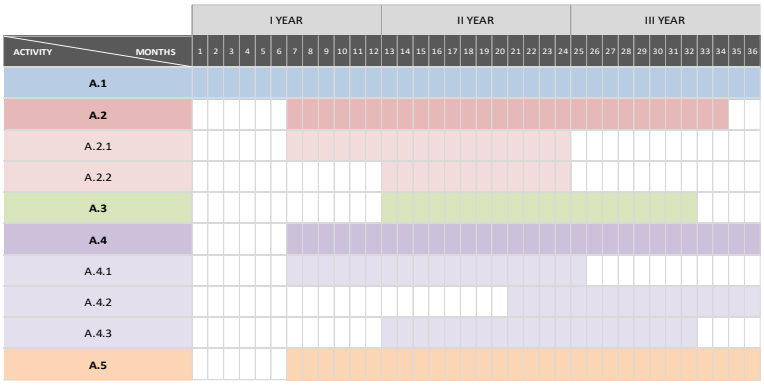
A.4.1 Study of the effects of the coating and film with the inclusion of natural additives. In addition to essential oils, the focus will be on extracts from herbs and wild plants. In particular, the Sicilian sumac. The bioactive principles of sumac will be extracted and inserted into the film with different inclusion techniques.

A.4.2 Investigate and experiment with different film making techniques, from the traditional one solvent casting to the most recent electrospinning, a method for producing from solutions polymeric thin fibers with diameters of nanometers and microns thanks to interaction electrostatic.

A.4.3 Study of the combined effect of film or coating and UV rays. In addition to determining microbial inactivation, UV rays could solve the problem of immediate evaporation of the solvent, which can be achieved thanks to an appropriate formulation of the coating recipe. We will try to formulate a coating that exposed to UV rays presents the immediate evaporation of the solvent and at the same time the effectiveness of UV rays on the shelf life of the product will be evaluated.

A.5 Statistical data processing, scientific papers, writing and editing of PhD thesis

Table 1



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Apulian Salicornia: from medicinal plant to innovative nutraceutical supplement

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Salicornia is a halophytic weed that grows abundantly on the Mediterranean coast and whose health-promoting potential is known in the literature. The project aims to extract, characterise and quantify the bioactive compounds present in the plant. The work plan is to explore the antioxidant and antinfiammatory profile through chemical and *in vitro* assays on HepG2 and A549 cell lines. In line with the experimental design, the collected data will be used to candidate the plant for the formulation of a potential nutraceutical to be proposed to the national and international food supplement market.

Salicornia: da pianta medicinale ad innovativo integratore nutraceutico

La Salicornia è una pianta alofita infestante che cresce abbondantemente sulle coste del Mediterraneo. Il progetto ha lo scopo di estrarre, caratterizzare e quantificare i composti bioattivi presenti nella matrice vegetale. Il piano di lavoro prevede di esplorare il profilo antiossidante e antinfiammatorio mediante saggi chimici e *in vitro* su linee cellulari HepG2 e A549. In linea con le finalità progettuali, i dati sperimentali raccolti saranno utilizzati al fine di candidare la matrice vegetale per la formulazione di un potenziale nutraceutico da proporre al mercato nazionale e internazionale dell'integrazione alimentare.

1. State-of-the-Art

Nowadays, there has been considerable attention toward the recovery of the waste plant matrices as possible sources of functional compounds with health properties. In this regard, we focused our research program on *Saliconia*, a halophyte plant that grows abundantly on the coasts of the Mediterranean area. The project aims to investigate this plant by transforming it from a weed waste to a health resource. It is used not only as a seasoned vegetable, but also in traditional medicine for the treatment of diseases such as obesity, diabetes and cancer and its consumption has been extended as a functional food for its many beneficial effects. The overall literature studies suggest that *Salicornia* is rich in numerous bioactive components such as pentadecyl ferulate, phytol, stearolic acid, c-linolenic acid, (3Z, 6Z, 9Z) -tricosa-3,6,9-triene, linoleic acid, stigmasterol, ergosterol, dioctyl phthalate, dibutyl phthalate, vanillic aldehyde and scopoletin; it was found that pentadecyl ferulate has a strong antioxidant activity as well as phytol has an antiproliferative action on HepG2 cells.[1,2] To this purpose, we focus our attention on the health properties evaluation of *Apulian Salicornia* by innovative green extraction procedures, and biological activity on HepG2 and A549 cells. In addition, the study may be extended to other biological activities so as antimicrobial profile.[3] Finally, bioaccessibility and bioavailability studies useful will be carried out to produce a nutraceutical supplement with a health antioxidant and antiproliferative profile.
The proposed project is granted as PhD research on REACT EU XXXVII cycle and was funded under the Green topic. It will take place in collaboration with an Apulian Start up, Sestre srl, which produces nutraceuticals. The study started in January 2022 with the first WP concerning the feasibility study based on literature data and market analysis.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:
WP1) After a thorough analysis of scientific literature, it will be developed an experimental design for green and efficient extraction methods. Afterwards we will proceed to analytical characterization of extract components through HPLC.
WP2) Evaluation of antioxidant activity through antiradical assays like ABTS, DPPH and analysis of their antinfiammatory activity
WP3) Bioavailability and bioaccessibility studies of the extracted matrice.
WP4) Nutraceutical formulation with a health antioxidant and antiproliferative profile
WP5) Writing and Editing of the PhD thesis, scientific papers and poster communications.

Table 1 *Gantt diagram for this PhD thesis project.*

WP	TASK	I	II	III	IV	V	VI
WP1	**Task 1.1** Analysis of scientific literature						
	Task 1.2 Sampling of Salicornia						
	Task 1.3 Experimental design for extraction methods						
	Task 1.4 Analytical characterisation of extract compounds						
WP2	**Task 2.1** Chemical evaluation of antioxidant activity						
	Task 2.2 *In vitro and in vivo* analysis of extracts						
WP3	**Task 3.1** Bioavailability and bioaccessibility studies						
WP4	**Task 4.1** Nutraceutical formulation						
WP5	**Task 5.1** Writing and Editing of the PhD thesis						

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Use of meta-omics approaches for characterization of microbiota isolated from different ecological niches

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This PhD thesis research project is aimed to characterize microbiota inhabiting different ecological niches trough meta-omics approaches. This approach, applied to food, including food waste and by-products, could be a great opportunity to increase knowledge about microbiota. By-products from different fruit (e.g., pomegranate) and vegetables may be regarded as an interesting secondary raw material, but still not investigated in depth. The characterization of the microbiota in these niches would also help to lay the basis for an efficient exploitation.

Approcci meta-omici per la caratterizzazione del microbiota in diverse nicchie ecologiche

Questo progetto di tesi di dottorato mira all'applicazione di approcci meta-omici al fine di caratterizzare il microbiota di diverse nicchie ecologiche. Questo approccio, applicato agli alimenti, includendo scarti e sottoprodotti alimentari, potrebbe essere una grande opportunità di incrementare la conoscenza del microbiota. I sottoprodotti derivanti della lavorazione di diversi tipi di frutta (es., melograno) e verdura possono essere considerati come un'interessante materia prima seconda, di cui si sa ancora molto poco. La caratterizzazione del microbiota in queste nicchie aiuterebbe anche a porre le basi per uno sfruttamento efficiente.

1. State-of-the-Art

Climate change, also caused by high concentrations of carbon dioxide in the atmosphere, induces higher levels of non-structural total carbohydrates (e.g., starch and easily digestible carbohydrates) and lower levels of protein in most crops used for food production (Dietz, 2020). Furthermore, high concentrations of carbon dioxide cause a decrease in many minerals (e.g., iron and zinc), not only in whole plants (especially C3 plants, such as rice and wheat), but also in the edible organs of plants. All these aspects are contributing to the increase the prevalence of health problems in the world population, such as:
- Overweight (caused by the excessive introduction of food to compensate for the deficiency) (Loladze, 2014);
- Malnutrition (in subjects characterized by exclusion diets linked to allergies or intolerances) (Schreiner *et al*., 2020).
Hence, the project idea of developing food products enriched in proteins, minerals and vitamins, starting from a second raw material, through the fermentation with microorganisms isolated from the matrix. The study of scientific literature concerned especially pomegranate seeds, a by-product of pomegranate juice processing. Compared to arils, pomegranate seeds contain even higher amounts of nutritionally valuable components, such as proteins, fibers, ellagitannins, polysaccharides and minerals (Gül and Sen, 2017; Venkitasamy *et al*., 2019). Studies conducted so far have looked at the enrichment of food and feed with pomegranate seed meal (Table 1). However, no studies have characterized the microbiota of pomegranate seeds and juice yet. The use of microorganisms as starters of fermentation processes could improve the nutritional aspects of foods (Ahmed *et al*., 2017).

Table 1 *Main applications of by-products from pomegranate juice production in the food and feed sectors.*

By-products	Example of Case Study	Beneficial proprieties	References
Pomegranate peels	Fortification of macaroni	High level of dietary fiber and natural antioxidant compounds useful for treating and reducing cholesterolhemia	Khojah and Hafez, 2018
Pomegranate peels	Fortification of wheat bread	Antioxidant capacity	Altunkaya *et al*., 2013
Pomegranate seeds	Fortification of gluten-free pasta	Enrichment in bioactive compounds	Dib *et al*., 2018
Pomegranate seeds	Fortification of wheat bread	Dough rheology and bread quality	Gül and Şen, 2017
Pomegranate peels and seeds	Supplemented feeds	Improving the nutritional quality and shelf-life of broiler meat	Ahmed *et al*., 2017

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above, this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 2:

A1) **Collection and characterization of by-products** (microbiological characterization using culture-dependent and meta-omics approaches; chemical characterization and evaluation of health-promoting activities through *in vitro* analysis).
A2) **Isolation and identification** of microorganisms from spontaneously fermented by-products.
A3) **Controlled fermentation** of by-products with microorganisms isolated from the matrix.
A4) **Characterization of fermented by-products** (microbiological characterization using meta-omics approaches, chemical characterization and evaluation of health-promoting activities through *in vitro* analysis).
A5) **Technological application** (production of a novel food with fermented by-product).
A6) **Writing and editing** of the PhD thesis, scientific papers and oral and/or poster communications.

Table 2 *Gantt diagram for this PhD thesis project.*

Months / Activity	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1) ***Collection and characterization of by-products***	■	■	■	■																				
A2) ***Isolation and identification***					■	■	■	■	■															
A3) ***Controlled fermentation***										■	■	■	■											
A4) ***Characterization of fermented by-products***														■	■	■	■	■						
A5) ***Technological application***																			■	■	■	■		
A6) ***Writing and editing of thesis and papers***	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■

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Advanced Emulsions as Bioactive Compound Carriers for Functional Food Design: Technological and Nutritional Aspects

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This PhD research project is aimed at studying at a technological level different types of advanced emulsions enriched with a bioactive compound by applying the Design of Experiment and Response Surface Methodology techniques. Subsequently, the optimized emulsion formulations will be tested in some food matrices, different for production process technology and storage conditions, to verify the effect of the emulsions on the quality of the final products. Finally, *in vitro* tests will be performed to evaluate both the emulsion stability during all the digestion steps and the protection and bioavailability of the bioactive compound.

Emulsioni complesse come sistemi di trasporto di composti bioattivi per lo sviluppo di alimenti funzionali: aspetti tecnologici e nutrizionali

Questo progetto di ricerca di dottorato mira allo studio a livello tecnologico di alcune tipologie innovative di emulsioni arricchite con un composto bioattivo, attraverso l'applicazione delle tecniche di disegno sperimentale e di superfici di risposta. Successivamente, le formulazioni ottimizzate saranno applicate in matrici alimentari che differiscono per processo di produzione e condizioni di conservazione per verificare l'effetto sulla qualità dei prodotti finiti. Infine, verranno condotti test *in vitro* per valutare sia la stabilità dell'emulsione durante tutte le fasi della digestione, sia il livello di protezione e la biodisponibilità del composto bioattivo.

1. State of the Art

Food emulsions consist of two immiscible phases, one as a continuous phase and one as a dispersed phase in small droplets. Conventional emulsions are thermodynamically unstable, limiting certain applications. Moreover, they have a limited ability to control the release profile of encapsulated ingredients and often a high amount of fat is required to form oil-in-water emulsions and achieve a viscous or semi-solid texture (Tan & McClements, 2021). Advanced emulsion systems have been developed to overcome these issues, including double or multiple emulsions, able to encapsulate and protect both hydrophilic and lipophilic bioactive molecules (Aditya et al., 2015). Another delivery system particularly interesting is that of emulsion gels, i.e., soft solids in which emulsified lipid droplets are entrapped within a gel matrix (Dickinson, 2012). These systems have good physical stability and mechanical properties, and they can deliver lipid-soluble ingredients. The latest trend is on Pickering emulsions, i.e., particle-stabilized emulsions that have unique properties due to the almost irreversible adsorption of particles at the oil-water interface. Recently it has been found that some environmentally friendly particles, such as modified starch granules (Li et al., 2020) and β-cyclodextrin esters (Leclercq et al., 2020), can efficiently stabilize Pickering emulsions.

In the last decade, the interest in the fate of emulsions after digestion has increased, as well as the need to understand the role of the emulsion structure and composition to better design foods and beverages. Different *in vitro* methods have been developed to simulate the digestion process, considering digestive enzymes, their concentration, pH, and digestion time. There are several types of *in vitro* digestion methods (e.g., static, semi-dynamic, and dynamic), which differ for the simulation conditions, such as pH gradients during the gastric and intestinal phases, food flows, and intestinal uptake. However, to overcome differences among laboratories' procedures, an international network of multidisciplinary experts harmonized the digestion conditions by publishing the INFOGEST protocol (Minekus et al., 2014).

Because most bioactive compounds have a highly unsaturated structure that is susceptible to chemical degradation, encapsulation within emulsions might be a way to protect and increase their stability (Jain, Winuprasith, & Suphantharika, 2020). Only a few studies were found on the application of emulsions as bioactive compound carriers in real food products, and none of them performed a technological evaluation of the effect on the final food structure and digestion fate. The application in food products of these delivery systems can exploit bioactive compounds extracted from food wastes and losses (Marinelli et al., 2020), thus enhancing the sustainability of the production chain.

2. PhD Project Objective and Milestones

The overall objective of this PhD research project is the development and optimization of advanced emulsions as bioactive compound carriers for the design of functional foods. It will be reached by performing the following activities according to the Gantt diagram shown in Fig. 1:

A1) **Literature survey about emulsions.** Study of the different emulsion-based systems (e.g., single, double, gel, and Pickering) in terms of composition and process conditions. The most suitable methods for emulsion characterization will be also defined. **Deliverable (D) 1**: Report on emulsion characterization methods. **Milestone (M) 1**: Choice of the experimental factors.

A2) **Study and optimization of the advanced emulsion systems.** Design of Experiment (DoE) techniques and Response Surface Methodology (RSM) will be applied for the evaluation of the main and interaction effects of composition (e.g., emulsifier/solid particle type, dispersed and continuous phase composition, type of bioactive compound) on the properties of different emulsion systems. The technological evaluation will include rheological behaviour, microstructure, droplet size distribution, and creaming stability during storage. RSM results, coupled with the desirability function, will be used for the optimization of formulation and processing conditions of the different emulsion systems considered. **D2**: Optimized formulations of the advanced emulsions. **M2**: Choice of the advanced emulsions to be used in food design. **Risk (R) 1**: Difficulties in optimizing stable advanced emulsions; mitigation action: adjustment of formulation and processing/storage conditions.

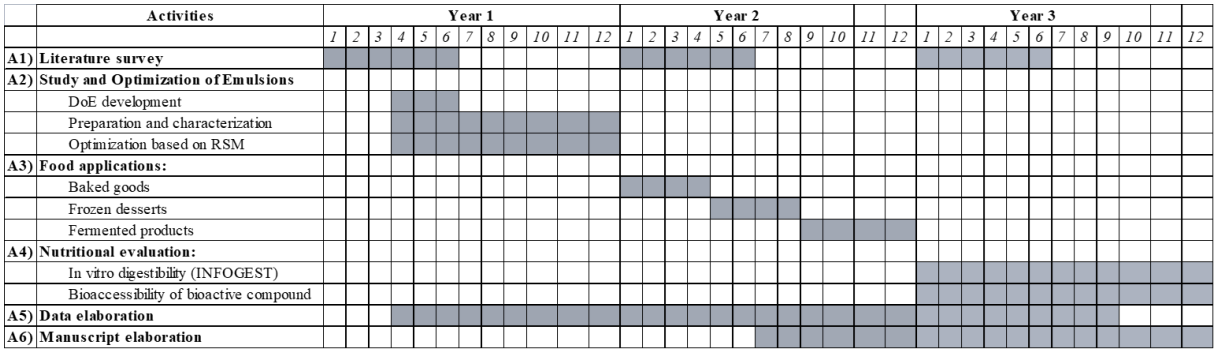
A3) **Food applications.** The optimized delivery systems will be applied to three different food products (e.g., biscuits, ice cream, and kefir). Technological parameters for each food matrix will be evaluated to design enriched food products. **D3:** Functional food formulations. **M3**: Choice of the functional foods to be subjected to nutritional characterization. **R2**: Difficulties in the design of foods with the chosen emulsions; mitigation action: revision of the emulsion formulation/process and/or choice of different food products.

A4) **Nutritional evaluation.** Both the optimized emulsion systems and the food prototypes developed will undergo *in vitro* tests to assess the digestibility and the bioaccessibility of the bioactive compound. In particular, *in vitro* digestion will be performed according to the INFOGEST protocol. **D4**: Nutritional profile of the functional foods.

A5) **Data elaboration.** The most suitable statistical tools will be applied to all the collected data.

A6) **Manuscript elaboration.** During the three-year project, scientific papers and oral/poster communications will be prepared, thus assuring proper dissemination of the results. **D5**: At least three scientific papers besides the PhD thesis.

Figure 1 *Gantt chart of the PhD project*



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Development of multiresponse kinetic models for the prediction of stability and shelf life of dry foods

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Today, the use of novel bio-based materials for food packaging is increasing exponentially. However, their application could dramatically affect food stability, especially when dealing with long-life low-moisture foods. The higher sensitivity of novel packaging to moisture compared to that of conventional packaging materials results in a less effective barrier against water uptake, ultimately compromising the stability, and thus the shelf life, of low-moisture food. This Ph.D. project aims to develop multiresponse kinetic models for the shelf life prediction of dry foods.

Sviluppo di *multiresponse kinetic models* per la previsione della stabilità e s*helf life* di alimenti a basso valore di umidità

Al giorno d'oggi l'utilizzo di nuovi materiali *bio-based* per l'imballaggio di alimenti sta aumentando esponenzialmente. Tuttavia il loro impiego potrebbe influenzare sostanzialmente la stabilità degli alimenti, soprattutto nel caso dei prodotti secchi e a lunga durabilità. La maggiore sensibilità di questi materiali al vapore acqueo rispetto ai materiali convenzionali determina una minor efficacia in termini di barriera contro l'assorbimento di acqua, compromettendo così la stabilità, e pertanto la durabilità, degli alimenti secchi. Questo progetto di dottorato si prefigge l'obiettivo di sviluppare dei *multiresponse kinetic models* per la previsione della *shelf life* degli alimenti secchi.

Keywords: dry foods; shelf-life, multiresponse modeling, packaging, biopolymers

1. State-of-the-art

Dry food stability and, hence, shelf life mostly depends on water uptake during storage. Water may be responsible for undesired physical changes or trigger chemical reactions whose extent depends on the dynamic change in moisture content and, thus, in water activity. For this reason, these foodstuffs are usually protected by employing high water barrier packaging materials such as petroleum-derived polymers. Their barrier properties against water vapor guarantee a negligible water uptake during storage. Despite their worldwide extensive application in food packaging, their use brings about severe environmental impacts, due to poor biodegradability, micro-plastic pollution, and leaching of chemical compounds (*e.g.*, plasticizers) in oceans and lands. To overcome these issues, several compostable and biodegradable packaging solutions have been developed. Even though this new class of bio-based polymers (or biopolymers) represents a promising alternative to conventional ones, their application is critical due to their moisture sensitivity. The transition from conventional to bio-based packaging materials is thus expected to be responsible for modifications of food stability, bringing about the need for a careful re-assessment of product shelf life. To date, moisture uptake in packaged dried foods has been evaluated based on a canonic modeling approach which aims to estimate the time needed to reach a "critical moisture content", corresponding to food water monolayer (Labuza, 1980). This approach, introduced by Heiss (1958) is based on the following two hypotheses: (i) food water content and water activity affect the rate of quality decay and the relevant kinetic model; (ii) the water vapor permeability of packaging material does not depend on the partial pressure gradient between the relative humidity inside and outside the film. However, until now this last assumption has been demonstrated only for conventional polymers. By contrast, based on the few data available in the literature, biopolymers exhibit a humidity-dependent behavior, whose effect on food quality and stability is still unknown and difficult to predict based on conventional kinetic models (Lee and Robertson, 2021). The development of mathematical models, taking into account the humidity-dependence of the packaging materials, is thus required to predict both the performances of biopolymeric materials during storage and the stability and shelf life of food. Based on these considerations, this Ph.D. project aims to develop multiresponse kinetic models (Bahmid et al., 2021) accounting for the conjoint behavior of biopolymer and dry food under different environmental conditions (*e.g.*, temperature and relative humidity). These models will be able to simulate the shelf-life evolution of dried foods while considering dynamic changes in food properties (e.g. water activity, glass transition temperature, micro-structure), packaging characteristics (e.g., water vapor permeability, film thickness, head-space volume), and different environmental conditions (*e.g.*, temperature and relative humidity). For this purpose, different food matrices and different bio-based packaging materials will be considered, and, depending on food features, different quality decay reactions will be monitored.

2. Ph.D. Thesis Objectives and Milestones

During the first year, the evaluation of physical and physicochemical properties (*e.g.*, a_w, T_g) of different dry food matrices (*i.e.*, potato chips, corn flakes, whey protein isolate powder, instant and ground coffee) were assessed. Indicators suitable to monitor food quality decay and relevant acceptability limits were also identified. Considering the next 24 months, according to the Ph.D. thesis project, the following activities will be pursued (Table 1):
A0) **Literature review**;
A1) **Permeability assessment** of conventional and bio-based materials as a function of different parameters (e.g., temperature, relative humidity) to investigate their behavior in the whole environmental conditions range;
A2) **Kinetic data collection** relevant to quality decay rates of the chosen food matrices under different conditions (e.g., temperature, relative humidity);
A3) Based on A1 and A2, different **multiresponse kinetic models** accounting for the conjoint behavior of packaging materials and food matrices under different environmental conditions will be developed and validated;
A4) **Mobility period**;
A5) **Papers and thesis writing**.

Table 1. *GANTT Diagram for this PhD thesis project*

Months / Activity		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A0)	***Literature review***																								
A1)	***Permeability assessment***																								
	1) Permeance evaluation of petroleum-based materials at different temperatures																								
	2) Permeance evaluation of *bio-based* materials under different conditions																								
A2)	***Kinetic data collection***																								
	1) Kinetic modeling and stability assessment																								
	2) Modeling the dependence of the environmental variables on quality decay rates																								
A3)	***Multiresponse kinetic model development and validation***																								
A4)	***Mobility period***																								
A5)	***Papers writing and thesis development***																								

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Fruit waste reduction through post-harvest early damage detection using 3D imaging methods

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The aim of this Ph.D. project is the reduction of fruit waste by early, in-line and precise fruit damage detection, using a combination of bioimpedance and 3D imaging methods. The novel method will be validated by coupling the electrical data, collected from non-invasive impedance measurements, with the optical techniques available at the industrial partner. Given the industrial nature of this Ph.D., the project will have a strong focus on the development of non-invasive methods and machines, for the fast and real-time measurement of fruit quality in both the sorting and post-harvest storage stage of the fruit supply chain.

Riduzione degli sprechi di frutta attraverso il rilevamento precoce dei danni in post-raccolta utilizzando metodi di imaging 3D

L'obiettivo di questo progetto di dottorato è la riduzione degli scarti di frutta attraverso il rilevamento precoce, in linea e preciso dei danni alla frutta, utilizzando una combinazione di bioimpedenza e metodi di imaging 3D. Il nuovo metodo sarà convalidato accoppiando i dati elettrici, raccolti con misure di impedenza, con tecniche ottiche disponibili presso il partner industriale. Data la natura industriale di questo dottorato, il progetto sarà fortemente incentrato sullo sviluppo di metodi e macchinari non invasivi, per la misurazione rapida e in tempo reale della qualità della frutta sia nella fase di selezione che in quella di conservazione post-raccolta.

1. State of the Art

Fruit quality evaluation can be implemented through each step of the fruit supply chain. More specifically, the fruit sorting happening in the post-harvest stage can be carried out based on both internal (e.g., interior defects) and external (e.g., modification in color) quality attributes and defects, mostly related to damages. Here, the most common type of damage, mostly caused by a mishandling of the fruit, are temperature (e.g., freezing, thermal shocks) and mechanical-related (e.g., pressure, impact). Freezing can happen during cultivation and preservation (Jha *et al.*, 2019), while mechanical damage or bruising can happen by dynamic forces during transportation (Mahanti *et al.*, 2022). The purpose of sorting, next to the identification of quality classes, is to identify fruit that are unsuitable for the market due to the presence of damages. There are different techniques for quality measurement, which can be categorized into two main groups: destructive and non-destructive methods. Destructive methods are slow, time-consuming, and require sample preparation, thus they cannot be used for large-scale measurement. On the other hand, non-destructive methods are fast and provide immediate measurement which is suitable for industry. In this context, non-destructive methods can be divided into two subcategories: electrical impedance spectroscopy (or bioimpedance when applied to a biological tissue) and a variety of optical methods.

Bioimpedance is the response of biological tissue to an applied, frequency-dependent, electrical field and it is measurable by contacting the fruit by means of electrodes placed directly on its surface. This method, despite being accurate, is slow and time-consuming which makes it more suitable for laboratory research purposes. With increasing demands for real-time detection of fruit quality at the industrial level, it is necessary to develop a non-destructive and non-contact detection system (Srivastava and Sadistap, 2018). The currently available non-contact quality detection technologies for postharvest products are based on optical methods. The optical techniques, that are based on the interaction of the light with the sample under the test, include colorimetry (Reid, 2012), visible imaging (Jha *et al.*, 2019), visible and near-infrared (VIS-NIR) spectroscopy (Cortés *et al.*, 2019), hyperspectral and multispectral Imaging (Qin *et al.*, 2013).

Other non-invasive methodologies that are based on the interaction of the electromagnetic field with the sample for evaluating fruit quality are Magnetic Resonance Imaging (MRI) (Barreiro *et al.*, 2000) and Computer Tomography (CT)(Du *et al.*, 2019). The aim of this green Ph.D. project is to focus on the development of a novel non-invasive method for fruit quality assessment by combining the two above-mentioned electrical and optical approaches. Furthermore, the development of a novel non-contact approach, based on the interaction of the fruit with an electromagnetic field, will be considered.

2.Ph.D. Thesis objective and milestones

The main milestones, corresponding to the three stages of this project, are the following:
M1 – Literature research: research on most recent literature on the study topic.
M2 – Stage 1: measuring bioimpedance with available instruments in the laboratory and collecting bio-impedance.
M3 – Stage 2: The bio-impedance measurements will be coupled with existing optical techniques in the Biometic/Microtec company. The results of the first stage will be coupled with the outcomes of this stage, in order to develop a new method for non-destructive measurement.
M4 – Stage 3: Finally, within this stage, the development of a new and optimized non-destructive method for bio-impedance measurement will be finalized. The optimized model will allow for the detection of the defected fruit during fruit sorting.

Table 1: *Gantt diagram for this Ph.D. thesis project*

Milestones	**1st year**				**2nd year**				**3rd year**			
Terms	I	II	III	IV	I	II	III	IV	I	II	III	IV
M1 – Literature research												
M2 – Stage 1												
M3 – Stage 2												
M4 – Stage 3												

3.Selected References

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Development of extracts and fermented officinal plants for food use endowed with sensory, antimicrobial and nutraceutical properties

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This PhD research project is aimed at developing extracts and fermented officinal plants, evaluating their sensory, antimicrobial, nutraceutical functions and their potential use inside food products.

Sviluppo di estratti e fermentati di piante officinali per uso alimentare con funzione sensoriale, antimicrobica e nutraceutica

Questo progetto di dottorato mira allo sviluppo di estratti e fermentati di piante officinali, valutandone le funzioni sensoriale, antimicrobica, nutraceutica e il potenziale inserimento in prodotti dell'ambito alimentare.

1. State-of-the-Art

Fermentation is an ancient food technology, still today subject of study and improvement, that can enhance sensory, organoleptic, nutritional quality and shelf-life of products and recently, this bioprocess has been applied to the production and extraction of bioactive compounds by food, chemical and pharmaceutical companies (Hur *et al*., 2014). Fermented foods have unique functional properties due to the presence of microorganisms capable of improving nutraceutical aspects, eliminating unwanted components, preventing food spoilage and fortifying food with bioactive compounds.
In this research project, plant extracts are used for fermentation tests with microorganisms capable of developing on these substrates and after fermentation. Products will be evaluated by checking the presence of new properties or potentially beneficial molecules. Plants are well known as source of bioactive compounds and indigenous microflora, that can have beneficial effects on human health and more and more plant natural products have been identified for different applications, especially in nutritional and pharmaceutical fields (Zhou *et al*., 2014).
The choice of microorganisms for fermentation depends on yield production and desired final products (Table 1); certainly, the bioprocess can be optimized by identifying the suitable plant-microorganism combination and the optimal incubation time and conditions (Hur *et al*., 2014). In this respect, products containing lactic acid bacteria are gaining more and more importance (Gupta and Abu-Ghannam, 2012).

Table 1 Main microorganisms and enzymatic activity used in food fermentation (Hur et al., 2014).

Microorganisms	Species	Active enzymes
Lactic acid bacteria	*Lactobacillus acidophilus*	Amylase, lactate dehydrogenases, peptidases, proteinase
	Lactobacillus casei	Amylase, lactate dehydrogenases, peptidases, proteinase
	Lactobacillus fermentum	Amylase, lactate dehydrogenases, peptidases, proteinase
	Lactobacillus lactis	Amylase, aminotransferases, decarboxylase, esterase, lactate dehydrogenases, peptidases, proteinase
	Lactobacillus plantarum	Amylase, β-glucosidase, decarboxylase, lactate dehydrogenases, peptidases, phenolic acid decarboxylases, phenol reductase, proteinase, tannase
	Lactobacillus rhamnosus	Amylase, cellulases, esterase, glucoamylase, β-glucosidase, lactate, dehydrogenases, peptidases, proteinase
Fungi	*Bacillus cereus*	Amylase, cellulases, tannase
	Bacillus subtilis	Amylase, cellulases, hydrolases, levansucrase, peptidase, proteases, xylanase
	Bacillus thuringiensis	Amylase, cellulases, tannase
	Aspergilus niger	Amylase, cellulases, estrase, fucoidanase, glucoamylase, β-glycosidase, invertase, lipase, mannanase, pectinases, phytase, protease, tannase, β-xylosidase, xylanase
	Aspergilus oryzae	Acid protease, α-galactosidase, amylase, invertase, lignin peroxidase, tannase
Yeast	*Cryptococcus flavus*	Amylase, β-glucanases, β-glucosidase, catalase, esterase, lipase, proteases, xylanases
	Cryptococcus sp. S-2	Amylase, β-glucanases, β-glucosidase, catalase, esterase, lipase, proteases, xylanases
	Rhodotorula glutinis	Alcohol dehydrogenase, amylase, β-glucosidase, epoxide hydrolases, invertase, lipase, pectinase, tannase, thiamine hydroxylase
	Saccharomyces cerevisiae	Alcohol dehydrogenase, amylase, β-glucosidase, invertase, maltase, proteases

The fermentation of plant-based extracts can give an improvement in antioxidant activity and bioaccessibility of natural bioactive compounds (Hur *et al*., 2014), as well as a reduction of antinutritional components and toxic substances in plant foods (Reddy and Pierson, 1994; Gupta and Abu-Ghannam, 2012).
This kind of fermented products and any final derived substances can be potentially used in the formulation of novel foods or be subjected to the extraction of bioactive substances produced, potentially beneficial for the human body (Hur *et al*., 2014). Functional foods are becoming increasingly popular since modern consumers are interested in their own health and expect the food they eat being healthy or even preventing illness. In the last years there has been an increase in the marketing and in consumption of fermented foods as functional foods, nutraceutical-based foods and organic foods (Gupta and Abu-Ghannam, 2012).

2. PhD Thesis Objectives and Milestones

Based on the overall objective mentioned above this PhD project can be subdivided into the following activities, according to the Gantt diagram given in Table 2:

A1) **Selection plant extracts:** starts with optimization of heat treatment in order to maintain natural components of plants (A1.1) and continues with fermentation tests of different plant extracts by some microorganisms (A1.2).
A2) **Choice food-grade microorganisms:** continuing fermentation tests in order to identify which microorganisms conduct a good fermentation depending on vegetable matrix (A2.1) and find out the optimal incubation time and conditions (A2.2).
A3) **Identification of plant-microorganism:** finding out the best plant-microorganism combinations (A3.1) and checking potentially beneficial molecules from fermentation products (A3.2).
A4) **Identification of technological processes:** in collaboration with a company stability studies of selected fermented extracts will be carried out (A4.1) and tested for insertion in final food products (A4.2).
A5) **Thesis and Paper Preparation**, writing and editing of the PhD thesis, scientific papers and oral and/or poster communications.

Table 2 Gantt diagram for this PhD thesis project.

Activity \ Months		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**
A1)	***Selection plant extracts***																								
	1) Optimization heat treatment																								
	2) Fermentation tests																								
A2)	***Choice food-grade microorganisms***																								
	1) Fermentation tests																								
	2) Optimization incubation time																								
A3)	***Identification plant-microorganism***																								
	1) Optimization fermenting combinations																								
	2) Check potentially beneficial molecules																								
A4)	***Identification technological processes***																								
	1) Stability study																								
	2) Making final products																								
A5)	***Thesis and Paper Preparation***																								

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Insight into *in-vitro* digestibility of leavened baked goods

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This PhD research aims to deepen the investigation of the main drivers that characterize the digestibility of bread employing a static *in vitro* simulation of gastrointestinal food digestion and simulator of the Human Intestinal Microbial Ecosystem (SHIME®). Mainly focusing on mapping the factors affecting the digestibility of sourdough breads and their impact on the human gut microbiome and metabolome.

Approfondimenti riguardo la digeribilità *in vitro* dei prodotti da forno lievitati

Questo progetto di dottorato mira ad approfondire le conoscenze riguardo i principali fattori che caratterizzano la digeribilità del pane mediante una simulazione *in vitro* del percorso gastrointestinale. Inoltre, il funzionamento dell'ecosistema microbico intestinale umano sarà simulato mediante l'apparato SHIME® (Human Intestinal Microbial Ecosystem). Nello specifico, le analisi riguarderanno i principali fattori nutrizionali del pane a lievitazione naturale, comparato al pane con lievito di birra, durante ogni fase della simulazione della digestione *in vitro* e il loro impatto sul microbioma intestinale umano e sul metaboloma.

1. State-of-the-Art

Sourdough bread is a staple food essential for human nutrition. Primarily, research concerning sourdough biotechnology focused on specific technological properties such as natural leavening, texture, taste, and shelf-life. However, now research is redirected to the nutritional aspects and possible health benefits of sourdough biotechnology. Fermentation of cereals and pseudo-cereals with lactic acid bacteria improves the taste and enriches the substrate with several nutritional factors such as peptides and free amino acid (FAA), as well a greater *in vitro* protein digestibility level. However,, a long fermentation time leads to a lower glycemic index and decrease in phytate content and other anti-nutritional factors (Arora *et al*. 2021).
In addition, many studies have shown that the long fermentation time of the sourdough biotechnology improves the digestibility of bread. The digestibility is linked to multiple factors both of gastrointestinal nature and bioavailability of starch and protein, as well as appetite perception (Rizzello *et al*. 2019). Nevertheless, a clear definition of bread digestibility concept is difficult to find. In the recent years, a number of *in vitro* digestion models have been developed. They aim to investigate the paths of foods during digestion within the upper gastro-intestinal tract and their physiological effect on human health. Simulated digestion methods typically include the oral, gastric and small intestinal steps and try to mimic physiological conditions *in vivo* by using digestive enzymes and taking into account several parameters, such as pH and digestion time (Li *et al*. 2020). The use of the Simulator of the Human Intestinal Microbial Ecosystem (SHIME®) is the only, up to date, *in vitro* validated system able to map the impact of various food components on the human gut microbiota (Da Ros *et al*. 2021). Thus, my PhD project aims to carry out a precise and in-depth characterization of the main digestibility factors of sourdough bread compared to baker's yeast bread, during each segment of the gastrointestinal path tested *in vitro*. In addition, the large intestine will be simulated by means of a TWIN SHIME® experimental set up in order to have an overview on sourdough bread impact on the gut microbiota.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above, this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) **Experimental design of sourdough and baker's yeast breads.** Sourdough bread will be characterized by fermentation at 30°C for 24 h using *Lactiplantibacillus plantarum* CR1, *Furfurilactobacillus rossiae* CR5 and *Saccharomyces cerevisiae* E10. The sourdough bread will be compared with a baker's yeast bread prepared with 1.5% (w/w) of baker's yeast and fermentation lasting 2 h at 30 ◦C used as the control.

A2) ***In vitro* pre-digestion process of bread samples.** The *in vitro* simulation of human gastrointestinal digestion consists of 3 phases: the oral phase, the gastric phase and the small intestine phase. The oral pre-digestion includes food dilution with simulated salivary fluid and the exposure (2 min) to salivary amylase. Then, the oral bolus is diluted with simulated gastric fluid and gastric enzymes (pepsin and gastric lipase) and incubation lasting for 2 h at 37 ˚C, using a pH gradient from 6.0 to 2.0. Finally, the gastric chyme is diluted with simulated intestinal fluid, bile salts and pancreatic enzymes (pancreatin, lipase, trypsin and

chymotrypsin), and incubation at pH 7 is extended for further 3 h at 37 ˚C under static dialysis with a membrane of 14 kDa.

A3) **Analyses *in vitro* digestibility index of sourdough and baker's yeast bread at different time points.** Samples during *in vitro* pre-digestion simulation will be analysed for several digestibility indexes. Sampling will be done in seven different time points representing each step of the gastrointestinal tract. The first time points are as follows: T_0 bolus, T_1 chyme, T_2 kilo and permeate. The analyses will concern: Protein hydrolysis markers (total free ammino acids and peptides), total starch determination, starch hydrolysis products (amylose, amylopectin, reducing sugar, dextrins), resistant starch and soluble and insoluble fiber.

A4) **Determination of the *in vitro* correlation between the factors affecting the in vitro digestibility of breads and the human gut microbiota and metabolome**. Samples will be further analyzed using the SHIME®, to evaluate the effect of breads on the gut microbiota functionality. The set-up will be a TWINSHIME® (UGent/ProDigest) configuration consisting of four consecutive bioreactors simulating stomach and small intestine together, and ascending (AC), transverse (TC), and descending (DC) colon. Before starting, all the bioreactor that mimic the colon tracts will be inoculated with a representative healthy fecal sample from the same donor. Samples will be collected from the SHIME® bioreactors at T_3 fecal material collected from ascending colon tract, at T_4 from traversal colon tract, at T_5 from descending colon tract and at T_6 the descending colon waste. The analyses will involve protein hydrolysis markers (total free ammino acids and peptides), resistant starch, soluble and insoluble fiber, short chain fatty acids (SCFA), volatile organic compounds (VOC) and microbiota composition.

A5) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

Table 1 *Gantt diagram for this PhD thesis project.*

Activity	Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	***Experimental design sourdough breads***																								
A2)	***In vitro pre-digestion process of sourdough breads***																								
A3)	***Analyses in vitro digestibility index***																								
	1)T_0 bolus, T_1 chyme, T_2 kilo and permeate,																								
A4)	***Determination of the in vitro correlation between the factors affecting the in vitro digestibility of breads and the human gut metabolome***																								
	1)T_3 AC fecal material, T_4 TC fecal material, T_5 DC fecal material, T_6 descending colon waste																								
A5)	***Thesis and Paper Preparation***																								

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Improvement And Optimization of Eco-Friendly and Sustainable Packaging Materials for Meat Products

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This Ph.D. thesis aims to study, optimize, and validate innovative packaging materials designed for cured meat products packaged under a modified atmosphere (MAP) with an improvement in the sustainability and circularity degree.

Miglioramento e ottimizzazione di materiali di imballaggi sostenibili ed eco-friendly per prodotti carnei

L'obiettivo di questa tesi di dottorato mira a studiare, ottimizzare e validare materiali di confezionamento sostenibili ed eco-friendly, ideati per prodotti carnei trasformati conservati in atmosfera modificata.

1. State-of-the-Art

In Italy, pork meat represents one of the most expanding sectors among all the cured meat products nationwide according to the annual ASSICA Report. The main purchased cured meat products are cooked ham (27,2%) followed by Prosciutto Crudo (21,8%) (ASSICA Report, 2020). Packaged cured meat products are very sensitive to microbial growth, discoloration, and fat oxidation, especially due to residual oxygen and water vapour concentration in the headspace of the package. Therefore, packaging plays a crucial role to avoid chemical, enzymatic and physical spoilage through efficient barrier properties against humidity, gases (O_2, CO_2, odours), and UV radiations (Baele *et al.*, 2021).

The traditional packaging for cured meat products combines a modified protective atmosphere (MAP) and a plastic tray, usually made of polyethylene terephthalate (PET) together with multi-layered packaging composed mainly of polyethylene (PE) and ethylene-vinyl-alcohol (EVOH). Multi-layered plastic packaging is characterized by excellent processing performances such as mechanical strength, transparency, low permeability, and heat sealability. All these properties ensure a high level of quality and safety of the meat. However, multi-layered plastic materials are not environmentally sustainable since they are assembled from non-renewable sources, i.e. fossil fuels and natural gases (Peelman *et al.*, 2014) and the recycling process is still an open issue due to challenges with layers separation (Cinelli *et al.*, 2016).

The massive consumption of such materials further contributes to environmental challenges such as depleting natural resources, pollution of terrestrial and marine ecosystems, and global warming. Therefore, thanks to recent European environmental policies and the growing public awareness regarding environmental challenges, there is an urgent demand to switch to suitable and effective sustainable packaging materials capable of ensuring the same food quality and safety as conventional packaging materials (Van Crevel, 2016).

The outlined strategies of the European Commission aiming to swift toward a green transition, include firstly the reduction of over packaging and food waste. Secondly, all plastic packaging inside the EU market must be reusable or recycled in a cost-effective manner by 2030. Moreover, also biodegradable and compostable polymers could represent a possible and alternative solution for achieving sustainable development goals of the Circular Economy (European Commission, 2020).

Based on this information, this Doctoral project aims to study, optimize, and validate packaging materials designed for cured meat products in MAP conditions, with an improvement of the sustainability and circularity degree. Suitable solutions could be the mono plastic materials and bio-based plastics that are both fully recycled, but also bio-based and compostable materials such as bioplastics (PLA, PHA, PBS). However, to our knowledge, only limited information is available regarding sustainable packaging for cured meat products in MAP conditions, and to date, the most sustainable packaging applications in the market are restricted to the use of paper combined with plastic layers, which ensure the lowest levels of permeability. For this purpose, sustainable packaging materials will be selected and the required properties for cured meat products stored in MAP will be investigated. In addition, shelf life analyses will be performed to validate the effectiveness of this alternative packaging, thus comparing it with traditional packaging's shelf life.

2. Ph.D. Thesis Objectives and Milestones

This Ph.D. thesis project can be divided into the following activities according to the Gantt diagram given in Table 1:

1) **Bibliographic research;**
2) **Study of packaging materials** through the following analyses: seal and tensile strength, elongation break resistance, thermal behaviour, colour and opacity, oxygen and water vapor permeability, and wettability of the film;
3) **Mobility period** focused on the improvement of barrier and mechanical properties of the selected packaging materials;
4) **Shelf-life studies** of the cured meat products stored in MAP conditions with the most efficient packaging materials examined in the previous phase. This step will be characterized by microbiological analyses, water activity, pH, colorimetric analysis, variation of the O_2 and CO_2 concentration in the headspace during the shelf life, sensorial and volatile organic compounds evaluation;
5) **Internship at the company;**
6) **Industrial validation** of the innovative and sustainable packaging;
7) **Thesis and papers preparation.**

Table 1. Gantt diagram of the Ph.D. thesis project.

Activity / Months		2	4	6	8	10	12	14	16	18	20	22	24	26	28	30	32	34	36
1)	Bibliographic research																		
2)	Study of packaging materials																		
3)	Mobility period																		
4)	Shelf-life studies																		
5)	Internship at the company																		
6)	Industrial validation																		
7)	Thesis and Papers Preparation																		

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Identification of Aggregate Metabolic Phenotypes for Dietary (Poly)phenols and Assessment of the Factors associated with their Formation: Development of an Oral (Poly)phenol Challenge Test (OPCT)

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This PhD thesis research project aims at identifying aggregate metabolic phenotypes (metabotypes) for the main dietary (poly)phenols, evaluating the determinants of inter-individual variation leading to different phenotypic profiles and building predictive algorithms for a faster identification of metabotypes. An intervention study carried out on 300 volunteers, which undergo an oral (poly)phenol challenge test (OPCT) in the form of (poly)phenol-rich tablets and provide personal information as well as their health status, gut microbiota composition and genotyping, will allow to find an association between cardiometabolic health and (poly)phenol metabolism. Metabotyping may thus represent a promising attempt for cardiometabolic health promotion through personalised nutrition initiatives.

Identificazione dei fenotipi metabolici aggregati dei principali (poli)fenoli della dieta e valutazione dei fattori determinanti la loro formazione: sviluppo di una challenge nutrizionale per lo studio del metabolismo dei (poli)fenoli

Questo progetto di tesi di dottorato mira ad identificare i fenotipi metabolici aggregati (metabotipi) per i principali (poli)fenoli della dieta, valutare i fattori della variabilità inter-individuale che portano ai diversi profili fenotipici e costruire algoritmi predittivi per una più rapida identificazione dei metabotipi. Uno studio di intervento condotto su 300 volontari, sottoposti ad una challenge nutrizionale (OPCT) consumando compresse ricche di (poli)fenoli e fornendo informazioni personali, nonché sul loro stato di salute e includendo profilazioni genetiche e di microbiota intestinale, fornirà indicazioni sull'associazione tra salute cardiometabolica e metabolismo dei polifenoli. La metabotipizzazione potrà essere funzionale alla promozione della salute cardiometabolica attraverso approcci di nutrizione personalizzata.

1. State-of-the-Art

Increasing evidence suggests that plant-based foods play an important role in the prevention of chronic diseases, as they are rich sources of a variety of biologically active phytochemicals with known health-promoting effects (Liu, 2013). (Poly)phenols or phenolic compounds are the most abundant category of phytochemicals and are almost ubiquitous in fruits, vegetables, legumes, cereals, and beverages like wine, coffee, tea, and beer (Del Rio et al., 2013). Modest long-term intakes of (poly)phenols can reduce the risk of chronic diseases, especially cardiovascular diseases and type 2 diabetes (Rodriguez-Mateos et al., 2014). Nevertheless, the role of (poly)phenols in cardio-metabolic protection has not been consistently demonstrated yet (Gibney et al., 2019), mainly due to the heterogeneity of each individual response to the consumption of dietary (poly)phenols, that modulates their efficacy (Manach et al., 2017). The inter-individual variability in the physiological response is mainly influenced by differences in the absorption, distribution, metabolism, and excretion (ADME) of (poly)phenols (Gibney et al., 2019), along with other factors, including genetic background, gut microbiota, sex, age, ethnicity, lifestyle (diet, smoking, and physical activity), (patho)physiological status and medication (Gibney et al., 2019; Cassidy & Minihane, 2017). After ingestion, (poly)phenols are poorly absorbed in the small intestine and reach the colon, where they undergo modifications by the gut microbiota, being converted to smaller catabolites that are easily absorbed into the blood stream where they circulate principally as conjugated phase II metabolites, which can act as mediators of diet-induced effects on health (Del Rio et al., 2013). The inter-individual differences in gut microbial composition and functionality can lead to quantitative and qualitative differences in the production of specific metabolites, influencing the bioactivity of (poly)phenols in the host (Manach et al., 2017). The different catabolite production patterns are related to metabolic phenotypes (the so called metabotypes) clearly defined by the presence/absence of specific metabolites of colonic origin in the circulation. This results in different excretive phenolic profiles, which can be clustered to form phenolic metabotypes.

This acute human intervention study is directed to understand the association between aggregate metabolic phenotypes for the main dietary (poly)phenols and the factors determining their formation. Subjects will be clustered according to their aggregate metabotype, representative of the production and urinary excretion of relevant dietary phenolic metabolites. Moreover, to establish the main determinants of inter-individual differences in metabolic profiles, the relationships between the aggregate metabotypes and individual characteristics such as age, sex, ethnicity, dietary habits, lifestyle (physical activity, smoking, and sleeping habits), cardiometabolic health status (defined by anthropometrics, body composition, blood pressure, and plasma and urine biomarkers),

genotype, gene expression, and gut microbiota composition will be evaluated. Lastly, predictive algorithms for the identification of each aggregate metabotype will be built by using all the information collected for each subject (both metabolomics data and individual characteristics), which defines individual's makeup.

2. PhD Thesis Objectives and Milestones

Specific objectives of this PhD thesis project can be divided into the following activities according to the Gantt diagram given in Table 1:

1. ***Development of the challenge:*** developing a dietary challenge for the study of the metabolism of (poly)phenols, producing the tablets, managing the beginning of the intervention;
2. ***Deployment of the OPCT:*** recruiting 300 volunteers and managing the collection of data/samples;
3. ***Sample analysis:*** analysing biological samples (blood, urine, feces) to assess the different outcomes;
4. ***Existence of metabotypes:*** investigating the existence of comprehensive (aggregate) phenolic metabotypes using multivariate statistics;
5. ***Determinants of variation:*** evaluating the determinants of inter-individual variation leading to different phenotypic profiles through different statistical tools;
6. ***Association among outcomes:*** associating aggregate phenolic metabotypes to determinants of individual variability and to markers and scores of cardiometabolic health;
7. ***Building predictive models:*** building predictive algorithms for the identification of metabotypes;
8. ***Thesis and Paper Preparation:*** writing and editing of the PhD thesis, scientific papers and oral and/or poster communications.

Table 1 Gantt diagram for this PhD thesis project.

Activity	Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1)	***Development of the challenge***																								
2)	***Deployment of the OPCT***																								
3)	***Sample analysis***																								
4)	***Existence of metabotypes***																								
5)	***Determinants of variation***																								
6)	***Association among outcomes***																								
7)	***Building predictive models***																								
8)	***Thesis and Paper Preparation***																								

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Sensory and technological improvement of hemp seed flour. Strategies for food applications

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In line with the circular economy principles and considering the significant growth trend of the global hemp food market, this PhD thesis project aims to improve the sensory, technological, and functional properties of hemp seed flour, an underutilized by-product of hemp oil production, through a biotechnological approach. Different food formulations will be then developed using the ameliorated flour in a percentage higher than that currently used, achieving processed hemp food products of high quality and well appreciated by consumers.

Miglioramento delle proprietà sensoriali e tecnologiche della farina di canapa. Strategie per il settore alimentare

In linea con i principi dell'economia circolare e considerando il significativo trend di crescita del mercato globale dei prodotti alimentari canapicoli, questo progetto di tesi di dottorato mira al miglioramento delle caratteristiche sensoriali, e delle proprietà tecnologiche e funzionali della farina di canapa, sottoprodotto - oggi ancora poco utilizzato - del processo di estrazione dell'olio, attraverso un approccio biotecnologico. Verranno poi sviluppate diverse formulazioni alimentari utilizzando la farina migliorata in una percentuale superiore a quella attualmente in uso, ottenendo prodotti alimentari a base di farina di canapa di elevata qualità ed apprezzati dai consumatori.

1. State-of-the-Art

Industrial Hemp Market size was valued at USD 6.2 Billion in 2021 and is projected to reach USD 19.9 Billion by 2030**,** growing at a compound annual growth rate (CAGR) of 14.01% from 2022 to 2030**.** The industrial hemp market is driven by factors such as increasing legalization in the cultivation of industrial hemp, rising application of industrial hemp in diverse industries - such as, among others, textile, pharmaceutical, food, construction & material - and raising awareness among the consumers about the benefits of industrial hemp. Among the various industrial sectors, the food sector is expected to grow significantly, thanks to the functional properties of hemp seed and hemp seed oil and their increased use in different food applications (Verified Market Research, 2022).
The increasing demand for hemp seed oils will result in a large availability of hemp seed flour, a by-product of the oil production process. Hemp seed flour is a rich source of nutrients, such as protein, dietary fiber, minerals, unsaturated fatty acids, and vitamins, that make it a valuable component for human consumption. Indeed, hemp seed flour has been used as fortifying ingredient for improving the nutritional value of bakery products (Mikulec et al., 2019), pasta (Teterycz et al., 2021), and meat (Zając et al., 2019) or, also, as a structure-enhancing ingredient in gluten-free bread (Korus et al., 2017).
Despite its undoubtable nutritional value, hemp flour is still an underutilized ingredient due to its poor sensory and rheological properties. Its bitter taste and herbaceous flavor allow using hemp seed flour at a percentage not exceeding 5% in meat products (Zając et al., 2019), and up to 20% of the conventional flour in baked products (Hayward et al., 2020); in the case of fortified pasta, high percentages of hemp seed flour also detrimentally affect the structure of pasta reducing its cohesiveness (Teterycz et al., 2021).
There is evidence that fermentation of cereal, legume, and seed flours positively affects the nutritional, sensorial, technological, and functional properties of flours by reducing the antinutritional factors responsible, *inter alias*, of bitter taste, decreasing off-flavors compounds - such as, for ex., medium-chain aldehydes - enhancing protein digestibility and bioavailability, and modifying flour functional properties (Garrido-Galand et al., 2021). The use of commercial enzymes can also be effective in improving the nutritional, functional, and sensory quality of vegetable flour by increasing protein digestibility and availability, lowering the insoluble fiber amount, enhancing the antioxidant activity, and releasing protein fragments with different biological activities and taste. To the best of our knowledge, very few are the information reported in the literature on the applications of these strategies to hemp seed flour and aiming mainly to improve the antioxidant properties and the protein digestibility of whole hemp seeds (Pontonio et al., 2020).
As regards the food system, the Sustainable Development Goals of the United Nations 2030 Agenda and the European Commission Circular Economy Action Plan imply reducing the amount of waste, re-use of food, utilization of by-products and food waste, nutrient recycling, and changes in diet toward more diverse and more efficient food patterns. So, the conversion of the hemp oil by-product into flour, and its readmission into the productive food chain are of great interest, but it is equally important to boost the use of this ingredient to avoid that the low demand for hemp flour makes it become surplus and waste.
In this context, the aims of this PhD thesis project are: 1) improving the sensory, technological and functional properties of hemp seed flour through a biotechnological approach, i.e. spontaneous or guided lactic acid

fermentation and enzymatic treatments with commercial food-grade proteases, transglutaminases, hemicellulases, and cellulases; 2) using the ameliorated flour in food formulations in a percentage higher than that currently used; 3) and achieving hemp food products of higher quality and well appreciated by consumers.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above, this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1 **Production of hemp flour from fermented hemp cake (FHC)**. A.1 Development of a submerged method for the lactic acid fermentation of unmilled hemp cake; assessment, and monitoring of fermentation parameters (temperature, moisture, pH, aeration). Spontaneous fermentation (A1.1.1) or guided fermentation with selected LAB starters (A1.1.2) will be performed. Starter cultures will be selected according to the following criteria: 1) technological characteristics (growth, acidification); 2) capability of lowering antinutritional factors; 3) proteolytic activity; 4) β-glucosidase activity; 5) degrading activity toward plant cell wall polysaccharides. Selected cultures will be used as single strains or as mixtures of strains. A1.2 Drying of fermented hemp cake, milling into flour, and sieving.

A2 **Production of fermented hemp flour (FHF).** A2.1 Development of a submerged method for the lactic acid fermentation of hemp flour (i.e. milled hemp cake) as reported in A1; A2.2) Drying of fermented hemp flour.

A3 **Production of hemp sourdough (HSD).** A3.1 Set up of the fermentation conditions for spontaneous fermentation (A3.1.1) or guided fermentation with selected LAB starters (A3.1.2). Criteria for Starter selection are reported in A1.

A4 **Production of enzymatically treated hemp flour (ETHF)** using commercial food-grade proteases (A4.1), transglutaminases (A4.2), hemicellulases, and cellulases (A4.3).

A5 **Characterization of FHC, FHF, HSD,** and **ETHF** from A1-A4 through physicochemical and chemical analyses (A5.1), sensory analyses (A5.2), technological and functional attribute determination (A5.3)

A6 **Design and development of hemp-enriched** or **hemp-based food products** using the ingredients from A1-A4, such as pasta (A6.1), bakery products (A6.2), gluten-free products (A6.3), and meat analogs (A6.4).

A7 **Characterization of food products** from A6 through nutritional value, protein digestibility, glycemic index, antioxidant compounds and activity (A6.1), color and texture profile (A6.2), sensory features (A6.3), and consumer acceptability (hedonic test, CATA test, survival analysis, preference maps) (A6.4).

A8 **Writing and Editing** of the PhD thesis, scientific papers, and oral and/or poster communications.

Table 1 Gantt diagram for this PhD thesis project.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1	Production of FHC																								
A2	Production FHC																								
A3	Production of HSD																								
A4	Production of ETHF																								
A5	Characterization of FHC, FH, HSD, ETHF																								
A6	Design and development of hemp-enriched or hemp-based food products																								
A7	Characterization of food products																								
A8	Thesis and Paper Preparation																								

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Production of added value bakery products by upcycling oenological by-products

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The aim of the research project is to enhance the by-products from winemaking by using them as functional ingredients in the production of baked goods to improve their nutritional composition and quality. The best performing conditions of use will be selected to obtain functional and technological advantages, and then produced on an industrial scale. Subsequently market and consumer acceptance will be evaluated with direct surveys carried out with the final consumers.

Produzione di prodotti da forno ad alto valore aggiunto mediante il recupero dei sottoprodotti enologici

Il seguente progetto di dottorato è volto alla valorizzazione dei sottoprodotti enologici mediante loro reimpiego come ingredienti funzionali da inserire nel processo produttivo di prodotti da forno con lo scopo di migliorarne la qualità ed il profilo nutrizionale. Le condizioni di utilizzo più performanti saranno selezionate per ottenere diversi vantaggi funzionali e tecnologici e utilizzate per la produzione in scale-up industriale. Conseguentemente verrà effettuata una analisi di mercato e della preferenza dei consumatori.

1. State-of-the-Art

The wine sector represents one of the most important economic sectors of Italy, which leads the world's wine production (19% of the total) producing over 47 million hL per year (Sabetta *et al*., 2022).
However, in addition to wine, the production process also generates by-products such as marc, grape seeds, stems, and wine lees. In most cases, these are not treated as resources but as waste, and this creates problems related to their disposal (Maner *et al*., 2015). Although treating them as secondary raw materials would have several advantages because they are rich in functional molecules such as polyphenols, which inhibit the oxidation of cells, tissues and the peroxidation of low-density lipoprotein, thus fighting cardiovascular disease, cancer and diabetes, and also contain fibers and vitamin E, which improve and stimulate the human immune system (Gunes-Bayir *et al*., 2019) (Table 1).

Table 1. Functional compounds of oenological by-products.

By-product	Bioactive compounds	Author
Grape pomace	Protocatechuic, gallic and vanillic acid, quercetin, myricetin, anthocyanins.	Pintać *et al*., 2018
Seeds	Vitamin E, proanthocyanidin, phytosterols.	Troilo *et al*., 2020
Grape stalks	Tannins, hydroxycinnamic acid, stilbenes.	Ping *et al*., 2010
Wine lees	Dietary fibers, tartaric acid.	Rivas *et al*., 2021

Moreover, these compounds can increase the nutritional value of foods. In particular, grape pomace added to biscuits has an antioxidant action that delays the rancidity of the product (Ahmed *et al*., 2020), and increases the fiber content, which could lower the glycemic index of baked products (Kuchtova *et al*., 2018; Rainero *et al*., 2021). The mannoproteins contained in wine lees have an emulsifying and foaming activity, (De Iseppi *et al*., 2021), which will be exploited to replace and reduce the lipids used in biscuit production.
For these reasons, the objectives of the project are:

(i) to create nutritionally improved bakery products by using oenological-by-products; to test the improvement in the nutritional characteristics of the products made in laboratory, the protocols will be transferred to an industrial scale where sensory evaluation tests will be performed to ensure the acceptability of the products obtained;
(ii) to meet the current needs of consumers, who are increasingly attentive to a healthy diet (due to increased awareness of the connection between genuine food and psycho-physical well-being) and the environmental sustainability of agri-food production.

In this context the project also aligns with the 2030 Sustainable Development Goal of eradicating world hunger while ensuring food safety and security. In addition, the use of by-products in the form of flour could replace a part of the wheat flour used in bakery products to partially alleviate the current critical issues of cost and supply of wheat due to the international geo-political situation.

2. PhD Thesis Objectives and Milestones

The objectives of the following PhD project will be achieved via the following activities:

A0) **Literature study**.

A1) **Formulation of baked goods fortified with by-products**: doughs with different percentages of by-product flours will be prepared on a laboratory scale.

A1.1) Treatment and characterization of by-products: grape pomace, grape stalks, seeds and wine lees will be subjected to pretreatment and subsequent analysis of proximate chemical and nutritional composition.

A1.2) Characterization of bakery products: analysis of nutritional composition (protein, ash, fibers, fats and carbohydrates), texture profile, ABTS, DPPH, Folin-Ciocalteu, determination of volatile compounds and sensory analysis.

A2) **Industrial scale-up of fortified baked goods**: selected products will be produced with industrial scale-up optimizing the technological parameters.

A2.1) Evaluation of nutritional and sensory characteristics: evaluation of proximate composition (proteins, ashes, lipids, carbohydrates, dietary fiber) and organoleptic characteristics (panel test).

A2.2) Market analysis and acceptability assessment: environmental impact assessment (evaluate that all components of the by-products are reused) and consumer test.

A3) **Shelf-life evaluation**: study of different storage conditions.

A4) **Data processing and writing of scientific paper**.

Table 2 Gantt diagram for this PhD thesis project.

Activity	Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A0)	**Literature study**																								
A1)	**Formulation of baked goods fortified with by-products**																								
	A1.1) Characterization of by-products																								
	A1.2) Characterization of bakery products																								
A2)	**Industrial scale-up of fortified baked goods**																								
	A2.1) Evaluation of nutritional and sensory characteristics																								
	A2.2) Market analysis and acceptability assessment																								
A3)	**Shelf-life evaluation**																								
A4)	**Data processing and writing of scientific paper**																								

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Non destructive methods for predicting cheese making traits

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This PhD thesis research project aims to explore innovative and non destructive methods to monitor the cheese making using a broad range of vibrational spectroscopy techniques.

Metodi non distruttivi per la predizione dei caratteri relativi alla caseificazione

Questo progetto di tesi di dottorato mira ad esplorare metodi analitici innovativi e non distruttivi per il monitoraggio del processo di caseificazione, applicando un'ampia gamma di tecniche di spettroscopia vibrazionale.

1. State-of-the-Art

The cheese production is nowadays one of the main ways to add value to the milk along the dairy chain. In Italy, more than 80% of the milk production is processed into cheese, among which more than 50% are Protected Designation of Origin (PDO) cheeses. Among the PDO cheese production, the Grana Padano and Parmigiano Reggiano are taking the biggest share. Yet, the production and quality control of the processes involved in the production of those cheeses are still poorly monitored. Moreover, most of those analyses routinely used for cheese making characterization is costly and time consuming.
For this reason, the development of in situ methods to assess the milk quality and cheese-making could improve the quality control and possibility to promptly adjust the running process. The development of nondestructive spectral sensors could be a useful tool for monitoring and improving cheese making efficiency.
While the milk payment system and the quality control are still based on traditional traits (i.e., milk protein and fat), the cheese industry is interested in more practical phenotypes such as the cheese yield (%CY): fresh, solids and retained water ($\%CY_{CURD}$, $\%CY_{SOLIDS}$, $\%CY_{WATER}$); and the recovery traits (%REC): milk fat, protein, solids and energy in the curd ($\%REC_{FAT}$, $\%REC_{PROTEIN}$, $\%REC_{SOLIDS}$ and $\%REC_{ENERGY}$) which represent the ratio between the weights of the fat, protein, and total solids in the curd, respectively, and the corresponding components in the milk. Energy recovery, REC_{ENERGY}, represents the percentage energy content of the cheese compared with that in the milk (Cipolat-Gotet et al., 2013). One of the aims of this PhD project is to allow their prediction at an early stage of the process, preferentially using a quick and user-friendly technique.
To make this monitoring possible at a cheese factory level, it is necessary to provide handheld or inline solutions. In the last years, several studies have been carried out to develop portable Near Infrared (NIR) devices allowing to carry on site analysis. The development of calibrations using the NIR spectra allows to determine the chemical composition in a faster, cheaper, and more environmentally friendly way than the humid chemical analysis that it replaces (Holroyd, 2013).
Even though the use of NIR has been proved to be reliable and useful especially for on-line application in the dairy sector, the interpretation of the spectra remains an issue, as well as the intrinsic low sensitivity. Therefore, the possibility to integrate signals from NIR and other emerging techniques such as Hyperspectral Imaging (HSI) or Raman spectroscopy could enable the collection of a larger amount of information about cheese quality. Indeed, the HSI is a novel technology in the field of food safety while not yet fully investigated at dairy chain level (Priyashantha et al., 2021). However, HSI has great potential to be employed within the dairy sector, as it is a rapid, reliable, and inexpensive technique which requires minimal sample preparation (Roberts et al., 2018). Therefore, the combination and/or comparison of signals from NIR spectroscopy and HSI could bring new scientific breakthroughs and provide with an effective tool to monitor the quality and safety of dairy products.
Beside this, the Raman spectroscopy is raising further attention for being a quick and non destructive technique to analyze milk and cheese, "*although many publications describe several applications of Raman spectroscopy and chemometric analyses, such approach are not yet employed in standard laboratories and industries. Usually they prefer NIR spectroscopy because is cheaper and simple. However, Raman spectroscopy provides the higher information content, since the spectrum contains information of each one of the chemical components*". (de Sá Oliveira et al., 2016)
This method could be studied in combination with the others to provide the most useful information for the cheese factory.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above, this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) **Exploration of different non destructive methods used for milk and cheese analysis** . In order to determine which method/combination of methods could be applicable on the field, as a balance between costs and results.
A2) **Assessment and modelling of the cheese making traits** to identify the mathematical model capable of building the most accurate predictions as functions of the milk traits that can be analyzed on the field (composition, spectra, …).
A3) **Development of the statistical predictions and trials on several dataset** to assess the accuracy, robustness, and limits of use of the calibrations.
A4) **Development of built-in tailored application** to give the predictions on the field in cooperation with sensor manufacturer.
A5) **Writing and Editing** of the PhD thesis, scientific papers, workshops and conferences, and oral and/or poster communications.

Table 1: Gantt diagram for this PhD thesis project.

Activity	Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1	**Exploration of different non destructive methods** • IR Spectroscopy • Hyperspectral Imaging • Raman Spectroscopy																								
A2	**Assessment and modelling of the cheese making traits**																								
A3	**Development of the statistical predictions and trials on several dataset**																								
A4	**Development of built-in custom application**																								
A5	**Thesis and paper preparation**																								

3. Selected References (APA)

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Exploring barriers and enablers for the environmental sustainability communication "Zero-waste supply chain": insights from the Italian vegetables supply chain trough the LOWINFOOD project

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The aim of this PhD thesis project is to explore enablers and barriers for the introduction of innovations direct to reduce food waste in the case of an Italian Vegetables supply chain. The impacts are measured in the framework of the "Zero-Waste Supply Chain" which is defined during the study. This framework is designed to assess which are the effects on different actors of more sustainable food systems. Finally, the acceptance of a sustainability communication (*ecolabel*) on the final product by the consumer will be assessed.

Esplorare fattori abilitanti e barriere riferite alla comunicazione di sostenibilità ambientale "Filiera Zero-rifiuti": approfondimenti per la filiera italiana di Frutta & Verdura attraverso il progetto LOWINFOOD

L'obiettivo di questo progetto di tesi di dottorato è di esplorare fattori abilitanti e barriere per l'introduzione di innovazioni dirette a ridurre lo spreco alimentare nel caso di una filiera italiana di verdure. Gli impatti sono misurati nel framework della "Zero-Waste Supply Chain" definita durante lo studio. L'obiettivo è di valutare quali sono gli effetti di sistemi alimentari più sostenibili sui diversi stakeholder sulla supply-chain. Infine, sarà valutata l'accettazione di una comunicazione di sostenibilità (*ecolabel*) sul prodotto finale da parte del consumatore.

1. State-of-the-Art

Reduction of Food Loss (FL) and Waste (FW) along the Food Supply Chain (FSC) is one of the most urgent challenges of this century. In the recent years this topic has achieved a strategical importance in governments and organizations' Agenda as part of the efforts to fight global hunger and improve food security. Food Waste & Loss (FWL) means losses of food which still contains nutritional value and that could have been used for human consumption. In September 2015, United Nations together with other 193 member states, reached an agreement on establishment of 17 new "Sustainable Development Goals" (SDGs) that aim to promote sustainability concept at all levels. In particular, the Goal 12.3 aims *"to halve per capita global food waste at the retail and consumer level, and reduce food losses along production and supply chains by 2030".* In addition to this The Food and Agriculture Organization (FAO) has a vision to make food and agriculture more sustainable so that nutritious food can be accessible for everybody. In doing so, natural resources should continue to be managed to be able to sustain ecosystem functions. In terms of the FAO, move to sustainable food value chains involve three pillars, which are (i) economic sustainability where it is profitable, (ii) social sustainability where it is beneficial for society, and (iii) environmental sustainability where it is compatible with nature (*FAO,2019*). FLW can be measured in five key stages of the agri-food supply chain: agricultural production, post harvesting handling and storage, processing, distribution and consumption. For each phase several actors are involved in the operations. It is possible to distinguish between FL, generated in the initial stages of the supply chain, and FW which occurs in the final stages of the food supply chain (*Porat et al., 2018*). In the case of the global Fruit & Vegetable Supply Chain (F&VSC), FAO reported that in 2019 between 25 and 55% of all Fruit & Vegetables (F&V) produced worldwide got lost or wasted along the supply chain (*FAO, 2011; FAO, 2019*). FWL are generated in different quantities in each step of the F&VSC but one of the limits in the literature is the lack of a shared and harmonized quantification methodology that allows to identify where FWL are generated the most. In addition, in the review of *Tort et al.* it is assessed that the design of a SC, or the relationship among parties, are root causes of the majority of the problems (*Tort et al., 2021*). To tackle this systemic problem, adopting innovations is one of the action which may change the structure of the SC and improve the cooperation among parties to tackle common problems . The same innovation is not likely to follow the same process or diffusion in different context, so to maximize the efficacy of innovations it is crucial to find key-drivers among parties to enhance the innovation process (*Avolio, 2014*). Recent literature on FW reported that innovations have a high potential in reducing and preventing FWL in all stages of the agri-food supply chain (*Aramyan et al., 2021*). It is possible to distinguish between three macro-categories of innovation (*Aramyan et al., 2019*). The traditional concept of innovation in firms distinguishes product and process innovation and is defined as *technological innovation* (*Schmidt and Rammer et al., 2007*). An *organisational innovation* is the creation or adoption of an idea or behaviour new to the organisation. *Marketing innovation* is the implementation of an innovative marketing method involving significant changes in product design or packaging, product placement, product promotion or pricing (*OECD, 2018*). The introduction of innovations in the agri-food sector it is a complex process of development and change, affected by a number of variables (*Avolio et al., 2014)*.

At the same time, it is still an open question if the value generated by the reduction of FWL shall be included in the attributes of the final product and whether this is recognized as an added value from the final consumer, since sustainability is increasingly viewed as a quality attribute, enabling firms to garner price premiums from the market.

2. PhD Thesis Objectives and Activities

The aim of this PhD thesis project will be direct to explore enablers and barriers for the introduction of sustainability innovations, together with the impacts on the stakeholder, in the case of an Italian vegetables supply chain. The impact assessment will be made by the development of the framework "Zero-waste supply chain" which will be defined during the project. To include real case-studies assessment, the innovations included in the research are part of the European Project LOWINFOOD.
This PhD thesis project can be subdivided into the following activities, according to the Gantt diagram given in Table 1:
A1) Mapping problems and solutions in the F&V supply chain: To give an overview on the State of Art on the quantification of FWL in the Italian F&VSC and on the available solution in the F&VSC to reduce FWL.
A2) Driver and barriers to the introduction of sustainability innovation inside an organization: This is the core part of the project where will be defined a framework for the "*Zero-Waste supply chain*" assessment.
A3) Assessment of sustainability communication "Zero-waste Supply Chain": Impact assessment of the "Zero-waste supply chain" framework. Including the consumer' perception of the *ecolabel* communication on the final product.
A4) Thesis and Paper preparation. Writing and Editing of the PhD thesis, scientific papers and oral and/or poster communications

Table 1 *Gantt diagram for this PhD thesis project.*

Activity \ Months		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	***Mapping drivers and enablers***																								
	State of Art of Food Waste in the Italian F&V Supply Chain																								
	Identification of one vegetables supply chain to study																								
A2)	***Development of the framework***																								
	Define the "Zero-waste supply chain" framework																								
	Selection of the case studies from the LOWINFOOD project.																								
A3)	***Impact assessment***																								
	Measure the efficacy of innovations to increase sustainability																								
	"Zero-waste supply chain " ecolabel																								
A4)	***Thesis and Paper Preparation***																								

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Sustainable innovation for improving the quality of meat products in the era of the *Green Deal*

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This PhD thesis research project is aimed to select a microbial starter with antioxidant and bioprotective properties in order to reduce the use of synthetic antioxidants in meat products, also combining in a new formulation microbial strains with bioactive molecules obtained from waste from the food chain (in particular oil and wine) with antioxidant and nutritional activity.

Innovazione sostenibile per migliorare la qualità d prodotti carnei nell'era del *Green Deal*

Questo progetto di dottorato mira a produrre uno *starter* microbico con attività antiossidante e di bioprotezione per ridurre l'uso di antiossidanti di sintesi in prodotti carnei fermentati. Inoltre, si vuole arricchire lo starter con composti estratti da scarti della filiera alimentare (in particolare dell'olio di oliva e del vino) con attività antiossidante e nutrizionale.

1. State-of-the-Art

One of the main causes of meat quality depletion after microbial alteration are oxidative reactions, which cause colour changes, development of off-flavors, and accumulation of toxic compounds, which can lead to the onset of non-transmissible chronic diseases (Carocho et al., 2018). The use of molecules with antioxidant activity is common in food preparation because it enables the control of the onset of such reactions. Antioxidants are substances that, when administered in low concentrations compared to the oxidable substrate, significantly delay or prevent oxidation. They include free radical scavengers, reducing agents, inactivators of peroxides or other reactive oxygen species, and metal chelators (Yang et al., 2018). Recently, the safety of several synthetic antioxidants, including butylated hydroxyanisole and butylated hydroxytoluene, has been called into question due to liver damage and carcinogenicity, moreover, consumers prefer products with a "clean label" (Wang et al., 2017). Microorganisms, particularly probiotics, have been studied for their health benefits because they inhibit pathogen adhesion and invasion, stimulate the host's immune system, and produce bioactive compounds such as amino acids, peptides, short-chain fatty acids, vitamins, exopolysaccharides, and antioxidants (Chugh & Kamal-Eldin, 2020). On the other hand, the major issue of food waste is current, and the importance of the circular economy in food production is even more relevant. Food waste, particularly waste from the olive oil and wine production chains, as well as waste from other vegetable production chains, is a rich source of bioactive compounds and antioxidant molecules. Antioxidant compounds of microbial or vegetable origin, particularly bioactive peptides, are useful as a natural alternative to synthetic antioxidants and also have bioactive activity, affecting both food quality and human health. Microbial starters can be studied to improve food shelf life and sensory properties, while also lowering the risk of chronic disease emergence (Lorenzo et al., 2018).

This is especially concerning for meat products, which are an important source of high-quality dietary protein and contribute to micronutrient intake. Until now, there have been no alternatives to meat consumption because cultured meat, plant-based meat alternatives, insects, and novel protein sources necessitate high levels of transformation and processing, limiting environmental sustainability gains (van der Weele et al., 2019). It is estimated that 23% of total meat production is lost and wasted, and as a result, cured meat products require radical changes to reduce their environmental impact. As a result, strategies for increasing the shelf life of meat products, while limiting oxidative alterations and improving nutritional value, must be developed.

Several methods to assess the *in vivo* antioxidant activity of intact cells, cell-free supernatants or lysates have been developed. The methods monitor the stages of lipid oxidation or the scavenging of electrons or radicals. It is recommended to use a combination of more than two assays both for monitoring lipid peroxidation and radical scavenging activity to assess the antioxidant potential of a sample as these methods do not reflect the real antioxidant potential in food or in *in vivo* but they are interesting sources of information about the intrinsic antioxidant potential with minimal environmental interference (Munteanu & Apetrei, 2021). As a result, it is not enough to carry out a single test to study this ability but it is necessary to combine different types of methods to obtain a better overview of the antioxidant activity of the different bacterial or yeast strains.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

I. Literature review
II. Isolation and phenotypic and genotypic characterization of about 200 microbial strains belonging to different genera, such as *Lactobacillus* spp., *Lacticaseibacillus* spp., *Saccharomyces* spp. and non-*Saccharomyces*
III. Evaluation of antioxidant activity of selected microbial strains through different *in vivo* methods.
IV. Evaluation of antioxidant and genotoxic effects of selected strains on human health.
V. Antioxidant potential evaluation of strains and bioactive compounds on food matrix compared to conventional antioxidants in order to reduce the use of conventional additives such as NO_2 and NO_3 trough antioxidant assays.
VI. Evaluation of the impact of selected strains and bioactive compounds on the microbial ecology of the product.
VII. Construction of a predictive model and definition of biomarkers to ensure authenticity and sustainability of innovative meat products.
VIII. Evaluation of the impact of selected strains on sensory properties of food products.
IX. Assessment of sensory properties of food products and definition of the relationship between functional properties and innovative meat products taking into consideration the rules for the correct labelling of novel foods.
X. Evaluation of the impact of stress induced by technological and digestive processes on the production of antioxidants and bioactive compounds by selected microbial strains and bioactive compounds.

***Table 1**. Gantt diagram for this PhD thesis project:*

		Months																		
Activities		0	2	4	6	8	10	12	14	16	18	20	22	24	26	28	30	32	34	36
I	Literature review																			
II	Isolation, phenotypic and genotypic characterization																			
III-IV	Evaluation of antioxidant activity of microbial strains *in vitro* and *ex vivo*																			
V	Evaluation of antioxidant potential on food matrix																			
VI	Evaluation of the impact on the microbial ecology of the product																			
VII	Definition of biomarkers and construction of a predictive model																			
VIII-IX	Evaluation of the sensory properties and labelling of meat products																			
X	Omic approach (transcriptomic, proteomic, metabolomic)																			
XI	PhD didactic activities and papers/thesis preparation																			

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The potential of the cheese microbiome to control cheese fermentation and its repercussions for the industry

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Cheese is considered as highly nutritional food that hosts different kinds of microorganisms. The diversity, functionality, and interaction of these microbial communities, associated with milk or cheese, are the most important parameters for the dairy industry to improve the nutritional and sensory properties as well as the fermentation of cheese. My aim is to contribute to these improvements and therefore to obtain an advantage in controlling cheese fermentation as well as improving the nutritional properties of cheese by using alternative protein sources.

Potenzialità del microbioma del formaggio sul controllo della fermentazione e ripercussioni sull'industria casearia

Il formaggio è considerato un alimento altamente nutritivo che ospita un ecosistema complesso costituito da diversi microrganismi. La biodiversità, la funzionalitá e l'interazione delle comunità microbiche del latte o del formaggio, sono considerati i parametri più importanti per l'industria lattiero-casearia. Questi, infatti, rappresentano dei fattori di fondamentale importanza, al fine di migliorare le proprietà nutrizionali e sensoriali del formaggio insieme al suo processo di produzione. L'obbiettivo del mio progetto di ricerca è quello di ottimizzare il processo di produzione del formaggio mediante una fermentazione controllata e implementando le sue proprietà nutrizionali attraverso l'utilizzo di fonti proteiche alternative.

1. State of the Art

Lactic acid bacteria (LAB) serve several functions in cheese manufacturing and ripening that directly or indirectly impact cheese flavor (Olson, 1990). LAB (primary starters) are also responsible for the acidification and the conversion of milk to curd along with rennet. Thus, the interaction along with the function and the diversity of these microbial communities are important drivers for the dairy industry in order to control the fermentation and the final cheese attributes (Ferrocino et al., 2022). Recent advances in next generation sequencing methods followed by the advancement of sophisticated bioinformatics tools have provided deeper insights into the composition and the potential functionality of microbiota which allow us to optimize flavor, appearance, and overall quality and safety in cheese (Yeluri Jonnala et al., 2018). Along with these parameters mentioned above, one of the primary objectives of cheesemaking is to preserve and fortify the nutritional potential of milk. Mammalian milk is one of the first food products which has been described as a rich source of vital nutrients (Yu et al., 2017). Apart from these nutrients, cow's milk also contains a high number of functional nanoparticles referred as exosomes. Exosomes are a type of extracellular vesicles (EVs), that are ranged between 30-120 nm in diameter and contain cell-surface proteins, lipids as well as nucleic acids such as microRNAs (Yu et al., 2017, 2019). Recent studies suggest that exosomes have a role in regulating intercellular communication. Thus, their effect on the bacteria population could be promising, accelerating growth or upregulating other biological functions during cheese manufacturing. Although cow's milk is considered an essential component of human nutrition, milk consumption has been declined rapidly during the last decade due to the increase in cow's milk allergies, lactose intolerance, cholesterol issues and ethical reasons. In the last years, there is a growing trend toward the production of plant-based products. Some studies focused on making cheese substitutes with mixtures of cow and soy milk, which are called partial dairy cheese analogues, in order to improve their nutritional value. However, further research should be done to determine whether the addition of plant-based protein can actually improve the nutritional properties of conventional cheese (Short et al., 2021; Wei & Yano, 2020).

2. PhD Thesis Objectives

Within the overall objectives mentioned above my PhD thesis project can be divided into the following activities according to the Gantt diagram given in Table 1.

A1 - Literary review; Experimental design

A2 - Selection of autochthonous lactic bacteria to be used as natural starter cultures for the production of semi-hard cheeses

The aim of this study is the selection of the best autochtonous strains belonging to the South Tyrolean biobank for the formulation of natural starters. A mathematical model was designed and 2094 isolates were grouped creating 680 combinations (3 isolates per combination) which were screened for the ability to produce peptides during milk fermentation, acidification and volatile compounds production. Based on these results, three final combinations were selected to be tested in a pilot plant scale to produce semi-hard cheese. Cheeses will be evaluated for their microbiological and physicochemical characteristics, throughout the ripening stage, in order to select the best combination.

A3 - Evaluation of the impact of exosomes in the bacterial growth

The aim of this project is to investigate what is the impact of exosomes on the bacterial growth and how they can influence the final population of bacteria during cheese ripening. Exosomes are going to be isolated from unpasteurized cow milk and characterized for their morphology and size. LAB strains will be injected in a 96 well-plate, containing cheese broth, and their growth rate will be measured, with and without the presence of exosomes.

A4 - Partial dairy cheese analogues

The aim of this study is to use flours from different plant sources in order to make partial dairy cheese analogues. Trials will be performed using different percentages of red lentil, chickpea, quinoa and faba flours and protein isolates and cheese samples will be evaluated for their physicochemical properties. A second screening will be performed using different pairs of starter cultures in order to establish their performance. After selecting the best combos, concerning the type and the percentage of the flour as well as the starter cultures, final cheese attributes, such as organic acids, proteins and antinutritional factors, will be evaluated in order to see whether there is a benefit concerning the nutritional value.

A5 - Thesis and Paper Preparation

Table 1. *Gantt diagram for this PhD thesis project.*

	1st year				2nd year				3rd year			
Activities Months	I	II	III	IV	I	II	III	IV	I	II	III	IV
A1 - Literary review; Experimental design												
A2 - Selection of autochthonous lactic bacteria to be used as natural starter cultures												
A3 - Evaluation of the impact of exosomes in the bacterial growth												
A4 - Partial dairy cheese analogues												
A5 - Thesis and Paper Preparation												

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Industry 4.0 in the agri-food sector: innovative sensors and smart logistics to support the sustainability of the supply chain

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The aim of this PhD research project is to find solutions that objectify the quality of some high value-added productions through the creation of simplified portable devices that exploit the technology of vis/NIR spectroscopy. This technology is already widely used in these fields but it exploits very complex and expensive instrumentation. Implementing new smart sensors could lead to improvements in production, an increase in the awareness of supply chain operators, better management of resources and also a reduction of food waste, one of the final objectives of the entire agri-food chain.

Industria 4.0 nel settore agroalimentare: sensori innovativi e logistica smart a supporto della sostenibilità di filiera

Lo scopo di questo progetto di ricerca di dottorato è quello di trovare delle soluzioni che oggettivino la qualità di alcune produzioni ad alto valore aggiunto attraverso la creazione di dispositivi portatili semplificati che sfruttano la tecnologia della spettroscopia vis/NIR. Questa tecnologia è già ampiamente utilizzata in questi ambiti ma sfrutta strumentazione molto complessa e costosa. Implementare nuovi sensori smart potrebbe portare a miglioramenti nella produzione, a un aumento della consapevolezza degli operatori di filiera, a una migliore gestione delle risorse e anche a una riduzione degli sprechi, uno degli obiettivi finali di tutta la filiera agroalimentare.

1. State-of-the-Art

A significant shift in measurement technologies is currently taking place in the agri-food sector, and the chance to replace consolidated analytical techniques (based on laboratory analyses) with methods based on rapid, non-destructive (Nicolai et al., 2007) and environmentally sustainable approaches is of great interest. Among the available technologies, visible and Near Infrared (vis/NIR) spectroscopy and imaging techniques are effective tools for monitoring of qualitative parameters and for maturation control of agri-food products (Shah et al., 2020). These are analytical techniques that have been investigated and utilized in this sector for a long time because they offer various advantages, including the aforementioned non-destruction of samples, the speed with which findings are obtained, and the ability to conduct checks throughout production processes.

It is common knowledge that the instruments needed to apply these techniques available on the market are very expensive, difficult to use, often heavy and cumbersome, and therefore challenging to use in setups other than a specialized laboratory. Through the creation of new networked sensors, the goal is to develop and identify solutions that can suit the needs of the agri-food sector in terms of production processes and quality control (IoT approach) (Misra et al., 2020). The Internet of Things (IoT) offers exciting possibilities for monitoring the quality of perishable cargo in real time while on the move, which could help to prevent large-scale product losses. Just to give some examples, sensors and wireless communication devices could be installed on containers, pallets, crates, or single products to provide real-time product data.

To achieve this, research is focusing on the development of low-cost prototypes that can have similar characteristics to reliable benchtop instruments, even if lower performance, while also being able to be used in larger numbers to provide punctual controls at various points along the supply chain due to their low cost. Carrying out punctual controls has as its ultimate goal the achievement of products of the highest quality, not only from the point of view of healthiness but also from the point of view of sustainability. As a matter of fact, many customers currently have a strong interest in these two topics.

Furthermore, the application of this type of techniques allows the creation of a considerable amount of data per sample. However, this instrumental data may not yet yield the intended result; it must be processed to produce a final decision-making answer. In line with the principle of parsimony, the capacity to remove non-informative and/or redundant signals can lead to models with improved accuracy, resilience, and chemical interpretability (de Araújo Gomes et al., 2022). For these reasons, the use of spectroscopic techniques requires the use of chemometrics for data analysis: to be able to correctly interpret the data, for the creation of predictive models and for the selection of variables (fundamental step in the simplification process of the devices) (dos Santos Costa et al., 2019).

This PhD program aims at offering a possible solution to the needs of high value-added supply chains: the implementation and application of a simplified spectroscopic device, with a few numbers of wavelengths, which can be used for punctual and real-time analyses that guarantee both the achievement of a finished product of the highest quality and a waste reduction.

2. PhD Thesis Objectives and Milestones

This PhD thesis project fits into this state of the art and can be divided into the following activities (illustrated in Table 1):

A1) **Application of the first version of a simplified prototype on grapes and on larger fruits and related data analysis.**
Milestones: creation of predictive models and identification of the structural changes to be made to the prototype.

A2) **Hardware modifications with additive manufacturing.**
Milestone: obtaining a version of the prototype suitable for matrices of different sizes and structures (also a version suitable for liquids such as wine).

A3) **Validation of the prototype for solid matrices and application on liquid matrices.**
Milestone: validation of prototype for solid matrices of different sizes (and validation of predictive models) and experimentation on complex liquid matrices.

A4) **Application of the prototypes in real scale conditions.**
Milestone: assessment of the capabilities of predictive models in real conditions.

A5) **PhD project reporting and dissemination:** PhD thesis, scientific papers, and oral and/or poster communications.

Expected result: design and test of the prototypes, validation on different matrices, application in different points of the supply chain due to the characteristics of the devices (portable, low cost, user-friendly).

Table 1. Gantt diagram for this PhD thesis project.

Activities/Months:	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1: Application of a first version of the simplified prototype on grapes and on larger fruits and related data analysis.																								
M1: creation of predictive models.																								
M2: identification of structural changes to be made.																								
A2: Hardware modifications with additive manufacturing.																								
M1: version of the prototype suitable for matrices of different sizes.																								
M2: version of the prototype suitable for liquids.																								
A3: Validation of the prototype for solid matrices and application on liquid matrices.																								
M1: validation of prototype and validation of predictive models.																								
M2: experimentation on complex liquidi matrices.																								
A4: Application of the prototypes in real scale conditions.																								
M1: assessment of the capabilities of predictive models.																								
A5: PhD project reporting and dissemination																								

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Response of plant holobiont to bacterial invasion: a focus on plant growth promoter and antibiotic resistant bacteria

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This PhD project is focused on plant holobionts' responses towards bacterial invasion, previously focused in the literature on a pathogen perspective. The phenomenon will be investigated in the frame of beneficial (i.e., Plant Growth Promoting strains) or neutral (i.e., antibiotic resistant) plant-bacteria interaction. The study foresees the design and administration of strains or synthetic bacterial communities on plant model spp. of relevance for food systems, and the study of the holobiont response both considering the performances from the plant perspectives and focusing on the plant microbiome assembly when the community is altered by the addition of the invading bacteria.

La reazione dell'olobionte pianta all'introduzione di microrganismi invasori: uno studio specifico sui batteri promotori della crescita vegetale e su quelli antibiotico-resistenti

Questo progetto si focalizza sulla risposta dell'olobionte pianta all'invasione batterica, precedentemente focalizzata soltanto su una prospettiva patogenica. Questo progetto di dottorato mira ad investigare questo fenomeno all'interno della cornice dei batteri benefici (i.e. promotori della crescita vegetale) o neutrali (i.e. antibiotico resistenti). Questo verrà investigato attraverso la progettazione e somministrazione di comunità batteriche sintetiche su specie di piante modello rilevanti per i sistemi alimentari, e attraverso lo studio della risposta del sistema sia considerando le prestazioni dal punto di vista della pianta, sia concentrandosi sullo sviluppo del microbioma della pianta quando la comunità è alterata dall'introduzione di un invasore.

1. State-of-the-Art

Plants provide a multitude of niches for the growth and proliferation of a diversity of microorganisms. These microorganisms can form complex co-associations with plants and have important roles in promoting the productivity and health of the plant in natural environments. It has been proposed that plants and the associated microbiota form a 'holobiont'. Bacteria are qualified as Plant Growth Promoting (PGP) strain when they can produce a positive effect on the plant upon inoculation (Stefan et al., 2013). The use of PGP microorganisms in agriculture as biofertilizers and biostimulants represents an eco-friendly alternative to the chemical products that are excessively used to obtain high yields, which is also a new approach to the practice of organic farming (Inculet et al., 2019). However, there is growing concern that these 'alien' microorganisms have properties that make them likely to become invasive (Mawarda et al., 2020). Moreover, invading allochthonous microbes can include Antibiotic Resistant Bacteria (ARB), which importance rises in consideration of the role of antibiotic production and/or resistance for the colonization of environmental niches, including the rhizosphere. ARB, together with Antibiotic Resistant Genes (ARGs), can enter the agri-food ecosystems using municipal sewage treatment plants (STP) effluents (Christou et al., 2017) and horizontal gene transfer (HGT) plays a crucial role in the spreading of ARGs (Santala et al., 2016). Literature shows that *in situ* plasmid transfer in natural environments occurs in specific hot spots, where appropriate growth conditions, colonization, and interaction of bacteria occur. These environmental niches include the rhizosphere and plant surfaces. In this context, our research group is studying the spread of AR within bacterial communities naturally associated to lettuce, a crop cultivated for raw consumption. The idea behind this project relates to studies focused on plant holobionts' responses towards bacterial invasion, previously only focused on a pathogen perspective. Given the importance of the plant microbiome and beneficial bacteria for food production, and the risk of antibiotic resistance spread through HGT in food systems, I will focus on the intersections with plant growth promoting and antibiotic resistant bacteria, in compliance with the One Health concept. This will be seen from a holistic approach, considering both the plant and the bacterial community response to the addition of specific bacterial strains or consortia. In fact, while natural microbial communities are composed of a mix of microbes mostly with unknown functions, the construction of synthetic microbial communities allows for the generation of defined systems with reduced complexity (Großkopf and Soyer, 2014). A further objective of this study would be the exploitation of the gained knowledge to develop an effective system for bacterial delivery to plants, working on a selection of the most promising strain or consortium according to their ability to improve the plant resilience to abiotic stress. Vegetable-derived biopolymers are good candidates for PGPB encapsulation, according to literature (Power et al., 2011, Vejan et al., 2018), and they could be obtained from food industry wastes in a circular economy approach.

2. PhD Thesis Objectives and Milestones

The main tasks of this project are, specifically: I) the selection of beneficial and/or antibiotic-resistant bacteria from relevant plant spp./environments and the design of SynComm; II) SynComm administration to the plant spp. of interest to study bacterial invasion and measure the response of the holobiont under different growth conditions; III) the developing of novel delivery systems to facilitate the holobiont invasion by beneficial bacteria in a sustainable agriculture perspective; IV) the dissemination of the scientific results. According to the Gantt diagram (Table 1), the activities will be subdivided as follows:

A1) **Definition of plant microbiome** under specific environmental conditions to evaluate the prevalence of taxonomic groups and address further isolation efforts to **create robust synthetic communities**, representative of the specific plant microbiome **(**M1.1: depiction of plant microbiome under different conditions, M1.2: design of SynComm).

A2) **Setup of reshaped plant holobiont and its assessment** in terms of microbiome assembly and plant performance, trough the administration of SynComm, represented by plant-growth promoting and/or antibiotic resistant bacteria previously isolated in A1. A2.1: assessment of antibiotic resistance spread in the plant microbiome. A2.2: plant holobiont response to beneficial bacteria invasion (M2.1: description of plant microbiome response to invasion by ARB, M2.2: Description of holobiont response to invasion by PGP bacteria)

A3) **Prototype delivery systems for beneficial bacteria application to plants** to improve effectiveness and establishment of biofertilizer/biostimulants in agriculture (M3.1: setup of protocols to improve beneficial strain delivery to plants)

A4) **Dissemination** of the scientific research outputs will be done pursuing the open diffusion of scientific results. (M4.1: participation to 3 national/international conferences and publication of 3 open-access articles to peer-review journals)

Table 1. *Gantt diagram for this PhD thesis project*

Activity/month		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	Definition of plant microbiome and Synncom design																								
	A1.1 depiction of plant microbiome under relevant environmental conditions																								
	A1.2 design of SynnComm																								
A2)	Setup of reshaped plant holobiont and its assessment																								
	A2.1 assessment of AR spread in the plant microbiome																								
	A2.2 plant holobiont response to beneficial bacteria invasion																								
A3)	Prototype delivery systems for beneficial bacteria application to plants																								
A4)	Dissemination																								

3. Selected References

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Computational approaches integrated to biomolecular technologies to study foodborne viral infections: virulence factors and identification of food-grade components with potential anti-viral activity

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This PhD thesis research project revolves around innovative ways to study foodborne viruses. Specifically, computational approaches such as molecular modelling, molecular docking and molecular dynamics will be exploited to both characterize virulence factors and possibly identify anti-viral food-grade components. The *in silico* results will be then evaluated on a selection of strong candidates through experimental trials.

Approcci computazionali integrati a tecnologie biomolecolari nello studio di infezioni virali di origine alimentare: fattori di virulenza e identificazione di costituenti alimentari a potenziale attivita antivirale

Questo progetto di ricerca per la tesi di dottorato si concentra su modi innovativi per lo studio di virus di origine alimentare. In particolare, verranno sfruttati approcci computazionali quali la modellazione molecolare, il docking molecolare e la dinamica molecolare per caratterizzare fattori di virulenza ed eventualmente identificare componenti antivirali per uso alimentare. I risultati *in silico* saranno successivamente valutati su una selezione di candidate che verranno testati in laboratorio.

1. State of The Art

As reported by the World Health Organization (WHO), around 10% of people worldwide get sick due to the ingestion of contaminated food and roughly half a million of them die. These deaths are not normally distributed throughout the population, with children under 5 years old accounting for 30%.
Despite foodborne diseases are especially spread in low/middle income countries, the more frequent international trades and the consequent lengthening of food chains have increased the risk of food contamination in all geographical areas. In this context, foodborne viruses represent 20% of the total foodborne-related illnesses accounting for the 8% of the total number of deaths, being particularly threatening for kids, immunocompromised, elders and pregnant women (WHO, 2015).
There are several foodborne viruses such as Norovirus, Hepatitis A and E viruses, Rotavirus, Astrovirus and many others. Their *in vitro* culture is frequently hard to achieve, indeed mammalian cell cultures carry along lots of weaknesses which would be avoided running *in silico* analysis (Bosch et al., 2018; Yeh et al., 2008; D'Souza et al., 2016). However, computational approaches are still quite rare in the Food Science field although they could bring lots of advantages, either as standing alone techniques or integrated to *in vitro* approaches.
The first advantage of computational approaches is the possibility of evaluating the activity and the underpinning mechanism of action of a huge number of compounds in relatively short times (Dellafiora et al., 2018; Badawy et al., 2019). The *in silico* screening allows the planning of targeted experiments, once again saving time and money. Secondly, such approaches permit the rational design of new possible antimicrobial natural-derived compounds (Palmieri et al., 2016).
This PhD thesis project will initially focus on Hepatitis E Virus (HEV) to possibly move to other foodborne viruses applying the same pipeline adopted for this first virus. HEV is the first chosen foodborne virus being the cause of more than 50 000 deaths/year, accounting for the 3% of the mortality due to hepatitis, and being optimal cell culture models not available yet.

2. PhD Thesis Objectives And Milestones

The main activities of the PhD Project, reported in the below Gantt diagram (Table 1), are the following:
1) **Characterizing the virulence factors of HEV and others foodborne viral pathogens**. The virulence factors (*i.e.* crucial proteins for the virus proliferation) will be retrieved from the Protein Data Bank (https://www.rcsb.org/) or, if they are not available, modelled through homology/comparative modelling procedures or by exploiting new deep-learning based tools.
2) **Collecting natural compounds both from literature and available databases**. The first step will be the collection of already known viral inhibitors and secondly a ligand-based similarity search will be exploited to discover new chemical structure potentially endowed of antiviral activity.
3) ***In silico* evaluation of the virulence factors models and of the natural compounds' activity**. This step is the most time-consuming. Firstly, the *virulence factor – natural compound* complexes will be built through molecular

docking and secondly molecular dynamics simulations ran to estimate the complex stability as a mean to predict ligand activity. The *in silico* results will be evaluated and ranked based on the docking scores and mostly on the results of molecular dynamics simulations.

4) ***In vitro* testing of the *in silico* chosen natural compounds**. This step will be crucial to either confirm or reject the outcomes collected from previous step. The testing of food-grade compounds will be privileged in the light of their possible future use along the food and feed production chains.

5) **Collecting literature, writing scientific papers, preparing scientific communications and the PhD thesis**.

Table 1 Gantt diagram highlighting the further steps of the PhD project

	Activity	***Month***																							
		01	**02**	**03**	**04**	**05**	**06**	**07**	**08**	**09**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**
1)	***Characterizing virulence factors***																								
	Virulence factor identification																								
	Recover the 3D structure or *in silico* model it																								
2)	***Collecting natural compounds***																								
	Recover already known inhibitors																								
	Find new ones via similarity search																								
3)	***In silico testing***																								
	Molecular Docking																								
	Molecular Dynamics simulations																								
4)	***In vitro evaluation of in silico results***																								
5)	***PhD thesis and papers preparation***																								

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Applying and testing of "on site on time" sensors and tools for the sustainability of wine and extra virgin olive oil processes

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This PhD research project aims to apply sensors on site and on time, using technologies that allow the cost reduction in the application. The enological and elaiotechnical industries in fact require simple, rapid and reliable methods for an objective assessment of the quality of wine and oil in real time during the process. Optimizing the phenolic extraction process for wine or oil extraction means increasing the energy coefficient, high output with low input. The application of new technologies can allow the reduction of the use of energy within the company and favor the quality of the transformation processes, thus leading to produce materials with higher quality, and to have more safety in working operations.

Applicazione e sperimentazione di sensori e strumenti "on site on time" per la sostenibilità dei processi del vino e dell'olio extravergine di oliva

Questo progetto di dottorato di ricerca mira ad applicare sensori in loco utilizzando tecnologie che consentano la riduzione dei costi nell'applicazione. Le industrie enologiche ed elaiotecniche richiedono infatti metodi semplici, rapidi ed affidabili per una valutazione oggettiva della qualità del vino e dell'olio in tempo reale durante il processo. Ottimizzare il processo di estrazione fenolica per l'estrazione di vino o olio significa aumentare il coefficiente energetico, alta resa con basso input. L'applicazione di nuove tecnologie può consentire la riduzione del consumo di energia all'interno dell'azienda, favorire la qualità dei processi di trasformazione, aumentare la qualità dei prodotti e garantire maggiore sicurezza nelle operazioni di lavoro.

1. State of the Art

The use of sensors and new technologies can help reduce costs and increase the safety of analysis results with very low margins of errors. Generally, the errors are correlated with the sampling or with the sample itself, but they are hardly correlated with the new technology instruments. The instruments clearly have to be subjected to a calibration model directly related to a mathematical format and then the response is established. In recent years, attention has been drawn to the use of SAWs to drive microfluidic implementation and a variety of other processes. Due to the mismatch of the sound velocities in the substrate and the SAW fluid, SAWs can be efficiently transferred into the fluid, creating significant inertial forces and fluid velocities.
Another instrument is the Near infrared (NIR) spectroscopy, based on molecular nuances and combined vibrations. NIR radiation induces the vibrational transition of molecule bonds. In addition, the NIR region contains the nuances and combinations of OH, NH, CH and SH bonds of organic molecules. It presents the possibility of working on leaves, fruit and liquids. Scans the range of the NIR region between 1100-2300 nm, with 2 nm of resolution and 1 spectrum plus the average of 100 scans. This tool allows to evaluate the progress of alcoholic fermentations (di Egidio et al., 2010), the influence of the activity of yeasts (Marsico et al., 2018), the ripening process of grapes (Cozzolino et al., 2006) and the prediction of anthocyanins and polyphenols content in wine and olive oil (Can et al., 2018).
Another application has been carried out in the research of volatile compounds and its use also allows the recognition of matrices coming from the same material (Martelo-Vidal & Vázquez, 2014). The application of biosensors, analytical devices consisting of a biologically active sensitive element (enzymes, cells, antibodies, etc.) and an electronic part, has also give the possibility to reduce the chemical analysis operations. These exploit the work of various components. A bioreceptor is related to the analyte, which can be an enzyme or an antibody, then there is the product and finally the transducer, which can be electrochemical, acoustic, optical or mechanical. Their application has been implemented in the determination of polyphenols (Gomes et al., 2022), in the control of fermentation kinetics, and the behaviour of the yeasts (Samphao et al., 2022).
QCM-D was already used for wine analysis, with ad hoc experiments exploring the role of positively and negatively charged functional groups (Mierczynska-Vasilev et al., 2016) and different polymeric functionalizations (Mierczynska-Vasilev, Molecules, et al., 2016). Its chemical functionalization based on proteins and peptides allowed to quantify the content of two families of tannins and procyanidins (Krishnamoorthy et al., 2022). This tool has also been used to check the presence of ochratoxins in wines. Another situation is the use of these sensors for the quantification of sulfite, that is based on the detection of an enzyme (Situmorang et al., 1999). Selection of appropriate kinetic models and data-analysis techniques are essential to predict the future quality, to account for variability in environmental conditions, and to allow real-time monitoring.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above, this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) **Application of new technologies tools and sensors**: An evaluation of the application of different tools within specific research plans. An itinerary that helps to carry out destructive analyzes in order to be able to calibrate the various tools to be applied. The installation of sensors on site in order to evaluate analytic parameters and subsequently compare their efficiency in terms of reading and economics. Creation of a protocol to apply on future research and quantification of the adaptability. Study of the ability to speed up work and save money without making mistakes. Direct quantification of substances during winemaking and olive oil extraction process.

A2) **Evaluation of the efficiency of non destructive instruments:** Quantification of data reliability in comparison with the destructive analysis Statistic correlation with the destructive analysis, comparison with the credibility of the analyzes and demonstration of their sustainability and cost-effectiveness. Check if thoose tools help in increasing the quality of the final product and reduce costs for the wineries and olive oil companies.

A3) **Writing and Editing:** the PhD thesis, scientific papers and oral and/or poster communications and statistical analyses of data.

Table 1: *Gantt diagram for this PhD thesis project.*

Activity	Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1	***Application of new technologies tools and sensors***																								
	1) Itinerary process for the calibration function																								
	2) Installation of sensors on site to evaluate analytic parameters																								
	3) Direct quantification of substances during winemaking and olive oil extraction																								
A2	***Evaluation of the efficiency of non destructive instruments***																								
	1) Quantification of data reliability in comparison with the destructive analysis																								
	2) Statistic correlation and inerpretation of data																								
A3	***Thesis and Paper Preparation***																								

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Study of oenological practices for the production of long-lived white wines

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This PhD thesis research project is aimed to find one or more strategies to help winemakers to produce long-lived white wines. The parameters that will be taken into consideration are the maturation of the grapes, the type of pre fermentation operations such as the mashing methods, the management of oxygen, the maceration and the kind of yeasts used for the fermentation. After the fermentation process the refining techniques that includes the use of lees, the use of wood and the use of fining adjuvants will be analysed.

Studio delle pratiche enologiche per la produzione di vini bianchi longevi

Questo progetto di tesi di dottorato è mirato a trovare le pratiche enologiche migliori per aiutare gli enologi nella produzione di vini bianchi longevi. I parametri che saranno presi in considerazione sono la maturazione dell'uva, le tecniche pre-fermentative ovvero la tipologia di pressatura, la gestione dell'ossigeno, la macerazione e i diversi tipi di lieviti impiegati per la fermentazione. Successivamente, nella fase post fermentativa saranno valutate le tecniche di affinamento dei vini, come la permanenza sulle fecce di lievito, l'utilizzo del legno e l'impiego di coadiuvanti.

1. State-of-the-art

Over the last decade, a new frontier has emerged in the world of oenology concerning the ageing potential of white wines. In fact, studies on this subject have been almost non-existent up to recent times. The change in the consumers' awareness and knowledge of the world of wine has led the market to a development of a new front. In addition, there is a technological problem regarding the distribution sector and its increasingly complex structure. The wines put on the international market reach the final consumer after following a tortuous supply chain almost without any control by the producer.

In the large-scale distribution market wines are often subjected to stress conditions which can determine either the formation or the loss of their volatile

molecules. Since white wines have a lower polyphenol content than red wines, they are more susceptible to oxidation and therefore to the loss of their fruity, fresh, and floral characteristics (Silva Ferreira et al., 2003). If on the one hand evolution leads to the formation of new molecules which enrich the wine bouquet, on the other it also involves the development of volatile substances characterized by unpleasant and trivializing odours (Silva Ferreira et al., 2002). It is likely that several processes simultaneously interact for the success of a good ageing, first of all the reactions of oxygen with the various components of the wine matrix (Oliveira et al., 2011). Metals, above all iron and copper, activate oxygen setting off a cascade reaction. The simple phenolic fraction also comes into play in oxidation reactions.

The world of oenology has refined its techniques through changes in primary production, processing, and packaging. This has been made possible by the evolution of knowledge and strategies of the industries in the wine sector. This evolution, however, has not always been followed by an update of the analytical techniques useful for objectively evaluating the different processing stages.

2. PhD Thesis Objectives and Milestones:

This project involves the study of techniques which can be applied to obtain long-lived white wines. It can be subdivided into the following activities according to the Gantt diagram given in Table 1:

1) Adapting to the wine chain and developing analytical methods for monitoring the evolution of white wines over time, introducing analytical and non-conventional methods such as cyclic voltammetry (Gonzalez et al., 2018) for assessing susceptibility to oxidation will make it possible to test the effectiveness of the different oenological practices in the wine evolution (Martins et al., 2008)
2) Adapting and developing analytical techniques for evaluating both the reactivity and stability of the products
3) Evaluating the impact of mashing methods

4) Evaluating the influence of refining procedures
5) Identifying the critical points of wine conservation
6) Writing and Editing of the PhD thesis, scientific papers and oral and/or poster communications

Table 1 Gantt chart for this PhD thesis project.

Activity \ Months		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1)	***Developing analytical methods***	■	■	■	■	■	■	■	■																
2)	***Using analytical techniques for evaluating the stability of the products***	■	■	■	■	■	■	■	■	■				■	■	■	■	■	■	■	■	■			
3)	***Evaluating the impact of mashing methods***										■	■	■	■									■	■	■
4)	***Evaluating the influence of refining procedures***	■	■	■	■	■	■	■	■	■					■	■	■	■	■	■	■	■			
5)	***Identifying the critical points of wine conservation***					■	■	■	■	■	■	■	■	■					■	■	■	■	■	■	■
6)	***Thesis and Paper Preparation***	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■

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New strategies to face antibiotic resistance in healthcare and food sectors

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This PhD Thesis Research Project aims to develop methods with high specificity, short-time response and practical applications for antibiotic-resistant *Staphylococcus aureus* and *Escherichia coli* detection. Furthermore, it aims to test alternative antibacterial agents.

Nuove strategie per combattere la resistenza agli antibiotici nei settori sanitario e alimentare

Questo Progetto di Ricerca per Tesi di Dottorato mira a sviluppare metodi con elevata specificità, risposta in tempi brevi e applicazioni pratiche per il rilevamento di *Staphylococcus aureus* ed *Escherichia coli* antibiotico-resistenti. Inoltre, mira a testare agenti antibatterici alternativi.

1. State-of-the-Art

Antimicrobial Resistance (AMR) is one of the biggest threats to human and animal health. In 2019, 1.27 million deaths were ascribed to AMR, the prediction made by the World Health Organization (WHO) hypothesizes 10 million people deaths per year within 2050 and the antibiotic ineffectiveness can cause a financial cataclysm translated into a $100 trillion loss (Dadgostar, 2019).
The spread of AMR along the food chain from the raw materials to the final products is being studied as a concrete possibility.
The consumers are put at risk through AMR contaminated food consumption, which enhances the transmission of antibiotic-resistant genes (ARGs), providing a likely route for their entry into the community.
Food and Drug Administration data showed that 62% of antibiotics significant for human health were sold to feed producers, enabling the diffusion of AMR in animal husbandry and food final products (FDA, 2015). AMR bacteria were isolated in a variety of foods and outbreaks of foodborne diseases (Hudson et al., 2017).
AMR diffusion paths along the food chain are not completely known, but there is growing concern over them and the resistance transmission in the food chain must be considered.
Different types of AMR bacteria have been identified in the food context such as methicillin-resistant *Staphylococcus aureus* (MRSA) and extended-spectrum beta-lactamase (ESBL) producing Enterobacteriaceae.
Classical AMR surveillance is based on isolation of indicator microorganisms, phenotypic characterization and antimicrobial susceptibility testing (AST), with a long-time required, therefore, methods for fast and specific detection of ARGs to detect AMR bacteria are urgently needed to face the global threat of AMR.

2. PhD Thesis Objectives and Milestones

S. aureus and ***E. coli*** resistant to the antibiotics most responsible for AMR (*i.e.*, **β-lactam antibiotics**, **fluoroquinolones**, **tetracyclines** and **macrolides**) will be isolated and analyzed.
The ARGs associated with the antibiotics will be studied, including *bla* genes and *mecA*, *mecC* related with β-lactams, *norA* (ARGs against fluoroquinolones), *tetL*, *tetM* (tetracyclines), ermB (macrolides) on *S. aureus* (Oniciuc et al., 2018; Thames et al., 2012) and β-lactamases *bla* genes, ARGs encoding resistance to fluoroquinolones (*qnrS1*, *qnrB19*) and to tetracyclines (*tetA*, *tetB*) on *E. coli* (Oniciuc et al., 2018; Poirel et al., 2018).
This PhD Thesis Research Project is organized into phases, according to the Gantt Chart (*Table 1*):

1) **State of art upgrade (literature review)** and **experimental design**.
2) **Microbiological analyses**.
   2.1) *S. aureus* and ***E. coli*** **isolation and characterization**. About 200 strains of each microorganism from food (*i.e.*, milk and dairy products) and environmental sources (*i.e.*, working surfaces and waters) will be isolated through selective agar plates (Baird-Parker agar and Coli ID agar, respectively) and subjected to classical microbiological tests.
   2.2) AMR profiling. The tests will be performed on Mueller Hinton agar plates using filter paper discs containing a specific amount of the antibiotics (disk diffusion antibiotic sensitivity test) according to the EUCAST indications.
3) **Molecular analyses**.
   3.1) DNA extraction and **PCR confirmation**. *S. aureus* and *E. coli* DNA extracts will be used for species confirmation by conventional PCR using specific primers (Javid et al., 2018; Maheux et al., 2009).
   3.2) Whole Genome Sequencing (WGS) will be applied for a comprehensive picture of all ARGs present in

the isolates.

3.3) Specific primers for ARGs detection will be selected/designed and end point PCR/qPCR protocols will be optimized to characterize *S. aureus* and *E. coli* isolated resistant strains. **DNA probes** specific to ARGs will be designed for the construction of a biosensor which will replace the utilization of PCR in the ARGs detection.

3.4) Electrochemical analyses. Gold electrodes after functionalization with DNA probes will be utilized in an electrochemical device (biosensor) using Differential Pulse Voltammetry (DPV) measurements.

4) Alternative solutions to antibiotics test.

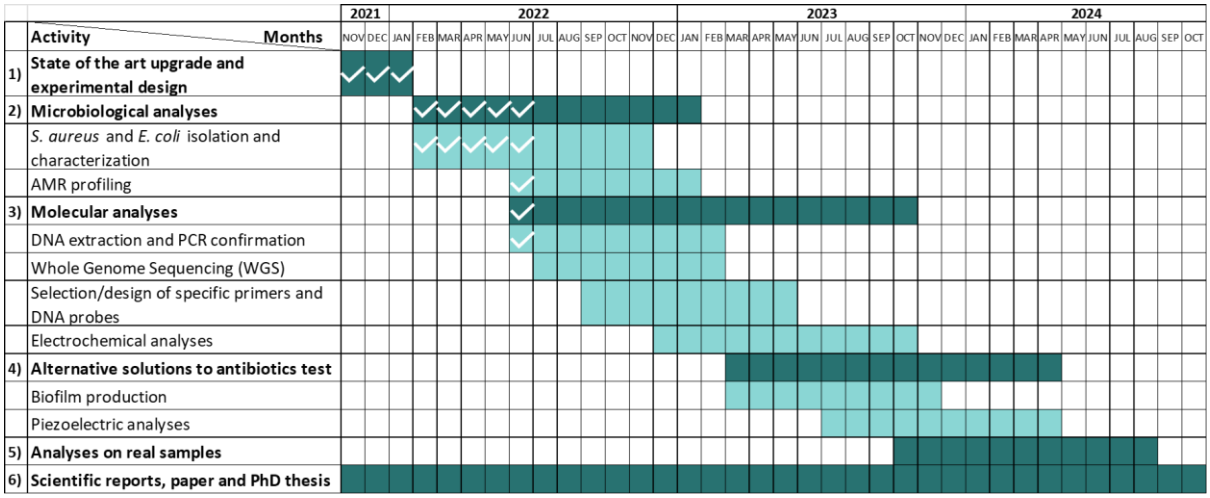
4.1) Biofilm production. The capability of the AMR isolated bacteria of biofilm formation will be studied using the microtiter plate assay (Merritt et al., 2005).

4.2) Piezoelectric analyses. The Quartz Crystal Microbalance, QCM (biosensor) will be used to record formation and removal of amounts of AMR bacterial mass (biofilm) from the electrode surface showing the effectiveness of alternative molecules as a substitute for antibiotics.

5) Analyses on real samples will be performed through the optimized biosensors in order to implement future miniaturized and automated devices.

6) Scientific reports, **papers** and **writing PhD thesis**.

Table 1 *Gantt Chart for this PhD Thesis Research Project.*



The ✓symbol indicates the activities already carried out.

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Grapevine-associated microorganisms as biocontrol agents against the proliferation of pathogenic fungi

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This PhD project aim at selecting potential biocontrol agents able to prevent, or limit, the infection of grapevine and grape (table and wine) by pathogenic fungi, in pre- and post-harvest periods, in order to set an eco-friendly plant protection strategy as alternative to reduce both the use of chemicals fungicides in vineyard and possible residues in wine. The project will investigate the putative mode of actions of selected epi- and endophytic species to counteract the pathogen proliferation and will evaluate how to exploit these metabolic traits to optimize the antagonistic effect under different plant growth conditions and grape management.

Microorganismi associati alla vite come agenti di biocontrollo contro la proliferazione di funghi patogeni

Questo progetto di dottorato mira a selezionare potenziali agenti di biocontrollo in grado di prevenire, o limitare, l'infezione della vite e dell'uva (da tavola e vino) da funghi patogeni, in pre- e post vendemmia, al fine di impostare una strategia fitosanitaria eco-compatibile per ridurre l'uso di fungicidi chimici in vigna e di eventuali residui in vino. Il progetto studierà le presunte modalità d'azione di specie microbiche epi- ed endofitiche selezionate per contrastare la proliferazione del patogeno, valuterà come sfruttare queste caratteristiche metaboliche per ottimizzare l'effetto antagonista in diverse condizioni di crescita delle piante e di gestione dell'uva.

1. State-of-the-Art

Directive 2009/128/EC of the European Parliament and of the Council of 21 October 2009 established a framework to achieve the sustainable use of pesticides by reducing their risks and impacts on human health and the environment. Afterwards, in September 2015, at the United Nations General Assembly, countries around the world signed the 2030 Agenda for Sustainable Development ant its 17 Sustainable Development Goals (SDGs); specifically, goal nr. 12 aims to ensure a sustainable consumption and production patterns. Thus, the future objective is the reduction in the use of synthetic pesticides by 50% by 2030.
Biocontrol is defined as a "*set of alternative strategies to the use of chemical products fighting fruit and vegetable diseases*" (Newman *et al.*, 2015). Some epiphytic and endophytic microorganisms can work as biocontrol agents (BCAs), inhibiting a wide spectrum of plant pathogens through specific biocontrol mechanisms (competition for space and nutrients and minerals, biofilm formation, elicitors/killer toxins/enzyme/volatile organic compounds (VOCs) production. This latter mode of action includes the release of terpenes that show a broad range of the biological properties, including cancer chemopreventive effects, antimicrobial, antifungal, antiviral and antiparasitic activities (Chen *et al.*, 2021).
Vitis vinifera is a perennial woody crops, with an estimated 78 million tons of grapes produced annually. The grapes are used for winemaking (57%), about 36% are consumed fresh and 7% are consumed as dried fruits (OIV 2020). Commercial cultivars are greatly affected by many pathogens, therefore the project aims at selecting potential BCAs against *B. cinerea, Aspergillus and Penicillium* spp. infection of grapevine and grape, in pre- and postharvest periods. Other more specific objectives include: i) the determination of capability of BCAs to inhibit the pathogens; ii) the evaluation of the biofungicide effect of BACs in grapevine cell cultures and in pot trials. Also, particular attention will be given to grapevine-associated yeasts and VOC release. Indeed, endophytic yeasts belonging to *Metschnikowia, Pichia* and *Hanseniaspora* species have been isolated from the plant (Ghanbarzadeh *et al.*, 2020) and VOCs have been previously shown to be involved in quorum sensing and morphological transitions in *Saccharomyces cerevisiae* and some other yeast species and can stimulate plant responses (biotic and abiotic stress), including growth promotion.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into Work Packages (WP), Tasks (T) and Milestones (M) according to the Gantt diagram given in Table 1:
WP1. Evaluation of endophytic community in *V. vinifera* and *V. sylvestris*:
T1.1 *Set up of protocols for the cultural isolation of endophyte*s (M1.1, Sterilization step optimized)
T1.2 *Isolation of culturable microorganisms* from grape flowers, leaves and fruits (T1.1). One international *V. vinifera* cultivar cultivated under different vineyard management will be considered. Two Georgian cultivars resistant to downy mildew infection will be also analysed (M1.2, Isolation of at least 10 culturable species)
T1.3 *Assessment of the grapevine microbiome signature* along the phenological states of the plant through

metagenomics (the cultivar will be selected in T1.2) (M1.3, DNA extraction step optimised)

WP2. Selection of potential BCAs:

T2.1 *Screening of potential biocontrol capability of microorganisms* (from T1.2/previous collections, by cultural inhibition tests on grape berries and leaves to evaluate their efficacy in controlling the mould infections) (M2.1 Selection of at least 1 potential BCA against each mould under study)

T2.2 *Characterisation of BCA candidates* (T2.1) for their capability of surviving under adverse environmental conditions and the resistance to copper and commercial agrochemicals. To evaluate the spectrum of action of BCA candidates, dual-culture methods against different mould strains of each species under study will be carried out (M2.2 Report on the spectrum of action of BCA candidates selected in T2.1)

T2.3 *Determination of the mechanisms of action of BCAs*. The biocontrol mechanisms of action of BCAs will be evaluated (space/nutrients/iron competition, biofilm/elicitors formation, volatile organic compounds/killer toxins/enzyme release). In case of VOC production, the corresponding molecules will be identified by GC/MS analysis (M2.3 Report on the main mode of action of at least 1 potential BCA against each mould under study)

WP3. Investigation of BCA-plant interaction

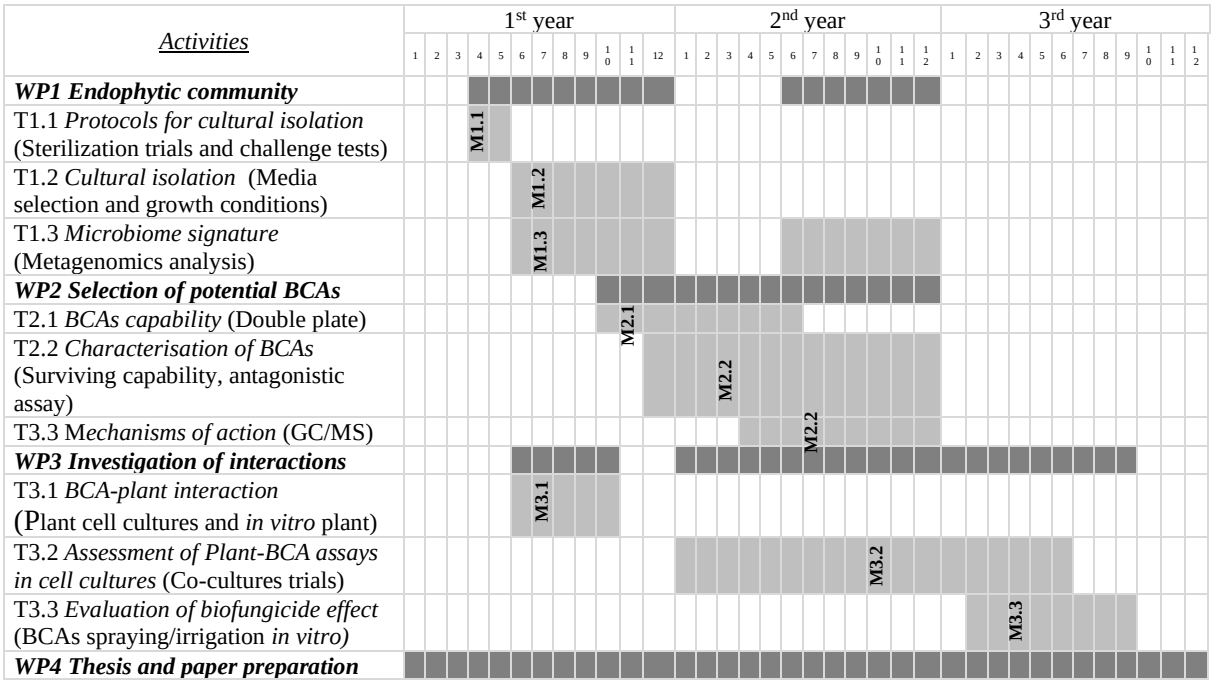
T3.1 *Set up of protocols for BCA-plant interaction*: i) plant cell culture production from *V. vinifera* and *sylvestris*: ii) *in vitro* plant experiment (M3.1 Identification of the best cultivar for grapevine cell cultures)

T3.2 *Assessment of Plant-BCA assays in grapevine cell cultures*. BCAs selected in WP2 (up to 5 isolates) will be analysed to test their interaction with plant in presence/absence with the pathogens (microscopy observation of cell cultures, resveratrol production and/or the expression of specific target genes) (M3.2 Inoculum and cultural conditions for the preparation of plant-pathogen-BCA validated)

T3.3 *Evaluation of biofungicide effect of BACs* in *in vitro* plant experiment. The minimal inhibiting concentration of BCA will be evaluated in infected 2.5month-old *V. vinifera* plantlets in glass tubes. Appearance of symptoms, photosynthetic efficiency, gas exchanges. BCA delivery will be tested as well (spraying/irrigation) (M3.3 Plantlet infection step validated)

WP4. Bibliographic research/communication and dissemination.

Table 1 Gantt diagram for this PhD thesis project.



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Technological, sensory, and nutritional assessment of ecofriendly food lipids

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This PhD thesis project aims at the formulation of innovative fat blends to be applied within the food industry of bakery products. The project aims at the formulation of these mixtures using oils extracted from waste matrices typical of the Italian food industry (wheat germ, rice germ, grape seed) to: reduce the environmental impact caused by imports oils used today (palm oil and coconut oil) as well as strengthening the Italian industrial production chain from an economic point of view by valorising food wastes into by-products.

Valutazione tecnologica, sensoriale e nutrizionale di grassi alimentari eco-sostenibili

Questo progetto di tesi di dottorato mira alla formulazione di miscele di grassi, innovativi, da applicare all'interno dell'industria alimentare dei prodotti da forno. Il progetto mira alla formulazione di tali miscele tramite l'impiego di oli estratti da matrici di scarto tipiche dell'industria alimentare italiana (germe di grano, germe di riso, semi di vinaccioli) al fine di: diminuire l'impatto ambientale causato dall'import degli oli oggi usati (olio di palma, olio di cocco) nonché rafforzare da un punto di vista economico la filiera produttiva industriale italiana tramite la valorizzazione di scarti alimentari a sottoprodotti.

1. State-of-the-art

The food industry makes intensive use of oils, fats and derivatives of both animal and vegetable origin for a myriad of different products. Worldwide, in 2014, 165 million tons of fatty substances were consumed, and the estimate is that this quantity will double over the next 30 years. After the establishment of the European Regulation 2019/649 (in modification of the previous 1925/2006), the food industry had to pay particular attention to the use of hydrogenated fats to avoid the use of trans fatty acids. Thus, has grown the consumption of palm oil. In 2011, 77,000 tons of palm oil for food use were imported into Italy, which corresponded to 8.4% of the total imported, while over 90% was destined to the production of biofuels. The import of this fat into our country has progressively increased from 40,000 tons / year in the period 2005-2008 to 75,000 in 2009 and 76,000 in 2010 (ISTAT data). This high consumption is linked both to its productivity (4.5 tons of oil / hectare against 1 ton of oil / hectare of the sunflower oil) and to its technological characteristics. This oil has been the subject of criticism both for its nutritional and environmental aspects: the former linked to the presence of toxic newly formed molecules, have disappeared, thanks to the improvement of production and refining techniques which have led to comparable MCPD values. (Arris et al., 2020; Knutsen et al., 2018) for all refined oils. The environmental aspects were addressed with the establishment of the Roundtable on Sustainable Palm Oil (RSPO) which is a multi-stakeholder capable of supplying and certifying sustainable palm oil. Both because certified palm oil is available in fixed quantities and at increased prices, and because the consumer has remained a certain mistrust towards this fat, a new page has opened in the procurement of fatty substances to increase the safety and sustainability of this supply chain and those connected to it. Net of these considerations, and to reduce the ecological, economic and ethical impact of the industry, it is necessary to enhance the by-products from other supply chains (wine, cereal and tomato) increasing the sustainability of the recovery of fatty substances also by virtue of of the content in bioactive compounds (tocopherols and sterols in particular). It is therefore important to develop mixtures of oils and fats from by-products, also with the addition of other fats as such or fractionated, suitable for giving the formulations the overall safety characteristics and the required shelf life, with particular attention to lipid oxidation. Particularly interesting, is the possibility of enhancing the by-products of the Italian cereal sector such as rice germ and soft and hard wheat germ, historically an integral part of Italian agricultural production. ISTAT data tell us that in 2020 the production of durum wheat in Italy was about 4 million tons, which must be degeminated in the early stages of milling. Wheat germ represents about 2% of the weight of the caryopsis and contains a quantity of valuable extractable fat (about 15%), with molecules of high organoleptic and industrial interest, such as cis-linoleic acid (C18: 2 cis, about 59.7% of total fats) and the set of monounsaturated fatty acids (MUFAs, about 29% of total fats) (Orsavova et al., 2015). The same consideration can be made for the rice germ, residue of rice husking, which currently amounts to about 2.5 million tons per year in Italy (ISTAT data); rice germ, just like its wheat analogue, has a high quantity of linoleic acid, but also a valuable quantity of palmitic acid (C16: 0, about 20%) (Orsavova, 2015 et al.). Furthermore, Italian agriculture also produces large quantities of grape seeds, from the wine and tomato seed supply chain, from the canning industry. The first is very rich in unsaturated fatty acids, as well as antioxidants and is therefore rather resistant to the thermal stresses typical of frying; the second, on the other hand, in addition to lycopene and beta carotene, contains 25% palmitic acid which, being saturated, has excellent stability. The study of these matrices could lead to the development of mixtures useful for industry, capable of reducing the waste of "good" fats with a consequent increase in the sustainability of the supply chain, also thanks

to the reduction of pollution caused by transport.
However, to achieve this goal, it is necessary to study the raw materials, individual oils, extraction and / or co-extraction and possible refining methods at a chemical, sensorial and industrial level, as well as their evaluation in model products compared with food industry standards. Another important part of the research in this area would also be represented by the development of rapid screening methods, possibly non-destructive, and therefore in the construction of reference systems for the evaluation of the quality of fats, with the aim of avoiding or minimizing the use of solvents and reagents. These innovative techniques could, in the future, represent a solid control and shelf-life forecasting system to guarantee production quality, the reduction of industrial losses and a greater health and environmental guarantee for both consumers and operators, who should not more to work with polluting substances, which are expensive to dispose of, and even dangerous to health in chronic conditions of intake.

2. PhD Thesis Objectives and Milestones

This research project aims to study innovative vegetable oil blends to be used in baked products (both sweet and salty) to replace the oils commonly used today by the food industry (palm oil and olive oil).
The PhD thesis project can be divided into the following activities, summarized in the Gantt chart shown in Table 1:

A1) Study of innovative vegetable oil blends.
A1.1) Compositional and qualitative characterization of the oils obtainable from the cereal, oenological and tomato supply chains, also as a function of the quality and type of raw material and process conditions.
A1.2) Formulation of previously characterized oil mixtures, also by fractioning the starting samples.
A2) Production of bakery products with prepared oil blends (A1) and comparison with analogues obtained with fats currently in use in the food industry.
A3) Evaluation of the rheological, technological, nutritional, sensory and safety of use characteristics of the products obtained.
A4) Economic evaluation and environmental sustainability of the use of alternative oil blends to oils currently used to produce bakery products.
A5) Evaluation of innovative techniques for the analysis of the products obtained, as well as of the mixtures to build models that can be used at company level and of better quality than traditional analyses.

Table 1. *Gantt chart of the PhD research activity*

Activity	Month	2	4	6	8	10	12	14	16	18	20	22	24	26	28	30	32	34	36
A1)	***Study of innovative vegetable oil blends***																		
	1) Compositional and qualitative characterization of oils obtainable from the cereal, wine, and tomato supply chains																		
	2) Formulation of blends of previously characterized oils, also, by fractioning the starting samples																		
A2)	***Production of bakery products with the oil blends developed***																		
	1) Study of oil mixtures replacing palm oil																		
	2) Production of bakery products with the aforesaid oils																		
A3)	***Characterization of the obtained products***																		
A4)	***Economic and ecological evaluation of the alternative oil blends***																		
A5)	***Evaluation of innovative techniques for the analysis of the products and mixtures obtained***																		
A6)	***Bibliographic research***																		

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Antioxidant activity of ancient vs conventional cereals

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Ancient cereals have been recently rediscovered by consumers because they are considered healthier, more sustainable, and more resistant to biotic/abiotic stress than conventional analogues. However, despite such interest, wheat species like spelt (*Triticum spelta*) have still limited use. Also, very little is known about their extrudability into products like flakes. Accordingly, this PhD project aims to test their functional properties (i.e., antioxidant activity, content of vitamin, and free/bound phenols) during hot melt extrusion. The results will support local producers to characterize the functional properties of ancient cereals, and in their manufacturing of flakes for breakfast.

Attività antiossidante di cereali antichi vs convenzionali

I cereali antichi sono stati recentemente riscoperti dai consumatori perché considerati più sani, più sostenibili e più resistenti agli stress biotici/abiotici rispetto agli analoghi convenzionali. Tuttavia, nonostante questo interesse, specie di grano antico come il farro (*Triticum spelta*) hanno un uso ancora limitato. Inoltre, si sa molto poco sulla loro estrudibilità in fiocchi. Di conseguenza, questo progetto di dottorato mira a testare le loro proprietà funzionali (attività antiossidante, contenuto di vitamine e fenoli liberi/legati) durante l'estrusione a caldo. I risultati sosterranno i produttori locali nella caratterizzazione delle proprietà funzionali dei cereali antichi e nella produzione di fiocchi per la colazione.

1. State-of-the-Art

1.1 Imporance of cereals and antioxidant compounds

Ancient cereals are defined as "*forms which are represented by populations not subjected to any modern breeding or selection, and sometimes retaining characteristics of wild ancestors, such as individual variability, height, brittle rachis, low harvest index and, in some taxa, hulled kernels*" (Giambanelli et al., 2013). Unique nutraceutical values of ancient cereals include polyphenols, which are important bioactive compounds that exert antimicrobial and antioxidant activity *(Cory et al.,* 2018). The classification of "ancient" is used when the type of cereal has not been genetically modified for more than one hundred years, and the term "modern" cereals, such as common wheat (*Triticum aestivum*), is used when it has been modified or cross-bred in recent years (Dinu et al., 2018). The global production of modern wheat in 2020 was just over 760 million tons. The consumption and use of modern cereals is due to their high yield and gluten content to improve bread and pasta quality. However, these genetical and breeding changes have led to a detrimental effect on the nutritional aspects of wheat and led to the replacement of ancient cereals with modern ones. As consumer awareness towards a healthier lifestyle is increasing, it is necessary to provide bakery products with more nutritional added value, which can be obtained from ancient cereals such as spelt, rye, millet or Kamut wheat. Even though ancient cereals are only grown in small areas and have lower grain yields, they tend to tolerate biotic and abiotic stresses from the environment, such as diseases and drought. In addition, ancient wheat species like spelt (*Triticum spelta* L.) are not only beneficial for human health, but also for the environment as they are also resistant to some fungal diseases and can be used in organic agriculture where chemical pesticides are prohibited (Góral et al., 2017). A recent study indicated that the ancient wheat variety of Gentil Rosso contained more total phenols (1137 µg/g dry weight), then modern wheat (783 µg/g dry weight) (Balli et al., 2022). The ability for these ancient cereals to tolerate different environmental stresses, could be the reason behind their high content of phytochemicals and phenolic compounds.

Polyphenols can be present in free soluble and insoluble forms. Free soluble phenols include hydroxybenzoic and hydroxycinnamic acid derivatives, like chlorogenic, vanillic, *p*-coumaric, ferulic, and *p*-hydroxybenzoic. Although free soluble phenols are readly available for adsorption in the small intestine, the bound phenols are by far the most abundant, and constitute the greatest source of antioxidants present in cereals. The drawback is that bound phenols seems having also a very limited bioavailability. Since ancient cereals contain a large amount of phenols, consuming them can contribute to the prevention of cardiovascular diseases.

1.2 Extraction and characterization of polyphenols

To obtain and quantify phenols, sample preparation steps are required and may differ depending on the nature of the food item. Chemical or enzymatic treatments are necessary to free the polyphenols from the food or plant matrix. Cereals are soluble in polar solvents, and the most commonly used solvent for the extraction of polyphenols from cereals are ethanol, methanol, ethyl acetate, and acetate. Alkali hydrolysis is the most effective hydrolysis treatment used in cereals, as the acidic hydrolysis tends to degrade hydroxycinnamic and benzoic acids. For the determination of the total phenolic content, Folin-Ciocalteu assay can be applied. As for the determination of the polyphenolic profile, liquid or gas chromatography coupled with mass spectrometry or an electrochemical detector

are needed. Since polyphenols exhibit antioxidant activity, they are considered electroactive compounds due to the hydroxyl groups present.

1.3 Effect of processing and extrusion technology on the antioxidant activity of cereals

To meet the needs of today's health-conscious customers, processed foods must contain bioactive chemicals. Extrusion is a fast processing technology that involves high temperatures, pressure, and a short processing time. It is used to make a wide range of processed foods, such as snack foods and read-to-eat cereals. The utilization of different cereals in extruded foods, can help increase consumer acceptance due to the presence of bioactive ingredients, in this case, polyphenols. Extrusion cooking and other processing methods tend to have dramatic changes towards the polyphenol content and antioxidant activity of cereals, such as barley, as reported by De Paula et al. (De Paula *et al.*, 2017). The investigation of the extrusion temperature, pressure and moisure can give us an idea on the alteration of the antioxidant activity of extrudates from different cereals. Thermal processing results in a darker colour of food, due to the Maillard reaction. These dark pigments, known as melanoidins, have antioxidant activity and by studying the effect of extrusion, we can have an explanation on whether or not the antioxidant activity can increase after the thermal processing, and know which compounds are responsible for this phenomenon.

2. PhD Thesis Objectives and Milestones

The research objectives of this PhD project can be achieved through the following activities and working plan as shown in the Gantt diagram given in Table 1:

A1) **Training and optimization of the main methodologies**
The quantitative extraction of free and bound phytochemicals. The quantitative analysis of vitamins and phenols in flours by using HPLC-DAD-MS. The application of antioxidant activity assays and electrochemical detection with the coupling of HPLC with Coulometric Array detection.

A2) **Bound vs Unbound phytochemicals in cereals products**
The study of the antioxidant activity of bound and unbound antioxidant compounds present in cereals and their behavior during different processing methods.

A3) **The investigation of extrusion cooking towards the functional properties of cereal flakes.**
Studying the effect of temperature, time and ingredient composition on the percentage loss of phytochemicals during cereal-based flakes manufacturing. Comparing the antioxidant activity of ancient vs commercial cereals after extrusion cooking.

A4) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

Table 1: Gantt diagram for this PhD thesis project.

Activity	Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	***Optimization and Analysis***																								
	1) Literature Review & Extraction																								
	2) Quantitative Analysis																								
A2)	***Bound vs Unbound phytochemicals***																								
	1) Characterization																								
	2) Processing applications																								
A3)	***Investigation of Extrusion cooking***																								
	1) Application of Extrusion																								
	2) Investigation of antioxidant activity																								
A4)	***Thesis and Paper Preparation***																								

3. Selected References

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ALTERTASTE: improving food pleasure and intake in oncology patients receiving chemotherapy

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This Ph.D. research project aims at understanding the impact of chemotherapy on taste and smell and the repercussions of these alterations on patients' food behaviors. Furthermore, it is oriented to develop guidelines to test these variables that can be used routinely and to collect information useful to innovate food products targeted to patients' needs and to provide them personalized nutritional interventions.

ALTERTASTE: migliorare il piacere del cibo e la sua assunzione in pazienti oncologici in chemioterapia

Questo progetto di dottorato mira a capire meglio l'impatto della chemioterapia su gusto ed olfatto e le ripercussioni delle loro alterazioni sui comportamenti alimentari dei pazienti. Inoltre, è orientato a sviluppare linee guida per la misurazione di queste variabili da utilizzare routinariamente, ed a raccogliere informazioni utili per innovare prodotti alimentari rispondenti ai bisogni dei pazienti e per fornire loro indicazioni nutrizionali personalizzate.

1. State-of-the-Art

Malnutrition is frequently reported in cancer patients receiving chemotherapy (CT) and predicts a poorer response to the treatment, an increased frequency and severity of chemotherapy-related toxicity episodes, an increased risk of postoperative complications, and a consequent deterioration of the patient's overall quality of life (Spotten et al., 2017; Ruiz-Ceamanos et al., 2022).

Taste (45-84%) and smell (5-60%) alterations are amongst the most distressing side effects of chemotherapy reported by patients, and they may contribute to malnutrition, loss of appetite, food aversions, fatigue, nausea and decreased in energy intake, with implications for food behaviors including food enjoyment, food preferences and liking (Boltong & Keast, 2012; de Vries et al., 2018; Drareni et al., 2019). It has been suggested that chemotherapy-related taste and smell changes (TSCs) may be attributable to the fact that the drugs attack rapidly dividing cancerous cells, but also mucosal, gustatory, and olfactory receptor cells that have a frequent turnover rate (de Vries et al., 2018; Ruiz-Ceamanos et al., 2022). Evidence showed that chemotherapy may also induce the development of oral mucositis and dry mouth that could impact food behaviors, as a decrease in salivary flow makes the detection of sapid molecules more difficult. Literature also showed that the timing of onset of TSCs following chemotherapy can vary, reporting short and long-term alterations that usually do not resolve uniformly with time. Normally TSCs appear to be transient and to decline with time post-treatment. Smell seems to be less affected by treatment than taste: most studies reported the greatest extent of taste changes after 3-8 weeks of treatment and the recovery to baseline after 6-12 months, while smell returned to baseline after 6-9 months (Boltong & Keast, 2012; Spotten et al., 2017; de Vries et al., 2018).

Literature provided insufficient evidence to suggest that chemotherapy influences taste and smell in a significant uniform way, pointing to a need for further investigations also to understand its impact on food preferences and behaviors. This inter-individual variability could be attributable to several putative factors including clinical variables such as the cancer diagnosis, the type of treatment, and the occurrence and severity of treatment-related symptoms, but also individual differences in perception resulting from taste responsiveness (taste perception, responsiveness to PROP), psychological traits (food neophobia, sensitivity to disgust, sensitivity to punishment and reward), and dietary exposure. Also, the study design, the method of assessment, the variables of interest, and the stage of chemotherapy at the time point of assessment may influence the results and explain the lack of consistency between studies. Not all patients describe TSCs in quite the same way, nor suffer them with quite the same intensity, so three different types of profiles can be evidenced: patients exhibiting unaltered taste and smell functions, patients exhibiting reduced sensitivity (hyposensitivity) to taste and smell, but also patients exhibiting an increased sensitivity (hypersensitivity) to taste and smell. This may lead to an individual "food trajectory" that differs between patients and throughout the treatment (Boltong & Keast, 2012; Drareni et al., 2019; Ruiz-Ceamanos et al., 2022).

To date, clinicians lack standardized tools with a high clinical utility to routinely assess and treat TSCs in oncology patients and the impact of chemotherapy on food behaviors. Future studies should consider a homogeneous sample composition on grounds of disease state and chemotherapy regimen, in order to minimize the impact of these variables. Data should be collected at multiple time points (before, during, and after CT) to determine the magnitude of alterations in taste, smell, and food preferences across the treatment trajectory. Future

multidisciplinary studies must be conducted to design uniform methodologies to rapidly assess chemosensory perception, food behavior, clinical and individual variables, to develop a patient-centered intervention approach that meets the needs of each patient.

2. PhD Thesis Objectives and Milestones

The objective of the thesis is to understand the impact of chemotherapy on taste/smell and its repercussion on food preferences and behaviors in order to prevent malnutrition and improve patients' food pleasure and intake. The information collected will be used to: 1) develop proofs-of-concept of new products and recipes tailored to the needs of cancer patients undergoing chemotherapy; 2) develop an easy and reliable procedure to test taste/smell changes in oncology patients to allow a routine measurement with a high clinical utility.
The following activities will be included according to the Gantt diagram given in Table 1:

A1) **Data collection following a multidisciplinary approach** involving 50 colorectal cancer and 50 breast cancer patients at six-time points (for a total duration of 9 months for each patient). Questionnaires will be administered to assess socio-demographic, anthropometric, health status, quality of life information, but also psychological traits, attitudes towards food, food preferences and emotional responses to foods, food habits, and self-report taste and odor responsiveness (A1.1). Taste responsiveness will be also measured using sensory stimuli (A1.2). Saliva samples will be collected to analyze the oral microbiota composition (A1.3).

A2) **Conduction of data analyses** with univariate methods to test the effect of the variables, such as data collection time and treatment and their interactions on taste, smell, BMI, and food preferences and habits but also with multivariate methods to explore the relationship between and within treatment, time, taste/smell changes, psychological traits, and food preferences. Segmentation techniques will be applied to identify groups of subjects differing in their taste/smell alterations. Saliva samples will be analyzed to characterize patients' oral microbiota composition and to understand the role played by this variable.

A3) **Writing and editing** of the Ph.D. thesis, scientific papers, and oral/poster communications.

Table 1 Gantt diagram for this Ph.D. thesis project.

Activity / Months		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**
A1)	***Data collection (Multidisciplinary approach)***																								
	1) Administration of questionnaires																								
	2) Taste responsiveness																								
	3) Oral microbiota																								
A2)	***Data analyses***																								
A3)	***Thesis and Paper Preparation***																								

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Influence of grape-born yeasts on wine colour

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This PhD thesis research project is aimed at selecting *Saccharomyces* and non-*Saccharomyces* yeast strains which affect the wine colour. The adsorption of polyphenols on yeast's cell wall will be investigated. The contribution of specific protein related to anthocyanin adsorption during the alcoholic fermentation will be researched through both proteomic and metabolomic approaches. This project will also characterize the volatile and non-volatile polyphenolic fractions of wines and will define the interactions between yeast cell wall, yeast metabolism and anthocyanins.

Influenza dei lieviti sul colore di vini

Questo progetto di dottorato ha come obiettivo di valutare l'effetto di lieviti *Saccharomyces* e non-*Saccharomyces* sul colore dei vini. Al tal fine verrà valutato l'assorbimento dei polifenoli sulla parete cellulare. Inoltre, verranno determinati proteine e metaboliti specifici coinvolti nell'assorbimento degli antociani attraverso tecniche di proteomica e metabolomica. Il progetto si focalizzerà anche sulla caratterizzazione della frazione fenolica volatile e non volatile dei vini e definirà le interazioni tra la parete cellulare dei lieviti, il loro metabolismo e gli antociani.

1. State-of-the-Art

Grape-associated microbiota plays an important role during the winemaking process, since microbes metabolize the sugars from the grapes and produce a whole set of secondary metabolites (Fleet, 2003). Microbial interactions influence the nutritional, hygienic, safety, and organoleptic characteristics of the final product. The major sensorial attribute perceived by the consumer is the colour of wines. In fact, coulor intensity and tonality are considered one of the main parameters contributing to the quality of wine and a matter of concern to winemakers. In general, the colour of young red wines mainly relies on the concentration of monomeric anthocyanins and related compounds, which are extracted from grape skins during the maceration process (Morata et al., 2016). Grape phenolic compounds, such as anthocyanins, also known as anthocyanins, are water soluble flavonoid pigments which are present in must and wine in different molecular structures: monomeric, polymerized, and co-polymerized. These anthocyanins interact with other compounds during fermentation, maturation, and aging, and yield the more complex polymerized and copolymerized pigments. Monomeric anthocyanins are converted in different anthocyanin-derived pigments, such as pyroanoanthocyanins, which influence the wine colour (Tofalo et al., 2021). There are two main types of pyranoanthocyanins: visitin A and visitin B. Visitins are more resistant to bisulfite bleaching and oxidation and are the main responsible of observed colour in aged red wines (Morata et al., 2019). Yeasts play a key role in the definition of wine colour. In fact, they can reduce colour intensity and modify wine tonality by deglycosylation of anthocyanins catalyzed by β-glycosidase or anthocyanidase enzymes (Manzanares et al., 2000), through the direct adsorption of pigments on yeasts' cell wall, and producing metabolites such as pyruvic acid and acetaldehyde that have been found to react with different phenolic compounds (Morata et al., 2006). One of the main enzymatic activity of yeasts involved in the modulation of wine colour is the hydroxycinnamate decarboxylase (HCDC) activity involved in the production of vinylphenolic pyranoanthocyanins (Benito et al., 2011). The absorption of polyphenols on the yeast cell wall entraps a large portion of these compounds during vinification. This property is a strain- specific feature, since the physical structure of mannoproteins (total charge, charge distribution, and accessible surface area) of the cell wall differs among yeast species and strains (Caridi et al., 2007). The aim of present PhD project is to further explore the interactions between yeasts and grape polyphenols during alcoholic fermentation and to screen wine yeast strains for their ability to affect the wine colour.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) Screening and selection of yeasts. *Saccharomyces* and non-*Saccharomyces* strains isolated from organic grapes and musts, belonging to the collection of Faculty of Bioscience and Technology for Food, Agriculture and Environment, will be screened for their ability to adsorb anthocyanins on the cell wall (A1.1). Moreover, the HCDC activity of the strains will be evaluated (A1.2). The production of pyranoanthocyanins by yeasts with high HCDC activity will be monitored using HPLC-DAD and confirmed using HPLC-DAD-MS/MS (A1.3). The strains with better behavior will be selected for the next steps.

A2) Small-scale fermentation of selected strains. Laboratory fermentations will be carried out using organic

must. Fermentation trials driven with single and mixed cultures will be performed. Moreover, the acetaldehyde and pyruvic acid synthesis will be detected (A2.1). Wine colour parameters will be evaluated based on CIELab methodology at the end of fermentation (A2.2).

A3) Cellar vinification. The most promising strains will be used for cellar vinifications. In particular, fermentation trials will be conducted using several combinations of the *Saccharomyces* and non-*Saccharomyces.* Skin maceration together with novel procedures based on the control of anthocyanins and tannins concentration will be applied to stabilize the wine colour.

A4) Proteomics and metabolomics analyses. These approaches will be used to monitor the protein and metabolite markers involved and related to the mechanisms underlying yeast pigment/anthocyanins adsorption during alcoholic fermentation. LC-MS/MS (FUSION Orbitrap mass spectrometer, Thermo Scientific). Mass spectrometry data will be deposited to the ProteomeXchange Consortium via the PRIDE partner repository (A4.1). Proteomics data analysis will be performed using Proteome Discoverer™ Software or MaxQuant (version 1.4.0.5) with the integrated search engine Andromeda. For peptide and protein identification, raw files will be searched against the UniProt database. All data sets will be adjusted to 0.05 peptide-spectrum match (PSM) and lower than 1% protein false discovery rate (FDR). MaxQuant output, will be analyzed through Perseus framework for statistical analysis of proteomics data (A4.2).

A5) Physical-chemical and sensorial characteristics of wines. The main oenological parameters will be determined by FOSS WineScan (FT-120) rapid scanning Fourier Transform Infrared Spectroscopy with FOSS WineScan software version 2.2.1. (A5.1). Wines were subjected to sensory analysis. Sensory tests were performed at room temperature (20 °C) by a group of 10 trained tasters on the basis of 10 descriptors using a scale from 1 to 10 (ISO, 2014) (A5.2).

A6) Writing and Editing of the PhD thesis, scientific papers and oral and/or poster communications.

Table 1: Gantt diagram for this PhD thesis project.

	II YEAR												III YEAR											
	MONTHS																							
ACTIVITIES	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Bibliografic research																								
A1) Screening and selection of yeast																								
A1.1) Evaluation anthocyanins adsorbtion on the cell wall																								
A1.2) Determination of HCDC activity																								
A1.3) Determination of pyranoanthocyanins production																								
A2) Small-scale fermentation																								
A2.1) Evaluation acetalydede and piruvic acid production																								
A2.2) Colour assessment																								
A3) Cellar vinification																								
A4) Proteomics and metabolomics analyses																								
A4.1) Monitoration of protein and metabolite markers involved in yeast anthocyanins adsorption																								
A4.2) Proteomic and metabolomic data anlysis																								
A5) Physical-chemical and sensorial characteristics of wines																								
A5.1) Main oenological parameters																								
A5.2) Sensorial analysis																								
A6) Writing and editing of PhD thesis and papers																								

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Technologies for reducing saturated fats in food formulations

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Despite the increased risk for human health posed by lipids rich in saturated fatty acids, such as palm and coconut oil, they are widely used in food industries. At the same time, there is a rising demand for "clean-label", trans-fat-free, and sustainable foods from consumers. Among current possibilities for reducing saturated fats usage, the encapsulation of less saturated fats into saturated using "Particles from Gas Saturated Solutions" (PGSS) and the increase of solid fat fractions content through enzymatic glycerolysis are considered prominent. Both technologies could provide an alternative way to design new fat ingredients with enhanced textural and oxidative stability.

Tecnologie per la Riduzione di Grassi Saturi nelle Formulazioni Alimentari

Nonostante l'alto rischio per la salute umana, alimenti ricchi di acidi grassi saturi, come l'olio di palma e di cocco, sono ampiamente utilizzati nelle industrie alimentari. C'è una crescente domanda da parte dei consumatori di prodotti alimentari "*clean-label*", e più sostenibili. Tra le possibilità per ridurre l'utilizzo di grassi saturi, l'incapsulamento di grassi meno saturi in saturi utilizzando "*Particles from Gas Saturated Solutions"* (PGSS) e l'aumento del contenuto di frazioni di grasso solido attraverso la glicerolisi enzimatica sono considerati importanti. Entrambe le tecnologie, potrebbero fornire un modo alternativo per progettare nuovi ingredienti grassi con una maggiore stabilità fisica e ossidativa.

1. State-of-the-Art

1.1. Saturated fats and their influence on human health

Lipids rich in saturated fatty acids are widely used in food industries for the production of meat, and dairy products, and especially for bakery and confectionery products. The most widespread used saturated fats are palm and coconut oil (CNO) fractions. The industrial use of these lipids is increasing year by year despite the dispute that is taken on the health benefits provided by those lipids. In past years, coconut oil was promoted as a solution to weight loss and healthy saturated fat. It was also implied, that CNO consumption could improve the lipid profile as well as act as glycemic control. Recent studies prove that coconut oil consumption results in notably higher low-density- and high-density lipoprotein cholesterol levels as well as serum total cholesterol than non-tropical vegetable oils, which may increase the risk of cardiovascular diseases (Jayawardena et al., 2021). Further meta-analysis on the influence of CNO on glycemic control found that coconut oil increases postprandial glucose levels by decreasing insulin and thus can be detrimental for long-term blood glucose control as it increases insulin resistance (Dhanasekara et al., 2022). The study of the CNO consumption influence on the gut microbiota revealed negative effects on most of the evaluated parameters: the decrease in bacterial diversity, insulin sensitivity, as well as in mitochondrial function and fatty acid oxidation. At the same time hepatic steatosis, the highest low-grade inflammation, and low-density lipoprotein (LDL-cholesterol concentration) were found, while the opposite result was shown for the olive and soybean oil (López-Salazar et al., 2021).

1.2. Saturated fats and the challenge of the industry

Nowadays, consumers are increasingly interested in both health and sustainability aspects of their life that is reflected in their demand for the industry. It can be described as more natural, organic, less processed, and 'free from' certain ingredients food. All that has a general definition of "clean-label" foodstuffs, that are considered to be health-promoting. This phenomenon has driven the food producers to communicate whether a certain ingredient or additive is not present or if the food has been produced using a more "natural" production method (i.e. organic agriculture) (Asioli et al., 2017). Modern technologies offer a wide range of solutions to reduce the consumption of solid fat through oil structuring, but the use of non-label-friendly structurants poses a challenge to the food industry. As most of those solutions could be found feasible for a variety of industrial applications, however, will not be considered clean-label ingredients. Thus, microencapsulation for saturated fat fraction reduction using PGSS technique and lipid structurization by lipase-catalyzed glycerolisys are considered feasible for this research.

1.3. Prospective methods of saturated fats reduction

Lipid structuring through lipase-catalyzed glycerolysis converts native triglycerides into partial glycerides (mono- and diacylglycerides) and has been shown to be an effective method for structuring plant-based oils into fats without changing the fatty acid composition (Nicholson and Marangoni, 2022). It is expected to influence the acylglycerols composition, solid fat content, thermal behavior, regiospecific distribution, microstructure, and rheological properties of selected lipids. The resulted trans-free fat could be used as a substitution for common saturated fats in a large variety of food products, such as margarine, confectionery fillings, bakery fats, cacao butter

substitutes in chocolates, etc. The use of alternative unsaturated fats may result in enhancing the lipid composition of the final product, lowering the production price, as well as switching to more sustainable raw materials.
Another approach to be applied is microencapsulation of less saturated oils into saturated ones for saturated fat fraction reduction using PGSS technique. The possibility of the use of nature-derived fats like palm and coconut oil as microcapsules wall material has to be assessed. The use of supercritical fluids for encapsulation could have certain benefits over other methods, since, it has the potential to produce microcapsules free of solvent traces, with high encapsulation efficiencies and high inner component loads. Saturating the supercritical fluid with a mixture of the bioactive ingredient and the wall material is the general approach for obtaining encapsulated particles. The saturated solution is then rapidly expanded at atmospheric pressure. Rapid CO_2 release produces an intense cooling effect, resulting in the creation of solids or liquid particles depending on the process parameters and the components' properties (Klettenhammer et al., 2022). As a result, it is expected to obtain a stable, natural, sustainable, less-calorie, easily stored, and transportable material, that could pose health benefits for the consumer by balanced lipid composition. Successful implementation will allow industries to use less-stable oils in production without jeopardizing the final quality and shelf-life of the final products. Microencapsulation is limiting the oxygen access to the encapsulated material thus creating conditions for enhanced stability of the oil located in the inner part of the capsule.

2. Ph.D. Thesis Objectives and Milestones

Presented below (Table 1) represents the overall structure of the Ph.D. project.
A1 – **Lipid structuring through enzymatic glycerolysis.** Reaction parameters, such as temperature, duration, agitation, and enzyme concentration to be tested (A1.1) in order to obtain fat from oil. With the following study of oxidative stability, thermal and rheological behavior, and quality parameters of the resulted matrices (A1.2).
A2 – **PGSS-obtained lipid development** and powders production process optimization (A2.1). The quality parameters, oxidative stability, as well as thermal and rheological behavior of the resulted powders have to be assessed (A2.2).
A3 – **Incorporation of the developed ingredients into foodstuffs** and the characterization of the final product.
A4 – **Writing and editing** of the papers, conference communications, and posters as well as the thesis dissertation.

Table 1 Gantt diagram for this PhD thesis project.

Activity	Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	***Lipid structuring through enzymatic glycerolysis***																								
	1) Production of structured fats and optimization of the process																								
	2) Study of the obtained lipids																								
A2)	***PGSS-obtained lipid development***																								
	1) PGSS powders production process optimization																								
	2) Powder characterization																								
A3)	***Lipids incorporation into foodstuffs***																								
	1) Recipe development and optimization																								
	2) Foodstuffs' quality characterization																								
A4)	***Thesis and Paper Preparation***																								

3. Selected References

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Microbial interactions during fermentations in winemaking

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Grape must's microbiome is composed, among others, by *Saccharomyces cerevisiae* and non-*Saccharomyces* yeasts. They are naturally present on grapes and winery environment, playing a key role in spontaneous fermentations. They could also be used as starter cultures in mixed inoculated fermentations to produce wines with specific characteristics. Yeasts interact with each other through various mechanisms like production of metabolites, quorum sensing, or cell-to-cell contact. Limited molecular information related to yeasts' interactions involved in mixed fermentations is available. Transcriptomic analysis applied on wine fermentations could help to further explore the mechanisms of interactions among yeasts and their contribution to wine characteristics.

Interazioni microbiche durante la fermentazione in vinificazione

Il microbioma del mosto d'uva è composto, tra altri, da lieviti *Saccharomyces cerevisiae* e non-*Saccharomyces*. Essi sono naturalmente presenti sulle uve ed in cantina, giocando un ruolo chiave nelle fermentazioni spontanee, mentre possono essere usati come colture starter nelle fermentazioni miste inoculate per produrre caratteristiche specifiche. I lieviti interagiscono tra loro attraverso meccanismi come la produzione di metaboliti, quorum sensing o *cell-to-cell contact*. Vi sono scarse informazioni molecolari disponibili delle interazioni tra lieviti coinvolti in fermentazioni miste. L'analisi trascrittomica applicata alle fermentazioni vinicole può aiutar ad esplorare i meccanismi di interazione ed il loro contributo per le caratteristiche del vino.

1. State-of-the-art

The wine is the product obtained by bio-chemical process that lead to the conversion of sugars present in grape must into carbon dioxide, ethanol, parallelly to the production of a wide set of other metabolites. This process is conducted mainly by yeasts that could be naturally present or artificially inoculated. Wine yeasts are generally subdivided into *Saccharomyces cerevisiae* and non-*Saccharomyces*. The former is the responsible of the full conversion of sugars into ethanol, while the latter are considered as producers of secondary compounds. The use of non-*Saccharomyces* species as part of mixed starters together with *S. cerevisiae*, has been suggested as a way to mimic spontaneous fermentations enhancing wine complexity, avoiding the risks linked to this practice. The efficiency of this technique is due to the capacity of non-*Saccharomyces* yeasts to modulate specific wine metabolites and characteristics. Mixed yeast starter cultures can also avoid the risks of stuck fermentation and contribute to better control the alcoholic fermentation, as well as the production of more uniform and balanced wines, tailoring them to the new global trends. (Jolly et al., 2006) (Ciani et al., 2009) (Tufariello et al., 2021).
The presence of specific species or strains during wine production is influenced by a wide variety of factors. The physico-chemical characteristics of the medium have been found to play a significant role: nutrients concentration and composition could determine competition for them affecting the dominance or the disappearance of some species. The must conditions are effectively responsible for the presence of some yeasts based on the availability of nutrients or metabolites produced. The management of temperature could favor or inhibit the presence of a species, leading to different final products. The presence of metabolites or added substances could negatively affect the development of both non-*Saccharomyces* and *S. cerevisiae* yeasts; it's the case of the addition of sulfur dioxide or other compounds with negative impact on yeasts viability. A result that could be easily shown by classical microbiology is the dominance of some species over the others during the different fermentation phases. The yeast-yeast interactions are also considered responsible of the dominance of a species over others directly affecting yeasts growth, but also the production of some metabolites. A wide set of microbial interactions need to be studied as tool to interpret the succession of yeasts' consortium. The more discussed mechanisms are related to killer factor and cell-to-cell contact. The first one has toxicity against sensitive species due to the production of toxic molecules. Quorum sensing is correlated to the production of signaling molecules directly correlated to genes' expression regulation. Cell-to-cell contact mechanism has also been identified as responsible for yeasts interactions during fermentation (Englezos et al., 2019).
Yeasts interactions are not totally explained by the classical microbiology using conventional methods. One of the limits associated to plate counts is related to right dilutions necessary: in case of mixed cultures, especially at middle-end stages of alcoholic fermentation, it's difficult to quantify the species less abundant. Bioinformatics based tools could help to further explore the nature of the interactions. Up to date, there are some works done on wine yeasts transcriptome analysis performed exclusively on synthetic medium (miming grape must). In addition to this, studies have been done principally on *S. cerevisiae*'s genes changes using RNA seq as a function of the modification of medium conditions (nitrogen availability, low temperature). The novel parts of this thesis project

are related to the use of real red grape must and the analysis of both yeasts behavior during mixed fermentations using transcriptome analyses.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above, this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A) **Bibliographic research** focused on microbial interactions during fermentation, RNA seq analyses and bioinformatics tools. This part is fundamental to understand the focal concept of this PhD project, to identify the reached goal and the future perspectives.
B) **Protocol improvement** and definition of critical steps is a key point of this project. Grape must and wine are difficult matrices due to the RNA-interfering compounds like polyphenols, tannins, and polysaccharides.
C) **Transcriptome analysis** performed under different fermentation trials (with pure and mixed cultures), sampling and RNA extraction, preparing and sequencing the RNA obtained from each sample.
D) **Data elaboration** is articulated in the arrangement of the data obtained by the sequencing and its development. Particularly in this phase, it is fundamental to learn and apply bioinformatic and biostatistics tools to compare the results of different conditions.
E) **Writing** and paper preparation for thesis and presentations.

***Table 1**: Gantt diagram of this PhD thesis*

			10-22	11-22	12-22	1-23	2-23	3-23	4-23	5-23	6-23	7-23	8-23	9-23	10-23	11-23	12-23	1-24	2-24	3-24	4-24	5-24	6-24	7-24	8-24	9-24	10-24
A		Bibliographic research																									
B		Protocol improvement																									
C		Transcriptome analysis																									
	.1	fermentation trials																									
	.2	RNA extraction																									
	.3	sequencing																									
D		Data elaboration																									
E		Writing																									

3. Expected results

The main goal of this thesis is the exploration of genes expression among *S. cerevisiae* and non-*Saccharomyces* yeasts involved in mixed fermentation. Thus, information will be groundbreaking for what concern the mechanisms of yeast-yeast interaction. Finally, the obtained knowledge achieved from transcriptomic analysis could help to modulate yeast contribution during fermentation and therefore wine quality (Simó et al., 2019).

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Less common grains in bakery industry: product and process optimization

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The main goal of my PhD project is to investigate how to improve the characteristics of baked goods enriched in less common grains. Despite their positive agricultural and nutritional traits, the use of minor cereals, pseudocereals and legumes is so far limited due to the several issues, including structural properties of starch and/or proteins that compromise their transformability into widely used food products, as well as sensory traits that reduce product acceptability. Enrichment of baked goods with less common grains will involve the development of (bio-)technological processes with a low environmental impact to improve the technological characteristics of these grains.

Impiego di cereali minori, pseudocereali e legumi nell'industria dei prodotti da forno: ottimizzazione di prodotto e di processo

Il presente progetto si propone di studiare come migliorare le caratteristiche dei prodotti da forno arricchiti in cereali minori, pseudocereali e/o legumi. L'utilizzo di queste materie prime nelle formulazioni alimentari è ad oggi limitato a causa di diverse problematiche, come le proprietà strutturali di amido e/o proteine che ne compromettono la trasformabilità in prodotti di largo consumo, nonché le caratteristiche sensoriali che ne riducono l'accettabilità del prodotto. L'arricchimento di prodotti da forno con cereali minori, pseudocereali e/o legumi prevederà la messa a punto di processi (bio-)tecnologici a basso impatto ambientale atti a migliorare le caratteristiche tecnologiche di tali materie prime.

1. State-of-the-Art

In a scenario of demographic and climatic changes, decreasing arable lands and water resources, grains could play an important role in promoting a sustainable diet. Indeed, in order to encourage the transition to a sustainable food production, the harvest of crops other than wheat should be increased. In this context, minor cereals, pseudocereals and legumes are characterized by good climatic adaptability, low production costs and water requirements (Kakabouki *et al*., 2021). One aspect that makes these grains attractive for food uses is their nutritional profile, such as fiber, phenolic compounds and resistant starch (Ragaee *et al*., 2006). Moreover, the high protein content allows to consider legumes as a viable alternative to the consumption of animal proteins (Rochfort and Panozzo, 2007). In order to increase the consumption of these grains, a feasible strategy could be their use in the bakery industry, as ingredients in products with different physical features (e.g., bread and biscuit). However, their use in food formulations is limited due the presence of specific anti-nutritional compounds and sensory traits, that compromise their acceptability to consumers. Moreover, the structural properties of the macromolecules (starch and protein) influence their transformability into widely used food products, including baked goods.
To solve these problems, the application of biotechnological (i.e., germination or fermentation) and technological (i.e., air classification) processes to grains could be functional to improve the technological, sensory and nutritional properties of end products. Germination (or sprouting) is the process that converts seeds into plants, a phenomenon triggered by intrinsic enzymatic activity (e.g., amylase and protease) which leads to the breakdown of proteins, carbohydrates and lipids into simpler forms, and improves the bioavailability of micronutrients and the digestibility of protein and starch (Lemmens *et al*., 2019). Another biotechnological process used to improve the properties of grains is fermentation, a process that causes biochemical changes through the activity of microorganisms and their enzymes (Kohajdová and Karovičová, 2007). Finally, among the technological processes, air classification is an inexpensive approach that exploit air currents in combination with centrifugal force to separate flour particles into different fractions according to size and density, and thus chemical composition. Therefore, it allows to collect fractions with higher content of different compounds of interest, including phenolic compounds, proteins, and fiber (Assatory *et al*., 2019).

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:
A1) **Literature review.** The purpose of this activity is to keep updated the literature review that started during the preparation of the proposal and of the present report.
A2) **Set up and scale up of the processes.** Grains (oat, buckwheat, red lentils and beans) will be subjected to germination, fermentation and/or air classification on a pilot scale under controlled conditions. Enzyme

activities (α-amylase and protease) will be monitored during germination (AACC 22-05.01; AACC 22-62.01), whereas starch (AACC 76-13.01), protein (AACC 46-12.01), lipid (AACC 30-10.01) and dietary fiber (AACC 32-07.01) contents will be measured before and after all the considered processes. The functionality of flours will be evaluated as: water absorption capacity, oil absorption capacity and swelling power according to thmethods reported by Cardone *et al*. (2021). The starch pasting properties will be evaluated using the Micro-Visco-Amilograph (MVAG; Brabender GmbH & Co.KG, Duisburg, Germany). Based on these results, optimal processing conditions will be selected and used in an industrial plant.

A3) **Effect of the processes on the functionality of blends.** Commercial wheat flour will be used as a base. Different levels of enrichment (from 5 to 20%) will be tested in medium (55%) and high (120%) hydration level systems, typical of a natural and chemical leavening product, respectively. These two systems are representative of the project partner's production. Rheological characterization of the mixtures will include the evaluation of mixing properties with GlutoPeak (Brabender GmbH & Co.) and Farinograph (Brabender GmbH & Co.; AACC 54-21.01), uniaxial extensional (Brabender GmbH & Co.; AACC 54-10.01), and three-dimensional (Chopin, Villeneuve La Garenne, France; AACC 54-30.02). and leavening properties (Chopin).

A4) **Products development and defining products quality.** The most suitable formulations will be selected and used to produce a biological and a chemical leavened product on a laboratory and industrial scale. Image analysis techniques will be used to assess physical characteristics (e.g., size, color, porosity). The specific volume of the products (AACC 10-05.01) and the crumb softness (AACC 74-10.02) will also be evaluated including during storage.

A5) **Thesis and paper preparation.** Writing and editing of the thesis, scientific papers and oral/poster communications.

Table 1 Gantt diagram for this PhD thesis project.

Activity	Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	***Literature review***																								
A2)	***Set up and scale up of the processes***																								
	1) Set up of the process conditions																								
	2) Flour composition and functionality																								
	3) Scale up of the processes																								
A3)	***Effect of the processes on the functionality of blends***																								
A4)	***Products development and defining products quality***																								
	1) Lab-scale production																								
	2) Industrial-scale production																								
A5)	***Thesis and Paper Preparation***																								

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Development of a decision support system (DSS) to predict and extend food shelf-life

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The goal of this PhD project is to develop a decision support system (DSS) that can simulate and forecast the shelf-life of food from distribution to consumption. The main research work will be on minimally processed fruits and vegetables products. To calibrate prediction models, quality decay will be evaluated as a function of process and storage parameters. The DSS will help with the implementation of new technologies to extend the shelf life of food and minimize waste.

Sviluppo di un sistema di supporto alle decisioni (DSS) per prevedere ed estendere la shelf-life degli alimenti

L'obiettivo del progetto di dottorato è quello di sviluppare un sistema di supporto alle decisioni (DSS) in grado di simulare e predire la shelf life degli alimenti dalla distribuzione al consumo. Il principale lavoro di ricerca riguarderà i prodotti ortofrutticoli minimamente processati. Per calibrare i modelli predittivi, il decadimento della qualità sarà valutato in funzione dei parametri di processo e di conservazione. Il DSS contribuirà all'implementazione di nuove tecnologie che prolungheranno la shelf life degli alimenti e ridurranno al minimo gli sprechi.

1. State-of-the-Art

Fruits and vegetables are key components of the human diet and there is substantial evidence that they provide health and nutritional advantages. The demand for the minimally processed fruits and vegetable has been expanded because of busy lives, rising purchasing power and increasingly health-conscious customers. A broad variety of minimally processed fruits and vegetables (MPFVs) have been produced in response to consumer demand for nutritious, fresh-like, and easy-to-prepare goods (Ramos *et al.*, 2013).
DSS is software that can simulate and anticipates the consequences of a process or distribution using mathematical models and real-time data obtained by sensors to assist food industry operators in selecting the best process parameters or storage condition to accomplish the intended goals (Zhai *et al.*, 2020). A model is often used to explain a physical process by utilizing the relationship between input and output characteristics, or it is used to construct or test 'what if' scenarios, which aids in avoiding arduous and time-consuming experiments (Wynn & Clarkson, 2018). Alteration reactions are mostly (bio)chemical and physical in nature. These changes occur at a certain rate and with specific kinetics. Kinetic modeling allows us to quantify these changes and their rates. We have a great tool in kinetic modeling that can help us understand basic reaction processes. Understanding the fundamental processes is critical for quality modeling and quality control (Datta & Sablani, 2007). They have been used to describe variations in fruit quality, including visible features such as firmness and color, as well as interior properties. It is a well-established and dependable approach for predicting quality changes in a variety of food commodities. For shelf-life modeling, empirical models based on reaction order, such as zero-, first-, second-, or higher order reaction modeling, are extensively used (Table 1).

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 2:

A1) **Mathematical models** (primary and secondary) will be identified for the description of the alteration kinetics of highly perishable food products (A1.1) and data available in the literature will be used to preliminary calibrate the selected models (A1.2).

A2) **Assessment of impact of environmental storage factors** on quality indices changes of minimally processed fruits and vegetables will be studied to predict their shelf -life under different distribution conditions (A2.1, A2.2).

A3) **Development of a decision support system (DSS)** will be carry out based on the results get by experiment and implementation of the model. Validation of the DSS will be performed by shelf-life test in real storage condition (A3).

A4) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications (A4).

Table 1. *Example of quality indices, models, and value of parameters of kinetic modelling apply to MPF&V*

Product	Quality index	Models	Parameters		References
Kiwifruits		Zero order and Ball Model	z °C	D_{ref}	
	i. Weight loss	$log_{10}\left(\frac{D}{Dref}\right) = -\left(\frac{T - Tref}{z}\right)$	29.371	3.820	(Zhang *et al.*, 2021)
	i. Firmness		30.535	1.556	
	i. Soluble solids content		29.370	4.522	
	v. Ascorbic acid		26.605	0.368	
	v. Titratable acidity		29.049	57.574	
	i. Total color change		28.922	1.300	
	i. Sensory score		29.176	3.531	
Fresh-cut Potatoes		Zero Oder reaction and Arrhenius Model	Activation energy E_a (J/mol)	Pre-exponential factor k_0	(Zhao *et al.*, 2022)
	i. Weight loss	$k = ko\ exp\ (\frac{-Ea}{RT})$	1.35×10^5	1.21×10^{24}	
	ii. PPO activity		3.07×10^4	3.45×10^4	
Fresh-cut Melons		Weibull Model	α	β	
	i. Appearance	$S(t) = \exp\ [-(\frac{t}{\alpha})\beta]$	7.354	1.967	(Amodio *et al.*, 2013)
	ii. Aroma		13.78	0.7694	
	iii. Translucency		12.52	1.456	
	iv. Firmness		8.575	1.241	
	v. Vitamin C		17.09	0.6291	

Table 2 *Gantt diagram for this PhD thesis project.*

Activity \ Months		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	***Mathematical models of quality decay for perishable food products***																								
	1) Selection of mathematical model and implementation of model																								
	2) Preliminary calibration of the selected models																								
A2)	***Impact of environmental storage factors and development of DSS***																								
	1) Effect of oxygen, carbon dioxide and temperature on quality decay of MP Apple																								
	2) Effect of oxygen, carbon dioxide and temperature on quality decay of MP strawberry																								
A3)	***Model calibration and DSS implementation***																								
	1) Implementation of the model into the DSS																								
	2) Prediction of shelf life																								
	3) Shelf-life test in real storage condition																								
	4) DSS validation																								
A4)	***Thesis and Paper Preparation***																								

3. References

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Improving the grape pressing for a sustainable wine production chain (GrapePress 4.0)

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The improvement and optimization of grape pressing are pivotal for obtaining white wine with balanced chemical and sensory profiles, the presence of varietal aromas and their precursors, and the preservation of the peculiar characters during shelf life. This PhD research project aims to implement the knowledge concerning the impact of pressing on must composition, based on both grape and wine characteristics. Suitable chemical and/or physical markers will be identified and selected for monitoring the pressing which will be optimized to achieve the desired wine features. The wine production with minimal intervention will be investigated in precision and sustainable oenology perspectives.

Perfezionamento della fase di pressatura dell'uva per implementare la sostenibilità della filiera enologica (GrapePress 4.0)

Il miglioramento e l'ottimizzazione della pressatura dell'uva sono fondamentali per ottenere vino bianco con caratteristiche chimiche e sensoriali equilibrate, con aromi varietali e relativi precursori, ed il mantenimento delle peculiarità desiderate durante la shelf life. Questo progetto di dottorato mira ad implementare le conoscenze relative all'impatto della pressatura sulla composizione del mosto correlata alle caratteristiche dell'uva e del vino. Saranno identificati e selezionati marcatori chimici e/o fisici adeguati per il monitoraggio della pressatura che sarà ottimizzata per ottenere un vino con le caratteristiche desiderate. Sarà indagata la produzione di vino con minimo intervento per accrescere l'enologia sostenibile e di precisione.

1. State-of-the-Art

The pre-fermentation steps are among the winemaking procedures playing an important role in the composition of must and, consequently, of wine produced with it being decision factors especially to obtain high-quality white wines (Boselli *et al.,* 2006). In particular, the grape pressing conditions are pivotal for the production of white wines having balanced chemical and sensory characteristics, the presence of varietal aromas and their precursors, and the preservation of the desired peculiarities during the shelf life. It is necessary to clarify how grape pressing should be carried out by identifying the conditions that emerge from possible correlations of the composition of grape, must, and wine. The pressure gradient applied for grape pressing affects the extractions of aroma precursors and phenolic compounds. To ensure the desired aromatic potential of the wine, an adequate amount of aromas and their precursors should be present in the must, particularly in the case of aromatic and semi-aromatic grape varieties (Gawel *et al.,* 2014). The phenols are involved in juice oxidation and wine stability (Darias-Martín *et al.,* 2004). The skin contact time needs to be properly managed as flavonoids and non-flavonoids are extracted in higher quantity with longer skin contact influencing the enzymatic oxidation and browning of must (Maggu *et al.,* 2007). Moreover, the quality of must can be affected by its turbidity, pH, and total acidity (Darias-Martín *et al.,* 2004).
The role of pressing on must characteristics is well-known by winemakers, but the knowledge currently available in the scientific literature is rather limited. The improved management of the pressing cycles can have a positive impact on the sustainability of the wine production chain since it can limit the use of chemicals, in particular sulfur dioxide, during the pre-fermentation steps and, potentially, on the finished product. The pressing improvement can favor the production of wine with minimal intervention, one of the priorities of the strategic plan of the International Organization of Vine and Wine (OIV, 2020). The monitoring of the pressing process will allow to understand the ideal conditions for pressing a specific grape having a certain composition in a specific vintage, guaranteeing a high-quality final product. These aspects can permit the efficient use of resources and promote green development and innovation of the wine production chain which can grow taking into account the three 'Es' of sustainability (Environmental, Economic, and social Equity) (Borsellino *et al*., 2016).
The main goal of the GrapePress 4.0 project is to identify the pressing cycle(s) being adequate and defined for the specific characteristics of grapes and wines that will be produced. The specific objective of this project is the identification and selection of chemical and/or physical markers suitable for precisely defining the optimal conditions of the pressing cycle, allowing for the optimized management of this process related to the raw material. In this way, it will be possible to define the pressing conditions while also obtaining the maximum expression of the grape variety, the must, and the wine through an accurate and innovative approach that up to now, to the best of our knowledge, has not been addressed. Green analytical methods (i.e. spectroscopy, hyperspectral imaging, Nuclear Magnetic Resonance) will be applied to strengthen the use of these emerging techniques for the analysis of grape, must, and wine (Silva *et al.,* 2018; Herbert-Pucheta *et al.,* 2019). Winemaking trials with the minimal intervention will be also carried out allowing to further increase the sustainable and precision oenology.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above, this PhD project can be subdivided into the following activities according to the Gantt diagram reported in Table 1:

A1) **Identification of chemical/physical parameters of wine grape.** Different white grape varieties will be pressed under controlled conditions (e.g. lab conditions) simulating different pressing processes (e.g. air pressing, pressing in anoxic conditions) (A1.1). The composition of the grapes and the must be obtained under laboratory conditions will be evaluated in order to identify the parameters that are most influenced by the pressing process.

A2) **Investigation of the impact of the different pressing cycles on must.** The production of musts on an industrial scale, accordingly to the pressing conditions adopted by the wineries involved, will be considered (A2.1). Contemporarily, optimized pressing conditions, defined based on the results obtained during the first year of the PhD project, will be explored (A2.2). Must samples will be collected during the pressing (e.g. draining, 20%, 40%, and 60% must yield, end of pressing) and analyzed.

A3) **Evaluation of the relationship between the pressing cycle and wine characteristics.** Winemaking trials will be carried out using the clarified musts collected in A2 and in vintage 2022 (PhD first-year). Vinification protocols (i.e. starter yeast, the addition of additives, and adjuvants) will be developed also considering a minimal intervention approach (A3.1) and for the production of sulfur-free wines (A3.2).

A4) **Application of green analytical methods.** Emerging techniques will be applied for the analysis of grape, must, and wine throughout the activities A1-A3. Specifically, attention will be given to the analysis by means of Nuclear Magnetic Resonance (NMR, A4.1) and spectroscopy (A4.2) methods.

A5) **Bibliographic research, writing & editing** of the PhD thesis, scientific papers, and oral and/or poster communication for the dissemination of the results deriving from the GrapePress 4.0 project.

Table 1 Gantt diagram for this PhD thesis project.

Activity \ Months		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	***Identify must chemical composition***																								
	1) Grape pressing trials																								
A2)	***Pressing impact on must***																								
	1) Industrial-scale pressing																								
	2) Pressing with optimized condition																								
A3)	***Winemaking trials***																								
	1) Wine with minimal intervention																								
	2) produce sulfur-free wine																								
A4)	***Green analytical methods***																								
	1) Nuclear Magnetic Resonance																								
	2) Spectroscopy methods																								
A5)	***Thesis and paper writing & editing***																								

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Untargeted/targeted UHPLC-HRMS approach for quality assessment in hazelnuts and finished products of the confectionery industry

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The PhD thesis project is oriented towards the implementation of new approaches for quality assessment in raw materials and finished products of the confectionery industry, based on the combination of the analytical techniques of Ultra-High Performance Liquid Chromatography (UHPLC) and High-Resolution Mass Spectrometry (HRMS). The research activities focus on the characterization of the rotten and "*cimiciato*" defect on different origins and matrices of hazelnuts, as well as on the qualitative and quantitative determination of different classes of polyphenolic compounds during the shelf-life of finished products.

Approccio untargeted/targeted UHPLC-HRMS per la valutazione della qualità in nocciole e prodotti finiti dell'industria dolciaria

Il progetto di tesi di dottorato è orientato all'implementazione di nuovi approcci per la valutazione della qualità di materie prime e prodotti finiti all'interno dell'industria dolciaria, basandosi sull'accoppiamento delle tecniche analitiche di Cromatografia Liquida ad Ultra-Alta Prestazione (UHPLC) e di Spettrometria di Massa ad Alta Risoluzione (HRMS). Le attività di ricerca si focalizzano sulla caratterizzazione del difetto avariato e "cimiciato" su diverse origini e matrici di nocciola, oltre che sulla determinazione quali-quantitativa di diverse classi di composti polifenolici durante la *shelf-life* di prodotti finiti.

1. State of the Art

According to the latest FAO data (FAOSTAT, 2020), Turkey (60%) is the largest hazelnut producer in the world, followed by Italy (13%), the United States, Arzebaijan, Chile and Georgia. About of 90% of the hazelnuts produced are processed by the food industry, imposing strict requirements in terms of quality, from the shape and the size of nuts to the aromatic and nutritional profile (Romero et al., 2021).
The rotten defect is the strongest sensory off-note of hazelnuts, which every year threatens their availability and marketability, inducing yield losses as a consequence of non-compliance with the quality standards required for their industrial use (Battilani *et al*., 2018; Arciuolo *et al*., 2020). The term "rotten hazelnuts" refers to hazelnuts with necrotic spots and / or internal browning, which at worst give a black nut. The incidence of the rotten defect on harvested hazelnuts generally ranges between 1% and 15%, but even a small presence of damaged fruit could be negative for the organoleptic properties (Arciuolo *et al.*, 2020; Valeriano *et al.*, 2022) A study conducted by Battilani *et al*. (2018) identified the genus *Diaporthe* as the main one in the rotten hazelnuts in the Caucasian region, as well as other fungal species (such as Alternaria spp., Cladosporium spp., Fusarium spp., and Colletotrichum spp.).
A second common defect of hazelnuts, sometimes related to rotten, is the *"cimiciato"* due to a bug attack. A study conducted by Singldinger *et al*. (2018) highlighted *"cimiciato"* infection as the main inducer of biosynthesis in the hazelnut kernel of the *cyclic diarylheptanoid asadanin*, which is identified as the main cause of bitter off-taste in hazelnuts. The PhD project aims to identify the molecules responsible for the defects mentioned above, through the development of HPLC-HRMS analytical methods, trying to provide new knowledge and tools for quality assessment.
Another activity of the PhD project concerns the qualitative and quantitative determination of the main phenolic compounds in two different botanical extracts contained in finished products of the confectionery industry. The botanical extracts of interest are "green tea and "grape seeds ". Different families of compounds have been mentioned in the literature for both types of extract, such as flavan-3-oils, flavanoids and hydroxybenzoic acids (Engelhardt *et al.*, 2010; Ma *et al.*, 2017; Tita *et al*., 2021)

2. PhD Thesis Objectives and Milestones

Regarding the activities mentioned above, the PhD thesis project can be divided into the following activities according to the Gantt diagram shown in *Table 1*:

A1) **Determination of the *"cimiciato"* defect:** target UHPLC-HRMS approach for the quantification of the main defect indicator (*cyclic diarylheptanoid asadanin* and eventual isomers) on different cultivars and hazelnut matrices (A1.2). Research possible correlations between other off-taste molecules and "*cimiciato*" defects (A1.2) and evaluate possible downgrade of the method towards faster and cheaper techniques (A1.3).

A2) **Determination of the rotten defect**: untargeted UHPLC-HRMS approach for the screening of the potential common markers responsible for the rotten defect on different hazelnut origins and cultivars (A2.1) and MS^n for qualitative analysis of the most significant ones (A2.2). Target UHPLC-HRMS approach for the quantitative determination of selected compounds (A2.3).

A3) **Determination of polyphenolic compounds on botanical extracts**: development of a multi-component target screening using UHPLC-HRMS on botanical extracts, such as "grape seeds" and "green tea" (A3.1), for research and qualitative-quantitative determination of different classes of polyphenolic compounds on finished products during their shelf-life (A3.2).

A4) **PhD thesis preparation and other activities**: writing of the PhD thesis. Writing of scientific papers, poster communication and sector conferences.

Table 1. Gantt diagram for activities of PhD project.

Activity \ Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1 Determination of *"cimiciato"* defect																								
1) Target UHPLC-HRMS on different hazelnut matrices and cultvivars																								
2) Untargeted/targeted UHPLC-HRMS: correlation between "cimiciato" and other off-taste compounds																								
3) Downgrade of technique																								
A2 Determination of rotten defect																								
1) Untargeted UHPLC-HRMS: screening of potential common "markers"																								
2) MS^n: qualitative analysis																								
3) Target UHPLC-HRMS: quantitative analysis of selected compounds																								
A3 Determination of polyphenols in botanical extracts																								
1) Multi-component target UHPLC-HRMS on botanical extracts: grape seeds and green tea																								
2) Quali-quantitave analysis of polyphenols on finished products																								
A4 PhD thesis preparation and other activities																								

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Use of sourdough for the recycling of agro-food by-products

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This PhD project is aimed at setting up a protocol for the recycling of agro-food by-products, and more specifically wasted bread, through the sustainable, low-cost and green biotechnology of fermentation. Tailored sourdough fermentation represents one of the oldest example of food biotechnology that has been studied. Recently, it was rediscovered as a very feasible option to enhance the technological, nutritional, sensory and functional features of agro-food by-products, responding to the modern vision of circular economy.

Utilizzo del lievito madre per il riciclo dei sottoprodotti agroalimentari

Questo progetto di ricerca è finalizzato al recupero dei sottoprodotti agroalimentari, e più specificamente al riutilizzo degli scarti di pane, attraverso biotecnologie low-cost, green e sostenibili. La fermentazione con lievito madre rappresenta uno dei più antichi e studiati esempi di biotecnologia alimentare. Recentemente è stato riscoperto come strumento per recuperare e/o migliorare le caratteristiche tecnologiche, nutrizionali e funzionali di questi sottoprodotti, rispondendo alla moderna visione dell'economia circolare.

1. State of the art

Nowadays it is estimated that one-third of the food produced for human consumption is wasted or lost every year from retailers, food service providers, and consumers. It's difficult to quantify individually the different categories of food wastes. However, the Food and Agriculture Organization of the United Nations (FAO) estimated that one of the most important category of food wastes is represented by leavened baked goods such as bread, which includes whole loaves discarder for visual defects and bread crust (ratio between crust and crumb = 1:1) derived from the production of sandwiches.

1.1 Processing of bread waste

Over the last decade, many researchers attempted to recycle such bread by-products to obtain, through microbial fermentations, chemicals for pharmaceutical or food industries, biofuels, enzymes, or directly using them as feed for livestock. Wasted bread has been proposed as a substrate for the cultivation of starters used in the food industry through the setting up of a protocol for obtaining a growth medium (Verni, M. et al., 2020). Nevertheless, recycling surplus bread for human food purposes, such as new bakery ingredients would be a novel and more sustainable practice. Since bread wastes contain high concentration of starch (more than 70% on dry matter) and proteins (up to 14% on dry matter), their pretreating through enzymatic hydrolysis is necessary before fermentation. This typical pretreatment method may be carry out with enzymes such as amylases, amyloglucosidases and proteases, which cause the substrate to release compounds (mainly sources of carbon and nitrogen) available for the microbial growth, making bread waste suitable as a fermentation substrate.

1.2 Sourdough fermentation

Fermentation is a biotechnology process for converting carbohydrates into alcohol or organic acids using microorganisms (yeasts and/or lactic acid bacteria), under almost anaerobic conditions. Lactic acid bacteria, which are the main players of bread fermentation, cause rapid acidification of the raw material, producing organic acids, aroma compounds, bacteriocines, exopolysaccharides (EPS), and enzymes.

Sourdough fermentation has been largely studied and recently rediscovered for the possibility of using it to reuse bread waste. The main secret for sourdough performance lies in its microbial diversity (Gobbetti, M. et al., 2019). The most abundant species of lactic acid bacteria in sourdough are *Lactiplantibacillus plantarum*, *Levilactobacillus brevis*, *Fructilactobacillus sanfranciscensis* and *Lactobacillus fermentum*, while the main species of yeasts usually identified is *Saccharomyces cerevisiae*. Generally, a sourdough contains a variable number of lactic acid bacteria and yeasts, ranging from 10^7 to 10^9 cfu/g and 10^5 to 10^7 cfu/g, respectively, with a ratio of about 100:1 or 10:1 (Arora, K. et al., 2021).

Regarding the effects of fermentation, several lactic acid bacteria strains also produce EPS that increase the viscosity of the surrounding matrix. Some EPS have already been found to enhance the textural properties of breads, and hence, can be beneficial for enhancing baking properties of recycled waste bread (Immonen, M. et al., 2020). Thus, sourdough fermentation might represents a realistic option for the valorization of bread waste, responding to the modern vision of circular economy.

2. PhD Thesis Objectives and Milestones

This PhD project can be subdivided into the following activities according to the diagram given in Table 1:
A1) **Review of the scientific literature** in the field of bread waste recycling, focusing on what has already been done and what could be done instead. Definition of a project proposal and preliminary work.
A2) **Definition of an experimental design** taking in consideration the analyses on the bread waste (A2.1), the preparation of the samples (drying, grinding, rehydration) (A2.2) and the enzymatic hydrolysis to enhance the release of compounds (source of carbon and nitrogen) available for the microbial growth (A2.3).
A3) **Production of sourdough** by mixing treated bread waste with new flour and water, and fermented with a specific starters combination (A3.1) of lactic acid bacteria and yeasts, selected according to protechnological properties. Definition of microbiological, chemical and functional properties of sourdough both at T_i (at the beginning of the fermentation) and T_f (at the end of the fermentation) (A3.2). HPLC-MS (High Performance Liquid Chromatography – Mass spectrometry) will be used to identify metabolites changes during fermentation.
A4) **Scaling-up of the process for new bread production**, evaluating the new bread composition (A4.1) and its structural features regarding the gluten network and the viscosity. Determination of the maximum percentage of treated bread waste that can be used as an ingredient for sourdough and new bread production, allowing the maximum level of recovery, while still taking in consideration the maximum quantities that avoid the deterioration of technological and sensory properties. Characterization, through sensory analysis with panel tests (A4.2), of the sensory profile of bread in terms of quantitative and qualitative descriptors that can make the products acceptable.
A5) **The setting up of the protocol for the re-use of bread waste** includes the definition of the amount of water, bread waste and flour, the type of enzymes and their degree of hydrolysis, the type of the starters and time-temperature parameters for the fermentation. Sourdough deriving from the recycling process is used as natural starter for making bread.
A6) **Writing and Editing** of the three scientific papers, oral and/or poster communications in both National and International Conferences and final PhD Thesis.

Table 1. *Activities diagram for this PhD thesis project*

Activities		1st year				2nd year				3rd year			
		I	II	III	IV	I	II	III	IV	I	II	III	IV
A1)	**Literary review and working plan**												
A2)	**Design of bread waste experiments**												
	1) Bread waste analyses												
	2) Samples preparation												
	3) Enzymatic hydrolysis (yes or no?)												
A3)	**Sourdough fermentation**												
	1) Starters screening and combination												
	2) Analyses of sourdough (T_i and T_f)												
A4)	**Final bread production and analyses**												
	1) New bread composition												
	2) Sensory analysis with panel test												
A5)	**Protocol development**												
A6)	**Papers and Thesis preparation**												

3. Expected outcomes

One of the main potential outcomes to achieve during this research project is the setting up of a biotechnological protocol for the re-use of bread wastes after by exploiting sustainable, low-cost and green fermentations processes. The optimal amount of water, the type of the enzymes (amylase, amyloglucosidase and protease) and their degree of hydrolysis, the type of starters and time - temperature for the fermentation will be determined.

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Innovative approaches for the valorization of apple by-products

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This PhD thesis research project propose valorization strategies for apple by-products (peel, flesh and seeds). Traditional applications includes direct disposal or addition to animal feed, while modern applications promote extraction of valuable compounds and applications into food products. The concept of full-component utilisation is proposed to improve economic efficiency and apple-by products utilisation ratio as much as feasible, effectively laying the groundwork for acquiring sustainable food products and increasing the value-addition to food wastes.

Approcci innovativi per la valorizzazione dei sottoprodotti della mela

Questo progetto di ricerca di tesi di dottorato propone strategie di valorizzazione dei sottoprodotti della mela (buccia, polpa e semi). Le applicazioni tradizionali includono lo smaltimento diretto o l'aggiunta ai mangimi per animali, mentre le applicazioni moderne promuovono l'estrazione di composti preziosi e le applicazioni nei prodotti alimentari. Il concetto di utilizzo completo dei componenti viene proposto per migliorare il più possibile l'efficienza economica e il rapporto di utilizzo dei prodotti derivati dalla mela, ponendo efficacemente le basi per l'acquisizione di prodotti alimentari sostenibili e aumentando il valore aggiunto ai rifiuti alimentari.

1. State-of-the-Art

Apple pomace (AP) is the leftovers from apple processing, peel and flesh together make up the major portion of AP being 95%, small proportion of seeds (2-4%), and stems (1%) (Van Dyk et al. 2013). AP is rich in bioactive compounds, carbohydrates along with small amount of proteins, minerals and vitamins. Fructose, glucose and galactose accounting for 23.6%, 22.7% and 6 to 15%, respectively are simple sugars, while cellulose, hemicellulose and lignin accounting for 127.9, 7.2 to 43.6 and 15.3 to 23.5 g/kg dry are the insoluble sugars that makes up the carbohydrates in AP (Dhillon, Kaur, and Brar 2013). It also contains a high proportion of polyphenols (31 to 51%), including cinnamate esters, dihydrochalcones, and flavonols. Additionally, it has been shown to contain a variety of natural antioxidants, including quercetin glycosides, phloridzin, and other phenolic components with significant antioxidant activity. As a result, apple pomace has a high nutritional content and is beneficial to one's health. According to many studies, apple pomace not only aids in the prevention of indigestion and hypertension, but it can also scavenge several toxic compounds in the human body, including free radicals. Seeds, which are rich in proteins (49.5%), oil (24%) and fibers (20%), are another component of AP (Bolarinwa, Orfila, and Morgan 2015). But, the physico-chemical composition of AP largely depends on the apple variety involved and the type of juice extraction method used, mainly how many times the fruits are compressed. Despite the fact that composition of AP is influenced by several factors, these investigations revealed the abundance of useful components recovered from AP. It was estimated that approximately 5-7 million tons of AP is needed to be treated every year. As a result, developing a proper utilization method for this substantial and valuable material should be prioritised.

Traditional methods of AP usage mostly concentrate on either direct disposal, utilization in animal feed or elementary fermentation conversion, depending on whether or not fermentation included separation and purification, resulting in environmental pollution and severe effects on local populations over the long natural biodegradation process. The reprocessing of AP for future use is a promising strategy that is linked to circular economy, but it is important to consider whether drying is required, as drying AP is a limited procedure in terms of economic cost and energy consumption, as well as environmental impact (Zhang et al. 2021). Extraction and conversion to high-value products are more practical approaches to realising the industrial application of AP in order to improve financial viability and reduce environmental pollution. Taking into account the composition characteristics of the AP as well as the present utilization method, a full-component utilization mode is needed to tackle the proposed limitations, such as the disposal of secondary solid wastes generated during high-value product extraction (Zhang et al. 2021). Referring to several research studies, the full-component utilization method consisted primarily of three components: pre-treatment, liquid utilization, and solid utilization.

Pre-treatment is a necessary aspect to almost all AP utilization modes, and it plays a major role in achieving higher product yield and conversion efficiency. As a result, the first module of the full-component utilization mode is pre-treatment. The second module, liquid utilization, focuses primarily on the separation and purification procedure. Most soluble substances, such as pectin, polyphenol, triterpenes, and some degradation products from macromolecules, are retrieved in liquid medium based on pre-treatment. As an example, in the extraction of polyphenol and pectin, the polar and nonpolar substances are separated using the alcohol precipitation process. Pectin would precipitate at high alcohol concentrations, but polyphenol has good solubility in alcohol solutions and could be separated further by resin adsorption or another method. Following pre-treatment and high-value

extraction, the solid residue is primarily composed of cellulose, hemicellulose, and lignin, and is appropriate to be used as a source of carbon for fermentation following enzymatic hydrolysis. Following that, varied strains and fermentation types can be used to produce enzymes, organic acid, pigment, and biofuels. As a result, more applications must be investigated to address the problem of excessive AP waste in an environmentally friendly manner, as well as to improve the quality and health aspects of a variety of food products through the incorporation of valuable functional ingredient.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) **Apple seed oil as a source of antioxidants for the development of stable mayonnaise.** Isothermal calorimetry and oximetry analysis of mayonnaise prepared using apple seed oil and 4 other oils (sunflower, corn, extra virgin olive and grapeseed oil) to determine oxidation kinetic and thermodynamic parameters (A1.1), chemical composition, antioxidant activity and phenolic content of oils (A1.2) and correlation of oxidation kinetic and thermodynamic parameters with chemical compositions, antioxidant and phenolic content of oils (A1.3)

A2) **Effects of processing technologies and storage on the loss of functional components in apple by-products.** Antioxidant activity, total phenolic, flavonoid, anthocyanin content and polyphenolic profile by HPLC-Coularray-UV and MS/MS detector of free and bound fractions from peel, seeds and flesh of 5 apple varieties will be studied (A2.1) and physico-chemical properties (A2.2)

A3) **Innovative post-processing approaches to effectively convert apple by-products into valuable products.** Based on the findings of above objectives suitable processing approach (A3.1) and value added products will be prepared using apple by-products (A3.2)

A4) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

Table 1 Gantt diagram for this PhD thesis project.

Activities \ Months		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	***Oxidation kinetics of apple seed oil mayonnaise***																								
	1) Isothermal calorimetry and oximetry analysis																								
	2) Chemical, antioxidant and phenolic content of oils																								
	3) Correlation analysis																								
A2)	***Effect of processing and storage***																								
	1) Bioactive compounds analysis																								
	2) Physico-chemical properties																								
A3)	***Processing and value addition***																								
	1) Processing approaches																								
	2) Value added products																								
A4)	***Thesis and Paper Preparation***																								

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Development and preclinical testing of a functional beverage for boosting the immune system in the elderly

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This PhD thesis research projects aims to design fermented and functional foods/beverages capable of modulating human immune function via the gut microbiome. The experimental activities will focus on characterizing the bioactive compounds profile of these novel fermented ingredients and measuring their impact on the gut microbiota of elderly subjects and human immune cells in vitro and ex vivo.

Sviluppo e test preclinici di una bevanda funzionale per potenziare il sistema immunitario negli anziani

Questo progetto di ricerca di tesi di dottorato mira a progettare cibi / bevande fermentati e funzionali di modulazione della funzione immunitaria umana attraverso il microbioma intestinale. Le attività sperimentali si concentreranno sulla caratterizzazione del profilo dei composti bioattivi di questi nuovi ingredienti proteici fermentati e sulla misurazione del loro impatto sul microbiota intestinale di soggetti anziani e cellule immunitarie umane in vitro ed ex vivo.

1. State of Art

The SARS-Cov-2 pandemic has exposed our vulnerability to new illnesses and novel viruses that attack our immune systems. COVID-19 is more susceptible against older people. Aging is associated with significant changes in that physiology, which reduce the ability to fight infection or, once infected, to develop effective immune responses to limit symptoms and disease progression. Cellular aging can induce metabolic imbalance by increasing the synthesis of inflammatory products. Mucous secretion alterations, reduced motility, and notably gut microbiota changes have a negative impact on the intestinal physiology. Dietary changes and protein deficiency eventually result in a loss of lean muscle mass and an increase in fat deposits, which contribute to inflammation, systemic and metabolic dysregulation, like obesity. It is becoming increasingly evident that metabolic health is closely intertwined to immune function, particularly the regulation of inflammation (Santos., et al., 2021). The malfunction and inflammation associated with age, determine the risk of chronic diseases, frailty progression and the risk of serious disease in the case of viral infection (De Oliveira., et al., 2021). It is worth noting that dietary intervention can reduce the risk of both metabolic and associated inflammatory diseases (Rizzetto, et al., 2018). In this regard, older people who followed a Mediterranean-style diet, rich in polyphenols and dietary fiber, performed better physically and mentally. This demonstrates the importance of the human gut microbiome in transforming complex dietary macromolecules into most biologically available and active nutrients (e.g., short-chain fatty acids, small phenolic acids, derivatives of amino acids), which subsequently help to regulate metabolism and both intestinal and systemic immune function (Meir et al., 2021). The role of lactic acid fermentation is prominent also as a powerful tool for improving the nutritional profile of human diet by releasing nutrients and boosting the complex bioactive compounds and vitamin content. According to several clinical trials (De Oliveira., et al., 2021), 16 studies on probiotics, 88 on vitamin D and 100 on antioxidants against COVID-19 are currently under investigation or completed. As a result, the formulation of a multi-bioactive nutritional support based on fermentation aiming at immune modulation of the intestinal microbiome for elderly, represents a great promise in generating natural formulations to improve and to reinforce the immunological state.

2. PhD thesis objectives and milestones

Within the overall objective mentioned above, this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in table 1:

A1. Literature review and experimental design

A2. Selection of functional ingredients

Depending on the class of bioactive compounds, protein, dietary fibers and beneficial effects on health, targeted matrices will be selected (e.g., legumes, nuts, moringa, whey and fruit juices).

A3. Set up of a technological process for making functional yogurt-like beverage

Selected prototypes will be pre-gelatinized then fermented with lactic acid bacteria (e.g., *Levilactobacillus brevis* and *Lactiplantibacillus plantarum*) and other commercial starters.

A4. Nutritional and functional characterization of functional yogurt-like beverage

Multi-omics platform will be used to characterize the novel fermented beverage prototypes in terms of amino

acids, bioactive peptides, vitamins, dietary fiber, fatty acids and polyphenols profiles. The *in-vitro* protein digestibility (IVPD) will be determined according to the method described by Akeson and Stahmann (1964).

A5. Effect of functional yogurt-like beverage on elderly gut microbiota

The impact of selected prototypes on the gut microbiota of elderly peoples will be measured using *in vitro* models of the human gut microbiome. Microbiome, metabolome, and impact on microbiome bile acid signaling will be investigated.

A6. Immune-modulatory ability of functional yogurt-like beverage

The immune-modulatory activity of supernatants collected from gut generated after ingesting yogurt-like beverage prototypes will be measured using human immune cells *in vitro* and *ex vivo*.

A7. Writing and editing of PhD thesis, scientific papers and oral and poster communications

Table 1. *Gantt diagram for this PhD thesis project*

	Activity / *Months*	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**
A1)	*Literature review, Experimental design*																		
A2)	*Selection of functional ingredients*																		
A3)	*Set up of a technological process for making functional yogurt-like beverage*																		
A4)	*Nutritional and functional characterization of functional yogurt-like beverage*																		
A5)	*Effect of functional yogurt-like beverage on elderly gut microbiota*																		
A6)	*Immune-modulatory ability of functional yogurt-like beverage*																		
A7)	*Thesis and Paper Preparation*																		

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Innovative formulation and process approaches for new plant-based ingredients with enhanced techno-functionalities

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This PhD thesis research project is aimed to develop different process approaches and structuring methodologies to obtain innovative formulations. Different innovative technologies will be explored for the development of new plant-based ingredients with enhanced techno-functional properties in order to obtain food products with higher qualitative and sensorial properties.

Approcci innovativi di formulazione e processo per lo sviluppo di nuovi ingredienti di origine vegetale con funzionalità tecnologiche potenziate

Questo progetto di ricerca di tesi di dottorato mira a sviluppare diversi approcci di processo e metodologie di strutturazione per ottenere formulazioni innovative. Saranno esplorate diverse tecnologie innovative per lo sviluppo di nuovi ingredienti di origine vegetale con proprietà tecno-funzionali al fine di ottenere prodotti alimentari con proprietà qualitative e sensoriali più elevate.

1. State-of-the-Art

Currently, the increased awareness of the modern consumers about the impact of food on human health and on the sustainability of their production process has prompted them to consider which components are used in the food products they eat in everyday life thus, a new trend has emerged, often summarized under the umbrella of the so-called “clean label” which refers to products characterized by easy-to-understand ingredient lists, free of chemicals additives and with limited processing.
The food industry, in response to the growing consumer demand for these products, has started to supply food products by decreasing, eliminating or replacing the use of synthetic additives in their formulations (Asioli et al., 2017). As a result, numerous research and technological applications have switched to focus on natural counterparts, such as bioactive compounds extracted from plants, herbs or industrial food by-products. In this frame, hop cones, inflorescences of the female plant of *Humulus lupulus L.*, besides their main use as flavouring, bittering and stabilizing ingredients for the beer industry, have attracted the attention of researchers and food industries for the high content in secondary metabolites with biological activity, i.e. resins, essential oils, and polyphenols (Moir et al., 2018). For the extraction of bioactive compounds, a tailored combination of extraction technology and solvent for each type of plant material and target compound should be employed. Additionally, thanks to recent technical advantages, more efficient and greener extraction techniques have been developed. An example in hops has been recently reported by (Santarelli et al., 2022) who, comparing conventional and innovative extraction methods for the production of food-grade hop extracts, demonstrated that the use of low-power ultrasound successfully extracted bioactive compounds with shorter time and energy consumption than conventional extraction method underling the enormous potential of study others innovative extraction technologies. However, the instability of these compounds during typical food processing conditions, such as exposure to high temperatures, pH changes, presence of light and oxygen have stimulated the use of different encapsulation techniques which protect, improve or maintain the properties of compounds increasing their stability and facilitating their prolonged release (Mandaij et al., 2022). This in turn has allowed their application in different food products therefore in several studies, plant extract compounds have been encapsulated to be applied as functional ingredients within the food matrix, and also for the preparation of food coatings, edible films, and active packaging materials. To date, the use of hops, in powder or as an infusion in different types of food, has shown positive effects such as increasing the stability of colour and lipids in lamb patties (Villalobos-Delgado et al., 2015) and antimicrobial activity against Gram-positive spoilage microorganisms in vacuum packed refrigerated chicken breast (Nieto et al., 2020). However, there is a lack of information on the use of hops as encapsulated form and the possible effects of its use in food formulations hence the desire to investigate different technologies to first produce and then use encapsulated food-grade hop extracts to assess their possible techno-functional properties in food products.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in *Table 1*:

Table 1. Gantt diagram for this PhD thesis project

		II YEAR												III YEAR											
		MONTHS																							
	ACTIVITIES	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
	Bibliographic research																								
A1	**A1:** evaluation of innovative extraction technologies for the production of food-grade hop extracts																								
	A1.1: application of ultrasonic bath extraction																								
	A1.2: application of ultrasonic probe extraction																								
	A1.3: application of rapid solid-liquid dynamic extraction (RSLDE)																								
	A1.4: application of dynamic high-pressure homogenizer extraction																								
	A1.5: application of Pulsed Electric Field (PEF) assisted extraction																								
A2	**A2:** study of technological functionality and sensory properties of hop extracts																								
	A2.1: chemical characterization and antioxidant activity evaluation																								
	A2.2:emulsifying and stabilizing properties evaluation																								
	A2.3:antimicrobial activity evaluation																								
	A2.4: volatile organic compounds profile																								
	A2.5: sensory analysis																								
A3	**A3:** extracts stabilization by encapsulation technologies																								
	A3.1: encapsulation by freeze-drying																								
	A3.2: encapsulation by spry-drying																								
	A3.3: physiochemical characterization of encapsulated powders and storage stability test																								
A4	Writing and Editing of the PhD thesis, scientific papers and oral and/or poster communications																								

A1) Evaluation of innovative extraction technologies for the production of food-grade hop extracts: Application of ultrasonic-assisted extraction: optimization of extraction using an ultrasonic bath (A1.1) and an ultrasonic probe (A1.2). Application of pressure-based extraction technologies: optimization of extraction using a rapid solid-liquid dynamic extraction (RSLDE) (A1.3) and dynamic high-pressure homogenizer (1.4). Pulsed Electric Field (PEF) assisted extraction application (1.5).
A2) Study of technological functionality and sensory properties of hop extracts. Chemical characterization and antioxidant activity (AoA) evaluation (A2.1). Extracts will be characterized by spectrophotometric analysis for the total phenolic content (TPC), total flavonoids content (TFC), total carotenoids content (TCC), chlorophyll α (CHLα) and chlorophyll β (CHLβ) content, Ferric Reducing Antioxidant Power (FRAP) and ABTS radical scavenging activity. Chromatographic methods will be used to evaluate the content of single and total polyphenols, α and β acids. Emulsifying and stabilizing properties will be evaluated (A2.2) studying surface and interfacial properties. Antimicrobial activity will be assessed by *in-vitro* tests (A2.3). Volatile organic compounds profile (A2.4) will be obtained by GC-MS analysis and sensory analysis (A2.5).
A3) Extracts stabilization by encapsulation technologies. Different microencapsulation techniques such as freeze-drying (A3.1) and spray-drying (A3.2) will be tested. For both techniques, different coating materials (i.e. maltodextrins, cyclodextrins, Arabic gum) and their different blends will be assessed. Physico-chemical characterization and storage stability tests (A3.3) of the resulting powders will be performed analysing the following parameters: water activity, moisture content, solubility, colour, water sorption behaviour, glass transition temperature, TPC, antioxidant capacity (FRAP and ABTS assay), load yield, encapsulation efficiency, α- and β-acids and xanthohumol content by HPLC-DAD, volatile organic compounds profile by GC-MS.
A4) Writing and Editing of the PhD thesis, scientific papers and oral and/or poster communications

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Use of chitin and its derivatives obtained from sustainable sources as an alternative to the use of chemical additives in the food production

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The aim of the project is to study and improve the knowledge about the use of chitosan, an innovative and natural polymer obtained from the deacetylation of chitin, as antimicrobial compound for food applications.
In particular, it will be explored the use of insects as sustainable source of chitosan and the potential use of this compound as alternative to the sulphur dioxide (SO_2), a chemical additive used for the microbiological control of wines, during all the steps of winemaking process, in order to meet the market demands for wines with a reduced content of sulphites.

Utilizzo di chitina e suoi derivati ottenuti da fonti sostenibili in alternativa all'utilizzo di additivi chimici nella produzione alimentare.

L'obiettivo del progetto è studiare e migliorare le conoscenze sull'uso del chitosano, un polimero innovativo e naturale ottenuto dalla deacetilazione della chitina, come composto antimicrobico da impiegare nel settore alimentare. In particolare, sarà approfondito l'utilizzo di insetti come fonte sostenibile per l'estrazione di chitosano e il potenziale impiego di questo composto in alternativa all'anidride solforosa, additivo chimico utilizzato per il controllo microbiologico dei vini durante tutte le fasi del processo di vinificazione, al fine di soddisfare le richieste del mercato di vini a ridotto contenuto di solfiti.

1. State-of-the-Art

The microbiological control of the winemaking process has been achieved for many years using chemical additives, among which $SO_2$is the most widely used. In addition to the antimicrobial action, this compound has other advantages, such as the control of oxidation processes, both of chemical and enzymatic nature, and the solubilizing action, which favors the extraction of pigments from the grape skins.
Nowadays the market requires the production of wines with a low sulphite content, in consequence of its effects on health problems induced in sensitive individuals (Lester, 1995; Vally and Thompson, 2001) and on sensory defects in the finished product, due to the production of hydrogen sulphide and mercaptans (Li et al., 2008; Guerrero and Cantos-Villar, 2015). The European Union (EU) has established with the EU regulations No. 606/2009 and 479/2008 the maximum doses of SO_2 that can be found in the finished wine and to be indicated on the bottle label with the words "contains sulphites".
Especially due to the attention paid to health issues, alternative methods to the use of SO_2 are continuously proposed and, among the various studied and authorized compounds, considerable importance has been found in chitosan. This is a biopolymer obtained from the deacetylation of chitin, the most abundant polysaccharide in nature after cellulose (Rinaudo 2006).
The International Organisation of Vine and Wine (OIV) has authorized the use of chitosan at different doses for various purposes (oiv-eno-338a-2009), as reported in Table 1.

Table 1 Main applications of Chitosan at different doses of use.

Dose of use	Purpose
100 g/hl	Reduce the concentration of heavy metals (Fe, Pb, Cd, Cu) and prevent hazing in wine
500 g/hl	Reduce any contamination by ochratoxin A
10 g/hl	Reduce the concentration of unwanted microorganisms, especially *Brettanomyces spp.*

In particular, the OIV has authorized the use of chitosan derived from the fungus *Aspergillus niger*. However, other sources of chitin, and consequently of chitosan, are available, such as crustaceans and insects. Unlike crustaceans and fungi, the insects, such as the Diptera *Hermetia illucens*, represent a sustainable source for chitin extraction. In fact, they are bioconverters capable of feeding on organic substrates (even waste), converting them into noble proteins, lipids and chitin (Scala et al., 2020). Furthermore, unlike crustaceans, they are not subject to seasonality and can be easily reared, as the conditions favorable to their development and survival can be easily reproduced (van Huis, 2013). The aim of the present PhD project will be the evaluation of chitosan effect on undesirable microorganisms involved in the different steps of the winemaking process and the role of this compound on the final quality of the wine. Furthermore, the effect of chitosan extracted by insects will be evaluated, in order to find a sustainable source for obtaining an antimicrobial compound alternative to SO_2.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram in Table 2:

A1) **Screening of a wide number of non-*Saccharomyces* strains belonging to the main wine yeast species for chitosan resistance.** In this step, the antimicrobial effect of chitosan will be evaluated, by testing non-*Saccharomyces* strains in synthetic media added with increasing doses of commercial chitosan. As control, it will be used SO_2, added in the concentrations usually added in the winemaking.

A2) **Chitin extraction from insects.** Chitin will be extracted from different life stages of *Hermetia illucens*, using chemical and biological extraction methods. The chitosan, obtained from the deacetylation of the chitin, will be used for the subsequent fermentation tests.

A3) **Insect feeding on pomace**. In order to increase the sustainability of chitosan production in the optic of zero waste circular economy system, the bioconverter insect *H. illucens* will be reared on pomace.

A4) **Evaluation of effect of chitosan addition in laboratory-scale fermentations.** In this step, chitosan extracted by different origins will be tested, in order to evaluate the effect of chitosan chemical structure on its activity. Indigenous yeasts, selected in the previous steps, will be tested in fermentation trials at laboratory scale, added with chitosan and SO_2 (used as control). The evaluation of microbial viable count and the final wine characteristics, such as content of aromatic compounds, polyphenols, antioxidant power, will be useful to evaluate the suitability of chitosan as an effective alternative to SO_2.

A5) **Fermentation trials at pilot scale.** The chitosan showing the best performance during laboratory scale fermentation will be evaluated in fermentation at pilot scale performed in the cellar involved in the industrial doctorate project.

A6) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

Table 2 Gantt diagram for this PhD thesis project.

Activity / Months		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	***Screening of a wide number of non-Saccharomyces yeast species for chitosan resistance***																								
	1) SO_2 resistance screening																								
	2) Chitosan resistance screening																								
A2)	***Chitin extraction from insects***																								
	1) Chemical extraction																								
	2) Biological extraction																								
A3)	***Insect feeding on pomace***																								
A4)	***Evaluation of effect of chitosan addition in laboratory-scale fermentations.***																								
	1) Microbial viable count																								
	2) Evaluation of final wine characteristics																								
A5)	***Fermentation trials at pilot scale***																								
A6)	***Thesis and Paper Preparation***																								

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Microbiomes in food systems

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This Ph.D. research project aims at investigating the microbial communities found in different fermented food matrices and to select potential starter cultures, starting from naturally fermented table olives. The experimental plan consists of integrating traditional culture-based techniques with a more advanced multi-omics approach, including metagenomics and metataxonomics. The use of MALDI-ToF MS Biotyper will also contribute to the identification of our isolates and will implement the current databases. The combination of these approaches will provide a complete overview of the complexity of the microbial populations that colonize fermented food matrices.

Il microbioma nei sistemi alimentari

Questo progetto di tesi di dottorato mira a far luce sulle comunità microbiche presenti in diverse matrici alimentari fermentate e di selezionare potenziali colture starter, a partire da olive da tavola fermentate al naturale. Il piano sperimentale consiste nell'integrare le tradizionali tecniche cultura-dipendenti con un approccio multiomico più avanzato, comprensivo di metagenomica e metatassonomica. L'utilizzo del MALDI-ToF Biotyper contribuirà inoltre all'identificazione dei nostri isolati e implementerà i database esistenti. La combinazione di questi approcci fornirà quindi una panoramica completa sulla complessità delle popolazioni microbiche che colonizzano le matrici alimentari fermentate.

1. State of the Art

Food fermentations mainly rely on mixed cultures of microorganisms, which may include bacteria, yeasts, and molds. Given the complexity of the microbial populations and their interactions in fermented foods, the combination of culture-based and culture-independent techniques is recommended when studying their microbial ecology (Sieuwerts et al., 2008). Culture-dependent strategies consist of traditional microbiological methods that lead to isolation and subsequent molecular analysis of autochthonous isolates. On the other hand, culture-independent techniques (e.g., next generation sequencing) rely on direct extraction of nucleic acids from a food matrix. The latter approach provides enormous insights into the microbial structure and functionality by determining the presence of viable but non culturable populations, thus allowing a more accurate definition of the drivers involved in the fermentation process (Botta & Cocolin, 2012). Although molecular approaches have been widely used for the identification of microorganisms (e.g., 16S rRNA and ITS sequencing for bacteria and yeasts, respectively), an emerging biotyping technique has proved to be easy-to-use and efficient for taxonomic purposes, that is to say matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-ToF MS). The basic principle relies on the comparison of the obtained spectra from an unknown isolate with an already available database. MALDI-ToF MS is routinely applied for the identification of clinical microorganisms, but insufficient data is available for the differentiation of food-related microorganisms. For this reason, an in-house database needs to be created and validated prior to the experiment (Agustini et al., 2014; Quintilla et al., 2018).
Table olives are produced from the raw drupe fruits deriving from varieties of the cultivated olive tree (*Olea europaea* L.). However, the raw fruit is inedible and highly bitter due to the presence of oleuropein, which needs to be degraded. Treatments to reduce the fruits bitterness involve brining, acidification, enzymatic hydrolysis, and spontaneous fermentation. Natural fermented olives are obtained by directly placing the fruits in brine with a salt concentration of about 6%-10% (w/v) without pre-treatments. Oleuropein is hydrolyzed by ß-glucosidases and esterases of indigenous microorganisms and the fermentation process can last up to 12 months. This spontaneous process mainly involves lactic acid bacteria (LAB) (e.g., *Lactiplantibacillus plantarum*, *Lactiplantibacillus pentosus*) and yeasts (e.g., *Saccharomyces cerevisiae*, *Candida boidinii*). LAB are responsible for oleuropein degradation thanks to their enzymatic activities and cause the acidification of the final product, providing microbial stability and elongating the shelf-life of the olives. Yeasts are involved in the production of volatile compounds and metabolites that increase the overall quality and preserve their sensory features (Perpetuini et al., 2020). Despite being one of the oldest fermented vegetables, there are still several challenges in processing and commercialization of natural table olives and their fermentation is not quite predictable (Tofalo et al., 2013). For these reasons, MALDI-ToF MS typing coupled with molecular methods will be useful to provide a reliable description of the olives microbial ecology and will help guide the selection of starter cultures.

2. Aims and Key Milestones

Within the overall objectives mentioned above this PhD thesis project can be divided into the following activities according to the Gantt diagram given in Table 1.

A1 – Literature review and experimental design

A2 – Sampling: culture-based techniques

Spontaneous fermented table olives deriving from a local company were analyzed at different fermentation stages, at which both pH and microbial counts were monitored. So far, a total of 582 yeasts isolates were collected from brine and olives.

A3 – Molecular identification (culture-based and culture-independent)

Isolates will be identified by means of DNA extraction followed by rep-PCR/RAPD-PCR for a preliminary screening and clustering of fingerprinting profiles. This step will be followed by the analysis of obtained fingerprints through BioNumerics software and subsequent sequencing of representative isolates from each group. After direct DNA extraction from olives and brines, amplicon sequencing (metataxonomics) and shotgun metagenomics on total DNA will be performed.

A4 – MALDI-ToF Biotyper: creation of in-house database and microbial identification

An in-house mass spectra database will be generated and used as reference for our unknown isolates (flexControl, flexAnalysis software for calibration and data processing). The database will consist of multiple food-related yeast culture collections previously identified by means of molecular methods (ITS and/or D1/D2 rRNA regions). Afterwards, our olives- and brines-related isolates will be extracted and analyzed by the MALDI Biotyper software 3.0 (Bruker).

A5 – Selection of potential starter cultures

Starter combination will be outlined using the generated omics data. In a second step, they will be inoculated in a synthetic model media at lab-scale level and screened for their protechnological and functional properties (e.g., survival and growth under simulated gastrointestinal conditions, antimicrobial activity...)

A6 – Paper and final thesis

Table 1. *Gantt diagram for this PhD thesis project.*

	1st year				2nd year				3rd year			
Activies Months	I	II	III	IV	I	II	III	IV	I	II	III	IV
A1 – Literary review; Experimental design												
A2 – Sampling: culture-based techniques												
A3 – Molecular identification (culture-based and culture-independent)												
A4 – MALDI-ToF Biotyper: creation on in house database and microbial identification												
A5 – Selection of potential starter cultures												
A6 – Paper and final thesis												

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Encouraging the consumption of alternative protein sources through the design of novel fermented foods

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My Ph.D. research project aims to design novel fermented food enriched with alternative protein sources generated from microalgae, cyanobacteria, and legumes flour. The experimental activities will focus on investigating the efficiency of combining lactic acid bacteria, yeasts, acetic bacteria as stable consortia for fermenting mixed substrates consisting of selected plant food matrices, agro-industrial wastes, microalgae, cyanobacteria biomass and legumes flour, in order to generate new fermented products enriched with single-cell protein sources.

Incoraggiare il consumo di fonti proteiche alternative attraverso la progettazione di nuovi alimenti fermentati

Il seguente progetto di ricerca di dottorato mira a progettare nuovi alimenti fermentati arricchiti con fonti proteiche alternative generate da microalghe, cianobatteri e farine di leguminose. Le attività sperimentali si concentreranno sullo studio dell'efficienza della combinazione di batteri lattici, lieviti, batteri acetici come consorzi stabili per la fermentazione di substrati misti costituiti da matrici alimentari vegetali selezionate, scarti agroindustriali, le biomasse di microalghe e cianobatteri e farine di leguminose, al fine di generare nuovi prodotti fermentati arricchiti con fonti proteiche monocellulari.

1. State of the Art

Novel food is an emerging trend in food and nutrition research and may represent an innovative food category derived from different sources (plant and animal based-products) that can meet individuals interest. Novel foods are defined as foods that do not have a history of consumption or that are developed by a unique, innovative method of production that meet safety and nutritional requirements. Consumer preference toward novel foods depends on the functional characteristic associated with them (Frewer et al., 2003). Furthermore, efforts have been made to find and exploit different sources of protein, due to the increase in consumer demand for higher nutritional and functional foods and changing in dietary habits. Indeed, as recently reported by Khan et al., (2022), fruits, vegetables and their by-products, represent good sources of bioactive compounds, carbon and nitrogen, whereas the protein content is limited.

On the other hand, the application of microalgae and cyanobacteria into food is a trend in the last years. Bioactive compounds, such as protein, polyunsaturated fatty acids, carotenoids, vitamins and minerals, found in commercial form of microalgal and cyanobacterial biomass (e.g., powder or liquid) might be a promising strategy to design novel food with novel features, in terms of nutritional, sensory and functional attributes (Beheshtipour et al., 2013). Besides, microalgae and cyanobacteria may generate new protein sources, that are qualified as single-cell protein sources (Sharif et al., 2021). Single-cell protein is the term for microbial protein biomass produced by the fermentation process using different carbon and nitrogen sources available in food matrices or agro-industrial wastes. Nevertheless, research on microalgae and cyanobacteria sector is limited and their use as food substitute is not thoroughly investigated, mainly due to the difficulties in cultivation processing (Caporgno & Mathys, 2018). Therefore, considering even other products rich in protein may increase the possibility to answer the protein request. Legumes represent a suitable alternative to plant-based food and are rich protein sources. Unfortunately, their nutritional values are reduced due to the presence of antinutritional factors, poor protein digestibility and low level of sulfur-containing essential amino acids. In this regard, fermentation may enrich food matrices by increasing the bioavailability and bio-accessibility of bioactive compounds, promoting the growth of beneficial microbiota (e.g., probiotics), and delivering microbial metabolites (e.g., post-biotics) (Anaemene & Fadupin, 2022). Microorganisms such as lactic acid bacteria, acetic acid bacteria, yeasts and their forms of symbiotic cultures, such as kombucha SCOBY, and kefir grains, are chiefly responsible for most food fermentations. Recently, microbe-driven processes are proposed as efficient and cost-effective techniques to design novel food.

2. PhD Thesis Objectives

Within the overall objectives mentioned above this PhD thesis project can be divided into the following activities according to the Gantt diagram given in Table 1.

A1 – Literary review and experimental design

A2 – Selection of potential plant material and potential substrates

Selection of the plant matrices will include fruits, vegetables and their by-products, depending on the bioactive compounds content and beneficial effects on health. Whereas, the selection of potential substrate will include *Chlorella vulgaris* and *Arthospira plantensis* species, legumes flours, concentrates and isolates.

A3 – Screening and selection of LAB and yeasts species and symbiotic culture as a starter

Lactic acid bacteria, yeasts, kombucha SCOBY and kefir grains will be taken for our collection and will be screened for pro-technological and functional properties and biomass production. Each strain will be inoculated in a formulated synthetic media, in which environmental adaptation, ,acidification, RNA production, the metabolic response and the capacity to increase protein content will be characterized.

A4 – Fermentation set up

The selected matrices will be subjected to a pre-treatment before fermentation including thermal treatment and enzymatic digestion. Whereas the selected fruit and/or vegetable by-products will be transformed into a powder before the fermentation. Afterward, powdered selected microalgae and cyanobacteria at a concentration of 1.5% (w/v) or legumes flours, or concentrate and protein isolates will be added to the product, and inoculated with selected strain of LAB and/or yeast and with selected symbiotic culture. Trials will be carried out before setting the final incubation time and temperature.

A5 – Characterization of bioprocessed products

New bioprocessed products will be subjected to further microbiological and chemical characterization. Sensory features of the novel product will be assayed and several potential prototypes will be proposed. The presence of endogenous or microbial bioactive compounds and in particular protein sources will be searched. Digestibility of the protein will be investigated. Bioactivity will be further assayed through in vitro experiments and ex vivo analysis on intestinal Caco-2/TC7 cells by the Department of Food Safety and Veterinary Public Health (Istituto Superiore della Sanità, Roma, Italy).

A6 – Paper and final thesis

Table 1. *Gantt diagram for this PhD thesis project.*

	1st year				2nd year				3rd year			
Activies Months	I	II	III	IV	I	II	III	IV	I	II	III	IV
A1 – Literary review; Experimental design												
A2 – Selection of potential plant material and substrate												
A3 – Screening and selection of LAB and yeasts species and symbiotic culture as a starter												
A4 – Fermentation set up												
A5 – Characterization of bioprocessed products												
A6 – Paper and final thesis												

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Green sensors and smart services for the optimization of agri-food supply chains with a view to industry 4.0: greater sustainability of production, business competitiveness and reduction of food waste

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This PhD thesis research project is aimed at building and optimizing a portable, user-friendly, and low-cost hyperspectral sensor that will be applied on different fresh-cut products under laboratory conditions. A chemometric approach will be used to evaluate some qualitative parameters. Finally, possible applications (in-line or on-line) of this sensor in the fresh-cut product chain will be evaluated with an LCA (Life Cycle Assessment) approach to assess the sustainability of the device application.

Sensori green e servizi intelligenti per l'ottimizzazione delle filiere agroalimentari in un'ottica di industria 4.0: maggiore sostenibilità della produzione, competitività delle imprese e riduzione degli sprechi alimentari

Questo progetto di ricerca di tesi di dottorato ha lo scopo di costruire e ottimizzare un sensore iperspettrale portatile, facile da usare e a basso costo che verrà applicato su diversi prodotti di IV gamma, in condizioni di laboratorio. Verrà utilizzato un approccio chemiometrico per valutare alcuni parametri qualitativi. Infine, le possibili applicazioni (in-line o on-line) di questo sensore nella filiera della IV gamma verranno valutate con un approccio LCA (Life Cycle Assessment) per valutare la sostenibilità dell'applicazione del dispositivo.

1. State-of-the-art

The market of fresh-cut products is growing because these foods represent a new opportunity for consumers to buy high-quality, healthful, and ready to consume fresh produce. In fact, these products are a source of carbohydrates, vitamins, natural fibers, and phenolic and antioxidant compounds. On the other hand, they represent a good opportunity for the industry to expand market sales by a high added value product. Fresh-cut products are prepared and conditioned in such a way to provide a whole series of services for the consumer (i.e., cleaning, hulling, washing, cutting into units or sub-units ready to use), while maintaining the product freshness characteristics (Colelli & Elia, 2009). However, these products do not maintain their nutritional content, look, or flavour over time since they go through metabolic processes throughout the pre- and post-harvest periods (Lu et al., 2020). As a result, both the agricultural and food industries must build precise, quick, and objective inspection systems along the product chain to assure consumer satisfaction, and food quality and safety. Currently, human visual inspections and analytical chemical analyses are the principal methods used for the evaluation of quality of fresh-cut products, but they are destructive, time-consuming, expensive, they can investigate a small number of samples, and sometimes they require sample preparation (Lu et al., 2017, 2020; Wu & Sun, 2013). In the last two decades, researchers are looking for the so called "green technologies", which are non-destructive, rapid and chemical-free detection methods to evaluate automatically quality and safety properties of foods. Among the available technologies, Visible-Near InfraRed (Vis/NIR) spectroscopy and hyperspectral imaging (HSI) are valid tools for monitoring qualitative parameters (Özdoğan et al., 2021). In Vis/NIR spectroscopy, a light source illuminates the sample, and the reflected or transmitted radiation is measured and translated into a spectrum. While the radiation penetrates the product, its spectral characteristics change through wavelength dependent scattering and absorption processes. This change depends on the chemical composition of the product, as well as on its light scattering properties which are related to the microstructure (Nicolaï et al., 2007). This technique is usually used to investigate the chemical composition of food; however, it is a point measurement method that cannot analyse the spatial properties of the sample (Lu et al., 2017). To remedy this, we can use hyperspectral imaging (HSI). HSI technique is commonly used in remote sensing and integrates both imaging and spectroscopy to obtain spatial and spectral information, at the same time. Furthermore, it is a non-destructive and rapid method that can be used for the evaluation of physical and chemical properties of the target. A hyperspectral imaging system consists in a light excitation source, a wavelength dispersion device, and an area detector. Light sources are a very important component because they have the function of illuminating the sample, while the detector will acquire the light after its interaction with the sample. Hyperspectral imaging produces three-dimensional data cube, generally called "hypercube", which is composed of "vector pixels" containing spectral information (of λ wavelengths) and two-dimensional spatial data (of x rows and y columns) (Özdoğan et al., 2021; Wu & Sun, 2013). Hypercubes are generally obtained in three sensing modes, which are reflectance, transmittance, and interactance. The reflectance mode is principally applied for the evaluation of external quality properties, such as size, surface texture, and

physical defects, while the transmittance mode is used for evaluating chemical compositions or internal defects. Finally, a chemometric approach, including data pre-processing methods and multivariate statistical analysis (e.g., PCA – Principal Component Analysis; PLSR – Partial Least Squares Regression) are applied on the data collected to extract the required information and create data modelling for classification or regression. This PhD project consists in the application of hyperspectral imaging on fresh-cut products, in order to evaluate and monitor some qualitative parameters. The main goal of this project consists in the construction and optimization of a portable, easy-to-use, and cheap hyperspectral sensor, relying on the work of Salazar-Vazquez and Mendez-Vazquez (2020). In fact, this kind of devices have emerged recently and can provide convenience and new opportunities for fast, and on-site inspection, avoiding chemical analyses, reducing the environment impact and food waste, and satisfying consumers expectations about quality and healthy foods.

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) **Testing phase in laboratory condition** in terms of identification of fresh-cut products to be analysed (A1.1), identification of quality parameters to be investigated (A1.2), development of a software for images acquisitions using MATLAB® (A1.3), and sampling and application of hyperspectral imaging (A1.4). Moreover, the use of the hyperspectral device will be repeated during the years in order to optimize the application and to improve the prediction models.

A2) **Application of chemometric techniques** using a software developed with MATLAB® (A2.1) in order to analyse the hyperspectral images acquired. On the data collected we will apply pre-processing techniques (A2.2), multivariate statistical analyses (A2.3) and, finally, we will calibrate and validate the built model (A2.4). Chemometrics will be applied after every application of hyperspectral imaging.

A3) **Application of LCA (Life Cycle Assessment) methodology** to evaluate the environmental and economic impact of the hyperspectral imaging technique potentially applied in fresh-cut products chain (A3.1). This analysis will be compared to LCA of chemical analyses (A3.2).

A4) **PhD project management activities**, regarding PhD thesis, scientific papers, and oral and/or poster communications preparation.

Table 1. *Gantt diagram for this PhD project*

	Activities	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1	***Testing phase in laboratory condition***																								
	1) Identification of the fresh-cut products to be analysed																								
	2) Identification of the quality parameters to be investigated																								
	3) Software creation																								
	4) Sampling and hyperspectral image acquisitions																								
A2	***Chemometrics***																								
	1) Software creation																								
	2) Image and data pre-processing																								
	3) Multivariate statistical analysis																								
	4) Model calibration and validation																								
A3	***LCA (Life Cycle Assessment)***																								
	1) LCA of HSI applications in fresh-cut products chain																								
	2) LCA comparison between chemical analysis and HSI applications																								
A4	***Thesis and Paper preparation***																								

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Development of strategies aiming to ameliorate the nutritional impact and feasibility of a sustainable plant-based dietary pattern rich in bioactive compounds

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This PhD research project aims to implement strategies to promote sustainable dietary patterns and increase their adoption identifying advantages and criticalities associated to plant-based diets. To this aim, a specific sustainable dietary pattern intervention will be carried out on a group of adult volunteers. The impact of the dietary pattern on nutritional, metabolic and functional status will be investigated together with the evaluation of micronutrients and bioactive compounds bioavailability. In addition, activities will be devoted to the study and improvement of the feasibility and acceptability of the diet, including the development of plant-based preparations according to the Italian dietary habits.

Sviluppo di strategie volte a migliorare l'impatto nutrizionale e la fattibilità di un modello alimentare sostenibile *plant-based* ricco di composti bioattivi

Il progetto di ricerca di dottorato mira a sviluppare strategie per promuovere modelli alimentari sostenibili, identificando i fattori critici e positivi associati ai pattern dietetici più ricchi in prodotti vegetali con lo scopo di aumentarne l'adozione. Uno studio di intervento verrà condotto su un gruppo di adulti a cui verrà chiesto di seguire un modello alimentare sostenibile per studiarne l'impatto nutrizionale, metabolico e funzionale oltre che per valutare la biodisponibilità di micronutrienti e composti bioattivi. Inoltre, saranno effettuate attività per migliorare la fattibilità e l'accettabilità della dieta attraverso lo sviluppo di nuove preparazioni vegetali in linea con le abitudini italiane.

1. State-of-the-Art

In the EU, the current food trend is associated with higher consumption of low-quality diets characterized by elevated intake of energy, red meat, sugars, salt and fats. On the contrary, plant-based foods rich in nutritional compounds, such as micronutrients, important bioactive compounds and dietary fiber, remains insufficient and lower than the national dietary recommendations, also in Italy. While micronutrient deficiencies have been correlated with an increasing incidence of health-related non-communicable diseases (e.g., obesity, diabetes and cardiovascular diseases), consuming whole-grain cereals, fruit and vegetables, legumes and nuts, could exert health benefits. In addition, an increased consumption of plant-based diets could decrease the emissions by up to 80% since plant foods seem to have a lower environmental impact than animal food sources. Indeed, the food system is one of the major contributors to environmental impact, not only in terms of gas emissions, but also of land and water use. In view of all of this, promoting healthy and sustainable diets represents a current urgent need, both at global and EU level (Springmann et al., 2018).

In 2009, the EAT- Lancet Commission developed a reference dietary pattern (ELCRD) on plant-based foods which could exert positive effects both on health and the environment. The ELCRD is a planetary diet that needs to be tailored to food cultures and cuisines of the different populations (Willet et al., 200000). Tucci et. al. (2021) have adapted it to the specific Italian food context and studied a sustainable and health-promoting plant-based dietary pattern, the EAT-IT diet.

Furthermore, the significant substitution of animal with vegetal products poses important questions on feasibility of the diet and on actual impact on nutritional status in relation to specific micronutrients content and availability. A very recent review has demonstrated that most of the studies assess the sustainability of a diet using just *environmental parameters* such as greenhouse gas (GHG) emissions, but those do not determine the nutritional adequacy of a dietary pattern which has to be assessed by *nutrition metrics* (such as dietary diversity and nutrient adequacy ratios, nutrient bioavailability and density of foods, recommended nutrient requirements, etc.). The sum of all these metrics makes possible to assess sustainable diets in the right way (Dave et al., 2021).

While a pilot study has been performed on a subgroup of volunteer (n=10) to verify the level of adherence to the EAT-IT diet and to estimate its acceptability, adaptability and feasibility, this PhD project research aims to implement a randomized cross-over controlled trial on 45 subjects to achieve the statistical power in order to validate the impact of the diet on nutritional, metabolic and functional status. The main expected result is the reduction of the environmental impact with an amelioration of the sustainability of the diets based on a higher consumption of plant-based foods which will have a positive effect on the modulation of the health-related markers and will be able to promote health.

The data collected will help finding useful strategies to increase the nutritional adequacy of the EAT-IT diet and to make it the most suitable and adaptable for the Italian population. In particular, the potential exploitation of different foods rich in micronutrient or the use of different methods of food preparation that can increase the bioavailability of specific nutrients will be explored. This will be enabled also thanks to the activities performed within the Mind Foods Hub project contributing through the development of plant-based products (e.g., cereals, legumes, vegetables, tubers, fruits) with an excellent nutritional profile, especially focusing on the improvement of the nutritional characteristics, bioavailability and acceptability. Bioavailability evaluation of micronutrient and bioactive compounds would help integrate plant-based foods capable of minimizing potential nutritional inadequacy that could occur in a plant-based pattern (e.g., vitamin B12, calcium or iron).

2. PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 1:

A1) **Selection of volunteers**: 45 healthy subjects aged between 18-60 years old with a normal BMI. **Collection of blood, urine and faecal samples:** before and after each period of intervention biological samples will be collected for the analysis of metabolic and functional markers, while fecal samples will be stored for the evaluation of microbial ecosystem.

A2) **Plant-based dietary intervention** through a cross-over randomized controlled dietary intervention. One arm will follow the EAT-IT diet, the other the habitual diet and, after a wash-out period, they will exchange the two diets.

A3) **Analysis of the biological samples, questionnaires, and bioavailability assessment**

A4) **Identification of criticalities and/or risks and development of strategies** aiming to ameliorate the plant-based dietary pattern in terms of nutritional adequacy, adaptability, acceptability and feasibility.

A5) **Writing** of the PhD thesis.

A6) **Dissemination of the results** through scientific papers and oral and/or poster communications

Table 1 *Gantt diagram for this PhD thesis project.*

Activity	Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A1)	***Volunteers***																								
	1) Selection of participants																								
	2) Samples and records collection																								
A2)	***Dietary intervention***																								
	1) Personalized diets elaboration																								
	2) Dietary intervention																								
A3)	***Analysis***																								
	1) Markers and questionnaires evaluation																								
	2) Bioavailability assessment																								
A4)	***Identification of criticalities and/or risks to be managed***																								
	1) Identification of critical factors																								
	2) Development of strategies																								
A5)	***Thesis and Paper Preparation***																								
A6)	***Dissemination of the results***																								

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The flat breads of the Mediterranean area: valorisation and innovation

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This PhD thesis research project is aimed to the valorisation and innovation of flat breads, traditional products of the Mediterranean area. Greater attention will be given to Italy, characterised by a long tradition and variety of flat breads. The innovation will consist in an improvement of the nutritional profile by fortifying flat breads with unconventional ingredients - principally legume flours - considering both gluten-free and conventional flat breads. Furthermore, partially-baked fortified flat breads will be produced and stored in different conditions, to evaluate their changes during the shelf life. This PhD project is part of the PRIMA "Flatbread Mine" project.

I pani piatti dell'area Mediterranea: valorizzazione e innovazione

Questo progetto di tesi di dottorato ha lo scopo di valorizzare e innovare i pani piatti prodotti e consumati nell'area Mediterranea. Particolare attenzione sarà rivolta all'Italia, caratterizzata da una lunga tradizione e varietà di pani piatti. L'innovazione consisterà nel migliorare il profilo nutrizionale dei pani piatti, fortificandoli con ingredienti non convenzionali - principalmente farine di legumi - considerando sia pani piatti senza glutine che convenzionali. Inoltre, pani piatti fortificati parzialmente cotti saranno prodotti e conservati in condizioni diverse, per valutarne i cambiamenti durante la shelf life. Questo progetto di dottorato è inserito nell'ambito del progetto PRIMA "Flatbread Mine".

1. State-of-the-Art

Bread is a traditional staple food and flour, water and salt are the principal ingredients, with or without the use of yeast (Boukid *et al.*, 2019). Among the hundreds of typologies of bread, the oldest are the flat breads, products characterised by a thickness between a few centimeters and a few millimeters (Pasqualone, 2018; Boukid, 2022). In the past the cultural importance of flat breads, especially in the Mediterranean Basin, was connected to the needs of the rural populations and the nomadic style of life, based on the necessity to transport few things and foods ready to cook or eat (Pasqualone, 2018). Today these reasons are still valid, considering the modern pace of life, which dramatically reduced the amount of time available for the preparation of meals and induced the need of healthy ready to eat meals (Boukid, 2022). Flat breads can be categorized in different ways, in relation to the presence of a leavening phase, the consistency of the dough (semi-fluid or semi-solid), the thickness or structure (thick or thin, single-layered or double-layered) and the baking method (under a layer of sand or embers, on fire plates, in horizontal or vertical ovens). This last aspect is related to cultural factors and the typical properties of the final products (Pasqualone, 2018). In particular, Italy has a long tradition of flat breads, some of which are well-known at global level, such as Piadina Romagnola and Pizza Napoletana (Pasqualone, 2018). A survey on the flat breads produced and consumed in the Mediterranean area, and the compilation of a database reporting all the surveyed types will be the starting activities of the PhD project, aimed at having an insight of the technical and cultural aspects of these breads as well as to select and prepare the suitable products to innovate. Flat breads are very versatile products, usually formulated with wheat flour, but also with other cereals, pseudocereals, chestnuts, and legume flours (Pasqualone, 2018; Boukid, 2022). In addition, their versatility relates to the numerous combinations of ingredients which can constitute the topping of the garnished flat breads (Pasqualone, 2018). The researchers have progressively proposed new flat breads, improving their nutritional, technological and sensorial properties (Boukid *et al.*, 2019; Boukid, 2022). Recently, the use of legumes as food ingredients has increased, considering the health benefits related to their consumption (Boukid *et al.*, 2019). Legumes are a good source of proteins, rich in essential amino acids complementary to those of cereals. The combination of legume flours with cereal flours could nutritionally improve bread (Boukid *et al.*, 2019). On the other hand, legumes contain some antinutritional compounds (Boukid *et al.*, 2019). However, treatments such as cooking, soaking and extrusion may reduce their content. Extrusion-cooking achieves also the gelatinization of starch, the denaturation of proteins, and the increase of soluble fibers, by combining high temperature, pressure and mechanical actions (Pasqualone *et al.*, 2020). In addition, legume-based sourdough may partially or totally eliminate phytates, and improve the sensory characteristics (Samtiya *et al.*, 2020). Modern consumers are increasingly interested to substitute animal proteins with vegetable alternatives, such as legumes: the fortified flat bread could be a good way to increase their consumption, overcoming the main obstacle related to the long cooking time of legumes (De Angelis *et al.*, 2021). Considering the need of sustainable means to obtain legume proteins, the dry fractionation is an interesting process based on micronization and air classification (De Angelis *et al.*, 2021). The finer fraction, rich in protein, could find application in the reformulation of flat breads. Moreover, being legumes naturally gluten-free, their incorporation is also suitable to prepare gluten-free flat breads (Pasqualone *et al.*, 2022). Finally, the Bake Off Technology (BOT), consisting of producing bread from industrial refrigerated, frozen or non-frozen bakery goods

(partially-baked bread or "part-baked" bread) and selling them for domestic baking, recently increased its market share indicating a growing interest by consumers (Najafabadi *et al.*, 2014).
In this context, the aims of this PhD project are to study and valorise the flat breads produced and consumed in the Mediterranean area and to improve their nutritional profile, without affecting the sensorial characteristics. The use of legume flours will be experimented, to offer new flat breads, also partially-baked, with particular attention to the environmental sustainability, nutritional and health benefits. In addition to the conventional flat breads, also gluten-free versions will be considered, to fulfil the expectations of celiac consumers for healthy food products.

2. PhD Thesis Objectives and Milestones

The Gantt diagram, illustrated in Table 1, reports the activities of the PhD project:

A0) **Bibliographic search** on the different types of flat breads in the Mediterranean area and on the application of legume flours in flat breads.

A1) **Definition and development of flat breads,** to set up recipes and processes for conventional and gluten-free flat bread, considering the products spread and consumed in the Mediterranean Basin, principally in Italy. The flat breads will be selected considering the results of the survey and FB database, two activities conducted during the first year of PhD and crucial for their valorisation.

A2) **Production of legume fortified flat breads** to improve the nutritional properties, without affecting the technological and sensorial features (A2-1). The fortification will take place by native, dry- fractionated and extruded legume flours. Legume fortified flat breads will be submitted to nutritional characterisation and consumer test, to evaluate their acceptability (A2-2). Partial-baked fortified flat breads will be stored in different conditions, to evaluate their changes during the shelf life (A2-3).

A3) **Industrial flat bread production tests.** A selection of formulations of conventional and gluten-free legume fortified flat breads will be prepared at industrial scale in collaboration with a local company partner of the project**.**

A4) **Data analysis, Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

Table 1 *Gantt diagram for this PhD thesis project.*

Activity \ Months		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A0)	***Bibliographic search***																								
A1)	***Definition and development of flat breads***																								
A2)	***Production of legume fortified flat breads***																								
	1) Fortification trials of conventional, GF and partially-baked flat bread																								
	2) Nutritional characterisation and Consumer test																								
	3) Shelf-life study																								
A3)	***Industrial flat bread production tests***																								
A4)	***Data analysis, thesis and papers writing and editing***																								

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Innovative strategies aimed at improving the sustainability of the oenological process

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The aim of this PhD-project is to promote the use of adjuvants and/or additives for a more sustainable management of the winemaking process in the frame of precision oenology. The effect of different oenological products will be evaluated on phenolic-related parameters and volatile organic compounds (VOCs) in musts/wines, focusing mainly on VOCs preservation and volatility. Green analytical techniques will be adapted, simulating fermentation and mouth-tasting conditions, to avoid organic solvents usage and reduce VOCs analysis time: headspace solid-phase microextraction gas chromatography-mass spectrometry (HS-SPME-GC-MS) for musts/wines analysis. In addition, near-infrared (NIR) spectroscopy will be applied for estimating the grape aromatic potential.

Strategie innovative finalizzate a migliorare la sostenibilità del processo enologico

Dall'unione dei concetti di enologia sostenibile e di precisione è nata l'idea di valutare, con scopo di preservazione, come l'aggiunta di coadiuvanti e additivi possa modificare in mosti e vini le composizioni fenolica e volatile. Per quanto riguarda quest'ultima, l'adattamento della microestrazione in fase solida dello spazio di testa accoppiata alla gascromatografia-spettrometria di massa (HS-SPME-GC-MS) permetterà di simulare le condizioni di fermentazione e degustazione, contemporaneamente eliminando l'impiego di solventi organici e riducendo i tempi di analisi. Inoltre, la spettroscopia nel vicino infrarosso (NIR) sarà applicata alla predizione del potenziale aromatico dell'uva in quanto tecnica analitica rapida a basso impatto ambientale.

1. State-of-the-Art

1.1. Oenological adjuvants and additives: impact on the phenolic and volatile composition

During winemaking, for preservation or to meet quality standards, it can be necessary to use adjuvants and/or additives whose dosage follows food safety and production competitiveness dynamics. Among the available products, yeast derivatives and oenological tannins are particularly interesting because they can be obtained by the recycling of vine and wine industry wastes. Oenological tannins are used during the vinification and storage of red wines for antioxidant and/or colour stabiliser purposes. Concerning white wine production, oenological tannins are usually used at the beginning of vinification for their antioxidant ability as an alternative to sulphur dioxide, which is responsible for intolerances and allergies. Among the commercially available formulations, hydrolysable tannins have a higher oxygen consumption rate in model solution, ellagitannins showing the greatest antioxidant potential (Vignault *et al.*, 2018). Nevertheless, the addition of tannins or other oenological products can interact with the matrix components of grape must and wine, altering the VOCs solubility, and therefore affecting the perceptibility of odour active molecules. The volatility of a specific compound can be estimated calculating, in a simple system, its partition coefficient (k_i) as the ratio between its concentration in the gas and liquid phases. The kinetic nature of k_i makes it affected by physical and compositional parameters (Mouret *et al.*, 2012). It is therefore considered mandatory, with the purpose of strengthening the sustainability of the production process, to investigate the role that oenological adjuvants take during white winemaking in modifying the phenolic and volatile composition, sensory profile, and colour characteristics, as well as to assess the varietal effect.

1.2. Green analytical technologies

1.2.1. Chromatographic analysis of VOCs on grapes, musts, and wines

The preparation of grape, must, and wine samples for GC analysis requires long extraction times with organic solvents increasing the environmental burden of the analytical process. In this sense, the PhD project objective is to adapt GC methodologies based on HS-SPME technique for improving the separation of VOCs and subsequent quantification by mass spectrometry of a greater number of compounds under experimental conditions similar to those of fermentation or tasting. The use of adsorbent fibers to directly extract VOCs from sample headspace requires minor sample preparation and completely avoids organic solvents usage (Marín-San Román *et al.*, 2020).

1.2.2. Near-infrared spectroscopy on grapes and grape homogenates

Near-infrared reflectance spectroscopy is a non-destructing and green analytical technique that correlates reflected NIR radiation with sample chemical and physical properties. The complex nature of IR spectra due to the high number of compounds absorbing near-infrared radiation requires spectral pre-processing procedures and chemometric analysis to extract latent compositional information. NIR has shown good potentiality to predict wine and grape chemical components. However, the prediction of the volatile composition is difficult due to the low concentration in which VOCs are present in grapes and wine, and the high number of chemical families involved. Furthermore, metals and/or cuticular waxes have been shown to be potentially coded into the IR spectra (Dambergs

et al., 2015). Therefore, it is considered mandatory to quantify the impact of these factors with the purpose of obtaining a grape/wine aromatic potential index.

2. PhD Objectives and Milestones

As represented by the Gantt diagram (Table 1), the PhD project will be divided into the following main activities:

A1) **Effect of oenological adjuvants and additives during alcoholic fermentation.** Exogenous tannins (gallotannins, proanthocyanidin tannins), chitosan, and yeast extracts have been tested on white grape must, as possible alternatives to sulphur dioxide. Colour characteristics, antioxidant activity, total polyphenols, and volatile compounds have been determined. In these first months of the PhD thesis, the experimental research has been completed, and nowadays the data treatment is being carried out.

A2) **Impact of oenological tannins on the volatility of odour active compounds during alcoholic fermentation and tasting**. The ability of exogenous tannin formulations (hydrolysable gallotannins and ellagitannins, proanthocyanidins from exotic woods like acacia and quebracho, proanthocyanidins from grape seeds and skins) to preserve VOCs during fermentation will be evaluated on the basis of antioxidant activity and partition coefficient (k_i) for the most relevant volatile compounds between gas and liquid phase. In addition, the effect of adding these tannins at bottling will be investigated during *shelf-life* on the volatility of VOCs at wine tasting considering the interaction VOCs-tannins-salivary proteins. The variety and matrix composition influence will be assessed according to the possible retention and salting out effects. HS-SPME-GC-MS methodologies will be adapted for improving VOCs separation and quantification simulating fermentation and in-mouth conditions.

A3) **Exploring the NIR capability of discriminating grape berries according to their volatile composition.** Whole red and white grape berries, as well as grape homogenates, will be analysed by NIR to develop a fast and green analysis technology aimed at predicting aromatic maturity. Samples will be analysed fresh (just after harvest) and after different storage times (3 and 10 months). GC-MS methodology will be used as reference. In addition, the impact of grape cuticular wax solvation to reduce the spectral variability and the presence of paramagnetic ions in solution will be evaluated on the NIR predictive capability.

A4) **Corporate Internship.**

A5) Writing PhD thesis and scientific articles, participation to scientific congress.

Table 1. *Gantt diagram for the second and third year of this PhD project.*

Activity		Months																							
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**
A1)	***Alternative oenological formulations***																								
	1) Data treatment																								
A2)	***Exogenous tannins−VOCs volatility***																								
	1) HS-SPME-GC-MS optimisation																								
	2) Tannins, must/wine characterization																								
	3) Fermentation trials																								
	4) VOCs determination																								
	5) Partition coefficient determination																								
	6) Interaction VOCs-tannins-saliva																								
	7) Wine tasting																								
A3)	***VOCs prediction by NIR***																								
	1) NIR and GC-MS analysis																								
	2) Spectral treatment																								
	3) Correlation studies																								
A4)	***Corporate internship***																								
A5)	***Thesis and papers preparation***																								

3. References

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Workshop contributions

2nd year: Poster Communications

Synergistic and antagonistic interactions between food antioxidants

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Nowadays, many antioxidant assays are available. However, most of them lack standardization and are unable to determine the presence of synergistic vs antagonistic interactions between mixture of antioxidants. Accordingly, this work proposed a novel kinetic approach based on the reaction mechanism between antioxidants (or natural extracts) and DPPH radicals. Through the numerical solution of a set of ordinary differential equations (ODE), the signal decay of DPPH was fitted. The fitting leaded to the expression of the rate constant for the reaction between antioxidants and the DPPH radicals. This constant represented a direct expression of the radical scavenging activity. Such approach was successfully applied to determine the antioxidant activity of (1) standard antioxidants, but also of (2) mixture of antioxidants and even (3) natural extracts. Furthermore, the approach was able to determine not only the existence of side reactions, but also the presence of synergisms between mixture of antioxidants. All the results of this approach were validated by HPLC-MS/MS analysis. Overall, the proposed approach was simple, rapid and inexpensive, and it allowed to gain better insights into the reactivity of antioxidants under standardized experimental conditions.

Interazioni sinergiche e antagonistiche tra gli antiossidanti negli alimenti

Ad oggi esistono molti saggi antiossidanti. Tuttavia, la maggior parte di essi non è standardizzata e non sono quindi capaci di determinare la presenza di interazioni sinergiche o antagonistiche tra miscele di antiossidanti. Di conseguenza, questo lavoro ha proposto un nuovo approccio cinetico basato sul meccanismo di reazione tra antiossidanti (o estratti naturali) e i radicali del DPPH. Attraverso la soluzione numerica di un set di equazioni differenziali ordinarie, è stato fittato il segnale di decadimento del DPPH. Il fitting ha portato all'espressione della costante per la reazione tra gli antiossidanti e il DPPH. Questa costante rappresenta un'espressione diretta dell'attività di scavenging del radicale. Questo approccio è stato applicato con successo per determinare l'attività antiossidante di (1) antiossidanti standard, ma anche (2) miscele di antiossidanti e (3) estratti naturali. In più, l'approccio è stato capace di determinare non solo l'esistenza di reazioni secondarie, ma anche la presenza di sinergismi in miscele di antiossidanti. Tutti i risultati di questo approccio sono stati validati con analisi HPLC-MS/MS. Complessivamente, l'approccio proposto è stato semplice, rapido e poco costoso e ha permesso di acquisire nuove conoscenze sulla reattività degli antiossidanti in condizioni sperimentali standardizzate.
Key words: antioxidant activity, kinetic model, side reaction, synergism, HPLC-MS, DPPH.

1. Introduction

In accordance with the PhD thesis project previously described (Angeli 2021), this poster reports the main results of the first two activities concerning:
(A1) the development of a stoichiometric and kinetic model for the DPPH assay to determine the activity of common antioxidants and complex mixtures.
(A2) the development of a kinetic model to determine the interaction between binary mixtures of antioxidants.

2. Materials and Methods

The absorption decay of DPPH was monitored at its maximum absorbance of 515 nm with a Cary 60 uv-vis spectrophotometer (Agilent, Santa Clara, USA). A 100 μM solution of DPPH usually had a maximum absorbance of 1.1±0.4 (ε in methanol = 11,700±300 $M^{-1}cm^{-1}$). This solution was studied against several concentration (10-50 μM) of gallic, ferulic, caffeic, sinapic, chlorogenic acid and Trolox. The method was also implemented with a stopped-flow system (the RX2000 coupled to a RX pneumatic drive, Applied Photophysics, Leatherhead, UK), which allowed to monitor the DPPH absorbance decay in the first seconds of the reaction, with a lag time of 6 ms. Moreover, the products formed after the reaction of antioxidants and DPPH were analyzed with a Dionex Ultimate 3000 HPLC (ThermoFisher Scientific, Waltham, MA, USA) coupled to a QExactive orbitrap mass spectrometer (ThermoFisher Scientific, Waltham, MA, USA) following the method of Berton et al with some modifications (Berton et al., 2020). The approach was finally extended to binary equimolar mixtures of antioxidants and natural extracts. Also, the reaction between α-tocopherol and ascorbic acid was studied by cyclic voltammetry, chronoamperometry and HPLC-MS.

3. Results and Discussion

3.1 Determination of the antioxidant activity of common molecules and complex mixtures with a novel stoichio-kinetic model for the DPPH assay

The reaction mechanism of DPPH with common antioxidants follows this scheme:

$$AOH + n \cdot DPPH \xrightarrow{k_1} AO^{\cdot} + DPPH - H \quad (1)$$

where AOH is the antioxidant, n is the stoichiometric factor of the reaction, DPPH is the radical, k_1 is the rate constant of the reaction expressed as $M^{-1}s^{-1}$, $AO^{\cdot}$ is the oxidized form of the antioxidant and DPPH-H is the reduced form of DPPH (Foti et al., 2004). Eq. 1 represents a simplified mechanism, which can be used to express the antioxidant activity (i.e., in terms of rate constants) of the reaction between DPPH and certain antioxidants. However, when eq. 1 fails to fit the DPPH signal decay, then it is necessary to include in the model of a further reaction, which keeps into account also side reactions between the DPPH radicals and the oxidized form of antioxidants. This is described by Eq. 2.

$$AO^{\cdot} + DPPH \xrightarrow{k_2} Products \quad (2)$$

Eq. 2 expresses another rate constant (k_2), which represents the ability of the antioxidant to further react with the radical. It is relevant to highlight that if such side reaction (Eq.2) is not taken in consideration, the resulting antioxidant activity is greatly underestimated, whereas the stoichiometric factor of the reaction becomes unrealistic. To validate our conclusions, the products of the reactions were analyzed by HPLC-MS. Only for those antioxidants showing the side reaction (Eq.2) products were found (Angeli et al., 2021). Moreover, the model was applied to herbal extracts to show its effectiveness of assessing the antioxidant activity in complex mixtures. Figure 1 shows the fitting of the experimental data of gallic acid and its mass fragmentation spectrum.

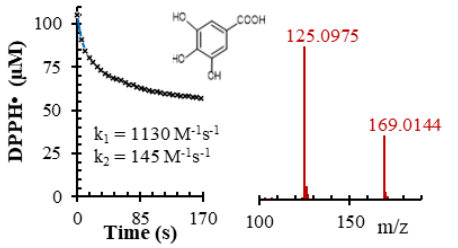

Figure 1 *Fitting of the experimental data for the concentration decay of 100 μM DPPH against 10 μM of gallic acid. The rate constants and the fragmentation mass spectrum are reported.*

3.2 Determination of the interaction between binary mixtures of antioxidants

The model was then applied to binary equimolar mixtures of antioxidants, to see if it was possible to observe a synergistic or antagonistic interaction. The single rate constants (k_1) obtained from Eq.1 were compared to those obtained in the new model (k'_1), also involving another equation, Eq.3.

$$AO^{\cdot}_1 + AO^{\cdot}_2 \overset{K_{eq}}{\longleftrightarrow} AOH + Products \quad (3)$$

Where $AO^{\cdot}_1$ is the oxidized form of the first antioxidant, $AO^{\cdot}_2$ is the oxidized form of the second antioxidant, AOH is the reduced form of one of the two antioxidants and K_{eq} is the equilibrium constant of the reaction. In each of the cases studied, it was observed that Eq.1 was not sufficient to study the mechanism. Indeed, by mixing two antioxidants together, a change in the kinetic mechanism and, eventually, also in the stoichiometry of the reaction could be observed. Therefore, only admitting another reaction where a recycling of one of the two antioxidants was possible, a good fitting could be obtained (R^2 >0.99). K_{eq} represents the ratio between the two rate constants obtained by the reversible reaction (Eq. 4). From the K_{eq} value it was also possible to calculate the Free Gibbs Energy as thermodynamic evidence of the presence of synergism/antagonism (Eq. 5).

$$K_{eq} = \frac{[AOH][P]}{[AO^{\cdot}_1][AO^{\cdot}_2]} = \frac{k_f}{k_b} \quad (4)$$

$$\Delta G^{\circ} = RTlnK_{eq} \quad (5)$$

When the $\Delta G°$ is < 0, a synergistic interaction is admitted. When $\Delta G°$ is > 0, no synergism is highlighted, therefore the interaction is antagonistic. The synergistic interaction is being studied also with cyclic voltammetry, chronoamperometry and mass spectrometry.

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Effect of different cooking treatments on polyphenols content and antioxidant activities of apples grown in South Tyrol

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This Ph.D. project has developed a novel kinetic method for determining the antioxidant activity of fruit extracts. The method consisted in a modification of the classical ORAC assay (oxygen radical absorbance capacity), which allowed to determine the absolute rate constants of the reaction between antioxidants and free radicals, together with their stoichiometry value. The method was successfully applied to investigate the effects of five cooking methods on the resulting antioxidant activities of five varieties of apples, typically grown in the South Tyrol. The results of this work will improve our understanding of the activities of antioxidant present in apples and will promote the diffusion of those cooking practices of apples that are traditionally used in South Tyrol.

Effetto di diversi trattamenti di cottura sul contenuto di polifenoli e sulle attività antiossidanti delle mele coltivate in Alto Adige

Questo progetto di dottorato ha avuto come scopo lo sviluppo di un nuovo metodo cinetico per determinare l'attività antiossidante di estratti di frutta. Il metodo consiste in una rielaborazione del classico saggio ORAC (Capacità di assorbimento dei radicali dell'ossigeno), con il fine di determinare le costanti di velocità assolute della reazione tra antiossidanti e radicali liberi, insieme al loro valore stechiometrico. Il metodo è stato applicato con successo allo studio degli effetti di cinque metodi di cottura sulle attività antiossidanti di cinque varietà di mele, tipiche dell'Alto Adige. Questi risultati potranno migliorare da un lato la nostra comprensione sulle attività antiossidanti delle mele, dall'altro promuoveranno l'utilizzo gastronomico delle mele attraverso la riscoperta delle tradizioni culinarie dell'Alto Adige.

Key words: Antioxidants, extraction, ORAC assay, cooking methods, kinetics.

1. Introduction

The PhD project was structured in three main experimental activities:
(A1) The determination of the most suitable method for the extraction and quantification of free antioxidants in apple
(A2) The development of a kinetic assay that could quickly determine the antioxidants activity of the extracts and their apparent stoichiometry.
(A3) The identification of the main phenolic compounds present in apple samples using advanced analytical methods like HPLC-DAD-MS and Coularray detector.

2. Materials and Methods

The extraction method consists of an ultrasound assisted extraction using 80% methanol or ethanol (Kalinowska et al. 2020). The resulting extracts were analyzed by ORAC assay (classical vs kinetic approach) and the results were expressed in terms of antioxidants capacity and activity. Apple samples consist of 5 varieties (Gala, Golden delicious, Grany smith, Fuji and Renetta), harvested in South Tyrol region. Five cooking methods were used to test the effects on antioxidant activity, respectively, boiling, deep-frying, microwave heating, oven heating and steaming.

Table 1. *Summary of cooking treatments with time, amount of liquid (water, oil) used and measured temperature in the samples.*

Cooking methods	Time (min)	Water	Oil	Measured temperature (° C)
Raw (R)	_	_	_	RT
Boiling (B)	5	500 ml	_	100
Deep-frying (DF)	3-5	_	2 L	>130
Oven-heating (Oven)	30	_	_	>130
Microwave -heating (Micro)	5	_	_	100
Steaming (Steam)	5	2 L	_	100

3. Results and Discussion

3.1 ORAC kinetic model

In order to implement a kinetic version of the classical ORAC assay, it was firstly applied to define a kinetic model. This consisted in a system of three reactions as follows:

R	2ROO• + N2	…….. k_i	(1)
ROO• + FH	ROOH + F•	……. k_f	(2)
ROO• + AH	ROOH + A•	…….. k_{inh}	(3)

Where R, FH and AH refer, respectively, to the azo-dye radical generator (AAPH), fluorescein, and antioxidant. Instead, ROO• corresponds to the peroxyl radicals, while F• and A• are radical products. The fluorometric signal recorded for the oxidation of fluorescein was fitted by solving the differential equations corresponding to the reaction in eq. 1-3 by a numerical fitting routine. As a result, three rate constants were obtained, respectively, k_i, k_f and k_{inh}. The model was successfully applied to 13 antioxidant standards, giving rate constants values ranging from 2000 to 20000 $M^{-1}s^{-1}$. The model was finally applied to measure the antioxidant activity of 5 apple varieties treated with 5 different cooking methods (analysis fully replicated in double). The values for apple samples ranges from 2500 to 5200 $M^{-1}s^{-1}$. This study provides a new approach to determine the antioxidant activity of foods in terms of kinetic values.

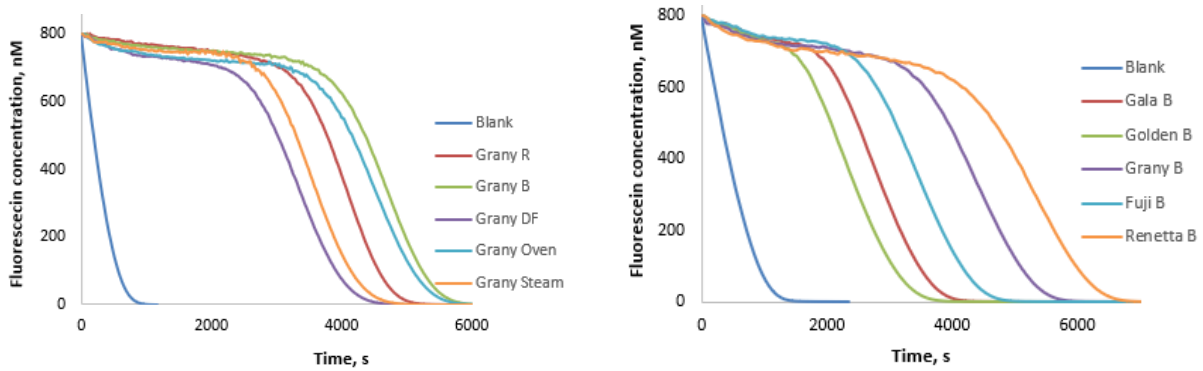

Figure 1. *ORAC kinetics of different cooking treatments of single variety (Grany smith) vs boiling treatment of five apple varieties*

The cooked samples specially treated with boiling, steaming and microwave heating treatments showed the highest antioxidant activity ($p < 0.05$, ANOVA test) in comparison to the control sample (Raw sample). This happened likely because of the release of some weakly bound polyphenols that can be liberated during cooking (Thuengtung and Ogawa 2020).

3.2 Characterization of main phenolic compounds present in apple

The main phenolic compounds in apple extracts were measured using high-performance liquid chromatography coupled with diode-array detector (280 nm, 320 nm) and confirmed by high resolution mass spectrometry in ESI^- ionization mode. The major phenolic compounds found on the flesh of apple samples are the phenolic acids, flavanols and dihydrochalcone (Table 2). Similar compounds have been reported in other studies (Kschonsek et al. 2018).

Table 2. *Characterization of main phenolic compounds, (retention time [t_R] and precursor ion* $[M-H]^-$ *in apple samples.*

Phenols group	Phenolic Compound	t_R (min)	$[M-H]^-$
Flavanols	(+)-Catechin	4.1	289.0717
	(-)-Epicatechin	8.9	289.0717
Phenolic acids	Chlorogenic acid	5.5	353.0878
Dihydrochalcones	Phloridzin	15.1	435.1297
	Phloretin	18.1	273.0769

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Cultivation and metagenomics for the assessment of microbial biodiversity in grapes and musts from different viticultural areas of Italy

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In this study, yeasts populations present on the surface of grape berries and resulting musts from cultivars Aglianico and Cabernet Sauvignon were assessed. Grapes were harvested in three different regions of Southern Italy. A culture-dependent method based on sequencing of the D1/D2 region of 26S rDNA of yeasts grown on solid medium was coupled with Next-Generation Sequencing (NGS) of the Internal Transcribed Spacer 2 (ITS2) with the Illumina MiSeq platform to evaluate differences within cultivars and geographic areas.

Studi coltura-dipendenti e metagenomici per la valutazione della biodiversità microbica in uve e mosti di diverse aree vitivinicole italiane

In questo studio è stata analizzata la popolazione microbica dei lieviti presenti sulla superficie degli acini d'uva di due diverse cultivar (Aglianico e Cabernet Sauvignon) e nei mosti derivanti da essi. Le uve provenivano da vigneti collocati in tre differenti regioni del Sud Italia. A questo scopo, è stato utilizzato un approccio coltura-dipendente, basato sull'identificazione mediante sequenziamento della regione D1/D2 del 26S rDNA di isolati microbici cresciuti su substrato solido, abbinato al sequenziamento di nuova generazione (NGS) della regione ITS2 (Internal Transcribed Spacer 2) attraverso la piattaforma Illumina MiSeq al fine di valutare possibili differenze legate alla cultivar e all'area geografica di provenienza.

Key words: culture-dependent isolation, grapes, musts, next-generation sequencing, yeast.

1. Introduction

The microbiological characteristics of must and, consequently, wine derive primarily from the microbial population present on the surface of grapes, which is strongly influenced by climate conditions (Belda *et al.*, 2017). In accordance with the PhD project, this poster reports the main results of the activities concerning (i) the isolation and identification of cultivable yeasts present on the grape skins of two different cultivars and in their resulting musts and (ii) the exploration of microbial communities in the samples by using the Illumina MiSeq technique.

2. Materials and Methods

Grape berries from cultivars Aglianico and Cabernet Sauvignon were collected in three different regions of Southern Italy (Sicily, Campania and Molise) at the right maturation time (between September and October 2020). Their resulting musts were sampled after 7 days of spontaneous fermentation at 22°C. Yeasts from grape skins and musts were isolated on WL-agar (Sigma-Aldrich, Milan, Italy) added with100 mg/L ampicillin sodium salt and 400 mg/L biphenyl (Sigma-Aldrich, Milan, Italy) to prevent bacterial and mould growth, respectively, following the protocol of Vaudano *et al*. (2019). After incubation at 25°C for 8 days, single colonies with different morphologies were taken and purified. DNA was extracted with the "Fungi/Yeast Genomic DNA Isolation Kit" (Norgen Biotek Corp., Thorold, ON, Canada) and amplified by specific PCR assays using NL1 and NL4 primers targeting the D1/D2 domain of 26S rDNA. PCR products were purified with the "MinElute PCR Purification Kit" (Qiagen, Hilden, Germany), diluted and sent to a commercial facility for sequencing (Eurofins Genomics, Germany). The obtained sequences were compared with BLAST through the NCBI website.
Genomic DNA was extracted from berries and must samples using the "Stool DNA Isolation Kit" (Norgen, Biotek Corp., Thorold, ON, Canada) according to the manufacturer's instructions. The fungal communities were accessed through sequencing of the ITS2 region. Illumina MiSeq amplification and sequencing were performed through an external service (Biodiversa s.r.l., Rovereto, Italy). Fungal ITS2 amplification was performed with primers 2024F and 2409R (White *et al.*, 1990). For data analysis, removal of adapters and filtering of raw reads were performed with Trimmomatic v.0.40. Classification of reads was performed using the DADA2 v.1.18 pipeline, and taxonomy was assigned using UNITE fungal ITS database v.8.2 (min. coverage for species identification ≥ 99%) within Qiime2 tool v.2020.2.

3. Results and Discussion

3.1 Culture-dependent identification

Different yeast species were detected on the skins of both grape varieties and they were identified as *Hanseniaspora uvarum*, *H. opuntiae*, *Metschnikowia pulcherrima* and *M. fructicola*. In Cabernet Sauvignon grapes, *Pichia terricola* and *H. guilliermondii* were identified only in Sicilian and Campanian sample, respectively. In all must samples, *H. uvarum*, *H. guilliermondii*, *M. pulcherrima* and *M. fructicola* were identified. Only in musts from Cabernet Sauvignon *Saccharomyces cerevisiae* and *S. pastorianus* were also identified in all samples.

3.2 Metagenomic characterization

At *phylum* level, fungal populations were very similar for both cultivars and mainly included Ascomycota (96%, 79.8% and 95.1% for Aglianico [A] samples and 83.5%, 77.15% and 88.95% for Cabernet Sauvignon [C] samples taken from Campania [BN], Molise [CB], and Sicily [CT] regions, respectively) followed by Basidiomycota (4%, 20.05% and 4.87% for Aglianico [A] samples and 16.48%, 22.54% and 11.02% for Cabernet Sauvignon [C] samples taken from Campania [BN], Molise [CB], and Sicily [CT] regions, respectively). NGS sequencing showed high heterogeneity among samples at genus level. High percentages of the genus *Hanseniaspora* spp. were recorded in Aglianico grapes from Campania and Sicily, while the genus *Sporobolomyces* spp. was detected in Cabernet Sauvignon grapes from Molise and Campania and in Aglianico grapes from Molise. A high percentage of the genus *Pichia* spp. was only observed in Aglianico grape samples from Campania.
In musts, fungal populations included mainly Ascomycota (99.91%, 93.17% and 100% for the Aglianico samples and 95.7%, 97.83% and 97.51% for Cabernet Sauvignon samples taken in Campania, Molise, and Sicily, respectively) followed by Basidiomycota (0.11% and 6.77% for Aglianico samples and 0.7%, 2.01% and 2.04% for Cabernet Sauvignon samples). NGS sequencing of the musts showed a high prevalence of the genus *Hanseniaspora* spp. in all samples. A high prevalence of the genus *Saccharomyces* spp. was observed in musts obtained from Aglianico grapes from Campania and Sicily and from Cabernet Sauvignon grapes from Molise. The greatest presence of the genus *Debaryomyces* spp. was observed only in musts obtained from Aglianico grapes from Molise. In Cabernet Sauvignon musts, a higher prevalence of the genus *Pichia* spp. was detected in comparison to Aglianico musts, where this genus was detected in low percentages only in samples from Campania. Figure 1 shows the relative abundances (%) of the microbial communities of the samples.

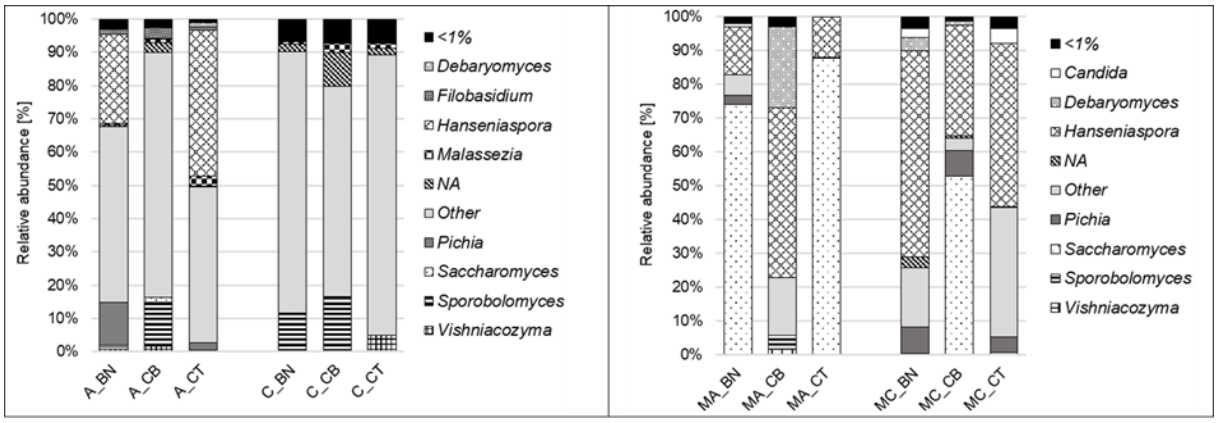

Figure 1. *Relative abundances (%) of microbial communities in grape samples (left side) and must samples (right side).*

Comparing the two approaches, substantial differences were found in terms of detected yeast populations in grapes and musts from different viticultural areas. The analyses will be supplemented with further data deriving from climatic indices and chemical analyses.

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Effect of the modified atmosphere packaging on the shelf-life of Tuscan DOP Bread

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The first activity of the PhD thesis project is described, where I focused the attention on the optimization of the M.A.P. (modified atmosphere packaging) system able to preserve the food products and delay their spoilage. In this regard, I considered the potential use of Nitrogen or Argon as storage atmospheres to improve the shelf-life of sourdough bread, while Air was selected as control.

Effetto del confezionamento in atmosfera modificata sulla shelf-life del Pane Toscano DOP

Viene descritta la prima attività del progetto di tesi di dottorato, dove ho focalizzato l'attenzione sull'ottimizzazione del sistema M.A.P. (confezionamento in atmosfera modificata) in grado di preservare i prodotti alimentari e ritardarne il deterioramento. A questo proposito, ho considerato il potenziale utilizzo di Azoto o Argon come atmosfere di stoccaggio per migliorare la shelf-life del pane a lievitazione naturale, mentre l'Aria è stata selezionata come controllo.

Key words: Bread Shelf-life, Modified Atmosphere Packaging, Tuscan DOP Bread.

1. Introduction

In accordance with the PhD thesis project previously described (Different Strategies for the Evaluation and Extension of the Shelf-life of Different Foods, 2021), this poster reports the main results of the first activities concerning:
A1) Evaluation of shelf-life: A preliminary test will be carried out in order to identify the critical chemical-physical parameters and their acceptable limits related to shelf-life of food product (A1.1) and to evaluate the optimal combinations of storage parameters (e.g., temperature, gas composition, type of package and closure) (A1.2).

2. Materials and Methods

For the production of bread, three different flours (A, B, C) obtained from a mix of different varieties of wheat (Bolero, Bologna, Verna and Pandas) were used. The milling process was made with a mill (Industry-Combi, Waldner Biotech, Lienz, Austria) and the breadmaking process starter from a pre-ferment as leavening agent prepared with sourdough. The "biga-preparation", as well as baking protocol and operating conditions (time and temperature) adopted, were performed as described by Venturi et al., 2016.
After baking, bread loaves were cooled for 2 h at room temperature, then sliced with an automatic slicing machine to 20 mm thickness. The slices were stored at 23 °C, under different M.A.P. (100% Nitrogen; 100% Argon; Air), in plastic bags (two plastic layers, outer nylon layer, Food saver, Moncalieri, Torino, Italy) with an industrial packing machine (Lavezzini 450 GAS, Fiorenzuola d'Arda, Piacenza, Italy). The sliced samples were inspected daily for weight loss, water activity, compressibility, presence of mold and sensory tasting following the methods described below. We determine: the compressibility with a penetrometer PNR-12 (Anton Paar, Rivoli (TO), Italy) according to the method described by Al Omari et al., 2016; water activity was measured with a HygroPalm HP23-AW-A equipment (Rotronic AG, Bassersdorf, Switzerland).
In order to evaluate the differences over time, the kinetic constants (k_c) for the percentage of water loss were calculated by adopting the equation of order zero ($[\%H_2O]_{t=t} = [\%\,H_2O]_{t=0} - k_c t$). At the end, all data obtained were elaborated with a statistical analysis and the significance of differences among means was determined by one-way ANOVA (CoStat, Version 6.451, CoHort Software, Pacific Grove, CA, USA).
Both at the end of the baking and daily at the time of each sampling, more tastings were carried out on the slices of bread so as to evaluate the evolutionary trend. The data obtained from these tastings were analyzed with the Big Sensory Soft 2.0 (BSS) program capable of carrying out a statistical analysis of sensory data.

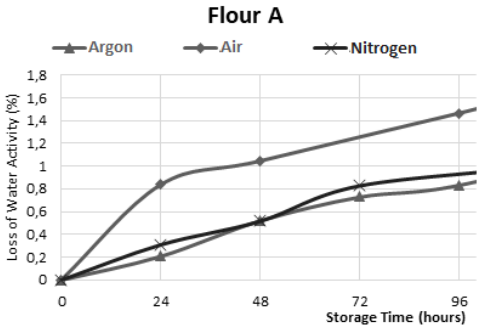
3. Results and Discussion

The statistical comparison of kinetic constants (Table 1) has confirmed how the use of Nitrogen and Argon as storage atmospheres compared to Air, reduces water loss and allows the bread to last longer. There were no significant differences in the three flours used, which showed the same behaviour, with the same gas used in storage, for all the parameters evaluated.

Table 1 *Kinetic constant (kc) related to bread water loss, in the three different storage atmospheres (Air, Nitrogen, Argon) for the three flours (A, B, C) used. Different letters indicate statistically different values (p <0.05).*

A			B			C		
Air	**Argon**	**Nitrogen**	**Air**	**Argon**	**Nitrogen**	**Air**	**Argon**	**Nitrogen**
0,0195±0,0011 a	0,0160±0,0008 b	0,0158±0,0012 b	0,0204±0,0011 a	0,0165±0,0009 b	0,0169±0,0009 b	0,0184±0,0015 a	0,0153±0,0008 b	0,0165±0,0007 b

The results of water activity have the same trend of water loss, in fact the effect of gases as storage atmospheres is confirmed. After 48 hours (Figure 1a) the Air showed a reduction of 1 % compared to the gases which is half (0.5%). The same happens for the penetrometer, index of the softness of the bread, where the data (Figure 1b) show that all the tests with the M.A.P. system compared to Air, the loss of softness is slower and in this case the bread maintains its characteristics for longer.



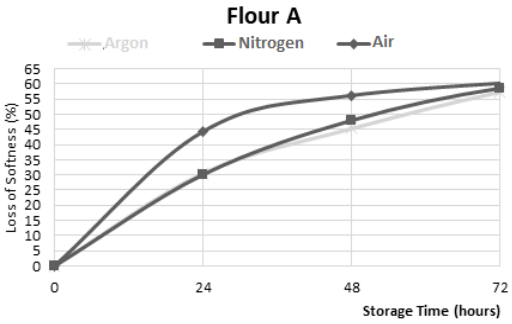
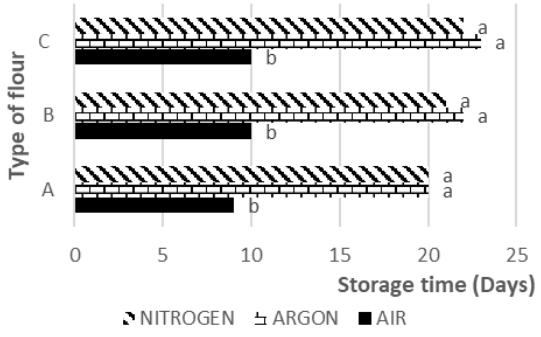


Figure 1a (left) *Percentage decrease of water activity relative to bread stored in Argon, Air and Nitrogen atmosphere for the flour A.*

Figure 1b (right) *Percentage decrease of softness relative to bread stored in Argon, Air and Nitrogen atmosphere for the flour A.*

The appearance of mold on the surface of the bread slices was selected as a parameter to define the limit of bread-shelf-life during storage. As shown in Figure 2a, the duration of the bread is extended with Nitrogen e Argon about twice as much of Air atmosphere. The shelf-life is not linked only to the chemical-physical parameters, but also to the sensorial ones. In the sensory profile after 4 days of storage (Figure 2b), only breads with Argon e Nitrogen still have a level of acceptability greater than 5 points (taken as a reference standard). Comparing the two selected gases, the use of Argon appears better not only from a chemical-physical point of view but also sensory. Because it showed a higher hedonic index of Nitrogen, managing to maintain the initial aromas of the bread.
In conclusion, the results seem to be important for the extension of the shelf-life of sourdough bread, especially with a view to reducing food waste.



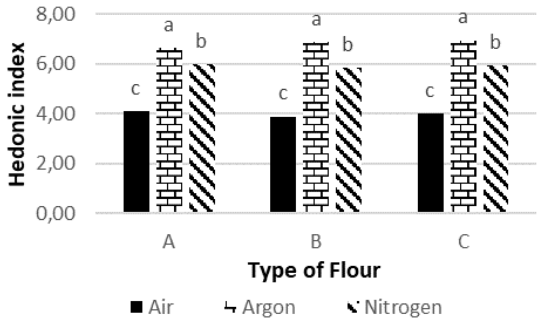


Figure 2a (left) *Shelf-life period (appearance of the first mold) in days relative to bread stored in Argon, Air and Nitrogen atmosphere for the three types of flours. Different letters indicate statistically different values (p <0.05).*

Figure 2b (right) *Hedonic index after 4 days storage relative to bread stored in Argon, Air and Nitrogen atmosphere for the three types of flours. Different letters indicate statistically different values (p <0.05).*

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Microbial exopolysaccharides as postbiotics for the development of new functional foods: optimization of yields

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Exopolysaccharide (EPS)-producer strains were screened from a database of lactic acid bacteria (LAB) cultures isolated from fermented foods. Qualitative and quantitative evaluation of EPS production from different carbon sources was carried out, and best-performing strains were identified. The effect of growth conditions (temperature, pH, sugar concentration) on EPS yields was studied.

Esopolisaccaridi di origine microbica come postbiotici per lo sviluppo di nuovi alimenti funzionali: ottimizzazione delle rese di produzione

Ceppi produttori di esopolisaccaridi (EPS) sono stati selezionati da una collezione di batteri lattici isolati da alimenti fermentati. È stata effettuata una valutazione qualitativa e quantitativa della produzione di EPS a partire da diversi zuccheri e sono stati identificati i ceppi maggiormente produttori. È stato quindi esplorato l'effetto delle condizioni colturali (temperatura, pH, concentrazione di zuccheri) sulla resa in EPS.

Keywords: Exopolysaccharides (EPS), lactic acid bacteria (LAB), sugar source, yield

1. Introduction

In accordance with the Ph.D. thesis project (Bisson, 2021), this communication reports the main results of the first two activities concerning:

(A1) **Screening of new EPS-producing microbial strains** using phenotypic methods
   a) Isolation of EPS-producing strains from different food sources
   b) Identification of positive strains using 16S rRNA sequencing

(A2) **Selection and characterization of the most interesting molecules**
   a) Small-scale cultivation of stains and application of different fermentation conditions
   b) EPS extraction, purification, and yield evaluation

2. Materials and Methods

105 LAB strains isolated from fermented foods of animal and plant origin were qualitatively screened for EPS production by observing the mucous or ropy appearance of the colonies on MRS agar containing 20 g/L of different carbon sources (glucose, sucrose, lactose, and fructose). Subsequently, positive strains were grown for 48 h at 30 °C in liquid media (MRS added with the relevant sugar), cells removed by centrifugation, and EPS precipitated with three volumes of cold ethanol at 4 °C overnight. The pellet was then separated by centrifugation at 3,000 x *g* for 10 min, washed thrice with cold ethanol, and finally resuspended in distilled water. EPS was quantified by phenol-sulphuric acid protocol (Dubois et al., 1956). Best-performing strains were then identified by 16S rRNA sequencing. The effect of cultural conditions on EPS production was then assessed in 20 mL cultures by growing the strains for 48 h in MRS adjusted for pH (6, 7 or 8), temperature (25 and 30 °C), and sugar concentration (20 and 40 g/L lactose, 100 and 150 g/L sucrose).

3. Results and Discussion

3.1 Screening of EPS-producing strains

Firstly, the strains were qualitatively screened, and 43 out of 105 strains showed to be able to produce EPS starting from at least one sugar source. 10% of strains showed to be quite versatile, producing EPS from all the carbon sources tested. Most strains produced EPS starting from sucrose, whereas fructose was less used. The quantitative screening revealed a normal distribution of the producer strains based on EPS amount, and most of the strains produced between 300 and 1,000 mg/L (Fig.1). After the quantitative screening, some producer strains were selected for identification (Table 1). The strains belonged to *Leuconostoc mesenteroides*, *Lactococcus lactis*, and *Lactiplantibacillus plantarum* species, which ability to produce EPS is widely documented (Zhou et al., 2019).

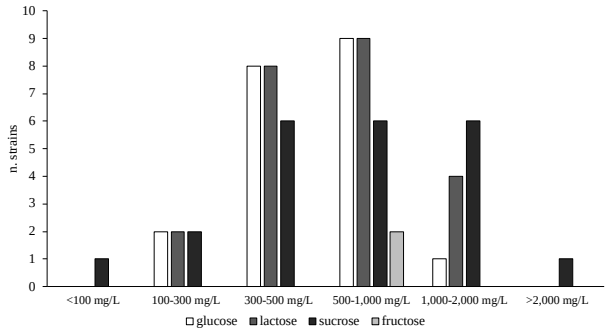

Figure 1 *EPS production in presence of different carbon sources*

Table 1 *Identification of best-performing strains and EPS yields*

Strain	Species	Carbon source	EPS yield (g/L)
FP_01A4	*Lactococcus lactis*	Lactose	0.72±0.05
FP_02A2	*Lactococcus lactis*	Lactose	0.79±0.01
F_02A5	*Leuconostoc mesenteroides*	Sucrose	1.55±0.49
B3	*Leuconostoc mesenteroides*	Sucrose	1.88±0.59
PM_01A5	*Leuconostoc mesenteroides*	Sucrose	1.35±0.23
BRO_01A7	*Lactiplantibacillus plantarum*	Lactose	0.59±0.04

3.2 Optimization of EPS yield for selected strains

The effect of incubation temperature, sugar concentration, and pH on the EPS yield of six selected strains (Table 1) was studied. For all the strains, no statistically significant effect of incubation temperature on EPS production was observed, and the same for sugar concentration (data not shown). Instead, the initial pH was found to be a parameter that significantly influenced the EPS yield. In *Leuc. mesenteroides* grown in presence of 100 g/L sucrose as pH increased, EPS yields significantly rose (Fig. 2a). At pH 8, the yield doubled in *Leuc. mesenteroides* PM_01A5 and F_02A5 respect to pH 6, and in the case of *Leuc. mesenteroides* B3 the yield increased by more than four times.

As for *Lc. lactis* and *Lb. plantarum*, the effect of pH on EPS amount was studied in MRS with 20 g/L of lactose (Fig. 2b). For *Lc. lactis* the pH effect was strain-dependent. *Lc. lactis* FP_01A4 yields were significantly higher at pH 6 and 8, while *Lc. lactis* FP_02A2 yielded the maximum EPS amount (1.11±0.06 g/L) when the initial pH was 8. These results are of interest because for this species lower yields are more frequently observed (Li et al., 2022). The effect of initial pH was also appreciable for *Lb. plantarum*. The highest EPS yields (0.85 and 0.8 g/L) were detected when the initial pH was 6 and 8, respectively.

These results are of relevance since yield is one of the limiting factors in EPS exploitation for technological and functional uses. In the following steps of the research activity, it will be relevant to identify the microbial polymers and characterize them both chemically and in terms of bioactivities.

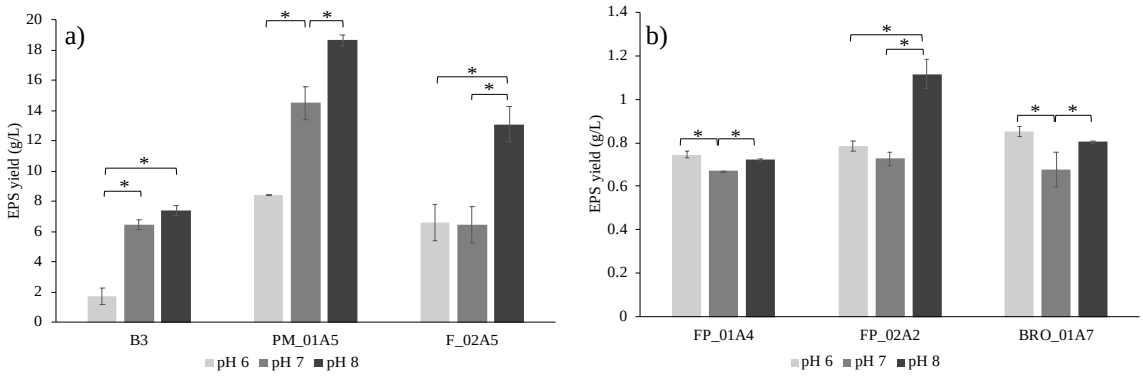

Figure 2 *Effect of pH on EPS yield by Leuc. mesenteroides (a), and Lc. lactis and Lb. plantarum (b)*

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Are phenolipids able to counteract lipid oxidation in oil-in-water emulsions?

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The aim of this PhD project is to develop innovative healthy foods using lipophenols as a strategy to preserve bioactive compounds, such as phenolic compounds and saturated short chain or polyunsaturated fatty acids, from degradation and increase their bioavailability. Nevertheless, since the foods represent a complex system, preliminary studies in model systems are required; thus, oil-in-water emulsions were selected to best model the food that will contain them. Therefore, the results related to the first part of the project, concerning the evaluation of the physical and oxidative stability of 1% oil-in-water emulsions added with lipophenols with different chain lengths, are reported.

I lipofenoli sono in grado di contrastare l'ossidazione lipidica in emulsioni olio-in-acqua?

L'obiettivo di questo progetto di dottorato è sviluppare alimenti salutari e innovativi utilizzando la lipofenolizzazione come strategia per preservare dalla degradazione composti bioattivi, quali composti fenolici, e acidi grassi saturi a corta catena o polinsaturi, e aumentarne la biodisponibilità. Tuttavia, risulta di primaria importanza lo studio del comportamento dei lipofenoli in sistemi modello, come le emulsioni olio-in-acqua, al fine di selezionare al meglio gli alimenti che li veicoleranno. Vengono quindi riportati i risultati relativi alla prima parte del progetto, riguardante, la valutazione della stabilità fisica e ossidativa di emulsioni olio-in-acqua addizionate con diversi lipofenoli caratterizzati da differente lunghezza della catena alchilica.

Key words: Lipophenols, oil-in-water emulsions, oxidative stability, bioactive compounds, healthier foods.

1. Introduction

Bioactive compounds such as saturated short chain or polyunsaturated fatty acids (PUFAs) and phenolic compounds have become of particular interest as a result of consumer demand for healthier foods. However, the intake of PUFAs and phenolic compounds is dramatically low since they are prone to oxidation and poorly assimilated at the intestinal tract due to their hydrophilicity, respectively. Their conjugation (the so-called lipophenols) could therefore represent an effective delivery system, potentially increasing their intake and beneficial effects in the human body. Considering that hypothesis, the present poster provides the main results of the evaluation of two different synthetic phenolipids, namely butyl ferulate (BF) and octyl ferulate (OF), to counteract lipid oxidation in model systems such as 1% oil-in-water emulsions at pH 7 and pH 3.5.

2. Materials and Methods

The oil-in-water (O/W) emulsions were prepared according to Cardenia et al. (2011) employing 1.0% (*w/w* of continuous phase) of stripped sunflower oil, 10 mM phosphate buffer solution (pH 7 and 3.5), and 0.10% of Tween 20 (*w/w* of dispersed matter*)* as non-ionic emulsifier. BF and OF were added at different concentrations: 0.05%, 0.10%, 0.15%, 0.20%, and 0.25% on the total oil weight (*w*/*w*), although samples without lipophenols (control) were also prepared. A coarse emulsion obtained by Ultra-Turrax (18000 rpm, 2 min) was homogenized by ultrasonic sonicator (200 W; 100 Hz) for 3 min and maintaining the temperature of emulsions lower than 35 °C. One millilitre of each emulsion was added into 20 mL headspace vials (previously acid washed) and stored in darkness at 35 °C for 14 days. The particle size distribution and droplet surface charge (ζ-potential) measured by dynamic light scattering (DLS) were used to define the physical behaviour of emulsions, while the oxidative stability was evaluated by determining the peroxide value (PV) according to Shantha and Decker (1994) and hexanal and nonanal content by headspace solid-phase microextraction (HS-SPME) combined with gas chromatography coupled with mass spectrometry (GC/MS). Three independent experiments (n=3) were conducted. Statistical analysis of data was performed using SPSS 25.0 (IBM-SPSS Inc., Chicago, Illinois, USA). Analysis of variance (ANOVA) was carried out to investigate the effect of storage and concentration on physical and oxidative emulsions behavior as related to the type of lipophenol. Duncan's honest significance test was carried out at a 95% confidence level to separate means of parameters; p-values < 0.05 were considered statistically significant.

3. Results and Discussion

At pH 7, the particle size distribution was affected by the presence of lipophenols (p<0.05). In fact, progressively smaller particles were obtained by increasing the concentration of both BF and OF (Fig. 1), making the emulsions more physically stable. Nevertheless, no change in particle sizes was observed during the whole experiment (p>0.05), showing that aggregation, coalescence, or flocculation did not occur during the storage. On the contrary, at pH 3.5, no differences were observed between the control and the emulsions with BF and OF (p>0.05), even if a general decrease in particle size from day 0 to day 14 was found (p<0.001). It might be hypothesized that at pH 3.5 lipophenols are concentrated behind the surfactant layer, playing a crucial role on the reduction of droplets superphysical tension (McClements and Decker, 2018). All the emulsions displayed negative ζ-potential at both pH, which significantly (p<0.05) negatively increased during the storage. As expected (Cardenia et al., 2011; McClements and Decker, 2000; Tian et al., 2022), higher ζ-potential values were reached in neutral environment (-58 mV) than in the acidic one (-6 mV), which lead to higher physical stability, but could worsen the oxidative one by attracting positively charged transition metals, initiators of oxidative reactions (McClements and Decker, 2018; Tian et al., 2022). However, the increase in negative surface charge was significantly less pronounced in samples with BF or OF, possibly suggesting that they locate at the O/W interface. In fact, the pK_a of tested lipophenols is about 8.5, thus maintaining a positive charge at both pH values.

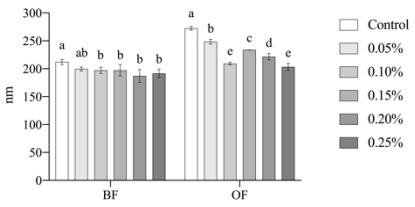

Fig. 1 Particle size distribution of emulsions added with BF and OF at day 14 at pH 7. Each bar represents the mean ± standard deviation (n=3), and results of ANOVA with Duncan's post hoc test are reported.

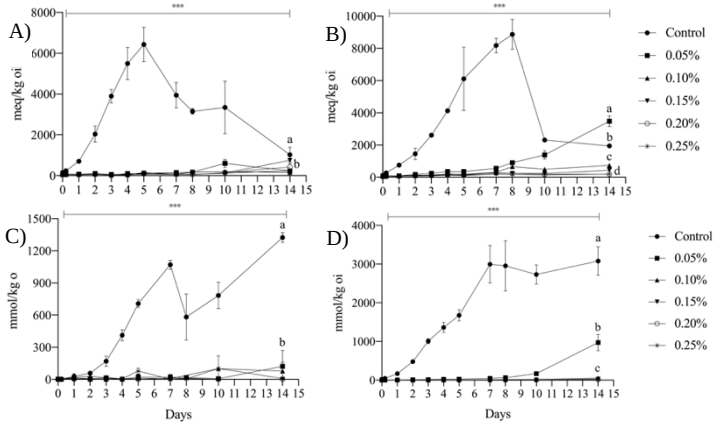

Fig. 2 Hydroperoxides content (meq/kg oil) and hexanal content (mmol/kg oil) in the 1% O/W emulsions without (control) and with butyl ferulate (A and C, respectively) and octyl ferulate (B and D, respectively) at different concentrations at pH 3.5 throughout the storage. Each point represents the mean ± standard deviation (n = 3), and results of ANOVA and Duncan's post hoc test (between samples, within day 14) are reported.

The phenolipids proved to effectively retard lipid oxidation at both pH values, and for both BF and OF; in addition, the antioxidant activity was significantly correlated to their concentration. BF was particularly effective in maintaining low PV during the entire experiment, elongating the lag phase from day 1 (control) up to day 10 and day 14 when added at more than 0.20% at pH 7 and pH 3.5, respectively. On the other hand, after 4.5 h of storage, in control samples at pH 3.5 the PV was 5 times higher (238.7 ± 4.67 meq/kg oil) than that of emulsions with 0.25% BF (52.24 ± 8.13 meq/kg oil) (Fig. 2). The acid environment was again the most favourable for the antioxidant activity of OF; however, OF led only to a slight extension of the induction time, but still keeping the PV much lower than the control (Fig.2). In fact, the maximum PV reached by the control was more than 45 times higher than the maximum PV observed in the emulsion with 0.25% OF (193.95 ± 27.06 meq/kg oil) (p<0.001). A similar behaviour was found between BF and OF for both hexanal and nonanal, which were detected at high levels in the control already after 4.5 h of storage, while their development was drastically reduced (p<0.001) as the phenolipids concentration raised (Fig. 2). In conclusion, both BF and OF proved to be successful in counteracting both primary and secondary oxidation products of PUFAs, especially in acidic environments. In addition, higher physical stability at pH 7 was also observed when the lipophenols were employed. Prospects include the study of additional lipophenols with different alkyl chain length, to keep investigating its potential impact on antioxidant activity, as well as the study of lipophenols recovered from natural sources such as wheat bran, naturally rich in alkyl resorcinols. The latter is currently under analysis and, for this purpose, a method for the extraction and purification of alkyl resorcinols, as well as a fast GC/MS method for their identification and quantification is in the process of being validated.

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Assessment of bioactive and contaminant compounds in underexploited agri-food wastes and new food products through targeted and non-targeted approaches

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The first two activities of the PhD thesis project are described. Raw extracts rich in bioactive compounds (stilbenes) were obtained from the vine shoots of Italian grape varieties. Characterization of extracts was initially performed by applying traditional targeted approaches (HPLC-DAD) to acquire a reference dataset. Hence, a direct non-destructive determination method is being developed through an untargeted technique such as fluorescence spectroscopy.
The second activity involved monitoring the presence of acrylamide in innovative foods, as it is considered a potential human carcinogen and one of the process contaminants of greatest interest.

Valutazione di composti bioattivi e contaminanti in scarti agroalimentari sottoutilizzati e nei nuovi prodotti alimentari attraverso approcci targeted e non-targeted

Vengono descritte le prime due attività del progetto di tesi di dottorato. Dai tralci di varietà di vite italiane sono stati ottenuti estratti grezzi ricchi di composti bioattivi (stilbeni). Gli estratti sono stati caratterizzati inizialmente attraverso metodi tradizionali (HPLC-DAD) per acquisire un dataset di riferimento. Quindi, si sta procedendo allo sviluppo di un metodo di caratterizzazione diretta e non distruttiva mediante spettroscopia di fluorescenza.
La seconda attività ha riguardato il monitoraggio della presenza di acrilammide in alimenti innovativi. L'acrilammide è ritenuta un potenziale cancerogeno per l'uomo ed uno dei contaminanti di processo di maggiore interesse.

Key words: wine shoots, stilbenes, resveratrol, viniferin, fluorescence spectroscopy, acrylamide.

1. Introduction

In accordance with the PhD thesis project, this poster reports the main results of the activities carried out, as follows:

(A1) Targeted and untargeted characterization of vine shoots rich in bioactive compounds, such as stilbenes. The predominant stilbenes in vine shoots are *trans*-resveratrol (Rsv) and *ε*-viniferin (Vf);

(A2) Sustainable food risk mitigation strategies: assessment of acrylamide (AA) contamination in innovative foods.

2. Materials and Methods

2.1 Extraction and characterization of stilbenes.

The extraction and characterization steps of stilbenes were performed as reported by Noviello *et al*. (2022) on different Italian varieties of *Vitis vinifera* L. Subsequently, the excitation/emission matrices (EEMs) of the extracts were collected in "right angle" acquisition geometry by a Fluoromax 4 spectrometer (Horiba Scientific, New Jersey, USA). The excitation and emission slits were 2 nm. The acquisition interval and the integration time were maintained at 5 nm and 1 s respectively. EEMs were obtained by recording emission spectra in the range from 240 to 500 nm and excitation wavelengths from 220 to 360 nm. During each measurement, the raw signal (S), the corrected one (Sc) and the corrected reference signal (Rc) were acquired. The final signal used for further elaborations was the corrected and normalized one (Sc/Rc). A final dataset of 52 samples was obtained.

2.2 Assessment of acrylamide contamination in innovative foods.

A total of 19 plant-based protein ingredients (PBPI) of different origin and type were collected and then analyzed for their content of acrylamide. The specimens are divided in four categories as follows: 3 native legume flours (F), 6 dry-fractionated protein concentrated (DF), 6 protein concentrates/isolates produced by wet extraction (WE), 4 texturized vegetable proteins produced by extrusion cooking (TVP). All the sample, once collected, were stored at -20 °C until the analysis. Acrylamide extraction was carried out according to Mastovska *et al*. (2006) with minor modifications. Acrylamide analysis was performed using an UltiMate 3000 UHPLC system (Thermo Fisher Scientific, MA, USA) interfaced with an electrospray ionization chamber (H-ESI) and an LTQ Velos Pro mass spectrometer. Data were acquired in positive ionization mode (ESI+) using the optimized instrument parameters obtained from the tuning procedure. The transitions m/z 72 → 55 and 75 → 58 were monitored for acrylamide and for d_3-acrylamide, respectively. A signal-ratio calibration curve was built up using the relative response of

acrylamide vs d_3-acrylamide. The accuracy of the method was verified by using a certified reference material (Acrylamide in crispbread, ERM®-BD272, BAM, Berlin).

2.3 Statistical analysis.

The analysis of fluorescence data was performed in MATLAB R2021a (The MathWorks, Inc.) using the PLS-toolbox (Eigenvector Research, Inc.). Preliminary results were obtained by using parallel factor analysis (PARAFAC) to decompose EEMs in the contributions of different fluorescent compounds. Non-negative constraints have been applied to all the modes. Core consistency diagnostics and split-half analysis were used for finding the optimal number of components in the PARAFAC models. N-way PLS regression (NPLS) have been used to model the relationship between fluorescence data and concentrations.

For the assessment of acrylamide contamination levels, the descriptive statistics of the dataset were calculated by using Minitab 17 (Minitab Inc., State College, PA, USA). Then, the data were subjected to the one-way analysis of variance ANOVA followed by Tukey's honestly significant differences test for multiple comparisons at a significance level $\alpha = 0.05$.

3. Results and Discussion

3.1 Extraction and characterization of stilbenes.

The results obtained from the characterization of vine shoots extracts carried out by the reference approach (HPLC-DAD) showed an average stilbenes concentration of about 4500 mg kg^{-1} DW, with a significant difference between the selected varieties, ranging from 2700 to 6400 mg kg^{-1} DW. This is consistent with literature results, where variation in stilbenes concentrations is strongly dependent on variety. The characterization of vine shoots extracts served to create a reference dataset for the subsequent development of an untargeted method of analysis of *trans*-resveratrol. Indeed, raw extracts were then analyzed by using fluorescence spectroscopy. A standard *trans*-resveratrol solution (100 mg L^{-1} ethanol:water 80:20 v/v) showed a maximum fluorescence intensity at the excitation/emission wavelengths of 350 nm and 380 nm, respectively, consistent with the data reported by Vitrac *et al*. (2002). However, the EEMs of raw extracts showed shifts of the absorption maximum toward longer excitation/emission wavelengths respect to the reference standard. Therefore, they were diluted by a factor of 1:100 to reduce phenomena related to the "inner filter" effect. A promising PARAFAC model with three components was identified. The first component was characterized by excitation/emission maxima at 300/380 nm, showing a fluorescence signal similar to that of standard *trans*-resveratrol. The scores contribution profile (Fig. 1) and the N-PLS results confirmed the promising relationship between the first component and *trans*-resveratrol concentration in raw extracts.

3.2 Assessment of acrylamide contamination in innovative foods.

The presence of AA has been well monitored for some well-known product categories (EFSA, 2015). However, there are innovative food products on the market of which limited information are available. This is the case of PBPI from cereals and pulses which are increasingly used in the food industry as ingredients for plant-based foods. The highest acrylamide content was found in TVP, while the lowest content was found in native legume flour (F). An intermediate level was found for the other two classes. A possible explanation of the considerable variability of acrylamide content can be traced back to factors such as: i) the content of precursors presents in raw materials and ii) process variables (e.g., temperature, time, humidity, pressure, extrusion) used in production technologies. To date, investigations are underway to confirm the data.

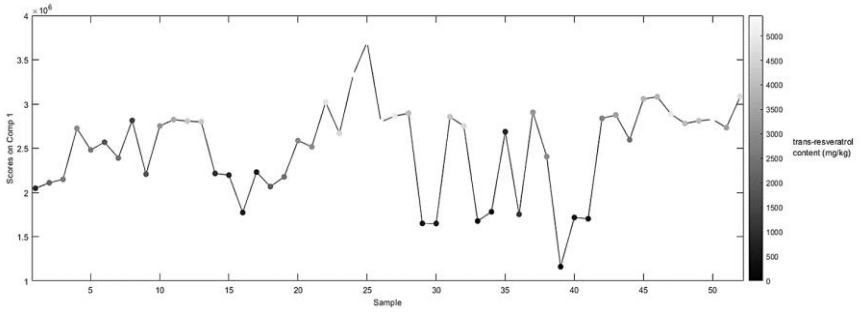

Figure 1 *Results of the PARAFAC analysis. Relation between the scores on component 1 and the trans-resveratrol content of the analysed samples.*

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Biotechnological valorization of residues and by-products from agro-food industries

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Some of the activities of my PhD thesis project are described. The chemical characterization of food by-products (*i.e.* pomegranate and citrus residues and *Equisetum arvense*) such as their phenolic content and volatile profile was carried out; these results were related with their functional activities as the antimicrobial and the antioxidant ones. The most promising by-products were used as ingredients directly added to a food, *i.e.* 'Primo Sale' cheese.

Valorizzazione biotecnologica di residui e sottoprodotti dell'industria agro-alimentare

In questo documento sono descritte alcune delle attività del mio progetto di tesi di dottorato. In, particolare, è stata effettuata la caratterizzazione chimica di alcuni sottoprodotti alimentari (residui di melograno e agrumi e *Equisetum arvense*) quali il contenuto fenolico ed il profilo in molecole volatili; questi risultati sono stati messi in relazione con le loro attività funzionali (antimicrobica e antiossidante). I sottoprodotti più promettenti sono stati utilizzati come ingrediente aggiunto direttamente ad un alimento: il formaggio "Primo Sale".

Key words: By-products, antimicrobial activity, antioxidant activity, volatile compounds, 'Primo Sale' cheese

1. Introduction

According to Eurostat (2020), the agro-food industry yearly generates 22.4 million tons of by-products only in Europe, which are disposed, used as feeds or for energy production. However, they are still rich in bioactive compounds having functional importance, *e.g.* antimicrobial and antioxidant activity (Agourram *et al*., 2013). Their valorization as functional ingredients can be a way to reduce industrial food waste and the basis to formulate novel foods or improve safety and shelf-life of traditional ones. According to the PhD thesis project, the main results related to citrus and pomegranate by-products (BPs) and a weed plant *i.e. Equisetum arvense* are reported:

1. Chemical and functional characterization to understand how they work *in vitro*
2. Their use as ingredient for the formulation of a 'Primo Sale' cheese

2. Materials and Methods

Peels and seeds of edible and ornamental pomegranate (*Punica granatum*), collected in 2 different Italian regions, were used: edible fruits' peel from a) Emilia-Romagna (Ed_Pome_ER) and b) Marche (Ed_Pome_MA), c) edible fruits' seed from Marche (Seeds_MA), d) mixed peels and arils of ornamental plants from Marche (Orn_Pome_MA). Citrus pomace (Citrus) was from a local industry producing orange juice. *Equisetum arvense* was collected in 2019 (Eq_2019) and 2021 (Eq_2021). Samples were air dried (Eq) or freeze-dried (Citrus and pomegranate BP) and grinded. The methanolic extracts (Ferioli and D'Antuono, 2016) were tested for total phenolic content (TPC, Folin-Ciocalteau assay) and DPPH (0.1mM) scavenging activity (Abid *et al.*, 2017). The results were expressed as gallic acid equivalent (GAE (mg/l)) and IC50, respectively. The extracts were tested against 48 strains (starters/probiotics, pathogens and yeasts) belonging to the collection of DISTAL- University of Bologna, with the agar diffusion method (Rao *et al*., 2016). The ability to support the growth of 12 lactic acid bacteria (LAB) (commercial probiotics and DISTAL collection strains) was assessed on TPY agar medium added with the dried (not extracted) BPs (2% w/v). Glucose (1.5% w/v), fructooligosaccharides (FOS, 2% w/v) or inulin (2% w/v) were supplemented as controls. Dried samples were characterized for volatile compounds through SPME-GC/MS analysis (Burns *et al.*, 2015). Citrus (2% w/w) and Eq (1% w/w) were used as ingredients for the production of "Primo Sale" cheese added also with LAB strains (*Lactiplantibacillus plantarum* B39.1.4A, *Pediococcus pentosaceus* B39.2.2A, *Enterococcus faecalis* B39.2.2B) (~ 7 logCFU/g). A challenge test was performed by inoculating the cheeses with *Listeria monocytogenes* 56LY (~ 4 logCFU/g), and viability of the pathogen and native microbiota was monitored during 29 days of storage at 4°C.

3. Results and Discussion

3.1 By-products characterization

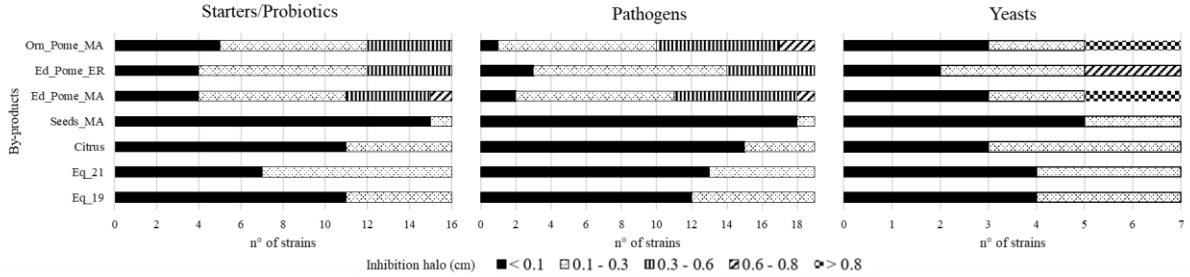
Pomegranate peel extracts were the samples with the highest TPC values ranging between 3500 and 6300 GAE (mg/l), while seeds showed the lowest content with 112 GAE (mg/l), being such values comparable to those reported by Abid *et al.* (2017) (Table 1). Intermediate values were detected for both Eq samples close to 430 GAE (mg/l). The TPC data are in accordance with the scavenging activity: in fact, the samples with the highest phenol

contents presented the lowest IC50 values (Table 1). Regarding the antimicrobial activity, almost all the tested strains were sensitive to all the BPs with differences in relation to the microbial species. Ed_Pome_MA, Ed_Pome_ER and Orn_Pome_MA were the most active ones against the pathogens which showed the highest mean inhibition halos (Figure 1). On the other hand, Eq and Citrus samples were more effective against yeasts. Overall, LAB strains were resistant to most of the tested substances (Figure 1). When cultivated in the presence of Citrus and Eq, used as carbon source, LAB showed a behavior similar to that with the commercial prebiotics (FOS and Inulin). However, 3 out of 12 strains (also including a commercial probiotic) showed limited growth with Ed_Pome_MA, Ed_Pome_ER and Orn_Pome_MA. Overall, such data can be explained by considering the TPC values and the analysis of volatile compounds of these BPs showing high amounts of terpenes, *e.g.* D-limonene and (S)-D-carvone, which are widely recognized as antimicrobial agents (Aggarwal *et al.*, 2002).

Table 1 *Comparison between total phenolic content (GAE (mg/l)) and IC 50 (µl) of methanolic extracts of the by-products.*

	Pomegranate				Citrus	*E. arvense*	
	Ed_Pome_MA	**Ed_Pome_ER**	**Orn_Pome_MA**	**Seeds_MA**	**Citrus**	**Eq_19**	**Eq_21**
TPC	4725 ± 1332	3501 ± 420	6328 ± 484	112 ± 16	233 ± 67	446 ± 37	448 ± 21
IC50	1.45	1.91	0.87	298.00	2221.00	101.40	94.54
R^2 (IC50)	0.953	0.959	0.970	0.979	0.988	0.994	0.998

Figure 1 *Mean inhibition halos (cm) of the by-products against starters/probiotics, foodborne pathogens and yeast strains.*



3.2 Real system – "Primo Sale" cheese

Regarding "Primo Sale" cheese, the viability of the functional strains during storage did not change in cheeses added with Citrus or Eq similarly to the control ones (Figure 2). On the other hand, the supplementation with Citrus limited the growth of *L. monocytogenes* 56LY during storage with final counts of 1 log unit lower than the control samples (Figure 2). Such preliminary data suggests that such BPs are promising functional ingredients to increase safety and quality of "Primo Sale" cheese.

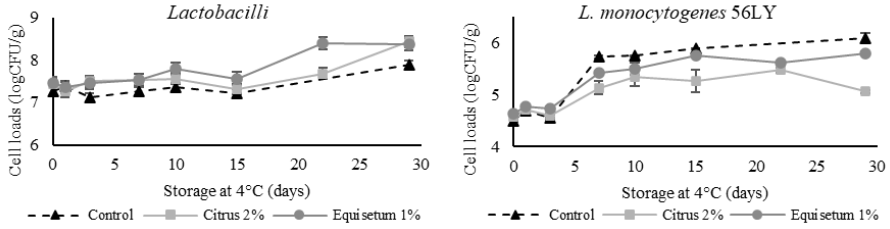


Figure 2 *Cell loads of Lactobacilli and* L. monocytogenes *56LY during storage of "Primo Sale" cheese added with Citrus (□), Equisetum (○), compared with control (Δ).*

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Physical, and microstructural properties of dairy dessert creams: effect of composition and frozen storage

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The first activity of the Ph.D. focused on the characterization of the physical, physicochemical and microstructural properties of dairy dessert creams having as main ingredient mascarpone cheese prepared according to the formulation and procedure typical of a traditional Italian recipe ("tiramisu"). Products were prepared by using two different mascarpone cheeses. Properties of mascarpone and corresponding dessert creams were analysed just after the opening and preparation, respectively; Although, creams were frozen (-18°C) and evaluated after 7- and 30 days of storage.

Studio delle proprietà fisico-chimiche, fisiche, e microstrutturali di desserts a base di latte: effetto della composizione e dello stoccaggio in congelamento

La prima attività della tesi di dottorato è incentrata sulla caratterizzazione delle proprietà fisiche, fisico-chimiche e microstrutturali di creme dessert a base di latte in cui il mascarpone è l'ingrediente principale, preparate secondo formulazione e procedura di una ricetta italiana tradizionale ("tiramisù"). Per la preparazione sono stati utilizzati due diversi mascarponi commerciali. I mascarponi e corrispettivi desserts sono stati analizzati appena dopo l'apertura e la preparazione, rispettivamente. Inoltre, le creme sono state congelate (-18°C) e valutate dopo 7 e 30 giorni di stoccaggio.

1. Introduction

In accordance with the aim of the Ph.D. thesis project previously described (1st telematic workshop Palermo, 2021), this poster reports the main results of the first activity:(A1): Study of the role of the ingredients and effect of solutes on freezing.

2. Materials and Methods

Two dairy dessert creams (D1, D2) were made at laboratory scale according to recipe of the traditional Italian dairy dessert "tiramisu" according to the following formulation: mascarpone cheese (61.3%), fresh egg white 17.2 %, fresh egg yolk 9.2 %, sugar 12.3 %). Two commercial mascarpone cheeses (M1, and M2) were used, respectively. The D1 and D2 dessert samples, were characterized, just after preparation and after 7 and 30 days of frozen storage (-18°C) by evaluating chemical, physicochemical, physical, and microstructural properties. Moisture and a_w were assessed by gravimetric analysis (oven, 105°C, overnight), and dew point water activity meter, respectively. pH value and total soluble solids (TSS) of the aqueous phase, obtained after centrifugation (5200 rpm, 10 minutes) were determined by using a pH meter and a refractometer, respectively. Rheological properties were assessed by using a rheometer (MCR 302, Anton Paar, Graz, Austria) equipped with a plate-plate geometry (d: 50 mm, 2 and 1 mm gap for M and D samples, respectively) at 4°C. Frequency sweep analyses were carried out in the 0.1 to 10 Hz range and 0.2% of strain, preliminarily determined by amplitude sweep test. Flow curves were determined by steady shear measurement, from 1 to 100 s^{-1}. Experimental results modeled with the Herschel-Bulkley equation (Arancibia et al., 2015):

$$\tau = \sigma_0 + k\dot{\gamma}^n \quad (1)$$

Thermal analyses were carried by Differential Scanning Calorimetry (DSC, Perkin Elmer DSC 8500, Waltham, Massachusetts, US) by using the following thermal conditions and steps: (1) from 30 to 60°C (20°C/m), (2) 1 min at 60°C, (3) from 60°C to -80°C (10°C/m), (4) 5 min at -80°C and (5) from - 80°C to 100°C (10°C/m). Particle size analysis was performed on samples preliminarily dissolved (0.1% (p/v) in water Cand (2) 1% sodium dodecyl sulfate (SDS) solution. The flocculation degree was also evaluated and calculated by the following equation (Balaghi and Senge, 2014):

$$Flocculation\ degree\ (\%) = \left[\frac{D_{43W} - D_{43SDS}}{D_{43SDS}}\right] * 100 \quad (2)$$

Confocal laser scanning microscopy (CLSM, mod. A1r+, Nikon Wetzlar, Germany) was carried out after the staining of samples with 10 µl of a 0,02g/L Nile Red (NR) in PEG 200 solution to dye the fat phase. All analyses have been performed in triplicate and data are expressed as mean ± standard deviation. One-way ANOVA

(XLSTAT software) was applied to the results of the samples during frozen storage. Significant differences between individual samples were determined by Tukey's test (P = 0.05).

3. Results

3.1 Fresh mascarpone cheeses and dessert creams characterization

Table 1: *Chemical and physicochemical properties of mascarpone cheeses (M1 and M2) and corresponding dairy creams (D1 and D2)*

Sample	*Moisture (%)*	*TSS (°Bx)*	*pH*	a_w
M1	55.45±2.36^{a}	8.67±0.46^{b}	6.40±0.01^{c}	0.993±0.001^{b}
D1	53.22±0.95^{ab}	28.33±0.51^{b}	6.78±0.09^{a}	0.981±0.000^{c}
M2	53.92±0.15^{ab}	8.33±0.06^{a}	5.97±0.03^{d}	0.998±0.000^{a}
D2	50.39±4.08^{b}	28.23±0.15^{b}	6.64±0.02^{b}	0.982±0.001^{c}

M1 and M2 samples presented significant moisture and physicochemical properties index of two different process conditions that affected also the results of the corresponding dairy dessert creams (Table 1)
Frequency sweep analyses evidenced a significantly higher and almost double elastic module G' of M2 in respect to M1 ($6*10^4$ Pa and $4*10^4$ Pa, respectively) but almost similar tanδ and results could be related to the corresponding moisture and pH. The rheological properties of the corresponding dessert creams highlighted a significant decrease of G' due to the effect of the other ingredients while tanδ resulted the same for D1 and D2(data not shown). Flow curves results modeled by Herschel-Bulkley highlighted that D2 expresses higher pseudo-plasticity, with lower σ0 values due to lower rigidity.
Thermal analysis was used to characterize M and D samples for the first- (fat crystallization and melting, water solid-liquid transition) and second-order (glass transition temperature Tg, and ΔCp) that were observed with some differences in all samples. M1 and M2 samples showed a single fat crystallization peak and two melting peaks with no significant difference in enthalpy and peak temperatures; similar behavior occurs in D samples with only a decrease of the enthalpy, meaning that egg fats do not influence the fat state transitions; on the contrary, D samples showed a significant increase of the glass transition onset temperature and increase of ΔCp, along with a significant decrease of the freezable water content.
Mascarpone samples showed a similar size of the lipidic dispersed phase with D[4,3] values lower than 10μm when dissolved in SDS solution. . Dissolution in water showed a similar increase resulting in a flocculation index higher than 100% for both samples, higher for M1. The D[4,3] values of the D1 and D2 were significantly higher than the corresponding mascarpone samples due to the presence of egg's fats, with a flocculation index higher than 50% (higher for D1).
CLSM images (Figure 1) confirmed the particle size measurements and rheological evaluations with higher particle dispersion for M1 in respect to M2, probably due to different process conditions. A similar trend could be observed for the corresponding dessert creams, with D1 showing a higher particle dispersion degree.

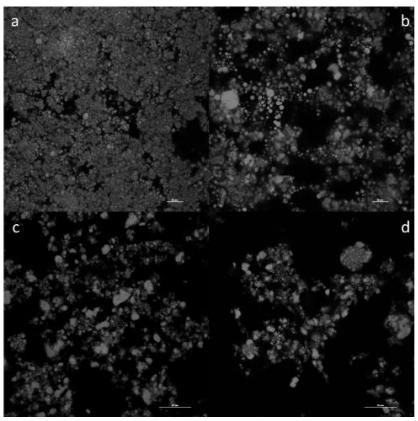

Figure 1: *CLSM images of mascarpone cheese (a: M1; b: M2) and dessert (c:D1; d:D2) samples. Fats, stained with nile red, are shown in grey. Scale bar: 10 μm (a and b), and 20 μm (c and d).*

3.2 Effect of frozen storage on dessert samples

Freezing and frozen storage of dessert cream samples didn't affect moisture and a_w while a time-dependent increase of pH was observed. In both D1 and D2, an increase of G' modulus and consequent decrease in tanδ values was noticed after 7 days followed by a decrease after 30 days of frozen storage for both samples even if of different entity, likely due to protein aggregation and degradation induced by the frozen state No significant differences on the particle size distribution and DSC analysis were seen.

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Oil structuring for improving healthy and sustainable diets: The case study of extra virgin olive oil oleogelation

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The aim of this PhD research project is to develop novel and sustainable strategies to structure liquid oils into pseudoplastic materials, called oleogels, to be used as saturated fat substitutes or as functional components able to modulate lipolysis during human digestion as well as deliver bioactive lipophilic molecules. In this context, the use of extra virgin olive oil (EVOO) as a target oil to be gelled would be particularly interesting due to its well-recognized health-promoting capacity. In this case study, EVOO-based oleogels were developed by using different gelation strategies. The oleogels, after structural characterization, were *in vitro* digested to study the impact of oil structure on the free fatty acids (FFA) release and polyphenols' bioaccessibility.

Strutturazione di olio come strategia per favorire diete salutari e sostenibili: il caso studio della gelificazione dell'olio extra vergine di oliva

Lo scopo del progetto di ricerca del dottorato consiste nello sviluppare strategie innovative e sostenibili volte alla strutturazione di oli, liquidi a temperatura ambiente, in materiali pseudoplastici, chiamati oleogel, ed utilizzabili come sostituti di grassi saturi o come ingredienti funzionali capaci di modulare la lipolisi durante la digestione e veicolare molecole bioattive. In questo contesto, l'impiego di olio extravergine di oliva come olio da gelificare risulta particolarmente interessante in virtù dei suoi ben noti effetti benefici sulla salute. In questo caso studio, oleogel di EVOO sono stati sviluppati utilizzando differenti gelificanti. Gli oleogel, dopo caratterizzazione delle proprietà fisiche, sottoposti a digestione *in vitro* per valutare l'effetto della struttura sul rilascio di acidi grassi e la bioaccessibilità dei polifenoli.

Keywords: oleogel, extra virgin olive oil, bioaccessibility, lipolysis, structure, polyphenols.

1. Introduction

In accordance with the PhD thesis project previously described (Ciuffarin, 2021), this poster reports the main results of the following activities:

(A1) Study of strategies for oil structuring (e.g., methods and selection of gelators).
(A3) Evaluation of the effect of oleogelation on the gastrointestinal behavior of oleogels by determining the bioaccessibility of selected bioactive molecules (e.g., polyphenols) as well as the lipolysis degree by using *in vitro* digestion methodologies.

2. Materials and Methods

Oleogels were obtained by adding 10 % (w/w) of saturated monoglycerides (MG), rice bran waxes (RW), sunflower waxes (SW), and β-sitosterol/γ-oryzanol mixture (PS) in EVOO heated at temperatures higher than the melting temperatures of the different gelators. Additionally, a whey protein-based oleogel (WP) was prepared by mixing EVOO with a WP aerogel prepared following the methodology of Plazzotta et al. (2020). The final oil content in WP-based oleogels was 80% (w/w). Oleogels were characterized for their structure using a texture analyzer (35 mm-diameter cylindrical probe for 5 mm of distance at a crosshead speed of 1.5 mm/s, TA. XT Plus, Stable Micro Systems Ltd, Godalming, UK) and an accelerated release test by centrifuging samples (10000g for 15 min, Mikro 120, Hettich Zentrifugen, Andreas Hettich GmbH and Co, Tuttlingen, Germany).
Unstructured EVOO and oleogels were then subjected to *in vitro* digestion according to the protocol proposed by Brodkorb et al. (2019). The FFAs released during digestion were assessed by titration (pH-stat). The bioaccessibility of tyrosol (T) and hydroxytyrosol (HT) was evaluated as the percentage ratio between the concentration of these components included in the micellar phase after intestinal *in vitro* digestion and their concentration in the undigested sample. The polyphenols were determined by HPLC.

3. Results and Discussion

3.1 Oleogel physical properties

Table 1 shows the oil retention capacity and firmness of the considered oleogels. All the samples presented a very high oil retention capacity upon centrifugation (<99%) despite the different firmness. The MG-based oleogel was the weakest gel, followed by WP, RW, SW, and PS. These mechanical properties can be associated with the

different natures of the networks structuring EVOO. In agreement with the literature, MG, RW, and SW formed a crystalline network (da Pieve et al., 2010; Doan et al., 2015), PS generated a fibrillar structure (Scharfe et al., 2019) and protein aerogels absorbed oil in the protein porous structure (Plazzotta et al., 2021).

Table 1. *Oil retention and firmness of oleogels prepared using MG, RW, SW, PS, and WP gelators.*

Sample	Oil retention (%)	Firmness (g)
MG	99.97 ± 0.01 [a]	33.6 ± 2.5 [e]
RW	100.0 ± 0.00 [a]	115.0 ± 3.0 [d]
SW	99.97 ± 0.03 [a]	532.7 ± 23.8 [b]
PS	99.96 ± 0.01 [a]	2534.0 ± 71.6 [a]
WP	100.0 ± 0.00 [a]	96.7 ± 6.8 [c]

Superscript letters (a–e) indicate significant differences among oleogels ($p < 0.05$).

3.2 In-vitro digestion: FFA release and bioaccessibility

Figure 1 shows the FFA release as a function of the digestion time of structured into oleogels and unstructured EVOO. The typical curve of lipid hydrolysis was obtained. The unstructured oil presented FFA release % of about 68%, followed by PS, SW, RW, and MG with 59.1, 50.8, 50.7, and 42.8% respectively. A different behavior was acquired for WP-based oleogels showing the complete digestion of the oil. These results clearly show that the extent of lipid lipolysis was significantly affected by oil structure. In the case of liposoluble gelators (i.e., MG, RW, SW, PS), it can be inferred that the lipase activity was hindered by the presence of a structuring network behaving like a physical barrier to the access of the enzyme to the substrate sites. On the contrary, WP probably completely dissolved in the gastrointestinal environment thus favouring the emulsification of the oil and thus the lipase activity. In summary, the results demonstrated that the digestibility of the oil can be steered by selecting the proper oleogelator.

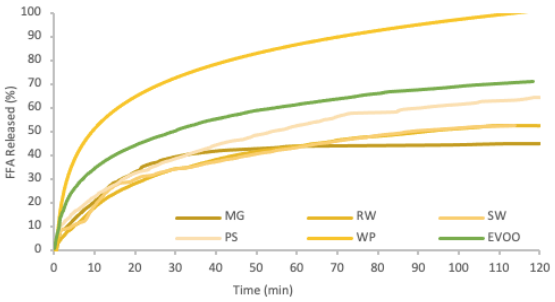

Figure 1. *Free fatty acids release of oleogels prepared using MG, RW, SW, PS, and WP gelators.*

In the next part of the study, the bioaccessibility of the major EVOO polyphenols (i.e., tyrosol and hydroxytyrosol) was assessed (Table 2).

Table 2. *HT and T bioaccessibility of oil and oleogels prepared using MG, RW, SW, PS, and WP gelators.*

Samples	Hydroxytyrosol (%)	Tyrosol (%)
MG	7.0 ± 1.75[b]	26.1 ± 0.97[bc]
RW	5.4 ± 0.49[b]	22.8 ± 1.77[c]
SW	5.3 ± 0.66[b]	31.6 ± 3.25[b]
PS	1.2 ± 0.08[c]	25.6 ± 1.81[c]
WP	17.0 ± 4.50[a]	52.3 ± 1.96[a]
Oil	n.d.	48.1 ± 3.38[a]

Superscript letters (a–c) indicate significant differences among oleogels ($p < 0.05$). "n.d." means "not detected".

Despite the higher content of hydroxytyrosol (HT) in EVOO than tyrosol (T) (HT: 248 mg/kg, T: 96 mg/kg), the bioaccessibility of T was significantly higher than that of HT. This result can be explained by considering the different susceptibility to oxidation of the two molecules during digestion (Alberdi-Cedeño et al., 2020). Moreover, differences were recorded among oleogels. Unstructured oil and WP presented the higher T bioaccessibility values, followed by SW, MG and RW, and PS. Since it is impossible to observe a direct effect of gel strength on polyphenol bioaccessibility, it can be speculated a possible interaction between the polyphenols and oleogel network structures. In fact, as well-known, polyphenols are surface-active molecules with the potentiality to interact with other food components.

In conclusion, the results reported in the present study confirm that oleogelation could be a profitable strategy to modulate lipid digestion while delivering bioactive molecules.

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Soil and legumes of the Sibillini for high quality pasta

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The main aim of the present PhD thesis is to investigate the relationship between the health of agricultural soil and the quality of its food products. To assess the former, several chemical-physical and biological indicators will be applied. For the latter, an analysis of the food products (legume flour and pasta) from a nutritional, technological, and organoleptic point of view will be carried out. In this poster, data related to the biological analysis of the soil of four organic farms involved in the production of legumes are discussed.

Suolo e legumi dei Sibillini per una pasta di qualità

L'obiettivo principale della presente tesi di dottorato è quello di indagare la relazione tra salute del suolo agricolo e qualità dei suoi prodotti alimentari. Per valutare il primo aspetto verranno applicati diversi indicatori chimico-fisici e biologici. Per il secondo verranno effettuate analisi dei prodotti alimentari (pasta e granella) dal punto di vista nutrizionale, tecnologico e organolettico. In questo poster vengono discussi i dati relativi alle analisi biologiche del suolo di quattro aziende agricole biologiche coinvolte nella produzione di legumi.

Key words: legume pasta; soil health, soil biodiversity; agroecosystem; organic; legumes

1. Introduction

"Healthy food from healthy soils" is the statement of the Farm to Fork Strategy. It's known that 95% of the food we consumed comes from soil, its health condition is one of the main factors determining food nutrient quality. Moreover, soil is a non-renewable resource, thus food security depends on soil preservation. This concept has been underlined by the Horizon Europe Mission: Soil Health and Food which title is: "Caring for Soil is caring for life". But many aspects of this fundamental link still need to be further investigated. Based on these assumptions, the project I am presenting aims to investigate the relationship between the health of agricultural soil and the quality of its crops and derived food products, specifically organic legume flour and pasta. Fields involved in the production of organic legumes will be monitored for three years. The farms, to which they belong, have supply chain agreements with Azienda Agricola Monte Monaco S.r.l. (our partner in this study), a local company specialized in the production of organic legume pasta. The project was funded within the Piano Stralcio for "Research and Innovation" 2015-2017 program and is part of the National Strategy for "Aree Interne", the main objective of which is the restoration and the enhancement of national areas characterized by loss of essential services and demographic reduction. During the past year, I focused on the assessment of soil health condition through several chemical-physical and biological indicators, considering climatic, and management factors as well as landscape effects and crop yields. In collaboration with the Company, the fields to be involved in the biomonitoring activities were select. A transect from the Sibillini mountains up to the Adriatic coastline will be monitored, to better evaluate possible differences in soil health, thus, both farms located outside and inside the inner area will be considered. Afterwards, flour and pasta obtained from each field will be evaluated for nutritional composition, technological functionality, and sensory characteristics.

2. Materials and Methods

2.1 Study Area

During the past year, four fields cultivated with chickpeas (*Cicer arietinum* L.) and located in the province of Fermo (Italy), were considered. Samplings were conducted during two seasons (June and September 2021). In the centre of each field soil samples for chemical-physical and biological analysis were collected according to Marche Region MOSYSS project sampling design. Interviews with field owners, geographic coordinates, environmental parameters, and field management information about the previous three years were collected. Additionally, during the summer sampling, in each field ten chickpea plants (phenological stage: podding) were collected to evaluate morphological characteristics and chlorophyll content.

2.2 Calculation of Soil Biological Quality Index (QBS-ar)

Among the biological indicators, the Biological Quality of Soil index based on arthropods (QBS-ar) has been applied. The method consists in the identification of the main functional groups (i.e., biological form - BF) through the evaluation of some specific morphological characters, which are related to their adaptation to belowground life (Parisi *et al*., 2005). In each field three replicates of soil cores (10x10x10 cm) were collected. In the laboratory, microarthropods' extraction was performed by Berlese-Tullgren funnel for 10 days. The extracted specimens were collected and preserved. A stereoscopic analysis of the "selections" was performed to determine the BF found and

assign to it an ecomorphological score (EMI), ranging between 1 and 20 depending on the adaptation to soil. At the end, for each replicate, the QBS-ar value was calculated as the result of the sum of the highest EMI values for each BF. It goes from 0 to more than 200, in relation to the use of soil considered. The structure of the microarthropod communities was evaluated also through biodiversity indices (Shannon-Wiener and Evenness), abundance and density (ind/m^2), number of biological forms (BF), ratio between the most abundant groups (mites and collembola), and percentage of oribatid mites on total mites. In addition to the classical "qualitative" (i.e., no abundance-based) approach of QBS-ar, the novel approach QBS-ab (Mantoni *et al*., 2021), that includes in the calculation of the index also the microarthropods' abundance, was determined.

2.3 Statistical Analysis

For the statistical analyses data obtained from the three replicates of the samples were used. Data were checked for normality (Shapiro-Wilk test) and homoscedasticity (Bartlett test) before analyses. Data were compared for season (Paired Student's T-test) and for sites (ONE-WAY ANOVA). Differences within groups were evaluated through Tukey's Honest Significant Difference test. The statistical analyses were performed using R 4.2.0 software. The heatmap of the abundances (Figure 1) was created using Heatmapper (www.heatmapper.ca).

3. Results and Discussion

Results show that farms 1 and 4, in both seasons, have an excellent soil biological quality. The QBS-ar values are higher than 120, the threshold that defines the best quality for arable lands. Significant differences in QBS-ar values are found between farm 2 and 4 ($p < 0.05$). The two farms differ in soil texture (silty clay and clay loam), but other aspects related to chemical-physical analysis, location, environment, and management are going to be considered. Despite the lower QBS-ar values, farm 2 shows an A/C ratio significantly higher compared to the other farms ($p < 0.01$), underling an excellent stress-free condition. Microarthropods abundance ($p < 0.05$) and % oribatid mites ($p < 0.01$) are significantly higher in autumn compared to summer (Table 1). This is due to the ecological behaviour of mesofauna, in autumn, moisture level was more favourable for the population of the surface layers of the soil and the organic matter increased because of the crop residues left in the field after the harvesting, this led to the increase of the oribatid mites, which prefer organic recalcitrant fraction.

Table 1 - *Data related to the biological parameters examined in the farms during the first year (June and September 2021).*

	Farm 1		Farm 2		Farm 3		Farm 4	
	Summer	**Autumn**	**Summer**	**Autumn**	**Summer**	**Autumn**	**Summer**	**Autumn**
QBS-ar value	129	136	133	98	115	161	143	186
QBS-ab value	147	174	134	124	154	172	175	184
Total density (ind/m^2)	16533	34100	36400	62033	27700	68533	27900	30100
A/C ratio	8	21	95	51	9	32	19	18
% oribatid mites	11%	17%	6%	21%	14%	17%	8%	29%
Shannon-Wiener (no A/C)	1.63	1.40	2.03	0.51	2.01	2.20	1.93	1.41
Evenness (no A/C)	0.68	0.68	0.92	0.25	0.87	1.00	0.84	0.61

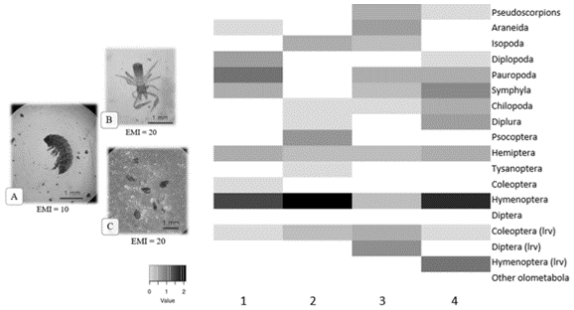

Figure 1 - *The heatmap represents the total abundance of the different BF collected during the autumn sampling, acarina and collembola are not included. The pictures represent some of the BF found in the samples. The EMI value for each of them is reported. A. Isopoda, B. Pseudoscorpion, C. Acarina*

As soon as the products obtained from each field will be processed, I will perform the analyses on flour and pasta. During the next year, according to crop rotation, I will consider other fields cultivated with legumes. The previous analyses, including those on food products, will be repeated. I am planning to implement the study with the analysis of the rhizospheric community by metabarcoding approaches (16S rRNA gene) to have a wider overview of the differences in soil biodiversity between the sites and their effects on plants and food health.

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Enrichment of Extra Virgin Olive Oil for the Development of Functional Oil to Special Consumers

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This PhD research project aims to enhance the traditional foods typical of rural areas by identifying their compositional and healthy peculiarities. To achieve this goal, extra virgin olive oil (EVOO) from Calabria Region (southern Italy), was enriched with bergamot (*Citrus bergamia* Risso). The obtained flavoured oil (FVOO) was found to be pleasant from a sensorial point of view and with marked health properties.

Funzionalizzazione di un olio extravergine di oliva per la creazione di prodotti destinati a categorie speciali

L'attività di ricerca di dottorato si propone di valorizzare gli alimenti tradizionali tipici delle aree rurali individuandone le peculiarità compositive e salutistiche. Per raggiungere questo obiettivo, l'olio extra vergine di oliva (EVOO) del territorio calabrese, è stato arricchito con bergamotto (*Citrus bergamia* Risso). L'olio aromatizzato (FVOO) ottenuto è risultato gradevole dal punto di vista sensoriale e con spiccate proprietà salutistiche.
Key words: Bergamot, extra virgin olive oil, flavoured olive oil, antioxidant activity.

1. Introduction

In accordance with the PhD thesis project previously described (Custureri IMG, 2021), this poster reports the main results of the first activities concerning:
A4) EVOOs enrichment with *Citrus bergamia* Risso
A5) EVOOs and FVOOs physical-chemical analysis
A8) Determination of antioxidant activity by multi-target approaches and enzyme inhibitory properties
A11) Statistical data analysis

2. Materials and Methods

The olive fruits (cv Ottobratica) were harvested in Calabria Region during the oil crop season 2021.The oil extraction was performed by a mini-pressing apparatus. Bergamot fruits, farmed in the Ionian side of the Reggio Calabria province (Italy), were added into halves to the mini-pressing apparatus together with the olives (35 kg) for crushing, malaxation and pressing of the paste. Free acidity, peroxide value and extinctions parameters were performed according to EUC 2016 Regulation. Total Phenolic Content (TPC), Total Carotenoid (TCC) and Chlorophylls Contents (ChlC) were spectrophotometrically determined following the methods reported by Piscopo *et al*., 2018. The effect of additions on antioxidant and enzyme inhibitor activity was evaluated by multi-target approaches: DPPH, ABTS, β-carotene bleaching t30 and t60, FRAP, Lipase carbohydrate hydrolysing enzyme, α-Amylase and α-Glucosidase. Experimental results were expressed as mean ± standard deviation (n=3). The significant differences among samples were determined by ANOVA and Tukey's post hoc test. To investigate the similarities and dissimilarities among the formulations PCA analysis was applied.

3. Results and Discussion

The EVOO and FVOO's analysis aims to evaluate the stability of the products all over the time. The addition of bergamot fruit at different percentages seemed to strongly affect the qualitative characteristics of oil. The content of organic acid in bergamot fruit might explain the increment of acidity level of FVOO (Sacchi *et al*., 2016). The more acidic environment during malaxation caused by the acids released from bergamot, promotes the hydrolysis of triglycerides and therefore higher free acidity values. Considering that the addition of bergamot ranged between 0.11 and 0.25 mg kg^{-1} of olive paste, the higher acidic condition also allows the hydrolysis of secoiridoid aglycons with a consequent production of simple phenyl alcohols more likely lost in the olive mill wastewater. The concentration of TPC in FVOO samples resulted significantly lower than in EVOO. Different trend was observed for ChlC and TCC, which were characterized by initially increasing trend, probably due to the solubilisation of these compounds, and a subsequent decrease. Table 1 shows as the enzymatic activity is higher in the FVOOs. Promising results were obtained with FVOO 20% that reached in lipase test at T60 an IC_{50} values of 100.12 µg ml^{-1}, against the EVOO's IC_{50} values of 156.76 µg ml^{-1}. TPC (Table 2) in both FVOOs are positively correlated with FRAP value (P=0.80 and 0.92 for FVOO 10% and FVOO 20%, respectively), whereas TPC of EVOO is

negatively correlated with the antioxidant activity assays. The TCC in both FVOOs are positively correlated with lipase IC_{50} value (P=0.98 and 0.91 for FVOO 10% and FVOO 20% respectively). By the PCA analysis, the first two principal components (PCs) explained 87.64% of the total variance observed in the original variables. PC1 explains about 51.88% and PC2 about 35.76%. The first PC (PC1 67.30 % of the variance) was positively correlated with the variables: acidity, chroma, TPC oil; acidity, chroma, DPPH, ABTS, β-carotene bleaching test, α-amylase, α-glucosidase for FVOO 10%; ChlC, TCC, ΔK, chroma, DPPH, ABTS, β-carotene bleaching test, α-amylase, α-glucosidase, lipase for FVOO 20%. The second principal component (PC2) is highly positively correlated with: ChlC, TCC, β-carotene bleaching t60, lipase for oil, ChlC, TCC, TPC, lipase for FVOO 10%; acidity and TPC for FVOO 20%.

Sample	α-Amylase[1]	α-Glucosidase[1]	Lipase[1]
		EVOO	
T0	72.45±3.77^{D}	70.32±3.73^{Db}	123.45±4.85^{Db}
T15	75.23±3.86^{Ca}	72.65±3.75^{Ca}	135.78±4.87^{Ca}
T30	78.89±3.92^{Ba}	76.69±3.82^{Ba}	142.92±4.91^{B}
T60	87.23±4.05^{Aa}	81.04±3.90^{Aa}	156.76±5.01^{Aa}
Sign	**	**	**
		FVOO 10%	
T0	72.66±3.68^{A}	71.23±3.67^{Aa}	124.24±4.78^{a}
T15	73.34±3.73^{Ab}	71.87±3.78^{Aa}	133.23±4.82^{b}
T30	70.17±3.45^{Bb}	70.07±3.90^{ABb}	130.15±4.80
T60	65.26±3.09^{Cb}	69.89±3.32^{Bb}	127.12±4.67^{b}
Sign	**	**	ns
		FVOO 20%	
T0	70.24±3.48	69.15±3.16^{Ac}	111.13±4.43^{Bc}
T15	68.14±3.37^{c}	69.07±3.08^{Ab}	114.27±4.45^{Ac}
T30	60.11±3.35^{c}	67.14±3.01^{Bc}	105.15±4.19^{C}
T60	58.21±3.21^{c}	66.26±2.99^{Cc}	100.12±4.08^{Dc}
Sign	ns	**	**

Table 1 *Carbohydrate hydrolysing enzyme and lipase inhibitory activities by EVOO and FVOO (10% and 20%).*

*[1]IC_{50} value (μg ml^{-1}); Acarbose was used as positive control in α-amylase and α-glucosidase tests (IC_{50} values of 50.18±1.32 and 35.57±0.99 μg ml^{-1}respectively). Orlistat was used as positive control in lipase test (IC_{50} value of 37.44±1.08 μg ml^{-1}). **: Highly significant; *: significant; ns: not significant. Results followed by different capitol letters in the same column and different lowercase letters show the differences among the samples at the same time.*

Table 2 *Antioxidant activity and total carotenoids and phenols content in EVOO and FVOO (10% and 20%).*

Sample	DPPH[1]	ABTS[1]	ß-carotene bleaching t30[1]	ß-carotene bleaching t60[1]	FRAP[2]	TCC[3]	TPC[3]
				EVOO			
T0	76.56±3.45^{Aa}	3.50±0.25^{ABa}	87.21±3.45$^{D.a}$	82.83±3.87^{Da}	27.03±1.20^{Cc}	2.12±0.22^{Db}	659.03±96.84^{Aa}
T15	63.23±3.12^{Ba}	3.43±0.28^{ABa}	89.54±3.85^{Ca}	92.41±3.92^{Ca}	27.67±1.48^{Cc}	5.52±0.23^{A}	571.56±11.87^{ABa}
T30	56.43±2.87^{C}	3.25±0.23^{Ba}	92.89±4.40^{Ba}	94.87±3.95$^{B.a}$	32.32±1.52^{Bc}	4.74±0.22^{Ba}	557.88±18.81^{Ba}
T60	57.83±2.82^{Ca}	3.97±0.31^{Aa}	95.91±4.42$^{A.a}$	97.72±3.98$^{A.a}$	38.91±1.56^{Ac}	3.98±0.20^{Ca}	251.25±21.08^{Ca}
Sign	**	*	**	**	**	**	**
				FVOO 10%			
T0	68.34±2.23^{Ab}	3.03±0.56^{Ab}	58.45±2.47^{Ab}	59.65±2.40^{Ab}	54.12±1.76^{Db}	2.01±0.23^{Db}	113.22±13.10^{Bb}
T15	63.52±2.18^{Ba}	2.97±0.34^{Ab}	57.22±2.52^{Bb}	55.22±2.48^{Bb}	56.31±1.78^{Cb}	5.82±0.16^{A}	160.56±31.74^{Ab}
T30	61.78±2.09^{Ca}	2.44±0.22^{Ab}	55.26±2.51^{Cb}	47.81±2.34^{Cb}	57.45±1.85^{Bb}	4.80±0.19^{Ba}	185.63±3.37^{Ab}
T60	49.22±2.47^{Db}	2.01±0.13^{Bb}	52.16±2.59^{Db}	45.78±2.31^{Db}	59.13±1.83^{Ab}	2.66±0.24^{Cb}	166.12±3.68^{Ac}
Sign	**	**	**	**	**	**	**
				FVOO 20%			
T0	62.13±2.24^{Ac}	3.00±0.26^{Ab}	56.12±2.61^{Ac}	52.38±2.43^{Ac}	63.67±1.92^{Da}	4.16±0.04^{Ba}	114.4±3.07^{Cb}
T15	60.11±2.32^{Bb}	2.52±0.18^{ABb}	54.32±2.40^{Bc}	50.14±2.47^{Bc}	69.83±2.00^{Ba}	5.38±0.25^{A}	190.98±12.16^{Bb}
T30	57.36±2.45^{Cb}	2.30±0.15^{BCb}	51.98±2.37^{Cc}	48.79±2.52^{Cc}	68.71±2.12^{C}	3.98±0.22^{Bb}	216.43±1.62^{Ab}
T60	47.09±2.65^{Dc}	1.98±0.12^{Cb}	50.82±2.35^{Dc}	41.43±2.59^{Dc}	70.31±2.27^{Aa}	3.85±0.03^{Ba}	207.88±1.44^{Ab}
Sign	**	**	**	**	**	**	**

*[1]IC_{50} value (μg ml^{-1}); [2]μM Fe(II) g^{-1}; [3]mg kg^{-1}. Ascorbic acid (IC_{50} values of 5.03±0.82 and 1.78±0.07 μg ml^{-1} for DPPH and ABTS test); Propyl gallate (IC_{50} values of 1.02±0.01 and 1.04±0.03 μg ml^{-1} for ß-carotene bleaching t30 and t60) and BHT (FRAP value of 63.26±0.81 μM Fe(II) g^{-1}) were used as positive control. **, *, ns see Table 1.*

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Traceability of Mediterranean anchovies with NIR spectroscopy

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Traceability and authentication of seafood is important to protect consumer's right and ensure food quality & safety. The chemical composition of animal tissue is a function of its growing environment & food and can be used to identify origin. The PhD thesis aims to authenticate anchovies (*Engraulis encrasicolus* L.) from different regions of Mediterranean Sea using NIR spectroscopy, Multielement profile and Stable isotope ratio. This poster submission focuses on the application of NIR spectroscopy. This PhD project is in the framework of SUREFISH PRIMA project: Fostering Mediterranean fish ensuring traceability and authenticity, https://surefish.eu/.

Tracciabilità delle alici del Mediterraneo mediante spettroscopia NIR

La tracciabilità e l'autenticazione dei prodotti ittici aiutano a proteggere i diritti dei consumatori e a garantire la qualità e la sicurezza degli alimenti stessi. La composizione chimica dei tessuti animali è funzione dell'ambiente di crescita e della nutrizione dei pesci e può essere investigata per identificarne l'origine geografica. La tesi di dottorato mira ad autenticare le alici (*Engraulis encrasicolus* L.) provenienti da diverse regioni del Mar Mediterraneo, utilizzando la spettroscopia NIR, il loro profilo multielementare e il rapporto isotopico stabile. Questa presentazione poster si concentra in particolare sull'applicazione della spettroscopia NIR. Il progetto di dottorato si inserisce nel quadro del programma SUREFISH PRIMA: Promozione dei prodotti ittici del Mediterraneo garantendo tracciabilità e autenticità, https://surefish.eu/.

Keywords: Food authentication, fish traceability, IR spectroscopy, Mediterranean diet, Anchovies

1. Introduction

Origin mislabeling, species substitution, selling old and/or frozen fish as fresh are the most common frauds in seafood industry. NIR spectroscopy has been used for food authentication for all kinds of food products. It has been to detect food fraud in honey, wine, oils, meat products, dairy products, fruits, etc. (Cozzolino, 2016). This poster describes and reports the application of NIR spectroscopy on Mediterranean anchovies (*Engraulis encrasicolus* L.). NIR spectroscopy was used to discriminate between anchovies based on their area of fishing in the Mediterranean Sea

2. Materials and Methods

Fresh anchovies fished in Tyrrhenian, Adriatic, Balearic Sea and Gulf of Tunis were procured from respective local markets by SUREFISH partners in 2021 in following months: Tyrrhenian: February, May, June, July, August, and September; Adriatic: April and November; Balearic: April and May; Gulf of Tunis: July and August. Anchovies were cleaned (removal of head, guts, bone, and internal viscera), washed with distilled water and homogenized in batches of 10-12 fish. The homogenate was freeze-dried and pulverised with ball-milling machine until 60% of it can pass through 100-mesh sieve and 80% can pass through 60-mesh size sieve. Perkin Elmer FT-IR 9700 with NIRA (Near-Infrared Accessory) was used for spectra acquisition. Total 108 spectra (interleaved) was acquired over a range of 1000-2500 nm (resolution of 4cm^{-1}, 64 scans/spectra).
The data analysis was performed using Unscrambler X (Version 10.4, 64 bit) and XLSTAT Version 2016.02.28451. NIR spectra were pre-processed with standard normal variate (SNV) followed by Savitzky-Golay (2nd degree, 1st order with 15 points of smoothening) pretreatment. Resulting spectra was analyzed with Principal Component Analysis (PCA) to reduce dimensionality in form of principal components (PCs). PCs obtained were used to build a discrimination model using Linear Discriminant Analysis (LDA). The robustness of the classification model was evaluated by a cross-validation test, using the "leave-one-out" procedure.

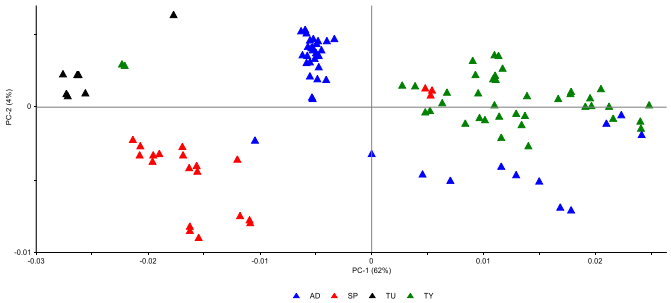

Figure 1: *PCA score plot of anchovies from Adriatic Sea (AD), Balearic Sea (SP), Gulf of Tunis (TU) and Tyrrhenian Sea (TY)*

3. Results and Discussion

Figure 1 shows the PCA score plot obtained from the pre-treated spectra. The variance explained by PC1 and PC2 was 59% and 4% respectively. Anchovies from similar region exhibited a natural tendency of natural grouping but it was uneven across the months of fishing. plane. The loadings plot from PC1 exhibited the most influential loadings in the range of 1450 to 1525 nm and 1935 to 2096 nm (Figure 2). These

regions in the NIR spectra are caused by the N-H stretching (first overtone) and O-H and N-H bonds combination present in amine I and II groups respectively (Khodabux et al., 2007). A clear separation was not observed among the anchovies from 4 different regions along any of the PCs. The Mediterranean region although largely oligotrophic in nature, has temperature and surface chlorophyll variations throughout the year. The temperature in all the regions at the given time of the year can differ up to 3-4°C. This leads to strict regional structuring of biophysical processes that controls the primary production (Basterretxea et al., 2018; Rossi et al., 2014) of plankton (phyto and zooplanktons), the main food source for the anchovies (Basterretxea et al., 2018; Mena et al., 2019). This variability in food changes keep the anchovy composition varied and distinct among all seasons across the Mediterranean region. Another reason could be the size of anchovies used for sampling. The chemical composition of anchovy tissue changes with age (Mais & Beach, 1981; Šimat & Bogdanović, 2012) and this makes anchovy sampling extremely un-homogenous. Finding the fish of same size in every sampling is not possible due to large anchovy populations across the Mediterranean Sea (Traina et al., 2011).

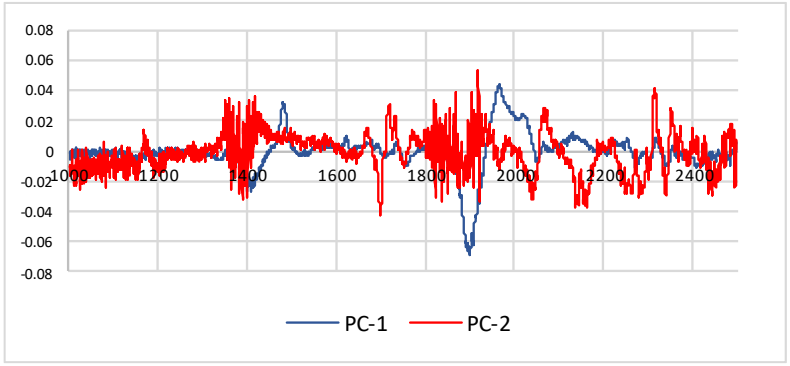

Figure 2: *Loadings from PC1 and PC2 of PCA analysis*

Unsupervised analysis followed by supervised LDA proved better in classifying anchovies based on their origin, irrespective of season, size, and frequency of sampling. The λ Wilks value of the classification functions was 0.016 indicating discriminatory ability of discriminant function. 91.67% correct classification was observed with LDA at 5% significance level (92.50% correct classification for Adriatic, 100% for Balearic, 85.71% f0r Gulf of Tunisia, and 87.50% for Tyrrhenian Sea). The result of cross validation is shown in Table 1. The 1st, 2nd, and 3rd discriminant factor (F1, F2 and F3) explained 73.2%, 19.5% and 7.3% of total variance. Although, PC6 only contributed to 1% variance in PCA, it exhibited highest discriminant ability followed by PC3 for both F1 and F2.

Table 1. *LDA confusion matrix for cross-validation of anchovies from Mediterranean Sea*

from \ to	Adriatic Sea	Balearic Sea	Gulf of Tunis	Tyrrhenian Sea	Total	% Correct
Adriatic Sea	35	0	0	5	40	87.50%
Balearic Sea	0	21	0	0	21	100.00%
Gulf of Tunis	1	0	6	0	7	85.71%
Tyrrhenian Sea	5	0	0	35	40	87.50%
Total	41	21	6	40	108	89.81%

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Olive cake as functional ingredient to improve the quality of gluten-free baked goods

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The richness of vegetable waste and by-products in bioactive compounds has led to a growing interest into their use as functional ingredients for the production of high added value foods. According with the first two activities of PhD project, lyophilized olive cake was characterised for its chemical and nutritional composition. Olive cake powder was added as functional ingredient into gluten-free breadsticks formulation, assessing its influence on nutritional, textural, and sensory properties of baked goods.

La sansa di oliva come ingrediente funzionale per il miglioramento dei prodotti da forno gluten-free

La ricchezza di scarti e sottoprodotti vegetali in composti bioattivi ha determinato un crescente interesse nel loro impiego come ingredienti funzionali per la produzione di alimenti ad alto valore aggiunto. Le prime due attività previste dal progetto di dottorato hanno riguardato la caratterizzazione chimico-fisica della sansa di oliva liofilizzata ed il suo impiego come ingrediente innovativo per la formulazione di grissini gluten-free, valutandone l'influenza sulle proprietà nutrizionali, strutturali e sensoriali nel prodotto cotto.

Key words: olive cake powder, gluten-free, dietary fibre, polyphenols, antioxidant, texture.

1. Introduction

In accordance with the PhD thesis project (de Gennaro, 2021), this poster described the main results of the first two activities concerning:
A1) Management of vegetable by-product and the evaluation of its physical-chemical properties and bioactive compounds;
A2) Application of vegetable by-products as functional ingredients into gluten-free (GF) breadsticks formulation, assessing their effect on nutritional, textural, and sensory properties.

2. Materials and Methods

Wet olive cake was lyophilized, grounded and the resulting powder was characterised for its proximate composition following the AOAC Official methods (2006). Moisture content was determined by an automatic moisture analyser at 105 °C. The total phenols content (TPC) and antioxidant activity (AA) were determined spectrophotometrically, by Folin-Ciocalteu, ABTS and DPPH assay. Tocopherols content was determined by reverse phase-ultra high performance liquid chromatography-fluorescence detector (RP-UHPLC-FLD) according with Difonzo et al. (2021). Control GF breadsticks (BSC) were prepared using rice (41%) and maize (9%) flour, water (35%), sunflower oil (11%), psyllium fibre (2%) and baking powder (1.5%). Fortified GF breadsticks were produced by replacing 1, 2, and 3% of maize flour with olive cake powder (OCP) into GF breadsticks production (BS1, BS2, and BS3). Psyllium fibre was mixed with water and oil producing a gel, which was added to the other ingredients, proceeding with manual kneading. Afterwards, the doughs were hand-shaped to form breadsticks, boiled and dried at room temperature. The boiled breadsticks were baked in a forced-air convention oven at 200 °C for 27 min and cooled at room temperature. Proximate composition, total phenols and antioxidant activities of baked GF breadsticks were carried out according with the methods previously described. Texture properties (hardness and brittleness) of GF breadsticks were evaluated through a three-point bending test, while colorimetric analysis (L*, a*, b*) using a colorimeter CM-600d. Volatile compounds were analysed through an headspace solid phase micro-extraction (HS-SPME), coupled with a gas chromatography/mass spectrometry as reported by Difonzo et al. (2018). Sensory evaluation of GF breadsticks was conducted by a semi-trained panel, which evaluated descriptors regarding the appearance, odour, texture, and taste. All the analysis were conducted in triplicate. Statistical analysis of data was performed by one way ANOVA followed by the Tukey's HSD test.

3. Results and Discussion

3.1 Proximate composition and bioactive compounds of OCP and GF breadsticks

OCP showed a good amount of ash (7.68 %), lipid (22.78%), and total dietary fibre (23.45%), a high amount of phenols and tocopherols equal to 78.23 mg GAE/g and 412.24 mg/kg, respectively, which contribute to the high antioxidant activity (285.24 ± 11.21 μmol TE/g DPPH and 346.12 ± 8.11 μmol TE/g ABTS). As it showed in

Table 1, the addition of OCP positively influenced the total dietary fibre and ash content, reaching the highest value in BS3 (3.59% ± 0.13 and 2.67% ± 0.04, respectively). In addition, the replacement of maize flour with OCP had a significant effect on the content of bioactive compounds of baked GF breadsticks. A dose-dependent increase of TPC was observed with higher replacement of OCP, reaching values of 1.79 ± 0.03 mg GAE/g in BS3; the same trend was observed for AA, with values in a range of 4.52 ± 0.19 µmol TE/g (BS3) and 0.88 ± 0.02 µmol TE/g (BSC) for DPPH assay, and in a range of 4.00 ± 0.15 µmol TE/g (BS3) and 0.60± 0.01 µmol TE/g (BSC) for ABTS assay. Thus, considering the results obtained, a positive correlation between OCP addition and the increase of TPC and AA into fortified GF breadsticks can be assumed.

Table 1 *Mean values, standard deviation, and results of statistical analysis of the proximate composition of GF breadsticks and the antioxidant profile.*

Parameters (%)	**BSC**	**BS1**	**BS2**	**BS3**
Moisture	2.90 ± 0.02c	5.02 ± 0.08a	4.18 ± 0.01b	4.98 ± 0.11a
Lipid	14.38 ± 0.03b	14.78 ± 0.23b	16.14 ± 0.16a	16.08 ± 0.17a
Total dietary fibre	2.72 ± 0.03c	2.90 ± 0.08c	3.16 ± 0.08b	3.59 ± 0.13a
Protein	6.23 ± 0.20a	5.58 ± 0.01b	5.69 ± 0.11b	5.57 ± 0.02b
Ash	1.83 ± 0.03c	2.43 ± 0.04b	2.49 ± 0.02b	2.67 ± 0.04a
Carbohydrate	71.94 ± 0.20a	69.29 ± 0.21b	68.34 ± 0.25c	67.11 ± 0.33d
Antioxidant profile				
TPC (mg GAE/g)	0.16 ± 0.01d	0.65±0.02c	1.38±0.03b	1.79±0.03a
ABTS (µmol TE/g)	0.60±0.01d	2.04±0.10c	3.69±0.13b	4.02±0.15a
DPPH (µmol TE/ g)	0.90±0.02d	2.30±0.03c	4.13±0.11b	4.5±0.19a

3.2 Physical and sensory characterisation

The colour of GF breadsticks was significantly influenced by the addition of OCP. Specifically, lightness (L*) and yellow index (b*) decreased with increasing OCP addition, due to the colour of OCP added and the production of melanoidins from Maillard's reaction during baking. The values of hardness and brittleness are reported in Table 2. BS3 showed the lowest value of hardness, while no significant differences were found for brittleness among the samples.

Table 2 *Mean values, standard deviation, and results of statistical analysis of the proximate composition of GF breadsticks and the antioxidant profile.*

Parameters	**BSC**	**BS1**	**BS2**	**BS3**
Colorimetric indexes				
L*	61.60 ± 0.13a	53.31 ± 0.38b	47.18 ± 0.24c	46.07 ± 0.20d
b*	31.89 ± 0.10a	27.22 ± 0.23b	27.33 ± 0.54b	26.05 ± 0.31c
a*	7.83 ± 0.10b	7.15 ± 0.17c	8.49 ± 0.22a	7.99 ± 0.02b
ΔE	-	9.55 ± 0.53a	15.15 ± 0.19b	16.60 ± 0.22c
Textural indexes				
Hardness (N)	19.12 ± 0.72a	19.40 ± 0.32a	18.91 ± 0.75a	12.68 ± 0.16b
Brittleness (mm)	0.08 ± 0.02a	0.09 ± 0.01a	0.10 ± 0.02a	0.07 ± 0.01a

Note: different letters indicate significant differences

Lipids oxidation represents one of the main causes of quality deterioration of baked foods. A general reduction of aldehydes, markers of lipid oxidation, was observed into enriched GF breadsticks (BS1, BS2, BS3) compared with the control, and the same trend was observed for Maillard's reaction products (furans, pyrazines and Strecker aldehydes). According to the literature, phenols compounds can exert an inhibitory action against aromatic compounds formation in the end products. Sensory analysis confirmed the results obtained from the instrumental evaluation of colour and texture, with a higher overall pleasantness of BS2 and BS3 samples, despite a higher perception of bitterness and astringency (data not showed).

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Late blowing defect in "Grottone" cheese: detection of clostridia and control strategies

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The initial focus of the Ph.D. thesis topic was on finding the optimal approach for determining spore-formers clostridia in dairy samples. The inhibitory action of several antimicrobial compounds was tested against *Cl. sporogenes* as the second target. Based on the findings, a protective culture was used for nine Grottone productions at the farm level.

Gonfiore tardivo in formaggio "Grottone": rilevamento di clostridi e strategie di contenimento

L'obiettivo iniziale della tesi di dottorato è stato quello di trovare l'approccio ottimale per la determinazione dei clostridi sporigeni nei campioni di latte. Come secondo obiettivo è stata testata l'azione inibitoria di diversi composti antimicrobici contro *Cl. sporogenes*. Sulla base dei risultati ottenuti, è stata utilizzata una coltura protettiva per nove produzioni di Grottone a livello aziendale.

Keywords: Grottone cheese; Late blowing defect; *Clostridium spp*., antimicrobial compounds.

1. Introduction

"Grottone" is a pasta filata hard cheese ripened for at least two months, traditionally produced in the Matese Regional Park (Campania region, Southern Italy) from cow's milk and with the addition of salt, rennet, lactic, and propionic ferments. The cheese is characterized by holes formation due to CO_2 development by lactate metabolism of Propionic Acid Bacteria (PAB). Actually, "Grottone cheese" might be defined as a medley between a traditional Caciocavallo from Campania and cheeses with holes such as Emmenthal. Due to the specific production technology, the contamination of raw milk with spore-forming clostridia represents a major concern for cheese producers since conditions supporting PAB development during ripening may favor clostridia as well. LBD is characterized by holes, cracks as well as an unpleasant flavor due to butyric acid development as well as protein catabolism. LBD control can be also performed by enumerating clostridial spores in raw milk to be processed, but reliable methods for spores' quantification are still needed. Therefore, the aims of the present work were: i) to compare the commonly used approaches for spore-forming clostridia detection of in dairy samples and to point out the most suitable procedure; ii) to evaluate the occurrence of *Clostridium* spp. spores in milk and to trace a correlation with LBD occurrence in Grottone cheese samples, and iii) to assess the inhibitory activity of several antimicrobial compounds, i.e. nisin, lysozyme, nitrite, and of a protective culture of *Lacticaseibacillus* (*Lb*.) *casei* against *Cl. sporogenes*.

2. Material and Methods

For the initial screening, 13 milk samples and 11 Grottone cheese samples with LBD were analyzed by using two procedures: the MPN technique in Bryant and Burkey medium (BBB - Bryant & Burkey, 1956) and CFU in modified Reinforced Clostridial Medium (mRCM - Hirsch & Grinstead, 1954). The inhibitory activity of nisin, lysozyme, nitrite, and *Lb. casei* Lyofast LC 4P1 (Sacco System, Como, Italy) was assessed against *Cl. sporogenes* ATCC1143 The MIC was determined as the lowest concentration that showed a complete inhibition of the growth of the assayed Clostridium strain. Tests were carried out according to Avila et al. protocol's (2014).
Grottone cheese manufactures were carried out at Iaquilat Trade srl on 200 L milk batches. In the three sets of trials, one vat was used to produce Grottone cheese according to the routine process adopted by the producer and served as control (C1, C2, and C3). Mofin Alce Group (Novara, Italy) provided both LAB and PAB cultures. In the six trials supplemented with the *Lb. casei* culture, LAB and PAB were provided by Sacco System. In one batch, the protective culture was added at the same time as the starters (A), while in the remaining five manufacturing, it was added in a batch of milk for a pre-maturation. The percentage of pre-fermented milk was 10 (B), 36 (C), 50 (D), 70 (E) and 100% (F). After brining, five Grottone cheeses for each production were randomly marked and cut in half after two months of ripening. The cheese's appearance was examined in terms of the existence, size, and distribution of holes. Ten well-trained panelists from the company's employees sensory examined the samples. Only two points were questioned for a 'yes or no' value judgment: LBD symptoms and the presence of the predicted sensory qualities.

3. Results and Discussion

3.1 Recovery of spore-forming clostridia in milk and cheese samples

Clostridial spores were found in 10 of the 13 milk samples. MPN spore levels in contaminated milk samples were quite low ranging from 0.03 to 95 cell/mL (mean MPN counts = 10,78 cell/mL). Indeed, even low quantities of clostridial spores have been shown to produce severe LBD in cheeses. Furthermore, the start of LBD is determined not only by the spore count since the cheese environment may promote the spores' germination as well (Burtscher et al., 2020).

3.2 Inhibitory effect of nisin, lysozyme, sodium nitrite and *Lb. casei* Lyofast LC 4P1

The MICs of nisin, lysozyme, sodium nitrite, and *Lb. casei* Lyofast LC 4P1 were assessed against *Cl. sporogenes* ATCC1143. The effectiveness of the four antimicrobial compounds varied with the culture media used. Even at the maximum tested concentration (8000 µg/mL), lysozyme did not inhibit the growth of *Cl. sporogenes* strains in Litmus milk, whereas, in Reinforced Clostridial Medium a variable effect was observed at a concentration equal or higher than 1.000 µg/mL (Data not shown). According to Ávila et al. (2014), lysozyme at the concentrations usually employed in cheese factories (400 µg/mL), does not inhibit the growth of *Cl. sporogenes*. The MIC of sodium nitrite was 20 mg/L in LM and only 10 mg/mL in RCM, while nisin did not inhibit the growth of *Cl. sporogenes* either in RCM or in LM. The supernatant of *Lb. casei* Lyofast LC 4P1 prevented the growth of *Cl. sporogenes* after a dilution 1/2, although a slight inhibition was observed up to dilution at 1/8. On the other hand, no activity was observed when LM medium was used.

3.3 Use of *Lb. casei* Lyofast LC 4P1 in 'Grottone' cheese manufacturing

Nine Grottone cheese manufactures were carried out at farm level. After two months, a total 45 wheels were cut in two halves and analysed for their appearance. At the date of analyses, none of the produced Grottone wheels, other than the 45 initially marked, presented obvious signs of LBD. Actually, this defect is known to occur even later during the ripening, and it is not always possible to assess its oncoming before the wheel's opening (Cosentino et al., 2013). . Holes' development was hampered likely for an inhibition of the PAB starter and the expected 'Grouviera-type' taste was not perceived by panelists. Based on the results, the use of protective cultures needs to be contextualized and interactions with starters needs to be evaluated case by case.

Manufacure conditions	Trial								
	A	B	C	D	E	F	C1	C2	C3
Protective	LC 4P1	LC 4P1	LC 4P1	LC 4P1	LC 4P1	LC 4P1	-	-	-
PAB	PB1	PB1	PB1	PB1	PB1	PB1	CSL	CSL	CSL
LAB	YHL 094F	YHL 094F	YHL 094F	YHL 094F	YHL 094F	YHL 094F	HD 097	HD 097	HD 097
Pre-ferm. vol.	-	20 L (10%)	72 L (36%)	100L (50%)	140 L (70%)	200 L (100%)	-	-	-
Pre-ferm. lenght	-	~3 h	~3 h	~2 h	~40 min	~20 min	-	-	-
Appearence	Few holes	Few holes	Few holes	Few holes	Few holes	Few holes and crackings	Cavernous	Compliant	Compliant
									

Figure 1. *Grottone cheese manufactures at farm level. Trial A: Lb. casei culture Lyofast LC 4P1 added with starters; Trial B, C, D, E and F: LC 4P1 used for milk pre-fermentation at 10, 36, 50, 70 and 100% of the total volume. C1, C2 and C3: controls.*

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Study and development of innovative seafood products through the application of emerging process technologies

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This report describes the research activities carried out as part of this PhD project. Considering the diversity of the different emerging technologies and seafood species, and the scarcity of published studies in the literature, resources were concentrated over the past year on testing the effect of Pulsed Electric Fields (PEF) and Cold Plasma (CAP) on dry salted cod, and on fresh/minimally processed fillets of sea bass, sea bream and salmon.

Studio e realizzazione di prodotti ittici innovativi attraverso l'applicazione di tecnologie di processo emergenti

In questo documento sono descritte le attività di ricerca svolte nell'ambito di questo progetto di dottorato. Vista la molteplicità di tecnologie emergenti e specie ittiche e la scarsità di studi pubblicati in letteratura, nell'ultimo anno sono state concentrate le attività di ricerca nel testare l'effetto di Campi Elettrici Pulsati (PEF) e Plasma Freddo (CAP) su merluzzo salato ed essiccato (baccalà), filetti di branzino e orata e filetti di salmone freschi/processati al minimo.

Key words: emerging technologies; innovative seafood products; cold plasma; pulsed electric fields.

1. Introduction

In line with the previously described doctoral thesis project (D'Elia, 2021), the following research was carried out over the past year: A1) Rehydration of dry salted cod (*Gadus morhua*) assisted by PEF; A2) Evaluation of the PEF effect on salting kinetics of fresh sea bass; A3) Use of PEF for the modulation of mass transfer during salting of salmon fillets; A4) Effect of CAP treatment on the shelf-life of sea bream fillets. As examples, A3 and A4 are shown in this report. Seafood products are among the main constituents of food diets, important for their content of vitamins, proteins and other essential constituents such as polyunsaturated fatty acids (FAO, 2020). The main concern associated with this matrix is the rapid deterioration of freshness and quality. Salting is one of the oldest and simplest methods for preserving large quantities of fish for long periods of time (Sigurjon *et al.* 2014). This processing step results in safer products with a better sensory appearance, but the salting kinetics require long times for the salt to diffuse into the product and to work properly. Among existing emerging technologies, (research A3), PEF was applied to salmon tissue samples as a pre-treatment to dry salting to test whether this technique could speed up processing time and achieve higher yields. Indeed, by the research A4, the use of CAP was tested, as an emerging sanitizing technology to extend the shelf-life of fresh fish fillets (Andoni et al., 2022 ; Olatunde et al., 2021).

2. Materials and Methods

2.1 Use of pulsed electric fields (PEF) for modulating mass transfer during salting of salmon fillets

For this study, 90 samples of size 4 x 4 x 2 cm were obtained from Norwegian and Scottish cut of salmon (*Salmo salar*) fillets. The samples were divided into 10 groups of 9 replicates each. The experimental design included 5 sample groups for each salting time (3 and 6 hours). The experimental groups were control (NT) and 4 types of differently PEF pre-treated samples (PEF1, PEF2, PEF3, PEF 4). At the end of the salting times, the samples were rinsed in running water, dried and then subjected to the following analytical determinations: weight change, water activity, NaCl content change, water content change, texture, colour and the level of thiobarbituric acid reactive substances (tBars). The data were analyzed by performing an analysis of variance with one-way ANOVA. Afterwards, the Tukey HSD post-hoc test (with $p < 0.05$) was applied on the averages to reveal the presence of significant differences between the various experimental groups.

2.2 Effect of cold plasma treatment on the shelf-life of sea bream fillets

The samples of seabream fillets were divided into three experimental groups, two of which were respectively treated in air (18 kV for 20 min), or argon (80%) and oxygen (20%) mixture (18 kV for 20 min), while the third one was not treated (control). A plasma source with an SDBD (surface dielectric barrier discharge) configuration was used for plasma generation. The samples were following packed in conventional MAP (80% N_2 and 20% CO_2) and subjected to refrigerated storage (4±1 °C) for 14 days. During this period, at different storage time, possible differences among the samples were investigated in terms of quality characteristics (e.g. O_2 and CO_2 in the package headspace, pH, water content, tBars, texture, colour, sensorial properties and microbes load).

Experimental data were subjected to two-way analysis of variance (ANOVA) to determine the significant differences among samples during the storage and in each sampling time. Tukey HSD multiple range test, at a significance level of $p < 0.05$ was applied.

3. Results and Discussion

3.1 Use of PEF for modulating mass transfer during salting of salmon fillets

The investigations conducted have shown that PEF of 0.64 kV/cm (PEF3), applied prior to the three hours of salmon salting, promoted the salt diffusion into the tissues, leading to increased NaCl retention by the muscle, probably thanks to the PEF permeabilization effect on cell membranes. These process parameters in fact generated a reversible electroporation capable of favoring a more homogeneous distribution of salt within the product, also allowing for a lower percentage of weight variation compared to untreated samples. PEF did not provide any advantages in terms of a faster reduction of the water activity of the samples, especially during the shortest salting times, but it did improve the water retention properties of the samples, probably because of a more permeable structure that allowed retention of more liquid within the tissue. It is also possible that the results obtained were the consequence of a conformational change in the proteins induced by the applied treatment, which allowed greater NaCl absorption and less water loss from the samples. As far as texture, color and lipid oxidation was concerned, the treatment did not provide any difference in the treated samples compared to the control. The result obtained may be of great importance to the salmon processing industry because the achievement of higher salt levels, in a product that simultaneously lost less water, can provide an important technological advantage. In fact, the salting process is thus more efficient as processing times are significantly reduced (to only three hours) and higher processing yields are achieved with attractive cost savings for companies, improving the performance of industrial processes. However, further studies are needed in order to better understand the physico-chemical and biological mechanisms responsible of the obtained results.

3.2 Effect of CAP treatment on the shelf-life of sea bream fillets

Considering the physico-chemical characteristics, by the application of cold air plasma for 20 min, a reduced pH value of the product was recorded during storage, probably caused by the formation of acids (HNO_3, HNO_2, H_2O_2 and O_3) in the aqueous phase of the fish flesh, and a simultaneous slowing down of the natural increase in pH during shelf-life, promoted by the reduction of enzymatic activities. All the samples did not show significant differences in terms of texture parameters, evidencing that the applied treatments did not promote protein oxidation and/or evident structural modification, also confirmed by the constant maintenance of the water content during storage for all samples. The major limitation of the application of cold plasma to fish matrices can be lipid oxidation. Cold plasma is constituted by various reactive species, responsible of its potential sanitizing effect can also stimulate the oxidation process in food products with a high fat content, having a negative impact on their sensory and nutritional quality. The present study showed that already by the sixth day of storage, the samples treated by argon plasma, reached the quality limit of tBars > 4 mg MDA/kg of fresh product compared to the samples treated by air plasma (2.4 mg MDA/kg) and to control samples (1.20 mg MDA/kg). Color measurements, following the application of both treatments, also revealed significant changes in the red-green index colour (a*), that could be correlated with lipid oxidation of the matrix. The presence of total mesophiles was evaluated, setting a limit of 6 Log10 CFU/g. Results showed that especially at day 10 and day 20 of storage, the treated samples had a lower total load than the control. In particular at day 10, the control and argon group exceeded the set limit, while the data obtained from the analysis of the air-treated samples showed that the value was still below the limit. At day 20, the differences were significant, but all experimental groups were microbiologically unacceptable. It could be useful to conduct further studies to investigate the application of the 'hurdles theory', which envisages the application of CAP in conjunction with other stabilizing factors, such as the use of antioxidants etc., in order to minimize the negative side effects on the food matrix of the CAP treatment (e.g. lipid oxidation).

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Development of biotechnological protocols for the valorization of alternative plant matrices as a strategy for the sustainability of agri-food systems

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Two of the activities included in the PhD project are described below. In the first, the microbiota of carob flour, obtained from the pod and pulp, was characterized. It is indeed possible to obtain this matrix without it undergoing pre-treatment, so that the indigenous microbiota is left unaltered. The lactic acid bacteria isolated from the raw matrix were then identified. In the second, carob flour was fermented by selected starters previously isolated from alternative plant matrices and autochthonous lactic acid bacteria from carob flour, aiming at investigating the effect of fermentation on the main characteristics of the matrix.

Messa a punto di protocolli biotecnologici per la valorizzazione di matrici vegetali non convenzionali come strategia per la sostenibilità dei sistemi agroalimentari

Di seguito sono descritte due delle attività previste dal progetto di dottorato. In primo luogo, è stato caratterizzato il microbiota di uno sfarinato di carrube, ottenuto dal baccello e dalla polpa. È infatti possibile ottenere tale matrice senza che subisca pretrattamenti, così da lasciare inalterato il microbiota autoctono. I batteri lattici isolati sono stati poi identificati. Successivamente, impasti ottenuti con lo sfarinato di carrube sono stati fermentati con starter selezionati, e precedentemente isolati da matrici vegetali alternative o con i ceppi precedentemente isolati da sfarinato di carrube, al fine di verificare gli effetti della fermentazione sulle principali caratteristiche della matrice.

Key words: unconventional plant matrices, carob, fermentation, biotechnological protocols

1. Introduction

In accordance with the PhD thesis project this poster reports the main results of the first two activities concerning:
(A1) characterization of carob flour microbiota and identification of lactic acid bacteria isolates;
(A2) characterization of the carob flour fermented with lactic acid bacteria previously selected to ferment alternative plant matrices and with isolated from carob flour.

2. Materials and Methods

2.1 Spontaneous fermentation of the carob flour and identification of autochthonous lactic acid bacteria

The proximal composition of carob pods was previously evaluated by Loullis and Pinakoulaki (2018) as reported in Table 1. Carob flour, obtained from the seedless pods, was mixed with water (dough yield, DY of 200). At the end of fermentation at 30°C for 16 hours, 3 refreshments at 20% were carried out, resembling the backslopping procedure used for obtaining a traditional sourdough. Before fermentation (T0) and for each refreshment, pH and total titratable acidity (TTA) were measured. Plate counts were also carried out using selective media for lactic acid bacteria (LAB), yeasts, enterobacteria, and fungi.

Table 1 *Mean values (%) of the biochemical measurements of carob pods*

Moisture	Protein	Fat	Fiber	Ash	Sugars	Polyphenol*
6,0 – 11,0	2,0 – 7,6	0,4 – 1,3	7,6 – 38,0	2,0 – 3,4	40,7 – 54,7	1,2 – 7,0

*expressed as gallic acid equivalents (GAE) g per 100 g dry weight

Putative LAB colonies were isolated from T0 and striked until pure cultures were obtained. Genomic DNA was used for 16S rDNA amplification, and the obtained post-purification amplicons were sequenced by Macrogen Europe B.V. and identified by using NCBI database.

2.2 Fermentation of carob flour with selected starters and characterization of the fermented matrix

A flour obtained from seedless pods was mixed with water to obtain doughs with DYs of 200 and 500. The doughs were singly inoculated with lactic acid bacteria strains (*Furfurilactobacillus rossiae* T0A16, *Lactiplantibacillus plantarum* T0A10, *Lactiplantibacillus plantarum* T6B10, *Pediococcus acidilactici* 10MM0, *Lactiplantibacillus plantarum* 18S9, *Leuconostoc mesenteroides* 12MM1, *Lactiplantibacillus plantarum* H22, *Pediococcus pentosaceus* H18, *Lactiplantibacillus plantarum* H64, *Lactiplantibacillus plantarum* LB1, *Furfurilactobacillus rossiae* LB5, *Enterococcus faecium* CA16) at final concentration of approximately 7 log10 cfu/g. All the strains were previously selected as suitable starters for plant-based matrices fermentation on the basis of different

technological characteristics. The doughs were fermented for 32 hours at 30°C. Viable LAB counts and pH measurements were performed before fermentation (T0) and after 4, 8, 16, 24 and 32 hours of incubation (T4, T8, T16, T24 and T32, respectively). The concentration of peptides was tested by the *o*-phthaldialdehyde methods (OPA, Church et al., 1983), total free amino acids (TFAA) with cadmium-ninhydrin assay and organic acids using K-DLATE and K-ACETAK Megazyme kits. Antioxidant activity was tested using 2,2-diphenyl-1-picrylhydrazyl (DPPH) reagent (Blois, 1958). All analysis were carried out in triplicate, after 24 hours of fermentation at 30°C. Carob flour dough (DY 500) not inoculated, not incubated (CNI) or incubated under the same conditions of the other samples (CI) were used as controls. The antioxidant activity was tested also after carob pods roasting for 10 minutes at 150 °C and fermentation with 5 of the previously used starters which showed higher antioxidant activity. The data were subjected to one-way ANOVA; comparison was obtained with the Tukey HSD test with $P < 0.05$, using Statistica 12.0 statistical software (StatSoft Inc., Tulsa, USA)

3. Results and Discussion

3.1 Spontaneous fermentation of the carob flour and identification of autochthonous lactic acid bacteria
Analysis of autochthonous population in the fermented carob flour revealed the presence of lactic acid bacteria, yeasts, and fungi. Specifically, before fermentation approximately 3 log 10 cfu/g of LAB were observed. This cell density increased by 1 logarithmic cycle during following refreshment (T3) but decreased to 2.60 log 10 cfu/g with the next refreshment (T4). The presence of yeasts and fungi was also detected. In the unfermented dough, these were 1.78 and 3.80 log10 cfu/g, respectively. After 2 refreshments, their cell density was reduced to 1.60 log 10 cfu/g for yeasts and 3.00 log10 cfu/g for fungi. The cell density of the enterobacteria, however, was less than 10 cfu/g. During backslopping, the pH values decreased from 5.17 to 4.81; an opposite trend was observed for the TTA values, which increased from 7.30 to 11.25 ml NaOH 0.1 M after 2 days of propagation. A total of 21 isolates were obtained from the spontaneously fermented carob flour. The comparison of the sequences of the 16S rDNA amplicons, carried out within the NCBI database showed that all LAB belonged to *Enterococcus faecium* species.

3.2 Fermentation with selected starters
Dough with DY 200 did not reveal any acidification of the matrix, while when DY was 500 the initial pH (5.15 ± 0.06) decreased to values between 4.78 ± 0.06 (*E. faecium* CA16) and 3.70 ± 0.06 (*L. plantarum* H22). At the same time, 24 hours after the start of fermentation, an increase in cell density of approximately 1 log-cycle was observed for all starters used, except for *P. acidilactici* 10MM0. The strains with better capability to grow during the incubation period were *L. plantarum* H64 and *E. faecium* CA16. No differences were found between pH and cell density values observed after 24 and 32 hours, therefore fermentation time was set-up to 24 h. At the end of fermentation, both peptide and amino acid concentrations decreased in all doughs compared to the not-incubated control, except for the dough fermented with *E. faecium* CA16, which had a peptide concentration of 5350 ± 274 mg/kg. The doughs fermented with *P. pentosaceus* H18 and *L. plantarum* H64 presented higher peptide concentrations, 4966 ± 196 and 4819 ± 118 mg/kg, respectively, in comparison to the incubated control (4677 ± 362 mg/kg). The concentration of lactic acid increased significantly in all the doughs, except for that fermented with *E. faecalis* CA16 in which a lower increase was detected. The acetic acid detected in the doughs fermented with *P. pentosaceus* H18 and *E. faecium* CA16 had values only slightly higher than that found in the incubated control. For the doughs fermented with the other starters, however, an increase in acetic acid concentration was observed. The strains with the higher acidification activity were *F. rossiae* LB5 (13.67 ± 0.52 mmol/kg dry) and *L. plantarum* 18S9 (13.71 ± 3.28 mmol/kg dry). The results of antioxidant activity are shown in Figure 1. Moreover, roasting treatment had increased the antioxidant activity of the matrix both with and without subsequent fermentation.

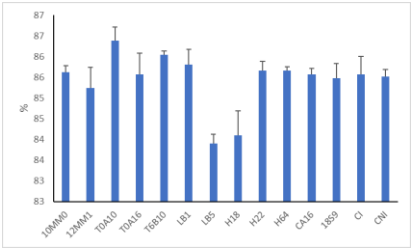

Figure 1. *Antioxidant activity expressed as DPPH radical scavenging activity (%) on methanolic extracts from carob flour fermented with selected starters after 24 hours of fermentation at 30 °C. CI: not-incubated; CNI: not-inoculated and non-incubated.*

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Modifications of vegetables subjected to conventional, innovative and non-thermal technologies

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For the processing and preservation of fruits and vegetable products, many conventional thermal processes were applied. Although these treatments are essential to stabilize the product, they could negatively modify the physico-chemical and organoleptic characteristics. One of the alternative emerging non-thermal technologies, readily accepted by consumers and successful, is high-pressure processing (HPP) (Cardello et al., 2007). The process involves the application of high pressures (up to 1000 MPa) for a short time (Chauhan et al., 2019) with the aim to reduce food pathogens at room temperature, to inactivate the deteriorative enzymes, and to extend the shelf life (Basak and Ramaswamy 1998; Zhou et al., 2014).

Analisi delle modificazioni nella microstruttura di vegetali sottoposti a tecnologie convenzionali, innovative e non termiche

I processi termici convenzionali sono stati da sempre applicati per la lavorazione e la conservazione dei prodotti ortofrutticoli. Sebbene questi trattamenti siano essenziali per stabilizzare i prodotti vegetali, essi potrebbero modificare negativamente le caratteristiche fisico-chimiche e organolettiche dei prodotti stessi. Un'emergente tecnologia alternativa di tipo non termico, , da subito ben accettata dai consumatori e risultata di gran successo, è rappresentata dal trattamento ad alte pressioni (HPP) (Cardello et al., 2007). Il processo HPP prevede l'applicazione di pressioni elevate (fino a 1000 MPa) per un breve periodo di tempo (Chauhan et al., 2019). Esso ha l'obiettivo di ridurre, a temperatura ambiente, la presenza di patogeni , di inattivare gli enzimi deterioranti e di prolungare la shelf life (Basak e Ramaswamy 1998; Zhou et al., 2014).

Keywords: HPP, Pumpkin, colour-texture, Histological analysis, bioactive and volatile components

1. Introduction

HPP technology was used in fruit and vegetable products; results indicate an impact on texture, colour and flavour, but the intensity of the changes depends on both process conditions and the type of plant tissue treated (Oey et al., 2008). From the anatomical point of view, HPP influences the microstructure (cells and tissues) too: there are many changes at the cellular and tissue level such as changes in cell shape due to loss of turgor, damage to the cell wall and membrane and formation of cracks in the plant tissues (Trejo Araya et al., 2007; Basak and Ramaswamy, 1998; Contador et al., 2014; Paciulli et al., 2021). All these changes are responsible for the loss of firmness of fruits or vegetables. The aim of this study is to evaluate the kinetic profile of effects of HPP from 100 to 600 MPa on pumpkin samples.

2. Material and methods

Pumpkin (*Cucurbita moschata* Duchesne ex Poir) samples were cut into small cubes from 1 to 1.5 cm (UNTR) and then vacuum packed in HDPE bags and subjected to high-pressure processing from 100 to 600 MPa for 3min at 20ºC. After the treatment, all samples were stored at 4°C, next day samples were used for the physical (colour and texture (texture profile analysis TPA) and chemical analyses (polyphenols and carotenoids (LC-MS), volatiles (HS-GC-MS), sugars (HPLC) and antioxidant capacity (DPPH)). For the microstructure examination, the fixed and dyed sections were observed by means of an optical microscope at different magnifications (5, 10, 20, 40 and 63X).

3. Results and discussion

3.1 Physical analyses

Moisture, total soluble solids (TSS) and pH were measured with no significant differences between UNTR and HPP treated samples. A noticeable difference was observed in HPP600 samples, with a difference in terms of colour (i.e., ΔE 11.3+1.9) due to enzymatic activity and breakdown of β-carotene and hardness (87.4+27.8 N) due to loss of turgor pressure, the loose skeleton of cell wall, cell wall and pectin bond breakdown compared to the UNTR ones (194.9+37.9 N). Treatments at other pressures changed the color and texture of the pumpkin samples, but less markedly.

3.2 Bioactive, volatiles, sugar and antioxidants component characterization

A higher number of extractable polyphenols was observed at middle pressure (200 to 400 MPa), whereas higher carotenoids content was observed at lower pressure (100 - 300 MPa) than UNTR. Regarding volatile compounds, some aldehydes (which included furan, 3-methyl (furan, 2- methyl, furan, 2-ethyl-, Pentanal, Hexanal, furan, 2-pentyl-, trans-2-(2-pentenyl) furan, 1-hexanal, benzaldehyde) significantly increased after HPP application. Sugar content decreased with HPP treatment because of the leaching effect. HPP at a pressure between 200 to 400 MPa significantly increased the antioxidants capacity (AC), as compared to other samples.

3.3 Histological analysis

Pumpkin tissue showed great structural modifications such as changes in cell size and shape, cell wall damage, increases cell wall thickness, cell detachment, cell dehydration and calcium ions deposition mainly at very high pressures (from 300 to 600 MPa) **(Fig. 1 and 2)** (Trejo Araya et al., 2007; Zhou et al., 2014;). UNTR samples showed the highest value of maximum and minimum cell elongation, perimeter segment and more regular cell wall thickness whereas HPP600 samples showed the lowest values for the same parameters (Zhang et al., 2015).

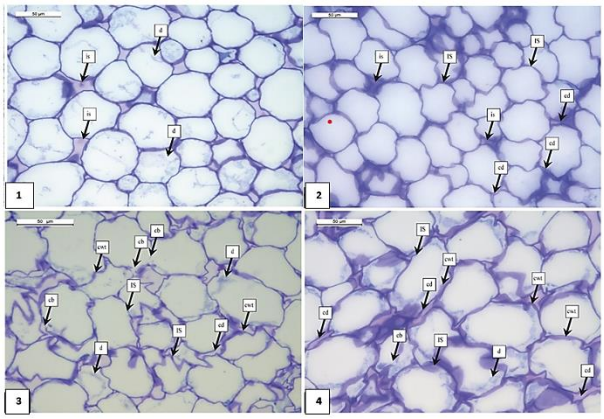

Figure 1: Light microscopy images of fresh pumpkin stained with TBO (Toluidine Blue): 1-HPP200; 2-HPP300; 3-HPP500; 4-HPP600**. Legends: d = dehydration; cd = cell detachment; Cd = cell damage; cwt = cell wall thickness**

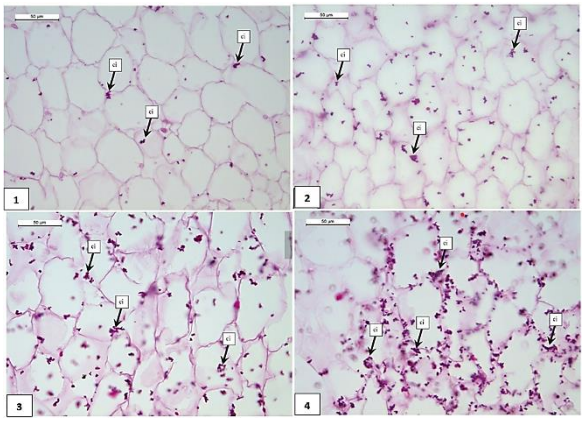

Figure 2: Light microscopy images of fresh pumpkin stained with Von Kossa (Bio-Optica kit, Milano): 1-UNTR; 2-HPP200; 3-HPP400; 4-HPP600**. Legends: ci = calcium ions.**

4. Conclusion and perspectives

High-pressure treatment from 400 to 600 MPa pressure negatively influenced the structural quality means texture and microstructure. On the contrary, from 200 to 400MPa could ensure a higher amount of "bioactive, volatiles, sugar and antioxidants components availability. On the basis of these results data, the next research will be at selective pressure ranges and at different time profiles. Samples will be tested for the same characterization to determine the quality perception of HPP technology on the vegetable.

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Sample Preparation for the Qualification and Quantification of Microplastics in some Food Matrices

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The activities carried out during the second year of the PhD course, partly carried out at Imperial College London, are described. Firstly, the pre-treatment of the food samples is studied to extract and determine microplastics content in them. Therefore, several digestion protocols are developed to identify the best digestion efficiency which guarantees a good recovery rate of microplastics. For this reason, both percentage digestion efficiency and percentage recovery rate are evaluated.

Preparazione del Campione per la Qualificazione e Quantificazione delle Microplastiche in alcune Matrici Alimentari

Le attività svolte durante il secondo anno del Corso di Dottorato, svolto all'Imperial College London, sono riportate di seguito. È stato studiato il pretrattamento dei campioni alimentari per estrarre e determinare il contenuto di microplastiche in essi contenuto. Pertanto, sono stati sviluppati diversi protocolli di digestione per identificare la migliore efficienza di digestione che garantisca un buon tasso di recupero delle microplastiche. Sono state valutate sia l'efficienza di digestione percentuale che il tasso di recupero percentuale.

Key words: microplastics, foods, contamination, plastic, digestion.

1. Introduction

In accordance with the PhD thesis project, this short communication reports the main the preliminary main results obtained during the second year concerning:

(A1) the determination of the best digestion agents for some food matrices, identifying the agents which is able to remove the organic matter of food as good as possible.

(A2) food matrices considered are spiked with polystyrene fluorescent microbeads to count them after the pre-treatment of the sample. This step is important to determine the recovery rate of microplastics after the pre-treatment and thus to quantify them.

2. Materials and Methods

On the basis of the scientific literature, the extraction of microplastics from biological matrices is commonly carried out using a digestion of organic matter with acid, base or oxidizing treatments are the most used (Avio et al., 2015). Potassium hydroxide, one and five molar in concentration, nitric acid (68%), hydrogen peroxide (30%) and Fenton's reagent are tested on pasta, red meat and fat, edible for human consumption. Samples, carefully milled prior to use, are placed into bottles (80 mL) are added with 50 mL of digestion agents and left to act for 24-40h, depending on the status of the digestion. Solutions are then filtered using Whatman glass membrane filters (pore size 1.6 µm, diameter 47 mm) with a vacuum system. All procedures are carried out under a fume hood to prevent air borne contamination. Samples are then spiked with yellow polystyrene fluorescent microbeads (diameter 9.9 µm) and undergone to digestion protocol to determine the percentage recovery rate using the visual counting (Catarino et al., 2018). Particles are counted before and after the pre-treatment using an optical microscope. For both percentage digestion and recovery rate, the experiments are conducted in triplicate.

3. Results and Discussion

3.1 Determination of percentage digestion efficiencies

For each food matrix, all digestion agents are tested and results are reported in the Table 1.
The digestion efficiency is given by the following formula:

$$ED\% = \frac{W1 - W2}{W1}\ x\ 100$$

Where W1 is the initial weight of the sample and W2 is the weight of the sample after the treatment.

***Table 1**. Percentage digestion efficiencies and relative standard deviation for pasta, red meat and fat tissues, using potassium hydroxide (KOH) one and five molar, nitric acid (68%), hydrogen peroxide (H_2O_2) and Fenton0s reagent.*

Sample	KOH 1 M	KOH 5M	Nitric Acid (68%)	H_2O_2 (30%)	Fenton's reagent
Pasta	-	-	95.0±1.0	-	93.7±5.9
Red meat	88.4±2.2	98.0±0.5	90.1±3.8	80.1±5.7	80.0±4.5
Fat	55.8±5.7	97.7±0.8	-	65.9±7.8	67.9±5.9

For pasta, the highest digestion efficiency is obtained using nitric acid 68%, whereas potassium hydroxide, one and five molar, have not provided a solution able to be filtered. After the treatment with potassium hydroxide and hydrogen peroxide, in fact, the solution was still dense and viscous. For this reason, the filtration was not performed. For red meat, the highest digestion efficiency is achieved using potassium hydroxide five molar, and, prior to filtration, ethanol (purity > 98%) was added to dissolve fat and make the filtration easier and faster. For fat tissues, edible for humans, the same treatments are tested. In this case, the suitable agent is represented by potassium hydroxide five molar.

3.2 Determination of percentage recovery rate of microplastics

For percentage recovery rate of microplastics, a counting using an optical microscope was used and the results are showed in the Figure 1.

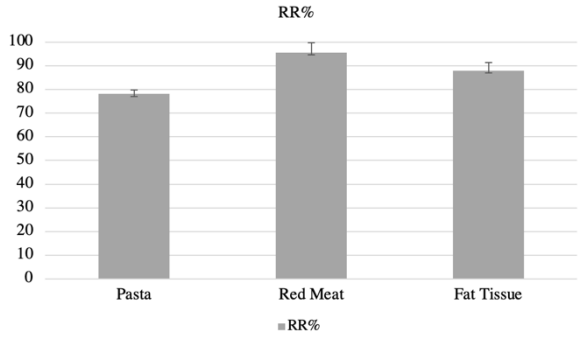

***Figure 1**. Percentage recovery rate of microplastics on pasta, red meat and fat edible tissues using as digestion agent nitric acid (68%) and potassium hydroxide (5 M), respectively.*

The percentage recovery rate is given by the following formula:

$$RR\% = \frac{NP2}{NP1}\ x\ 100$$

where NP2 is the number of particles after the treatment of the sample and NP1 represents the number of particles before the treatment of the sample. The highest recovery of microplastics is obtained for red meat sample, which was of 95.6% with a standard deviation of 4,2. For pasta and fat edible tissue, the recovery rate was of 78,1 and 87,9%, with a standard deviation of 1,7 and 3,4, respectively. For pasta, some organic matter was still visible on the filter, despite the digestion. The organic matter was thus able to cover the plastic particles, making them challenging to be seen under the optical microscope. As known from scientific literature organic matter compromises the analysis of microplastics, so far, because of its ability to generate interferences (Fabbri et al., 2020). Further researches will be carried out to optimize the digestion step and thus the recovery rate of particles to achieve a good estimation of plastic human intake via food.

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Stress response analysis of three different *Listeria monocytogenes* strains: a comparative study

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The poster shows the activities related to the PhD thesis project described previously. Three *Listeria monocytogenes* 1/2a strains were selected and analyzed applying a proteomics approach. The preliminary trials were focused on the immunoproteomics analyses of a *L. monocytogenes* strain by Western Blotting (WB) and in gel digestion combined with nLC-ESI MS/MS technique. A whole proteomics approach was applied to analyze the other selected strains in order to overcome side effects related to protein extracts resolved by 1D electrophoresis.

Analisi di risposta allo stress di tre differenti ceppi di *Listeria monocytogenes*: uno studio comparativo

Nel poster sono descritte le attività relative al progetto di dottorato precedentemente descritto. Tre ceppi di *L. monocytogenes* 1/2a sono stati selezionati e analizzati mediante tecniche di proteomica. Le prove preliminari sono state incentrate sullo studio dell'immuno-proteoma di un solo ceppo di *L. monocytogenes* mediante Western Blotting (WB) e digestione in gel seguita da analisi nLC-ESI MS/MS. Al fine di superare gli effetti negativi relativi alla separazione di estratti proteici mediante 1D elettroforesi, è stato analizzato l'intero proteoma dei ceppi selezionati.

Key words: *Listeria monocytogenes*, proteomics, food safety, data analysis, molecular biology.

1. Introduction

The following poster illustrates the main results obtained by the activities described in the relative PhD thesis project. Three different *Listeria monocytogenes* 1/2a strains, provided by the Italian National Reference Laboratory (It NRL Lm), were analyzed by proteomics techniques combined with bioinformatic tools to identify potential antigens. The latter were produced by such bacteria when they were exposed at different environmental stress conditions. Two strains were isolated from two different pork meat products, *Coppa di testa* (S1) and sausage (S2), while the third was a reference strain (S3). An immunoproteomics approach was applied to analyze S1. To overcome smearing results, a whole proteomics analysis was used to examine the other strains.

2. Materials and Methods

Listeria monocytogenes strains were cultivated at four different conditions like as C1 (control): T 37°C, pH 7.0, NaCl 0.5%; C2: T 37°C, pH 5.5, NaCl 7%; C3: T 12°C, pH 7.0, NaCl 0.5%; C4: T 12°C, pH 5.5, NaCl 7%. Biological and technical triplicates were carried out for each experimental condition. The bacterial pellet was collected and washed three times with sterile ice-cold 0.01 M phosphate-buffered saline (PBS) pH 7.0 by centrifugation at 5,600 x *g* for 10 min at 4°C. The proteins were extracted and purified by CelLytic B Cell Lysis Reagent (Sigma Aldrich) and CelLytic IB Inclusion Body Solubilization Reagent (Sigma Aldrich). The protein extracts were precipitated by TCA protocol (Koontz, 2014) and resuspended in solubilization buffer (7 M urea, 20 mM Tris–Cl, pH 8.0, 5 mM EDTA, 5 mM MgCl2, 4% CHAPS, 1 mM PMSF). Protein concentrations were quantified by PierceTM BCA Protein Assay Kit (Thermo Scientific). Protein extracts were resolved by SDS PAGE charging 12 μg of total proteins per well, by NuPage® 4-12% Bis-Tris pre-cast gels (Life Technologies Ltd) at 200 V. The WB was carried out analyzing only S1 (D'Onofrio et al., 2021). The immunogenic proteins highlighted by WB were cut from the corresponding gel to perform the in-gel digestion (Shevchencko et al., 1996). The protein extracts obtained from S2 and S3 were resolved by 1D electrophoresis, then the entire gel lane was digested in-gel. The peptide extracts were analyzed by nLC-ESI-MS/MS technique (Kidiyoor, et al., 2020). The proteins were identified by Proteome Discoverer (version 1.4.1.14, Thermo Fisher Scientific, Waltham, MA, USA), while the MS/MS spectra were searched by Mascot engine (version 2.6.0, Matrix Science, Boston, MA, USA) against the database "uniprot_listeria_monocytogenes". Scaffold (version Scaffold_4.3.3, Proteome Software Inc., Portland, OR) was used to validate peptide and protein identifications (Peptide identifications threshold: 95%; Protein identifications threshold: 99.0% with at least two identified peptides). The proteins subcellular localization was evaluated only for S1 according to Paci et al. (2021).

3. Results and Discussion

The WB highlighted different protein banding profiles for all conditions. The antigen-antibody immunocomplexes (Ag-Ab ICs) were visualized at 38 and 48 kDa for all trials, at 80 kDa for C1 and C3, at 60 kDa for C2 and C3 (data not shown). To identify *L. monocytogenes*, immunogenic proteins Ag-Ab ICs were visualized by WB and identified by nLC-ESI-MS/MS analysis. A total of 186 proteins were identified (Figure 1): 62 of which were non-cytosolic proteins, and 53 were characterized by epitopes exposed to solvent. Then, 32 proteins with high potential of antigenicity were characterized using Vaxign and Vaxigen tools (adhesion probability greater than 0.5). A total of five and three potential antigens were identified only for C1 and C2, respectively, while two for both C3 and C4.

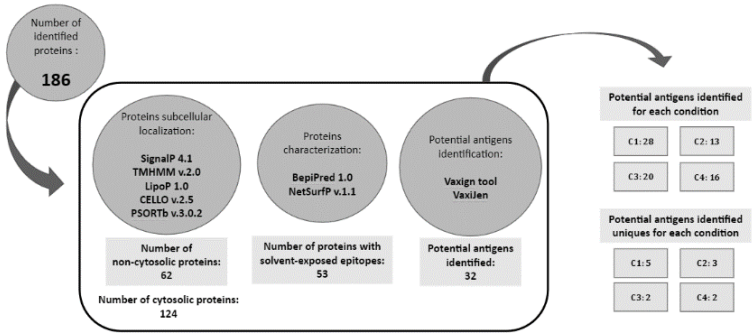

Figure 1 *Workflow of immunogenic candidates prediction.*

According to the validation criteria, the number of identified proteins by mass spectrometry were listed in Table 1. A total of 520 and 513 proteins were overlapped in S2 and S3, respectively. According to Gene Ontology (GO) analysis, the genes groups encoding for these proteins were related to the basal cell life cycle of such pathogen. The biological processes upregulated (GO analysis) in C1 were involved in the basal cell metabolism. In C2, C3, and C4 the gene groups related to response of external stimuli and cell damage repairment pathways were upregulated. Same trends seemed to be shown in S3. Such analysis allowed to understand that the pathogen can switch its metabolic functions in response to stress growth conditions. Data-analysis for S1 will be performed also for S2 and S3 to know if the potential antigens expression for each condition are the same in different strains isolated from different matrices.

Table 1 *Number of identified proteins in all experimental conditions*

Growth conditions	S2	S3
C1	831	903
C2	893	722
C3	1053	720
C4	696	734

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Sustainable production of bio-based surfactants from dairy waste using oleaginous yeasts

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Single-cell oils (SCO) are intracellular storage lipids produced by oleaginous yeasts and mainly composed of triacylglycerols (TAGs), that can be converted into industrially useful compounds such as bio-surfactants (sugar fatty acid esters, SFAEs) (Pappalardo et al., 2020). To fit the circular economy pillars, whey permeate (WP), a dairy by-product, was used as starting material for lipid production by *Cutaneotrichosporon oleaginosus* strain ATCC 20509. Processes performed on WP-based media containing different nitrogen sources and additives were compared to reach high lipid content and lipid concentration (45%, 38 g/L). To monitor the process almost in real time, flow cytometry was evaluated as a tool for the analysis of intracellular lipid content. At the end of the process, lipids were extracted, enzymatically hydrolyzed to free fatty acids (FFAs), characterized by GC-MS analysis. SCO will be used as precursors for the enzymatic esterification of alkyl glycosides into SFAEs.

Produzione sostenibile di bio-surfattanti da scarti lattiero-caseari attraverso l'uso di lieviti oleaginosi

Il permeato di siero di latte (WP), un sottoprodotto lattiero-caseario, è stato utilizzato per la crescita di lieviti in grado di accumulare TAGs. La produzione di lipidi da parte del ceppo *Cutaneotrichosporon oleaginosus* ATCC 20509 è stata ottimizzata in bioreattore, confrontando processi condotti su terreni di crescita a base di WP e contenenti diverse fonti di azoto e additivi. Sono state quindi individuate le condizioni che hanno permesso di raggiungere un elevato contenuto lipidico (45%, 38 g/L). Inoltre, la citometria a flusso è stata utilizzata per analizzare rapidamente il contenuto lipidico. Alla fine del processo, i lipidi sono stati estratti, idrolizzati enzimaticamente ad acidi grassi liberi (FFA) e caratterizzati mediante GC-MS. Tali lipidi saranno utilizzati come precursori per l'esterificazione enzimatica di glicosidi alchilici e l'ottenimento di bio-surfattanti SFAE.

Key words: Whey permeate, Single-cell oil, *C. oleaginosus*, flow cytometry, circular economy.

1. Introduction

In oleaginous yeasts, lipid accumulation occurs on sugar-rich media in concomitance with shortage of nitrogen, when sugar concentration is still high (Ageitos et al 2011). For this reason, the process is usually performed in two steps: the first one, the growth phase, was designed to provide defined amount of nitrogen and nutrients for biomass growth. The second step was designed to promote accumulation of intracellular lipids, foreseeing carbon source additions to increase C/N ratio of the medium.
This poster reports the main results concerning the process development:
A. optimization in 2L bioreactor of TAGs production by the best producer strain (*C. oleaginosus* ATCC 20509), starting from whey permeate (WP) as growth medium and comparing different nitrogen sources, medium additives and feeding strategies.
B. the assessment of flow cytometry as a fast tool to analyze the lipid content almost in real-time avoiding lipid extraction.

2. Materials and Methods

Fed-batch cultures were performed in a 2L bioreactor (Applikon Biotechnology, The Netherlands), with a starting volume of 1L. WP-based media containing different nitrogen and nutritional sources were sterilized by autoclaving at 112 °C (0.5 atm) for 30 min. Temperature was set at 28 °C, the air inlet at 1 vvm and the Cascade system allowed to maintain a constant level of dissolved oxygen (>40%). The pH was automatically adjusted and maintained at 5.5. Glucose 500g/L or diluted syrup from candied fruits (mango) manufacture (SVZ, Breda, the Netherlands), containing glucose 199 g/L and fructose 296 g/L and no nitrogen, were supplied as feeding.
Concerning flow cytometry analysis, firstly we tested different BODIPY 493/503 concentrations (0.75, 1.5 and 7.5 µM, from Thermo Fisher Scientific) and then, using BODIPY 493/503 1.5 µM, we demonstrated a strong linearity between the fluorescence intensity and the lipid content by building a calibration curve.

3. Results and Discussion

3.1 Screening of oleaginous yeasts on lactose and on WP.

To verify the presence of ß-galactosidase activity and the ability to use lactose, 16 strains were cultivated on YNB-

lactose plates. Some strains showed growth after 7 days of incubation: *Cutaneotrichosporon oleaginosus* ATCC 20508 and ATCC 20509, *Lipomyces lipofer* DBVPG 6630, *Lipomyces starkey* DBVPG 6637, *Lipomyces lipofer* ATCC 10742 and *Cryptococcus albidus* DBVPG 6110. These strains were then cultivated on WP to study how they use this waste as medium without nutritional additions. Lactose and nitrogen concentrations were on average 50±4.0 g/L and 0.28±0.01 g/L, respectively. Lipid content analyses revealed that the two C. *oleaginosus* strains synthetized the highest amount of biomass and lipids on WP, 5.33 g/L and 5.73 g/L respectively, corresponding to an intracellular lipid content of 40% and 47% (on DW).

3.2 Process optimization in bioreactor

C oleaginosus ATCC 20509 was selected as the best performing yeast, and the process was scaled-up in 2L bioreactor. In the first process, run on WP supplemented with yeast extract (WP+YE), *C. oleaginosus* developed high biomass concentration, 26.5 g/L with a final lipid concentration of 18.0±1.3 g/L, (Tab 1). For the second process, urea was chosen as an additional nitrogen source as it is considered more suitable for industrial use (WP+YE+urea). Due to the lower initial C/N ratio, *C. oleaginosus* accumulated a lower lipid content but, thanks to the higher amount of cells (65.5 g/L DW), final lipid concentration reached 29.4 g/L (Tab 1). With the aim to obtain a process completely based on waste utilization, we attempted to substitute YE by corn steep, maintaining urea as nitrogen source (WP+urea+corn steep). Under these conditions, *C. oleaginosus* produced a lower amount of total biomass with a lipid content of 43%, corresponding to a lipid concentration of 19 g/L (Table 1). In conclusion, despite the C/N ratio in this process was maintained similar to the one with YE, corn steep seems to represent a poorer substitute of YE for production of biomass. To furthermore improve the use of wastes as feedstock for the whole process, pure glucose feeding was replaced by a syrup from mango processing, an industrial residue coming from candied fruits production. In this process (WP+YE+urea+mango), with the purpose of further enriching the medium by essential nutrients, the initial amount of YE was increased, and urea was provided as nitrogen source. Cells grew at the highest growth rate, and the highest concentrations of biomass and lipids were obtained, 84 g/L and 38 g/L respectively (Tab1).

Table 1 *Process parameters by C. oleaginosus* ATCC 20509 *in WP media with different nitrogen and nutritional sources.*

	WP+YE	WP+YE+urea	WP+urea+corn steep	WP+YE+urea+mango
Growth rate (μ)	0.26	0.28	0.26	0.32
Final biomass (g/L)	26.5±0.27	65.5±1.21	46±0.51	84±1.27
Lipids (g/L)	18±1.3	29.4±0.6	19±0.4	38±1.0
Lipids (%)	68%	45%	43%	45%
Yield	0.21	0.16	0.12	0.10
Productivity (g/L/h)	0.20	0.31	0.20	0.57
Initial C/N	60.59	18.33	16.54	10.5

3.3 Lipid analysis

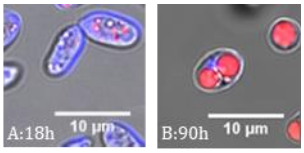
Lipid accumulation was evaluated by:

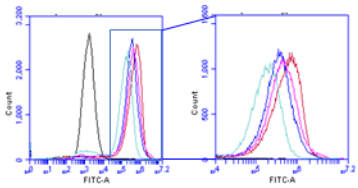
A. Confocal microscopy, by staining cells with Nile-Red (3 μM) at the beginning and at the end of the process. As shown in Fig. 1B, after 90 hours of process the cells appear full of lipid emitting in red.

B. Flow cytometry, by staining intracellular cellular lipids with BODIPY 493/503 (1,5 μM) (Fig. 2). It is clearly visible a shift in fluorescence intensity signal towards higher values in samples over time (from 174498.47 after 26 hours, to 603583.06 after 90 hours), indicating the progress of lipid accumulation.

C. CG analysis to obtain the lipid profile (Table 2), that resulted very similar to those obtained by the same strain on different media (Capusoni et al., 2017).

Figure 1 *Confocal microscopy, cells stained with Nile-Red (3 μM) after 18h (A) and 90h (B) of process in WP+YE (60x).*
Figure 2 *FACS histograms showing BODIPY 493/503 fluorescence (in FITC channel). Black: unstained sample, light blue: 25h, blue: 55h, pink: 75h, red: 90h.*
Table 2 *Fatty acid (FA) profile obtained after 90h in WP+YE+urea.*





Fatty acid	%
Oleic acid (C18:1)	45.49
Palmitic acid (C16:0)	27.24
Stearic acid (C18:0)	21.42
Linoleic acid (C18:2)	2,91

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Development of an electrical sensor to detect micro and nanoplastics in environmental and agri-food samples

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The aim of the project is to develop an electrical sensor for the detection of micro/nanoplastics. An electrolyte-gated field-effect transistor based sensor was fabricated, with carbon nanotubes as semiconducting material. The stability of the device was first tested in water, then tests with varying concentrations of polystyrene nanoplastics were carried out. In presence of the nanoplastics, the current flowing from drain to source electrodes increased, with increasing concentrations of nanoplastics. This leads to the assumption that nanoplastics and CNTs are interacting and this interaction causes a rearrangement in the CNTs network, which is shown in a higher current.

Sviluppo di un sensore elettrico per il rilevamento di micro e nanoplastiche in campioni ambientali

Lo scopo del progetto di dottorato è sviluppare un sensore elettrico per la rilevazione di micro-nanoplastiche. Un sensore basato su un transistore ad effetto di campo è stato fabbricato, con nanotubi di carbonio come materiale semiconduttivo. Come prima cosa, la stabilità del sensore in acqua è stata testata, poi sono stati fatti test con diverse concentrazioni di nanoplastiche di polistirene. In presenza delle nanoplastiche, si è visto un aumento nella corrente tra source e drain, proporzionale alle concentrazioni di nanoplastiche. L'interazione tra nanoplastiche e nanotubi causa probabilmente un riarrangiamento del network dei nanotubi, che quindi risulta in una corrente maggiore.

1. Introduction

In accordance with the PhD project, here are reported the first practical activities carried out and developed in the second year of PhD, which were: 1) Optimization of the fabrication process of the electrolyte-gated field-effect transistor (EG-FET) based sensor (such as the photolithography steps parameters and the carbon nanotubes (CNTs) spraying parameters); 2) Stability test with only deionized water (DI-water), on the fabricated EG-FETs; 3) Test with different polystyrene (PS) nanoplastics (NPs) concentrations to assess the device response.

2. Materials and methods

An EG-FET was fabricated on glass substrate, as shown in Fig 1, by employing negative photolithography process followed by thermal evaporation and lift-off steps, as described in (Shkodra et al. 2022). Semiconducting CNTs were spray coated on top of the interdigitated contacts (60 layers of CNTs, for details, refer to (Shkodra et al. 2022)). The electrical response of the devices to water and to increasing concentrations of NPs was tested (probe station - Keysight B1500A semiconductor device parameter analyzer); a plastic microchamber was put on top of the interdigitated contacts and part of the gate electrode, as a way of incapsulating the liquid solution and of covering with liquid only those parts of the device. The stability of the devices in DI-water was tested, and transfer curves (drain to source current (I_{DS}) measured, with fixed V_{DS} of -100mV and V_{GS} swept from 500 to -500mV) were taken every 5 minutes for 2 hours. To investigate the NPs (PS microbeads with 100nm diameter, Alpha Nanotech Inc., Canada), DI-water was first added, then 7 concentrations of PS were used (0.05, 0.1, 0.25, 0.5, 1, 2 and 5 mg/ml), for each concentration transfer curves were taken every 5 minutes for 45 minutes (every 45 minutes more volume was added to the microchamber to obtain the needed concentration of NPs). Atomic force microscopy (Nanosurf CoreAFM, Nanosurf AG, Switzerland) images of the interdigitated contacts were taken both before and after NPs test (PS solution was removed and the device was gently rinsed with DI-water), first to confirm the formation of a network of CNTs, and then to check whether NPs were present on top of CNTs (and interacting in some way).

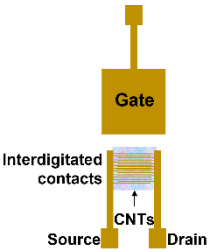

Figure 1. Schematic of the sensor's design. Gold electrodes (gate, source and drain) are patterned with photolithography process. CNTs are spray deposited on top of the interdigitated contacts.

3. Results and Discussion

a. EG-FET stability in DI-water

EG-FETs are sensors suitable for environmental monitoring applications (Elli et al. 2022) and thus were chosen for this project. Transfer curves of EG-FETs with DI-water were taken every 5 minutes, and after 2 hours I_{DS} stabilized. To understand whether a change in liquid volume would change the current, 4 additions of DI-water were done; the transfer curves (taken every 5 minutes) showed that the increase in volume did not affect I_{DS}. The normalized I_{DS} (%) was calculated (Normalized I_{DS} = (I_DX/I_D5min)*100) and in Fig 2, both I_DON (at V_{GS} -0.4V) and I_DOFF (at V_{GS} 0.8V), are shown (while V_{DS} is fixed at -0.1V). These results lead to the conclusion that addition of volume does not affect the electrical response of the devices, thus they can be used for biosensing applications, and in this case for the test with NPs. In fact, this behavior is consistent with the current literature, as (Molazemhosseini et al. 2021) previously showed an EG-FET which stabilized after 1 hour in DI-water and was then used for biosensing applications.

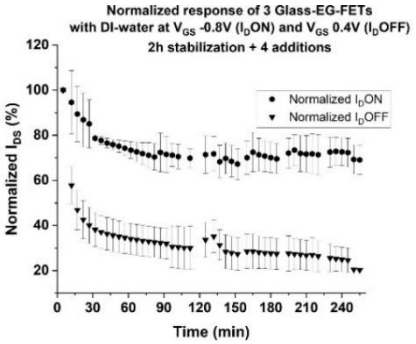

Figure 2. Normalized response of 3 EG-FETs (average and standard deviation) during stabilization test with DI-water. Black lines indicate the addition of more water volume. No increase could be seen after those additions.

b. Test with PS

Different concentrations of PS nanoplastics were tested on EG-FETs (after 2h of stabilization in DI-water). A small increase in I_DON was seen, starting from PS concentration of 0.25 mg/ml (an increase in normalized I_DON of about 3%), while for higher concentration the increase was higher and proportional to the concentration (at 0.5 mg/ml, the increase was of ~12%, while at 5 mg/ml the increase was of ~54%). In Fig 3, normalized I_DON and I_DOFF are shown, the average of 3 devices and standard deviation are depicted. From these preliminary results, PS seemed to interact with the CNTs and affect their electrical properties, we assume that NPs in the solution could be physically and/or chemically adsorbed by the CNTs network.

c. Morphological Characterization with AFM

In Fig 3, AFM images of the interdigitated contacts are shown (top images, defined as "before PS"). Both on the glass and on the gold part, a CNTs network was visible. AFM images taken after the PS test are also shown in Fig 3 (bottom images, defined as "after PS"), the NPs are visible and seem to interact with the CNTs. The physical interaction between NPs and CNTs is thus confirmed, however what is still missing is to understand whether there is also a chemical interaction or not.

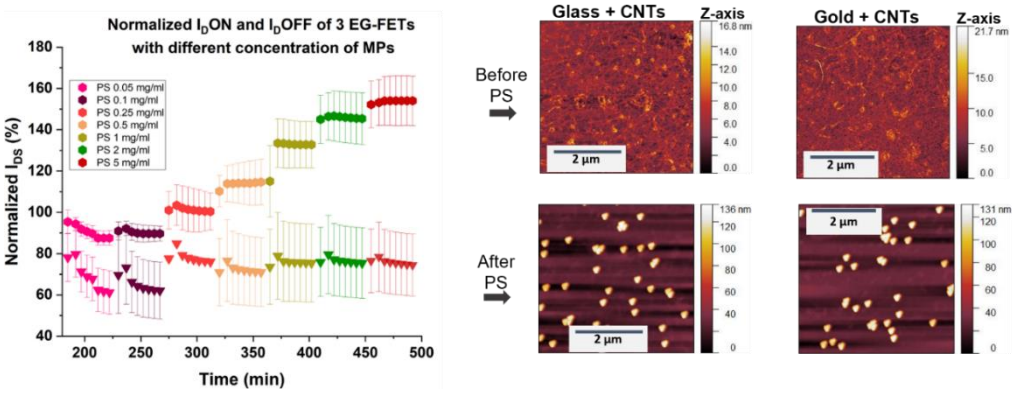

Figure 3. On the left; normalized I_DON and I_DOFF (average of 3 devices) when PS at different concentration was added on the devices. An increase in I_DON is visible starting from PS 0.25mg/ml. On the right; AFM images of the interdigitated contacts (gold and glass part) before and after PS test. Before PS, CNTs network is clearly visible; after PS the microbeads (100nm in diameter) are visible and seem attached to the CNTs.

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Food Combinations and its Quality as hallmarks for Biogenic Amines Exposure. A study on current People Habits in Specific Meals and Snacking Occasions

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Tutors: Prof. Dino Mastrocola, Prof. Maria Martuscelli

Here are presented some of the preliminary phases of this study including: the ideation of the survey, its distribution, and results collection. Furthermore, the biogenic amines' (BAs) presence in selected products is presented with the first outputs from questionnaires including a wider description of the participants involved. Subsequently, the next planned steps are briefly summarised, pointing out the main expected results.

Le combinazioni di alimenti e la loro qualità come segni distintivi per l'esposizione alle amine biogene. Uno studio sulle attuali tendenze nelle abitudini alimentari in specifici pasti e/o spuntini

Sono qui presentate alcune delle fasi preliminari di questo studio; l'ideazione dei questionari, la loro diffusione, e la raccolta dei risultati. Vengono inoltre forniti alcuni dati sulla presenza di amine biogene (AB) in prodotti scelti insieme ai primi *outputs* provenienti dai questionari che includono una più ampia descrizione dei partecipanti. In seguito, vengono riassunte le future linee di ricerca puntando l'attenzione sui risultati attesi.

Key words: Biogenic Amines, Food Combinations, Food Quality, Food Safety

1. Introduction

The everyday choices in diet are linked to our health as known for obesity, type 2 diabetes, and metabolic syndrome emergency. Foods and beverages expose us to many compounds which are still needing extensive research Russo et al. (2010). Among these, BAs are of high interest for their controversial nature. They can be contemporarily hazardous and fundamental for many bodily functions Martuscelli et al. (2021). Their negative action can be somehow amplified by dietary habits and meal composition above the personal sensitivity of subjects. At any rate, talking about BAs' risk, the potential hazardous profile of a food is primarily influenced by factors like its obtention (e.g., the process), and foods' intrinsic characteristics (e.g., the composition, the storage). So, the close interconnection among all these factors explains why food quality -of the raw ingredients and of the processing- is as important as personal choices and meal composition. These features are people-dependent, and few recommendations or protocols may be actuated on them. Finally, the puzzling picture coming out, clears that a holistic approach must be embraced. For this reason, this project is focused on understanding which is the real situation when evaluating the risk of BAs exposure of people on specific eating occasions and/or snack episodes.

2. Materials and Methods

2.1 Redaction of the online survey

A survey was designed asking people how they have snacking episodes during the day and specifically considering those occasions conducible to happy hour/*aperitivo*-like. The survey contains a section about general information and lifestyle (dietary patterns, drugs assumption, allergies, etc..), the other sections are divided in respect to food categories: meaty foods, cheeses, vegetables-based foods, seafood, and beverages. For each food category, it is required to select the quantity of defined products listed (e.g., salami, dry-cured ham, pecorino cheese, olives etc.) and the frequency of consumption (sometimes, 1-3 times a week, 4-6 times a week, every day). In the end, questions about the health status in relation to the consumption of these goods are posed. Before the survey's distribution, a commission of experts evaluated the clarity and the validity of the questions giving positive feedback.

2.2 BAs content of food products

The BAs' content was determined according to Latorre-Moratalla et al. (2009) for table olives (n=8), nuts (n=26) and beer (n=20) For fermented cold cuts (n=20), dry-cured ham (n=15), and mature cheeses (n=20) the method described by Chaves et al. (2016) was used. Finally, for wines (red, rosé, and white, n=15 for each) the BAs content was achieved according to Manetta et al. (2016). All the products were coming from the market retail, trying to simulate the closest scenario to the reality.

3. Results and discussion

Some information from respondents is summarized in Figure 1. By reviewing the outcomes from the questionnaire, it resulted that fermented cold cuts, dry-cured ham, mature cheeses, nuts, and olives, are popular choices in composing *aperitivo*- like meal occasions, while among beverages wine (red, rosé, and white) and beer. Figure 2 shows the frequency option of 1-3 times a week for all the food classes mentioned. This selection was done considering its constant rate for all the products listed. However, it should be noted that these extrapolations are incomplete as the survey distribution is still open. Data showed are referred to the lapse of time of 1 month (05/05/2022-06/06/2022). Table 1 lists the range contents and the median value of BAs of investigated samples. Aside from the great variability among different sources, it would be interesting to put in evidence the possibility of a cumulative effect obtained from the survey, as it is commonly accepted eating together these products in reduced portions (or even not) often adding units of alcoholic beverages. By crossing data from the frequency of consumption, the quantity of ingested foods, and the BAs' content, it is expected to calculate the real exposure of people during these meal occasions. Hopefully, soon it will be available a tool to attempt a deeper comprehension of this risk and introduce a different communication to consumers. Furthermore, this kind of approach will be used to reach other population segments such as children, toddlers, and elders. By means of questionnaires, other key information will be reached as the drugs' consumption and the possible interaction with declared symptoms and eating habits. Among other goals, the available dataset is continuously in updating, entering new food products (mainly vegetable-based goods fermented or not) and adding new entries of fermented foods and beverages

Figure 1. General information from respondents

General info about respondents

Number of participants: 282

Age: 41.14 ± 13.9

Gender: 63.5% women, 35.8% men

0.7% I prefer not to answer

Drug's use: 15.25% yes, 84.8% no

Figure 2. Frequency of consumption (%) of selected products (1-3 times a week) n=282

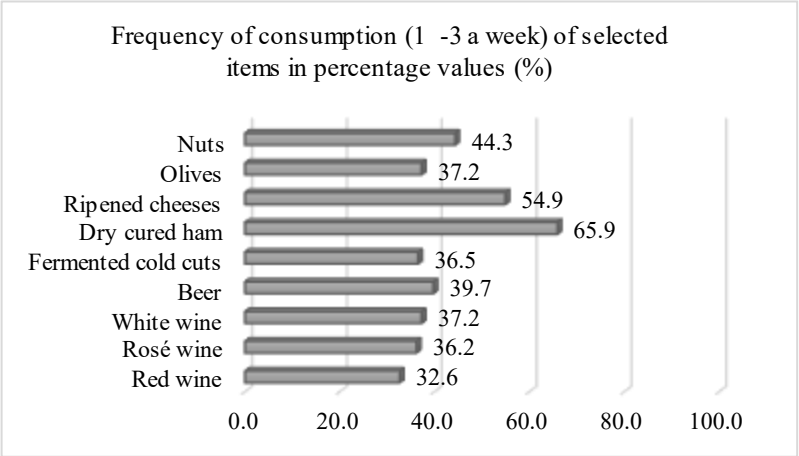

Table 1 content of BAs in some classes of products present in the survey. Range, and median value in brackets

BAs content in food samples (mean mg/kg)									
Product	TYR	PUT	CAD	HIS	SPD	SPM	PHE	SER	ETY*
Fermented cold cuts (n=20)	n.d.-556 (56)	n.d.-446 (35)	n.d.-271 (46.5)	n.d.-100 (39)	0-99 (49)	n.d.-97 (47)	n.d.-99 (19)	n.d.	n.d.
Dry cured ham (n=15)	n.d.	1-22.4 (8.28)	n.d.	0.5-7.83 (2.61)	5.8-34.1 (17.2)	12.1-174.4 (59.3)	0-11.1 (0)	5.3-85.6 (19.5)	n.d.
Mature cheeses (n=20)	n.d.-141.4 (5.05)	n.d.-85 (8.1)	n.d.-192 (2.3)	n.d.-40.8 (0.5)	n.d.-6.1 (2.4)	n.d.-25.3 (0.7)	n.d.-8.4 (0.85)	n.d.	n.d.
Nuts (n=26)	0-2.73 (0)	0-8 (1.71)	0-2.4 (0)	n.d.-0.75 (0)	0.1-58.3 (23.68)	0.1-23.8 (8.32)	n.d.-0.8 (0)	n.d.-3 (2.37)	n.d.
Olives (n=8)	n.d.-0.1 (0.1)	0.1-4.6 (2.7)	0-1.4 (0.1)	n.d.-0.1 (0.1)	n.d.-0.1 (0)	n.d.	n.d.	n.d.	n.d.
Red wines (n=15)	0-16.8 (4.1)	0.5-29.7 (7.1)	0-1.1 (0)	0-10.8 (0)	n.d.	n.d.	n.d.	n.d.	0.8-6.3 (3)
Rosé wines (n=15)	0-12.8 (0.4)	0.4-9.9 (2.8)	0.7-1.1 (0)	0-4.3 (0.9)	0-1.4 (0)	n.d.	0-1.5 (0)	n.d.	1.4-7.5 (2.3)
White wines (n=15)	0-8 (0)	0.8-9.6 (1.8)	0-1.2 (0.5)	0-3.4 (0)	n.d.	n.d.	n.d.	n.d.	1.1-8.3 (2.9)
Beer (n=20)	0-46.8 (2.37)	0.2-5.2 (0.4)	n.d.	0-21.6 (0.7)	0-35.3 (4.3)	n.d.	n.d.	n.d.	n.d.

*TYR, PUT, CAD, HIS, SPD, SPM, PHE, TRP, SER, ETY Abbreviations for tyramine, putrescine, cadaverine, histamine, spermidine, spermine phenylethylamine, tryptamine, serotonin, and ethylamine.
n.d.= not detected

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New tools for authentication and traceability to assure the integrity of food chain

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The use of label claims such as "antibiotic-free" or "raised without antibiotics" in the meat market leads to new challenges concerning tools for authentication and traceability. The objectives of this study were to evaluate metabolic changes and investigate putative metabolites related to antibiotic treatments, to identify potential biomarkers of antibiotic-free reared pig. To this aim, pig liver was investigated by a metabolomics approach and preliminary statistical results are presented here.

Nuovi strumenti per l'autenticazione e la tracciabilità per assicurare l'integrità della filiera alimentare

La diffusione nel settore delle carni di claim in etichetta, es. allevato senza l'utilizzo di antibiotici, conduce a nuove sfide concernenti strumenti per l'autenticazione e la tracciabilità. Gli obiettivi di questo studio sono stati: valutare differenze metaboliche tra gruppi di campioni e ricercare possibili metaboliti correlati all'utilizzo di antibiotici per identificare biomarker che garantiscano il claim "allevato senza l'utilizzo di antibiotici". Un approccio metabolomico untargeted è stato impiegato su fegato di suino e, di seguito, vengono presentati i risultati preliminari di questo studio.

Key words: Antibiotic free, Pigs liver, Metabolomics, ^{1}H NMR, multivariate data analysis.

1. Introduction

This poster reports the achievement of the first two milestones of the PhD thesis project concerning:
(A1) the development of an appropriate tissue extraction protocol that is suitable for NMR analysis;
(A2) the assessment of metabolic differences by using multivariate data analysis to identify discriminant metabolites among two groups of pigs.

2. Materials and Methods

41 heavy pigs belonging to 4 different farms of Northern Italy were selected for this study and divided into 2 groups. The groups were chosen according to the daily dosage of the active substance (mg) used to treat 1 kg of pig related to the biomass of the animals present in the farm, known as Defined Daily Dose Animal for Italy ($DDDAit_{biom}$) established by the Italian Ministry of Health (Ministero della Salute - Direzione Generale della Sanità Animale e dei Farmaci, 2022).

Table 1. *Overview of the experimental design. For each farm, $DDDAit_{biom}$ has been obtained from the Italian monitoring system ClassyFarm (www.classyfarm.it) related to the period under investigation, year 2020. The values reported are expressed as "dav/animal/vear".*

Treatment group (*n*=19)		**Control group (*n*=22)**	
Farm 1	Farm 2	Farm 3	Farm 4
16.3	37.4	0.38	0.14

The Bligh and Dyer (Bligh, E.G. and Dyer, 1959) method was employed for the recovery of both polar and non-polar metabolites of frozen liver sample. Prior to ^{1}H NMR analysis, the polar fractions were resuspended with sodium phosphate buffer in D_2O and TSP, while the non-polar fractions with deuterated methanol and chloroform. ^{1}H NMR spectra were collected on 600 MHz spectrometer and corrected for phase and baseline, thus calibrated. Data were subjected to Principal Component Analysis (PCA) and Orthogonal Partial Least Squares (OPLS-DA). The quality of the models was evaluated considering as performance indicators the goodness-of-fit (R2X) and the predictive ability (Q2). Further, we considered significant those signals in the range of ppm with Variable Importance in Projection VIP score ≥ 1.

3. Results and Discussion

Two data matrices N x R (N= samples; R= ppm range of integration pattern focused in the spectral region of 0-9 ppm) were built for a total of 3 444 and 3 116 values for polar and non-polar fractions, respectively. The presence of a grouping trend was observed in PCA, as depicted in **Figure 1** for score plots (**A)** and (**C)**. Related to PCA, the first 2 PCs explained 39 and 50.9% of cumulative variance for non-polar and polar phase, respectively. This

clustering became clearer when OPLS-DA was performed thanks to which the condition under investigation was strengthened since the two groups of samples were perfectly separated in the bi-dimensional space, the control samples were distributed along the positive t[1] component and the treated samples along the negative one. As shown in **Figure 1** for the score plots (**B)** and (**D)**, a better performance was observed for non-polar extracts (**B**) compared to polar extracts (**D**) which showed a mild degree of heterogeneity within samples of the same group. Seventeen and eleven NMR signals of polar and non-polar fractions, respectively, were found to be characterised by VIP score ≥ 1 and selected to perform the identification of the discriminant signals. Also, regarding the assignment of ^{1}H NMR peaks, polar spectra were predominantly characterized by amino acids, while for non-polar fraction different classes of lipids were identified.

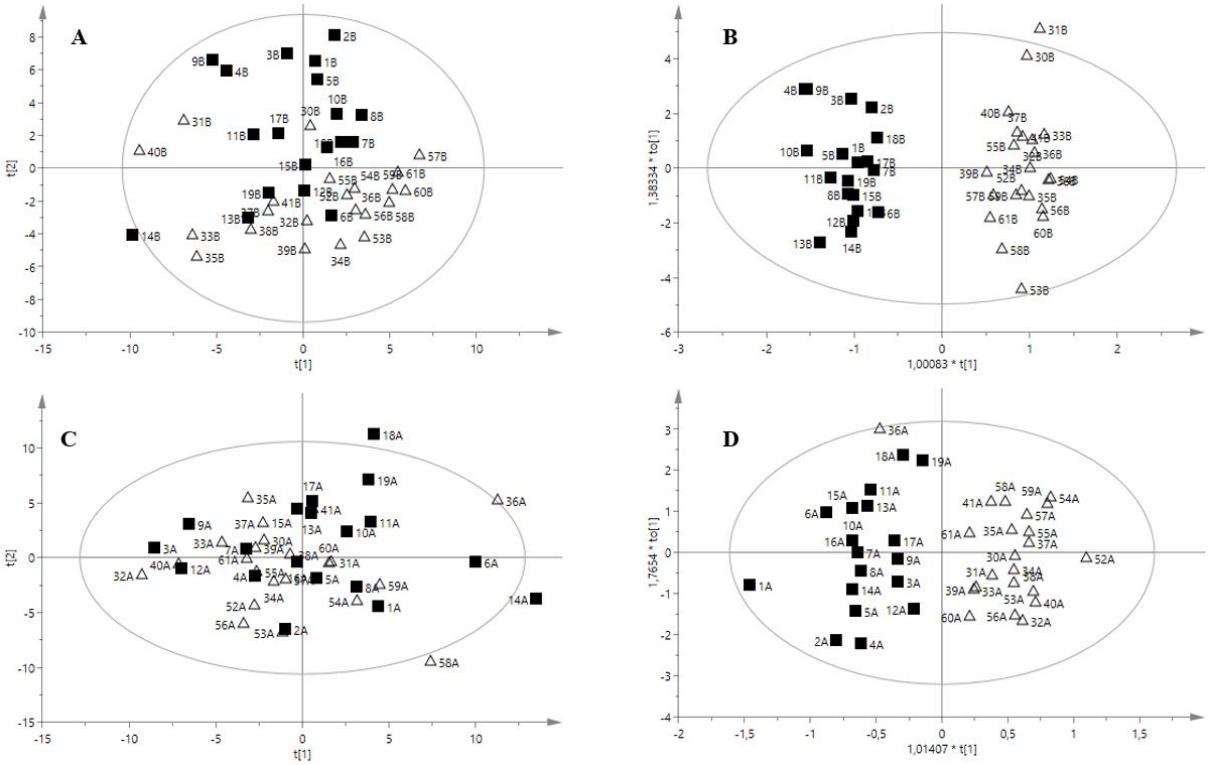

Figure 1. *PCA score plots (**A**-**C**) and OPLS-DA score plots (**B**-**D**) of non-polar and polar extracts are reported here. The black boxes represent positive group while the empty triangles the control group. Regarding liver non-polar fraction, the performance indicators were for (**A**) PCA (R2X= 0.725; Q2=0.419) and for (**B**) OPLS-DA (R2X= 0.939; R2Y=0.948; Q2=0.869); for polar fraction were for (**C**) PCA (R2X =0.751; Q2= 0.529) and for (**D**) OPLS-DA (R2X= 0.892; R2Y= 0.774; Q2= 0.613).*

In conclusion, metabolic differences between pigs subjected to antibiotic treatment from those untreated have been observed in liver; these preliminary results provide high potential with regard to the identification of treatment biomarkers useful for meat authentication and traceability.

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Sensory methods ensuring authenticity and fostering Mediterranean fish

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Tutor: Prof.ssa Rossella Di Monaco

The aim of this PhD project is to use sensory analysis to foster Mediterranean fish products, ensuring authenticity and improving consumers' trust. It will be achieved through a multidisciplinary approach concerning the study of consumer behavior toward seafood products, and through the development and the optimization of descriptive sensory methods to assess the authenticity and quality of Mediterranean seafood products. The final objective is to reduce fishing fraud and improve consumers' trust in fish products by providing all the useful information to consumers, both on traceability and authenticity of fish and on sensory and nutritional aspects.

Nuovi metodi sensoriali per garantire l'autenticità e valorizzare i prodotti ittici del Mediterraneo

L'obiettivo del seguente progetto di dottorato è quello di utilizzare l'analisi sensoriale al fine di valorizzare i prodotti ittici del Mediterraneo. L'obiettivo sarà raggiunto attraverso un approccio multidisciplinare riguardante lo studio del comportamento del consumatore nei riguardi dei prodotti ittici, e attraverso lo sviluppo e l'ottimizzazione di metodi sensoriali descrittivi capaci di garantire autenticità e qualità ai prodotti ittici del Mediterraneo. Lo scopo ultimo è quello di ridurre le frodi ed aumentare la fiducia dei consumatori nei riguardi dei prodotti ittici, fornendo tutte le informazioni utili sia riguardo la tracciabilità e l'autenticità, sia su aspetti sensoriali e nutrizionali.

1- Introduction

Fish products are consumed all around the world thanks to their nutritional composition rich in protein, long-chain fatty acids (EPA and DHA), vitamins, and poor in carbohydrates (Carlucci et al., 2015). Thus, given the growing interest of the population in a healthy lifestyle, world pro-capita fish consumption increases in the last 50 years, from 9.9 kg in 1960 to 19.2 in 2012 (FAO 2014). This increasing trend in fish consumption has led to a negative ecological impact and can threaten the natural fish stocks in the sea (Verbeke et al., 2007). For the reasons above, the aim of the following study was to investigate several individual variables possibly driving fish consumption among two European Mediterranean countries in which fishing provides an important income and commercial opportunities. The investigation was carried out through an online questionnaire submitted to a big number of consumers in both Italy and Spain. Then, the second aim was to characterize two species of tuna through the descriptive analysis. Finally, the influence of sustainability labels on consumers' fish perception was studied.

2- Materials and methods

The questionnaire, which consisted of 50 questions, was sent to 1000 Italian and 1000 Spanish consumers, but only 1917 of them were selected (963 in Italy and 954 in Spain) since some of the subjects were not compatible with the objectives of the research (different diet habits, no fish liking, no fish consumers). Subjects were equally distributed for social-demographic information. Two species of tuna were analysed by a classical descriptive method with seven trained assessors, evaluating the sensory properties of the samples by using 10cm linear scales. The consumer test was carried out with 104 subjects. in two experimental conditions: they first evaluated in blind the tuna samples, and second, after one week, they evaluated the same samples, accompanied by the sustainability label information. A 9-point hedonic scale (1: Extremely disliked; 9: Extremely liked) was used for the acceptability evaluation. The respondents to the online questionnaire were grouped for provenance (Italy and Spain), gender, and age (4 age groups) for 16 observations. Multifactorial analysis (MFA) was used to study the relationship between the observations and all the collected variables. Paired sample t-test was used to characterize and discriminate the two tuna species, and also to assess the influence of the sustainability label on acceptability. The XLSTAT statistical software (v.2016.02, Addinsoft) was used for data analysis.

3- Results and discussion

Figure 1a is a representation of the subjects on the first two dimensions extracted by MFA accounting for 62.94% of the variance. Subjects were well separated in terms of provenance and age. Figure 1b allows observing the variables which characterize the subjects and the associations among them. Old Spanish subjects consume more fish than Italian ones, on the other hand, Italian subjects like fish more than Spanish. Also, old Spanish subjects consume more fresh fish, while old Italian subjects are more familiar with frozen and canned fish. Focusing the attention on sustainability and traceability, young subjects are more familiar with the MSC label than the adult and

old ones, according to Salladarré et al. (2010). Notably, the importance of traceability and the Mediterranean Sea provenance are perceived as more important for old subjects than young ones. The descriptive analysis allowed us to characterize the two species of tuna, it also highlighted how the two raw species differed significantly from each other in all sensory attributes ($p \leq 0.05$) (Figure 2). Regarding the influence of the sustainability label on consumer fish perception, the consumer test showed as liking scores increased when the sustainability label information was provided to consumers, in particular, the overall liking score increased significantly ($p \leq 0.05$) (Figure 3). Despite subjects demonstrated to have a limited knowledge of ecolabels and the different aspects they cover (Hoek et al., 2021), the sustainability label information increased the evaluated scores. As Di Monaco et al. (2004) highlighted, external information provided at purchase time, plays a key role in consumers' choices and liking judgments.

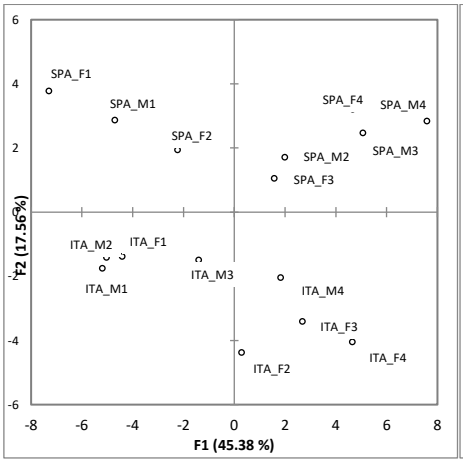

Figure 1a- Observation map – MFA results

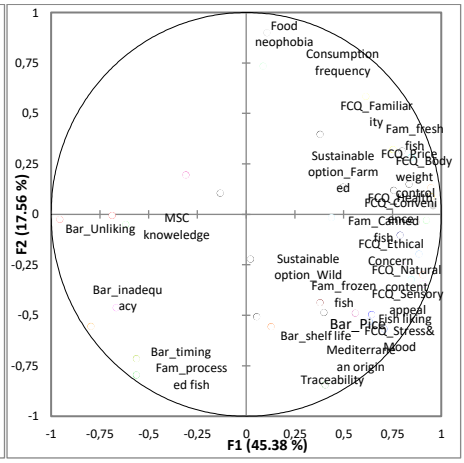

Figure 1b- Variables map – MFA results

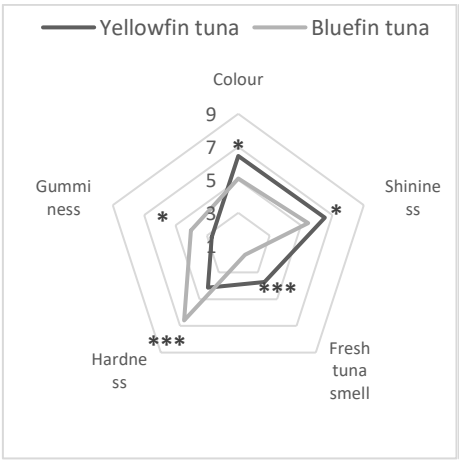

Figure 2- Raw tuna sensory profile

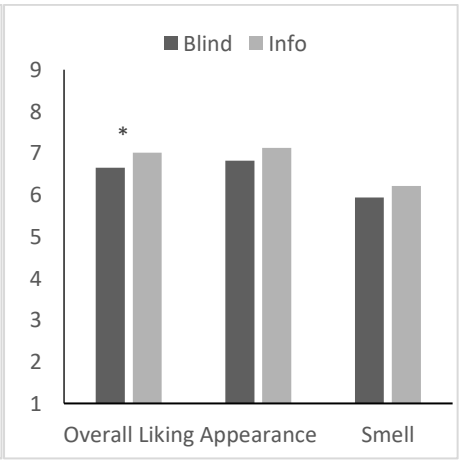

Figure 3- Consumer test evaluation

Asterisks indicate significant differences (* $p<0.05$; ** $p<0.01$; *** $p<0.001$)

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Application of functional molecules recovered from bergamot by-products: development and improvement of food systems

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The present poster communication reports the first activities of the PhD thesis project. Bergamot pomace, a citrus by-product, has been studied optimizing phenolic compounds' extraction using different food-grade extraction techniques (conventional, microwave, ultrasound). The selected extract has been used to enrich vegetable fat to use as a new ingredient for the formulation of functional bakery product. The aim is to valorise citrus by-products to a circular economy perspective. Following this approach, this by-product can be exploited in the food field to obtain natural antioxidants and additive, functional, innovative, and sustainable products.

Applicazione di molecole funzionali recuperate da pastazzo di bergamotto: sviluppo e miglioramento di sistemi alimentari

Il presente poster riporta le prime attività del progetto di tesi di dottorato. Il pastazzo di bergamotto, un sottoprodotto degli agrumi, è stato studiato ottimizzando l'estrazione dei composti fenolici utilizzando diverse tecniche di estrazione "food-grade" (convenzionale, microonde, ultrasuoni. Il miglior estratto ottenuto è stato utilizzato per arricchire un grasso vegetale da utilizzare come nuovo ingrediente per la formulazione di un prodotto da forno funzionalizzato. L'obiettivo è di valorizzare i sottoprodotti agrumicoli in un'ottica di economia circolare. Seguendo questo approccio, questo sottoprodotto può essere sfruttato in campo alimentare per ottenere antiossidanti e additivi naturali, prodotti funzionali, innovativi e sostenibili.

Key words: Citrus by-products, phenolic extraction, bioactive compounds, functional foods, vegetable fat, bakery product.

1. Introduction

In accordance with the PhD thesis project, the main results of the activities are reported:
(A1) Extraction of bioactive compounds;
(A2) Analytical characterization of obtained extract, in terms of basic chemical and physical characteristics and identification and quantification of phenolic compounds;
(A3) Formulation of a super-ingredient (vegetable fat) and its application on a functional food (bakery products).

2. Materials and Methods

The aim of this work was to optimize the recovery of the phenolic compounds from Bergamot Citrus pomace (BCP), in order to obtain a natural antioxidant extract (AE) to use in food system. BCP represents an important by-product and it was supplied by a citrus company located in Reggio Calabria. Before extraction, the BCP was pre-treated at 50°C to reduce the moisture content (12%); milled and after subjected to different extraction techniques (Conventional "C", Ultrasound "UA" and Microwave "MA"), using two food-grade solvents (H_2O and H_2O/EtOH 50%) and different times (30-60 min for C and UA; 5-15 min for MA). The best extract (AE) was selected and used for the formulation of a functionalized ingredient, called vegetable fat (VF). VFs were obtained from sunflower oil and soymilk replacing 1% (VF1%) and 2% (VF2%) of soymilk with the AE (Tab. 1a). In the next step, the VFs were used for the biscuits (Bs) formulation (Tab. 1b). All the samples were evaluated for physicochemical, antioxidant activities (ABTS and DPPH), total (TPC) and individual phenolic content (UHPLC), total flavonoid content (TFC) and oxidative stability properties (OXITEST) (Imeneo et al. 2021). Statistical analysis was performed by analysis of variance (one-way ANOVA) and Tukey's comparison test.

Table 1: *Vegetable fats(a) and biscuits composition (b)*

* (a)	Sunflower oil	Soymilk	AE	* (b)	Flour	VFC	VF1%	VF2%	H_2O	Yeast	Sugar
VFC	150	75	-	**CB**	400	96	-	-	96	8	96
VF1%	150	72.75	2.25	**B1%**	400	-	96	-	96	8	96
VF2%	150	70.5	4.5	**B2%**	400	-	-	96	96	8	96

All the ingredients are reported in grams

3. Results and Discussion

3.1 Antioxidant properties of Antioxidant extract (AE)

Among the different antioxidant extracts (AE_s) obtained from bergamot pomace previously described, conventional extraction for 30 min at 70°C, was selected. This represents the best extract in terms of antioxidant properties and phenolic compositions and the results were reported in Tab. 2. The AE showed a high content of flavonoids where the most representative detected (using UHPL-DAD) were reported in Tab.2 in according to the phenolic compounds present in citrus peel reported by other authors (Anticona et al. (2020); Singh et al. (2020)).

Table 2:*AE antioxidant activity and phenolic composition*

TPC	TFC	ABTS	DPPH	1	2	3	4	5	6
7762±159	2698±50	932±41	2793±57	4.68±0.24	341±8.42	2.32±0.29	386±17	2255±56	125±9.91

*Total phenolic content (TPC: mg GAE 100 mL^{-1}); Total flavonoid content (TFC: mg CE 100mL^{-1}); ABTS (mmolTrolox 100mL^{-1}); DPPH (mmol Trolox 100mL^{-1}); **1)** Eriocitrin (mg 100 mL^{-1}); **2)** Neoheriocitrin (mg 100 mL^{-1}); **3)** Narirutin (mg 100 mL^{-1}); **4)**Naringin (mg 100 mL^{-1}); **5)** Hesperidin (mg 100 mL^{-1}); **6)** Neohesperidin (mg 100 mL^{-1}).*

3.2 Physical-chemical composition and oxidative stability of Vegetable Fat (VF)

Tab. 3 showed the differences among samples in terms of colour parameters. Samples did not show significant differences in lightness (L*) and redness (*a**) values. High significant differences were shown in the increasing positive b*, correlated to the quantity of extract, highlights the yellowness colour in the VF, probably due to the presence of carotenoids, and other pigments abundantly present in citrus peel extract (Montero-Calderon et al. 2019). The phenolic compounds concentrations detected showed a trend in relation to the quantities added, in accordance with the added extract ratios. The induction period (IP) obtained for the VFs, were 10:37 (VFC), 12:36 (VF1%) and 13:08 (VF2%). The concentration of the AE in the VFs, as observed in phenolic compound concentrations, significantly affects the oxidation (Tab.3). The results of oxitest were in accordance with El-aal and Halaweish (2010) who found that orange peel extract improve the oxidative stability of soybean oil.

Table 3: *Vegetable fats characterization (colour, oxidative stability, phenolic profile)*

Sample	L*	a*	b*	IP(h:m)	Eriocitrin (mg 100g^{-1})	Neoheriocitrin (mg 100g^{-1})	Naringin (mg 100g^{-1})	Hesperidin (mg 100g^{-1})	Neohesperidin (mg 100g^{-1})
VFC	88.56	-0.15	6.69^{b}	10:37^{c}	-	-	-	-	-
VF1%	88.77	-0.26	7.16^{a}	12:36^{b}	0.53±0.02	16.96±0.11	15.01±0.46	11.35±1.21	5.15±1.59
VF2%	87.67	-0.23	7.23^{a}	13:08^{a}	1.32±0.02	31.5±0.07	28.69±0.14	19.41±0.29	11.24±0.09
Sign.	**n.s.**	**n.s.**	**	**	**	**	**	*	*

3.3 Physical-chemical composition and oxidative stability of functionalized biscuits

The results of the colour coordinates (Tab.4) on the analysed biscuits showed that there were no statistical differences on colour and browning, with the advantage that there are no colour changes among the analysed samples. Therefore, the AE present in the VFs did not influence the colour of the cooked products. For the oxidation stability test, statistically significant differences in the IPs were observed among the functionalized biscuits (B1% and B2%) and the Control biscuit (CB), they are related to the results observed for VFs (Tab.3).

Table 4: *Colorimetric parameters; induction period (IP) and major flavonoids detected in Bs*

Sample	L*	a*	b*	Browning index	IP(h:m)	Neoheriocitrin (mg 100g^{-1})	Naringin (mg 100g^{-1})	Hesperidin (mg 100g^{-1})
CB	73.9	4.87	22.41	40.84	10:36^{c}	-	-	-
B1%	72.53	6.26	2286	44.92	15:51^{b}	4.41±0.81	5.42±1.03	0.32±0.14
B2%	72.09	6.71	23.19	46.21	13:13^{a}	9.85±0.17	8.77±1.12	3.04±0.95
Sign.	**n.s.**	**n.s.**	**n.s.**	**n.s.**	**	*	**n.s.**	**n.s.**

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Role of root-exudated secondary metabolites in beneficial interactions between plant and polychlorinated biphenyls-degrading bacteria

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The results of the first two years of PhD regard the modulatory effect of root-exudated flavonoids on rhizocompetence traits in the PCB-degrading strain *Paraburkholderia xenovorans* LB400. In particular, the impact of flavonoids in stimulating bacterial growth and influencing motility and chemotaxis was investigated. Moreover, the ability of LB400 to colonize the roots of *Arabidopsis thaliana* WT and of the *tt4* knock-out mutant for flavonoids biosynthesis was monitored *in vitro* and through epifluorescence microscopy using a *mScarlet*-tagged LB400 strain. The results showed a higher colonization rate of the strain in WT plants under PCB stress compared to *tt4* and to control conditions.

Ruolo dei metaboliti secondari essudati dalle radici nelle interazioni benefiche tra piante e batteri degradatori di policlorobifenili

I risultati di seguito illustrati riguardano l'effetto svolto dai flavonoidi essudati dalle radici delle piante sulla rizocompetenza del batterio degradatore di PCB *Paraburkholderia xenovorans* LB400. È stato studiato l'impatto dei flavonoidi nella stimolazione della crescita batterica e nell'influenzare la motilità e la chemiotassi. Inoltre, la capacità del batterio di colonizzare le radici di *Arabidopsis thaliana* WT e del mutante *tt4*, knock-out per la biosintesi dei flavonoidi, è stata osservata *in vitro* e tramite microscopia utilizzando il ceppo LB400 marcato con *mScarlet*. I risultati hanno mostrato una maggiore colonizzazione da parte del ceppo nelle piante WT trattate con PCB rispetto a *tt4* e alle condizioni di controllo.

Key words: Polychlorinated Biphenyls, Flavonoids, Plant-Bacteria Interactions, Bioremediation, Root Exudates

1. Introduction

Understanding the role of secondary metabolites released by plant roots is important to unveil the complex interactions occurring between plants and bacteria in stressful conditions, like the presence of recalcitrant contaminants such as PCBs. Exploiting this crosstalk can have a positive impact on rhizoremediation to remove pollutants from the soil. According to the PhD thesis project formerly described (Ghitti, 2021) this report illustrates the outcomes of two activities, referred to the following sections: A2) investigation of the influence of root-exuded plant flavonoids on features related to bacterial root colonization using *in vitro* tests; A3) observation of *A. thaliana* root colonization, growing in presence and absence of PCB, by a *mScarlet*-tagged LB400 strain.

2. Materials and Methods

2.1 *In vitro* tests to assess the role of flavonoids on growth, chemotaxis and motility of LB400 strain

Fresh cultures of LB400 grown in 1/10TSB medium were collected at late exponential phase, washed and diluted 1000-fold in the different media used for the bioassay, consisting of 1/10TSB added with flavonoids (flavone, flavanone, naringin, naringenin and quercetin) at concentrations 10-100µM (Huang, 2019). The diluted cultures were aliquoted into transparent 96-well plates and bacterial growth was monitored by measuring the OD_{600} every hour for 24h at 30°C using a plate reader. Culture media with the solvents in which the flavonoids are solubilized were used as negative controls. The chemotactic activity of the strain was observed through a gradient plate chemotaxis assay as described by Reyes-Darias et al. (2016). Briefly, 1-100mM flavonoids, 0.5M sodium pyruvate (positive control) and solvents (negative controls), were spotted at the centre of the plate as chemoeffectors and incubated overnight at 4°C to allow gradient formation around the spot. On the next day a fresh culture of LB400 was washed and diluted to a concentration of 0.5OD/mL, and 2µL of the bacterial suspension spotted at 2.5cm distance from the chemoeffectors. After incubation for 72h at 30°C the plates were scanned and measures taken using ImageJ software. To assess swimming motility a plate experiment with 1/10TSB semi-solid medium (0.25% w/v agar) was performed. The medium was supplemented with 50-100µM flavonoids and 2µL of a 0.5OD/mL bacterial suspension were spotted at the centre of the plate and incubated for 24h at 30°C. The swimming area formed was then measured. Statistical analyses were conducted using the Mann-Whitney U test.

2.2 Root colonization on *A. thaliana* exposed to PCB stress using a *mScarlet*-tagged LB400 strain

The strain LB400 was tagged chromosomally with the fluorescent protein *mScarlet* expressed under a constitutive promoter through filter-mating conjugation, using as donor the *E. coli* S17-1 helper strain carrying the Tn5

transposon delivery plasmid (Schlecter, 2018). The red fluorescent LB400 obtained was used to perform colonization experiments on WT and *tt4 A. thaliana* plants, the latter being a knock-out mutant for flavonoids biosynthesis. Surface-sterilized *A. thaliana* seeds were colonized with LB400 on MS solid medium, vernalized in the dark for 3 days at 4°C and then incubated under controlled conditions for 5 days to allow germination. At day 0 the colonized plantlets were transferred on MS medium with 20µM PCB-18 or acetone (untreated control). At day 0, 7 and 14 plant roots were collected, weighed and LB400 cells reisolated. Additionally, at day 7, plant roots were observed at epifluorescence microscopy to visualize root colonization due to the fluorescent signal.

3. Results and Discussion

3.1 Flavonoids improve essential features involved in *P. xenovorans* LB400 rhizocompetence traits

The growth assay showed that flavonoids improve LB400 growth and final biomass accumulation at stationary phase (measured as OD_{600} at 24 or 30h). Increasing concentration of naringin, naringenin and quercetin led to significantly higher biomass accumulation compared to the respective controls (Fig. 1). In the second *in vitro* test, chemotaxis response index (RI) was calculated to quantify chemoattraction (Pham 2012). As shown in Fig. 2, 100mM naringenin and 50mM quercetin induced significant chemoattraction on LB400 compared to the negative controls, having RI mean values higher than 0.52. Finally, in the swimming assay, the addition of naringin 50 and 100µM led to a significant enhancement of bacterial motility compared to the control. The flavonoids used are naturally exuded by plant roots and mostly known for their role in the symbiosis between legumes and rhizobia. Their structural similarities with PCB and the observed positive effects on LB400 root colonization traits, however, could widen the perspectives on the role played by these molecules in plant-bacteria interactions. These flavonoids could be, in fact, good candidates as biostimulators of the biphenyl oxidative pathway in PCB-degrading bacteria. This aspect will be further investigated.

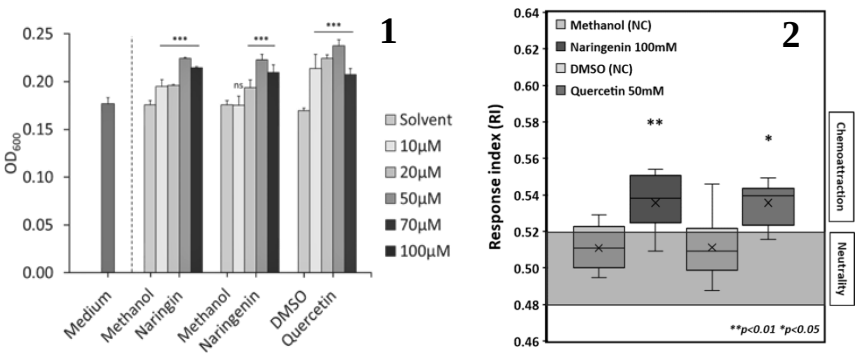
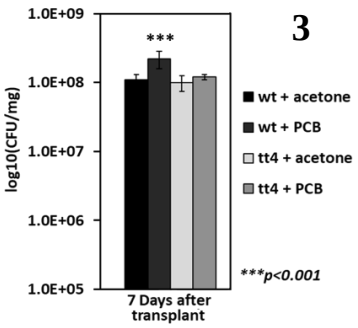


Figure 1 *Amount of total biomass (OD_{600}) of LB400 accumulated at the stationary phase in presence of different concentrations of flavonoids.*

Figure 2 *Chemotaxis response index of LB400 towards naringenin and quercetin.*

3.2 *P. xenovorans* LB400 colonization pattern under PCB stress



The ability of LB400 to colonize *A. thaliana* roots was examined by re-isolation of the strain from plant roots and was further confirmed by observing the fluorescence of the tagged strain at the confocal microscope. Both approaches showed a significantly higher colonization yield in WT plants exposed to PCB stress (Fig. 3). On the other end the colonization rate on the *tt4* mutant did not vary in presence or absence of PCB treatment. These results suggest that the release of flavonoids from plant roots is an important driver for an efficient colonization by beneficial strains like LB400 in plants growing in PCB contaminated environments.

Figure 3 *CFUs/mg of LB400 strain on Arabidopsis.*

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Metabolic attenuation of probiotics: a strategy for functional fruit juices development

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Plant-based beverages provide a suitable alternative to dairy products as vehicles for probiotics. The matrix/probiotic interaction induces changes that can be counteracted by attenuating the fermentative metabolism of beneficial bacteria. In the era of nonthermal technologies, the potential of ultrasound technology as an attenuation system has been evaluated. The response of the probiotic *Lacticaseibacillus casei* ATCC 393 to the treatment was first characterized in terms of acidifying capacity and cultivability. Further post-treatment analysis was then performed on the adhesive properties and membrane structure. In conclusion, the results obtained show a relationship between the parameters analyzed and the intensity of sonication.

Attenuazione metabolica dei probiotici: una strategia per lo sviluppo di succhi di frutta funzionali

Le bevande vegetali rappresentano una valida alternativa ai prodotti lattiero-caseari come veicoli di probiotici. L'interazione matrice/probiotico induce dei cambiamenti che possono essere contrastati attenuando il metabolismo fermentativo dei batteri benefici. Nell'era delle tecnologie non termiche, è stata valutata la potenzialità degli ultrasuoni come sistema di attenuazione. La risposta del probiotico *Lacticaseibacillus casei* ATCC 393 al trattamento è stata innanzitutto caratterizzata in termini di capacità acidificante e coltivabilità. Sono state, poi, effettuate ulteriori analisi post-trattamento relative alle proprietà adesive e alla struttura della membrana. In conclusione, i risultati ottenuti evidenziano una relazione tra i parametri analizzati e l'intensità della sonicazione.

Key words: attenuation, ultrasound, acidification, surface properties, cell membrane.

1. Introduction

In accordance with the PhD project previously described, the activities carried out can be categorized in two groups:

(A1) experimental design development and process optimization;
(A2) characterization of ultrasound effects on *L. casei* ATCC 393 metabolism, cultivability, surface properties (auto-aggregation, hydrophobicity, biofilm production), and cell membrane structure.

2. Materials and Methods

Bacterial suspension in deionized water was subjected to two sonication treatments. Main parameters were power (57 W), duty cycle (50 %) and duration, which varied from 6 to 8 min. Attenuation was assessed as pH decrease of MRS broth after 6 and 24 h of incubation at 37 °C (Racioppo et al., 2017). Probiotic cultivability was evaluated by spread plate count immediately after sonication and by growth index on overnight cultures. Auto-aggregation and hydrophobicity (cells affinity to iso-octane) tests were performed on bacterial suspension (OD_{600},t_0 0.6 ± 0.2) as described by Fonseca et al. (2021). Biofilm production was assessed by a colorimetric assay on 24 h-cultures (OD_{600} 1.0 ± 0.2) in 24-well microtiter plates. Attached cells, after being fixed, were stained with a crystal violet solution (10 %) and absorbance was read at 570 nm (Chen et al., 2020). Membrane damage was correlated to the release of intracellular components which were quantified by reading the absorbance at 260 (protein) and 280 (nucleic acids) nm and expressed as Absorbance Increase (Bevilacqua et al., 2019).

3. Results and Discussion

3.1 Acidifying capabilities and cultivability

Table 1 shows the results of ultrasound-induced attenuation and probiotic cultivability. The 6 min-treatment induced temporary attenuation while the 8 min-treatment induced complete attenuation. The findings imply that LC_S6 can restore its metabolism and that increasing the sonication intensity results in a stronger attenuation effect. Hypothetically, free radicals, formed due to implosion of cavitation bubbles, can interact with enzymes involved in the sugar transport and metabolization system leading to their oxidation and, consequently, alteration of normal function.

Table 1 *Attenuation and cultivability alteration induced by ultrasound. LC_S0:* L. casei ATCC 393 *non sonicated (control); LC_S6:* L. casei ATCC 393 *sonicated for 6 min; LC_S8:* L. casei ATCC 393 *sonicated for 8 min.**

Sample	Incubation time (ΔpH)		Log UFC/ml	Growth index		
	t_6	t_{24}		GI > 75 % No growth inhibition	25 < GI < 75 % Partial growth inhibition	GI < 25 % Complete growth inhibition
LC_S0	0.38 ± 0.05[a]	2.16 ± 0.18[a]	9.30 ± 0.02[a]	-	-	-
LC_S6	0.06 ± 0.02[b]	1.91 ± 0.12[a]	8.58 ± 0.12[b]	+	-	-
LC_S8	0.03 ± 0.02[b]	0.97 ± 0.14[b]	6.43 ± 0.04[c]	-	-	+

Sonication reduced the plate count by approximately 1- and 3-Log for the 6 and 8 min-treatment, respectively. The growth index was calculated as follows:

$$Growth\ index\ (GI)\ = \frac{A_{US}}{A_C} \times 100 \quad (1)$$

where A_{US} is the absorbance of sonicated samples, and A_C is the absorbance of the control.
Collected data show that there was no growth inhibition for sample LC_S6 and complete inhibition in the case of LC_S8. Therefore, probiotic cultivability was affected by sonication. In stress conditions, bacteria can enter in a viable but non-culturable (VBNC) state. However, further analysis is required to proper define the ultrasound-induced VBNC status. Results obtained from the growth index analysis suggest that LC_S6 resuscitate. Although the more intense treatment may have caused LC_S8 inactivation, Brandão et al. (2021) demonstrated that ultrasound inactivation does not impair the health benefits of probiotics.

3.2 Cell surface characterization

Cell surface properties of *L. casei* ATCC 393 and of the sonicated probiotic are summarized in Table 2.
Membrane damage is given by:

$$Absorbance\ increase\ (AI)\ \% \ = \left[\frac{(A_{US} - A_C)}{A_C}\right] \times 100 \quad (2)$$

Auto-aggregation and hydrophobicity were calculated as follows:

$$AA\ or\ HY\ \% \ = \left[\frac{(A_0 - A_t)}{A_0}\right] \times 100 \quad (3)$$

where A_0 is the absorbance of samples at time 0, and A_t is the absorbance after incubation.
Biofilm production was quantified by establishing a low cut-off (ODc) and comparing OD_{570} of the samples with it. Sonicated *L. casei* ATCC 393 resulted in increased membrane permeability due its damage and weakening. Upon sonication, a negative correlation was found between sonication treatment and probiotic auto-aggregation, while hydrophobicity increased, and biofilm production improved. Our results suggest that ultrasound alters the surface structure of *L. casei* ATCC 393, thus affecting its normal function and physiological activities. This could explain the different adhesive properties of the sonicated strains. Furthermore, comparing our data with those in the literature, it is evident that the response to ultrasound treatment is strain- and species-specific.

Table 2 *Adhesive properties and membrane structure evaluation before (LC_S0) and after sonication (LC_S6: 6-min treatment; LC_S8: 8-min treatment).**

Sample	Auto-aggregation (%)	Hydrophobicity (%)	Biofilm production	Membrane permeability (%)	
				A_{260}	A_{280}
LC_S0	23.98 ± 1.32[a]	6.29 ± 0.68[a]	Weak (OD ≤ ODc)	-	-
LC_S6	3.39 ± 0.78[b]	11.68 ± 2.65[b]	Strong (4ODc < OD)	216 ± 8.16[a]	140 ± 10.00[a]
LC_S8	0.65 ± 0.26[c]	15.01 ± 1.59[b]	Strong (4ODc < OD)	256 ± 10.69[b]	165 ± 9.64[b]

**Data are reported as means value ± standard deviation (n = 3). Statistical analysis (One-way ANOVA and t-Student tests) were performed by SPSS software (p < 0.05). Different letters in the same column indicate that the differences are significant.*

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Sustainability of technology, quality control and consumption of olive oil

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The two main activities of the PhD thesis project are herein described. Firstly, the set-up and application of rapid, innovative, and sustainable analytical methods for assessing the quality and purity of virgin olive oils. Secondly, the olive pomace valorization by developing sustainable methods for the extraction of phenolic compounds, as well as the characterization and shelf-life evaluation of the obtained extracts.

Aspetti di sostenibilità in relazione alla tecnologia, controllo qualità e al consumo dell'olio di oliva

Le due attività principali di questo progetto di dottorato sono di seguito descritte. La prima è focalizzata sullo sviluppo e sull'applicazione di metodi analitici rapidi, innovativi e sostenibili, per la determinazione della qualità e purezza degli oli di oliva vergini. La seconda, invece, è incentrata sulla valorizzazione della sansa di oliva sviluppando metodi sostenibili per l'estrazione dei composti fenolici, e conseguente caratterizzazione e studio della shelf-life degli estratti ottenuti.

Key words: virgin olive oils, analytical methods, olive oil by-products, quality control.

1. Introduction

This contribution reports the results and next expected activities of two activities concerning:

(A1) Rapid and sustainable analytical methods for quality and authenticity of virgin olive oils: development and application of easy-to-use, innovative and sustainable analytical approaches to evaluate the quality and genuineness of virgin olive oils. In particular, a focus is carried out on the study of the volatile fraction by gas-chromatographic analyses, as a relevant potential tool to support the sensory analysis (panel test) for the determination of the commercial category in virgin olive oils.

(A2) Olive pomace valorisation: set-up of sustainable methods for the extraction of phenolic compounds from olive pomace, as well as characterization and shelf-life evaluation of the so obtained phenolic extracts. This activity is aimed to produce sustainable extracts potentially usable in different industrial sectors, such as pharmaceutical, food and cosmetic.

2. Materials and Methods

2.1 Rapid and sustainable analytical methods for quality and purity of virgin olive oils (A1)

HS-GC-IMS (gas chromatography coupled with ion mobility spectrometry) analyses were performed on an olive oil set composed of 119 samples, in order to have a relevant and balanced variety in the commercial categories of virgin olive oils (VOOs) among extra virgin (EV, 42), virgin (V, 54) and lampante (L, 23), determined by sensory analysis (panel test). The analysis was performed using a GC-IMS Flavourspec® instrument (G.A.S. Dortmund, Dortmund, Germany) connected to a nitrogen generator for carrier/drift gas production (Microprogel, Pordenone, Italy). Samples were analyzed following the same method reported by Valli et al, 2020. 3D chromatograms (heat maps) were obtained. The samples were classified in the commercial category through a previously developed prediction approach, based on PLS-DA models, built on the maximum intensity of the areas belonging to 15 selected volatile compounds (monomers and dimers) as main responsible for fruity and sensory defects (Valli et al., 2020). In addition, on the same set a Flash-GC analysis was performed and the prediction of the classification of the samples into the three commercial category is now ongoing by applying a previously developed chemometric model (Barbieri et al., 2020).

2.2 Olive pomace valorisation (A2)

Firstly, several tests have been carried out on the olive pomace provided by a local mill and stored at -18 °C by using solvents, such as ethanol, that are less toxic for the environment than the usual ones adopted for extraction, and by applying techniques that could favour this process, e.g. use of ultrasound-assisted extraction.

The procedure, developed and selected for the phenolic compound extraction, is as it follows: 2 g of the freeze-dried and ground samples were weighed. Then, 20 mL of the ethanol/water mixture (80:20 v/v) were added. The mixture was shacked and placed in an ultrasound water bath, at room temperature for 30 minutes. Then the solution was centrifuged for 15 min at 3500 rpm. The polar phase was carefully removed, and the extraction process was repeated twice. Finally, the two extracts were combined, evaporated in a rotary evaporator until dryness and the residue was dissolved in 2 mL of methanol/water (1:1 v/v).

Secondly, a sustainable mechanical approach (using a lab scale screw-press) was applied on the olive pomace by

adding a mixture of water and food grade ethanol (80:20 v/v) and two types of samples were obtained, one more liquid drained from the lower part of the mill and one drier from the frontal part. These were freeze-dried and ground, and the phenolic extracts were obtained by applying the procedure detailed above. The study of the phenolic fraction was based on the determination of the total reducing molecules content (Folin-Ciocalteu method), then a HPLC-MS/MS analysis is now ongoing.

The A2 activity of this PhD thesis is developed in the framework of the project SUSTAINOLIVE "Novel approaches to promote the sustainability of olive groves in the Mediterranean" within the PRIMA programme supported by the EU (Grant agreement No 1811).

3. Results and Discussion

3.1 Rapid and sustainable analytical methods for quality and purity of virgin olive oils (A1)

The results are satisfactory in terms of percentages of correctly classified samples in the different commercial categories (see table 1) with respect to the sensory results and the related grade, in according to the previous study (Valli et al., 2020).

Table 1 *Results of the HS-GC-IMS prediction model on the olive oil samples set.*

COMMERCIAL CATEGORY	NUMBER OF SAMPLES CORRECTLY CLASSIFIED	%
EV	30/42	71.4
V	31/54	57.4
L	16/23	69.6
TOT	79/119	66.4

The next step is to combine data obtained by HS-GC-IMS and Flash-GC methods (data fusion) for providing new reliable chemometric models to estimate the VOO quality grade as tools to support the panel test.

3.2 Olive pomace valorisation (A2)

The total reducing molecules content analysis, by Folin-Ciocalteu method, was carried out on the extracts derived from the olive pomace as it is, and the two different samples obtained from the application of the mechanical approach.

Table 2 *Total reducing molecules content of TQ: extract obtained from olive pomace sample as it is, after freeze-drying and grounding; SI: extract obtained from the liquid pomace sample drained from the lower part of the mill screw press, after freeze-drying and grounding; SF: extract obtained from the drier pomace sample from the frontal part, after freeze-drying and grounding.*

SAMPLE	TOTAL REDUCING MOLECULES CONTENT (mg gallic acid/kg sample)	SD
TQ	3294.33^{b}	378.53
SI	7101.32^{a}	423.97
SF	3094.73^{b}	39.15

ANOVA, HSD Tukey, $p<0,05$

The results of the determination of the total content of reducing molecules, including phenolic compounds, show that the richest sample is extract obtained from the liquid pomace sample drained from the lower part of the mill screw press (SI), probably due to the effect of the mechanical approach and the addition of the mixture of $EtOH/H_2O$, that could favour the concentration of these compounds.

This suggests that "SI" could be the most promising sample for obtaining sustainable extracts potentially usable in different industrial sectors, such as pharmaceutical, food and cosmetic. Meanwhile, also further investigations of a potential application and sustainable recovery methods of the drier pomace sample from the frontal part (SF) could be interesting.

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Detoxifying effect of cold atmospheric plasma on pure *Alternaria* mycotoxins and naturally contaminated sun-dried tomatoes

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In the last decade, the attention towards *Alternaria* toxins, belonging to the group of so-called emerging mycotoxins, has increased therefore the European Union has published the Recommendation (EU) 2022/553 on monitoring the presence of *Alternaria* toxins in food. In this study, five different mycotoxins produced by *Alternaria alternata*, such as alternariol (AOH), alternariol monomethyl-ether (AME), tentoxin (TEN), altenuene (ALT), and tenuazonic acid (TeA) were selected. Initially, the detoxifying effect of the cold atmospheric plasma (CAP) treatment on pure molecules was evaluated. Subsequently, the detoxifying effect of CAP treatments on a naturally contaminated and artificially contaminated real matrix (sun-dried tomatoes) was assessed.

Effetto detossificante del plasma freddo atmosferico sulle micotossine dell'*Alternaria* e pomodori secchi naturalmente contaminati

Nell'ultimo decennio l'attenzione nei confronti delle tossine dell'*Alternaria*, appartenenti al gruppo delle così dette micotossine emergenti, è aumentata tanto che l'Unione Europea ha pubblicato la Raccomandazione (EU) 2022/553 relativa al monitoraggio della presenza di tossine dell'*Alternaria* negli alimenti. In questo studio sono state selezionate cinque differenti micotossine prodotte da *Alternaria alternata* quali alternariolo (AOH), alternariolo monometil-etere (AME), tentossina (TEN), altenuene (ALT), e acido tenuazonico (TeA). Inizialmente è stato valutato l'effetto detossificante del trattamento con il plasma freddo atmosferico (CAP) sulle molecole pure. Successivamente è stato determinato l'effetto detossificante del trattamento CAP su una matrice reale (pomodori secchi) naturalmente e artificialmente contaminata.

Keywords: Cold atmospheric plasma, *Alternaria* mycotoxins, alternariol, tenuazonic acid, alternariol monomethyl-ether, tentoxin, altenuene, sun-dried tomatoes, LC-MS/MS.

1. Introduction

In line with PhD thesis project previously described (Laika, 2021), the first activities were focused more on *Alternaria* mycotoxins rather than *Alternaria* fungus, and this poster communication reports the main results:

- efficacy evaluation of CAP treatments on *Alternaria* mycotoxins reduction and optimisation of treatment parameters;
- the assessment of CAP treatments on sun-dried tomatoes artificially and naturally contaminated by *Alternaria* mycotoxins.

2. Materials and Methods

Cold atmospheric plasma (CAP), generated by a Surface Dielectric Barrier Discharge (SDBD), previously described (Molina-Hernandez et al., 2022), was used. In order to determine the mycotoxin degradation by CAP treatments, aliquots of mycotoxins standard solutions (50 µg/kg) were deposited into polystyrene six-well plates that, after solvent evaporation at room temperature, were placed in the treatment chamber and subjected to CAP. The influence of CAP regimes (O_3 and NO_x) and exposure time were investigated. Mycotoxin determination was performed by LC-MS/MS analysis. 26 batches of commercially sun-dried tomatoes were screened for the occurrence of *Alternaria* mycotoxins. Mycotoxin extraction and determination from dried tomatoes were performed according to Tölgyesi and Stroka (2016). Then, a not contaminated batch of sun-dried tomatoes was spiked with all five mycotoxins at two different concentration levels (50 µg/kg and 200 µg/kg) and exposed to CAP in O_3 regime for 15 min. Finally, a batch of sun-dried tomatoes naturally contaminated by *Alternaria* mycotoxins was subjected to CAP treatments for different times (15, 30, and 60 min) and regimes (NO_x and O_3).

3. Results and Discussion

3.1 Determination of CAP treatment effects on pure molecules and artificially contaminated sun-dried tomatoes

Table 1 summarises the effect of CAP treatments on five *Alternaria* mycotoxins concentration. CAP treatments resulted in a significant reduction of all investigated mycotoxins, and the degradation was time-dependent, indicating that most of the action took place in the first 15 minutes. The O_3 regime showed to be the most effective,

especially for TEN and ALT. Table 2 displays the effect of 15 min CAP exposure in O_3 regime of sun-dried tomatoes fortified with two concentration levels of mycotoxins. In the spiked real matrix, CAP treatment reached detoxifying effect level lower than the standard molecules.

Table 1 *The results of cold atmospheric plasma (CAP) treatment on reduction of pure Alternaria mycotoxins.*

Plasma regime	Exposure time (min)	Residual mycotoxin %				
		TeA	AOH	AME	TEN	ALT
	0	100.00±1.59^{a}	100.00±4.37^{a}	100.00±1.01^{a}	100.00±3.85^{a}	100.00±1.13^{a}
O_3	15	31.55±2.87^{c}	47.37±3.11^{b}	46.61±5.36^{b}	48.87±2.02^{c}	20.25±2.37^{d}
	30	26.06±0.31^{c}	40.31±17.78^{bc}	45.22±6.71^{b}	37.04±11.70^{c}	15.02±11.19^{de}
	60	7.97±4.32^{d}	9.73±3.42^{d}	37.38±5.63^{bc}	9.52±0.61^{d}	4.17±3.59^{e}
NO_x	15	43.99±9.36^{b}	40.15±4.04^{bc}	34.12±3.26^{bc}	96.52±5.27^{a}	89.60±1.62^{a}
	30	10.95±1.76^{d}	26.11±2.07^{d}	24.52±9.16^{c}	83.81±2.42^{b}	66.51±6.62^{b}
	60	8.52±0.43^{d}	38.05±13.05^{bc}	29.67±7.58^{c}	83.40±2.65^{b}	43.99±3.47^{c}

Values are presented as means ± standard deviations (n=3). Values in the same column with different superscripted letters are significantly different ($p < 0.05$).

Table 2 *Effect of 15 min CAP (O_3 regime) treatment on artificially contaminated sun-dried tomatoes.*

Enrichment level (µg/Kg)	Exposure time (min)	Residual mycotoxin %				
		TeA	AOH	AME	TEN	ALT
50	0	100.00±3.49^{a}	100.00±0.88^{a}	100.00±2.58^{a}	100.00±1.72^{a}	100.00±4.06
	15	78.40±3.78^{b}	98.44±3.67^{a}	98.25±0.53^{a}	94.82±3.30^{b}	98.13±3.99
200	0	100.00±0.90^{a}	100.00±2.90^{a}	100.00±2.13^{a}	100.00±3.41	100.00±2.50
	15	63.67±3.33^{b}	89.48±1.46^{b}	88.86±2.23^{b}	87.69±1.40*	83±1.53*

Values are presented as means ± standard deviations (n=4). Values with different superscripted letters are significantly different ($p < 0.05$).

3.2 Effect of CAP treatment on naturally contaminated sun-dried tomatoes in comparison with standard mycotoxins

Sun-dried tomatoes (26 batches) were screened for *Alternaria* mycotoxins. The results evidenced that among the five mycotoxins analysed, more than 40% of the batches were contaminated by only tenuazonic acid, which concentration ranged from 1.3 µg/kg to 186 µg/kg. These results were in accordance with the findings of other authors (EFSA, 2011; EFSA et al., 2016). To evaluate the treatment efficacy on naturally contaminated sun-dried tomatoes was selected a batch contaminated with 40 µg/kg of tenuazonic acid and subjected to CAP for different times (15, 30, 60 min) and different regimes (O_3 and NO_x). At the same time, 40 µg/kg of pure tenuazonic acid was treated. Fig. 1 depicts the lower efficacy of CAP treatment on the real matrix, underling that food, being a complex matrix, contains several compounds that could have a protective role in mycotoxin degradation.

Our results suggest the potential of CAP treatment in reducing *Alternaria* mycotoxins naturally present in food. In perspective, we will proceed, through multi-omics approaches, to study metabolic and cellular changes triggered by atmospheric cold plasma treatment on *Alternaria alternata*.

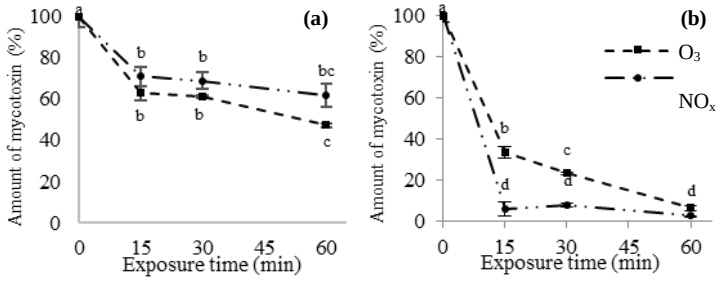

Figure 1 *Comparison between the effect of CAP treatments on (a) sun-dried tomatoes naturally contaminated with tenuazonic acid and (b) standard tenuazonic acid molecule, at the same concentration.*

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Metabolomics to investigate the effects of treatments on food and of food consumption on health

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The first three activities of the PhD thesis project are described. Firstly, preliminary research about the effects of stress conditions on the physiological and metabolism of food was studied, especially on seafood. Secondly, a specific SOP (Standard operation procedures) was set up and applied to deal with the metabolites of seafood. Thirdly, metabolomics-oriented experiments focusing on the consequences of high hydrostatic pressure (HHP) on seafood products were carried out.

La metabolomica per studiare gli effetti dei trattamenti sugli alimenti e del loro consumo sulla salute

Il presente lavoro descrive le prime tre attività del progetto di tesi di dottorato. Inizialmente, sono state condotte ricerche preliminari sugli effetti delle condizioni di stress sulla fisiologia e sul metabolismo degli alimenti, con particolare riferimento ai frutti di mare. Successivamente, è stata messa a punto procedura standardizzata per analizzare specificatamente i metaboliti dei frutti di mare via ^{1}H-NMR. Infine, sono stati condotti esperimenti orientati alla metabolomica, incentrati sulle conseguenze dell'alta pressione idrostatica (HHP) sui prodotti ittici.

Key words: Metabolomics; High hydrostatic pressure; ^{1}H-NMR; Microorganism

1. Introduction

In agreement with the PhD thesis project previously described (Lan, 2021), this presentation poster reports the main results of the first three activities concerning:

(A1) Literature review of the latest research related to the investigation of the effects of stress conditions on the physiological response and metabolism of food.

(A2) Set up and application of specific SOP to characterize and quantify the metabolites of seafood by ^{1}H-NMR.

(A3) Metabolomics-oriented experiments focusing on the consequences of an innovative treatment on food composition and quality of treatments. The effects of high hydrostatic pressures (HHP) on the metabolome of grey mullet *(Mugil cephalus*), striped prawn *(Melicertus kerathurus*), and deep-water rose shrimp (*Parapenaeus longirostris*) during chilled storage were investigated.

2. Materials and Methods

Striped prawns, rose shrimps, and grey mullets were fished in the Adriatic Sea. They were fast frozen at a temperature of -18°C for 24 h by the company Economia del Mare (Cesenatico, Italy). Seafood samples were thawed at 4°C for 16 h, then mechanically deboned and shelled. Flesh was manually diced and packed in polypropylene (PP) trays containing 6 monoportions of about 15-20 g each, that were packed under vacuum with a PP film. Vacuum packed samples underwent HHP treatments (400, 500, and 600 MPa) for 10 min performed at the premises of HPP Italia s.r.l (Parma, Italy). A further, untreated, sample was used as a control.

Samples were then stored at 2±1°C. Samples were subjected to analytical determinations at 1, 6, 9, 14, 21, 28, and 35 days. For each HHP treatment and at each storage time, 3 different packages were used. Shelf life was considered to end when microbial load reached 6 log CFU/g.

Microbial groups considered in this research were total mesophilic bacteria (TMB), *Lactobacillus spp*., *Pseudomonas*, sulfite reducing anaerobic bacteria, total Coliforms, *E. coli*, and coagulase positive staphylococci. The presence of *Salmonella spp*. and *Listeria monocytogenes* was also checked.

The samples were prepared for NMR analysis at each sampling time and HHP treatment. A trichloroacetic acid extraction (TCA) was performed on three fish samples, by following the procedure set up by Ciampa et al (2012). To register ^{1}H-NMR spectra an AVANCE III spectrometer (Bruker, Milan, Italy) was employed, operating at a frequency of 600.13 MHz and equipped with the software Topspin (ver. 3.5).

3. Results and Discussion

3.1 Microbiological determination

Results from microbiota analysis (Figure 1) show that the application of 600 MPa extended the microbial shelf life of the seafood samples up to 30 days. The application of lower pressures was able to inactivate *E. coli*, total

Coliforms, sulfite reducing anaerobic bacteria, *Pseudomonas*, and positive coagulase staphylococci. However *Lactobacillus spp.*, *Pseudomonas spp.* and/ or total Coliforms were able to recover during storage (data not shown). Taken together, these results suggest that the application of high pressures improved shelf life of the seafood products considered from a microbiological point of view.

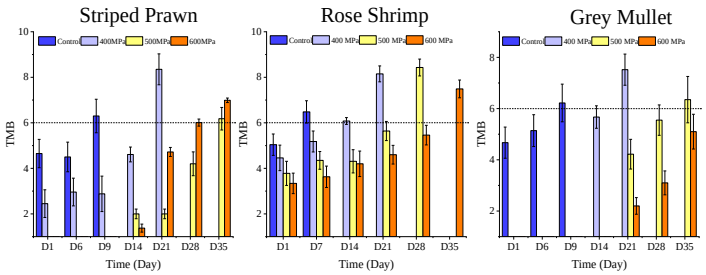

Figure 1 *Changes in microbial cell loads (log CFU/g) of total mesophilic bacteria (TMB) of packaged striped prawn, rose shrimp, and grey mullet in relation to the High Hydrostatic Pressure (HHP) treatments applied (400, 500, 600 MPa).*

3.2 Effect of HHP treatment on metabolites of seafood

^{1}H-NMR spectra representative of striped prawn, rose shrimp, and grey mullet are shown in Figure 2. It is apparent that some signals (such as putrescine and cadaverine) were only detected on the last day of storage and only in untreated samples. Moreover, the intensity of some signals (such as acetate, pyruvate, acetoin) showed different trends between treated and untreated samples during storage.

Among the molecules detected, nucleotides, trimethylamine (TMA) and biogenic amines are known to be related to freshness of seafood (Márquez-Ríos E et al., 2007; Visciano et al., 2020). Moreover, some water-soluble, low-weight molecules are known as taste-active components and contribute to the specific taste of seafood, which is classified into umami, sweet, sour and bitter (Sarower et al., 2012). Freshness and taste related metabolites observed though a single analytical platform can provide novel insights into the evolution of the overall quality of seafood as affected by high pressures as well as other innovative conservation treatments. The present results suggest that the HHP processing delays the degradation of nucleotides and amino acids and thus inhibits the decay of freshness and umami taste of seafood (data not shown).

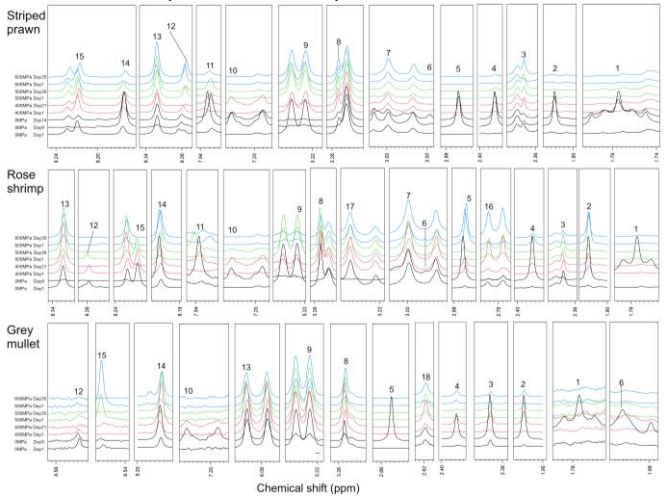

Figure 2 *A part of ^{1}H NMR spectra of tiger prawn, rose shrimp, and grey mullet untreated and treated with HHP (400, 500, 600 MPa) during storage. 1, putrescine; 2, Acetate; 3, Pyruvate; 4, Succinate; 5, Trimethylamine; 6, Cadaverine; 7, Lysine; 8, Trimethylamine N-oxide; 9, Glucose; 10, Tyramine;; 11, Xanthine; 12, adenosine-5'-triphosphate, adenosine-5'-diphosphate, and adenosine-5'-monophosphate;13 Ino, Inosine; 14, Hypoxanthine; 15 IMP, inosine-5'-monophosphate, 16, Aspartate; 17, Arginine; 18, Methionine.*

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Preliminary chemico-physical evaluation of soymilk fermented by *Lactiplantibacillus plantarum* LP 95

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Initially, some functional properties of five *Lactiplantibacillus plantarum* strains, isolated from bee bread, were evaluated *in vitro*. More in detail, the evaluated properties were: antioxidant and β-glucosidase activities, exopolysaccharides (EPS) production and the ability to synthesize γ-aminobutyric acid (GABA). Based on the results obtained, the *L. plantarum* LP 95 strain was selected to be used as a starter for laboratory-scale production of fermented soymilk.

Valutazione chimico-fisica preliminare di latte di soia fermentato da *Lactiplantibacillus plantarum* LP 95

In una fase preliminare, sono state studiate *in vitro* alcune proprietà funzionali di cinque ceppi di *Lactiplantibacillus plantarum* isolati dal pane d'api. Nello specifico, sono state valutate l'attività antiossidante e β-glucosidasica, la produzione di esopolisaccaridi e la capacità di sintetizzare acido γ-amminobutirrico. In base ai risultati ottenuti, è stato selezionato il ceppo *L. plantarum* LP 95 per essere utilizzato come starter nella produzione su scala di laboratorio di latte fermentato di soia.

Keywords: antioxidant activity, EPS, fermented soymilk, *L. plantarum*, β-glucosidase, γ-aminobutyric acid.

1. Introduction

While a substantial number of well-characterized probiotic strains are available worldwide, there is still great interest in the search for new strains with specific properties. In this work, we evaluated some bio-functional properties of five *Lactiplantibacillus plantarum* strains, isolated from bee bread in order to evaluate their possible application as food supplements or as microbial cultures for the production of functional foods. In the early phase, conducted *in vitro*, antioxidant and β-glucosidase activities, exopolysaccharides (EPS) production and the ability to synthesize γ-aminobutyric acid (GABA) were evaluated. Based on the results obtained, the *L. plantarum* LP 95 strain was selected and used as a starter for laboratory-scale production of fermented soymilk.

2. Materials and Methods

Antioxidant activity, β-glucosidase activity, EPS production and the ability to synthesize GABA from *L. plantarum* LP 8, LP 25, LP 86, LP 95 LP 100 were analysed according to previously published methods (Letizia *et al.* 2022). As reference strain *L. plantarum* ATCC 14917 was used. The best performing strain LP 95, was used as a starter for laboratory-scale production of fermented soymilk. Soymilk was prepared by soaking 200 g of mature yellow soybeans in 600 mL sterile distilled water for 12 hours. Subsequently, soybeans were drained, dried and blended with 700 mL of water. The resulting product was filtered and sterilized in autoclave (121°C for 15 min). The obtained soymilk was inoculated with *L. plantarum* LP 95 at a final concentration of about 10^7 CFU/mL. At the end of the fermentation process, the fermented soymilk was stored at 4°C and 22°C and the LABs survival was evaluated after 7, 14, 21 and 28 days by plating on MRS agar medium incubated at 37 °C for 48 h in anaerobiosis. The evaluation of pH, titratable acidity and water holding capacity (WHC %) of the fermented soymilk was performed according to Li *et al.* (2014). Apparent viscosity was measured using a rotational HAAKE™ MARS™ Rheometer (Thermo Scientific™) with a plate–plate measuring system (20 mm in diameter, 1 mm gap distance) at fixed shear rate of $50s^{-1}$ at 4°C and 25°C.

3. Results and Discussion

3.1 Functional properties from *L. plantarum* strains

As reported in Table 1, antioxidant activity values are significantly different among the tested strains. In detail, the strains LP 95 and LP 100 exhibited the highest values, 14.23 and 15.84 µg Trolox Eq./mg BSA Eq., respectively. About the β-glucosidase activity, it has been shown that all strains tested possess this specific enzymatic activity with the highest value obtained from *L. plantarum* LP 95. GABA production showed significant differences among the tested strains, with values within a range of 0.345 (LP 25) and 2.227 (LP 95) pmol GABA/µg BSA Eq./mg. Finally, the values of EPS production are reported in Table 2. In detail, the EPS-r values ranged from 2.84 (LP 25) to 3.43 (LP 8) µmol glucose Eq./mL while the values of EPS-b* varied from 38.95 (LP 86) to 223.42

(LP95) nmol glucose Eq./mL. About the EPS-b**, compared to cell proteins, the range of values was between a minimum of 0.016 (LP 86) and a maximum of 0.069 (LP 95) nmol glucose Eq./µg BSA Eq.

Table 1. *Antioxidant and β-glucosidase activities and GABA production of L. plantarum strains. Statistical analysis was performed using one-way ANOVA.All values are expressed as mean ± SD (n = 3). Different lowercase letters (a–d) in each row indicate significant differences (p < 0.05). Expressed as: *µg Trolox Eq./mg BSA Eq.; **nmol p-Nitrophenol/(min mL); ***pmol GABA/µg BSA Eq.*

	LP 8	LP 25	LP 86	LP 95	LP 100	ATCC 14917
*Antioxidant activity	6.46 ± 0.65 ^{c}	6.51 ± 0.38 ^{c}	3.89 ± 0.34 ^{d}	14.23 ± 1.60 ^{a}	15.84 ± 0.80 ^{a}	13.35 ± 0.26 ^{b}
**β-glucosidase activity	0.30 ± 0.06 ^{a}	0.39 ± 0.07 ^{a}	0.38 ± 0.08 ^{a}	0.45 ± 0.03 ^{a}	0.26 ± 0.08 ^{b}	0.26 ± 0.08 ^{b}
***GABA production	0.781±0.055 ^{c}	0.345±0.026 ^{d}	1.004±0.063 ^{c}	2.227±0.118 ^{a}	1.350±0.111 ^{b}	1.204±0.134 ^{b}

Table 2. *EPS-released (EPS-r), EPS-bound (EPS-b*) and EPS-bound related to cell proteins (EPS-b**). Statistical analysis was performed using one-way ANOVA. All values are expressed as mean ± SD (n = 3). Different lowercase letters (a–d) in each row indicate significant differences (p < 0.05). EPS-r: µmol glucose Eq./mL; EPS-b*: nmol glucose Eq./mL; EPS-b**: nmol glucose Eq./µg BSA Eq.*

EPS fractions	LP 8	LP 25	LP 86	LP 95	LP 100	ATCC 14917
EPS-r	3.43 ± 0.08 ^{a}	2.84 ± 0.15 ^{b}	3.29 ± 0.20 ^{a}	3.06 ± 0.17 ^{a}	3.03 ± 0.18 ^{a}	3.16 ± 0.13 ^{a}
EPS-b*	135.91 ± 5.67 ^{c}	136.23 ± 5.08 ^{c}	38.95 ± 1.91 ^{d}	223.42 ± 7.90 ^{a}	160.96 ± 7.94 ^{b}	51.45 ± 2.74 ^{d}
EPS-b**	0.048 ± 0.007 ^{b}	0.046 ± 0.005 ^{b}	0.016 ± 0.004 ^{c}	0.069 ± 0.011 ^{a}	0.050 ± 0.004 ^{b}	0.026 ± 0.006 ^{c}

3.2 Chemico-physical and microbiologic changes during production and storage of fermented soymilk

Monitoring of the fermentation process was carried out by measuring the pH after 0, 5, 24, 30 and 48 hours. At the end of the fermentation process (48 h) the following parameters were determined: pH, titratable acidity, viscosity and water holding capacity (WHC %). The initial and final values of these parameters are reported in Table 3 showing an increase in titratable acidity, viscosity and WHC, and a pH decrease from 6.47 to 4.22. After 21 days the viable cell count of *L. plantarum* LP 95 decreased by about one logarithmic cycle at 4°C and by two logarithmic cycles at 22°C (Table 4). Our results highlight that the properties examined are strain-specific and careful selection within a given species is of utmost importance to identify appropriate strains for specific biotechnological applications. The chemical and structural characteristics of the fermented soymilk produced, together with the viability and functional properties observed, encourage future applications of *L. plantarum* LP 95 as starter culture to optimize the organoleptic, technological and nutritional properties of fermented soymilk products.

Table 3. *Chemico-physical and microbiological changes of soymilk during fermentation process using L plantarum LP 95 as starter. *One-way ANOVA;**Paired t-test. All values are expressed as mean ± SD (n = 3). Different lowercase letters (a–c) in each row indicate significant differences (p < 0.05).*

Chemico-physical characteristics	Fermenting time (h)				
	0	5	24	30	48
*pH	6.47 ± 0.02 ^{a}	5.89 ± 0.10 ^{b}	4.22 ± 0.05 ^{c}	4.22 ± 0.06 ^{c}	4.22 ± 0.05 ^{c}
**Titratable acidity (°SH)	6.79 ± 0.12 ^{b}	/	/	/	25.63 ± 1.15 ^{a}
**WHC (%)	0.00 ± 0.00 ^{b}	/	/	/	33.12 ± 1.81 ^{a}
**Log (CFU/mL)	7.83 ± 0.01 ^{b}	/	/	/	9.09 ± 0.03 ^{a}
**Apparent viscosity 4 C° (mPa s)	133.80 ± 4.20 ^{b}	/	/	/	941.51 ± 68.07 ^{a}
**Apparent viscosity 25 C° (mPa s)	77.22 ± 3.20 ^{b}	/	/	/	517.00 ± 19.90 ^{a}

Table 4. *Viable counts (log CFU/mL) of L. plantarum LP 95 during storage at 4°C and 22°C of fermented soymilk. Statistical analysis was performed using one-way ANOVA. All values are expressed as mean ± SD (n = 3). Different lowercase letters (a–d) in each row indicate significant differences (p < 0.05).*

Temperature \ Days	7	14	21	28
Storage 4°C	9.15 ± 0.03 ^{a}	8.71 ± 0.02 ^{b}	8.09 ± 0.04 ^{c}	7.09 ± 0.02 ^{d}
Storage 22°C	8.72 ± 0.04 ^{a}	7.78 ± 0.03 ^{b}	7.74 ± 0.02 ^{b}	7.63 ± 0.03 ^{c}

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Ready-to-Eat food as a vehicle of microorganisms in the context of the *microbial deprivation hypothesis*

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This project is intended to test the idea that food is a key vehicle of microorganisms, whose quantity and diversity can be affected by modern industrial processes practiced by the food industry. According to the *microbial deprivation hypothesis*, reduced encounters between microorganisms and a host's immune system could have an impact on host's health, eventually leading to an increased incidence of autoimmune diseases and allergic disorders. The results originated from this Ph.D. project could help re-modulate our dietary regimens in order to integrate the "microbial quote" traditionally associated with food.

Alimenti di quarta gamma come veicolo di microrganismi nel contesto dell'*ipotesi dello svuotamento del bioma*

L'obbiettivo di questo progetto è quello di valutare l'ipotesi che il cibo possa essere un importante veicolo di microrganismi, la cui quantità e diversità può essere influenzata dai moderni processi industriali. Secondo l'*ipotesi dello svuotamento del bioma*, il minor contatto tra microrganismi e il sistema immunitario dell'ospite, può avere un ruolo sulla salute dell'ospite stesso, incrementando l'incidenza di malattie autoimmuni e disturbi allergici. I risultati di questo progetto possono aiutarci a rimodulare la dieta umana, al fine di integrare la "quota batterica" tradizionalmente associata con il cibo.

1. Introduction

In this poster, I want to show the main results of the activities previously described (Mantegazza, 2020) concerning (A 1.1) the ecological analysis of rocket salad, (A 1.2) the isolation of lactic acid bacteria (LAB), and (A 1.3) the capability of rocket salad-associated bacteria to survive simulated gastro-intestinal digestion.

2. Materials and Methods

This study involved the analysis of rocket salad (n=63, 27 vertical farming, 15 conventional farming, 12 organic farming, 6 integrated farming, 3 conventional farming, Conventional /Not Washed (CNW)) obtained from retailers in Milan, Italy. According to protocols ISO 4833 and ISO 15214, the viable count of mesophilic, total bacteria and LAB was performed through dilution and pour-plating onto Plate Count Agar and De Man, Rogosa and Shape Agar at pH 5.7, respectively. Colonies grown on MRS plates were streaked twice on MRS agar and single colonies were inoculated in MRS broth. DNA isolated from single isolates was then amplified with panbacterial primers P0-P6 targeting the 16S rRNA gene and sequenced using the primer 1100R. Total bacteria and plant DNA was extracted using the PowerLyser PowerSoil kit (Qiagen, Milan, Italy) following manufacturer's instructions. The V3 and V4 regions of the 16S rRNA gene were sequenced using Illumina MiSeq with plants Blocking Oligo (Lundberg *et al*., 2013). Pairing, filtering, taxonomic assignment, and taxonomic diversity analyses starting form raw amplicon sequencing data were carried out by means of the bioinformatic pipeline Quantitative Insights Into Microbial Ecology (QIIME2) version 2021.8 with the GreenGenes database (version 13_5). To evaluate the capability of rocket-associated bacteria to survive the human gastro-intestinal tract, *in vitro* gastro-intestinal tract transit was performed using the INFOGEST (Brodkorb et al., 2019).

3. Results and Discussion

3.1 Ecological Analysis of Rocket Salad

Agar plate count experiments revealed that rocket salads from traditional farming harbours much more bacteria than rocket salads from vertical farming according to the number of colonies on both PCA (mean ± standard deviation 7.41 ± 0.60 vs 4.77 ± 0.59; $p<0.001$) and MRS (1.88 ± 1.24 vs 0) media. No differences were found when comparing the impact of the different traditional farming methods on viable cell count on PCA (conventional: 7.41 ± 0.56; organic: 7.18 ± 0.67; integrated: 7.70 ± 0.29; CNW: 7.74 ± 0.86). Regarding the viable count of LAB, more colonies were found in unwashed rocket salads (3.37 ± 0.41) compared to conventional farming (1.61 ± 1.08; $p<0.05$) and integrated farming vs (1.08 ± 1.22 vs 3.37 ± 0.41, $p<0.05$), but not compared to organic (2.20 ± 1.03; $p=0.16$).

After taxonomic assignment, 16S rRNA gene profiling data were first used to assess the biodiversity of the bacterial communities associated to rocket salad. The analysis of α-diversity was carried out with four indexes that differently consider the evenness, richness, and phylogenetic distance of the bacterial taxa within each sample.

Pielou's (measure of evenness), Shannon's entropy (considering both evenness and richness) and observed features (measure of richness) indexes were significantly lower in vertical than traditional farming salads, whereas an opposite result was found for Faith's PD index, which measures biodiversity based on phylogeny. No significant differences were found among traditional farming salads. Therefore, α-diversity analysis revealed that the bacteria associated to rocket salads from vertical farming had a significantly reduced taxonomic richness, were more unevenly distributed, and possessed a wider phylogenetic distance. The marked difference in the bacterial community structure between vertical and traditional farming rocket salads was also evidenced by β-diversity analysis based on Weighted UniFrac algorithm. PCoA plot of Weighted UniFrac distances also revealed a much higher inter-sample diversity among vertical compared to traditional farming salads. On the contrary, samples from traditional farming could not be distinguished and showed a lower inter-sample diversity in their bacterial community structure.

Fig. 1

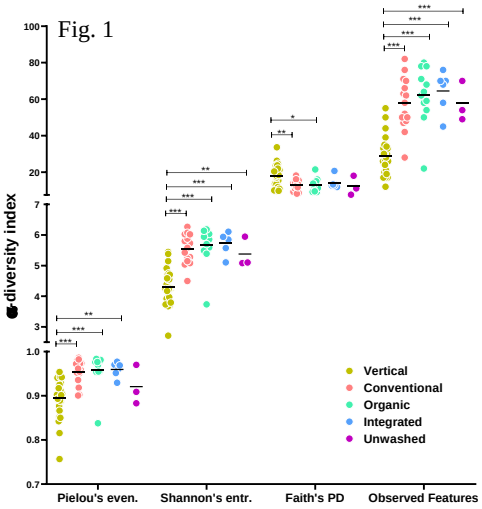

Figure 1 *α-diversity analysis of 16S rRNA gene profiling. data. The four parameter chosen for the analysis are: Pielou's evenness, Shannon's entropy, Faith's PD, and observed features.*

Fig. 2

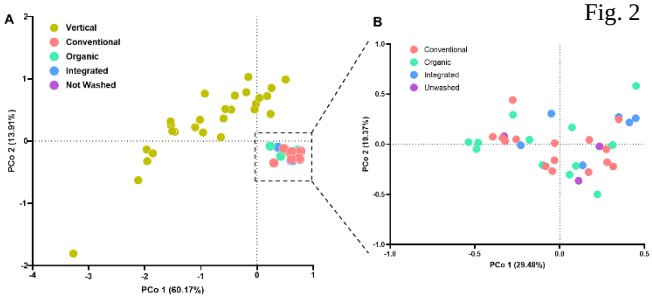

Figure 2 *β-diversity of the microbiota associated to rocket salads shown as principal coordinates analysis of Weighted UniFrac distances based on amplicon sequence variants (ASVs) abundances. A, all investigated rocket samples; B, rocket samples from traditional farming only.*

3.2 Isolation of Lactic Acid Bacteria

In order to define the taxonomic distribution of LAB associated to rocket salad, we isolated from MRS agar 237 colonies (95 from conventional, 84 from organic, 18 from integrated and 43 from unwashed salads). Sequencing of the 16S rRNA gene revealed that all isolates belonged to LAB species, with the only exception of two isolates from an organic farming salad sample that were taxonomically assigned to the Proteobacteria species *Herbaspirillum huttiense*. *Leuconostoc* was the prevalent genus in conventional (76% of the isolates), organic (59%) and unwashed (49%) rocket salads. On the contrary, *Levilactobacillus* and *Weissella* were the most frequent genera among isolates from integrated farming (33%) rocket salads. Less represented genera were *Latilactobacillus* (25 isolates), *Lactococcus* (16 isolates, 14 of which from organic salads), *Lactiplantibacillus* (2 isolates), and *Paucilactobacillus* (2 isolates). In total, the isolates were ascribed to 18 LAB species, none of which were found in all samples. Only 4 species were found in all kind of salads (conventional, organic, integrated and unwashed): *Latilactobacillus sakei*, *Leuconostoc mesenteroides*, *Leuconostoc miyukkimchii*, and *Weissella soli*.

3.3 Survival of rocket-associated bacteria to simulated gastro-intestinal transit

To assess the ability of bacteria colonizing rocket salads to potentially survive the passage through the gastrointestinal tract, we performed a simulated gastro-intestinal tract transit following the INFOGEST protocol. Agar plate count revealed a drastic reduction in the total mesophilic count (before and after simulated gastro-intestinal tract; 6.81 vs 3.04 for conventional rocket salad, 8.31 vs 2.85 for organic rocket salad). Interestingly, LABs seem to have a higher resistance (3.04 vs 2.18 for conventional, 2.85 vs 2.43 for organic). There results suggest that rocket-associated bacteria can survive to the human gastrointestinal transit and reach alive the human intestine. Finally, we isolated several colonies from APC plates inoculated with rocket samples after simulated gastrointestinal digestion. Taxonomic identification assigned the isolates to 8 different species: *Kocuria arsenatis*, *Oerskovia enterophila*, *Bacillus amyloliquefaciens*, *Priestia aryabhattai*, and *Enterococcus* sp. from conventional rocket, and *Pseudomonas fragi*, *Pseudomonas* sp., and *Rhodococcus* sp. from organic rocket.
In conclusion, in light of the results obtained in this study, we can speculate that the intake of fresh rocket salad from ready-to-eat commercial products may be a source of live bacteria that possess the ability to survive the gastrointestinal transit and can potentially counteract the microbial depletion occurring in Western-type diets.

4. References

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Mantegazza G. Ready-to-Eat Foods as a Vehicle of Microorganisms in the Microbial Deprivation Hypothesis. First Virtual Workshop on the Developments in the Italian PhD Research on Food Science, Technology and Biotechnology, Palermo (Italy), 15-16 September 2021, p. 114-115 (Poster Presentation)

Antimicrobial coatings to preserve microbiological quality of dried fruits

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Co-Tutor: Prof. Patrizio Tremonte

The main goal of the PhD project is to identify new protective cultures and develop biotechnological strategies for their use in ready-to-eat products. The active coating development able to preserve microbiological qualities of dried fruit is one of the specific aims of the project. For this purpose, *Lactiplantibacillus plantarum* and *Apilactobacillus kunkeei* were preliminary screened for their antimicrobial activity. Of the best performing strains, the post-biotic antagonist activity was tested, and the metabolite involved in inhibition was subsequently identified by high-performance liquid chromatography (HPLC). The bioactive metabolites were used to activate a carboxymethyl cellulose-based coating. The efficacy of the activated coating was validated in the preservation of dried fruit.

Rivestimenti antimicrobici a garanzia della qualità microbiologica della frutta secca

L'obiettivo principale del progetto di dottorato è individuare nuove colture protettive e sviluppare strategie biotecnologie per un loro ottimale utilizzo in prodotti ready-to-eat. Lo sviluppo di un rivestimento commestibile attivo che preservi le qualità microbiologiche della frutta secca costituisce uno degli obiettivi specifici del progetto. A tal proposito, *Lactiplantibacillus plantarum* e *Apilactobacillus kunkeei* sono stati scrinati per la loro attività antimicrobica. Dei ceppi con le migliori prestazioni è stata testata l'attività antagonista post-biotica e successivamente è stato identificato il metabolita coinvolto nell'inibizione mediante cromatografia liquida ad alte prestazioni (HPLC). I metaboliti bioattivi sono stati utilizzati per attivare un film edibile a base di carbossimetilcellulosa. L'efficacia del film edibile attivato è stata validata nella conservazione di frutta secca.

Key words: *Carboxymethyl Cellulose, Fungi, Lactiplantibacillus plantarum, Apilactobacillus kunkeei*

1. Introduction

The last years have been marked by an increase and diversification of dried fruits which, besides being widely used in the sweet industry, are proposed in single dose sizes as ready-to-eat foods. These are minimally processed products that do not require cooking and, as such, can directly deliver microbial contaminants to consumers. Regarding to dried fruits, nut drying process does not eliminate microbiological and therefore mycotoxigenic risks (Razavi, et al. 2021; Lombardi et al 2022). Tailored protective biotechnologies are strongly recommended for these products. According to my PhD project, this poster reports the first results of the activity (i) selection of post-biotic with the best antimicrobial and antifungal activity, (ii) identification of metabolite involved in inhibitory activity, (iii) add the metabolite at antimicrobial coating.

2. Materials and Methods

2.1Antimicrobial activity screening. Sixty-three LAB (*Lp plantarum* and *A. kunkeei*) previously biotyped by RAD-PCR, were evaluated for their antimicrobial activity. Post-biotics of each strain were obtained by centrifugation of the overnight culture and microfiltered (MINISART HIGH FLOW filters, 0.22µm pore size, Sartorius GE) to remove any residual cells. Antifungal activity was assessed by measuring the growth capacity of indicator moulds placed by a mycelium disc on PDA fortified with the post-biotics of the producing strains (5% v.v.) Antibacterial activity was assessed by agar well diffusion assay (Tremonte et all. 2017).
2.2 Post-biotics characterization. The post-biotic that showed the most inhibiting activity against indicator strains was analysed by high-performance liquid chromatography (HPLC) according to Almaforte et all. 2006.
2.3 Coating activation. Two molecules (phenyl-lactic acid and phenyl-acetic acid) found in post-biotics and recognized for their antimicrobial action were added to a mixture of water and carboxy-metil cellulose [1%]. The dried fruit was immersed in the edible film for 30 seconds and left to dry for 20 minutes under a laminar flow hood. To evaluate antifungal efficacy, a challenge test on dried hazelnuts was carried out. Hazelnuts were preliminarily inoculated (1% v/w) with 3 Log CFU/g of the multi-strain fungi cocktail and then were divided in three batches: NC without coating; CMC: with carboxy-metil cellulose based coating; CMC+A: carboxy-metil cellulose based coating activated with post-biotic compounds. Growth or inhibition of intentionally inoculated fungi was monitored at ideal incubation conditions on PDA plates

3. Results and Discussion

3.1 Antimicrobial activity of post-biotics. Primary screening showed different effects of post-biotics against indicator strains (mould and bacteria). The results highlighted that each RAPD_biotype was characterized by a specific antimicrobial profile. RAPD_biotype VI, which groups 7 producer strains (all isolated from bree-bread), showed the most interesting antimicrobial activity.

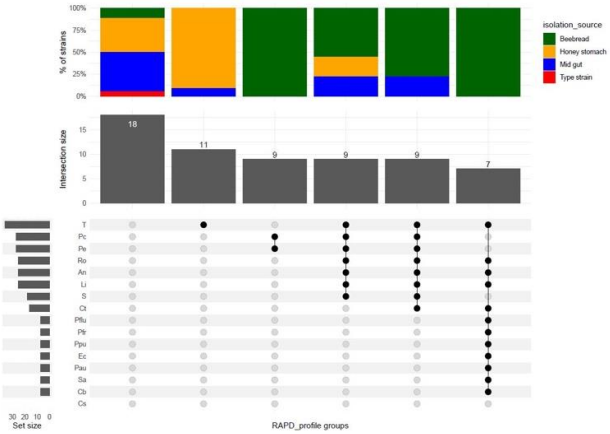

Figure 1. *Upset plot regarding the anti-microbial of the 6 RAPD biotype groups belonging to post-biotic of LAB. Indicators are: Penicillium commune (Pc), P. echinolatum (Pe),Pseudomonas fragi (Pfr), Tricoderma (T), Ryzopus oryzaea (Ro), Ps fluorescens (Pflu), Ps. aeruginosa (Pau), Clostridium tyrobutyricum (Ct), C. butyricum (Cb), C. sporogenes (Cs), Staphilococcus aureus (Sa), Listeria innocua (Li), Ps. putida (Ppu), Salmonella (S), Aspergillus niger (An) Escherichia coli (Ec),*

3.2 Post-biotic bioactive compounds and activated coating. The results of the HPLC analysis showed that the post-biotics of the strain with anti-fungal and antibacterial activity compared to those without activity were characterised by the presence of two neo-formation peaks corresponding to phenyl-lactic acid (PhLA) at concentration of 129.93 mg/mL and phenyl-acetic acid (PhAA) concentration of 62.43 mg/mL, respectively. The concentration of the two acids in the post-biotic is much higher than their MIC values, making it possible to apply the post-biotic compounds in the edible film for its activation (Figure 2).

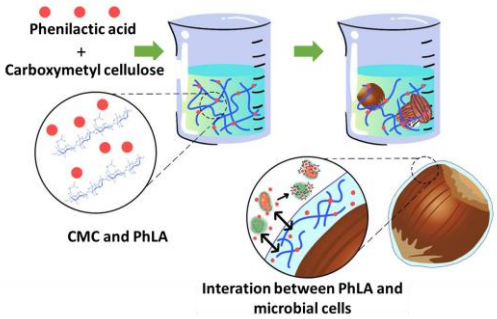

Figure 2. *Representation of Edible Film Active*

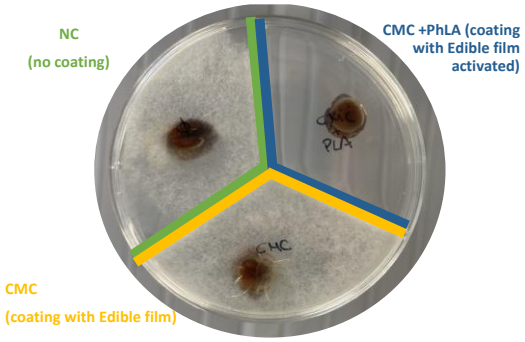

Figure 3. *Effectiveness in fungi control in uncoated (green) methylcellulose-coated (yellow) and in activated CMC- coted*

Figure 3 highlighted the effectiveness of the activated edible coating. Uncoated or methylcellulose-coated hazelnuts showed rapid growth of intentionally inoculated fungi whose mycelium rapidly invaded the plate. In contrast, the activated coating impeded the growth of intentionally inoculated fungi. This represents an important starting point for the development of new biotechnological strategies in the preservation of ready to eat dried nuts.

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Legume flours: characterization and food applications

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This PhD work aims the physico-chemical characterization of legume flours (red lentils, chickpeas and green peas) and verify their effect in food applications (e.g. bakery products, soups, meat analogues). I report the design and development of a snack for gut-brain axis health (formulation strategies, product's quality assessment, consumers' evaluation). The snack consists in a symbiotic chocolate coated cookie. Prebiotic rich cookie is legume based, contains specialty fibres (for sugar and saturated-fat reduction), is rich in fibre, slowly digestible carbohydrates and unsaturated fats, low in sugar. The cholate coat contains a mix of probiotic (with documented positive effect on gut-brain axis).

Farine di legumi: Caratterizzazione e applicazione alimentare

Questo lavoro di dottorato mira alla caratterizzazione delle farine di legumi (lenticchie rosse, ceci e piselli) e alla realizzazione di applicazioni alimentari da forno. Sono presenti i risultati del processo di sviluppo della formulazione per snack simbiotico per la salute dell'asse intestino-cervello, utilizzando come ingrediente farine di legumi come prebiotici, miscela di formulazione di probiotici (SLAB 51) e fibre speciali. Sono state valutate la qualità degli alimenti, l'accettabilità dei consumatori e l'efficacia dell'attività probiotica. I risultati suggeriscono che la durezza dei biscotti ai legumi è maggiore, gli attributi nutrizionali aumentano considerevolmente, la previsione dell'indice glicemico è bassa e il prodotto è stato molto ben accettato dai consumatori contro il controllo.

Key words: probiotics, prebiotics, cookie, legume flours, snack

1. Introduction

Aging and age-related neurodegeneration are among the most urgent challenges in modern society because of the progressive increase in the number of elderly people all over the world and are strongly dependent on the gut-brain axis (Oleskin *et al.* 2016*)*. Preservation of healthy gut microbiota is favoured by healthy diet (prebiotic rich) and probiotic supplementation is a promising perspective to prevent or delay neurodegeneration. Snacks could be an opportunity for improving dietary habits of consumers as snackification is one of the recent megatrends in food, in response to consumers' request for a combination of health and indulgence in food (Boukid *et al.* 2022). Cookies are a good candidate for snack as they demand is very high in the western world and in Italy and Spain, in particular. The aim of this work was, therefore, to develop a symbiotic functional snack (cookie) to promote healthy aging, with positive action on gut-brain axis, through the delivery of probiotics and prebiotics through proper food design

2. Materials and methods

A functional snack was developed containing chocolate coated (probiotic rich) cookie (prebiotic rich). Was developed starting from a conventional wheat "pasta frolla" cookie recipe that was modified by substituting wheat with legume flours (red lentils, chickpeas and their 50:50 combination; Martino Rossi SpA.), and by using specialty fibres that were proven easy sugar (Meltec®, Microsin®; Carcelli *et al.*, 2021) and fat/saturated fat reduction (WF fibre®, HI-Food SpA; Curti et al, 2018; Boukid *et al.* 2021). Were calculated the nutritional content (using the ingredients technical sheets and the European Institute of Oncology database, 2015), colour (Minolta Colorimeter), hardness [N], (cutting test TA1 Texture Analyzer, AMETEK, USA), and consumers' sensory evaluation (check-all-that-apply [CATA] tests with 60 consumers). The probiotic rich phase was obtained by inoculating multi-strain probiotic mix (SLAB51, Bonfilli *et al.* 2018), in tempered 75% dark chocolate (Socado®, Verona, Italy) to reach a probiotic concentration corresponding to RDA (recommended daily allowance) and 2RDA in the chocolate serving size (200 billion/30g chocolate). Chocolate was then characterized and evaluated with sensory attributes (consumer panel) and stability storage (whitening index [Minolta colorimeter], fat melting profile (DSC 8500, PerkinElmer) of the product. Data were elaborated using one-way analysis of variance (ANOVA) with a Tukey HSD tests to verify significant differences ($P \leq 0.05$) using a XLSTAT 2022 software.

3. Results and Discussion

3.1 Prebiotic-rich biscuit development, characterization, and optimization

Biscuits recipe development followed a series of recipe modifications aiming to evaluate the effect of substitution of wheat with legume flours on cookie characteristics, effect of specialty fibres and consumers 'acceptability of the final product. The results allowed to reach a pre-biotic carrier, compared to wheat control biscuit, reduction of

saturated fats, total carbohydrates and sugars and increased protein, fibre and unsaturated fat contents (Table 1). Legume flour increased cookies hardness, but the specialty fibres decrease it (Figure 1).

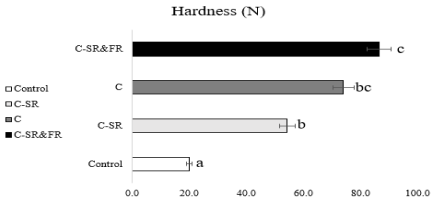


***Figure 1**. The different letters indicate significative differences among samples (P ≤ 0.05). Control: wheat biscuit original formula; C: Chickpeas; C-SR: Chickpeas sugar reduced; C-SR&FR: Chickpeas sugar and fat reduced*

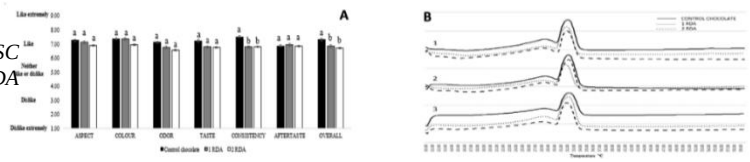
***Table 1**. Control: wheat biscuit original formula; C: Chickpeas; C-SR: Chickpeas sugar reduced; C-SR&FR: Chickpeas sugar and fat reduced*

Nutrients	Control	C	C-SR	C-SR&FR
Energy (kcal)	444	490	454	388
Fat (g)	15.7	21.40	19	12.6
Saturated fat (g)	8.7	10.5	9.4	1.9
Carbohydrates (g)	69.9	52.7	55.7	53.3
Sugars (g)	24.8	22.2	19.8	17.1
Fibre (g)	1.5	5.5	13.2	17.0
Proteins (g)	8,7	15.5	16.9	16.7
Salt (g)	0	0	0.0	0.0

3.2 Probiotic-rich chocolate characterization and consumers acceptability

The sensory analysis for the probiotic-rich chocolate indicated a slight reduction in acceptability (overall and texture) of the product but that the reduction was limited and product still acceptable (6.9 points; Figure 2A). Probiotics were vital in the fresh product and during storage (3 months). No indication of changes in fat polymorphism (Figure 2B) and blooming induced by presence of probiotics were recorded over 3-month period. The presence of probiotics in the chocolate did not alter sensory attributes or stability of chocolate.

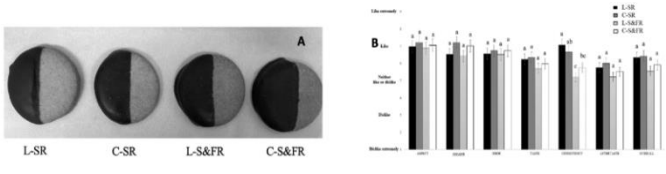
***Figure 2.** A) Acceptability and B) DSC thermograms of chocolate (control, 1 RDA and 2 RDA of probiotics)*



3.3 Functional snack (probiotic rich chocolate coated prebiotic cookie) characterization and consumer's acceptability

The best performing recipes identified were cookies containing either red lentil (L) or chickpea flours (C) in figure 3. Sensory evaluation of functional snack indicated a good consumers acceptability with scores among the categories Like (7) and neither like or dislike (5) Figure 3B. In particular, SR (Sugar reduced) cookies were generally better received by consumers than their S&FR (Sugar and fat reduced) counterpart for overall acceptability, consistency and aftertaste. The less preferred consistency of S&FR cookies was due to the harder and less friable texture of such cookie.

***Figure 3.** A) Functional snack B) Sensory acceptability. L-SR: Lentil sugar reduced; C-SR: Chickpeas sugar reduced; L-S&FR: Red lentils sugar and fat reduced; C-S&FR: Chickpeas sugar and fat reduced*



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Characterization and study on the recycling of coffee by-products for the manufacture of feed and functional food intended for human use and bioplastics

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Co-tutor: Dr. Francesco Esposito

Preliminary results obtained on the three main objectives of the PhD thesis project are given. The first was based on characterizing the qualitative, nutritional and functional aspects of Coffee Silverskin (CS) for the production of new healthy food products with a good nutritional profile for human diets. The second was based on the evaluation of CS as a food substrate for the growth of *Hermetia illucens* larvae for animal feeding. The third part of the project focused on the analysis of the physicochemical structure of the integument for bioplastics and paper production, with preliminary prototypes of functional bio-based coating.

Caratterizzazione e studio per il recupero di sottoprodotti della tostatura del caffè per la produzione di mangimi, alimenti funzionali ad uso umano e bioplastiche.

Vengono presentati i risultati preliminari ottenuti sui tre obiettivi principali del progetto di tesi di dottorato. Il primo è basato sulla caratterizzazione degli aspetti qualitativi, nutrizionali e funzionali del Coffee Silverskin (CS) per la produzione di nuovi prodotti alimentari sani con un buon profilo nutrizionale per la dieta umana. Il secodo è basato sulla valutazione del CS come substrato alimentare per la crescita di larve di *Hermetia illucens* per l'alimentazione animale. La terza parte del progetto si è concentrata sull'analisi della struttura del tegumento per la produzione di bioplastiche e carta, con prototipi preliminari di rivestimento funzionale a base biologica.

Keywords: Circular economy, food waste material, coffee by-products, recycling, functional food

1. Introduction

Following the PhD thesis project, this poster reports the main results of the first two activities concerning:
(A1) Determination of the nutritional and healthy properties of CS for the production of highly functional food products.
(A2) Development of *Hermetia illucens*-based feed
(A3) Characterization and production of paper-based packaging products and feasibility assessment of coffee silverskin-based bioplastics

2. Materials and Methods

Physico-chemical property analyses were performed on the CS sample to evaluate its nutritional profile and dietary healthiness. For characterization of the nutritional profile, analyses were performed on the protein fraction, total fat, saturated fatty acids, carbohydrates, sugars, lignin, cellulose, and hemicellulose content. The following analyses were carried out according to ISTISAN Report 96/34. Proteins of CS were isolated by adapting isoelectric precipitation technologically used for soy proteins and hemp proteins (Mamone et al., 2019). The resulting peptides were searched for their potential bioactivity through the Biopep software. Food safety and wholesomeness were verified by searching for the presence of environmental and process contaminants in the integument. Among the contaminants searched for were acrylamide (AA), following the Fernandes and Soares (2007) method of investigation with some modifications, polycyclic aromatic hydrocarbons (PAHs), following EPA 3550C:2007 + APAT CNR IRSA 5080 Man 29:2003 protocols, and heavy metals. The metal trace detection and quantification were performed using a Thermo ScientificTM ICAPTM RQ inductively coupled plasma mass spectrometer (Q-ICP-MS). Nutrient profile analyses were also used to evaluate CS as a growth substrate for *Hermetia illucens* larvae, with subsequent nutritional analyses on Hermetia biomass obtained. Lastly, characterization of the integument structure on spectral identification of its constituents, thermostability, and porosity was carried out by Fourier Transform Infrared (FTIR) spectroscopy, Solid-state CP/MAS 13C NMR spectroscopy, Thermogravimetric analysis (TGA), Scanning Electron Microscopy (SEM) and Volumetric nitrogen adsorption analysis

3. Results and Discussion

3.1 Determination of the nutritional and healthy properties of Coffee Silverskin

From the analysis, the levels of protein (18.9%), carbohydrate (42.0%), fat (3.0%), and fiber (34.7%) show the

potential for dietary use of CS as an additive to a fiber-rich, low-fat food. The wholesomeness of the CS was determined by evaluating the low levels of contaminants such as OTA and AA, heavy metals and PAHs (Table 1). The chemical profile of CS is also interesting from the point of view of bioactive compounds. The MS analysis showed the presence of 45 peptides inferred to protein involved in several metabolic pathways, among these: serine proteases, carboxypeptidase and neprosin. After the Biopep analysis of peptides found in CS extracts, several bioactivities have been highlighted. Among them, a key role could be played by peptides containing sequences with the angiotensin-converting enzyme (ACE) and dipeptidyl peptidase IV (DPP IV) inhibitory activity. This evidence could confirm the application of CS extract as a supplement to conventional drugs in the treatment of diabetes.

Table 1 *Levels of contaminants and trace elements in Coffee Silverskin*

Policyclic Aromatic Hydrocarbons (PAHs)	*Concentration (mg/kg)*	*Process Contaminants*	*Concentration (mg/kg)*	*Micronutrients*	*Concentration (mg/kg)*
Benzo(a)pyrene	< 0.01	Acrylamide (AA)	< 0.02	Manganese (Mn)	22.9
Benzo(b)fluoranthene	0.01	Furan	< 0.1	Copper (Cu)	58.6
Benzo(k)fluoranthene	< 0.01	Methyl-furan	< 0.1	Cobalt (Co)	1.02
Chrysene	< 0.01	*Mycotoxins*	*Concentration*	Zinc (Zn)	7.08
Dibenzo-Pyrene	0.86	Ochratoxin A	< 0.1	Iron (Fe)	349
Dibenzo Anthracene	0.02	*Heavy Metals*	*Concentration*		
Pyrene	0.02	Arsenic (As)	0.04		
Perylene	< 0.01	Cadmium (Cd)	0.05		
Naphthalene	0.04	Total Chromium (Cr)	0.86		
Acenaphthene	0.06	Mercury (Hg)	0.02		
Fluorene	0.03	Nickel (Ni)	1.78		
Fluoranthene	0.02	Lead (Pb)	2.63		

3.2 Development of *Hermetia illucens*-based feed

In order to evaluate CS as a growth substrate for arthropod larvae, two different substrate conditions (no treated CS and CS macerated with water) and nutritional values were considered, and nutritional values were compared with commercial growth substrates. The vegetal mix diet, with fruits and vegetables, and the standard diet, a balanced diet conventionally used to rear *Hermetia illucens* larvae, have 4.4% and 10.8% total fiber, respectively. Whereas untreated CS and macerated CS, have 29.5% and 37.2%. The protein content is also much higher in CS (20.72%) and macerated CS (21.60%) compared to the vegetal mix diet (10.3%) and standard diet (14.1%). Analyses concerning the nutritional value of Hermetia biomass obtained from the various growth substrates are ongoing.

3.3 Characterization and production of paper-based packaging products and feasibility assessment of coffee silverskin-based bioplastics

Through analyses of morphological-structural assessment of the CS, it was possible to identify the main functional groups present in the integument: cellulose, hemicellulose, and lignin. Lignocellulosic materials are widely recognized as interesting biosourced reinforcements for polymer-based composites (Ortega et al., 2021) for their excellent mechanical properties and wide availability. The presence of polyphenol aromatics like lignin and its derivatives is often associated with antioxidant properties that can be exploited in composites to confer functional properties of high applicative interest. Natural antioxidants can have beneficial effects on the stability of polymers during processing and/or reprocessing, avoiding the use of synthetic stabilizers (Kirschweng et al., 2017) and favoring recyclability. On the other hand, if composite films are produced for packaging applications, the migration of antioxidant molecules at the film surface can be promoted, extending the oxidative stability of the contained foods. From these analyses, preliminary tests were carried out to make a PLA/CS coatings.

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New technological approaches for improving the quality and shelf life of extra virgin olive oils

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This research activity is studying the application of new technologies in the extra virgin olive oil extraction process with the objective of improving the final quality and shelf life. The study tries to develop an industrial process in which there is the joint use of stoning technology with ultrasound technology for the treatment of olive pastes. The aim of this coupling is taking advantage of both technologies and mutually avoiding the disadvantages. Furthermore, the study is evaluating all the technological and qualitative improvements brought about by the most recent innovations in the extra virgin olive oil production's sector, such as the use of high vacuum in the malaxation phase and other innovations in the olive's crushing and paste's preparation before separation by centrifugation.

Nuovi approcci tecnologici per il miglioramento della qualità e della shelf life degli oli extravergini di oliva

Questo lavoro di tesi sta studiando l'applicazione di nuove tecnologie nel processo di estrazione dell'olio extravergine di oliva con l'obiettivo di andare a migliorare la qualità finale e la conservabilità dell'olio stesso. In modo particolare si cerca di mettere a punto un processo nel quale si utilizza la tecnologia di denocciolatura congiuntamente alla tecnologia ad ultrasuoni per il trattamento delle paste di olive. L'obiettivo è quello di trarre vantaggi da entrambe le tecnologie ed eliminarne reciprocamente gli svantaggi. Inoltre, lo studio sta valutando tutti i miglioramenti tecnologici e qualitativi apportati dalle più recenti innovazioni nel settore elaiotecnico, in modo particolare l'utilizzo dell'alto vuoto in fase di gramolatura ed altre innovazioni nelle modalità di frangitura e preparazione delle paste prima della fase di separazione per centrifugazione.

1. Introduction

In accordance with the PhD thesis project previously described in the former PhD workshop (2021), this poster reports the main results of the first two activities concerning:

A1) **Testing technology innovations.** In a pilot plant scale, there'll be test different technologies in combination due their expected effect on olive oil quality and yield. The tests comparing a normal crushing system with combination of pitting and ultrasound machine for olive paste treatment, in addition with thermal conditioning. It will try to see the effect on endogenous enzyme activities of fruit, such us lipoxygenase (LPO), peroxidase (POD), polyphenol oxidase (PPO) involved in regulation of biochemical reaction strictly related to phenolic and volatile fraction. Will be also tested the effect of high vacuum technology apply to malaxation phase and Ultrasound on EVO quality and yield.

A2) **Analysis of products and process.** The EVOO oils obtain by pilot plant are analyzed for legal quality parameter, phenols compounds following the method describe by Selvaggini et al. (2014), volatile compounds analysis will be performed by headspace-solid phase microextraction followed by gas chromatography-mass spectrometry (HS-SPME-GC-MS). Analysis will be repeated periodically to evaluate quality parameters and EVOO shelf life. Based on the results could be necessary the assessment of other minor compounds of EVOOs.

2.Results and Discussion

Concerning the result attended, the technology has showed a significative increase of phenols without negatively affecting the volatile fraction (Table 1 and 2). More tests should be carried out to confirm the increase of extraction's yield. The ultrasounds treatment doesn't affect the legal quality parameter, the destoning technology confirm the increasing of quality due to the system of fruit breaking.

Table 1. *Phenolic compounds of the extra virgin olive oil produced by different technology combinations: crushing (CTRL), crushing - ultrasound (US), destoning (DS), destoning - ultrasound (DS-US). Different letters in the same columns indicate significant differences.*

Cultivar	Process	Total phenols	Σ oleuropein derivatives	Σ ligstroside derivatives	Σ lignans
Peranzana	CTRL	586,6 ± 10,74 ^{a}	454,8 ± 10,36 ^{a}	114,3 ± 2,79 ^{a}	15,5 ± 0,64 ^{a}
	US	622,7 ± 9,63 ^{b}	486,4 ± 9,37 ^{b}	119,3 ± 2,5 ^{ab}	15,2 ± 0,37 ^{a}
	DS	679,4 ± 12,8 ^{c}	537,8 ± 12,19 ^{c}	124,2 ± 3,86 ^{bc}	15,4 ± 0,52 ^{a}
	DS-US	718,7 ± 10,98 ^{d}	517,5 ± 10,78 ^{d}	129,8 ± 2,05 ^{c}	15,7 ± 0,37 ^{a}
Canino	CTRL	583,5 ± 10,9 ^{a}	447,3 ± 9,5 ^{a}	92,7 ± 4,9 ^{a}	32,3 ± 0,8 ^{a}
	US	605 ± 17,5 ^{a}	468,3 ± 17,3 ^{a}	95,4 ± 2,7 ^{b}	31,5 ± 0,6 ^{a}
	DS	706,1 ± 20,1 ^{b}	556,4 ± 19,1 ^{b}	106,1 ± 6,2 ^{b}	31,4 ± 1 ^{a}
	DS-US	719,9 ± 25,8 ^{b}	559 ± 24,7 ^{b}	117,6 ± 7,1 ^{b}	31,1 ± 1,1 ^{a}
Coratina	CTRL	1137,2 ± 16 ^{a}	785,3 ± 15,6 ^{a}	290,6 ± 3,8 ^{a}	60 ± 0,9 ^{a}
	US	1157,5 ± 29,3 ^{a}	806 ± 27,6 ^{a}	290,4 ± 10 ^{a}	59,9 ± 1,4 ^{a}
	DS	1361 ± 31,9 ^{b}	976,8 ± 31,1 ^{b}	322,9 ± 7,1 ^{b}	60 ± 1,8 ^{a}
	DS-US	1379,2 ± 22,6 ^{b}	994,8 ± 22 ^{b}	325,1 ± 5,2 ^{b}	58,2 ± 1,2 ^{a}

Table 2. *Volatile compounds of the extra virgin olive oil produced by different technology combinations: crushing (CTRL), crushing - ultrasound (US), destoning (DS), destoning - ultrasound (DS-US). Different letters in the same columns indicate significant differences.*

Cultivar	Process	Σ C_5 and C_6 aldehydes	Σ C_5 and C_6 alcohols	Σ C_6 esters	Σ C_5 and C8 ketones
Peranzana	CTRL	15342 ± 553 ^{a}	1244 ± 36 ^{a}	1621 ± 50 ^{a}	356 ± 19 ^{a}
	US	15434 ± 320 ^{a}	1115 ± 22 ^{b}	1838 ± 55 ^{ab}	330 ± 12 ^{a}
	DS	17465 ± 323 ^{b}	1288 ± 52 ^{a}	1905 ± 108 ^{b}	369 ± 3 ^{a}
	DS-US	17306 ± 509 ^{b}	1230 ± 46 ^{a}	1827 ± 121 ^{ab}	380 ± 17 ^{a}
Canino	CTRL	22780 ± 1243 ^{a}	2815 ± 96 ^{a}	332 ± 4 ^{a}	312 ± 11 ^{a}
	US	23652 ± 1006 ^{a}	2482 ± 91 ^{b}	344 ± 31 ^{ab}	340 ± 27 ^{ab}
	DS	30390 ± 2057 ^{b}	2832 ± 89 ^{a}	403 ± 24 ^{b}	372 ± 17 ^{b}
	DS-US	30495 ± 2698 ^{b}	2565 ± 76 ^{b}	393 ± 31 ^{ab}	361 ± 23 ^{ab}
Coratina	CTRL	29548 ± 2091 ^{a}	2618 ± 80 ^{a}	11 ± 1 ^{a}	634 ± 45 ^{ac}
	US	29716 ± 1580 ^{a}	2527 ± 71 ^{a}	13 ± 1 ^{a}	629 ± 28 ^{a}
	DS	35332 ± 1405 ^{b}	2920 ± 90 b	13 ± 1 ^{a}	734 ± 35 ^{bc}
	DS-US	35186 ± 2217 ^{b}	2737 ± 82 ^{ab}	10 ± 1 ^{a}	726 ± 37 c

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Lacticaseibacillus casei: a bioprotective culture with antioxidant properties?

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The aim of the PhD project is to employ microorganisms and metabolites thereof to increase food product shelf-life exploiting their antioxidant activities, allowing the substitution or the reduction of synthetic additives. One of the experimental activities was to investigate the effect of respiratory metabolism of one strain of lactic acid bacteria for the production of fermented sausages.

Lacticaseibacillus casei: una coltura bioprotettiva con proprietà antiossidanti?

Lo scopo della tesi di dottorato è quello di impiegare microorganismi e loro metaboliti per allungare la shelf-life dei prodotti alimentari sfruttando la loro attività antiossidante, in modo da permettere la sostituzione o la riduzione di additivi sintetici. Una delle attività sperimentali è stata quella di indagare l'effetto del metabolismo respiratorio di un ceppo di batteri lattici per la produzione di salami.

Key words: Lactic acid bacteria, bioprotection, antioxidant, fermented sausages.

1. Introduction

Lactic acid bacteria (LAB) are used as starters, adjunct and/or probiotic cultures to improve food quality and promote human health, they are recognized as oxygen-tolerant anaerobes that used fermentative pathways for biomass and energy production. However, it has been demonstrated that some LAB are capable of using oxygen and producing a minimal electron transport chain in the presence of haeme and menaquinone, acquiring several physiological and stress response benefits such as oxidative stress tolerance. The activation of respiratory chain may be relevant in the reduction of oxidative processes on food components. The effect of the use of respiration-competent *L. casei* N87, cultivated under anaerobic and respiratory conditions, was evaluated during the production of fermented sausages.

2. Materials and Methods

L. casei N87 was adapted under anaerobic and respiratory conditions and used as starter cultures for fermented sausages production (about 6 Log CFU g-1). The respiratory condition was performed according to Zotta et al. (2014): briefly, the strain was cultivated in M17 broth added with 2.5 µg/ml hemin and 1 µg/ml menaquinone at 30 °C for 48 h. The production involved the following ingredients: pork meat 64.8 %, pork fat 25.2 %, sodium chloride 2.5 %, black pepper, sugar and red wine mixed with garlic (360 ml/45 kg). The meat batter was minced and divided in three aliquots to produce three different lots of fermented sausages: 1) control (C, fermented sausages without the addition of starter culture); 2) fermented sausages with the addition of *L. casei* N87 adapted under anaerobic conditions (A); 3) fermented sausages with the addition of *L. casei* N87 adapted under respiratory conditions (R). Sausages were ripened for 90 days and samples of each lot were taken in triplicate at various times (0, 2, 5, 7, 15, 30, 60 and 90 days) of ripening. The effect of strain cultivation was evaluated on the production and quality of the fermented sausages. During ripening, the evolution of the microflora and the physicochemical parameters (pH, a_w, weight loss and colour) were monitored. The effect of ripening and starter addition on the protein fraction was determined by SDS-PAGE. The volatiles profiles of the samples were determined at the end of ripening using GC-MS-SPME analysis. Statistical analysis was performed by analysis of variance with Tukey's test and two-way hierarchical analysis using R v 3.3.2.

3. Results and Discussion

Regarding physicochemical parameters, a_w and colour parameters did not show differences among samples, whereas, pH was significantly lower in samples A, as well as weight loss in R samples (2.5% less compared to the other). Initial counts of LAB were 4.08 Log CFU/g in the control samples and 7.18 and 6.43 in samples A and R, respectively. Throughout the period monitored, LAB counts were found to be higher in samples A on the days 0, 2, 7 and 30 and this could be enough to produce the pH lowering observed. Initial Enterococci counts were higher in the samples A, however, after 7 days and till the end of the ripening the counts were lower compared to the other. The large and rapid decrease in pH that occurred in the samples A may explain the reduction in the number of these bacterial groups. The proteolysis that occurred during sausages ripening indicated a differentiation of the samples A from the other (Fig. 1), which were characterized by a higher proteolytic activity. Proteolysis could be the result of the action of meat endogenous enzymes or the activity of the microorganisms involved in the sausages fermentation. Probably, the lower pH that characterized these samples contributed to the activation of endogenous

proteases (Xiao et al., 2020). Compounds resulting from protein breakdown and those generated from amino acids transformation have an important influence on the flavour development of fermented sausages. Principal component analysis (PCA) was performed to evaluate the relationships between the aroma profile and the effect of the starter cultures (Fig. 2). The first two principal components explained the 95.62% of the total variance. The meat mixture was clearly separated from the samples at end of ripening. In fact, it had higher alcohols concentrations, probably due to the addition of wine. The second principal component (PC2) explained 29.24 % of the variation and separated the samples at the end of ripening. CT90 samples were characterized by high levels of acids and hydrocarbons while AT90 samples had the highest level of aldehydes. Aldehydes are the main volatiles synthesized from lipid oxidation and in this study were represented by hexanal and nonanal (Ross & Smith, 2006). They were found at the highest level in samples AT90, which were more oxidised compared to the others.

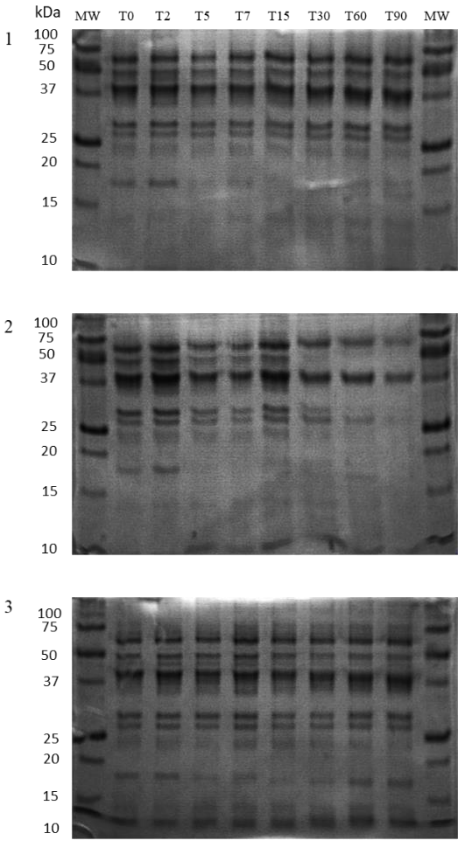

Figure 1 *SDS-PAGE profile throughout the ripening of fermented sausages: 1, control uninoculated (C); 2, sausages inoculated with L. casei N87 cultivated under anaerobiosis (A); 3, sausages inoculated with L. casei N87 cultivated under respiratory conditions (R).*

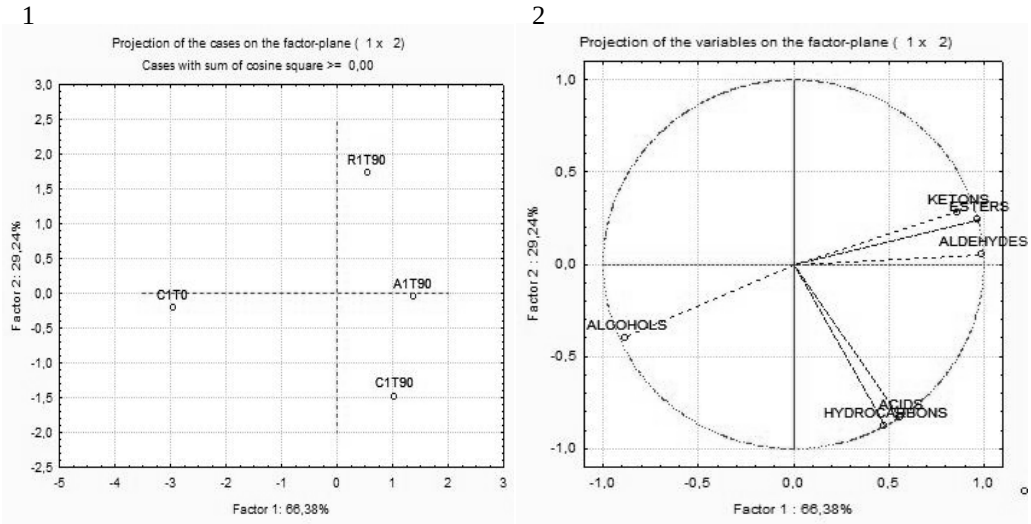

Figure 2 *Principal component anlysis (PCA) scores (1) and loadings (2) plots of fermented sausages (CT0, meat mixture; CT90, control uninoculated on day 90; AT90, sausages inoculated with L. casei N87 cultivated under anaerobiosis on day 90; RT90, sausages inoculated with L. casei N87 cultivated under respiratory conditions on day 90) and volatile compounds.*

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Technological development of new tropical fruit products and beverages

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Increasing consumer interest in beers with new flavours is promoting the introduction of innovative materials such as tropical fruit and novel yeast in brewing. In the first part of the PhD project, the effect of a *Saccharomyces* and a non-*Saccharomyces* yeast strain isolated from an ecological niche such as manna was studied for the production of fruit-based craft beer. The beer was made by adding loquat juice in all trials. The objective was to evaluate the effect of two novel yeast strains isolated as potential starter cultures for beer production and to enhance the aroma and taste profile of the final product. The results showed that these strains are promising as starter for beer productions.

Sviluppo tecnologico di nuovi prodotti e bevande a base di frutta tropicale

Il crescente interesse dei consumatori per le birre con nuovi sapori sta promuovendo l'introduzione di materiali innovativi come la frutta tropicale e nuovi lieviti nella produzione di birra. Nella prima parte del progetto di dottorato, è stato studiato il comportamento di un ceppo di lievito *Saccharomyces* e di un non-*Saccharomyces* isolati da una nicchia ecologica come la manna, per la produzione di birra artigianale a base di frutta. La birra è stata prodotta aggiungendo succo di nespola a tutte le prove. L'obiettivo è stato quello di valutare l'effetto di questi due nuovi ceppi di lievito come potenziali colture starter per la produzione di birra e di migliorare il profilo aromatico e gustativo del prodotto finale. I risultati hanno dimostrato che questi ceppi sono promettenti come starter per la produzione di birra.

Key words: Alcoholic fermentation; Loquat beer; *Lachancea thermotolerans*; *Saccharomyces cerevisiae*; Beer aroma.

1. Introduction

In accordance with the PhD thesis project, this poster reports the main results of the first two activities concerning:
(A1) Microbiological analysis and determination of main physicochemical parameters of samples collected at different stages of brewing;
(A2) Sensory analysis of the fruit beers produced.

2. Materials and Methods

Yeast strains used in this study are *Saccharomyces cerevisiae* MN113 and *Lachancea thermotolerans* MNF105. All strains belong to microbial culture collection of the Department of Agricultural, Food and Forest Sciences (SAAF; University of Palermo, Italy). MN113 and MNF105 were previously isolated from manna ash (Guarcello et al., 2019). The wort was made using only Pilsen malt (BestMalz, Heidelberg, Germany) to better understand the effect of the yeast inoculum. Experimental top-fermented beers were produced at a medium-scale level (5 L batch) using four different inoculums. Loquat juice was squeezed from fruits of the white-fleshed local cultivar "Claudia" (*Eriobotrya japonica* Lindl) following the procedure proposed by Tarantino et al., (2022). As reported by Gasiński et al., 2020; 20% loquat juice (w/w) was added to all trials at the end of alcoholic fermentation AF (day 10). The samples were collected at different stages of beer production, days: 0, 3, 6, 10, 11 and 16. Cell densities of yeast populations were evaluated as described by Iris et al. (2020). The fermentation was carried out at 18 °C under static condition. The determination of the main chemical parameters was performed following the procedure reported by Matraxia et al. (2021). The sensory analysis of the fruit beers produced was conducted in the laboratory of sensory analysis of the SAAF Department. Experimental design: TF1, inoculated with *S. cerevisiae* MN113; TF2, inoculated with commercial *S. cerevisiae* US-05, used as a control; TF3, inoculated with *L. thermotolerans* MNF105, TF4, inoculated with commercial *L. thermotolerans* Philly sour, used as a control.

3. Results

3.1 Determination of microbiological and the main physico-chemical parameters

The initial must had a pH value of 5.25 and 12 °Bx, whereas the loquat juice had a pH of 3.65 and 10.9 °Bx. The pH values recorded at the end of AF were between 3.78 and 3.44 for TF3 and TF4 trials, highlighting the ability of *Lachancea* to acidify the must. As a result, *L. thermotolerans* MNF105 yeast strain has the ability of low lactic acid production and a marginal influence on the decrease of pH compared to the commercial strain (0.52 g/L and 2.25 g/L respectively). The evolution of yeast populations during the AF is reported in Fig. 1. After inoculation,

yeast cell densities ranged between 6.20 and 6.95 Log CFU/mL. The persistence of the strains inoculated was phenotypically investigated by means of colony shape and cellular morphology to recognize typical members of *Lachancea* and *Saccharomyces* genera. Starter yeasts increased of about 0.5 Log cycles after 3 d for all trials and these results follow the general dynamics of yeast growth in fermenting must-beer. After day 3, trials TF3 and TF4 showed a decrease of presumptive *Lachancea* spp. populations. After loquat juice addition (day 11 of fermentation), the yeast populations levels increased for all trials. At the end of the AF, the highest cell counts were registered for *S. cerevisiae* MN113 in trial TF1 (6.80 Log CFU/mL). Instead, *L. thermotolerans* MNF105 in trial TF3 (6.05 Log CFU/mL) showed higher values respect the control (5.80 Log CFU/mL).

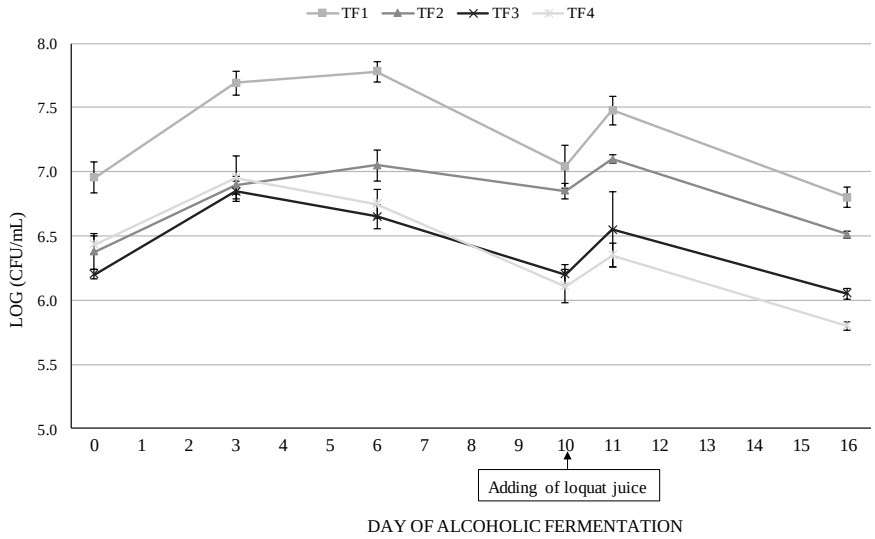

Figure 1. *Monitoring of yeast concentrations during alcoholic fermentation*

3.2 Sensory analysis

All panelists recognised the loquat aroma. The overall sensory investigation revealed a preference for the TF1 trial, showing good complexity and pronounced spicy notes and a greater ability to bring out loquat aromas. Instead, the TF3 test showed a greater preference than the control for spicy, sour and sweet notes, improving the mouthfeel of the produced beers. These results show that novel yeast strains from unconventional matrix, both *Saccharomyces* and non-*Saccharomyces*, can increase flavour complexity, in agreement with some studies. The application of these strains during brewing showed differences, indicating that *L. thermotolerans* MNF105 and *S. cerevisiae* MN113 strains are promising as starter in a wide range of beer productions. Thus, the results obtained agree with those expected and the original PhD thesis project can proceed with further experimental trials to evaluate the role of fruit and these strains during wort fermentation, especially with other types of wort

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Prolonged leavening to mitigate acrylamide formation in Neapolitan pizza

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The PhD thesis project aims to evaluate methods for acrylamide reduction in bakery products. To this aim, the evaluation of an extraction procedure and a HPLC-UV analytical method for acrylamide determination were firstly carried out; then, after the assessment of acrylamide content of Neapolitan pizza, the utilization of lactic acid bacteria and extended rising times as a possible mitigation strategy for acrylamide mitigation were checked.

Lievitazioni prolungate come strategia di mitigazione dell'acrilammide in Pizza Napoletana

Il progetto di ricerca di dottorato ha come obiettivo la valutazione di metodi per la riduzione dell'acrilammide nei prodotti da forno. In particolare, sono stati dapprima validati un metodo di estrazione e quindi di quantificazione dell'acrilammide mediate HPLC-UV; a seguire, la procedura analitica ottimizzata è stata utilizzata per la valutazione del contenuto di acrilammide in pizza Napoletana. L'utilizzo di batteri lattici e tempi di lievitazione prolungati sono stati valutati come possibile strategia di mitigazione della formazione di acrilammide.

Key words: acrylamide, Neapolitan pizza, lactic acid bacteria

1. Introduction

In accordance with the PhD thesis project previously described (Quiquero, 2021), this poster reports the main results of the activities concerning:
A2 (1) the development of an extraction procedure and a HPLC-UV method for acrylamide analysis.
A2 (2) the assessment of acrylamide content in Neapolitan pizza cooked in wood oven.
A3 (1) the evaluation of a method for acrylamide mitigation based on prolonged leavening and the use of lactic acid bacteria to control the acrylamide content in the Neapolitan pizza.

2. Materials and methods

2.1 Sample

Two pizza typologies (with a topping of tomato sauce and without any topping) were prepared following the Neapolitan recipe prescribed in the TSG disciplinary. Later, samples of pizza without any topping have been produced from dough left to rise for 16, 24 and 48 hours using a starter of lactic acid bacteria (*Levilactobacillus brevis* PA6, *Leuconostoc pseudomesenteroides* PD4, *Fructilactobacillus sanfranciscensis* SB52) combined with traditional yeasts for the evaluation of their possible effect on acrylamide formation. Pizza samples were lyophilized with Virtis Genesis 25ES freeze drying apparatus and ground with a refrigerated mill. For each sample, acrylamide was determined in external, internal part and slice. Samples extraction consisted in addition of water for acrylamide solubilization, stirring, centrifugation and purification with Oasis HLB SPE cartridges (6 ml, 200 mg) from Waters (Milford, USA). The samples were collected with 2 mL of water and analyzed with HPLC-UV.

2.2 Chromatographic analysis

For HPLC-UV acrylamide analysis, a standard stock solution (1000 mg L^{-1}) was prepared in H_2O:CH_3CN (94/6) solution. A calibration curve was generated using opportune dilution of the stock, allowing acrylamide quantification in the sample to be analyzed. The detection and quantification of acrylamide was performed with a HPLC-UV model Ultimate3000 of Dionex Corporation (Sunnyvale, CA, USA) equipped with an UV-VIS detector. The separation was obtained with a column IonPac ICE AS 1 Ion Exclusion 9 x 250 mm (Dionex Corporation) at 202 nm, using as mobile phase water/acetonitrile (v/v) H2O:CH_3CN (94:6) at the flow rate of 1,0 mL/min. The total run time was 30 min and the acrylamide peak appeared at 18 minutes. Chromatograms were elaborated with Chromeleon 6.6 (Dionex) data system.

2.3 Statystical analysis

All numerical data are averages of at least three independent replicates and values are represented as the mean ± standard deviation. Comparison of acrylamide content in the different samples was analyzed through the Unpaired t test (Welch's correction).

3. Results

3.1 Evaluation of HPLC analytical method performances

In order to define system performances, detection limit (LOD) and quantification limit (LOQ) using different concentrations of acrylamide standard solution were assessed. The calibration curves showed high linearity in the range of 0,0025-10 mg/L with a R^2=0,9999. The LOD was 0,00125 mg/L, and the LOQ 0,0025 mg/L.

3.2 Recovery test

Recovery tests on OASIS HLB SPE cartridges have been carried out using acrylamide standard solution and the extract from the samples. This test revealed that the optimal loading volume was 0.5 ml for the standard solution, and 2 mL for the samples' extracts. To amplify the signal and thus improve the sensitivity of the method, samples eluted from the cartridges with 2 mL of water were concentrated through a miVac Modular Concentrator Series and then re-suspended in 1 ml and 0.5 ml of water. The test showed a signal improvement of 2 and 4 times for the sample re-suspended in 1 e 0.5 mL, respectively.

3.3 Acrylamide content in different pizza typologies

Acrylamide content of different pizza typologies are reported in figure 1.

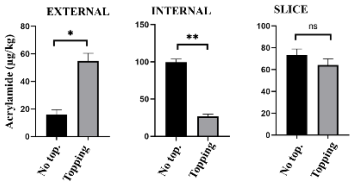

Fig.1 *Acrylamide content of different sections of pizza without topping and pizza with topping of tomato sauce (Unpaired t test (Welch's correction * p<0.05; **p<0.01).*

The results highlighted differences between the two pizza typologies: in the external part, pizza with a topping of tomato sauce had significant higher ($p<0.05$) amount of acrylamide; conversely, in the internal part it was pizza without any topping that had a significantly higher content of acrylamide ($p<0.01$). These different results might be due to the thickness of the product, which it is higher in the external part and it determines the degree of drying during cooking and hence the rate of acrylamide formation (Abt et al., 2019; Acar e Gokmen., 2009). Moreover, also tomato sauce could have an influence on the moisture of pizza, thus on the latency time necessary for the product to reach the oven temperature, delaying the Maillard reaction responsible for acrylamide formation. There is also to consider the possible effect determined by the topping related to pH lowering and the possible presence of antioxidant component that could affect acrylamide formation.

3.4 Prolonged leavening as a mitigation strategy for acrylamide reduction

As shown in Figure 2, a decrease in acrylamide formation was recorded as the leavening time increased, probably due to the consumption of acrylamide precursors (asparagine, reducing sugars) by the microorganisms' activity. Leavening carried out with both yeasts and lactobacilli contributes to decrease acrylamide content, which is most pronounced after 48 hours.

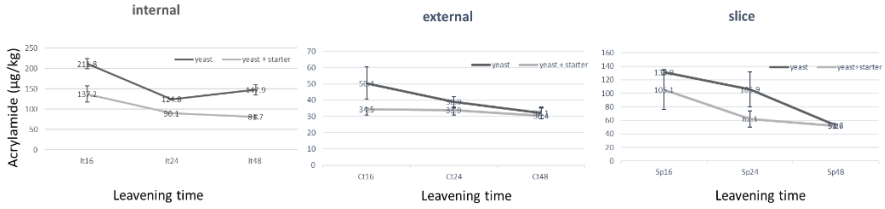

Fig. 2 *Acrylamide content in different sections of pizza after 16, 24 and 48 leavening hours.*

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Individual variation in food perception and implication in consumer preference of sustainable products

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This report presents the main activities carried out in the first two years of the PhD programme. The aim of the research is to study the determinants of liking of novel plant-based food formulations, exploring individual variation in perception and preference to foster the transition to a healthy and sustainable diet. Sensory data are combined with technological and instrumental ones to develop innovative improved foods.

Variazione individuale nella percezione del cibo e implicazioni sulla preferenza del consumatore per prodotti sostenibili

Questo lavoro presenta le principali attività condotte nei primi due anni di dottorato. La ricerca si focalizza sullo studio delle determinanti del gradimento di formulazioni alimentari a base vegetale, esplorando la variazione individuale nella percezione e preferenza del consumatore per favorire la transizione verso una dieta sana e sostenibile. I dati sensoriali sono associati a dati tecnologici e strumentali per sviluppare alimenti innovativi migliorati dal punto di vista nutrizionale e sensoriale.

1. Introduction

In accordance with the PhD thesis project previously described (Rabitti, 2021), this poster reports the main results of the first two activities, both part of the MIND FoodS Hub project (Lombardy Region): A1) Study of the drivers of liking and rejection of food formulations based on common and tartary buckwheat, a pseudocereal showing environmental (contributes to increased biodiversity) and nutritional (high levels of quercitin) benefits but reduced liking due to its bitter and astringent properties that may limit its consumption; A.2) Study of health and sustainable information on consumers liking and expectation for food formulations enriched with pulses (red lentils and chickpeas). Pulses are interesting from an environmental (require less water, land and energy for their production) and nutritional (rich in high nutritional value proteins) point of view but, to foster their consumption, consumer perception of new products should be explored.

2. Materials and Methods

A.1) Six gluten-free samples of a corn-based formulation were produced, enriched by the addition of 20%,30% and 40% of common buckwheat (CB) or tartary buckwheat (TB) flour (Raetia Biodiversità Alpine, Teglio, Sondrio, Italy). The overall liking and appropriateness of the sensory characteristics was assessed to explore the drivers of liking and rejection. Sensory data were related to various instrumental analyses: electronic tongue, colorimeter CIELAB and Texture Analyzer.
A.2) Whole-corn flour (Molino Filippini S.r.l., Teglio, Sondrio, Italy) was used to prepare one control sample (100% whole corn) and two experimental samples replaced with 20% pulse flour (either chickpea or red lentil). Samples were assessed for overall liking in blind (tasting without information), expected (only information without tasting) and informed conditions (tasting with information) to study the effect of information on consumer expectations. To explore the effect of information on consumer clusters, questionnaires on food neophobia (fear to try new foods), sustainable behaviour and health and taste attitudes were provided.

3. Results and Discussion

A.1) Samples were well accepted (Fig.1) and significantly different in terms of liking ($F_{5,694}$=6.40, $p<0.0001$). TB20 sample was the most liked and comparable to all other samples except TB40. The addition of increasing concentrations of tartary buckwheat produced a significant decrease in samples liking (Fig. 1) and an increase in the intensity of colour, overall flavour, bitterness and texture that were identified as drivers of disliking according to PLSR analysis (Fig.2). This confirms that polyphenols present in buckwheat may impart astringency and bitter taste (Soares et al., 2013) as well as influence colour (Khoo et al., 2017). Sensory data were supported by instrumental analyses (Fig. 3), whereby electronic tongue and CIELAB analyses revealed a higher intensity of astringency and a darker colour for samples with high percentages of common buckwheat (CB40) and tartary buckwheat (TB30; TB40). These results showed that by keeping tartary buckwheat concentration <40%, new sustainable and accepted products can be developed. Moreover, cluster analyses conducted on overall liking (results not shown), identified a non-negligible percentage of consumers (30%) who prefer the samples with the

highest tartary buckwheat additions, suggesting that there is a possible target of consumers willing to accept these products.

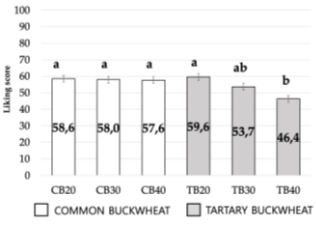

Figure 1. *Mean liking scores for Common Buckwheat (CB) and Tartary Buckwheat (TB) samples. Different letters indicate significant differences (p < 0.0001).*

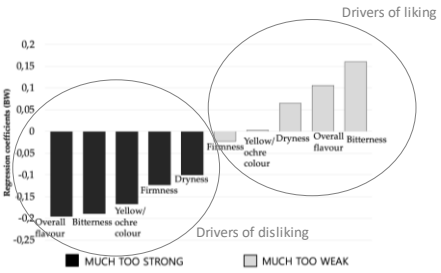

Figure 2. *Regression coefficients obtained by the PLSR model of the six samples (CB20, CB30, CB40, TB20, TB30, TB40). (CB= Common buckwheat; TB=Tartary buckwheat).*

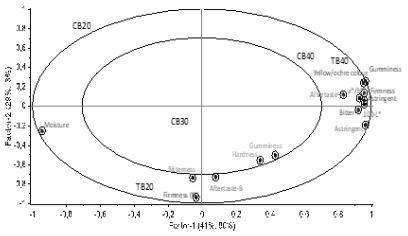

Figure 3. *Correlation plot obtained by the PLSR model of 6 samples (CB20, CB30, CB40, TB20, TB30, TB40) considering instrumental data and technological variables as independent variables and sensory profile data as dependent variables.*

A.2) One-way ANOVA showed that samples within each condition were comparable (results not shown), indicating no effect of type of flour on liking. Moreover, samples with addition of pulses produced a negative disconfirmation of expectation (the product is less liked that expected, E>B), which was associated with an incomplete assimilation effect (Tab.1). In other words, when consumers taste the products in informed condition, they increase the hedonic score (assimilation, I>B) but the effect of information does not compensate for sensory perception (incomplete effect, I<E). These results are consistent with previous research showing that information about health (Saba et al., 2010) and sustainability (Laureati et al., 2013) lead to increased consumer expectations and liking.

Table 1. *Mean hedonic scores provided by consumers (n=127) under the 3 experimental conditions (B=blind; E=expected; I=informed) and expectation effect on samples acceptability (ns, not significant; *, p<0.05; **, p<0.01; ***, p<0.001 based on paired t-tests). Disconf. = negative disconfirmation, Assim. = assimilation.*

Samples	Scores			E-B		I-B		I-E	
	B	E	I	Mean	p-value	Mean	p-value	Mean	p-value
CONTROL	62,7	76,2	65,0	13,5	*** (Disconf.)	2,3	n.s.		
20% CHICKPEAS	60,2	73,2	67,3	13,0	*** (Disconf.)	7,1	** (Assim.)	-5,9	* (Incomplete)
20% RED LENTILS	58,9	72,2	65,4	13,3	*** (Disconf.)	6,5	** (Assim.)	-6,8	** (Incomplete)

The health and sustainable information affected differently consumers hedonic responses according to their Food Neophobia level (results not reported). A negative disconfirmation of expectation was found only for Neophilics. This disconfirmation was associated with a complete assimilation effect for the sample with 20% of red lentils, indicating that the information was effective only for this type of pulse. No significant differences between blind and expected conditions were found for the Neophobic group showing no health and sustainable information effect at all. This finding is coherent with the fact that neophobic subjects are resistant to tasting new foods (Pliner & Hobden,1992) and might be less interested in new and healthy products even if accompanied by encouraging information. Moreover, these results show that for consumers more inclined to try new foods (Neophilics), promoting the benefits of pulses in new food formulations may be a strategy to encourage their consumption.

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Novel insights on the functional/nutritional features of the foods based on cereals, pseudocereals and legumes

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Below, are describer two activities carried out during the second PhD year. Firstly, based on the results obtained during the last year, which showed microbial diversity in sprouted grains compared to un-sprouted ones, the research carried out focused on the microbiological, biochemical and nutritional analysis of sourdoughs obtained by sprouted wheat and lentil grains. The second activity was aimed to study the nutritional and functional characteristics of unconventional gluten-free (GF) pasta.

Nuovi approfondimenti sulle caratteristiche funzionali/nutrizionali degli alimenti a base di cereali, pseudocereali e legumi

Di seguito, sono descritte due attività svolte durante il secondo anno di dottorato. In primo luogo, sulla base dei risultati dello scorso anno, i quali hanno evidenziato diversità microbica in granelle germinate rispetto alle non germinate, la ricerca svolta si è concentrata sull'analisi microbiologica, biochimica e nutrizionale delle paste acide ottenute da granelle germinate di frumento e lenticchia. La seconda attività di ricerca è stata finalizzata allo studio delle caratteristiche nutrizionali e funzionali di pasta gluten-free ottenuta da materie prime non convenzionali.

Key words: fermentation, germinations, pseudocereals, legumes, sourdough

1. Introduction

Cereals, pseudocereals, legumes and related foods are an important source of energy, as well as a range of non-nutrient bioactive components that provide health benefits. The main challenges for the near future include the exploration of non-conventional matrices and the implementation of processing and biotechnological strategies (such as germination and fermentation) finalized to improve their functional and nutritional properties. In accordance with the PhD thesis project, this poster reports the main results of the first two activities concerning:

(A1) evaluation of microbiological, biochemical and nutritional properties during the preparation and propagation of firm and liquid sourdoughs obtained by traditional fermentation (type I sourdough) and backslopped over 10 days with sprouted and non-sprouted flours in order to assess its potential use in bread making;

(A2) nutritional and functional evaluation of gluten-free pasta obtained from non-conventional matrices.

2. Materials and Methods

2.1 Set-up and characterization of the sourdough from sprouted and non-sprouted grains

Preparation of dough and propagation of sourdough was performed by traditional protocol without the addition of starter cultures or baker's yeast. Spontaneous wheat and lentil sourdough fermentations were carried out through backslopping, both in firm and liquid condition. Flours tested: (i) soft wheat flour refined (type 00); (ii) whole wheat flour; (iii) wholemeal flour obtained from germinated soft wheat; (iv) wholemeal lentil flour; (v) wholemeal flour obtained from germinated lentils; and (vi) mixture of wholemeal flours obtained from germinated wheat and lentils. Prior to fermentation, after the first 24 h of fermentation, and after each refreshment step, samples were taken from the ripe sourdoughs to monitor the diversity of prokaryotic microbiota through culture-independent and -dependent methods, and the biochemical and nutritional features. Culture dependent microbiological characterization by using plate count and catabolic profiles at community level (CLCP) by using OmniLog MicroStation were studied in dough before fermentation (D0) after the first fermentation of 24h (D24) and after 3, 6, and 10 refreshments. For metagenomic analysis 16S rRNA gene amplicons of the bacterial communities of doughs and sourdoughs were sequenced by using Illumina Miseq. Organic acids, total peptides, total free amino acids (FAA), antioxidant activity, total phenols and phytic acid were evaluated at begin and at the end of propagation.

2.2 Nutritional and functional evaluation of gluten-free pasta

The following types of pasta were selected: red lentil pasta, buckwheat pasta, mung bean pasta, rice/corn pasta, durum wheat pasta as a control. Nutritional and functional characteristics were evaluated through *in vitro* and *in vivo* analysis. In details, the *in vitro* analysis concerned: hydrolysis index (De Angelis *et al.*, 2007), predicted glycemic index (pGI), in vitro protein digestibility (IVDP) as previously described (Rizzello *et al.*, 2014**)**, anti-

nutritional factor (phytic acid, raffinose, saponins, tannins, trypsin inhibitors TIA).

3. Results and Discussion

3.1 Microbial ecology dynamics in spontaneous sourdoughs made from native and sprouted wheat and lentil flour

Culture-dependent approaches showed that dough day 0 was characterized for a low cell density of LAB (approx. 3.5 log CFU / g) and yeasts (approx. 2 log CFU / g) (Perri *et al.*, 2020). After six days of propagation, LAB dominated the sourdough, reaching a cell density of ca. 8.5 log CFU /g and remained at this level until the end of the 10-days propagation, indicating a stable bacterial community, as commonly observed for cereal sourdoughs (Arora *et al.*, 2022). The yeast initial cell density remained constant until day 3, but thereafter it increased to 5.0. As shown by plate count, after the sixth backslopping, Enterobacteriaceae decreased and were no longer detected on day 10 of sourdough propagation. Cell numbers of presumptive lactic acid bacteria moderately fluctuated in firm sourdoughs. Firm sourdoughs had slightly higher pH values than the liquid sourdoughs. Although there were slight variations between flours, sourdoughs achieved maturity during six days of propagation. The trophic relationships between lactic acid bacteria and yeasts during sourdough fermentation diversify the utilization of available carbon sources, as shown by the community level catabolic profiles microbial. All 5 chemical classes (amino acids, carbohydrates, carboxylic acids, amines, and polymers) were variously degraded during manufacture and propagation of dough and sourdoughs. Compared to doughs from non-sprouted flours, the highest ability to consume carbohydrates, polymers, amines, carboxylic acid and amino acids sources was found for the microbial communities of sprouted sourdoughs. The highest bacterial diversity estimated by Shannon index was found for D0 and sourdoughs after 24h of fermentation, in particular in sprouted samples. Proteobacteria, Cyanobacteria, and Firmicutes were mainly found in the doughs and showed differences in abundance between firm and liquid propagation condition. Just one fermentation was needed to completely turn the microbial diversity from mainly Proteobacteria to Firmicutes. Sprouting of flour enhanced and modified microbial diversity of sourdough ecosystem. Most of the OTUs classified at genus level were similarly between non sprouted and sprouted samples and firm and liquid sourdough propagation. After the first fermentation, the bacterial profile markedly changed, the microbial complexity simplified and the sourdoughs become more similar to each other, based on the presence and abundance of lactic acid bacteria driving the fermentation (*Weissella*, *Leuconostoc* and *Pediococcus*). An increase of total phenols and antioxidant activity was detected during propagations, with the highest values in the mature sourdoughs. Fermentation with LAB has an efficient method for reducing the presence of antinutritional factors (Perri *et al.*, 2021).

3.2 Nutritional e functional evaluation of gluten-free pasta obtained from unconventional matrices

In vitro analysis: all types of pasta have a lower % HI and pGI compared to wheat pasta. Pasta based on legumes and buckwheat have IVPD% values similar to each other and lower than those of rice/corn pasta. Higher resistant starch values were measured in legume-based pasta. As for the anti-nutritional factors, the types of pasta based on legumes and buckwheat have a significantly higher content of phytic acid and saponins, a high content of raffinose was found in pasta based on legumes while a high content of TIA and tannins was found in pasta based on buckwheat. As for the antioxidant activity, buckwheat-based pasta followed by legume-based pasta has the highest values compared to rice/corn pasta. Scientific evidence shows that these anti-nutritional factors can be reduced by the cooking process of the pasta.

In vivo analysis: Pasta having the high nutritional value, were taken by 40 healthy volunteers for 30 days. A good general approval rating was found for legume-based pasta (red lentils and green beans). Patients suffering from stuffiness and epigastric pain appear able to consume buckwheat or rice/corn pasta. Patients with alterations in bowel habits should prefer buckwheat, rice/corn and beans pasta. The glycemic index tends to be better in lentil / bean varieties than in buckwheat / corn. However, gut microbiota analyzes are still ongoing.

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Development of palm olein and high oleic sunflower seed oil-based oleogels and their physical characterization

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The aim of the present study was to investigate the physical behavior of palm olein (PO) or high oleic sunflower oil (HOSO) based oleogels containing different additives. The full characterization of acylglycerols profile was carried out. The physical behavior of oleogels was investigated by Differential Scanning Calorimetry (DSC) and fat solids content (SFC) evaluated by magnetic nuclear resonance (NMR). The better gelling proprieties were obtained using sterols, ethyl cellulose and fatty acids esters (1-2%). The use of waxes shown more criticality, good structuring agents (1%) but in larger amount led to a completely hard product (2-5%).

Sviluppo e caratterizzazione di oleogels a base di oleina di palma e olio di semi di girasole alto oleico

Lo scopo di questo studio è stato quello di indagare il comportamento fisico di oleogel a base di oleina di palma (PO) o olio di girasole alto oleico (HOSO) contenenti diversi additivi. È stata effettuata una caratterizzazione completa del profilo gliceridico, mentre il comportamento fisico degli oleogel è stato studiato mediante calorimetria a scansione differenziale (DSC) e il contenuto dei grassi solidi (SFC) mediante risonanza magnetica nucleare (NMR). Le migliori proprietà gelificanti sono state mostrate dagli steroli, dall'etil-cellulosa e dagli esteri di acidi grassi (1-2%); invece, l'impiego di cere è risultato più critico, poiché hanno dimostrato ottime *performance* come agenti strutturanti (1%), ma in elevate quantità (2-5%) hanno portato ad una eccessiva rigidità ai prodotti.

Key words: Oleogel, oleogelator, wax, palm olein, sunflower oil, low-calorie fats.

1. Introduction

In accordance with the main objective of the doctoral research project presented at the "25th Workshop on Developments in Italian PhD Research on Food Technology and Biotechnology" the present poster reports an excerpt of the advancements of research project in accordance with the following activities of the doctoral project:

A. Application of chromatographic technique to study the acylglycerols profile and enforce the study of physical behavior with a focus on crystallization and melting behavior through DSC and solid fat content (SFC) analysis.
B. Collection data related to fats raw materials and novel blend with different type of compound to understand the importance of modified fatty substances in processed food.

About the study carried out over the common "fats modification techniques", this research work reports a study on the physical behaviour of two oils blended with different kind of oleogelators (such as wax). Oleogels used in food are usually composed of a vegetable oil and a GRAS (Generally Recognized as Safe) additive or a combination of several of them. With their particular melting behaviour, oleogels can provide a suitable alternative to fully hydrogenated fats, margarines or cow's butter, in food production. There are a lot of works in literature where oleogels are used in different kind of finished products (such as meat products, bakery and confectionary products) (Kim et al., 2017; Yilmaz & Öٖütcü, 2015; da Silva et al., 2018). Many authors use to divide oleogelators into two broad categories: "low molecular weight oleogelators" (LMOGs), small molecules such as fatty acids, fatty alcohols, waxes, wax esters and phytosterols and "high molecular weight oleogelators" (HMOGs) (Davidovich-Pinhas, 2019; Marangoni et al., 2007), obtained by molecular network of polymers formed by chemical reaction or physical interaction (Palla, et al., 2017).

2. Materials and Methods

Two fatty substances were collected: refined palm olein - PO *(Elaeis Guineensis* L.*)* and a refined high oleic sunflower seed oil – HOSO *(Helianthus annuus* L.). The quality control of raw materials was carried out by characterization of the acylglycerol fractions by Shimadzu GC 2030 coupled with flame ionization detector (FID). Triglyceride profile analysis was performed as described by AOCS method *Ce – 5-86*; while the fatty acid methyl esters (FAMEs) analysis was performed as described in AOCS method *Ce 1a – 13*. LMOGs and HMOGs were collected from different suppliers (Table 1). Oleogelators were added at different concentration levels into fats substances and mixed with different temperature handling ranged between 38 °C (fatty acids esters) and 150° (ethyl cellulose), related to the melting point of each oleogelator. Differential Scanning Calorimetry (DSC)

technique was performed with DSC 3+ Metter Toledo and the evaluation of the Solid Fat Content (SFC) by a benchtop magnetic nuclear resonance (NMR) system – Bruker Minispec mq20 in a T° range 0-40 °C.

Table 1 *shows the commercial name, the type of compound, GRAS qualified and the ID code of the oleogelator agents considered in this study.*

Commercial Name	Type	Categories	GRASS	ID code
12- Hydroxy-stearic acid (HSA)	Fatty acids esters	LMOGs	NO	n.a.
Grinsted 110	Mono & Di - Glycerides	LMOGs	YES	E471
Grinsted 209	Mono & Di - Glycerides	LMOGs	YES	E471
Propymulus SPV	Fatty acids esters	LMOGs	YES	E477
Soy Lecithin	Phospholipids	LMOGs	YES	E322
Grinsted STD 30	Sorbitan esters	LMOGs	YES	E492
Grinsted SMS	Sorbitan esters	LMOGs	YES	E491
ETOCEL STD 20	Ethyl Cellulose	HMOGs	YES	E462
ETOCETL STD 45	Ethyl Cellulose	HMOGs	YES	E462
ETOCEL STD 100	Ethyl Cellulose	HMOGs	YES	E462
β Sitosterol	Sterol	LMOGs	YES	n.a.
γ Oryzanol	Sterols esters	LMOGs	YES	n.a.
Rice wax	Wax	LMOGs	NO	n.a.
Carnauba wax	Wax	LMOGs	YES	E903
Candelilla wax	Wax	LMOGs	YES	E902
Sunflower wax	Wax	LMOGs	NO	n.a.
Bee wax	Wax	LMOGs	YES	E901

3. Results and Discussion

The study of fatty substance was necessary in order to determine the glyceride fraction (>99.8%) as related to the physical behaviour of fats. Table 2 and table 3 report some importance evidence: HOSO and PO have a high content of unsaturated fatty acids and unsaturated triglycerides; they are liquids and own a melting point below room temperature.

Table 2. *Triglycerides fraction and fatty acid methyl esters of the Palm Olein. The melting point of the constituents are reported.*

PO	Compound	%		Melting point (°C)
FAME Profile	C16:0	36.2	±2.3	62.9
	C18:0	4.6	±1.1	16.3
	C18:1	44.8	±1.2	-5.0
Triglycerides profile	POP	18.2	±1.8	35.2
	POO	30.1	±2.1	22.0

Table 3. *Triglycerides fraction and fatty acid methyl esters of the Hight Oleic Sunflower seed Oil. The melting point of the constituents are reported*

P = Palmitic acid, S = stearic acid

HOSO	Compound	%		Melting point (°C)
FAME Profile	C16:0	9.3	±2.3	62.9
	C18:0	76.8	±1.1	16.3
	C18:1	7.4	±1.2	-5.0
Triglycerides profile	POO	10.1	±1.8	22.0
	SOO	6.8	±2.1	25.2
	OOO + SLO	60.5	±1.6	5.5

Oleogelators allow the formation of an assembled gel network structure and can form different shapes through noncovalent bonds, including hydrogen bonding, van der Waals interactions, and π-π stacking. Through the study of the physical behaviour, four typical cases were identified: non-gelling agent, such as soy lecithin and sorbitan mono and tri stearate; gelling agent**,** such as HSA (2-5%) and β-sitosterol and γ-oryzanol (5 %) blended; structuring agents, such as mono and diacylglycerols group; and solidifying agent such as waxes (3-5%). Based on the evaluation of DSC and SFC the HSA displayed the most attractive and interesting gelling properties, even at low concentrations, by impacting the crystallization and melting profile of a liquid fatty substance such as HOSO (Table 4). In the future, a deeper study using polarized light microscope and phase-contrast microscope would allow to better understand the interaction and the shapes between fatty substance and additives (crystal network).

Table 4. *shown an excerpt over the study of the influence of HSO mixed in HOSO on physical behavior*

	Crystallization behaviour		Melting behaviour		SFC (20°C)	
	Peak Max. (C°)	Hating (J/g)	Peak Max. (C°)	Hating (J/g)		
HOSO	-36.60	-42.545	-3.759	-81.588	0	
HSA	68.21	-164.862	80.235	-157.677	n.d.	
HOSO + HAS (1.5%)	-31.44	61.872	-2.779	-73.337	1.03	± 0.12
HOSO + HAS (2.5%)	-30.32	72.058	-2.791	-77.329	1.85	± 0.18

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Antimicrobial, anti-quorum sensing, and anti-virulence potentials of pomegranate (*Punica granatum* L.) peel extract against foodborne pathogenic bacteria

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The aims of this Ph.D. research project were to investigate the antimicrobial and antibiofilm capacity of the pomegranate peel (PPE) extracts on several foodborne pathogenic bacteria, together with fractionating the crude extracts and testing the anti-quorum sensing activity, based on this HPLC-MS is conducted to identify the chemical composition of the fractions with the highest antimicrobial activity. Finally, bacterial RNA extraction and gene expression analysis will be done where an evaluation of the PPE abilities to down-regulate the expression of several genes which are involved in virulence and biofilm formation of these bacteria species will be conducted.

Potenziali antimicrobici, anti-quorum sensing e antivirulenza dell'estratto di buccia di melograno (*Punica granatum* L.) contro i batteri patogeni di origine alimentare

Gli obiettivi di questo dottorato di ricerca progetto di ricerca mirava a studiare la capacità antimicrobica e antibiofilm degli estratti di buccia di melograno (PPE) su diversi batteri patogeni di origine alimentare, insieme al frazionamento degli estratti grezzi e al test dell'attività di rilevamento dell'anti-quorum, sulla base di questo HPLC-MS è condotto per identificare il composizione chimica delle frazioni a più alta attività antimicrobica. Infine, verrà effettuata l'estrazione dell'RNA batterico e l'analisi dell'espressione genica in cui sarà condotta una valutazione delle capacità dei PPE di sottoregolare l'espressione di diversi geni coinvolti nella virulenza e nella formazione di biofilm di queste specie batteriche.

Key words: Punica granatum, agrobiodiversity, OPLS-DA, punicalagin, *Staphyloccocus aureus*, LAB

1. Introduction

In accordance with the Ph.D. thesis project previously described (Amira, 2021), this poster reports the main results of the conducted activities concerning:

A1. Extraction of PPEs from pomegranate fruit peels
A2. Determination of the antimicrobial activity of the PPEs
A3. Phytochemical composition analysis of the PPEs bioactive compounds and antioxidant efficiency

2. Materials and Methods

Aqueous extraction was conducted for 7 pomegranate variates using two levels of temperature (20 and 40), PPE total phenolic content was assessed by Folin-Ciocalteu assay, and total flavonoids (TotF) were quantified by colorimetric assay according to the AlCl3 method and analysis of condensed tannins was carried out by vanillin assay (Deiana et al., 2019; Re et al., 2019). The antioxidant capacity of pomegranate extract was evaluated using both the DPPH (1,1-diphenyl-2- picrylhydrazyl) and ABTS (2,2-azinobis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt) methodologies. Finally, reverse-phase HPLC analysis of phenolic compounds was performed, and Chromatographic separation was achieved. The antimicrobial activities of PPE were quantitatively evaluated in vitro by measuring the minimum inhibitory concentration (MIC), and the quantitative analysis of the ability of pathogenic bacteria strains to form biofilms was evaluated by crystal violet (CV) assay (Xu et al., 2016; Petretto et al., 2018). For data analysis, orthogonal projections to latent structures discriminant analysis (OPLS-DA) was used to characterize each variety according to the chemical composition of the pomegranate peel extracts (PPE).

3. Results and Discussion

Eighteen phenolic compounds were identified by HPLC-DAD: hydrolyzable tannins, phenolic acids, chlorogenic acid, flavan-3-ols, flavonoid glycoside, and anthocyanins. The ellagitannins were the predominant components; with the two punicalagin anomeric structures, α and β, being the most abundant phenolics. The antioxidant activities of PPEs obtained at the two different extraction temperatures were generally similar. Moreover, according to the OPLS models, total flavonoids, are the chemical compounds with the highest antioxidant activity.

The antibacterial activities of the PPEs from the different cultivars were assessed by determining the MIC for each bacteria strain tested. Most of the PPEs showed some effectiveness at suppressing microbial growth, with a MIC ranging from 3 to 0.09 mg mL^{-1}, and Gram-negative bacteria were notably more resistant than Gram-positive bacteria. *Staphylococcus aureus* and *Listeria monocytogenes* showed high susceptibility to nearly all the PPEs tested. In contrast, *Salmonella bongori* showed resistance to all the PPEs tested, whereas the *Escherichia coli* strains showed resistance against most of the PPEs. PLS-DA analysis enabled us to identify the bioactive compounds contributing the most to the antimicrobial activities of PPE. It is worth noting that the two ellagitannin isomers exhibited good activity against all the bacterial species tested. Finally, high values of total tannins and total phenols were positively correlated with the PPE antimicrobial activity against most of the strains tested. Regarding the antibiofilm activity of the bacterial strains tested, *S. aureus* and *L. monocytogenes* showed the highest biofilm formation abilities, whereas *S. bongori* and *E. coli* strains showed the least biofilm formation ability.

In agreement with Whang et al. (2011), we found extraction with water at 40 °C for 4 hours to be an efficient method for the extraction of pomegranate peel antioxidants. However, our data also show that extraction at this higher temperature was not always accompanied by a higher concentration of extracted compounds compared with extraction at room temperature (20 °C). Previous studies have attributed the main antioxidant activity of PPE to punicalagins, punicalins, and ellagic acids (Rosas-Burgos et al., 2017). In contrast, our findings attributed a negligible role to the latter two compounds, and only a secondary role to punicalagins. Moreover, our results indicate overall antioxidant activity is partially related to the presence of flavonoids epicatechin, catechim, and rutin, and principally related to the levels of total flavonoids and total phenols. Rosas-Burgos et al. (2017) suggested punicalagin and ellagic acid to be the bioactive molecules principally responsible for the antimicrobial activity of pomegranate peel. This is partially in line with the results of the current study, where the inhibitory effect was primarily correlated with punicalagin α and β, but also with other flavonoid and phenolic molecules. However, the PLS-DA models investigating the relationship between PPE composition and relative antimicrobial activity only partially describe the biological process at play. In the present study, the susceptibility of microbial biofilms to PPEs was also evaluated. The results showed that PPEs were able to inhibit biofilm development at concentrations below the MIC of the tested isolates. The antibiofilm activity of PPEs could be attributed to the presence of compounds such as punicalagin and ellagic acid, which may exert their effects through different mechanisms of action (Balaban et al., 2021a).

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Metagenomics and Big-Data approaches for monitoring food quality and safety

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The relationship between foods and their microbiome is fundamental to ensure food quality and safety. For this purpose, data on food-associated microbiota accumulated in public repositories potentially represent a useful resource to be exploited in meta-studies, to improve our knowledge of food microbial consortia involved in food spoilage and on the influence of processing parameters on their development. Furthermore, omics-based monitoring of microbial contamination can be usefully applied for epidemiological tracking of foodborne pathogens, allowing a rapid identification of food products responsible for an outbreak.

Metagenomica e Big-Data per lo sviluppo di sistemi di monitoraggio della qualità e sicurezza alimentare

La relazione tra gli alimenti e il loro microbioma è fondamentale per garantire la qualità e la sicurezza alimentare. A tal fine, i dati sul microbiota associato agli alimenti accumulati nei repository pubblici rappresentano una risorsa potenzialmente utile da sfruttare nei meta-studi, per migliorare la nostra conoscenza dei consorzi microbici alimentari coinvolti nell'alterazione degli alimenti e dell'influenza dei parametri di processo sul loro sviluppo. Inoltre, il monitoraggio della contaminazione microbica basato sulle scienze omiche può essere utilmente applicato per il tracking epidemiologico dei patogeni di origine alimentare, consentendo una rapida identificazione dei prodotti alimentari responsabili di un focolaio.

Key words: Food metagenome, Food safety, Food spoilage, Omics, Food metagenome.

1. Introduction

In accordance with the PhD thesis project, this poster reports the main results of the first two activities concerning:
(A1) Meta-analysis of publicly available sequencing data on microbiota in three different food chains (raw meat, milk and fishery products), in order to identify the most important microbial species involved in food spoilage and/or potential pathogenic microbes spread across these food chains;
(A2) Shotgun metagenomics analysis to evaluate microbial dynamics and the genomic potential of the microbiome involved in fish fillet spoilage during storage in different packaging and temperature conditions.

2. Materials and Methods

For the meta-analysis, a database reporting microbial taxonomic composition in the different food chains was developed. The database contains taxonomic composition of 981 samples retrieved from 18 different studies, divided as follows: 601 from meat chains, including 306 fresh and 145 spoiled meat (336 stored aerobically, 44 stored under-vacuum, 71 stored under *modified atmosphere, 150 swabs of meat processing environment)*; 232 from fish and seafood chain, including 152 fresh and 80 spoiled samples (126 fish fillets, 40 cooked peeled shrimps, 66 small octopus); 148 milk (131 raw milk, 15 pasteurized milk, 2 thermized milk). Data were downloaded from public repositories and associated metadata were retrieved from the original papers. In detail, NCBI/SRA-toolkit was used for the download of raw sequence data from Sequence Read Archives and the Qiime 2 pipeline based on Amplicon Sequence Variants (ASVs) was used for data analyses (https://qiime2.org).
Furthermore, we used shotgun metagenomics to understand the functional potential of the microbial community in fish fillet, identifying the presence of spoilage-associated activities or genes related to pathogenesis. Fish fillets were stored at three different temperatures (0, 4, 10°C), under three different packaging conditions (aerobic, AIR; vacuum, VP; modified atmosphere, MAP) and analysed at different time points (up to 10 days for AIR and 16 days for MAP and VP). At each sampling point, shotgun metagenomics was carried out in order to understand microbial dynamics during food spoilage.

3. Results and Discussion

3.1 The raw meat chain

Meat and meat products are highly perishable, with colonization and development of a variety of microorganisms, especially bacteria. This is due to complex nutrient-rich environment with chemical and physical conditions favorable to bacterial development (Cauchie et al., 2020). Microbial spoilage of meat is a complex process affected

by competition among different microbial groups and their biotic and abiotic interactions.
The different storage conditions, such as gaseous atmosphere, storage temperature and the application of antibacterial compounds, may have a major effect on microbial growth and dynamics of different populations (LAB, *Pseudomonas*, *Carnobacterium*). However, regardless the packaging conditions, a common spoilage-associated core microbiome can be observed. These taxa are always present in fresh and spoiled meat, as well as in meat-handling environment, highlighting that they are well-adapted and able to become resident in this type of environment and can be transferred to the meat. During storage, extrinsic factors, mainly related to temperature and type of packaging, may exert a selective pressure, boosting the development of some of them. Therefore, using HTS for microbiota mapping in meat processing and handling environments is an interesting opportunity for food industry to identify possible contamination routes.

3.2 Milk chain

Milk is a highly nutritious food that can be obtained from a variety of animal sources such as cows, goats, sheep and buffalo. However, the high nutrient content, coupled to a near neutral pH and a high-water activity, makes it the ideal environment for the growth of many microorganisms (Quigley et al., 2013). The microbiota of raw milk is highly complex, including LAB, such as *Lactobacillus*, *Streptococcus*, *Enterococcus*, *Lactococcus* and *Leuconostoc*, besides several other Gram- positive bacteria (e.g., *Bacillus*, *Macrococcus* and *Corynebacterium*). However, also Gram-negative, such as *Enterobacteriaceae* (e.g., *Enterobacter*, *Hafnia*, *Klebsiella*) and *Pseudomonas* spp. are frequently found in raw milk.
Principal Coordinate Analysis (PCoA) based on Bray-Curtis distance and box plots showing the Simpson's diversity index of analyzed milk samples revealed that raw milk contain a high biodiversity, that may be influenced by several factors, such as farm practices, animal management and breed, milking hygiene, seasonality. On the contrary, pasteurized and thermized milk samples cluster together and have a more similar microbiota, due to a standardization of the microbial composition caused by the application of the heat treatment.

3.3 Fish and seafood chain

Seafoods are among the most popular and healthiest foodstuffs worldwide, containing a variety of essential elements for human diet such as proteins, vitamins, nutrients and long-chain polyunsaturated fatty acids, including omega-3. The modern dietary trends have led to a continuously increasing demand for seafood. Both high quality and extended shelf-life of seafood is required to satisfy the nowadays dietary tendency, as well as the industrial interest to increase the added value of such products. However, microbial spoilage is the main factor linked with the rapid seafood sensorial degradation, resulting in high food losses along the production and distribution chain and thus, noteworthy economic losses for seafood producing countries. Psychrotolerant Gram-negative bacteria (e.g., *Pseudomonas*, *Psychrobacter*, *Arcobacter*, *Rubritalea*, *Shewanella* spp.) dominate chilled fish aerobically stored, while vacuum or CO_2 packing selects for *Photobacterium*, *Brochothrix* and LAB (Zotta et al., 2019). The mean relative abundances of frequently detected genera (>1% of total microbiota) in fresh and spoiled fish stored in different packaging conditions were compared. *Carnobacterium*, *Photobacterium*, *Psychrobacter*, *Leuconostoc*, *Flavobacterium*, *Lactococcus* and families such as Moraxellaceae, *Spirochaetaceae*, *Fusobacteriaceae* were confirmed as the main players in fish spoilage. In addition, microbial diversity strongly decreases during fish storage, regardless the packaging type, highlighting the selection of a well-adapted and psychrotolerant microbiome.

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Optimization of the extraction techniques using Deep Eutectic Solvents for the recovery of biomolecules from food industry by-products

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The initial activities of this PhD project have been focused on the selection and evaluation of 11 natural hydrophobic deep eutectic solvents (HDESs) to be utilised for the extraction of carotenoids from plant by-products, namely the peels of carrots, yellow and red peppers and pumpkins. For all the matrices, the extraction efficiency was assessed for each HDES. Furthermore, the solvents were characterised for their physicochemical properties in order to acquire data not available in the literature and useful for optimising the extraction conditions.

Ottimizzazione delle tecniche di estrazione mediante Deep Eutectic Solvents per il recupero di biomolecole da sottoprodotti dell'industria alimentare

Le prime attività di questo progetto di Dottorato sono state focalizzate alla selezione e la valutazione dell'uso di 11 solventi eutettici profondi idrofobici (HDESs) naturali da impiegare per l'estrazione di carotenoidi da sottoprodotti vegetali, bucce di carota, peperoni gialli e rossi e zucca. Per tutte le matrici è stata valutata l'efficienza di estrazione per ogni HDES. Inoltre, i solventi sono stati caratterizzati dal punto di vista chimico-fisico allo scopo di acquisire dati non disponibili in letteratura e utili per individuare le condizioni di estrazione più idonee.

Key words: circular economy, food by-products, green extraction, hydrophobic deep eutectic solvents, pigments

1. Introduction

As reported by several authors (Silva et al., 2019; Stupar et al., 2020; Lazzarini et al., 2022) natural HDESs show a great potential as extracting media for carotenoids recovery. The global market of these pigments is growing constantly and is expected to reach US$ 2 billion by 2031 due to the rising interest on healthy and natural food along with the increase of dietary supplement consumption. Thus, the increasing request for naturally sourced carotenoids, now representing much less than half of the total demand, is an emerging issue, taking also into account the production costs and the processes environmental sustainability. Within this frame, identification of suitable HDESs for carotenoid recovery is of the utmost interest and the research has focused on the selection and evaluation of eleven natural HDESs to assess their potential use, working on real by-products of interest for industries located in Northeast Italy.

2. Materials and Methods

HDESs were prepared according to the method proposed by Dai et al. (2014), with slight modifications. The two solid components in exact molar ratios were placed in a bottle with a stirring bar and cap and heated in a water bath at 70 °C for 30-60 min, till a clear liquid was formed. Camphor (≥96%) and thymol (≥ 98.5%) were utilized as hydrogen bond acceptors (HBAs), while lactic acid (≥90%) and decanoic acid (≥98.0%) were selected as hydrogen bond donors (HBDs); furthermore DL-menthol (≥ 98.0%) was utilized as both HBA and HBD. Acetone (≥ 99.8%) was utilised as a reference extraction solvent. All the chemicals were of analytical grade (Merck KGaA, Darmstadt, Germany). Eleven HDESs were prepared at the following molar ratios: DL-menthol/lactic acid 1:2 (HDES 1), 1:1 (HDES 2) and 8:1 (HDES 3), DL-menthol/decanoic acid 1:1 (HDES 4) and 6.5:3.5 (HDES 5), thymol/DL-menthol 1:1 (HDES 6) and 1:2 (HDES 7), thymol/decanoic acid 3:2 (HDES 8) and 1:2 (HDES 9), camphor/decanoic acid 1:2 (HDES 10) and 3:2 (HDES 11). The extraction substrates consisted of by-products (peels) deriving from the processing of fresh carrots, yellow and red peppers and pumpkins, and were gently supplied from Ortonuovo Srl (Arbizzano-Santa Maria, VR). The collected samples were cleaned, comminuted, freeze-dried and stored at -20 °C until use. For the extraction tests, 0.1 g of lyophilised sample was added to 5 mL of each HDES (sample:solvent 1:50) vortexed at 25 °C for 60 s and then kept under continuous mixing for 30 min using a disc rotator (UniLOPMIX2, LLG-Labware, Meckenheim, Germany). Afterwards, the mixture was sonicated for 60 min at 45 kHz (2200 MH S3, SOLTEC, Milan, Italy), before being centrifuged at 3900 RCF for 10 min. The amount of carotenoids recovered was spectrophotometrically assessed diluting with acetone (1:5) a given aliquot of the supernatant and taking the absorbance at 450 nm. Carotenoids were quantified as β-carotene and the extracting trials were performed in triplicate. As a reference, the extractions were carried out also with acetone, an organic solvent that finds use in the recovery of carotenoids from vegetable matrices for food purposes. The HDESs were physicochemically characterized assessing their water activity (aw), density (ρ), viscosity (ν) and ionic conductivity (γ). Aw of HDESs and their constituents was measured at 25 °C using a HygroPalm HP23-

AW (Rotronic, Bassersdorf, Switzerland), while the HDESs' density was assessed in the temperature range 20 - 80 °C using a pycnometer (BLAUBRAND density bottle, Merck KGaA, Darmstadt, Germany). A First Pro Plus viscosimeter (Lamy Rheology, Champagne-au-Mont-d'Or, France) and a COND 5 tester (XS instruments, Carpi, Italy) were utilized to measure ν and γ, both in the temperature range 20-60 °C. Viscosity was assessed at different share-rates. Statistical analysis of the data was performed using the software XLSTAT Premium (Version 2020.3.1, Addinsoft, Paris, France). Data were analyzed by one-way ANOVA and significant differences among means were computed by Tukey's HSD test (Honestly Significantly Different) at a significance level of 0.05.

3. Results and Discussion

3.1. Selection of HDESs and physicochemical characterization

The literature review along with preliminary trials have allowed the individuation of the eleven HDESs object of the investigation. An initial selection was made based on the stability of the mixtures after their preparation and storage. Actually, HDESs 3, 8 and 11 showed instability with tendency to separate at room temperature, thus generating two layers and requiring subsequent heating for their use as extraction media. Therefore, they were excluded from the subsequent investigation steps. The selected HDESs were then physicochemically characterized for density, viscosity and conductivity. Density ranged from 0.918 to 1.031 g/cm^3 at 20 °C, showing HDES 9 and HDES 2 the lowest and the highest density values. As far as viscosity, HDES 9 exhibited a very low value, below the detection limit of the instrument (5 mPa). In Figure 1, the shear stress values are reported for HDES 6 and 7 as a function of the applied shear rate. The data show that the two solvents have a shear-thickening behavior, with a consequent foreseeable negative impact on their transport properties and extraction capability. This assumption is substantiated by the carotenoids extraction yields observed working with the four different vegetable substrates. Also for conductivity, reliable data could be acquired only for HDESs 1, 2 and 3, while all the other HDESs showed a conductivity lower than the detection limit of the instrument (<0.01 μS cm^{-1}), thus indicating a very high hydrophobicity of the selected solvents. Taking into account the carotenoids extraction yields obtained using the eight selected HDESs, significant differences could be highlighted among the various solvents. ANOVA analysis of the recovery data for each different vegetable matrix, allowed selecting HDESs 6-7 for carrot and yellow pepper peels, HDES 9 for red pepper peels and HDES 2 for pumpkin peels, as shown in Figure 2. In carrot and yellow pepper peels, HDESs 6 and 7 exhibited the highest yield, being significantly different ($p<0.05$) from the other HDESs. For red pepper peels, HDESs 6, 7 and 9 allowed similar yields, higher than that obtained with HDES 2. However, HDES 9 was chosen for further investigation on its hydrophobicity and the interaction with the target moieties, being obtained using decanoic acid as HBD. The research carried out allowed acquiring data not available in the literature with reference to the selected HDESs and fundamental to design an extraction procedure for the compounds of interest. On the basis of the obtained data, the research will then focus on the extraction process optimization and eventual recovery of the carotenoids from the extraction solvent.

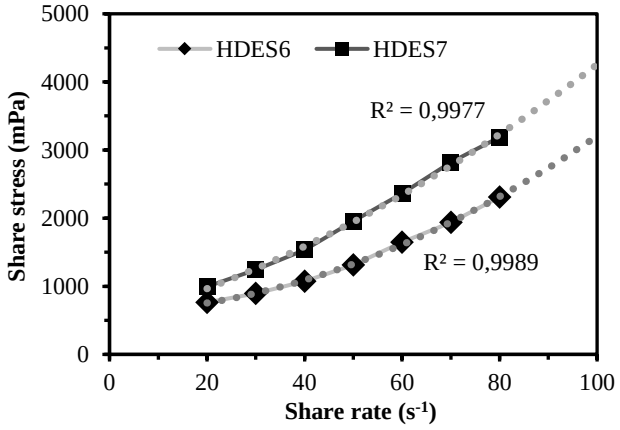

Figure 1. Plot of shear stress vs. shear rate for HDESs 6 and 7.

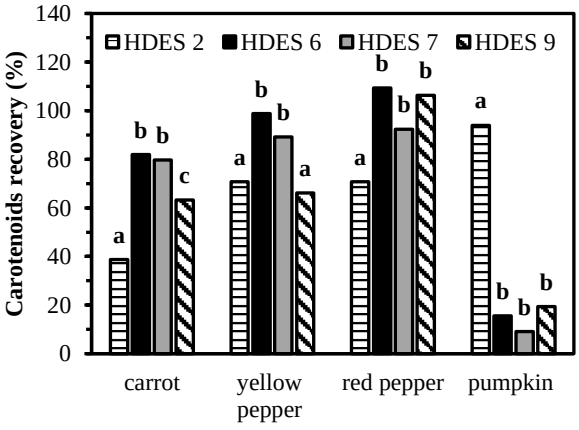

Figure 2. Carotenoids recovery from different matrices using different HDESs with reference to acetone extraction. For each matrix, values with different letters are significantly different for $p<0.05$.

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The effect of particle sizes and cell wall integrity on the cohesiveness and starch digestibility of durum wheat and rye bread

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During the first two years of the Ph.D. course, the role of the cell wall integrity on the starch digestibility and cohesiveness of durum wheat and rye bread was studied. From each cereal, three particle sizes were produced, i.e. small (<350 µm), medium (1000 µm-1800 µm), and large (> 1800 µm) flour. The starch digestibility of flour and bread was tested with the in *vitro* Englyst method (Englyst et al., 1992). The Confocal laser scanning microscopy was used to check the presence of intact cells in flour and bread. Texture analyses were conducted to evaluate the cohesiveness of the bread made with increasing flour particle sizes.

L'effetto della granulometria e integrità cellulare sulla coesività e digeribilità dell'amido in pani prodotti con grano duro e segale

Durante i primi due anni di dottorato, è stato approfondito l'effetto dell'integrità delle pareti cellulari sulla digeribilità dell'amido e la coesività nel pane di grano duro e di segale. Sono state prodotte da ogni cereale, tre farine con granulometrie differenti: piccola (<350µm), media (1000µm-1800µm), e grande (>1800µm). La digeribilità dell'amido della farina e del pane è stata testata *in vitro* seguendo il metodo Englyst (Englyst et al., 1992). Le immagini al microscopio confocale sono state utilizzate per verificare la presenza di cellule intatte nella farina e nel pane. La coesività del pane è stata valutata con il Texture Analyzer.

Keywords: cell integrity; durum wheat; rye; *in vitro* digestion; cohesiveness; confocal laser scanning microscopy.

1. Introduction

Bread is widely consumed all over the Europe as main source of energy, but, due to its porous structure and gelatinized starch, it is characterized by high glycemic index (GI). Reduction of GI in starchy foods, such as bread, is beneficial for lowering the risks for major non-communicable diseases, such as type 2 diabetes (Fardet et al., 2020). One approach studied to reduce the GI is to limit starch accessibility to α-amylase. In cereals and legumes, intact cell wall made up of indigestible polysaccharides, act as a physical barrier reducing the diffusion and access of digestive enzymes to starch substrates (Rovalino-Córdova et al., 2019). Increasing the flour particle size used for bread baking may help in lowering the starch digestibility. In accordance with the PhD thesis project previously described (Tagliasco, 2021), this poster reports the main results of the first two activities concerning (A1) the effect of increasing particle size on starch digestibility and texture of durum wheat flour and bread. (A2) the effect of increasing particle size on starch digestibility and cohesiveness of rye flour and bread.

2. Material & Methods

Durum wheat was purchased from Duru BakliyatTM (Hediklik Diş Buğdayı, Turkey) and rye grain from Tibiona (Villanova Mondovi, Italy). The grains were milled by Alpine® multi-processing system (Augsburg, Germany) to obtain three particle sizes: small < 350 µm, medium 1000 – 1800 µm, and large > 1800 µm. The bread was prepared following a standard recipe. The amount of water and the mixing time to obtain a final dough consistency of 500 Brabender Units (BU), were assessed with a water absorption test at 30 ºC and 63 rpm using a Farinograph (Brabender GmbH & Co KG, Duisburg, Germany). The digestibility of flour and bread was determined according to the *in vitro* Englyst's method (Englyst et al., 1992). The texture profile of the bread crumb was analyzed by TA-XT plus analyzer (Stable Micro Systems, Godalming, UK). The bread was squeezed twice with a 75.0 mm diameter cylinder probe P/75 and a load cell of 50 kg. Confocal laser scanning microscopy Zeiss 510 (Carl Zeiss microscopy, Oberkochen, Germany) was used to check the cell wall intactness in flour and bread. A solution containing 0.01% Calcofluor white (CFW) and 0.005% Rhodamine B (RB) was used to stain in blue the cell walls and, at the same time, the protein in red. All the analyses were repeated three times.

3. Results and Discussion

In this poster, the effect of cell wall integrity in modulating the starch digestibility of durum wheat and rye was explored. In order to confirm that the cell wall integrity was retained during all the bread processing, the confocal laser microscopy was used. The results showed that, for both durum wheat and rye, the integrity of the cell wall for the medium and large particle was kept during the whole bread processing, whereas cell walls were mostly destroyed in the small fraction samples. For what concern the starch digestibility of flour (Table 1), the rapidly

digestible starch (RDS) and the slowly digestible starch (SDS) decreased and resistant starch (RS) increased with the increase of particle sizes in both cereals. This could be mainly ascribed to the presence of a high fraction of intact cells in the medium and large flours. For what concern the durum wheat bread, these differences in RDS, SDS and RS were kept, but the magnitude of this effect was much smaller than that observed in the flour, probably due to the increased porosity of the cell wall during bread processing, as already suggested by Korompokis et al. (2021). Conversely, in rye bread, the bread made by small particle resulted the one with the lowest value of SDS and the highest of RS. As displayed in Table 1, the rye bread made with bigger particle size flours showed lower cohesiveness, since big fraction could inhibit the formation of a structured network (Lin et al., 2020). This resulted in a medium and large rye bread more susceptible to fracture or crumble. During the digestibility (data not shown), the physical breakdown of the rye bread made with medium and large particle size was higher, the surface area of the digesta was larger and this ended up in an increased accessibility of amylases. Nonetheless, bread made with small fraction maintained the structure and consequently, it exhibited lower starch digestibility. Also in durum wheat bread, increasing the particle size, the cohesiveness decreased, but the magnitude of this effect was lower due to the denser gluten network than the one formed in rye. This resulted, for durum wheat bread, in a less physical disintegration during digestion. To conclude based on our results, the main factor influenced the digestibility is the bread structure and physical breakdown during digestibility.

Table1. *Starch digestibility of flour and bread and cohesiveness of bread made with different particle sizes of durum wheat and rye.*

	RDS (g/100g starch)	SDS (g/100g starch)	RS (g/100g starch)	Cohesiviness
Small Wheat	$_{FLOUR}$ 30.5± 4.2^{a}	$_{FLOUR}$ 63.4 ± 2.6^{a}	$_{FLOUR}$ 6.8 ± 1.1^{c}	0.75± 0.02^{a}
	$_{BREAD}$ 68.4± 9.7^{A}	$_{BREAD}$ 22.5 ±6.4^{B}	$_{BREAD}$ 7.6 ± 1.6^{B}	
Medium Wheat	$_{FLOUR}$ 16.5± 3.2^{b}	$_{FLOUR}$ 60.4 ± 5.6^{a}	$_{FLOUR}$ 25.6 ± 3.9^{b}	0.71± 0.04^{b}
	$_{BREAD}$59.1±14.1^{AB}	$_{BREAD}$ 33.7 ± 4.6^{A}	$_{BREAD}$ 9.2 ± 0.2^{A}	
Large Wheat	$_{FLOUR}$ 8.9± 1.0^{c}	$_{FLOUR}$ 36.0 ± 1.8^{b}	$_{FLOUR}$ 56.9 ± 2.4^{a}	0.65± 0.07^{c}
	$_{BREAD}$ 55.7± 5.8^{B}	$_{BREAD}$ 37.2 ± 3.5^{A}	$_{BREAD}$ 3.7 ± 0.7^{C}	
Small Rye	$_{FLOUR}$ 26.1± 4.1^{a}	$_{FLOUR}$ 71.8 ± 7.4^{a}	$_{FLOUR}$ 2.1 ± 1.5^{b}	0.57± 0.03^{a}
	$_{BREAD}$ 56.2± 7.4^{A}	$_{BREAD}$ 32.1 ± 3.4^{B}	$_{BREAD}$ 26.1 ± 10.5^{A}	
Medium Rye	$_{FLOUR}$ 18.2± 2.7^{b}	$_{FLOUR}$ 60.3 ± 7.2^{b}	$_{FLOUR}$ 23.1 ± 7.7^{a}	0.45± 0.03^{b}
	$_{BREAD}$ 49.3± 3.6^{A}	$_{BREAD}$ 59.4 ± 9.3^{A}	$_{BREAD}$ 5.5 ± 5^{B}	
Large Rye	$_{FLOUR}$ 15.1± 1.0^{b}	$_{FLOUR}$ 55.5 ± 0.3^{b}	$_{FLOUR}$ 21.0 ± 6.2^{a}	0.38± 0.05^{c}
	$_{BREAD}$ 58.6±11.2^{A}	$_{BREAD}$ 48.2 ± 4.6^{A}	$_{BREAD}$ 6.1 ± 5.8^{B}	

Values are expressed in mean ± SD. The same small letter (flour digestibility), the same capital letter (bread digestibility) and the same letter in *italic* (cohesiviness) within the same column indicates no significant difference between mean values respectively of durum wheat and rye ($p < 0.05$, Tukey's test, n =9).

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Fusarium musae, a potential new food safety threat. Can a diseased banana be the source of a fungal disease for humans?

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The first two activities of the PhD thesis project are described. The worldwide collection of *F. musae* strains isolated from both banana fruits and human patients obtained during the first year was characterised. Firstly, I assessed the sensitivity of strains to different azoles widely used in agriculture and clinical settings. Secondly, I looked for genome and mitogenomes diversity between strains with different host and geographical origin.

Fusarium musae, una nuova potenziale minaccia per la sicurezza alimentare. Può una banana malata essere fonte di malattia fungina per l'uomo?

Le prime due attività del progetto di tesi di dottorato sono descritte. La collezione di ceppi di *F. musae* isolati da banane e pazienti ospedalieri ottenuta da diversi continenti durante il primo anno è stata caratterizzata. In primo luogo, ho valutato la sensibilità dei ceppi a fungicidi azolici ampiamente utilizzati in campo sia agricolo che ospedaliero. In secondo luogo, ho investigato la diversità a livello di genoma e mitogenoma di ceppi con provenienze geografica e di ospite diverse.

Key words: Fusarium infection, azoles, fungal characterization, genomics, strain diversity, *F. musae*.

1. Introduction

In accordance with the PhD thesis project (Tava, 2021), this poster reports the main results of the first activities:
(A1) fungicides sensitivity of a worldwide collection of *F. musae* strains obtained during the first year exploring public mycological repositories to eight DMIs used in crop protection and five medical antifungals;
(A2) sequencing of whole genome of all the *F. musae* strains (n= 17) in order to study fungal diversity in relation to the geographical origin and host. Mitogenomes of the entire population were assembled and comparatively analysed and I obtained a very complete genome of *F. musae* that will serve as reference for my future studies on comparative genomics and transcriptomics of this species.

2. Materials and Methods

(A1) All molecules were prepared at final concentrations ranging from 0.03 to 16 mg/L according to the Clinical and Laboratory Standards Institute guidelines for filamentous fungi (Reference CLSI M38-A2). Inoculum suspensions were prepared from 2–5-day-old cultures diluting to a final working inoculum of 0.5–5 × 10^4 CFU/mL. Plates were incubated at 28°C for 48 h. The minimum inhibitory concentration (MIC) value was the concentration of drug yielding no fungal growth at visual reading. Tests were performed in duplicate.
(A2) DNA used for sequencing was obtained from fresh mycelia of 17 strains according to a modified CTAB method (Pasquali *et al.*, 2004), followed by Genomic tips column purification (Qiagen, Germantown, MD, USA). Sequencing was carried out using Illumina Hiseq 2000 (151 bp x2) by Novogene (Cambridge, UK). Mitogenomes of NRRL25059 strain was obtained from the NCBI database. Assembly was carried out de novo with NOVOplasty 4.2. Mitogenomes were then annotated by integrating MFannot and RNAWeasel. Sequences were then aligned using the MAFFT alignment tool using Geneious prime software and manually checked and analyzed using Median Joining Network in PopArt (Degradi *et al.*, 2022).
Complete genome of one representative strain was obtained by combining Illumina with long-read sequencing using the MinION MIN101B platform, R9.4.1 flow cell (Nanopore). Assembly was performed using Canu v.2.1.1 + galaxy0. Autopolishing was performed using Medaka v.1.0.3 + galaxy2. Minimap2 v.2.17 + galaxy4 was used to align short reads on the obtained assembly. Manual correction was done using Geneious Prime software v.11 (Biomatters) and final assembly statistics were evaluated using Quast tool v.5.0.2 + galaxy1 (Degradi *et al.*, 2021).

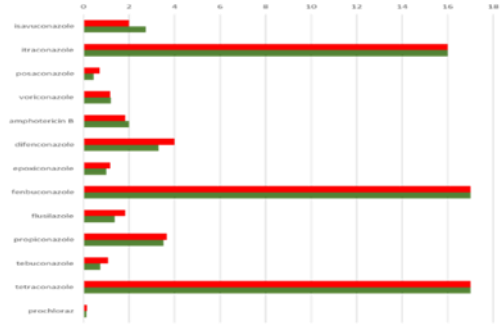
3. Results and Discussion

3.1 Sensitivity to antifungals

MIC values showed that among the medical azoles itraconazole presented high MICs (G-MIC = 16 mg/L) while posaconazole, voriconazole and isavuconazole showed lower values (respectively 0.54 mg/L; 1.2 mg/L and 2.4 mg/L); amphotericin B showed a G-MIC of 1.93 mg/L. For agricultural azoles the highest activity was shown by prochloraz and the tebuconazole (G-MIC of 0.14 mg/L and 0.83 mg/L respectively), while higher values were showed by difenoconazole (2–8 mg/L), propiconazole (2–4 mg/L) and flusilazole (1.55 mg/L). Tetraconazole and

fenbuconazole showed G-MICs > 16 mg/L for all strains. Results are comparable with data already present in literature and no statistically significant differences between the isolates of different origins were observed (Figure 1) for any azole (Tava *et al.*, 2021). Each azole has a unique spectrum and power, so it's important to study the sensitivity of novel species to azole derivatives in order to select the most appropriate molecule to use in different setting considering the risk of potential resistance occurrence within the Fusarium genus.

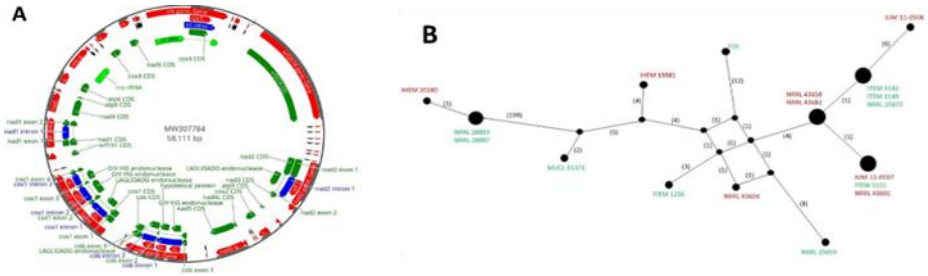
Figure 1 *Susceptibility to antifungal drugs of F. musae isolates from human (upper line) and bananas (lower line).*



3.2 Mitogenomes

Mitogenomes of *F. musae* strains ranged from 56,439 to 59,256 bp (Figure 2A). The analysis of codon usage in coding sequences did not reveal significant differences among the whole set of analysed strains, all mitogenomes showed a high similarity for protein-coding regions. However our study confirmed that intergenic regions and endonucleases may be exploited to identify subgroups within a species. Different nuclear gene haplotypes exist and at least one set of strains belonging to the same mitochondrial haplotypes included both human and banana derived strains (Figure 2B) suggesting that the species can travel as hypothesised by (Triest and Hendrickx, 2016).

Figure 2 *(A) Graphical representation of circular mtDNA of F. musae. (B) Representation of mitogenomes network haplotypes using PopArt software.*



3.3 Genome

Strain F31 presented a size of 44.07Mb divided into 12 chromosomes of which 11 have both telomers, the circular mitochondrial DNA (mtDNA), and one unplaced contig. Functional annotation led to a total of 13,963 annotated genes, of which 13,661 were proteins and 302 transfer RNA (tRNA) for nuclear DNA. This represents the first genome completed at chromosome level of *F. musae* and it will be a useful resource for comparative analysis of *F. musae* species, in addiction it represents an important reference for completeness and for understanding the genome evolution in the FFSC.

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Advancement and prospects of study of bioactive peptides during food fermentation

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Bioactive Peptides from novel protein sources are gaining scientific and industrial relevance due to their health-promoting and pro-technological potential. In this study, red lentils protein isolate was fermented with selected starter cultures of lactic acid bacteria and yeasts from the Micro4Food Culture Collection (UNIBZ, Italy), aiming to produce bioactive peptides featuring antifungal activity. Hyphal radial growth inhibition was observed for all the fermented peptides´ extracts (pWSE) to a different extent at different time points against *Penicillium roqueforti* P1. *Hanseniaspora uvarum* SY1 and *Lactiplantibacillus plantarum* LM1 resulted in the highest growth inhibition among the started samples.

Avanzamento e prospettive di studio dei peptidi bioattivi durante la fermentazione alimentare

I peptidi bioattivi ricavati da nuove fonti proteiche stanno acquisendo rilevanza scientifica e industriale grazie al loro potenziale salutistico e pro-tecnologico. In questo studio, l'isolato proteico di lenticchie rosse è stato fermentato con colture starter selezionate di batteri lattici e lieviti della Micro4Food Culture Collection (UNIBZ, Italia), con l'obiettivo di produrre peptidi bioattivi con attività antifungina. Per tutti gli estratti peptidici fermentati è stata osservata un'inibizione della crescita radiale ifale, in misura diversa e a diversi punti temporali, nei confronti di *Penicillium roqueforti P1*. *Hanseniaspora uvarum* SY1 e *Lactiplantibacillus plantarum* LM1 hanno prodotto la più alta inibizione tra gli starters.

Key words: Bioactive Peptides, started fermentation, lactic acid bacteria, yeast, green-biotechnologies.

1. Introduction

Bioactive peptides production through microbial fermentation is considered by several authors among which Gobbetti et al. (2007); and De Pasquale et al. (2020), to be one of the most effective non-thermal biotechnology to exploit the full biological potential of different protein sources. It is a process that meets the requirements of sustainability, innovation and functionality, but in order to be effective it must necessarily be monitored and design to obtain the metabolic pathways of interest (Tlais et al., 2021). The poster reports the main results of the first two activities of the first semester of the second year of PhD in Food Engineering and Biotechnology. In particular, after eight days of controlled fermentation, the peptides´ extracts were tested for:

- Protein and peptides content; peptides´ chromatographic profile via FPLC
- Preliminary screening for bioactivity: Hyphal radial growth rate assay

2. Materials and methods

The fermentation was held at 30°C for eight days, and included three red lentils protein isolates started with lactic acid bacteria (inoculum 10^7 log cfu*g^{-1}: *Fructilactobacillus sanfranciscensis* E10; *Lactiplantibacillum plantarum* LM1.3; and *Lactiplantibacillum plantarum* ATCC), two started with yeasts (inoculum 10^5 log cfu*g^{-1}: *Hanseniaspora uvarum* SY1; and *Kazachstania unispora* KFBY1), and one un-started sample. The extraction of the peptides´ fraction was obtained with the Water/Salt soluble extract of (50mM Tris HCl; pH 8.8), further treated with 5% (v:v) trifluoroacetic acid to precipitate proteins and amino acids, as described by De Pasquale et al., (2020). Protein and peptides concentration was assessed before precipitation via Badford and o-phtaldialdehyde (OPA) method, respectively.

Hyphal radial growth rate assay was used to determine the *in vitro* antifungal activity of the pWSE, as previously described by (Rizzello et al. (2011) with some modifications. Briefly, pWSE was added to mini-Petri dishes and Potato Dextrose Agar (PDA) (Oxoid Ltd, Basingstoke, Hampshire, England) medium was poured over and gently mixed to spread uniformly the extract in the growth media. *Penicillium roqueforti* P1 was previously selected as mold indicator and refreshed twice on PDA media before antifungal assay. A 5mm Ø of fresh fungal mycelia from the refreshed culture was placed at the center of the PDA/pWSE mini-Plates and used as inoculum for the antifungal assay. The hyphal radial growth rate was measured after 5 days of incubation at room temperature and the related inhibition percentage was calculated as follows:

$$Growth\ inhibition\ (\%) = \frac{(mycelial\ growth\ on\ PDA - mycelial\ growth\ on\ PDA\ /\ pWSE)}{mycelial\ growth\ on\ PDA} * 100 \quad (1)$$

3. Results & Discussion

Proteolytic activity of *Hanseniaspora uvarum* SY1 resulted the highest among all the fermented samples, with a decrease in the protein content of approx. 36% and a final peptide content of 321.09 ± 6.45 mg*g^{-1} d.m.. Moreover, the peptides´ profile after yeast fermentation, resulted in the highest variation in chromatographic peaks, when compared to the un-fermented flour. Among the samples started with lactic acid bacteria, *Fructilactibacillus sanfranciscensis* E10 and *Lactiplantibacillus plantarum* LM1.3 resulted in the highest protein hydrolysis and peptides production. The relative protein hydrolysis was about 24 and 22% and the peptides concentration after 8 days of fermentation was 244.04±6.21 and 228.33±5.16 mg*g^{-1} d.m., respectively. All the fermented hydrolysates were able to inhibit mold growth to some extent against *Penicillium roqueforti* P1 (Table 1), with the RLI hydrolysate fermented by Hanseniaspora uvarum SY1 and the spontaneously fermented showing the highest growth inhibition (60.4%). Furthermore, especially the fermented peptides extract by *H. uvarum* SY1 showed on average the best inhibition in all the sampling points, ranging from 41.7% at t2, 51.5% at t4 and t8, till the maximum inhibition reached after 8 days of fermentation. The result is in alignment with findings reporting different species among the *Hanseniospora* genus, able to produce a broad spectrum of antifungal compounds.

Table 1 Hyphal radial growth rate inhibition assay used to determine the antifungal activity of the fermented peptides´ extracts. The sampling occurred every two days for a total of eight days of fermentation. The inhibition (%) is expressed by comparing the growth of an untreated strain of *Penicillium roqueforti* P1, with the one of the samples treated with the fermented extracts.

Species	2 days	4 days	6 days	8 days
	Inhibition (%)			
Spontaneous fermentation	27.1	50	50	60.4
Lactiplantibacillus plantarum LM1.3	20.8	35.4	39.6	56.3
Fructilactibacillus sanfranciscensis E10	33.3	43.8	50	35.4
Lactiplantibacillus plantarum ATCC	43.8	54.2	47.9	50
Hanseniaspora uvarum SY1	41.7	54.2	54.2	60.4
Kazachstania unispora KFBY1	41.7	37.5	43.8	54.2

4. Conclusion and Recommendations

This study was one of the first to use controlled lactic acid and alcoholic fermentation of red lentils protein isolate as a green biotechnology to guide protein hydrolysis into potentially bioactive peptides with antifungal activity. Under investigation was a diversified pool of lactic acid bacteria and yeast form the Micro4Food Cultures Collection (Department of Science and Technology, UNIBZ, Italy). *Hanseniaspora uvarum* SY1 resulted one of the best starter culture to produce presumptive bioactive peptides via fermentation. Among the lactic acid bacteria strains, *Lactiplantibacillum plantarum* ATCC resulted in the highest hyphal radial growth inhibition among the tested lactic acid bacteria starters. All the fermented hydrolysates were able to inhibit mold growth to some extent against *Penicillium roqueforti* P1. The results of this preliminary screening for bioactivity sets a solid base for the identification of fermentation derived bioactive peptides featuring antifungal activity. In particular, the next steps of this project will include several steps of ultrafiltration and fractionation via FPLC. The purified fractions will be then tested individually in-vitro for antifungal activity in order to identify the active fraction. Finally, the presumptive bioactive peptides will be identified via Orbitrap mass spectrometry. The isolation and identification of new bioactive peptides from innovative food sources such as red lentils will allow to develop new products such as dietary supplements, novel food products and nutraceuticals**.**

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Evaluation of wine evolution, application of conventional and innovative approaches

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The aim of this PhD project is to create a model able to forecast wine evolution, based on conventional analysis as well as innovative approaches, such as cyclic voltammetry (CV) using screen-printed electrodes. To create the model different commercial white wines have been analyzed. The advantage of using CV for predicting wine evolution is that this analytical technique is fast, simple and it does not need sample preparation.

Valutazione dell'evoluzione del vino, applicazione di approcci convenzionali e innovativi

L'obbiettivo della tesi di ricerca è la creazione di un modello capace di predire l'evoluzione del vino, basato su approcci di analisi convenzionali, ma anche innovativi, come la voltammetria ciclica (CV). Per la creazione del modello sono stati analizzati differenti vini bianchi prodotti per il commercio. L'uso della voltammetria ciclica per predire l'evoluzione di un vino ha differenti vantaggi, in quanto è una tecnica veloce, semplice da eseguire e non necessita di fasi preparatorie per l'esecuzione dell'analisi.

Keywords: Evolution, white wine, cyclic voltammetry, screen-printed electrode, forecast model, oxidation.

1. Introduction

One of the crucial questions in modern enology is to improve the management of wine evolution. For this reason, the first step of this PhD project is to create a forecast model, based on the use of different approaches of analysis and different commercial white wines.
The model will be based on the correlation between cyclic voltammetry (CV) with conventional parameters, aldehydes and volatile compounds.
The final model will be an important instrument for the winemaker to improve and optimize the production chain, the use of antioxidants, packaging, storage and shipping condition, according to the enological project.

2. Materials and Methods

Different commercial white wines were kindly supplied by different wineries located in north-east Italy.
Different conventional parameters, like Sulphur dioxide content (methods OIV-MA-A323-04A R2018) (OIV, 2019), thiol compounds (Gallardo-Chacón et al., 2010), browning assay (POM test Müller-Späth 1992), colorimetric and spectrophotometric indices (CIELab, Abs 420 and 280) were determined on bottled wines during ageing. Analysis of volatile compounds was carried out by SPME-GC-MS. At the same time, cyclic voltammetry profile was also acquired on wines, using screen-printed electrode (SPCE) model 110 Metrohm Dropsense (Metrohm Italiana S.r.l., Origgio, Italy). Statistical analysis made by statistical software R 4.2.0.

3. Results and Discussion

3.1 Effect of the evolution on enological parameters

According to Hopfer et al. (2013), the packaging configuration, the storage and shipping conditions are the most important factors after the bottle stage. At the same time, it is difficult to establish the best timing for consuming and estimating the evolution of wine.
Our aim is to create a useful tool for the winemaker to reach the enological project. The model will be based on different types of analysis, to better understand and portray what happens in a bottle of wine.
After collecting the bottled wines, sampling was performed approximately every 4 months, from september 2021 to may 2023.
A strong decrease in free sulfur dioxide was observed, but this does not affect the level of total SO_2; in addition, a slight increase of ABS 420nm and an increase of POM test value were measured. Total polyphenolic index (IPT) remained stable.
Concerning cyclic voltammetry, all samples showed a decrease in the anodic curve during ageing time.
Regarding volatile compounds, there was an increase of aldehydes, which are ageing markers, organic acids, ethyl esters, and other esters (3-methylbutyl octanoate and 3-methylbutyl hexanoate) on the other hand, there is a decrease in acetic esters and alcohols.

Principal component analysis (PCA) well represents the distribution of samples and at the same time the distribution of the two sampling points.
Figure 1 shows the PCA performed on chemical data and volatile compounds; it demonstrates a good division for the sampling times, but the sum of the dimension one and two is not highly significant.
However, considering all the variables considered in figure 1, adding the anodic trace of cyclic voltammetry (Figure 2), there is a better representation for the dimensions, one and two, and there is a negative correlation between parameter L* of CIELab and the first part of voltammograms (up to 300mV), but also between aldehydes and the final part of the voltammetric trace. Finally, there is a correlation between the first part of voltammogram and IPT.

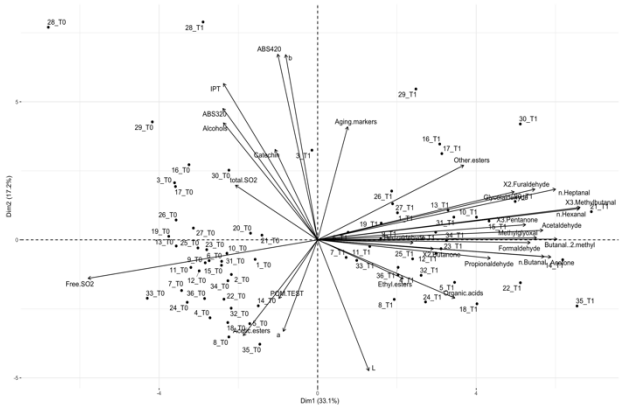

Figure 1. PCA plot generated with chemical parameters and volatile compounds

The preliminary data of the third sampling time confirm this trend furthermore, the PCA shows better discrimination of the different sampling times for the chemical analysis (data not shown).

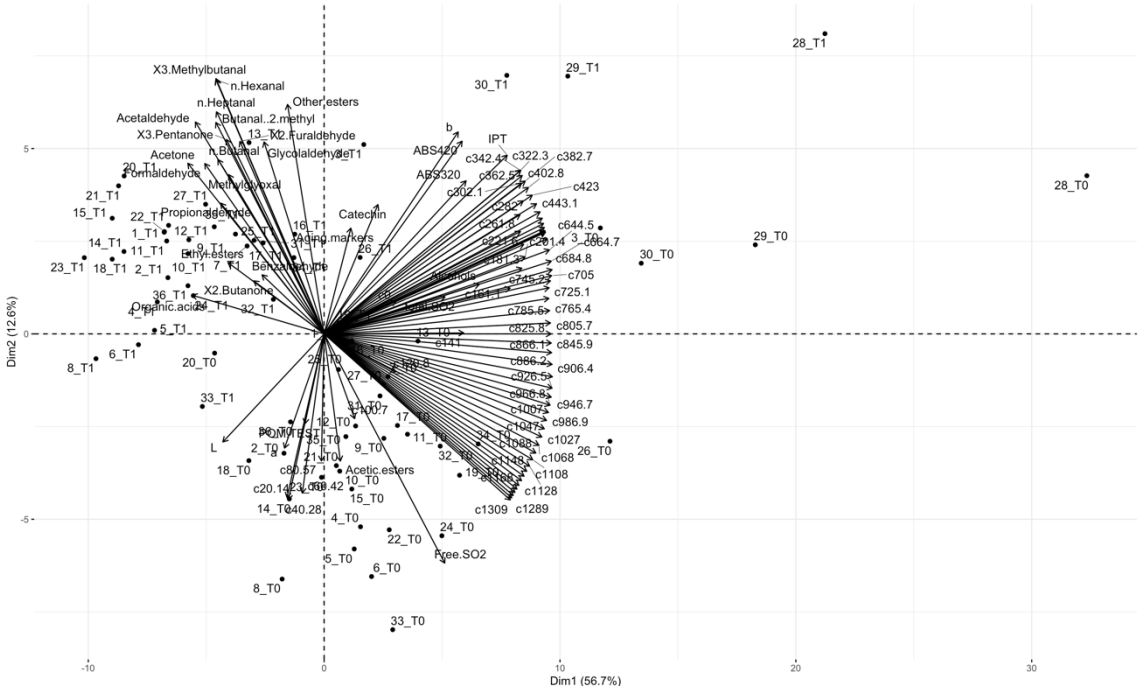

Figure 2. PCA plot generated with chemical parameters, volatile compounds and cyclic voltammetry data

To conclude, the expected results are in line with the experimental data obtained, so it is not necessary to modify the original PhD thesis project. Furthermore, in parallel with this project there is another study to understand how the oxygen transfer rate of closures and the management of gasses into the bottle could affect the wine evolution.

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Phenolic metabolites after acute and chronic consumption of different flavan-3-ol sources

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The first two activities of the PhD thesis project are described. Firstly, the influence of the flavan-3-ol structure on metabolite production was evaluated after acute consumption of different flavan-3-ol sources. Secondly, the phenolic profile and the inter-individual variability in the metabolism of flavan-3-ols and other (poly)phenols was determined after chronic consumption of cranberry.

Metaboliti fenolici dopo il consumo in acuto e in cronico di diverse fonti di flavan-3-oli

Sono descritte le prime due attività del progetto di tesi di dottorato. In primo luogo, è stata valutata l'influenza della struttura dei flavan-3-oli sulla produzione di metaboliti fenolici dopo il consumo in acuto di diversi estratti. Secondariamente, sono stati determinati il profilo fenolico e la variabilità interindividuale nel metabolismo dei flavan-3-oli e di altri (poli)fenoli dopo il consumo in cronico di mirtillo rosso americano.

Key words: flavan-3-ol, phenyl-γ-valerolactones, metabolism, bioavailability, metabotype.

1. Introduction

This poster reports the main results of the first two activities concerning flavan-3-ol metabolism:

(A1) the proof-of-concept study of the METANOLS project (*METabotypes in the urinary excretion of flavANOLS*), aiming to determine the influence of the flavan-3-ol structure on the bioavailability and metabolism of phenolic metabolites after acute consumption of different flavan-3-ol sources;

(A2) the COMBAT project (*The impact of Cranberries On the Microbiome and Brain in healthy Ageing sTudy*), aiming to assess the urinary phenolic profile and the inter-individual variability in the excretion of phenolic metabolites after chronic consumption of cranberry.

2. Materials and Methods

In the METANOLS project, a double-blind, randomized, cross-over, acute intervention trial was performed in 11 healthy subjects. Volunteers consumed 1000 μmol of flavan-3-ols from different food extracts: green tea rich in galloylated (epi)gallocatechins (GT), grape seed rich in (epi)catechins (GSM), and grape seed rich in procyanidins (GSP). Urine samples were collected before the intervention and during the 24/48 hours after the treatment. In the COMBAT project, a 12-week double-blind, randomized, placebo-controlled, parallel intervention trial was performed in 60 healthy older subjects. The intervention consisted in the intake of cranberry powder in a dose providing 587.5 mg (poly)phenols (281 mg proanthocyanidins). Spot plasma and urine samples were collected before and after the treatment (baseline visit, BL, and follow-up visit, FU). Samples from both studies were extracted with μSPE and analyzed with uHPLC-ESI-QqQ-MS/MS to quantify phenolic metabolites (Brindani et al., 2017). Up to 182 compounds for METANOLS and 74 for COMBAT were monitored in SRM mode:

• 1/2/3/5-carbon chain ring-fission metabolites (1/2/3/5C-RFMs): hippuric acids (HAs), benzaldehydes (BALs), benzenes (BZs), benzoic acids (BAs), phenylacetic acids (PAAs), phenylpropanoic acids (PPAs), cinnamic acids (CAs), phenylvaleric acids (PVAs), 4-hydroxy-PVAs (4-OH-PVAs), and phenyl-γ-valerolactones (PVLs);

• structurally-related flavonoid metabolites (SRFMs): (epi)catechins (ECs), (epi)gallocatechins (EGCs), diphenylpropan-2-ols (DPPOLs), flavonols.

Principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) were applied to analyze the differences between the treatments (GT, GSM and GSP) and to identify metabotypes in the urinary excretion of (poly)phenol metabolites, respectively. Data were pre-treated with mean-centering and UV scaling.

3. Results and Discussion

3.1 The METANOLS project: phenolic metabolites after acute consumption of different food extracts

A total of 76 compounds were quantified in urine. Flavan-3-ols from GT had the highest molar mass recovery in catabolites, and flavan-3-ol bioavailability was higher for GSM than GSP at 24h, but it was lower at 48h. The low yield of catabolites at 24h from procyanidins compared to flavan-3-ol monomers could be due to the steric hindrance limiting the access to the C-ring by the colonic microbiota, resulting in a lower microbial catabolism. On the contrary, monomers from GT and GSM were rapidly metabolized in the first 24h, while in the following 24h the metabolite production was more pronounced for GT, probably due to the slower metabolism of galloylated

flavan-3-ols, which need to be hydrolyzed by the colonic microbiota to be bioaccessible. For all treatments, the most representative classes were PVLs, followed by BAs. GT flavan-3-ols were metabolized to trihydroxylated (3',4',5'), dihydroxylated (3',4' and 3',5') and monohydroxylated (3' and 4') derivatives, while GS flavan-3-ols yielded only 3',4'-dihydroxylated and 3′/4′-monohydroxylated derivatives. PCA was applied considering all metabolites and phenolic classes, defined according to the aglycone moiety (Figure 1). Treatment with GT was characterized by the excretion of trihydroxylated metabolites, treatment with GSM by SRFMs and 5C-RFMs, and treatment with GSP by of 1/2/3C-RFMs. This could be due to the degradation of the upper unit, yielding PAAs.

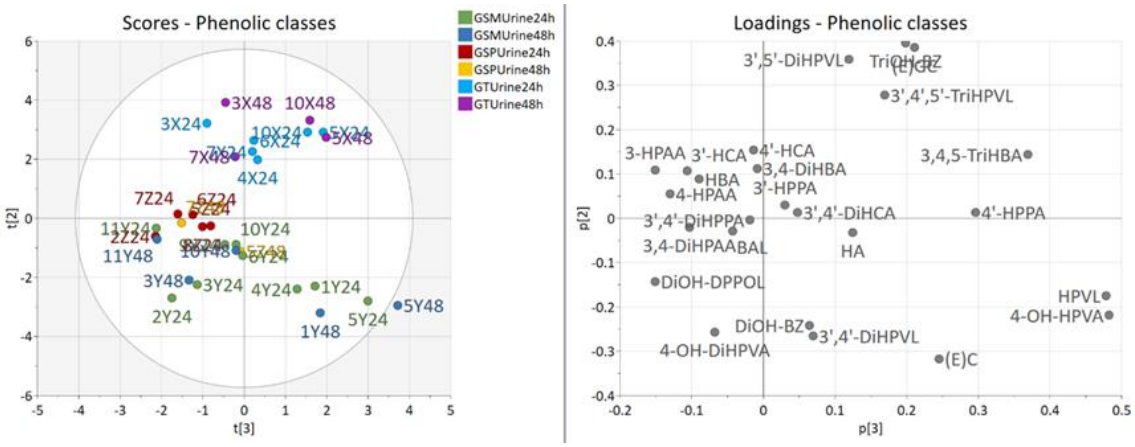

Figure 1 *PCA highlighting differences between treatments associated with the excretion of phenolic classes.*

3.2 The COMBAT project: phenolic metabolites after chronic consumption of cranberry

A total of 67 compounds were quantified in urine. Without considering HAs, PVLs and PAAs were the most represented classes. PCA was applied to explore the inter-individual and inter-group variability in the urinary excretion of phenolic metabolites. 3 different datasets consisting of 120 observations (samples) were considered, differing on the type of variables: all metabolites without HAs (n=65), phenolic classes defined according to the aglycone moiety (n=20), and metabolites belonging to HPPAs and PVLs (n=17). With all datasets, 3 clusters were identified according to the PC score. No distribution was observed according to the treatment. PLS-DA was applied to predict which metabolite(s) could better explain the inter-individual variability observed and to stratify subjects into groups sharing a common excretion profile (metabotypes, MTs). Results were similar for all datasets: MT1 is characterized by the excretion of PPAs and other 1/2/3C-RFMs (extensively metabolized catabolites), MT2 by the excretion of diHPVLs and other 5C-RFMs (poorly metabolized catabolites), and MT3 (the most numerous) by a low metabolite excretion (Figure 2). The most relevant classes differentiating MTs on a qualitative criterion (VIP value >1.25) were 3,4-diHPVLs and 4-HPPAs. These results are consistent with those obtained in other studies using flavan-3-ol sources (Mena et al., 2019, 2022), suggesting that PVLs and HPPAs could be representative of the excretion of all metabolites, and flavan-3-ol metabotypes could be predictive of (poly)phenol metabotypes.

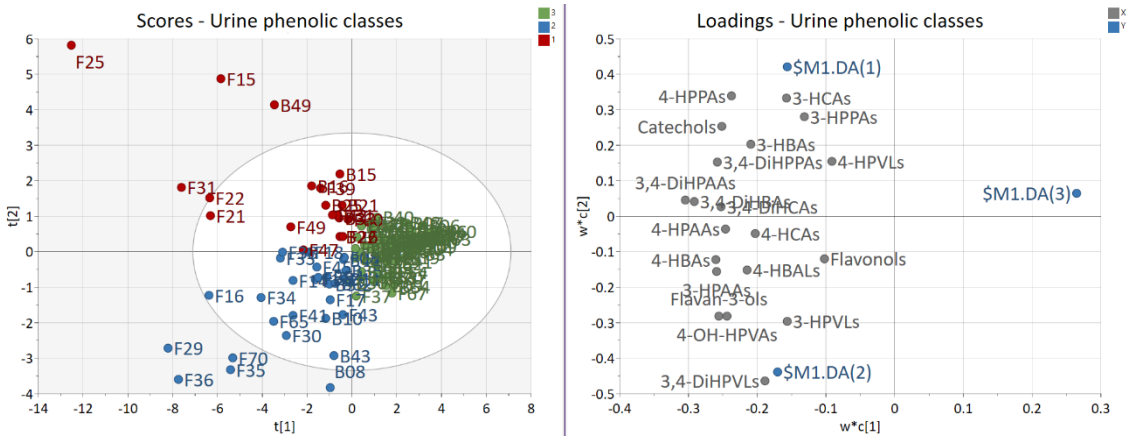

Figure 2 *PLS-DA highlighting inter-individual differences in the urinary excretion of phenolic classes.*

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Technological approaches to improve the quality of meat products

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The feasibility of using the Olive Leaf Extract (OLE) for the reduction of nitrate and nitrite level in the ripened meat products was one of the activities of my PhD project. Ripened sausages with different nitrate/nitrite and OLE ratios were produced in collaboration with a local farm and characterised in terms of residual nitrate and nitrite, microbial growth, colour analysis and induction time, compared with a control with only the synthetic additives added.

Approcci tecnologici per il miglioramento della qualità dei prodotti carnei

È stata valutata la possibilità di utilizzare l'estratto di foglie di olivo (OLE) nella formulazione ingredientistica di insaccati stagionati per ridurre il livello di nitrati e nitriti. Sono stati prodotti insaccati stagionati con diversi rapporti di nitrati/nitriti e OLE, e caratterizzate in termini di nitrati e nitriti residui, crescita microbica, analisi del colore e tempo di induzione, rispetto ad una tesi controllo in cui sono stati aggiunti solo gli additivi sintetici secondo i limiti legali.

Key words: Olive leaf extract; ripened sausages; nitrate; nitrite.

1. Introduction

Over time, the feasibility to reduce the use of nitrate and nitrite and the consequent formation of Nitrosamines in meat products by natural ingredients, such as essential oils, extracts from fruits, or vegetables spices have been investigated (Sun *et al.*, 2020). Furthermore, wastes and by-products from the agricultural and food industry sectors contain highly valuable bioactive substances. Among olive by-products, the olive leaves have been proposed for food preservation thanks to the abundant bioactive molecules with antioxidant and antimicrobial activities (Difonzo *et al.*, 2021). In accordance with the PhD project this poster reports the main results concerning the effects of the addition of OLE on the residual nitrate and nitrite content, microbial growth, colour analysis and induction time of ripened sausages.

2. Materials and Methods

The production and characterization of OLE was carried out as described in Difonzo et al. (2017). Pork meat was purchased from a local farm Salumi Martina Franca S.r.l. (Martina Franca, Italy) where preparation and ripening phases also took place. Potassium nitrate E252 and sodium nitrite E250 (SolMar, Italy) and salt tanning (30% dextrose, 70% salt; 40 g/kg of raw meat) were added. Seven different combinations (Tr) of nitrate and nitrite and OLE concentration were considered as reported in Table 1:

Table 1 *Formulations of OLE - nitrate and nitrite used for samples preparation.*

	Olive Leaf Extract (mg/kg)	Nitrate and nitrite (mg/kg)
Control	0	150 NO_2/150 NO_3
Tr 1	200	75 NO_2/75 NO_3
Tr 2	400	75 NO_2/75 NO_3
Tr 3	800	75 NO_2/75 NO_3
Tr 4	200	0
Tr 5	400	0
Tr 6	800	0

After ripening the residual nitrate and nitrite content, sulphite *Clostridia* and spores, *Coliforms*, *Escherichia coli* and *Staphylococcus* coagulase positive, colour analysis and induction time have been carried out, following the methods described in (Difonzo *et al.*, 2022). Data were subjected to statistical analysis and significant differences were determined at $p < 0.05$.

3. Results and Discussion

3.1 Nitrate and Nitrite Residual, Microbiological Analysis

In the samples investigated, all the microbiological parameters evaluated (sulphite-reducing *Clostridia* and spores, *Coliforms*, *Escherichia coli* and *Staphylococcus* coagulase positive) were within the law limits Reg. (CE) No. 1441/2007 (data not shown). A possible role of OLE in exerting an antimicrobial effect can be assumed since also in the samples without nitrate and nitrite added no growth was found. This could be due to the direct inhibitory action of OLE and the presence of polyphenolic compounds in OLE with antimicrobial properties. In Table 2 the residual nitrate and nitrite content of the samples under investigation are reported.

Table 2 *Residual nitrate and nitrite content (mg/kg).*

	Residual nitrate (mg/kg)	Residual nitrite (mg/kg)
Control	15	20
Tr 1	6	<10
Tr 2	10	<10
Tr 3	9	11
Tr 4	<5	<10
Tr 5	<5	<10
Tr 6	<5	<10

The highest residual nitrate values (15 mg/kg) were found in the Control sample. In the samples without synthetic additives, amounts of residual nitrate below 5 mg/kg were found. Similar results were found for nitrite determination, which were higher in Control (20 mg/kg) and were reduced in all other samples with an estimated maximum content of 11 mg/kg. It can be also observed that the residual nitrite content was higher than the nitrate, probably due to the conversion of nitrate to nitrite by nitrate-reductase bacteria.

3.2 Colour Analysis

The results of colour analysis are reported in Table 3. The lowest a* value was observed in sausages with only OLE, probably linked to the lack of synthesis of nitroso-myoglobin, which determines the red colour stability of the meat-based product. The reduction of the nitrate and nitrite doses caused a significant increase of the lightness (L*), while no significant effect was linked to the OLE variables (p >0.05). The OLE variable significantly affected the yellowness index (b*) generally associated with oxidation processes. In fact, a reduction of the parameter was observed to increase the OLE doses, since the presence of antioxidant compounds of OLE could have acted as efficient radical scavengers and metal chelators, reducing oxidative processes which promote colour changes.

Table 3 *Mean value, standard deviation, and results of the statistical analysis (two-way ANOVA) of the colour analysis (n=3).*

	Control	Tr 1	Tr 2	Tr 3	Tr 4	Tr 5	Tr 6
Lightness (L*)	32.58 ± 0.02^{B}	35.37 ± 2.92^{AB}	33.98 ± 0.39^{B}	33.25 ± 0.74^{B}	35.18 ± 0.62^{AB}	37.92 ± 0.58^{A}	35.43 ± 0.75^{AB}
Redness (a*)	8.27 ± 0.60^{A}	8.48 ± 1.58^{A}	8.10 ± 1.72^{A}	6.55 ± 1.27^{AB}	4.24 ± 0.19^{B}	3.35 ± 1.24^{B}	4.42 ± 1.06^{B}
Yellowness (b*)	7.55 ± 0.18^{AB}	9.27 ± 1.33^{AB}	6.85 ± 0.30^{B}	6.57 ± 0.30^{B}	8.26 ± 0.74^{AB}	9.90 ± 2.13^{A}	6.92 ± 0.03^{B}

Note: Different letters indicate significant differences at $p < 0.05$.

3.3 Induction Time

The oxidative stability of sausages was determined with a RapidOxy, and the results were expressed in minutes (induction time) (Figure 1). The samples Tr 3 and Tr 6, with the maximum amount of the OLE (800 mg/kg), showed the highest oxidative stability. In fact, the increase of the OLE amount corresponded to a greater induction time. However, the synergy with nitrate and nitrite cannot be excluded, since the sample with the lowest content of OLE and no added synthetic additives (Tr 4) showed the lowest oxidative stability, while the homolog with synthetic additives added (Tr 1) showed a higher induction time. Overall, the results indicated that OLE, both alone and together with nitrate and nitrite, exerted an antioxidant effect in ripened sausages and, interestingly, the OLE addition bolstered the antioxidant power of nitrate and nitrite.

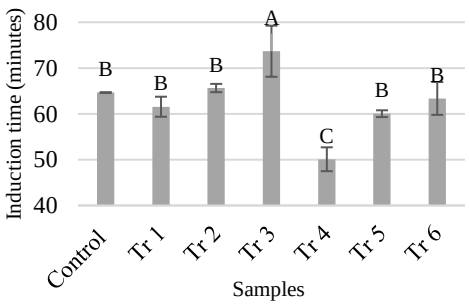

Figure 1 *Results of the RapidOxy Test of the ripened sausages. Different letters indicate significant differences at p < 0.05.*

4. References

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Pathogens-seeking Along Infant Food Production Chain: Could Untargeted Approaches go a long way towards Targeted ones?

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Tutor: Prof. Kalliopi Rantsiou

The aim of this PhD thesis research project is to comprehend how the untargeted metataxonomic analysis could unscramble pathogen's prevalence, in alignment with the targeted detection of each pathogen through enrichment, isolation and molecular approaches. Consequently, it is suggested that metataxonomic and other omics approaches could provide us with features and lead to the apprehension of pathogens distribution during food processing. The final scope is to construct accurate and applicable predictive models for the benefit of food safety. This PhD is part of the European Project "SAFFI - Safe Food for Infants in the EU and China".

Ricerca di Agenti Patogeni Lungo la Filiera di Produzione degli Alimenti per L'infanzia: Potrebbero Approcci non Mirati Aiutare la Loro Rilevazione?

L'obiettivo di questo progetto di ricerca di tesi di dottorato è capire come l'analisi metatassonomica non mirata possa decodificare la prevalenza di microorganismi patogeni. Questo diventerebbe possibile attraverso il suo allineamento con i risultati della ricerca mirata di ciascun patogeno, effettuata con l'arricchimento, l'isolamento e gli approcci molecolari. Di conseguenza, si vuole individuare come gli approcci metatassonomici e altri approcci meta-omici potrebbero fornirci informazioni per comprendere la distribuzione di agenti patogeni durante la lavorazione degli alimenti per l'infanzia, con lo scopo finale di costruire modelli predittivi accurati e applicabili a beneficio della sicurezza igienico-sanitaria. Questo dottorato fa parte del progetto europeo "SAFFI – Safe Food for Infants in EU and China".

Key words: pathogens, infant food, DNA, real-time PCR, metataxonomic analysis

1. Introduction

Foodborne diseases are still a significant public health issue worldwide with almost one in ten people each year suffering after the ingestion of unsafe food. This fact creates an immense challenge for food industries that can be abided by pathogens with the tendency to persist even after various stages of the process line. As a matter of fact, foodborne pathogens are considered a critical issue for infant food industries as the final consumer is characterised by a fragile immune system and can end up with severe illness and even death. Therefore, the determination of the presence, distribution and prevalence of foodborne pathogens is essential. In this research culture - dependent and culture - independent methods are combined to uncover the routes of contamination and investigate possible correlations among metataxonomic analysis and pathogens prevalence. (World Health Organization, 2015, 2017; Cho *et al.*, 2019; Rantsiou *et al.*, 2022)

2. Material and Methods

A total of one - hundred samples were collected during various stages of the infant food process line. More specifically swabs (ENV), raw material (RM), intermediate (IP) and final products (FP) were considered. On each sample, 16S rRNA amplicon - based sequencing was performed and the Amplicon Sequence Variants (ASVs) distribution was investigated. Additionally, the presence of five targeted foodborne pathogens – *Listeria monocytogenes*, *Bacillus cereus*, *Salmonella* spp., *Staphylococcus aureus* and *Clostridium perfringens* - was examined prior and subsequent to twenty - four hours of enrichment, whereupon pathogens isolation followed by species specific PCR and real - time PCR were applied.

3. Results and Discussion

Bacterial communities showed diversity among food samples, related to their composition, while environmental samples were less segregated (Figure 1). Furthermore, there was an increase in the biodiversity of the samples (Figure 3) throughout the time along with the complexity of the metataxonomic profiles. Concerning the most abundant species (Figure 2), they varied during the production, where *Bacillus* species showed increased abundance in the second and third production relative to the first, likewise *Clostridium* species and *Enterococcus faecium*. On the other hand, the third production was more characterised by the presence of *Lactococcus* species, *Streptococcus parasanguinis* and *Weissella paramesenteroides*. *Paeniclostridium sordelli* was the most abundant at the second production.

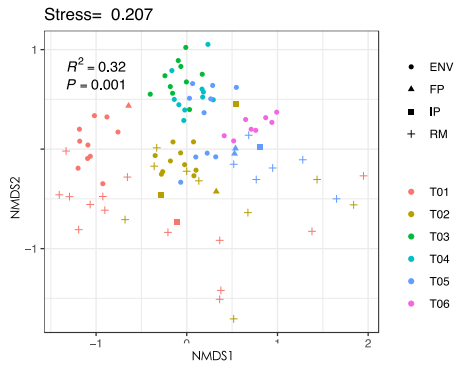

Figure 1. *Non-metric Multidimensional Scaling Ordination (PERMANOVA – Bray-Curtis Distance).*

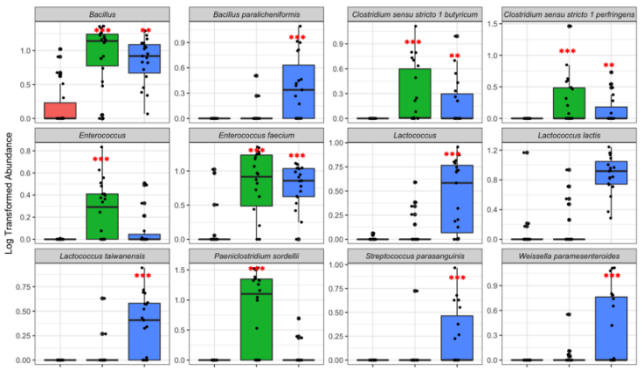

Figure 2. *Most Abundant Taxa (Pairwise Wilcoxon's Test, *p<0.01 **p<0.001).*

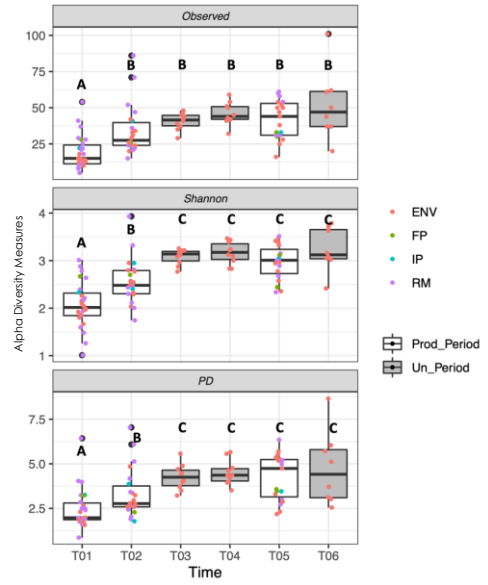

Figure 3. *α-Diversity (Kruskal-Wallis Test - Wilcoxon's Test (p<0.01).*

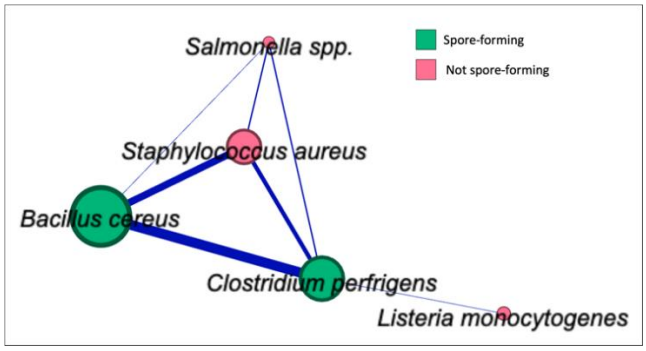

Figure 4. *Co-occurrence Network Displaying the Pathogens Related Presence by real-time PCR after 24 h. Nodes are made Proportional to the Detection Frequency; Edges Thickness to the Co-occurrence.*

The targeted pathogens are considered as low abundant microbial taxa, something that makes their direct detection not likely through metataxonomic analysis. Nevertheless, this fact does not exclude the possibility to correlate definite metataxonomic profiles or ecological parameters with evident pathogens presence. In the Figure 4 the detection of pathogens after twenty-four hours of enrichment and real-time PCR assay is shown. This simplified network indicates that the spore - forming bacteria were the most present, followed by *Staphylococcus aureus*. It was also observed that there was a correlation in their presence, as in samples that was present *Bacillus cereus* was present *Clostridium perfringens* and *Staphylococcus aureus* too. At this point of the research all the possible correlations are under research with the more in-depth integration of omics data.

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Identification and characterization of exopolysaccharides-producing *Liquorilactobacillus mali* T6 52

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Lactic acid bacteria (LAB) can be proven safe based on years of research. Because of their positive effects on the human body and food products, LAB producing exopolysaccharides (EPS) are gradually replacing chemical and synthetic polymers. In this Ph.D. research project RAPD-PCR was used to identify, at strain level, a group *L. mali* isolates, originated form grape pomaces. Subsequently, the bacterial growth was determined in different carbon sources. This allowed the identification of high EPS-producing strain T6 52. For this strain, the antibiotic minimum inhibitory concentration was determined and EPS were purified for future studies on their chemical composition and biological activities.

Identificazione e caratterizzazione del ceppo produttore di esopolisaccaridi *Liquorilactobacillus mali* T6 52

Anni di ricerca hanno dimostrato che i batteri lattici (LAB) possono essere considerati microrganismi sicuri. Grazie ai loro effetti positivi sull'uomo e sulla produzione alimentare, gli esopolisaccaridi (EPS) ottenuti dai LAB sono proposti come sostituti dei polimeri chimici e sintetici. In questo progetto di dottorato la tecnica RAPD-PCR è stata utilizzata per identificare, a livello di ceppo, un gruppo di isolati di *L. mali* proveniente da vinacce. La loro crescita è stata valutata utilizzando diverse fonti di carbonio. Ciò ha permesso l'identificazione del ceppo T6 52 ad elevata produzione di EPS. Per questo ceppo, è stato determinato il livello di resistenza ad antibiotici di interesse umano e gli EPS sono stati purificati per studi futuri sulla loro composizione chimica e attività biologica.

Keywords: *Liquorilactobacillus mali*, MIC, RAPD-PCR, Exopolysaccharides

1. Introduction

The exopolysaccharides (EPS) producing microorganisms can be proposed as functional starters, since they can improve the rheological characteristics of food products, contribute to increase the nutritional value, and confer health advantages. In particular, EPS producing species belonging to lactic acid bacteria (*LAB*) group are of great interest. Indeed, *LAB* are generally recognized as safe due to their traditional use in food production. The *LAB* EPS generally have two forms: cell-bound EPS or released EPS. They are long-chain, high-molecular weight, naturally produced biopolymers. They influence the texture and stability of foods and present anticancer, antibiofilm, and antimicrobial activity. Moreover, they possess immunomodulatory activities, and radioprotective properties. Hence they have potential in the field of agriculture, food processing, dairy industry, pharmaceutical, and chemical industry. *Liquorilactobacillus mali* is a homofermentative rod-shaped bacterium belonging to the *Lactobacillaceae family*, consistently isolated from fruit juice. It was formerly named *Lactobacillus mali* (Carr and Davies, 1970).

2. Materials and Methods

Fifity-three *LAB* isolates were generally cultured in sterile MRS agar (Sigma SIAL0596, St. Louis, MO, USA) and incubate at 30°C for 24h. The DNA was extracted by using DNeasy PowerSoil Kit (Qiagen, Hilden, Germany), and RAPD-PCR was performed using primer M13 (5′ GAGGGTGGCGGTTCT 3′); electrophoretic profiles were analyzed using the software package, Gel Compare Version 4.1 (Applied Maths, Kortrijk, Belgium) based on the Pearson product-moment correlation coefficient and the dendrogram was obtained by using Unweighted Pair Group Method with Arithmetic Mean (UPGMA). MIC evaluation was performed using the broth microdilution method by 96-well microtiter plates (Sigma, USA) (Wiegand *et al.*, 2008) testing the following antibiotics: ampicillin, chloramphenicol, ciprofloxacin, oxacillin, erythromycin, gentamicin, kanamycin, penicillin G, streptomycin, tetracycline, trimethoprim, vancomycin, neomycin, rifampicin, spectinomycin, and carbenicillin (Sigma, USA). Strains growth was performed in presence of different carbon sources and concentrations (10% sucrose, 2% sucrose, 2% lactose, 2% glucose and 2% fructose) to determine the best condition for EPS production. For EPS purification the protocol proposed by Wang *et al.*, (2015) was used.

3. Results and Discussion

3.1 Strain identification - RAPD-PCR analysis

The 53 isolates were compared based on their electrophoretic profiles (Fig. 1). Based on 80% similarity, 35 strains were found. The isolate T6 52 was further identified as *Liquorilactobacillus mali*.

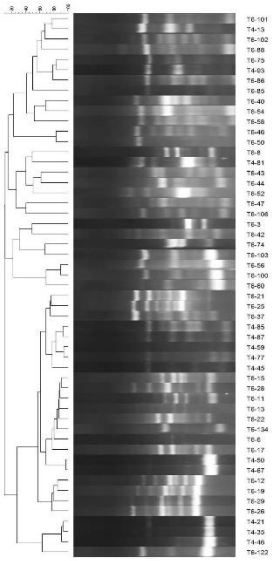

Figure 1 *UPGMA dendrogram derived from the combined RAPD-PCR patterns using the primer M13.*

3.2 Growth on different carbon sources

All the 35 strains were tested. *L. mali* T6 52 was found to produce the highest EPS amount with 10% sucrose.

3.3 Antibiotic resistance

The MIC values of antibiotics for *L. mali* T6 52 are reported in Table 1. According to the European Food Safety Authority (EFSA), it is sensitive to all antibiotics.

Table 1 *MIC (µg/mL) values for 16 antibiotics against L. mali T6 52*

	AMP	CHL	CIP	OXA	ERY	GEN	KAN	PEN G
T6 52	≤0.25	2	1	2	0.5	0.5	8	≤0.25
	STR	TET	TMP	VAN	NEO	RIF	SPT	CAR
T6 52	2	0.5	0.5	> 128	2	1	2	2

3.4 EPS extraction and purification of EPS

The crude EPS extract from ethanol precipitation consisted of 6.925 g/L. After removing the bacterial cells, proteins, and small molecular impurities, the concentration of lyophilized purified EPS was 660 mg/L.

4. References

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Workshop contributions

3rd year: Oral Communications

^{1}H-NMR-based metabolomics to assess the impact of the soil on the chemical composition of Nero d'Avola red wines

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This research project aims at investigating the effect of the soil type on wine sensorial quality. It consists of several steps, including the identification of study sites, soil surveys, soils chemical-physical analysis, grapes samplings, grapes chemical-physical analysis, vinification and wines chemical-physical analysis. Grapes were sorted according to their apparent density by means of flotation in solutions containing different concentrations of salts. Then, polyphenolic compounds of skin and seed extracts were analyzed by means of UV-Vis Spectroscopy and HPLC-DAD. Wines polyphenolic compounds and volatile organic compounds were analyzed by means of UV-Vis Spectroscopy and chromatographic techniques (HPLC-DAD and GC-MS). Finally, wine metabolomic analysis was performed by means of ^{1}H-NMR spectroscopy.

Analisi metabolomica mediante ^{1}H-NMR per determinare l'impatto del suolo sulla composizione chimica di vini da uve Nero d'Avola

Il progetto di dottorato mira a studiare l'effetto del suolo sulla qualità sensoriale dei vini. Il progetto è articolato in diverse fasi, tra cui: identificazione dei siti di studio, campionamento dei suoli, analisi chimico-fisiche dei suoli, campionamento delle uve, analisi chimico-fisiche delle uve, vinificazione e analisi chimico-fisiche dei vini. Le uve sono state sottoposte a flottazione in soluzioni saline a diverse concentrazioni. I composti polifenolici degli estratti di semi e bucce sono stati analizzati mediante spettroscopia UV-Vis e HPLC-DAD. I composti polifenolici ed i composti organici volatili dei vini sono stati analizzati mediante spettroscopia UV-Vis e tecniche cromatografiche (HPLC-DAD e GC-MS). Infine, l'analisi metabolomica del vino è stata condotta mediante spettroscopia ^{1}H-NMR.

Key words: terroir; wine; metabolomics; NMR; multivariate statistical analysis

1. Introduction

Wine sensorial quality results from a dynamic interaction among several factors, such as grape variety, climate, soil, metabolism of yeasts during alcoholic fermentation and human activities, including viticultural and oenological techniques (Mascellani et al., 2021). This dynamic interaction is referred to as the French concept of *terroir*. Among all the factors affecting *terroir*, soil plays a pivotal role because it provides the base for grapevine growth. Despite its importance, only few information is available about the effect of soil chemistry on wine chemical composition (de Andrés-de Prado et al., 2007).

Over the last decades, advances in the "omics" sciences significantly improved the understanding of the nature of vine-environment interactions and their effects on wine quality. Among them, metabolomics aims at identifying and quantifying the ensemble of the metabolites in a given organism or biological sample (Wishart, 2022) at a specific moment and under particular environmental conditions. Among the analytical methods that can be used to perform metabolomic analysis, ^{1}H-NMR spectroscopy plays the major role because it provides structural and quantitative information on almost all organic chemical classes, as based on the abundance of their hydrogen-nuclei. Two different metabolomic approaches have been developed. They are classified as targeted (TA) and non-targeted (NTA) metabolomic analysis. The former one, also referred to as *profiling*, aims at accurately identifying and quantifying a defined set of metabolites in biological samples. The latter one, also called *fingerprinting*, is a more exploratory technique: it does not necessarily deal with metabolite identification, but rather it focuses on the recognition of metabolite patterns by measuring and comparing as many signals as possible. Since NTA produces a large amount of data, multivariate statistical analysis is necessary to reduce data complexity and extract the most relevant information.

This study intends to achieve a better understanding of the role of soil chemistry on wine quality. To attain this goal, four vineyards with different soils and same *Vitis vinifera. L.* cultivar (*Nero d'Avola*) were chosen as study sites. Vines were trained with the same agronomic management in order to remove the variability associated with viticultural techniques. Grapes from each vineyard were separately vinified with a standard procedure, thus removing the variability associated with the winemaking techniques. Finally, due to the spatial proximity of the study sites, also the macroclimate variability is reduced. Thanks to these measures, the soil effect in its individuality, without the influence of climate and human activities can be investigated.

Moreover, in this study ^{1}H-NMR-based metabolomic analysis has been performed for the first time to classify wines according to different soil types.

2. Materials and Methods

Study sites

Four vineyards with different soils, located in the hilly landscape nearby Menfi, along the southwestern coast of Sicily (Southern Italy), were chosen as study sites. Sites were chosen according to homogeneity in vine cultivar (*Nero d'Avola L.*), vine age (26 years), vine rootstock (140 Ruggeri), pruning method (Guyot), planting system (2.50 m x 1.00 m), irrigation system (drip irrigation), altitude (100 m a.s.l.), slope (10-20%), sun exposure (south/south-east) and agronomic management (conventional).

Soil sampling and chemical-physical analysis

A soil survey for each vineyard was carried out and samples from each identified diagnostic horizon were taken. Different soil parameters were analyzed, including texture, pH, total carbonates, organic matter, cation exchange capacity, and electric conductivity.

Vinification process and wine physical-chemical analysis

Grapes from *Nero d'Avola L.* red cultivar belonging to the different vineyards were separately vinified with the standard procedure described by Squadrito et al. (2010). Musts/wines physical-chemical parameters were monitored during alcoholic fermentation by using a Winescan™ instrument (FOSS, Hilleroed, Denmark) calibrated by applying the EEC 2676 standard procedure (EEC, 1990). The obtained wines were subjected to spectrophotometric analyses to evaluate total anthocyanins and flavonoids, according to the method proposed by Di Stefano and Cravero (1991). Then, they were analyzed by means of HPLC-DAD to investigate anthocyanin, flavonol, and HCTA profiles, according to Squadrito et al. (2010). Finally, volatile organic compounds (VOCs) were determined by means of GC-MS, according to Corona (2010).

^{1}H-NMR Spectroscopic Analysis of wines

One milliliter of raw wine was analyzed by means of a Bruker Avance II 400 spectrometer operating at a proton Larmor frequency of 400.15 MHz. The ^{1}H-NMR spectra were acquired by applying the NOESYGPPS1D pulse sequence, to achieve water and ethanol signals suppression. 128 scans were used. Free induction decays (FID) were collected with a 64k time domain, a spectral width of 8012.82 Hz, a relaxation delay of 4 s and an acquisition time of 4 s. A line broadening of 0.3 Hz was applied to the exponential function used as filter to perform the Fourier transformation.

D_2O was used as an external reference to optimize the field frequency lock and the chemical shift reference. No quantitative internal standard was used. In order to maintain the absolute non-targeted character of the experiment and to avoid any kind of modification of the matrix, no artificial pH adjustment was carried out on wines. ^{1}H-NMR spectra were manually phased, and the baselines were manually corrected via the Whittaker smoother method by using MNova 14.2.3 software (Mestrelab Research, Santiago de Compostela, Spain). To correct vertical scale errors deriving from the residual water and ethanol signals, quantitative assessment of the spectra was done by normalizing to the total spectral area after having removed the spectral regions containing water protons signal (4.75 - 4.90 ppm) and ethanol methyl protons signal (1.15 - 1.20 ppm). The spectral region containing ethanol methylene protons signal (3.60 - 3.68 ppm) was not removed because it also contains signals produced by other metabolites present in wines.

Targeted analysis (TA): identification of compounds

Signal assignment in each spectrum was performed by comparison with the pure compounds spectra sourced from Biological Magnetic Resonance Data Bank (BMRB) (Ulrich et al., 2007) and Natural Products Magnetic Resonance Database (NP-MRD) (Wishart et al., 2022). The reference spectra were collected into a library by means of Simple Mixture Analysis (SMA) plug-in of MNova software, that identifies the compounds inside the spectra according to the signal chemical shift, signal multiplicity and relative coupling constants. The absolute integrals of the identified compounds were measured by means of qNMR plug-in of MNova software. The absolute integrals were used as indicative of the concentration since the area of a NMR signal is directly proportional to its concentration. Up to 69 different compounds were identified and quantified. The obtained dataset was subjected to the one-way analysis of variance (ANOVA) with Tukey's b post hoc test to highlight significant differences among wines. Differences with $p < 0.05$ were considered statistically significant. The ANOVA analysis was performed by means of Minitab™ statistical software (version 19.0 for Windows, Minitab, LLC, Pennsylvania State University).

Non-targeted analysis (NTA): data reduction and multivariate statistical analysis

The generation of the input variables was done via bucketing the spectra by means of MNova software. The bucketing was performed in the spectral range 0.5-9.5 ppm and divided the spectra into 890 buckets. The obtained dataset was imported into MetaboAnalist 5.0 web-based tool suite (Chong et al., 2018) to preprocess the data and to run multivariate statistical analysis. The preprocessing step consisted of log transformation, mean-centering, and Pareto scaling. To explore the chemical variability and to discriminate wines from grapes grown on different soils the unsupervised principal component analysis (PCA) was performed.

Wine-soil relationship analysis

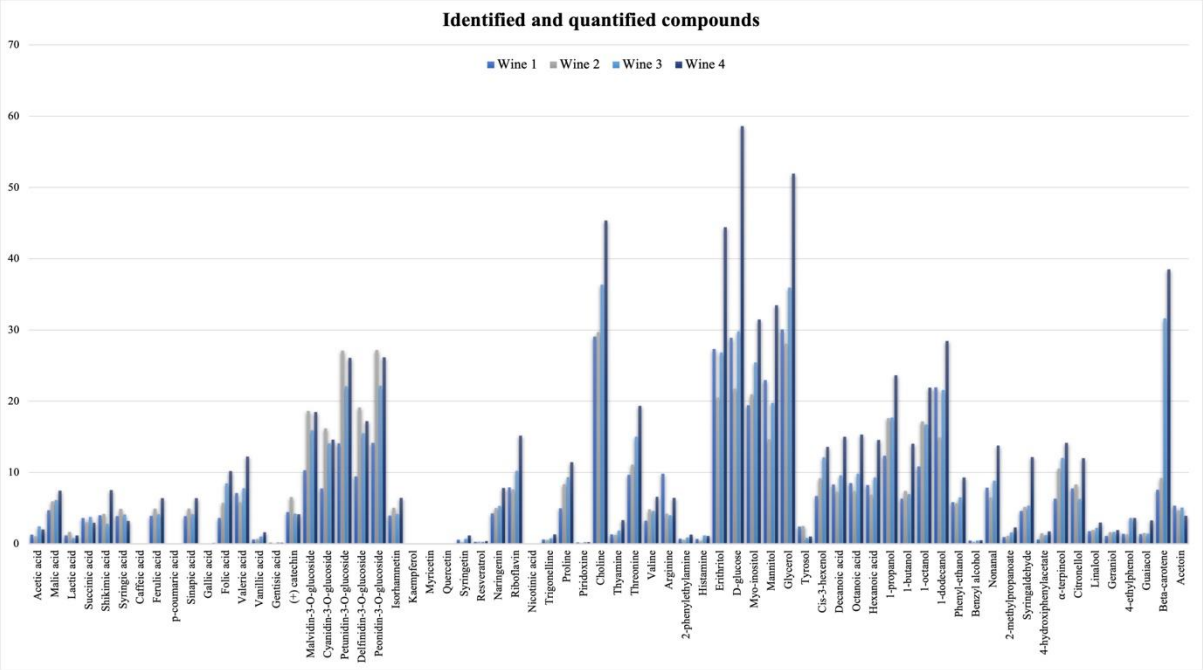
The data obtained from the TA were subjected, together with soil physical-chemical parameters, to the correlation analysis, to point out possible correlations. To visualize the existing relationships between soil and wine parameters a correlation heatmap was built (not shown). Then, the principal component analysis (PCA) provided a summary of the obtained information.

3. Results and discussion

NMR spectra evaluation: targeted analysis

The TA leaded to the *Nero d'Avola* wines *profiling* through the identification and quantification of 69 metabolites, all listed in Figure 1. The one-way analysis of variance (ANOVA) applied to the data matrix containing the absolute integrals of the identified compounds highlighted the existence of significant differences ($p < 0.05$) among the investigated wines. These differences involve organic acids, anthocyanins, flavonols, amino acids, polyols, and aroma compounds.

Figure 1. *Identified and quantified compounds*



NMR spectra evaluation: non-targeted analysis.

The NTA leaded to the *Nero d'Avola* wines *fingerprinting*. Principal component analysis applied to the spectral buckets reduced the number of original variables (890) into three principal components, PC1, PC2 and PC3, that, combined, accounted for 100 % of the total variance.

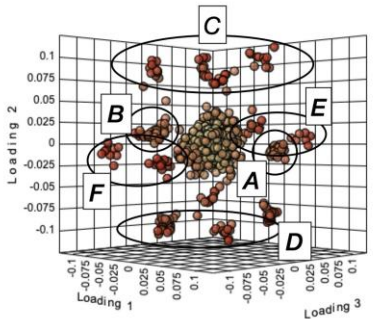
The 3D PCA loading plot shown in Figure 2 (A) revealed the fragments of spectra contributing mostly to each component. The A group included sinapic acid, p-coumaric acid, kaempferol, isorhamnetin, (+) catechin, 2-phenylethylamine, 4-ethyl-phenol, benzyl alcohol, 2-phenyl ethanol, riboflavin, and vanillic acid. The B group comprised higher alcohols (1-butanol, 1-propanol), volatile fatty acids (hexanoic, octanoic, decanoic acids), and monoterpenes (citronellol, linalool, and geraniol). The C group contained glycerol, mannitol, valine, threonine, and nicotinic acid. The D group contained caffeic acid, 4-hydroxy-phenyl acetate, and guaiacol. The E group included the flavonols quercetin, myricetin, and kaempferol. Finally, the F group comprised nicotinic acid and unidentified peaks.

The complementary 3D PCA scores plot, shown in Figure 2 (B), revealed a great separation among wine samples.

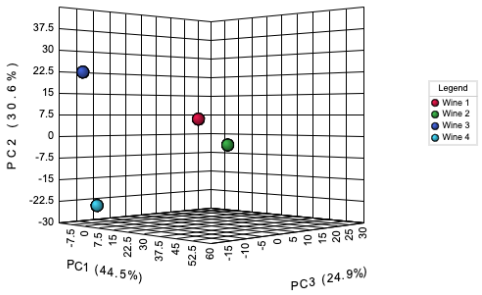
From an accurate visual inspection of chemical shift dispersions, it can be observed that spectral signals differ not only in terms of peak intensity but also for their chemical shift values. As a matter of fact, in the spectral portion hosting polyphenols (5.5-9.5 ppm), signals of wines 2 and 3 are misaligned with respect to wines 1 and 4. In particular, the signals in the spectrum of wine 2 are shifted towards higher chemical shift values, while those in

Figure 2. *(A) 3D PCA loadings plot of Nero d'Avola spectral buckets. (B) 3D PCA scores plot for the selected PCs. The explained variances are shown in brackets.*

A

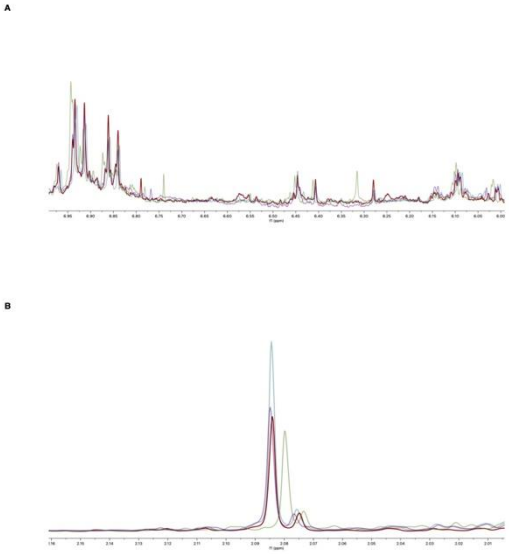


B



the spectrum of wine 3 are shifted towards lower values. Otherwise, in the aliphatic region (0.0-3.5 ppm), signals of wines 1, 3, and 4 are aligned to each other, while signals of wine 2 are shifted towards lower ones. Figure 3 shows the aforementioned ^{1}H-NMR chemical shift dispersion in a representative spectral portion of aromatic (A) and aliphatic (B) regions. Such chemical shift behavior cannot be explained by the sole difference in wine pH values. Indeed, ^{1}H-NMR chemical shift is extremely sensitive to intermolecular and intramolecular interactions, such as the hydrogen bonds. It was highlighted the existence of a correlation between hydrogen bond strength and chemical shift values. In particular, the strengthening of the hydrogen-bonding network corresponds to the enhancement of chemical shift values (and vice versa). The strength of hydrogen bonds depends on the ethanol content and on the nature of dissolved solutes (Nose et al., 2004). In particular, the hydrogen-bonding network is strengthened by small amounts of ethanol, while it is weakened when the amount of ethanol increases (Ickes et al., 2017). Acidic species strengthen the water-ethanol hydrogen bonds through proton donations. Polyphenols play a strengthening role inside the wine hydrogen bonding network. This depends on the structure of the polyphenolic compounds, and it is more effective as the -OH substitutions on the aromatic rings increase (Nose et al., 2004). Conversely, higher alcohols and esters seem not to exert any measurable effect on the H-bonding structure, while some inorganic anions (e.g., Cl^-, Br^-, NO_3^-) are known to weaken the structure of the hydrogen bond network (Nose et al., 2004; Conte, 2015). According to the abovementioned mechanism, we can hypothesize that wines 1 and 4, with aligned spectral signals, exhibit a very similar hydrogen bonding network. Conversely, wine 2 and wine 3, with misaligned spectral signals in the polyphenolic region, can be supposed to have different hydrogen bonding networks. In particular, in wine 2, polyphenols are involved in a stronger network of H-bond interactions, while in wine 3 the interactions were weaker. This, conceivably, depends on differences on the hydroxylation degree of the aromatic rings and, therefore, on polyphenols structure. On the contrary, given that in the aliphatic region the behavior of wine 2 signals is reversed, aroma compounds interact weakly with the matrix, as compared to wines 1, 3, and 4.

Figure 3. *^{1}H-NMR chemical shift dispersion in a representative spectral portion of aromatic region (A) and of aliphatic region (B). The red line represents wine 1, the green line represents wine 2, the light blue line represents wine 3, and the purple line represents wine 4.*

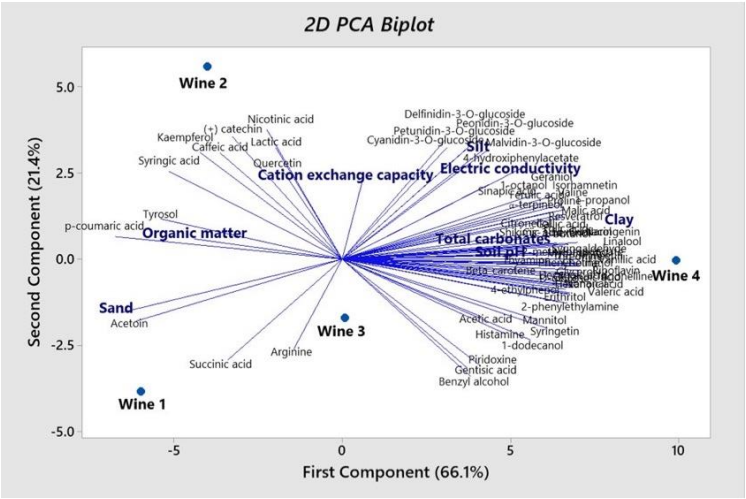


Changes in hydrogen-bonding structure seem to be related to modifications in wine sensorial quality (Nose et al., 2004). Some gustatory perceptions, such as mouthfeel and taste, as well as olfactory perceptions, that depend on aromas release, are supposed to be affected by the hydrogen bond network inside the wine. In fact, the type of interactions between solvent and solutes can modulate the way how solutes interact whit human sensorial receptors.

Wine-soil relationship

The correlation analysis performed on data obtained from the TA and on soil physical-chemical parameters highlighted the existence of significant correlations between wine composition and soil features. From the 2D PCA biplot shown in Figure 4 it can be observed that the first two components accounted for 87.5 % of the total variation in the dataset. PC1 was mostly driven by soil texture, as revealed by the fact that clay content leads the positive side while sand amount leads the negative side of PC1. Conversely, PC2 was mainly represented by the cation exchange capacity, the electric conductivity, and silt content, all contributing to the positive side of this principal component. Both the correlation analysis and the PCA revealed that large clay content in soils was significantly related to high concentrations of organic acids, flavonols, amino acids, polyols, fermentative and varietal aroma compounds. Conversely, high sand contents were significantly associated with low concentrations of hydroxycinnamic acids and aroma compounds. Finally, high silt contents were significantly related with high concentrations of anthocyanins. The

Figure 4. *2D PCA Biplot. The explained variances are shown in brackets.*



strong influence of soil texture on wine chemical composition is justified by its impact on several soil features, including nutrient adsorption capacity and water holding capacity. Soil texture affects water and nutrient dynamics primarily through its porosity. In fact, pore size distribution is directly affected by soil particle sizes. Clay-rich soils are mainly characterized by small-sized pores, or micropores. The micropore water is trapped into the soil system and it is scarcely available to plant nutrition. Conversely, big-sized pores, or macropores, are characteristic of sandy soils. Macropore water is rapidly drained away on behalf of air diffusion. This results in the leakage of dissolved nutrients. Finally, loamy soils are characterized by the presence of intermediate-sized pores, or mesopores, that can both retain and release water against gravity, making it available for plant nutrition. Grapevine development, yield, and berries composition are largely affected by water and nutrient dynamics in soil. In particular, high water and nutrient availability determine great vine vigor and canopy development. Great vegetative development and excessive leaves crowding determine low sunlight irradiation that causes low soluble solids accumulation in grape berries (Cortell et al., 2006). Clayey soils are characterized by low water and nutrient availability. Thus, they provide vines with low vigor, low canopy development, and, therefore, high sunlight irradiation for grape berries. Therefore, sunlight-induced metabolic processes are perturbated. Low water and nutrient availability also determine low berry weight and high skin weight per berry. Consequently, metabolites contained in skin cells, such as flavonoids and varietal aroma compounds, undergo to a concentration effect.
Soil texture is not the only soil parameter showing strong relationships with wine chemical composition. As a matter of fact, soils with high amounts of total carbonates and basic pH values produced wines with high contents of beta-carotene, cis-3-hexenol, and α-terpineol. Soil pH plays a pivotal role in the modulation of nutrient availability and, therefore, in soil fertility. To the best of our knowledge, no data are present in literature about aromas content dependence on soil pH. However, it appears likely that slightly high pH values play a stimulating role in the transcription of genes involved in aromas biosynthesis through the supply of some key nutrient.

4. Conclusions

This project achieved a better understand of the role of soil chemistry in wine sensorial quality. In particular, the effects of some soil chemical physical parameters, such as texture, pH, total carbonates, organic matter, cation exchange capacity and electric conductivity, on wine chemical composition were highlighted. The great relevance of these findings lies with the fact that, despite the crucial role played by soil in grapevine growth, only few information was available in literature about the influence of soil chemistry on wine quality. Moreover, for the first time ^{1}H-NMR-based metabolomic analysis has been applied to classify wines according to different soil types. Results obtained in this study highlighted that soil exerts a strong influence on wine chemical composition. Firstly, TA coupled with ANOVA unveiled significant differences among the concentrations of compounds detected in wines. Then, the NTA together with chemometrics, revealed that the differences among wines concerned not only the concentrations of the detected analytes but also the strength of the hydrogen bonds network in which the different compounds were involved. The correlation analysis carried out on soil physical-chemical parameters and wine compositional data revealed that high clay content increases the concentrations of organic acids, flavonols, amino acids, polyols, fermentative and varietal aroma compounds. A possible explanation to justify the observed correlations may lay in the effect of soil texture on water and nutrient dynamics in soils.

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Characterisation of new bio-protective and functional lactic acid bacteria isolated from spontaneously European fermented sausages

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This PhD thesis was focused on the study and development of innovative strategies to improve the quality and microbiological safety and to extend the shelf-life of fresh or fermented products. To this purpose, autochthonous lactic acid bacteria were isolated from traditional spontaneously fermented sausages. They were characterised for safety and technological features to select new bio-protective and functional cultures to valorise Mediterranean biodiversity through their application in food industry. Moreover, plant by-products and unconventional matrices were exploited to extract bio-active natural compounds with antimicrobial and antioxidant activities, that have been combined with strains to try to maximise the bio-protective effects.

Caratterizzazione di nuovi ceppi di batteri lattici bio-protettivi e funzionali isolati da salami Europei fermentati spontaneamente

Questa tesi di dottorato ha riguardato lo studio e sviluppo di strategie innovative volte a prolungare la shelf-life e migliorare la qualità e sicurezza microbiologica di prodotti freschi e fermentati, attraverso la valorizzazione della biodiversità di prodotti dell'area del Mediterraneo. In particolare, sono stati utilizzati salami tradizionali fermentati spontaneamente come fonte di isolamento di batteri lattici autoctoni. Successivamente al loro screening per aspetti di sicurezza e tecnologici, sono stati selezionati i ceppi più promettenti per applicazioni nell'industria alimentare. Inoltre, è stata testata la combinazione con composti naturali bio-attivi antimicrobici e antiossidanti, ottenuti da sottoprodotti vegetali, per massimizzare questi effetti bio-protettivi.

Key words: Bio-protection; bio-preservation; lactic acid bacteria; bio-active compounds; biodiversity valorisation; food wastes reduction.

1. Introduction

1.1 Food safety and sustainable strategies

The actual global population growth drove energy and food resources availability towards an ever-stronger pressure. In addition, in recent years there is an always increasing amount of food wastes in supply chains, that is strongly related to the microbial spoilage and resulted in a loss of food quality and microbiological safety (FAO, 2015). So, an improvement of these aspects and an extension of food shelf-life could be necessary to achieve an efficient waste reduction and an optimisation of the production yields, with a sustainable use of energy (Hoff, 2011). A strategy to obtain this goal is represented by Nexus approach, that promotes these concepts. Moreover, the widespread consumers demand for natural and innovative foods, as an alternative to synthetic ones, directed the research towards the study of new "green" and sustainable solutions, that need to be adapted regard the specific characteristics of the different food products. In particular, the attention is focused on bio-preservation and bio-protection techniques. Nowadays, a suitable solution against the use of synthetic additives is represented by bio-active natural compounds, that could be employed thanks to their strong antimicrobial and antioxidant activities (Calo et al., 2015). On the other hand, their application in food industry is limited by the possible organoleptic impact, that can alter the aroma profile of the products. To avoid this, a combination with other technologies could be employed, in order to reduce their amount.

1.2 Lactic acid bacteria in meat industry

In recent years, the use of LAB strains as starter cultures has become common in the meat industry, due to their technological features. This application allows an optimisation of the fermentations through a controlled process and a standardisation of the products, with a safety improvement. But, on the other hand, the typical characteristics of the traditional products can be impoverished due to the standardised application of a limited number of commercialised strains. Nevertheless, some local producers still achieve their products through a spontaneously fermentation and this represents an important heritage of biodiversity, also linked to the origin area. For this reason, these products can be used as a source of isolation of new autochthonous strains that are more suitable for adaptation in specific ecological niches. These could be applied in food industry, both as bio-protective cultures to enhance food safety and functional starters, to preserve this biodiversity and improve the peculiarities of traditional meat products. In particular, LAB strains can be characterised for both technological features, that make them able to be used as starter cultures, and bio-protection activities, due to their competition with spontaneous microbiota and foodborne pathogens and their ability to produce specific antimicrobial metabolites (bacteriocins), without affecting the organoleptic characteristics of the foods (Chikindas et al., 2018). The microbial spoilage and

safety concerns can be therefore hurdled with the application of these bio-protective and functional LAB strains, isolated from traditional naturally fermented sausages. This strategy could be used in combination with the addition of bio-active compounds to maximise the bio-protective effects and extend shelf-life of perishable foods, maintaining their peculiarities and organoleptic properties and reducing microbiological risks.

2. Experimental Procedure

During my PhD research project, two possible approaches to extend the shelf-life and improve the quality and microbiological safety of some fresh or fermented Mediterranean products were considered. In particular, the use of bioprotective cultures and bioactive compounds with antimicrobial properties were investigated. The experimental procedure is described in Figure 1.

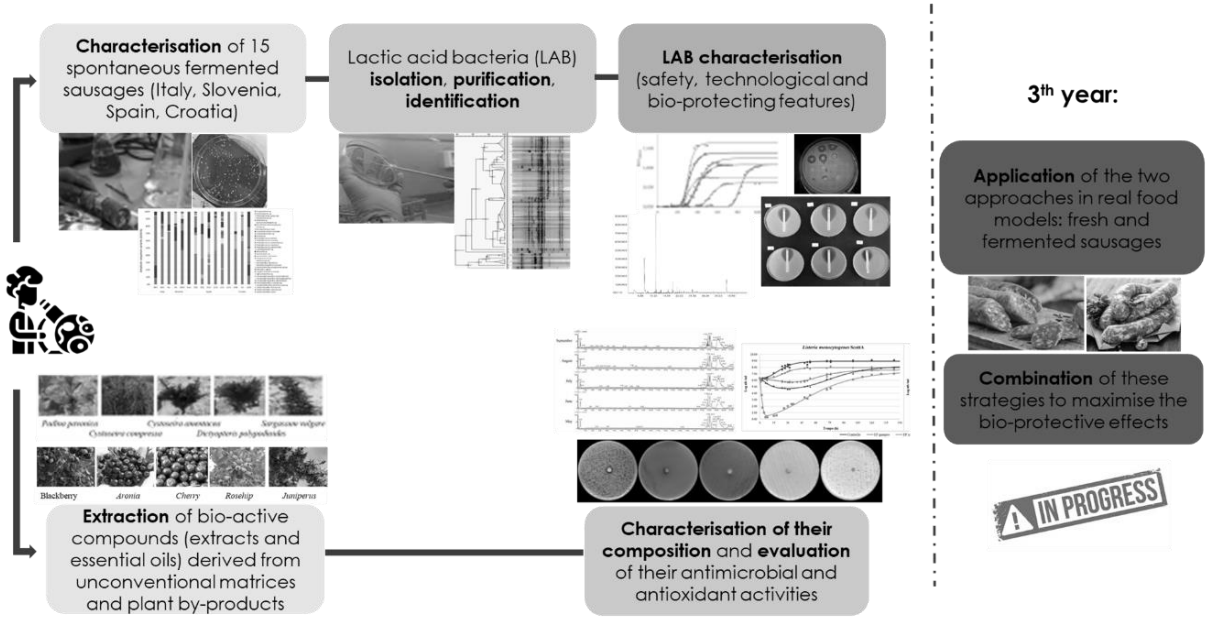

Figure 1 *Flow chart of the different laboratory activities performed during my PhD thesis.*

Fifteen artisanal spontaneously fermented sausages, that were previously characterised (Barbieri et al., 2021), were used as a source of isolation of presumptive LAB strains, that were then genetically identified. The core of the work was represented by the biotypes screening, firstly for their safety aspects and then for the technological features. In particular, the strains considered safe were tested *in vitro* for their antimicrobial activity against spoilage and pathogenic microorganisms. The most promising strains were selected for the technological potential, *i.e.* growth performances at different incubation temperatures and salt concentrations and aroma compounds production in meat batter. These screening were carried out with the final aim to select the best candidates to be applied in real food systems, such as fresh or fermented meat products or ready-to-eat foods, in order to achieve safety and food quality and preserve products peculiarity. Moreover, these strains have been combined with the use of bio-active compounds, in particular phenolic extracts and essential oils, obtained from plant by-products of traditional Mediterranean productions and endowed with antimicrobial and antioxidant activities.
In this oral communication I focused the attention on the characterisation of new bio-protective lactic acid bacteria isolated from spontaneously European fermented sausages and endowed with good technological properties. In particular, they were studied for their safety and their technological and bioprotective features and the most promising strains were selected to be tested *in vitro* against *Listeria monocytogenes*, a relevant foodborne pathogen, in order to evaluate their effect on the inhibition of its growth.

3. Materials and Methods

3.1 LAB strains characterisation for their safety aspects

After the genetic identification of the presumptive LAB, isolated from different spontaneously fermented sausages collected from Italy, Slovenia, Spain and Croatia (Barbieri, 2021), 141 biotypes were detected and tested for their safety aspects: in particular, antibiotic resistance profile and amino biogenic potential were evaluated. In the first case, the strains were assessed against 8 different antibiotics (Gentamycin = Gen, Kanamycin = Kan, Streptomycin = Str, Tetracycline = Tet, Erythromycin = Ery, Clindamycin = Clin, Chloramphenicol = Chlor, Ampicillin = Amp), according to EFSA indications (2012), and the minimum inhibitory concentration (MIC) was detected by using the protocol published by Dentice Maidana et al. (2020). Regarding the biogenic amines (BAs), Bover-Cid-

Holzapfel medium was employed to investigate the ability of the strains to produce histamine, tyramine, putrescine and cadaverine (Bover-Cid and Holzapfel, 1999). Then, the obtained samples were injected into HPLC to confirm results (Barbieri et al., 2021).

3.2 Antimicrobial activity of LAB strains considered safe

Based on the safety profile, some LAB strains were characterised for their bio-protective features. In particular, they were tested *in vitro* against foodborne pathogens or spoilage microorganisms to investigate their antimicrobial activity, assessing also the presence of bacteriocin-encoding genes (Dentice Maidana et al., 2020).

3.3 Characterisation of LAB strains for their technological features

The most promising strains, previously tested for their bio-protective traits, were finally analysed for some technological aspects. In particular, the acidification and the growth performances in relation to different salt concentrations (0%, 2.5% and 5%) and incubation temperatures (10°C, 20°C and 30°C) were evaluated. The data collected were modelled through the Gompertz equation (Zwietering et al., 1990). Moreover, the strains were characterised regarding their volatile organic compounds (VOCs) production, after a meat batter fermentation at 20°C for 48 h, by using GC-SPME-MS technique (Barbieri et al., 2021).

3.4 Evaluation of the effect of selected LAB strains and their cell free supernatants (CFSs) against *Listeria monocytigenes* growth kinetics

Based on the previous results about bioprotective features and technological characteristics, 3 strains were selected (two belonging to the species *Lat. sakei* (ZK39 and 2M7) and one to the species *Lat. curvatus* (KNK55) and tested *in vitro* against *Listeria monocytigenes* ScottA to evaluate their ability to inhibit its growth. Moreover, other two LAB strains belonging to *Pediococcus acidilactici* (SCT9) and *Lactiplantibacillus plantarum* (BPF2) species, isolated from additional Spanish products, were selected for this study due to their ability to produce interesting peptide compounds, which have not yet been identified, with a potential antimicrobial activity. The target strain was grown in BHI medium in the presence of LAB strains or with only their cell free supernatants. In particular, LAB strains were pre-cultivated in MRS medium at 20°C for 48 h and then inoculated in BHI medium at a final concentration of 6 log cfu/ml. In the same sample *List. monocytogenes* ScottA was inoculated at 3 log cfu/ml. The same cellular concentration was used in the case of the CFSs test. CFSs were collected after the growth of the selected LAB strains in MRS medium for 24 h at 30°C. Then the samples were centrifugated (10 min at 4000 rpm) and filtered at 0.22 µm with PES filters. CFSs were added the growth media at a ratio of 1:10 before target microorganism inoculum. The samples, incubated at 20°C, were collected overtime to assess growth performances, by using sampling onto selective media, and the obtained data were modelled through the Gompertz equation (Zwietering et al., 1990).

4. Results and Discussion

4.1 Evaluation of the safety aspects of the LAB biotypes detected

The previously genetic identification of the isolates showed the presence of 141 strains belonging to *Latilactobacillus sakei* (63.8%), *Latilactobacillus curvatus* (28.4%) and *Companilactobacillus alimentarius* (7.8%). These LAB strains were evaluated for their safety aspects. In the case of antibiotic resistance profile, the results showed a great variability based on the source of isolation: all the strains isolated from Italian and Slovenian samples were sensitive to the tested antibiotics, while more than half (57%) of biotypes isolated from Spanish and Croatian samples were resistant. In general, the resistance to streptomycin is widespread (33.1%), followed by tetracycline (17.4%), gentamycin (16.5%) and kanamycin (11.6%), even if a few strains carried at least one of the associated genetic determinants (tetM/tetS/ermB). Moreover, some strains were characterised by multiple resistance. Regarding the decarboxylase activity, as already observed for antibiotic resistance, the results were strongly dependent on the geographic origin of the isolates: the highest number of the amino biogenic strains were isolated from Spanish products, indicating an effect of raw materials and environmental conditions that apply a selective pressure on microbial populations and their metabolisms. However, this variability was based not only on the source of isolation, but also on the species. In fact, no BAs were detected among the *Lat. sakei* strains, while a high number of *Lat. curvatus* (67.5%) and *Comp. alimentarius* (63.6%) accumulated these compounds. Most of the amino biogenic *Lat. curvatus* strains were characterised by the accumulation of tyramine (19 out of 27) or putrescine (6 out of 27) and only one produced both BAs. Finally, one strain was characterised by histamine production. In the case of *Comp. alimentarius*, most of the strains produced tyramine, that sometimes was coupled with the production of histamine (3 strains) or putrescine (1 strain) and, again, one strains produced only histamine. Noteworthy, the spontaneously fermented sausages used as source of isolation of these biotypes presented BAs concentrations ranging from about 100 mg/kg to more than 1000 mg/kg.

4.2 LAB strains antimicrobial activity against foodborne pathogens or spoilage microorganisms

The strains considered safe (48), that were not able to produce BAs and sensible to the tested antibiotics (Figure 2), were characterised for their antimicrobial activity against *Escherichia coli* ATCC25922 and *Listeria innocua* UC8409 *in vitro*. The best results were observed for the strains isolated from Croatian products, that showed an

inhibition halo higher than 4 cm, probably due the ability to produce bacteriocins. In fact, these strains were resulted positive for the presence of possible sakacin or curvacin encoding genes.

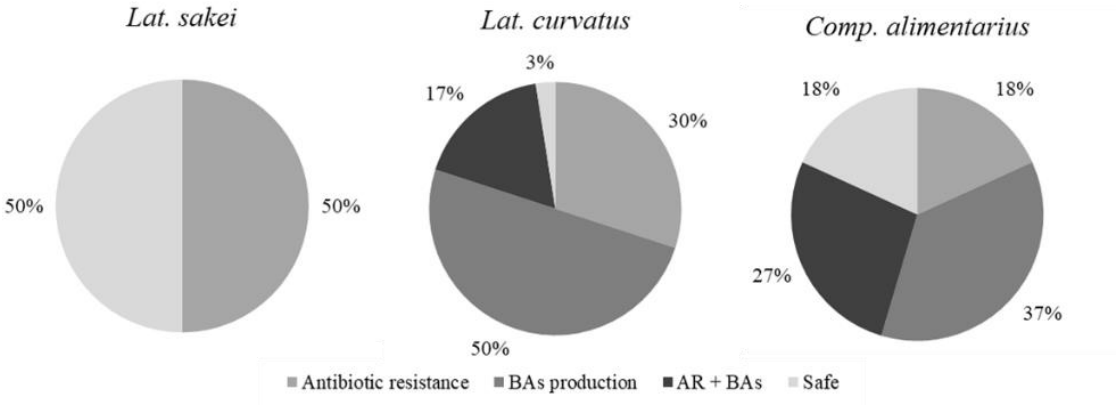

Figure 2 *Percentage resulted from the safety aspects investigation of the 141 LAB biotypes considered for the screening.*

4.3 Technological features characterisation

Based on the obtained results, the most promising strains were selected and characterised for their technological features. In particular, 23 biotypes, mainly belonging to *Lat. sakei*, were studied regarding their acidification and growth performances in the presence of different salt concentration and at different incubation temperature. The estimated parameters of growth kinetics evidenced an extreme variability among the strains of the same species, confirming *Lat. sakei* phenotypic diversity (Figure 3). However, as expected, there was a growth kinetics reduction in the case of increasing in salt concentration or decreasing temperature and these behaviours were characterised by a lower rate of growth with an increase in the lag phase. Moreover, the metabolic heterogeneity was highlighted also by volatile organic compounds (VOCs) analysis. The VOCs accumulation was mainly related to the metabolism of pyruvate (diacetyl and acetoin) and the beta-oxidation of fatty acids, that highlighted the activation of secondary metabolic pathways when fermentable sugars are depleted. Some strains were characterised by a low accumulation of these compounds and this could make them interesting candidates as bio-protective culture, due to their low organoleptic impact.

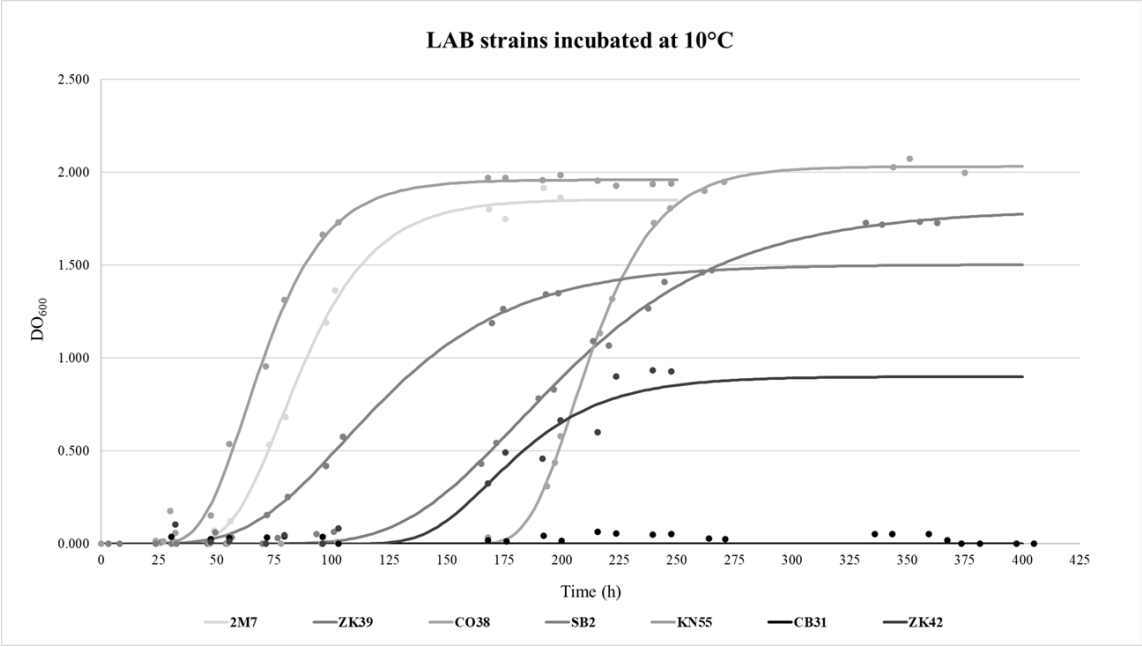

Figure 3 *Some growth kinetics profiles of LAB strains incubated at 10°C reported as an example to highlight the presence of an important phenotypic diversity among them.*

4.4 *In vitro* study of the effect of selected LAB strains and their cell free supernatants on *Listeria monocytogenes* growth kinetics

Another aspect considered in this PhD project was the study of the effect of selected LAB strains and their cell

free supernatants on *Listeria monocytogenes* ScottA growth kinetics. This microorganism was chosen as representative of foodborne pathogens because, due to its ability to grow in different conditions (*i.e.* low pH, low temperature) and in a wide array of environments as well as food matrices, it has become an emerging issue for human safety. In these trials, LAB viable cells or their CFSs were used. In the first case, the results showed that the best performances were observed in the presence of *P. acidilactici* STC9 and *Ltp. plantarum* BPF2. In fact, the effect of STC9 was characterised by a delay in the growth of *List. monocytogenes* with 3 times longer lag phase. In addition, the final cell load was at least 1 log cycle lower with respect to the control. BPF2, instead, stopped the growth of the target microorganism: the effect of this LAB strain against *List. monocytogenes* highlighted a bacteriostatic effect, that maintained the detected cellular level in the range of about 3 log cfu/ml. Regarding the other tested strains (KN55, ZK39 and 2M7), there was not a delay in the lag phase, but the growth speed in the exponential phase was reduced and, again, the maximum cells level achieved was about 7.7 log cfu/ml against 9.1 log cfu/ml in the control sample. On the contrary, the presence of CFSs obtained from *Lat. sakei* and *Lat. curvatus* did not affected the growth performances of *List. monocytogenes*, showing profiles similar to the control. However, the results confirmed the better activity also in the case of *P. acidilactici* and *Ltp. plantarum* CFSs. In both sample the detected level of *List. monocytogenes* was characterized by an initial preservation of the inoculum (3 log cfu/ml), that then dropped under the detection limit (< 1 log cfu/ml).

5. Conclusions and Future Perspectives

In conclusion, the traditional fermented sausages can represent an important source of autochthonous functional strains, that could be more suitable for adaptation in specific ecological niches. The application of the most promising strains in food industry depends on the possibility to achieve safety and food quality and preserve the typical characteristic of the products. So, to guarantee food safety and environmental sustainability of agri-food systems, different possible solutions are available, such as the valorisation of traditional foods, with the aim to find new bio-protective or functional strains. This strategy could be applied, also in combination with bio-active compounds, in food industry to maximise the bio-protective effects and extend shelf-life of perishable foods, maintaining their peculiar nutritional and organoleptic properties and reducing microbiological risks. On the basis of the results obtained until now in *in vitro* tests, the future trials will be focused on the application in real food systems (fresh and fermented sausages, or ready-to-eat foods) of the most promising strains, alone or in combination, to investigate the shelf-life extension and the improvement of the quality and microbiological safety of these products.

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Hyperbaric storage: An innovative and sustainable technology to extend stability and improve functionality of food

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This PhD thesis consisted of a multi-aspect investigation on hyperbaric storage at room temperature, which is an innovative approach to sustainable food preservation. In particular, the following aspects of the technology were considered: i) the capability of guaranteeing food hygiene and microbiological safety; ii) the possibility to steer the functionality of industrially relevant food proteins; iii) the effect on the kinetics and mechanism of food alterative phenomena (enzymatic and non-enzymatic browning); iv) the effect on food packaging materials.

Hyperbaric storage: Una tecnologia innovativa e sostenibile per estendere la stabilità e migliorare la funzionalità degli alimenti

Questa tesi di dottorato consiste in una ricerca multi-aspetto sull'*hyperbaric storage* a temperatura ambiente. Tale tecnologia rappresenta un approccio innovativo per la conservazione sostenibile degli alimenti. In particolare, sono stati considerati i seguenti aspetti della tecnologia: i) la capacità di garantire l'igiene e la sicurezza microbiologica degli alimenti; ii) la possibilità di orientare la funzionalità tecnologica di proteine oggetto di interesse nell'industria alimentare; iii) l'effetto sulla cinetica e sul meccanismo di sviluppo di fenomeni alterativi (*i.e.*, imbrunimento enzimatico e non-enzimatico); iv) l'effetto sui materiali per il confezionamento.

Key words: Hyperbaric storage, food hygiene and safety, protein structure and functionality, enzymatic activity

1. Introduction

Food storage under pressure has been studied during the last decade as a sustainable substitute for refrigeration. The technology, also known as hyperbaric storage (HS), consists of applying moderate hydrostatic pressure (P < 250 MPa) to foods packaged into plastic pouches and inserted inside steel autoclaves (Santos *et al*., 2020). As compared to refrigeration, the main advantage of HS is the capability to inhibit microbial growth in foods at a fraction of the energetic cost of cold storage (Bermejo-Prada *et al*., 2017; Jannasch *et al*., 1971). Recently, an increasing amount of evidence has surfaced, indicating that HS not only prevents growth of bacteria, but also induces substantial microbial inactivation (3-5 log reductions of Gram -, Gram + and sporogenic bacteria) and changes in structural and functional properties of food biomolecules (*i.e.*, proteins, lipids, enzymes, polysaccharides) (Santos *et al*., 2020). Although limited to safety and quality aspects solely, this basis of evidence allows to expand the scope of HS beyond that of sustainable food storage. HS could represent a multi-tasking technology, capable to concomitantly guarantee: (i) food pasteurization *via* microbial inactivation, (ii) functionality improvement *via* biomolecules structure modification, and (iii) food preservation *via* pressure-induced control of the kinetics of chemical and enzymatic alterations. Nevertheless, the technology has not been investigated from such standpoints yet. Moreover, the technical aspects of HS (*e.g.*, equipment design, feasibility of plastic packaging materials) still require adequate assessment before the technology can bridge the gap between research laboratories and the industrial context.

This Ph.D. thesis was intended as a multi-aspect investigation on hyperbaric storage. In particular, the technology was assessed for its multi-tasking character based on its capability to inactivate microorganisms, enhance food functionality, and control enzymatic and non-enzymatic browning. Attention was particularly paid to the influence of the native structural organization of proteins on the outcome of HS application, and on the relationship between protein structural changes and functionality. The same research approach was also adopted to study the effect of HS on food packaging materials.

In this presentation, the main results inherent to the effect of HS on food microbiological safety, protein functionality and enzymatic browning are critically overviewed.

2. Materials and Methods

2.1 Samples preparation and hyperbaric storage

Fresh hen (*Gallus gallus domesticus*) eggs and "Golden delicious" apples were purchased at a local retailer. Raw skim milk was obtained at a local dairy processing plant. Egg white and egg yolk were manually separated and homogenized by gentle stirring to obtain the samples. Apple juice samples were prepared according to Manzocco *et al*. (2013) with minimal modifications. Polyphenoloxidase (PPO) model solutions were prepared by dissolving mushroom tyrosinase (5771 U/mg, Sigma Aldrich, Milan, Italy) in 0.01, 0.05 and 0.1 M pH 4.5 sodium acetate and pH 7 potassium phosphate buffers. Samples were heat-sealed into polypropylene/ethylene-vinyl-alcohol/polyethylene pouches (Niederwieser Group S.p.A., Campogalliano, Italy) and subjected to HS in a pilot-scale working unit assembled by Comer Srl. (Bologna, Italy). The latter consisted of a water-tight steel autoclave (Hystat, Slaithwaite, Huddersfield, United Kingdom), filled with an aqueous solution containing 0.2% (w/w) potassium sorbate and 0.2% (w/w) sodium benzoate (Carlo Erba Reagents Srl, Milan, Italy), and pressurized by a Haskel International high-pressure pump (Burbank, CA, USA). Control samples were maintained under refrigerated (4 °C) or room temperature conditions (20 ± 1 °C) at atmospheric pressure (0.1 MPa).

2.2 Microbial counts

Total bacteria count (TBC) was evaluated by enumeration on Plate Count Agar (Oxoid, Milan, Italy) after incubation at 30 ± 1 °C for 48-72 h. *Salmonella enterica* subsp. *enterica* 9898 DSMZ, *S. aureus* 226 and *E. coli* 8048 were inoculated in egg white, egg yolk and raw skim milk and counted during HS. Counts of *Salmonella enterica* and *S. aureus* were performed after incubation at 37 ± 1 °C for 24-48 h onto Plate Count Agar (Oxoid). *E. coli* was counted on ColiID (bio-Merieux, Grassina, Italia) after incubation at 37 ± 1 °C for 24 h.

2.3 Physical properties, protein structure and technological functionality

Samples physical properties and protein structure was evaluated by determination of colour (tristimulus colorimeter), thermal properties (differential scanning calorimetry), infrared absorbance spectrum (FT-IR), particle size and zeta potential (dynamic light scattering). Samples protein fractions were further analysed for their structure by quantification of free SH groups, determination of absorbance at 280, 380 and 680 nm, and reverse phase-high performance liquid chromatography (RP-HPLC). Samples technological functionality was assessed by evaluating viscosity, solubility, and foaming, gelling, and emulsifying properties. Analyses were performed as described by Basso *et al*. (2021, 2022).

2.4 Enzymatic activity

PPO activity was assessed as described by Manzocco *et al*. (2013). The enzymatic unit (U) was defined as the amount of enzyme leading to 0.001/min increase in absorbance (420 nm) by adding 20 µL sample in 1980 µL of $1.5 \cdot 10^{-3}$ M L-Dopa (Sigma Aldrich).

2.5 Data analysis

Microbiological and RP-HPLC analyses were performed in single on two independent experiments and in duplicate, respectively. Other data were obtained by at least triplicate measurements. Data were reported as mean ± standard deviation and subjected to one-way analysis of variance (ANOVA) and Tukey's Honest Significant Differences test ($p<0.05$) using R for Windows (The R foundation for statistical computing).

3. Results and Discussion

3.1 Antimicrobial efficacy of hyperbaric storage

The efficacy of HS in guaranteeing food microbiological quality and safety was evaluated in egg white, egg yolk, apple juice, and raw skim milk (Table 1).

Table 1 *Time required for HS inactivation (t_i) below the detection limit (L.o.D.) of total bacteria count (TBC) and inoculated microorganisms in egg white, egg yolk, apple juice, and raw skim milk.*

Sample	Hyperbaric storage P (MPa)	TBC* Initial load	t_i (h)	*E. coli*** Initial load	t_i (h)	*S. aureus** Initial load	t_i (h)	*S. enterica** Initial load	t_i (h)
Egg white	200	N.a.	N.a.	N.a.	N.a.	3.96 ± 0.20	24	3.50 ± 0.07	3
Egg yolk	200	N.a.	N.a.	N.a.	N.a.	2.78 ± 0.19	48	3.35 ± 0.12	24
Apple juice	200	1.70 ± 0.05	24	N.a.	N.a.	N.a.	N.a.	N.a.	N.a.
Skim milk	150	3.89 ± 0.16	144	5.13 ± 0.33	48	5.66 ± 0.93	144	N.a.	N.a.

P: pressure; Initial load expressed as logCFU mL^{-1}; *L.o.D. = 1 logCFU g^{-1}; **L.o.D. = 0 logCFU g^{-1}; N.a. Not analyzed.

Egg white and yolk, which are sterile in normal conditions, were inoculated with *Salmonella enterica* and *S. aureus* (3-4 logCFU mL^{-1}) to simulate a process-related contamination. HS promoted the inactivation of both bacteria below the detection limit within few days (Table 1). In particular, *Salmonella enterica* was inactivated after just 3 hours at 200 MPa in egg white, whereas it took up to 1 day to reach the same effect in egg yolk. The higher

microbial resistance to pressurized conditions in egg yolk as compared to egg white was probably due to its richer composition, which probably exerted a protective effect on bacterial cells. As compared to *Salmonella enterica* (Gram -), *S. aureus* (Gram +) showed a far higher pressure resistance, which was most definitely due to its thick peptidoglycan layer.
The naturally present microflora of both apple juice and raw skim milk (*i.e.*, TBC) was completely inactivated upon HS, indicating the capability of the technology to guarantee optimal hygienic conditions regardless of food pH and microbial susceptibility (Table 1). The possibility of using HS as a novel approach for food pasteurization was also investigated in raw skim milk inoculated with *E. coli* and *S. aureus* (5-6 logCFU mL^{-1}) (Basso *et al.*, 2022). In this case, 150 MPa-HS completely inactivated *E. coli* and *S. aureus* below the detection limit after 2 and 6 days, respectively. This inactivation resulted to be irreversible, as demonstrated by no microbial recovery during 12 days of milk storage under refrigerated conditions (data not shown).
Results indicated that HS could be efficaciously used not only as a sustainable alternative to refrigeration, but also as an innovative and *quasi*-energy-free approach to achieve food decontamination and pasteurization.

3.2 Effect of hyperbaric storage on proteins

Based on the promising antimicrobial efficacy of HS, the possibility to apply the technology to steer the functional properties of egg white, egg yolk and raw skim milk was further investigated. Results allowed to summarize the main effects of HS on both protein structure and technological functionality of the tested foods (Table 2).

Table 2 *HS effects on the physical, structural and functional properties of egg white, egg yolk and raw skim milk.*

Matrix	Protein	Structure	Hyperbaric storage Pressure (MPa)	Time (days)	Structural effects	Functional effec	References
Egg white	Ovalbumin	Globular	200	28	Compression and electrical stabilization	Viscosity and foaming increase	Basso *et al.*, 2021
Egg Yolk	Apolipoprotein	Lipoproteins	200	28	Unfolding and swelling	Pressure-induced gelling	Basso *et al.*, *under review*
Skim milk	Casein and whey protein	Micellar and globular	150, 200	6	Casein-globulin complexation	Enhanced foaming capacity	Basso *et al.*, 2022

Egg white globular proteins (*e.g.*, conalbumin, ovalbumin) showed slight structural modification during pressurized storage, leading to an increase in viscosity and foaming properties. On the other hand, egg yolk proteins, which are primarily found embedded in lipoproteins membranes, were severely unfolded under the same pressure conditions, resulting in a fully gelled matrix after 28 days (Table 2) (Basso, 2021).
In the case of milk proteins, which occur either in highly organized micellar structure or as globular solvated proteins, the effects of HS were more complex (Table 3).

Table 3 *Size of micelles and submicelles, β-lactoglobulin concentration, proteose peptones RP-HPLC peak area and foaming capacity of raw skim milk during storage under refrigerated (0.1 MPa, 4 ± 1 °C) and hyperbaric (150 MPa, 20 ± 1 °C) conditions.*

Storage	Time (days)	Micelle size (nm)	Submicelle size (nm)	β-lactoglobulin concentration (g L^{-1})	Proteose peptones peak area (Arbitrary units · 10^7)	Foaming capacity (%)
Fresh	0	169.1 ± 2.6^{d}	N.d.	2.38 ± 0.28	1.40 ± 0.01	72.5 ± 4.4^{d}
Refrigerated	6	N.a.	N.a.	2.22 ± 0.08	2.27 ± 0.03	92.8 ± 5.1^{c}
Hyperbaric	1	237.0 ± 4.0^{c}	N.d.	0.44 ± 0.10	1.70 ± 0.21	119.5 ± 7.9^{b}
	2	275.8 ± 7.9^{b}	52.1 ± 8.0^{a}	0.56 ± 0.00	3.43 ± 0.20	123.4 ± 8.5^{b}
	5	377.9 ± 11.0^{a}	51.1 ± 2.8^{a}	N.a.	N.a.	N.a.
	6	371.1 ± 8.1^{a}	N.d.	0.11 ± 0.08	4.97 ± 0.08	267.3 ± 15.7^{a}

N.d. Not detected; N.a. Not analyzed; [a] Different letters in the same column indicate statistically different means ($p<0.05$; ANOVA).

Upon HS at 150 MPa for up to 6 days, milk casein micelles and globular whey proteins were highly destabilized. Dynamic light scattering and RP-HPLC analyses actually highlighted the release of sub-micellar particles (~ 50 nm) and a decrease in β-lactoglobulin content during 150 MPa-HS (Table 3). Consequently, destabilized casein micelles served as local aggregation points for unfolded β-lactoglobulin molecules, leading to a progressive increase in micelles size (Table 3). HS-destabilized casein micelles were also more susceptible to the attack of proteolytic enzymes (*i.e.*, plasmin), as clearly indicated by the enhancement of proteose-peptones, which typically derive from casein enzymatic hydrolysis (Table 3). Due to the extensive unfolding of β-lactoglobulin and enhanced formation of proteose peptones, HS increased the foaming capacity of milk to almost 4-fold that of fresh milk (Table 3). To this regard, it is reasonable that the formation of proteose-peptones was the primary driver of this

improvement, due to their exceptional bubble-stabilizing activity. Actually, a strong positive correlation (r=0.9085) was found between milk foaming capacity and proteose-peptones RP-HPLC peak area.
Results indicate that HS could be regarded as a novel approach to steer functional properties of protein-rich foods, with effects substantially influenced by the native organization of proteins.

3.3 Effect of hyperbaric storage on polyphenoloxidase activity

Based on the capacity of HS to modify protein structure and functionality, HS was further evaluated for its ability to inactivate food-spoiling enzymes. To this aim, PPO was specifically considered based on its well-known browning effect in fresh fruit derivatives viable for HS (*e.g.*, fresh-cut vegetables, fruit juices). The effect of HS at 100 and 200 MPa was firstly studied on PPO activity and color of apple juice, using samples kept under room conditions as a reference (Table 4).

Table 4 *PPO activity, luminosity and redness of apple juice during storage under room (0.1 MPa, 20 ± 1 °C) and hyperbaric (100, 200 MPa, 20 ± 1 °C) conditions.*

Storage	Time (h)	PPO residual activity (%)	Luminosity (L*)	Red point (a*)
Fresh	0	100.00 ± 0.00^{a}	67.12 ± 0.84^{a}	1.13 ± 0.14^{c}
0.1 MPa	24	71.43 ± 4.04^{b}	53.87 ± 1.86^{cd}	2.78 ± 0.53^{bc}
	48	57.14 ± 8.08^{c}	46.14 ± 2.95^{d}	7.00 ± 0.79^{a}
	144	19.05 ± 0.00^{e}	39.29 ± 1.12^{e}	6.39 ± 0.06^{a}
100 MPa	24	73.87 ± 3.12^{b}	N.a.	N.a.
	48	40.54 ± 3.82^{d}	N.a.	N.a.
	144	0.54 ± 2.36^{f}	N.a.	N.a.
200 MPa	5	51.43 ± 0.00^{cd}	62.11 ± 1.38^{b}	2.93 ± 0.35^{b}
	48	5.71 ± 0.00^{f}	53.76 ± 1.45^{cd}	2.49 ± 0.35^{bc}
	144	1.05 ± 1.98^{f}	53.82 ± 0.61^{c}	2.63 ± 0.08^{bc}

N.a. Not analyzed; [a] Different letters in the same column indicate statistically different means ($p<0.05$; ANOVA).

HS allowed for a much faster enzyme inactivation than room pressure storage. In addition, PPO inactivation was pressure-dependent, being significantly faster at 200 than at 100 MPa. As a result, HS allowed to slow down the browning of apple juice, whose change in luminosity (L*) and redness (a*) was significantly lower when stored under pressure (Table 4). Based on these results, HS would not only limit food microbial spoilage, but also prevent the quality decay deriving from enzymatic activity.
The effect of HS was further tested on the activity of mushroom tyrosinase (*i.e.*, PPO) in buffered solutions to understand the mechanism of PPO inactivation under pressure and the influence of environmental conditions. Firstly, the influence of enzyme crowding was tested by storing at 200 MPa solutions containing different amount of PPO (2, 6, 14, 26 U). The effect of HS on PPO activity resulted significant only in the 2 U samples (data not shown). In accordance with the literature, these results indicate that enzyme inactivation probably occurs following a structural unfolding mechanism, which takes place only if enzyme molecules have sufficient solvent volume around them. The influence of the environmental composition was then assessed by pressurizing 2 U PPO solutions at different pH and ionic strength (Figure 1). Results clearly indicate that the effect of pressure was particularly significant at neutral pH (Figure 1 A, B, C) and that the enzyme was substantially more stable at higher ionic strength. These results might be due to the stabilizing effect of acidic pH and salt ions on the molecular structure of PPO, which would make enzyme unfolding more difficult.

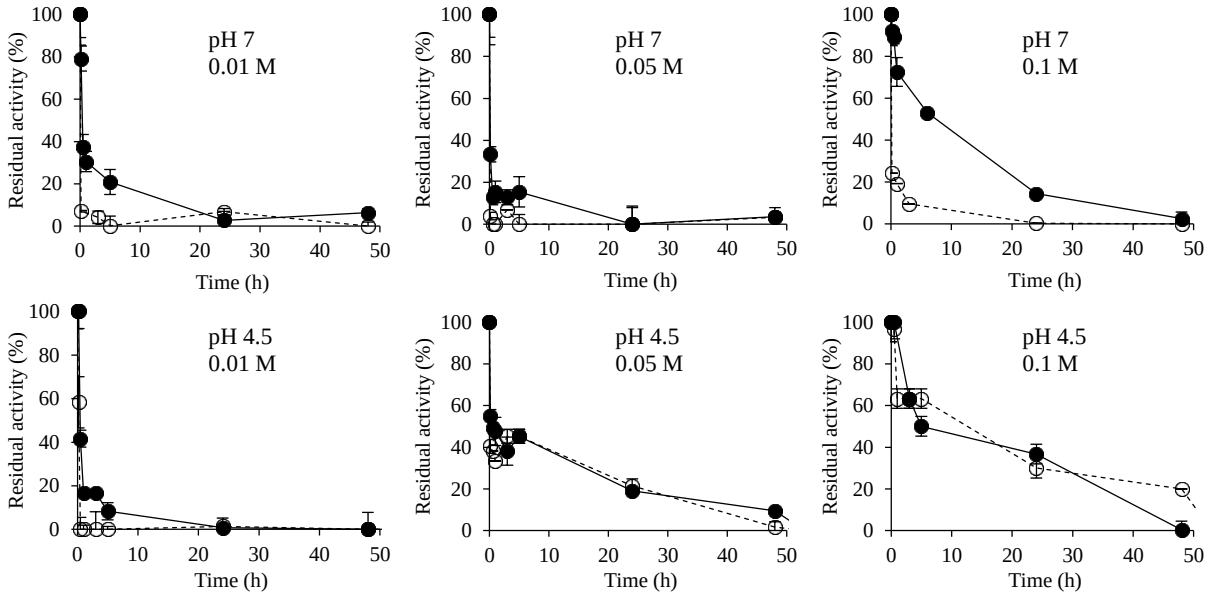

Figure 1 *Mushroom PPO activity at different pH (4.5 and 7) and ionic strength (0.01, 0.05 and 0.1 M) during storage under room (0.1 MPa, 20 ± 1 °C; ●) and hyperbaric (200 MPa, 20 ± 1 °C; ○) conditions.*

These results confirm the role of HS-induced unfolding in the loss of PPO activity during pressurized storage as well as the critical role of the electrical landscape of the enzyme.

4. Conclusions and future perspectives

The results acquired with this Ph.D. thesis clearly indicate that HS represents a promising innovative non-thermal food technology. Besides its sustainability, HS has a multi-purpose character, based on its concomitant capability to preserve, pasteurize, functionalize, and control alteration in food. This evidence strongly supports the urge to fulfill the research gaps that currently prevent the widespread application of the technology in an industrial context. To this aim, future research on HS should adopt a multi-disciplinary approach to boost TRL by aligning data achieved at laboratory level with those relevant to pilot and industrial scale. In particular, attention should be especially paid to the design of cost-effective steel or composite autoclaves, and to the development of mathematical models allowing to implement automated HS storage/treatment cycles.

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Use of non-destructive analysis techniques for the technological and chemical-physical characterization of fruit and vegetables and for monitoring of the drying process

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The present study aims to improve the competitiveness of the "Patata dell'Alto Viterbese" PGI (Protected Geographical Indication) (Latium, Italy) through i) the characterization and identification of cultivars suitable to be processed into fresh-cut and semi-dried products; ii) the optimization of the semi-drying process of sliced potato; and iii) the development of NIR-based models for the prediction dry matter content of tubers in pre- and post-harvest. The study identified Fontane as the cultivar more suitable for processing. The foundations were laid for a quality semi-dried product. NIR models showed high predictive performance on 15+ cultivars of the PGI area.

Impiego di tecniche di analisi non distruttive per la caratterizzazione tecnologica e chimico-fisica di ortofrutticoli e per il monitoraggio del processo di disidratazione

Il presente studio mira ad incrementare la competitività della "Patata dell'Alto Viterbese" IGP mediante: i) la caratterizzazione e la selezione di varietà idonee alla trasformazione in prodotto di IV gamma e/o semi-disidratato; ii) la ottimizzazione del processo di semi-disidratazione della patata a fette; iii) la messa a punto di modelli predittivi NIR per l'analisi della sostanza secca del tubero in pre-/post-raccolta. La cultivar Fontane è risultata idonea ad essere impiegata sia in IV gamma che in semi-disidratazione. Sono state poste le basi per un semi-disidratato di qualità. I modelli NIR hanno mostrato elevate prestazioni predittive su 15+ cultivar dell'areale IGP.

Keywords: *Solanum tuberosum* L., cultivar selection, dried products, NIR spectroscopy, chemometrics.

1. Introduction

According to the PhD project workplan, the oral communication reports the main results of the following activities:

(A1) Development of the research strategy; (A1.1) study of the state-of-the-art of cultivar selection for fresh-cut and dried potato processing, (A1.2) test and selection of fit-for-purpose classical and non-destructive analytical methods and (A1.3) preliminary tests for the optimization of the semi-/drying process parameters;
(A2) Characterization and selection of potato cultivars for their suitability as fresh-cut and semi-dried products;
(A3) Optimization of semi-drying process of potato slices using Smart drying technologies;
(A4) Development of Fourier Transformed NIR-based models for the prediction of dry matter content in potatoes, studying the possible impact of tuber tissue on the prediction performances.

2. State of art

Potato (*Solanum tuberosum* L.), one of the most widely grown crops in the world, is a staple food in many developed and developing countries. Nowadays, due to the consumer demand for high-convenience foods, fresh-cut and semi-/dried potatoes attract attention from the food industry. It is well-known that the quality of processed potatoes is strongly affected by both pre-and post-harvest factors. Among them, the genotype has a crucial role (Cabezas-Serrano *et al.*, 2009). In fact, potato cultivars for industrial processing (e.g., cutting, drying, frying, etc.) need to have specific physicochemical and enzymatic characteristics: i) as high dry matter (DM) content as possible (> 20 %), ii) good firmness and textural properties, iii) low susceptibility to post-cut enzymatic browning; and iv) low reducing sugar content, which is related to the occurrence of both non-enzymatic browning (i.e. Maillard's reaction) and acrylamide production during frying or roasting (Torres and Parreño, 2016). Considering all these aspects, the characterization and selection of cultivars, as well as the choice of proper chemical and/or physical treatments to the raw material, are crucial to obtain a high-quality product available all year round. In addition, process optimization, through monitoring and control, plays a key role to simultaneously ensure the quality and safety of the product and sustainability of the supply chain. In this context, the Process Analytical Technologies (PATs) are really promising for the food sector. They involve the use of tools (e.g., computer vision, near infrared spectroscopy, etc.) and practices for both real-time monitoring of food quality and dynamic control of process unit (Moscetti *et al.*, 2017).

In this context, the doctoral study aims to innovate the potato sector, with specific regards to the "Patata dell'Alto Viterbese IGP" consortium (Viterbo, Italy), by developing solutions to improve its competitiveness at regional and national level, through the resolution of the following specific issues: i.e., i) poor shelf-life of fresh-cut potatoes

and subsequent limited acceptance by large-scale retailers; ii) scheduling failures in tuber processing during storage due to poor cultivar characteristics records; iii) lack of knowledge for the production of semi-dried potatoes; iv) slowness in decision-making due to the non-timely monitoring of the maturity stage (DM content) of tuber in pre- and post-harvest, carried out by slow standard methods (e.g., oven and gravimetric methods) that cannot be performed *in-situ*.

3. Materials and Methods

The study was conducted on potato tubers (*Solanum tuberosum* L.) provided by the "Patata dell'Alto Viterbese IGP" consortium. The analyses were repeated along two production seasons (i.e., 2020-2021 and 2021-2022).

3.1 Physicochemical and enzymatic characterization during storage

Four potato cultivars were selected through preliminary tests: Fontane, Constance and Jelly as potential best cultivars and Gaudi as control. Tubers were sampled and analyzed every month for 8 months of storage at 5 °C, using ethylene as a sprout inhibitor. Samples were characterized for their i) dry matter content (DM); ii) total phenols content (TPC, mg of gallic acid equivalents per 100 g of dry weight); iii) reducing sugars content (RSC, mg of glucose equivalents per g of dry weight); iv) firmness (newton); v) post-cut color change (ΔE*, color difference developed after 2 h of air exposure); (vi) browning index (BI, Δabs after 2 h of incubation); and (vii) PPO enzyme activity (enzyme unit, 1 UEA was defined as an increase in absorbance of 0.001 min^{-1}). Data were subjected to one-way ANOVA and post-hoc test (LSD) ($p \leq 0.05$) using R software v4.1.0. Principal Component Analysis (PCA) was applied to evaluate inter-cultivar similarity.

3.2 Semi-drying tests

The cultivar selected during 3.1 activity was subjected to semi-drying tests. The following pre-drying treatments were tested on potato slices (6 mm thick): i) blanching at 90 °C for 30, 60, 90 and 120 sec; and ii) dipping in a) citric acid (CA) and b) sodium metabisulphite (SMB) solutions at 3 and 0.1 % (w/v) concentrations, respectively, for 5 min. The product:solution ratio was 1:10 and 1:5 (w/v) for blanching and dipping, respectively. Subsequently, potato slices were dried at 35 °C, 2.98 m s^{-1} air velocity, and 35 % R.H. using a smart drying unit. The process was stopped at a wet-basis moisture content of 60 %. The smart dryer consisted of i) a cabinet drying chamber; ii) a monitoring and control device mod. DICON touch (JUMO, Germany); iii) a load cell mod. PC42 (FLINTEC, Germany); and iv) a computer vision (CV) system placed in a black box on the top of the drying chamber. The CV system consisted of i) a CMOS camera mod. DFK 33UX264 (The Imaging Source Europe GmbH, Germany) equipped with a lens C 1/1.2, 8 mm, F/2.4; and ii) an illumination source of four LED strips (4200K). A Raspberry Pi 4 computer was connected to each device to acquire data in real-time on product (moisture loss; drying rate; color, shape and size changes) and process (temperature, air velocity and R.H.). Data were analyzed with the aim of selecting a drying pre-treatment capable of ensuring a high-quality dried product and predict the optimal drying time through CV as PAT tool. For the intended purpose, a combination of Matlab, Python, and R scripts was used.

3.3 FT-NIR-based predictive models for dry matter determination

The study was performed on 15+ cultivars to ensure data representativeness in terms of DM content (~8-25 %). Spectra were acquired using an FT-NIR spectrophotometer mod. Antaris II (ThermoScientific, USA) with a spectral range of 10000-4000 cm^{-1} (4 cm^{-1} resolution). The scans were performed on the following potato tissues: i) periderm; ii) cortex; iii) peripheral and iv) central parenchyma. Each scanned area was sampled, and oven dried at 105 °C for 72 h for DM analysis. Partial Least Squares (PLS) and interval PLS (iPLS) algorithms were used for developing DM prediction models based on the full spectrum and features selection, respectively. Models were developed by splitting data into calibration (70 %) and prediction (30 %) sets. A 10-split "Venetian blind" cross-validation was adopted. iPLS features selection was performed in stepwise-forward mode to select 10 intervals of 10 features each. The following spectral pretreatments were tested: Standard Normal Variate (SNV); Multiplicative Scatter Correction (MSC); and Savitzky-Golay 1st and 2nd derivatives with a 2nd or 3rd order polynomial fitted over a window of 11, 13 or 15 features. Mean-centering (MC) and Autoscaling (AS) normalization were also tested. Performances were evaluated in terms of the Root Mean Squared Error (RMSE), BIAS and R^2.

4. Results and Discussion

4.1 Physicochemical and enzymatic characterization during storage

The results obtained during the first production season revealed 'Fontane' as a cultivar subjected to less compositional changes during storage. Compared with the other tested cultivars, Fontane reported i) the highest DM content (~ 20 %) with no significant changes during storage, ii) the lowest TPC and RSC, and iii) the highest firmness. Jelly scored the second-best position, with good DM and firmness, but RSC and TPC values higher than Fontane. Fontane and Jelly had the lowest susceptibility to post-cut browning. The control cultivar (Gaudi) showed the highest ΔE*, BI, TPC and PPO activity values along storage, except for months 7 and 8 when the tubers sprouted, leading to a drop of about 70 % in both PPO activity and ΔE*. The Constance cultivar was characterized

by the highest RSC, and a PPO activity value lower than Gaudi, which however was higher than Fontane and Jelly. Consequently, Constance was also discarded as a potential candidate as fresh-cut or semi-dried product.

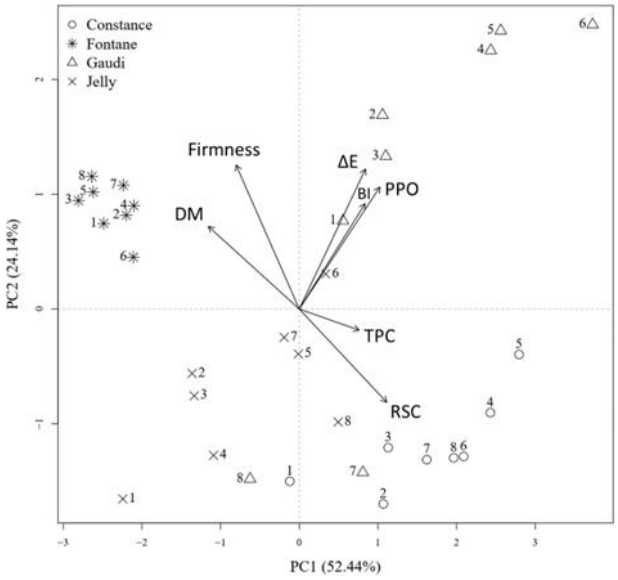

Figure 1. *Biplot of Principal Component Analysis computed using cultivar characterizations data acquired during storage.*

All the aforementioned results are summarized in Fig. 1, which showed the performance of each cultivar. The first two Principal Components (PCs) explained the 76.58 % of total variance. The biplot allows to identify pattern and cluster for each cultivar. In particular, Fontane (⁕) showed a tight cluster, positively correlated with both DM and Firmness loadings, and negatively correlated with both RSC and TPC loadings. The result highlighted the highest stability of the cultivar during the eight months. By contrast with Fontane, Constance (○) showed a positive correlation for RSC and TPC and a negative correlation for DM and Firmness. The control cultivar, i.e., Gaudi (△), showed a strong positive correlation with post-cut browning factors up to the 6th month (i.e., before sprouting). Jelly (×) was the only variety with a strong negative correlation with the susceptibility to browning, although it was positively correlated with RSC. Table 1 summarizes the most significant differences between the best (i.e., Fontane) and the control (i.e., Gaudi) cultivars, reporting the trends of tuber characteristics over storage months. At the harvesting time, Gaudi showed significantly higher values for all reported parameters. Over the storage period, the two cultivars showed different trends in all of them. Specifically, for TPC, Fontane showed a significant decrease after 1 month, while it notably increased in Gaudi close to sprouting. Regarding RSC, Fontane remained at an acceptable concentration throughout the storage period, while Gaudi exceeded the acceptability threshold (i.e., 8-10 mg/g d.w) after only 1 month, reaching the highest value at the 7th month. Regarding factors affecting the post-cut browning susceptibility (i.e., ΔE* and PPO), Fontane reported no significant changes during storage, whereas Gaudi showed a significant increase up to 6 month and then dropped to values similar to those of Fontane. Specifically, for the ΔE* value, Fontane always ranged between "minor" and "just perceptible" color differences, while in Gaudi ΔE* always corresponded to a "strong difference". The data acquired along the second production season seems to confirm the results discussed here (analysis still in progress - data not shown).

Table 1. *Comparison between the relevant characteristics of both Fontane and Gaudi cultivars during the 8 months. Statistical differences are reported as lower-case and upper-case letters for within-column and within-row values, respectively (P ≤ 0.05)*

	Fontane cultivar								***Gaudi cultivar***							
Months	**TPC**		**RSC**		**ΔE***		**PPO**		**TPC**		**RSC**		**ΔE***		**PPO**	
0	71.39	aB	3.03	cB	1.54	B	0.48	B	116.21	cA	10.57	eA	5.25	cdA	0.65	cdA
1	54.15	bcdB	2.58	cB	2.12	B	0.40	B	113.36	cA	32.74	dA	6.66	bA	0.73	bcA
2	55.17	bcdB	9.88	bB	2.10	B	0.47	B	89.72	dA	43.48	cdA	7.88	bA	0.83	abcA
3	46.16	dB	12.61	bB	2.44	B	0.46	B	111.89	cA	46.58	cdA	5.78	cA	0.82	abcA
4	51.90	bcdB	20.08	aB	2.28	B	0.53	B	120.37	cA	66.81	bA	9.14	bA	0.96	abA
5	47.02	cdB	14.38	bB	2.48	B	0.47	B	120.65	cA	69.09	bA	9.33	abA	0.97	abA
6	48.07	bcdB	19.29	aB	1.82	B	0.50	B	139.75	bA	75.19	bA	11.37	aA	1.02	aA
7	55.56	bcB	14.02	bB	2.19		0.48		156.76	aA	98.38	aA	3.78	de	0.46	cd
8	56.35	bB	10.63	bB	2.33		0.47		168.76	aA	48.11	cA	3.21	e	0.24	d
p-value	< 0.001		< 0.001		ns		ns		< 0.001		< 0.001		< 0.001		< 0.01	

4.2 Semi-drying tests

The drying tests performed using the smart processing unit allowed to select the dipping in citric acid solution (3 % w/v) as best treatment: it stabilized the hue angle (*h*) during drying (Fig. 2), preventing both enzymatic browning and after-cooking darkening (ACD), with results in line with the well-known effectiveness of the sodium metabisulphite solution (0.1 % w/v). The blanching treatment was not effective in preventing ACD.

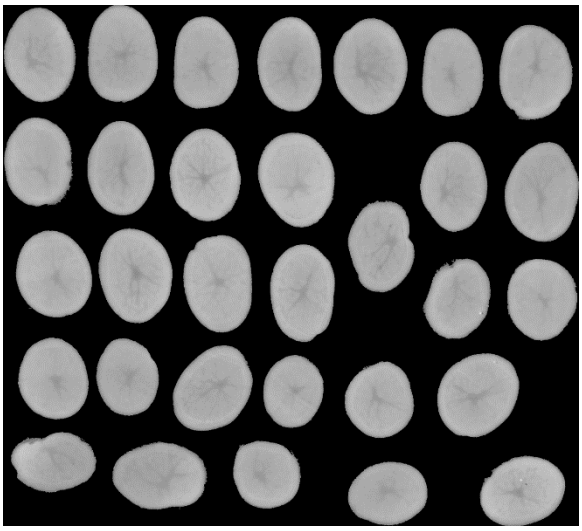

Figure 2. *Image of potato slices monitored during drying for changes in moisture, color, size and shape.*

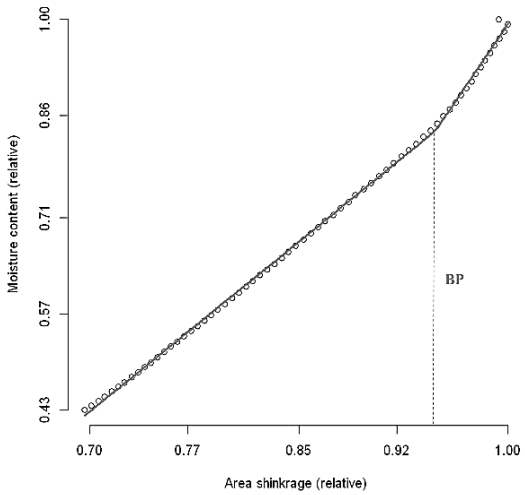

Figure 3. *Relationship between area shrinkage and moisture content during drying. BP, break point.*

The smart drying unit was also useful for developing drying models based on the relationship between the change in size (shrinkage) of potato slice during drying and its residual moisture content. For the intended purpose linear and segmented linear models were trained and validated. The segmented linear model was more effective in minimizing the moisture content prediction error as function of product shrinkage (Fig. 3). In fact, the Root Mean Square Error (RMSE) of the segmented model was 3.3 times lower than those of linear model (i.e., 0.006 and 0.020, respectively). The breakpoint (BP) of the segmented model was identified at a shrinkage of 0.94.

4.3 FT-NIR-based predictive models for dry matter determination

The DM content of potato tuber is heterogeneously distributed among tissues (Fig. 4): external tissues have higher DM content than the internal ones (Pritchard and Scanlon, 1997). Thus, the inner medulla always had the lowest

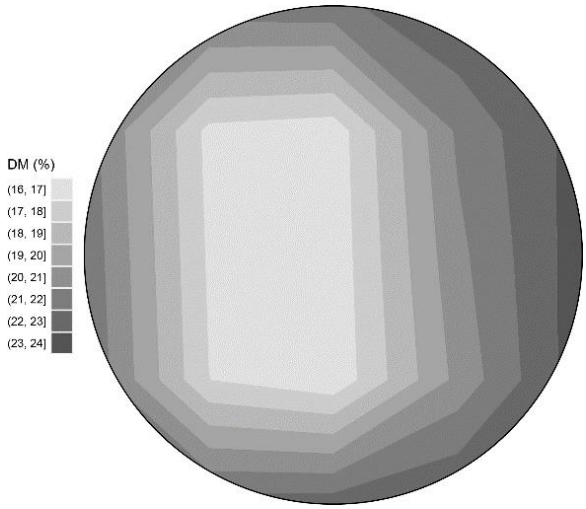

Figure 4. *DM distribution in a potato slice (cv. Fontane).*

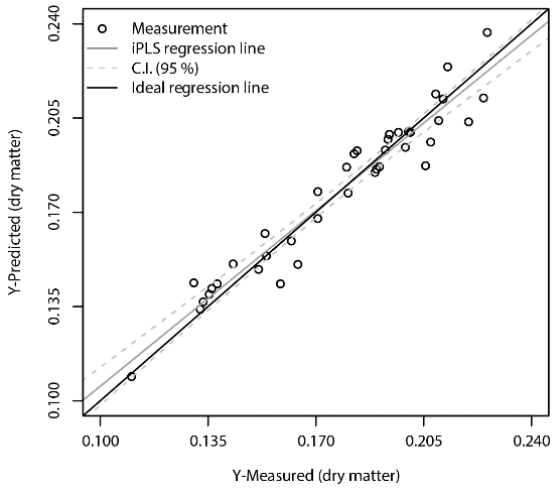

Figure 5. *iPLS regression plots of Y-measured and Y-predicted dry matter ratios in the inner medulla tissue.*

DM value followed by the outer medulla, periderm and cortex. Consequently, the thesis was focused on studying the impact of potato tuber tissues on the performance of FT-NIR-based DM predictive models. In general, PLS (full spectrum based) models showed poor performances (RMSEP > 2 % and $R^2P < 0.60$) irrespective of the tissue

(data not shown). Differently, feature selection through the iPLS algorithm allowed more accurate and reliable results (Tab. 2), especially when spectra were acquired from the scan of the inner medulla (RSMEP < 1 %; BIASP < 0.001 % and R^2P ~0.90) (Fig. 5). On the contrary, the iPLS model from spectra from periderm resulted in very poor performance metrics (data not shown). This was consistent with the results obtained by Han *et al.*, (2021) on the prediction of soluble solids in potato tuber using NIR spectroscopy. The Authors observed detrimental effects of surface impurities, spectral drift and light scattering on model performances. Poor metrics were also observed by scanning the cortex tissue, which is characterized by high density and low porosity values. Thus, further studies should focus on clarifying the impact of physical properties of tissue on model performances.

Table 2. *Summary of performance metrics for dry matter prediction models obtained using the iPLS algorithm*

Potato tissue	LVs	RMSE (%)		BIAS (%)		R^2	
		Cal. (C)	*Pred. (P)*	*Cal. (C)*	*Pred. (P)*	*Cal. (C)*	*Pred. (P)*
Periderm	*model performances too low to be considered*						
Cortex	5	1.36	1.66	$-2.78\ 10^{-16}$	$2.69\ 10^{-3}$	0.75	0.60
Outer medulla	5	1.08	1.12	$1.11\ 10^{-16}$	$-2.60\ 10^{-3}$	0.87	0.86
Inner medulla	6	0.79	0.93	$7.77\ 10^{-16}$	$-3.03\ 10^{-4}$	0.93	0.90

LVs, Latent Variables; RMSE, Root Mean Square Error; R^2, determination coefficient; Cal: Calibration; Pred: Prediction.

The spectral bands selected by the iPLS algorithm from the scan of the inner medulla were the following: i) 10000 to 9425 cm^{-1}, associated to the 2nd overtone of O-H and N-H groups and of the 3rd overtone of C-H group; ii) 8458-7690 cm^{-1}, corresponding to the 2nd overtone of C-H and N-H groups. The two selected wavebands were related to starch and moisture content (Workman and Weyer, 2012), which are major constituents in potato tuber. The NIR acquisitions during the second production season are in progress. New data are acquired in order to improve reliability and robustness of models, as well as to transfer models on a handled NIR spectrophotometer.

5. Conclusions and Future Perspectives

The physico-chemical characterization of the tubers afferent to the "Patata dell'Alto Viterbese" PGI district identified Fontane as the cultivar more suitable for processing as a fresh-cut and semi-dried product. It has high DM content and firmness; low RSC, TPC, and PPO enzymatic activity; and a physico-chemical profile stable during 8 months. The work lays the basis for planning the production chain of PGI area based on cultivar profiles.

The semi-drying tests performed using a Smart drying cabinet drier allowed to identify best antibrowning treatment (dipping in 3-% citric acid solution, w/v) and drying parameters through the real-time monitoring of changes in product quality. Further tests are needed to assess both shelf-life and oil uptake during frying of product.

Finally, the development of DM prediction models of different potato tissues (periderm, cortex, outer medulla and inner medulla) by scanning 15+ potato cultivars was successfully investigated. The iPLS algorithms with selected features (10000-9425, and 8458-7690 cm^{-1}) performed better than the full-spectrum PLS models (10000-4000 cm^{-1}). The inner medulla tissue resulted the most promising scanned area, reporting the following prediction metrics: BIAS tending towards zero, RMSEP and R^2P of 0.93 % and 0.90, respectively. The selection of specific bands can allow for transferability of the models to portable handheld instruments that could be very useful to i) define the optimal potato harvesting time; ii) monitor quality parameters during storage; iii) ensuring optimal quality of potatoes for each processing segment, thereby reducing food losses and allowing for better potato supply chain management. However, further studies should be carried out to, i) verify the effects of other cultivars and agricultural conditions on the robustness of models; and ii) evaluate the use of advanced algorithms like convolutional neural networks for improving the models.

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Nonstarter Lactic Acid Bacteria: origin and characterization for a potential targeted use in cheesemaking

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The nonstarter lactic acid bacteria (NSLAB) are a group bacteria found in cheese that originate mainly from raw milk. Some NSLAB species have a pro-technological role since they contribute to the final cheese characteristics. This PhD project is intended to deepen the knowledge about origin source, dynamic evolution in cheese, and geno-/phenotypic characteristics of NSLAB in order to predict and control their activity during cheesemaking as well as study their potential application as adjunct culture.

Batteri lattici nonstarter: origine e caratterizzazione per l'utilizzo in caseificazione

I batteri lattici nonstarter (NSLAB) sono un gruppo di batteri presente nei formaggi e che origina principalmente dal latte crudo. Alcune specie NSLAB hanno un ruolo pro-tecnologico in quanto contribuiscono alla formazione delle caratteristiche finali dei formaggi. Questo progetto di Dottorato ha lo scopo di approfondire le conoscenze riguardo le fonti d'origine, le dinamiche di sviluppo nel formaggio, e le caratteristiche geno-/fenotipiche dei NSLAB, al fine di predire e controllare il loro sviluppo durante la caseificazione, oltre che investigare il loro potenziale utilizzo come colture aromatizzanti.

Keywords: cheese quality, raw milk, nonstarter lactic acid bacteria

1 Introduction

The object of the present PhD project is to deepen the knowledge about NLAB to control their occurrence and activity during cheesemaking. The work done so far can be summarized in the following chapters:

- **The raw milk microbiota:**
  - Lactic acid bacteria in raw milk for cheese production: which and how many?
  - The interplay between farming system and raw milk quality: development of novel milk payment system
  - Selective enrichment of the raw milk microbiota in cheese production: investigation of a natural adjunct milk culture
- **NSLAB characterization:**
  - Geno- and phenotypic characterization of *Lacticaseibacillus* spp. strains isolated from raw milk for cheese production
  - Influence of raw milk low-temperature centrifugation on NLAB in long-ripened hard cheese

2 The raw milk microbiota

2.1 Lactic acid bacteria in raw milk for cheese production: which and how many?

Microbial colonization of raw milk occurs from different sources throughout the route from farm to cheese factory (Gobbetti et al., 2018), and it represents an ideal environment for the growth of many microorganisms (Quigley et al., 2013). Some of these bacteria will be important for the formation of the ripened cheese characteristics (Bottari et al., 2018; Gatti et al., 2014). Although many studies dealt with describing the complex dairy ecosystem focusing on LAB in raw milk, often reporting isolation, identification, and relative quantification of the most abundant species, they rarely provide absolute quantification. With the aim to answer the initial question: "which and how many lactic acid bacteria are present in raw milk used for cheese production?", this review described and commented on the studies available in the literature that addressed LAB (semi)quantification in raw milk used for the production of different cheeses, by means of culture-independent and culture-dependent techniques.

2.1.1 The culture-independent quantification of LAB in raw milk

In this review, we conducted a meta-analysis of taxonomic studies on raw milk using FoodMicrobionet [FMBN (De Filippis et al., 2018; Parente et al., 2019, 2016)], a collection of datasets created by 16S rRNA gene amplicon HTS studies of food bacterial communities. This tool was already used to widely review the microbiota of the dairy milk, discussing comparisons between the pasture and feed, farm environments, teat skin, teat milk (from different species, also affected by diseases), bulk tank milk and finally also high-temperature short time milk (Parente et al., 2020).

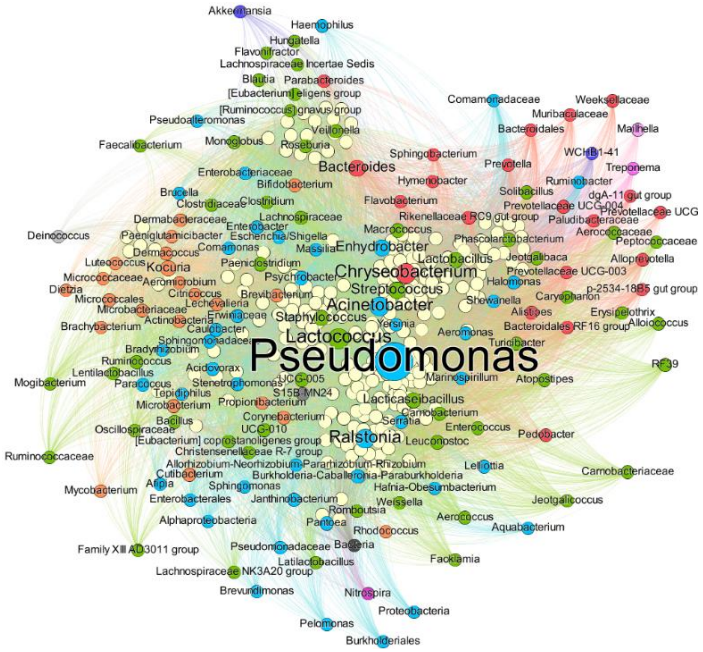

Fig 1 *Bipartite network showing the most abundant genus found in raw milk (nodes nameless). The bigger the genus name (OUT), the more abundant it is in raw milk.*

We used the updated version of FMBN 4.1.2 (Parente et al., 2022), integrated with the taxonomy reclassification for the genus *Lactobacilus* (Zheng et al., 2020) and further recent studies on the raw milk microbiota. Among the studies available in the database that analyzed cow raw milk, we selected the 10 in which whole raw milk was used for cheese production to conduct a bipartite network analysis (**fig. 1**).

After the filtering step, 149 taxa were retained, which belong to 10 different phyla (colors in the fig. 1). Part of the OTUs were classified only at the kingdom level (Bacteria). Among the most abundant genera, the psychrotrophic bacteria are present in significant proportions: these include Pseudomonas (23.85% of the tot weighted degree in the network), Chryseobacterium (6.56%), and Acinetobacter (6.01%). Their dominance is caused by the refrigerating temperature applied for raw milk storage which decreases bacterial diversity (Raats et al., 2011).

LAB relevant for cheese production are present as dominant taxa such as Lactococcus (7.24%), or subdominants such as Streptococcus (3.6%), Lacticaseibacillus (2.42%), Lactobacillus (2.35%), Leuconostoc (1.15%), and Enterococcus (0.41%).

2.1.2 The culture-dependent quantification of LAB in raw milk

The culture-dependent quantification of LAB can be performed by means of plate count on elective or selective agar media for LAB after incubation at different temperatures. This review retrieved, from 39 studies, concentration data of LAB in raw milk used for the production of 27 kinds of cheese. The main growth media used in the studies were de Man-Rogosa-Sharpe (MRS) elective for lactobacilli, and M17 elective for cocci LAB, incubated at 30-32°C. The average concentration of lactobacilli (3.33 ± 1.06 logCFU/mL) was found significantly lower ($p < 0.001$) than cocci LAB (4.48 ± 1.12 logCFU/mL).

This result is consistent with what we found in the meta-analysis of culture-independent studies on the raw milk microbiota, in which *Lactococcus* and *Streptococcus* genus were present in higher abundance with respect to *Lacticaseibacillus* and *Lactobacillus*.

2.2 The interplay between farming system and raw milk quality: development of novel milk payment system

Upon arrival to the cheese factory, milk is currently paid according to quality parameters, among which the microbial load, independently from the species present. Besides total microbial count, the biodiversity and the presence of specific LAB should represent an added value for milk quality; however, they are not considered.

The project aims to correlate farming practices (breeding and environmental factors) with LAB profile of raw milk, evaluating LAB's presence by impedometric analysis and Real-Time qPCR.

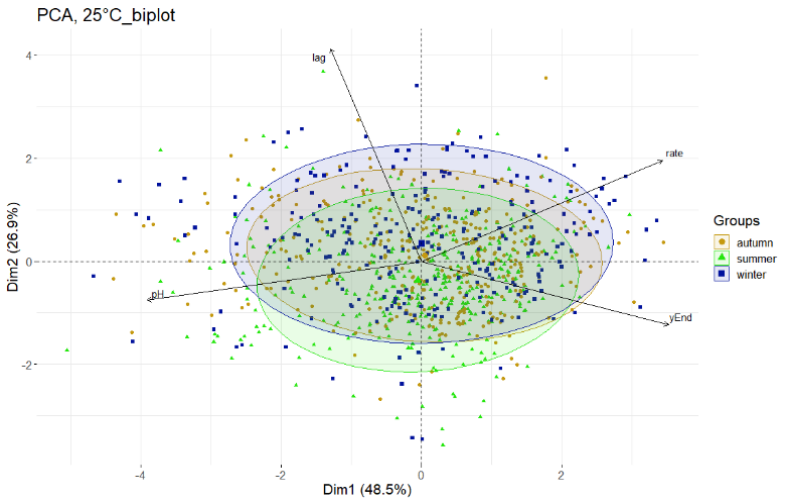

Fig 2 *Principal Component Analysis of impedometric results of raw milk grouped by seson of sampling. Ellipse confidence level = 80%*

The correlation between farming variables and milk microbiological and cheesemaking characteristics will make it possible to select the best drivers of milk quality. This will allow the definition and prediction of the most significant parameters to be considered in the milk payment system.

Preliminary impedometric results, elaborated by principal component analysis, allowed us to measure the effect of the seasonality on the microbiological quality of raw milk samples. As shown in the biplot in **fig. 2**, scores belonging to the summer season are separated, even if partially overlapped, on the second principal component with respect to winter milk samples; autumn samples are instead spread in the middle and completely overlapped with the winter season. The *lag* phase duration is the parameter that contributes the most to explaining the difference among the milk samples.
This factor will have to be controlled when evaluating the influence of different farming systems on raw milk quality.

2.3 Selective enrichment of the raw milk microbiota in cheese production: investigation of a natural adjunct milk culture

Microorganisms are often intentionally added at the beginning of the cheese making process, as so-called starters, where they are used for curd acidification, while secondary microorganisms are important during the ripening process. When produced with raw milk, an autochthonous non-starter flora are added to the cheese, resulting in added value for the final cheese characteristics. However, it is difficult to standardize this flora for two main reasons: their load and composition in raw milk are uncontrolled; their growth dynamics during cheese making and ripening are difficult to predict.

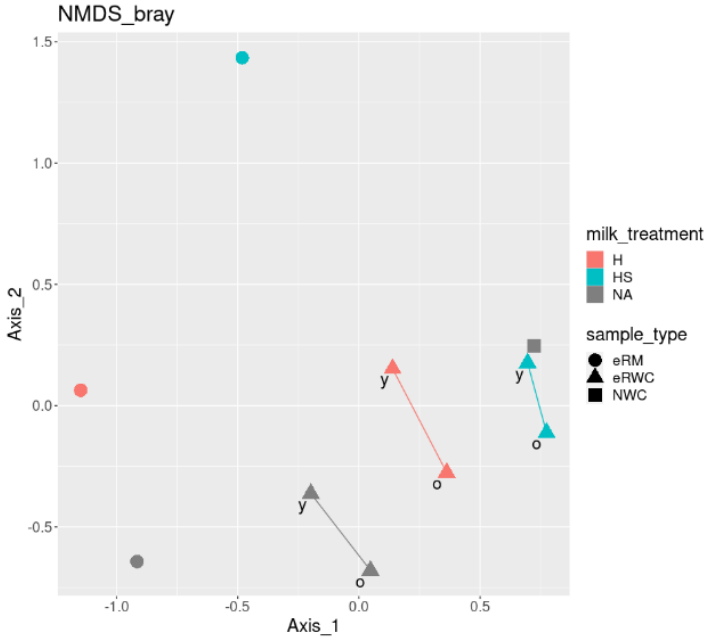

Fig 3 *Non-metric Multi Dimention Scaling with Bray-Curtis Dissimilarity of metataxonomics data of* ***enriched raw milk whey culture*** *(eRWC), obtained by mixing* ***enriched milk*** *(eRM) treated with* ***heat*** *(H),* ***heat and salt*** *(HS) and* ***no-treatment*** *(NA), with* ***natural whey culture*** *(NWC)*

With the aim of developing a practical tool for introducing a rich culture to the cheese, we investigated the production of an enriched raw milk whey culture (eRWC), a natural adjunct produced by mixing enriched raw milk (eRM) with a natural whey culture (NWC).
The raw milk was enriched by spontaneous fermentation for 21 days at 10°C. Three milk enrichment protocols were tested: heat treatment (H = 1h at 54°C) before incubation, heat treatment plus salt addition (HS = 5% NaCl, HS), and no treatment. After mixing with NWC at a ratio of 1:10, the cultures were further incubated at 38°C for 6h and 22h, then analyzed for their microbial diversity (colony forming unit; next-generation sequencing).
Results showed that the enrichment increased viable LAB (max. ~9 logCFU/mL).
Undesired Enterobacteriaceae, however, can also grow up to ~6 logCFU/mL, but they were inhibited by salt addition (~2.5 logCFU/mL). The final eRWCs harbour a more diverse microbiota than NWC, although NWC and eRWC.HS has a similar microbial profile (fig. 3). The final culture application in cheese making did not interfere with the acidification (data not shown).

3 NSLAB characterization

3.1 Geno- and phenotypic characterization of Lacticaseibacillus strains isolated from raw milk for cheese production

The need to standardize cheesemaking and accelerate ripening has driven the use of several NSLAB, often called as adjuncts or attenuated adjuncts secondary starters. Their proper targeted use, however, relies on the understanding of geno- and phenotypic characteristics at the strain level.
This study aimed to characterize NSLAB isolated from raw milk used for cheese production sampled from 20 different cheese factories. From a total of 122 isolates, 31 were screened as *Lacticaseibacillus paracasei* through specie-specific PCR (Savo Sardaro et al., 2016), and 10 strains from different Amplified Fragment Length Polymorphism (AFLP, fig. 4-A) clusters intended to cover the diversity of the species were selected. Strains were phenotypically characterized on the basis of their ability to grow in UHT milk under thermal, pH, NaCl% stresses by means of impedometric analysis, and their ability to grow on different substrates by Biolog MicroPlates (Biolog, Inc.).
Despite they belong to the same species, isolates showed different ability to use carbon and nitrogen source, as well as different resistance to chemical stresses, as revealed by Biolog metabolic assay (fig. 4-B). Principal Component Analysis was also able to measure differences in strains growth kinetics (fig. 4-D), as well as define the different influence on microbial growth of the different stresses applied (fig. 4-C)

According to their phenotypic characteristics, some strains may not perform well as adjunct culture for cooked cheese production (low resistance to thermal stress), while others seem to be promising thanks to their relative low acidifying capacity (important to avoid growth overlapping with SLAB)
Strains with the best phenotypic profile will be first tested for their aroma compounds production, and then applied in cheesemaking as adjunct culture.

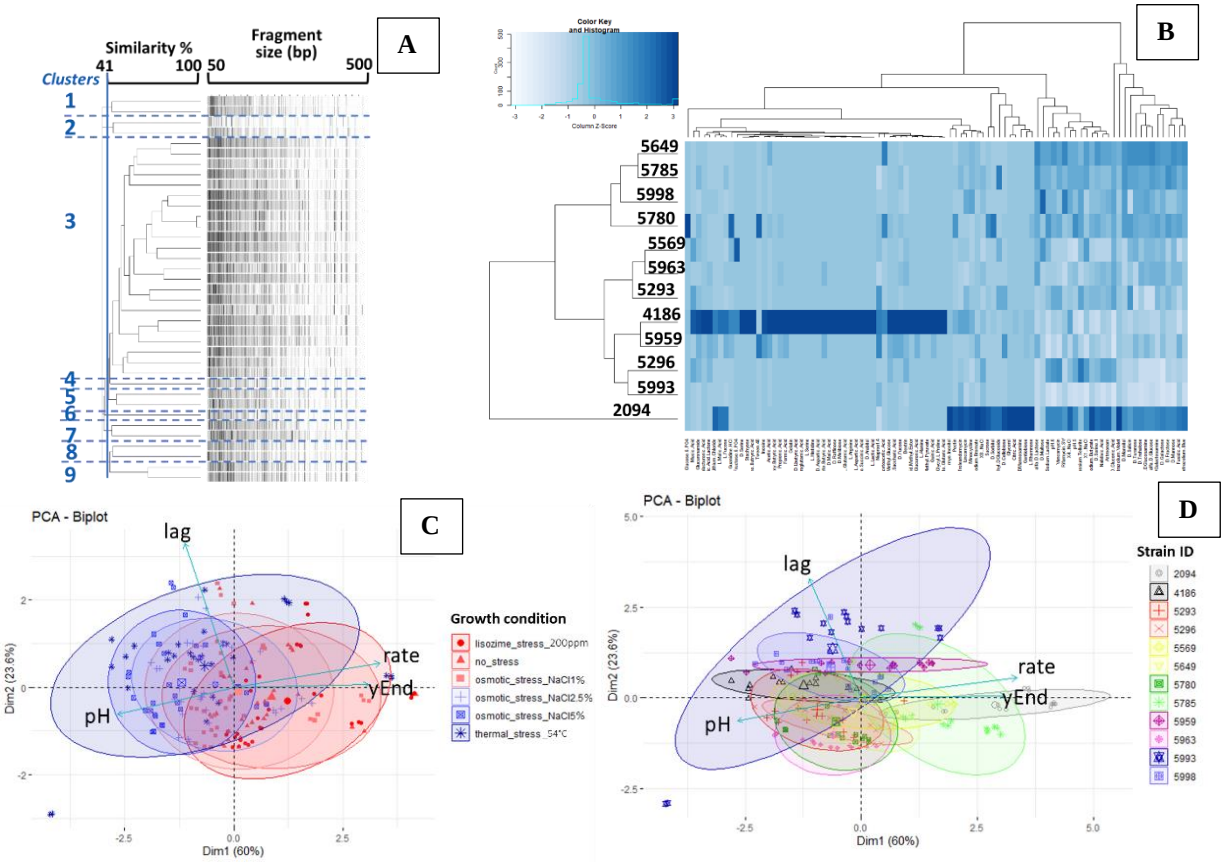

Fig 4 *A)AFLP dendrogram of 31 Lacticaseibacillus strain; B)Heatmap clustering the 10 selected strains in comparison with ref. strain Lacticaseibacillus casei(2094) and L. paracasei (4186); C) Principal component analysis of impedometric results, scores grouped by stress treatment; D) Principal component analysis of impedometric results, scores grouped by strain code*

3.2 Influence of raw milk low-temperature centrifugation on NLAB in long-ripened hard cheese

Low-temperature centrifugation is a milk treatment aimed to reduce the microbial spore concentration in milk. However, its side effect on other microbial populations has never been investigated.
This project analyzed the effect of raw milk low-temperature centrifugation on the microbial characteristic of long-ripened hard cheese. Cheeses produced with centrifugated and non-centrifugated milk, in two different centrifuge configurations (CC1, CC2), and ripened at 9, 15, and 20 months, were compared for their concentration of lactobacilli and more specifically *Lacticaseibacillus* spp., by means of CFU count on MRS agar medium and Real-Time qPCR, respectively.

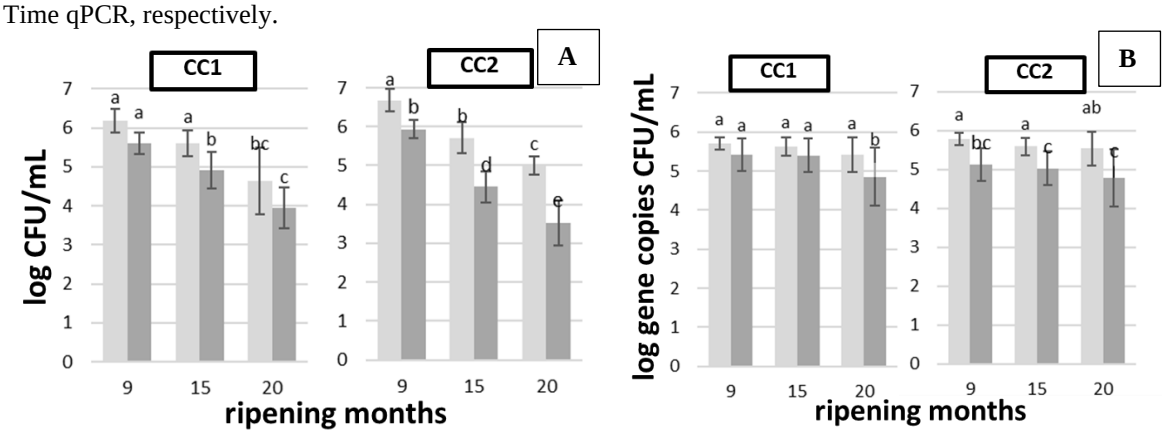

Fig 5 A) *Lactobacilli concentration (Colony Forming Unit on MRS medium) of cheese produced with centrifuge configuration 1 (CC1) and 2 (CC2); B) Lacticaseibacillus spp. concentration (Real-Time qPCR)*

All the samples show an expected diminution of LAB with the increase in the ripening time. Cheese produced with CC2 showed a significant ($p < 0.05$) lower concentration of viable lactobacilli and *Lacticaseibacillus* spp at all the ripening stages; this was less evident in cheese produced with CC1. These results are in line with concerns previously hypothesized by D'Incecco and colleagues (D'Incecco et al., 2020), whom analyzed the milk used for the cheese production finding a reduction in lactobacilli in the centrifugated milk. These preliminary results may suggest that a compromise has to be reached between spore removal efficiency and NSLAB reduction when treating raw milk with low-temperature centrifugation.

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Effects of processing on pulses and related products

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This report presents the main activities carried out during my Ph.D. project. The research aims at understanding the effect of processing on pulse functionality and its impact on product quality. Specifically, the functional properties of pulse flours after and before thermal and/or mechanical treatments were examined. Various extrusion conditions were applied to obtain three types of products: snacks, pasta and meat analogues. Processes have a different impact in changing the biopolymer characteristics leading to products with different features. Understanding the relationship between raw material and process allowed to improve product quality.

Effetto del processo sulle caratteristiche dei legumi e relativi prodotti

Questo lavoro mira a comprendere l'effetto del processo di estrusione sulla funzionalità dei legumi e qualità del prodotto. In particolare, sono state esaminate le proprietà funzionali delle farine di legumi prima e dopo trattamenti termici e/o meccanici. Sono stati applicati processi di estrusione con diverse condizioni di processo per ottenere tre tipologie di prodotto: snack, pasta e analoghi della carne. I processi hanno avuto un diverso impatto nel modificare le caratteristiche dei biopolimeri, dando origine a prodotti con caratteristiche diverse. La comprensione della relazione tra materia prima e processo ha permesso di migliorare la qualità del prodotto finito.

Keywords: legumes, extrusion, functional properties, pasta, snacks, meat analogues

1. Introduction

This report presents the main outcomes of the activities reported by Bresciani (2020):
A3) Setting up of the process conditions. In the previous report (Bresciani, 2021), the dry-extrusion for snacks production (A3.1) and wet-extrusion for pasta production (A3.2) were applied to corn with different amylose content. Here, the two processes were applied to pulses. In addition, high moisture extrusion was applied to produce meat analogues.
A4) Assessing biopolymer interactions. This activity aimed at understanding how the processes defined in A3 potentially affect the interactions among components. Specifically, starch (A4.1), proteins (A4.2), phenolic compounds (A4.3) were considered and their properties/contents were related to flour hydration properties (A.4.4) and product quality (A5)

2. Materials and Methods

2.1 Extrusion for pasta production

Pasta from conventional extrusion of chickpea, green pea and red lentil flours was produced on a pilot plant (Braibanti, Italy). Three processes were considered for yellow and red lentils (A3.2): 1) conventional extrusion of a commercial pregelatinized flour (sample 1), 2) conventional extrusion of native flour (sample 2) and 3) extrusion-cooking of native flour (sample 3). The experimental samples were then dried at 60 °C for 17 h. The effect of pre-gelatinization on starch (thermal and pasting properties; A4.1) and proteins (solubility, thiol accessibility, hydrophobicity; A4.2), as well as on hydration and mixing properties (A4.4) was assessed as reported by Bresciani et al. (2022). Pasta was characterized for (A5.2): color, surface heterogeneity, cooking behavior (water absorption, cooking loss and texture), starch properties (thermal and pasting properties, susceptibility to α-amylase hydrolysis) as reported by Bresciani et al. (2021a).

2.2 Extrusion for snack production

Snacks were produced on industrial scale (A3.1) using a twin-screw extruder (Fudex, Italy). Flours from chickpea, red lentil and green pea were transformed in co-extruded snacks as reported by Bresciani et al. (2021). Co-extruded snack based on rice was used as control. Moreover, coats from chickpea and green pea seeds were used to increase the fiber content of the snack based on rice. The effect of dry-extrusion on starch (pasting properties and starch susceptibility to hydrolysis; A4.1), absorption properties (A4.4), soluble phenolic acids (SPAs) and cell wall-bound phenolic acids (CWBPAs), as well as their antioxidant activity (A4.3) was assessed as reported by Bresciani et. al. (2021). The snacks were characterized (A5.1) in terms of shape, porosity, bulk density, and firmness as reported by Bresciani et al. (2021b). Finally, sensory analysis (A5.1) was performed as reported by Proserpio et al. (2020).

2.3 Extrusion for meat-analogue production

Meat analogues were produced by applying high-moisture extrusion (using a twin-screw extruder TwinLab-F 20/40; Brabender, Germany) to protein-enriched fractions obtained by applying air-classification process to chickpea, red lentils, yellow pea and fava bean flours. Functional (oil and water absorption, emulsifying and foaming ability and stability), pasting and mixing properties of flours were determined before and after air-classification. The extruded products were visually evaluated; specifically, the presence of a fibrous structure which can mimic that of meat was considered positively.

2.4 Statistical analysis

All the analysis were carried out in triplicate. One-way analysis of variance (ANOVA) was applied. The least significant differences were calculated by the Tukey-HSD test and the significance at $p < 0.05$ was carried out using XLSTAT statistical software (2010.5.02, Addinsoft, Boston, MA, USA).

3. Results and Discussion

Pulses considered in this study showed differences in rheological behavior, particularly in the mixing and pasting properties (data not shown). These differences were mainly due to differences in physico-chemical composition and might have affected their aptitude for being transformed into pasta, snacks or meat-analogues. Since starch gelatinization plays a key role in non-gluten pasta and snack production, the impact of processing on this component can greatly modify the characteristics of the final products. For this reason, in the discussion of the results, particular attention will be paid on the effect of processing on starch properties.

3.1. Pasta

Firstly, the suitability of chickpea, lentil and green pea for the production of pasta was studied. The best results were obtained for red lentils, whose pasta exhibited low cooking loss and high firmness (data not show). Thus, the second part of the study was carried out on lentils and the effect of pre-gelatinization was assessed. The pre-gelatinization treatment applied on red lentils was able to modify both starch and proteins. Specifically, a shift in starch gelatinization temperature and an increase in pasting properties was shown (Fig. 1).

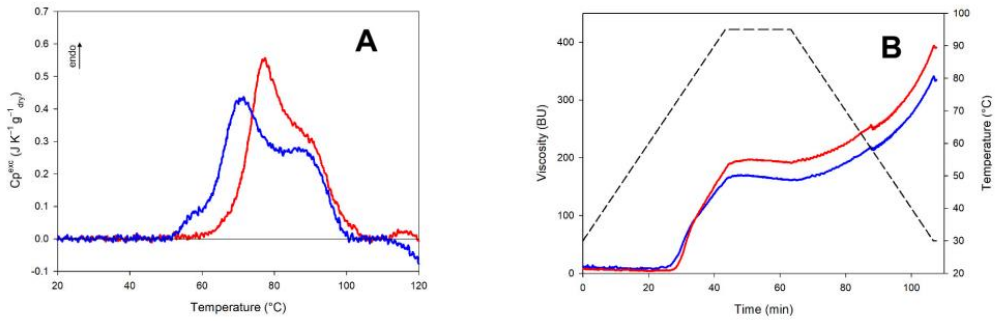

Figure 1. *Differential scanning calorimetry (A) and Micro Visco-Amylo-Graph (B) traces of native (blue line) and pre-gelatinized (red line) flours from red lentils.*

The effect of thermal treatment on proteins appear to be generally more pronounced. Denaturation was detected (lack of the first peak in Fig. 1A) and led to the formation of protein aggregates, stabilized mainly through hydrophobic interactions (Bresciani et al., 2022a). Changes in both starch and protein organization affected the hydration and mixing properties of the pregelatinized flour (Fig. 2).

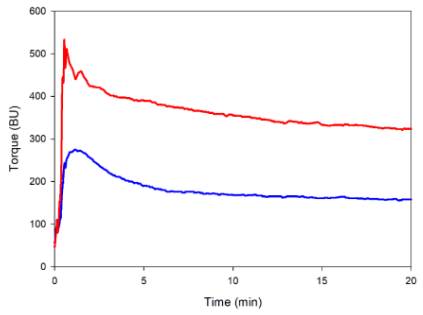

Figure 2. *Mixing properties of raw (blue line) and pre-gelatinized (red line) flours from red lentils at 50% hydration level.*

Specifically, the pregelatinized flour absorbed more water compared to the native flour (1.12 ± 0.02 versus 0.99 ± 0.02 g water/g flour) that, together with the greater protein aggregation properties, resulted in a dough with higher consistency during mixing (Fig. 2).

Regardless the type of raw materials (native or pre-gelatinized) and the pasta-making process (extrusion-cooking or conventional extrusion), pasta with acceptable cooking behavior was produced from red and yellow lentils (Table 1). However, pre-gelatinized flour performed better, resulting in a more homogeneous product with (i.e., low values of heterogeneity; Table 1). Heterogeneity followed the order: sample 2> sample3 > sample 1 (Table 1), suggesting that pre-gelatinization ensured good hydration during process and lead to a product with less white spots which are typical of an incorrect hydration.

Table 1 *Heterogeneity on uncooked pasta and cooking behavior of pulse pasta obtained from: conventional extrusion of pre-gelatinized flour (sample 1) or native flour (sample 2) and extrusion-cooking of native flour (sample 3).*

Sample	Heterogeneity (%)	Water absorption (%)	Cooking loss (g/100g d.m.)	Firmness (N)	Total work (N*mm)
Sample 1_yellow lentils	7a	74 a	7.4 b	636 b	2763 b
Sample 2_yellow lentils	49c	78 b	7.0 a	530 a	2448 a
Sample 3_yellow lentils	28b	76 b	7.1 ab	609 b	2898 b
Sample 1_red lentils	5A	77 B	5.7 A	637 B	2938 B
Sample 2_red lentils	45C	81 C	6.3 B	668 B	3216 B
Sample 3_red lentils	36B	66 A	9.4 C	548 A	2286 A

Means followed by different letters in the same column are significantly different (Tukey-HSD test, $p<0.05$). Separate one-way analysis of variance (ANOVA) was carried out for pasta from yellow (lowercase letters) and red (uppercase letters) lentils.

To better understand the effect of processing on pasta quality, the study focused on sample 2 and sample 3. Both processes applied on native yellow lentils led to pasta with similar cooking behavior at optimal cooking time (6.5 min; Table 1). However, in overcooking (8 min), pasta 3 showed a significantly higher firmness (513 versus 418 N) and compression energy (2898 versus 2448 N*mm). Such differences might be related to a different starch organization between the samples.
Data confirmed what was detected in rice pasta (Marti et al., 2011): starch processed by extrusion-cooking is characterized by an external region in which molecules are organized in an amorphous structure, and an internal core characterized by a crystalline structure. The combination of these two regions - or the two stages in which starch is organized - reflects product behavior during heating. The pasting profile showed that starch in sample 3 undergoes an increase in viscosity in two steps - at about 79 °C and 83 °C - and at higher temperatures than sample 2. Also starch reorganization - notable by the further increase in viscosity upon cooling - occurs at two stages, at about 70 °C and 55 °C. The higher gelatinization temperatures detected in sample 3 (onset temperature = 66.5 versus 63.2 °C) could indicate higher thermal stability in the product, resulting in better cooking behavior especially in overcooking. At the same time, in sample 3 the gelatinization process required less energy (enthalpy = 0.97 versus 1.07 J/g) and was completed in a limited temperature range, suggesting a lower crystalline order. Indeed, about 75% of the total starch was gelatinized during the process (compared to about 58% in sample 2). Overall results suggest that in sample 3 the non-gelatinized starch fraction might have a more organized structure.

3.2. Co-extruded snacks

Differences in physical features were observed among snacks, with chickpea led to the snacks with the smallest area (both the section and inner area) and the highest firmness (Fig. 3).

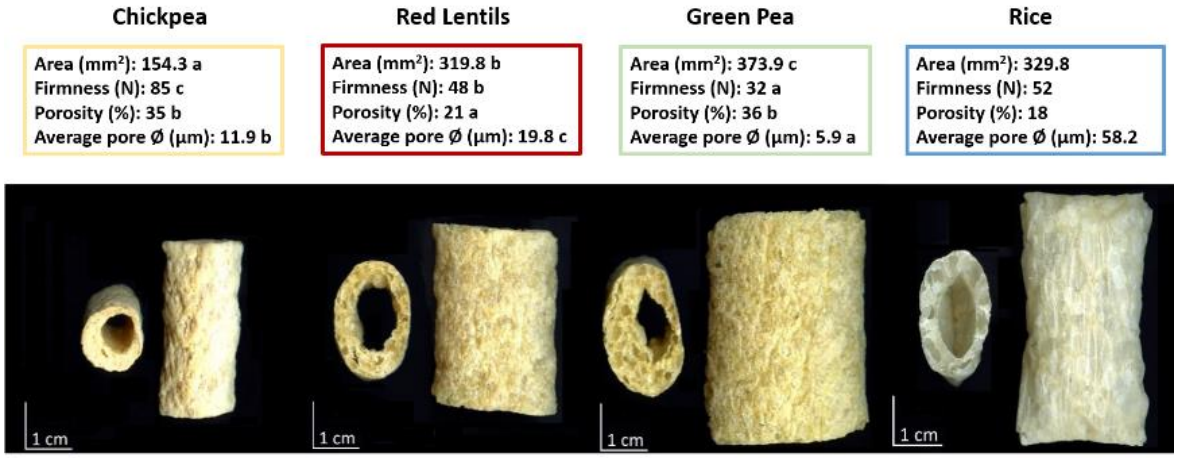

Figure 3 *Physical properties of co-extruded snacks and related images. Means followed by different letters in the same row are significantly different (Tukey-HSD test, $p<0.05$).*

This could be related to starch characteristics of chickpea that showed a lower susceptibility to amylase hydrolysis (2.2 g/100g of total starch) than red lentil and green pea (3.4 and 3.6 g/100g of total starch, respectively), thus, suggesting a high compactness of the starch granules. In addition, the higher lipid content in chickpea (6.1 g/100 g) than in red lentil and green pea (1.4 and 1.7 g /100 g, respectively) may have limited starch gelatinization because of amylose-lipid complex. In addition, lipids acting as lubricants in the screw, decreased the shear stress (Bresciani et al., 2022b). Red lentil snacks had a higher firmness and inner area than the green pea snacks. As regards the porosity, the green pea snacks showed the same porosity percentage as the chickpea products, but the pores had a lower average radius, thus it can be assumed that pores are distributed in a more homogenous way on the product surface. For this kind of product (i.e., co-extruded snacks), small pores led to a softer structure that is less resistant to breakage. Snack based on red lentil showed the lowest total porosity and the highest average pore

radius, but the high expansion rate contributed to mitigating the resistance to breakage. Comparing the data with those obtained from rice-based snacks (used as a control), it appears that the best pulse for this processing is red lentil. Extrusion-cooking reduced the soluble phenolic acid content (-45%) and flavonoids (-41%) but increased the cell-wall bound phenolic acids and antioxidant activity. The different pulses did not lead to a marked difference in the antioxidant activity of the extruded products, although the red lentils maintained the highest flavonoid content after both processes.

Finally, the effect of seed coat-enrichment was investigated, considering two types of seed coats (from chickpea and green pea) and two enrichment levels (15% and 30%). Such samples were compared with snacks from 100% rice flour, 100% chickpea flour and 100% green pea flour. Surprisingly, the control sample (100% rice) obtained the lowest liking score, whereas samples from 100% green pea, 100% chickpea 15% chickpea coats and 15% green pea coats were the preferred ones. Crumbliness and mild flavor attributes positively influenced hedonic scores, whereas stickiness, dryness, hardness, and to a lesser extent, visual aspect affected them negatively. Neophilic and neutral subjects preferred the snacks compared with the neophobic ones, while no differences in liking scores were found regarding food technology neophobia. Snacks with 15% chickpea coats showed a higher bulk density and a lower average pore radius than snacks with green pea (at the same enrichment level); however, such features did not affect either texture or porosity. At 30% enrichment, pea coats resulted in less dense snacks, more porous with smaller pores resulting in a firm product, even higher than the control formulation (i.e., 100% rice) (Table 2). The physical features did not impact on consumer liking: all the seed coat-enriched samples were comparable and preferred to the control. Extruded snacks with pulse flour and bran were moderately accepted by consumers involved in the present study, albeit to a lesser extent for neophobic subjects, and could represent an interesting sustainable source of fiber and high-value proteins, as well as a valuable alternative to gluten-free foods present on the market.

Table 2 *Physical characteristics and hedonic ratings of co-extruded snacks enriched in pulse seed coats.*

Parameter	Rice	15% pea coats	30% pea coats	15% chickpea coats	30% chickpea coats
Section area (mm^2)	330.3 c	190.8 b	177.3 a	334.8 c	191.1 b
Inner area (mm^2)	95.3 c	31.9 ab	37.4 b	35.4 b	28.5 a
Density (g/mL)	1.1 b	0.6 a	0.5 a	0.7 a	0.6 a
Porosity (%)	82.0 d	55.9 a	73.1 c	55.4 a	66.7 b
Average pore diameter (mm)	13.4 a	23.9 c	17.2 b	20.1 bc	27.3 d
Firmness (N)	47.4 ab	46.4 ab	55.9 b	47.4 ab	40.1 a
Hedonic ratings	42.4 a	51.5 b	49.8 b	55.2 b	49.2 b

Means followed by different letters in the same row are significantly different (Tukey-HSD test, $p<0.05$).

3.3 Meat analogues

The chemical composition of protein-enriched fractions obtained by air classification of pulse flours (chickpea, red lentil, fava bean, and yellow pea) is shown in Table 3.

Table 3 *Chemical composition of protein-enriched pulse flour (g/100g dry matter)*

Samples	Proteins	Carbohydrates	Fibers	Lipids
Chickpea	37a	25d	15a	12b
Red lentil	51b	20c	13a	4a
Yellow pea	52b	15b	17a	5a
Fava bean	58c	7.5a	14a	5a

Means followed by different letters in the same column are significantly different (Tukey-HSD test, $p<0.05$).

High moisture extrusion led to different results according on the starting raw material (Fig. 4). The best results were obtained from the extrusion of fava bean and yellow pea, in fact, these products showed “fibers” that could reproduce the characteristics of meat. These raw materials are characterized by the highest protein content and low starch content. High protein amount can guarantee an adequate quality of the final product. On the other hand, extrusion of red lentil (similar protein content of yellow pea) resulted in a product that was not fully textured and tended to expand. This could be related to the starch gelatinization during the process that led to a porous and alveolate structure, which was judged to be negative for this type of product. In contrast with other pulses, chickpeas do not seem to be suitable for this processing. This could be due to the lower protein composition compared to other raw materials and the higher lipid composition that acts as a lubricant during the process reducing specific mechanical energy (SME) and consequently mechanical stress leading to less protein denaturation and bonding.

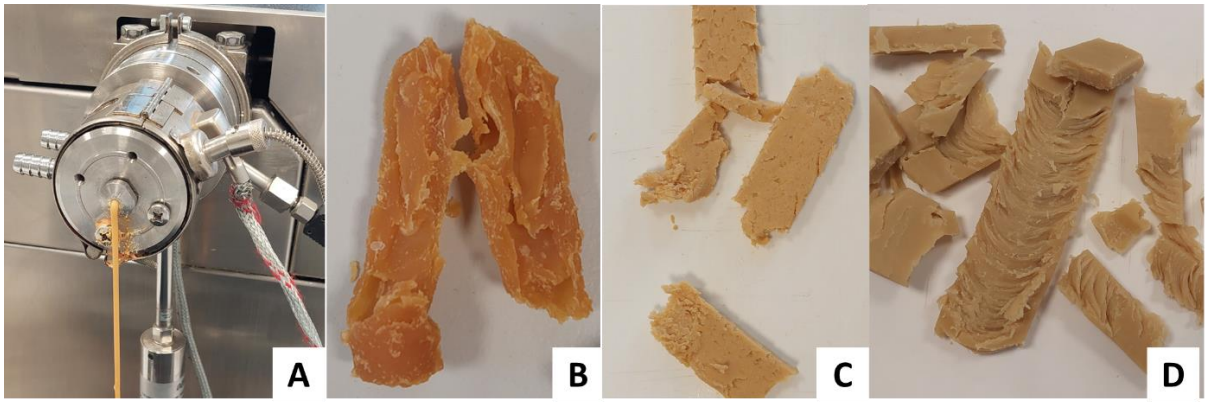

Figure 4 *Images of meat analogues obtained from protein-enriched fractions from chickpea (A), red lentils (B), yellow pea (C) and fava bean (D).*

4. Conclusions and Future Perspectives

This PhD project provides evidence of how the type of pulses (and their physico-chemical properties) and processing conditions (i.e., type of extrusion and pre-gelatinization process) greatly affect the features of the final products. For example, because of its high lipid content, chickpea resulted in firm snacks with low expansion properties, and it was not possible to be processed into a meat analogue product. Among the considered pulses, protein-enriched fractions from faba bean exhibited the best texturizing properties, followed by yellow pea, red lentil and chickpea. As regards pasta, the best results were obtained with red lentils. Although it is possible to produce pasta from lentils skipping the heat-treatment, pre-gelatinization enhances the hydration properties of flours and thus the aspect of the dry pasta, whereas pre-gelatinization during the extrusion-cooking process enhance the product stability to overcooking. Further studies will focus on the effect of pre-gelatinization level on product quality, as well as on the impact of processing conditions on both protein and starch digestibility.

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A Novel functional herbal tea containing probiotic *Bacillus coagulans* GanedenBC^{30}: an *in vitro* study using the Simulator of the Human Intestinal Microbial Ecosystem (SHIME)

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My Ph.D. thesis dealt with the study of the capability of probiotics to help and improve host health. This part of the analysis was directed to the assessment of a new way, such as tea infusions, to spread the probiotic strain *Bacillus coagulans* GanedenBC^{30}(*Bacillus coagulans* GBI-30, 6086 [BC30]), a spore-forming, Gram-positive, facultative anaerobic, nonpathogenic, lactic acid-producing bacterium with well-known probiotic properties across the gastrointestinal tract (GIT). This result was achieved using *in vitro* models to test its survival and behavior in the host digestive system.

Una nuova tisana funzionale contenente il probiotico *Bacillus coagulans* GanedenBC^{30}: uno studio *in vitro* utilizzando il simulatore dell'ecosistema microbico intestinale umano (SHIME)

La mia tesi di dottorato riguarda lo studio della capacità dei probiotici di aiutare e migliorare la salute dell'ospite. Nello specifico, questa parte delle analisi è stata indirizzata verso la valutazione di un nuovo modo, come infuso di tè, di diffondere il ceppo probiotico *Bacillus coagulans* Ganeden^{30}, un batterio sporigeno, Gram-positivo, anaerobico facoltativo, non patogenico, produttore di acido lattico con ben note proprietà probiotiche, attraverso il tratto gastrointestinale. Questo risultato è stato ottenuto usando modelli *in vitro* per testare la sua sopravvivenza e il suo comportamento nel sistema digerente dell'ospite.

Key words: Functional beverages; Herbal tea; *Bacillus coagulans*; Probiotics; SHIME; Colon colonization

1. Introduction

Following my Ph.D. thesis project previously defined, this oral communication reports the main results of the activities directed to:

A1) verify the survival of *Bacillus coagulans* GANEDED BC^{30} in a tea infusion at different temperatures;
A2) verify the resistance of *B. coagulans* GANEDED BC^{30} to the GIT conditions through an *in vitro* model (SHIME);
A3) verify the adherence of *B. coagulans* GANEDED BC^{30} to the intestinal mucosa.

2. SHIME Configuration

The Simulator of the Human Intestinal Microbial Ecosystem (SHIME) is a unique tool to get scientifically validated insights into the potential behavior of functional probiotics during GIT transit. Although clinical studies are anyway necessary to prove the health effects of probiotic products, the SHIME represents a suitable tool for a preliminary approach as it allows to bypass laborious, expensive, and ethically complex human challenges (Van den Abbeele et al., 2012). A mucosal-SHIME (M-SHIME) reactor configuration was adapted from the SHIME model (Prodigest, Belgium) and used for this study (Van den Abbeele et al., 2012) (Fig. 1).

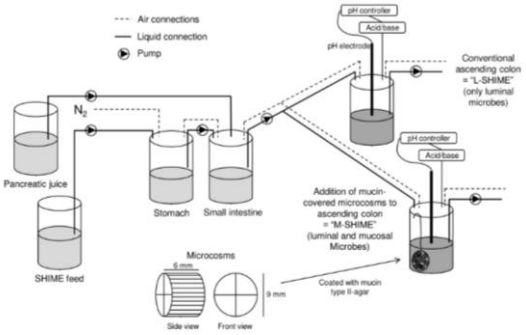

Figure 1 *Basic design of M-SHIME® system developed as an adaptation of the standard SHIME® by adding mucin-covered microcosms to the three colon regions. Bacteria that can stick to the mucus layer populate the microcosms and form a mucus compartment in the reactor. The intestinal replenishment of the mucus layer is reproduced by replacing half of the microcosms every three days, allowing the mucosa compartment to be modeled continuously. Additionally, adherent bacteria can be removed from harvested microcosms, allowing to characterize of the adhered community (www.prodigest.eu)*

The M-SHIME setup consisted of five double jacketed bioreactors representing the stomach (ST), small intestine (SI), and three main colon tracts: ascending (AC), transverse (TC), and descending colon (DC). Each colon vessel contained a mucosal environment, consisting of 60 mucin agar-covered microcosms placed into a polyethylene netting (Van den Abbeele et al., 2012). AC, TC, and DC bioreactors were filled with 500, 800, and 600 mL of adult M-SHIME growth medium (Prodigest, Belgium), respectively. Ranges of pH values in the different colon bioreactors were 5.7–5.9, 6.2–6.4, and 6.6–6.9 for AC, TC, and DC, respectively, and the pH was computer-controlled using 0.5 M HCl and 0.5 M NaOH to mimic the colon physiological conditions. The ST and SI reactors were set at pH 2.0 and 6.5–7.0, respectively. The temperature of 37 °C was maintained in all bioreactors.

3. Materials and Methods

B. coagulans GANEDEN[30] was first cultured at a laboratory scale starting from the tea bags provided by the producers and the cell density was evaluated. The genomic DNA extracted from the isolated colonies and small fragments were amplified using different sets of primers. The survival of *B. coagulans* GanedenBC[30] was investigated by simulating real brewing conditions and assessing different brewing temperatures. The capability of *B. coagulans* GanedenBC[30] to survive the GIT conditions was preliminarily assessed *in vitro* using simulated gastric and intestinal fluids. An in-depth investigation using the SHIME model which incorporated mucin-covered microcosms was performed. A q-PCR with targeted primers was used to understand the persistence of *B. coagulans* GanedenBC[30] in the mucosal compartments. Plate count methodology combined with 16S rRNA sequencing was used to understand *B. coagulans* GanedenBC[30] behavior in the luminal compartments.

4. Results and Discussion

4.1 Cell density of *B. coagulans* Ganeden[30] in tea bags and its surival after infusion

The cell density of *B. coagulans* GanedenBC[30] in the dry tea bag (9.7 ± 0.1 Log CFU/2 g of tea bag) was more than 3 Log cycles higher than the minimum probiotic dose (6 Log CFU/g) claimed for a functional food (Gobbetti et al., 2010). Therefore, one tea bag might be assumed as the safe daily dose for delivering this probiotic. Preliminarily, the assessment of the survival after infusion (3 min) at different temperatures (Fig. 2) showed that also the highest temperature (100 °C) guaranteed living cells (8.5 ± 0.2 Log CFU/2 g of tea bag) to exert a probiotic effect (Keller et al., 2010). To meet national guidelines that are more demanding and recommend higher daily dose to guarantee a temporary gut colonization such as the Italian one (Log 9 living cells per day) (Linee Guida Su Probiotici e Prebiotici. Direzione Generale per L'igiene e La Sicurezza Degli Alimenti e La Nutrizione – Ufficio 4, 2008), two options are available. Based on the calculation of D values, the initial cell density on herbal tea needs to be increased to 10.0 Log CFU per bag if the infusion is kept at 100 °C for 3 min (Bigelow, 1921) or a lower infusion temperature (e.g., 90 °C) and/or time needs to be used which did not interfere with the sensory and nutritional attributes of the beverage. As preliminarily shown under laboratory conditions, *B. coagulans* GanedenBC[30] sequentially exposed to high temperature of infusion and GIT environment did not or just slightly suffers such adverse environmental pressures.

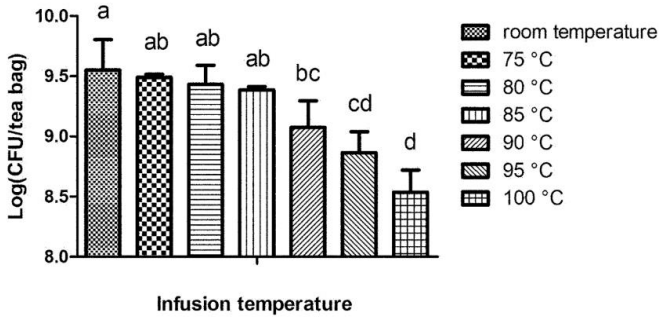

Figure 2 *Survival of Bacillus coagulans Ganeden[30] (Log CFU/tea bag) after 3 min of infusion at different temperatures. One-way ANOVA and individual post hoc comparisons with the Tukey-Kramer were performed. Bars with different superscript letters differ significantly (P < 0.05).*

4.2 Resistance to simulated gastrointestinal transit

The survival of *B. coagulans* GanedenBC[30] under laboratory GIT simulation in shown in Fig. 3. Before starting the assay (0 h), the total numbers, corresponding to a tea bag after infusion for 3 min at 100 °C, were 8.1 ± 0.0 Log CFU/tea bag. After 3 h of incubation in simulated gastric fluid, the resulting number was 7.7 ± 0.0 Log CFU/tea bag, which persisted (7.7 ± 0.3 Log CFU/tea bag) at the end of the intestinal transit (6 h). One-way ANOVA analysis demonstrated that such decreases were not statistically significant (P > 0.05). The final survival rate was 94.8 ± 2.8%. The high survival rate during transit through the simulated upper GIT agreed with previous reports and relied upon the spore-forming nature of *B. coagulans* GanedenBC[30] (Maathuis, Keller, & Farmer,

2010). Indeed, these spores are protected by the integument, a hardened coating mainly consisting of proteins, that withstands gastric acid and bile salts for delivery to the small and large intestines (Ara et al., 2002). The life cycle of germination by *B. coagulans* spores in humans was clearly described (Cao et al., 2020). Spores enter the body orally and safely transit to the stomach. Here, the spores do not germinate due to hostile environmental conditions (e.g., low pH, presence of gastric juices and bile), but they survive. After the stomach transit, the spores begin to germinate in the duodenum and proliferate in the upper part of the small intestine. Germinated cells reach the large intestine and become metabolically active as a part of the facultative anaerobic population and sporulate in the lower part of the colon due to nutrient deficiencies. Because of these promising results both in terms of survival under the most common and highest infusion temperature and after laboratory GIT simulation, the performances of *B. coagulans* GanedenBC^{30} were further investigated using the SHIME model.

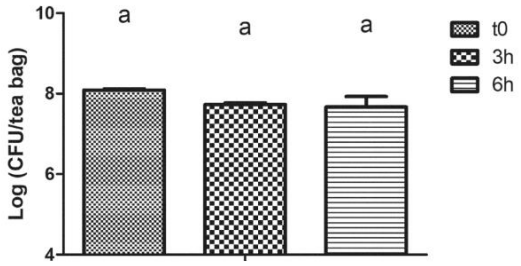

Figure 3 *Survival of Bacillus coagulans GanedenBC^{30} (Log CFU/tea bag) after in vitro simulation of the gastrointestinal transit. Determinations were carried out before the simulation, just after tea bag infusion (3 min at 100 °C) (t0), and after gastric (3 h) and intestinal (6 h) transits. One-way ANOVA and post hoc comparisons with the Tukey-Kramer were performed to highlight significant differences in cells survival during the simulation. Bars with different superscript letters differ significantly (P < 0.05).*

4.3 Fecal donor and stability of the microbial community in the SHIME bioreactors

The reliability of the M-SHIME model mainly depends on the choice of the fecal donor and on the assumption of getting a stable microbiota (Possemiers, Verth´, Uyttendaele, & Verstraete, 2004). The fecal donor, a healthy 37-year-old volunteer resulted from a nutritional screening and an in-depth anamnesis based on stringent criteria of exclusion. The stability of the microbial community in the M-SHIME colon compartments was established through the evaluation of short-chain fatty acids (SCFA), important metabolites from the intestinal microbiota, the level of which is a reliable marker for microbial stability (Liu et al., 2018). The SCFA analyses demonstrated that all bioreactors were stable (overall reproducibility >90%) for 6 consecutive samplings before starting the intake of the herbal tea.

4.4. Colonization of colon mucosal layers

Compared to earlier in vitro models, which only mimic the luminal microbiota and its functionality, the M-SHIME incorporates mucosal microcosms to mimic the mucosal ecosystem (Van den Abbeele et al., 2012). To the best of our knowledge, only a few reports previously used the human GIT *in vitro* model to assess the *B. coagulans* GanedenBC^{30} persistence (Maathuis et al., 2010; D. Keller, van Dinter, Cash, Farmer, & Venema, 2017). Nevertheless, these reports used a TIM-1 model and only focused on probiotic survival and its role in protein digestion. Although giving insights into the stability and activity of probiotics, the TIM-1 model only reproduces the upper GIT, allowing short-term simulations. On the contrary, the M-SHIME model emphasizes the ecological traits during longer timeframe experiments, which allowed to demonstrate of the gradual adaptation of *B. coagulans* GanedenBC^{30} to the colon environment, and its resilience and capability to colonize both the luminal and mucosal compartments. The q-PCR analyses of mucosal layers demonstrated that *B. coagulans* GanedenBC^{30} has the quick capability to colonize the mucosal microcosms of ascending, transverse, and descending colon. On the contrary, previous animal studies using rats reported that *B. coagulans* affected the intestinal microbiota only by temporary proliferation, being quickly eliminated through the feces (Abhari, Shekarforoush, Sajedianfard, Hosseinzadeh, & Nazifi, 2015). These findings brought the authors to consider necessary the daily consumption of the probiotic preparation. The results of this study highlighted the capability of *B. coagulans* GanedenBC^{30} to permanently colonize mucosal niches even after the interruption of the herbal tea intake. Therefore, a probiotic effect by *B. coagulans* GanedenBC^{30} might be hypothesized even after a relatively short intake period. Nevertheless, long-term studies with longer intake and wash-out periods are needed to confirm such a hypothesis.

4.5. Persistence in luminal compartments

Because of the complex microbial community inhabiting the colon bioreactors, all data from plate counts were confirmed in each colon luminal compartment through the identification of the *B. coagulans* species based on 16S rRNA sequencing. Consequently, data are referred to the species and not to the specific strain GanedenBC^{30}. Before herbal tea intake (T0), all luminal compartments (AC, TC, and DC) harbored values of *B. coagulans* species which were at least 1–2 Log cycles lower concerning those further detectable during the intake. This confirmed that herbal tea was the only source of viable *B. coagulans* GanedenBC^{30} cells. Summing the cell numbers

detectable on mucosal and luminal compartments after 4 days of wash out, *B. coagulans* GanedenBC^{30} abundantly persisted (9.7 ± 0.0 Log CFU/bioreactor both in AC and TC bioreactors) in all colon tracts, especially because of the sessile community. These results are following the capability of *B. coagulans* GanedenBC^{30} to persist within a complex mixture of gut bacteria in continuous culture fermenters, even after the cessation of supplementation (Keller et al., 2010), and with slow excretion of *B. coagulans* cells after discontinuation of their consumption (Adibpour et al., 2019). After interrupting the herbal tea intake (wash-out), almost the same values persisted in luminal compartments, the exception being a slight but significant ($P < 0.05$) decrease in AC bioreactor at T5.

5. Conclusions and Future Perspectives

In modern society, functional beverages, non-alcoholic drinks, mainly containing nontraditional ingredients, like minerals, vitamins, amino acids, dietary fibers, added raw fruits, and probiotics, have attracted consumer interest because they contribute to the nutritional well-being (Fior Markets, 2021). Within the emerging area of fruit-derived beverages, herbal teas have a longtime tradition but especially renewed interest. Currently, various strains of *B. coagulans* are used as probiotics with the overall potential claim of improving and/or preserving the ecological balance within the human intestinal microbiota, together with the capability to decrease the cholesterol level, the antagonism toward intestinal pathogenic microorganisms, and the decrease of abdominal pain, duration of diarrhea and frequency of defecation in individuals suffering from acute diarrhea (Adibpour et al., 2019). In particular, claims referred to strain *B. coagulans* GanedenBC^{30} is a probiotic strain with well-known beneficial effects for human health and wellness. (Keller et al., 2010). Nowadays pasta, chocolate, and ice cream are some of the most common functional foods to deliver it (Cao et al., 2020). Nevertheless, the capability of *B. coagulans* GanedenBC^{30} to permanently colonize colon tracts is still debated and its use in herbal teas is a novel challenge. Although clinical studies are anyway necessary to prove the health effects of probiotic products, the SHIME represents a suitable tool for a preliminary approach as it allows to bypass laborious, expensive, and ethically complex human challenges (Van den Abbeele et al., 2012). This study corroborates the potential of a novel functional beverage, which combines the consumption of herbal tea to deliver the probiotic *B. coagulans* GanedenBC^{30}.

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In vitro study of short-term effect on gut microbiota of foods and ingredients for specific consumer categories

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This PhD thesis is based on different sets up of an *in vitro* intestinal model to study applications related to the impact of whole food or food supplements on gut microbiota (GM) of specific categories of consumers. Some representative examples of the most popular sostitutions, fortifications and additions in the context of meat and dairy products and also a specific prebiotic ingredient obtained from industrial by-products for the bakery were considered.

Studio *in vitro* dell'effetto a breve termine sul microbiota intestinale di alimenti e ingredienti per categorie specifiche di consumatori

Questa tesi di dottorato ha riguardato la messa a punto e l'applicazione di un modello intestinale *in vitro* per lo studio degli effetti sulla microflora intestinale di alimenti o ingredienti destinati a specifiche categorie di consumatori. Sono stati presi in considerazione alcuni esempi rappresentativi delle più diffuse sostituzioni, fortificazioni e aggiunte nell'ambito dei prodotti carnei, prodotti lattiero-caseari e prodotti da forno. Sono stati considerati anche ingredienti specifici contenenti prebiotici.

Key words: Gut microbiota; *in vitro* model; short-term; prebiotics.

1. Introduction

In accordance with the PhD thesis project, this oral communication reports the main results of the *in vitro* study of short-term effect on gut microbiota of:
A1) hemp bran and its alcalase hydrolysate (under review for Scientific Reports);
A2) innovative formulation of salami with substitution of nitrates and nitrites with ascorbic acid and vegetal extract (submitted to Antioxidants – MDPI);
A3) lactose-free milk (in submission to International Journal of Food Science and Technology).

2. New consumption tendencies

Over the past 30 years, in many European countries the diffusion of a diet rich in fat and animal proteins and poor in fibres, together with an incorrect lifestyle, contributed to the increase of cardiovascular diseases incidence (Belc et al., 2019). In addition to more appropriate diets, e.g. Mediterranean diet, one of the solutions proposed by food industries is the balance of the diet on the basis of maximum or minimum levels of specific food component. This strategy led to the reformulation of many foods, modifying the eating habits by adding to the traditional diet fortified food, substitute ingredients, supplements, etc, whose effect on gut microbiota is unknown. An other important cause of changes in eating habits is the increase of intolerance to specific components of foods, such as gluten and lactose. Moreover an increasing number of not intolerant people are changing their diet because of the common perception that a diet without these components is healthier (Dekker et al., 2019). Despite the high technological quality of products for intolerant consumers, it has not been clarified how those dietary shift are related to the gut microbiota changes and their effects on health of intolerant or tolerant hosts. For all these reasons, the interest of the scientific community to the gut microbiota and its role in the interaction with the host has accelerated the evolution of alternative systems to the study on animals.

3. *In vitro* intestinal model

In vitro intestinal models are useful tools to study the impact on gut microbiota of many factors such as dietary compounds, microbial pathogens, bioactive compounds, pharmaceuticals, toxic substances. The rationale of *in vitro* models is to cultivate to the more representative way the human gut microbiota under regulated environmental conditions and study its metabolism and shifts over time. The state of the science is that the most competitive models shall include a complet gut microbiota, small working volumes, distinct interconnected compartments, and rigorous bio-chemical and ecological settings, controlled by a computer, as well as a free-hands accessibility, not to contaminate the mock microbiota ecology (Nissen et al., 2021).

4. Experimental Procedure

In this PhD thesis a Multi-Unit Colon Model (MICODE), an *in vitro* colon model, was used in order to mimick the microbe-driven colon fermentations occurring in humans. MICODE was used with fecal inoculum from at least two donors and batch cultures were run for 24 h after the adaptation of the fecal inoculum, definied as the baseline (BL) and definied on the first pH changes detected by Lucullus, a dedicated software of MICODE. Samples of the different timepoints were used for qPCR, SPME GC-MS, and Sequencing Illumina analyses. Technical replicas of analyses were conducted in duplicate for SPME GC-MS and in triplicate for qPCR, both from two independent experiments.

5. Materials and Methods

Fermentations were done using an *in vitro* gut model, MICODE, obtained through the assembly of Minibio Reactors (Applikon Biotechnology BV, Delft, NL) and controlled by Lucullus PIMS software (Applikon Biotechnology BV, NL). Samples were thus obtained:
A1) Hemp bran was previously prepared and characterized (Setti et al., 2020; Nissen et al., 2021). Briefly, alkalase-treated hemp bran (HBPA) was produced by the enzymatic digestion of the chemically extracted hemp bran (HB) protein isolate. Then, both HB e HPBA were lyophilized with a Savant Lyolab 3000 lyophilizer apparatus (Thermo Fisher Scientific, USA) and the powder was used to test the prebiotic potential;
A2) Innovative formulation of salami was produced in a SSICA (Stazione Sperimentale per l'Industria delle Conserve Alimentari – Fondazione di Ricerca, Parma, Italy) pilot plant as part of the project MiMe4Health – Innovative Milk and Meat products for Consumer's Health – Emilia Romagna region funded project;
A3) Lactose-free (LF) and lactose-containing milk (L) was purchased from Granarolo S.p.A. (Bologna, Italy)

6. Results and Discussion

6.1 Hemp bran and its alcalase hydrolysate

In vitro colonic fermentation with MICODE was used to study the prebiotic potential of hemp bran (HB) and alkalase-treated hemp bran (HBPA). The results from the Illumina MiSeq (data not shown) highlighted a reduction in microbial richness, probably caused by the transition from the *in vivo* to the *in vitro* condition (Isenring et al., 2021). The diversity between samples (beta diversity) indicated the ability to maintain a good biodiversity contributing to eubiosis both in HB and HBPA samples, with a higher HBPA capacity than in HB. At species level, the results indicated that HB transformed samples in an healthy ecological condition of the human colon are able to increase the abundance of beneficial bacterial groups, such as *Bifidobacterium* and *Akkermansia*, and to limit opportunistic and proteolytic bacteria, such as *Collinsella* and *Desulfovibrio.*

The results relative (**Figure 1)** to the class of alchohols of the whole volatilome showed that the main descriptors of fermentation with HBPA were mainly complex terpenoid alcohols, such as 4-Terpineol and Borneol, the major terpenoids found in hemp seed, that were increased after lactobacilli fermentation of HB.

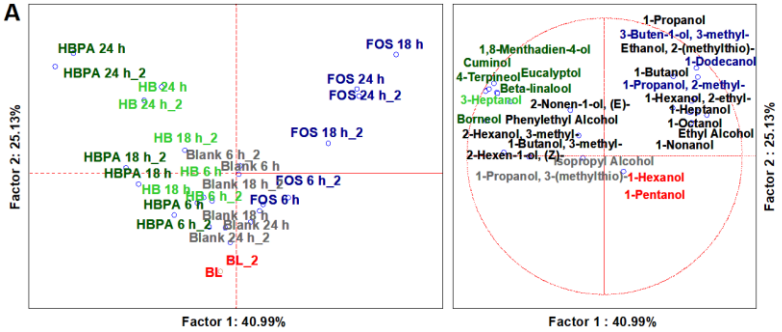

Figure 1 *PCAs of significant alcohols (ANOVA p < 0.05). Principal Component Analysis (PCA) of variables (**A**) and cases (**B**) on VOCs. Variables with different colors are the main descriptors of the respective group of cases by MANOVA (p >0.05)*

In order to evaluate the prebiotic potential of HB and HBPA, the qPI (qPCR Prebiotic Index) was used, an equation based on quantitative values expressed as Log_{10} cells/mL. Considering the results (**Table 1**), the fermented substrate with the best prebiotic activity was HBPA.

Table 1 *qPCR Prebiotic Index (qpI) of colonic fermentations on the substrates HBPA, HB, FOS, and on blank control, after 24h of fermentation.*

Substrate type	qPCR Prebiotic Index (qPI) (24 h)
HBPA	0.866 ± 0.07[a]
FOS	0.989 ± 0.11[a]
HB	0.566 ± 0.11[b]
BC	0.038 ± 0.03[c]

Normalized values by mean centering method. Scale 0 – 1.

6.2 Innovative formulations of salami: nitrites and nitrates substitution

In vitro colonic fermentation with MICODE was used to study the effect on GM of salami reformulated by substituting nitrites (CNO2) with Ascorbic acid (SA) and vegetal antioxidants (SMA) in comparison to not added control (CO). Results in **Figure 2A** shows the increase of organic acid following the colonic fermentation of salami in respect to the baseline. During the fermentation, the formulation with Ascorbic acid (SA) produced about 7 times more Acetic acid and 4 times Pentanoic acid and Hexanoic acid. The formulation with a mix of antioxidants and Ascorbic acid (SMA), on the other hand, produced lower amounts of each of the compunds mentioned above. **Figure 2B** shows that the fermention of all samples produced detrimental VOCs. The sample that generated the largest amount was the nitrite-free (CO) control. Compared to this control, SA, SMA and CNO2 produced similar overall amounts of any compound ($p > 0.05$), except Indole ($p < 0.05$) and Skatole (not detected in SA), approximately 6 times lower than CO ($p < 0.05$).

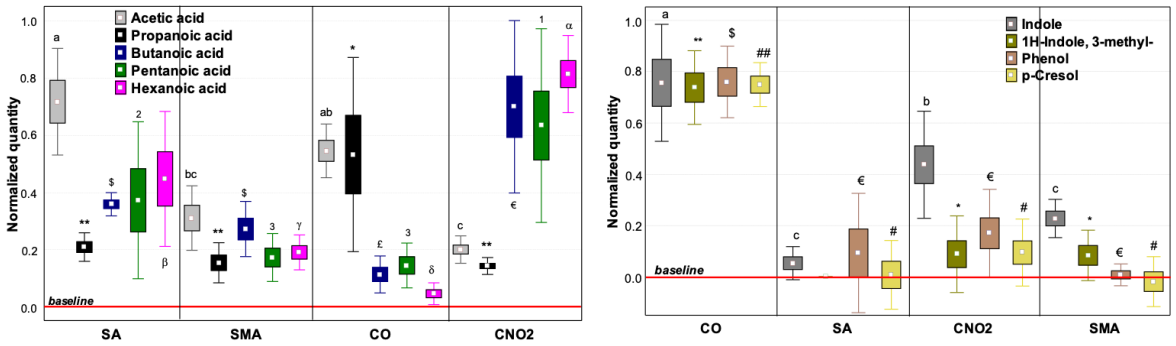

Figure 2 *Shift of the relative abundance of beneficial microbial metabolites (A) and potentially toxic metabolites (B), expressed as relative abundance at baseline after 6, 18, and 24 h of fermentation. Cases with different letters or numbers or symbols within a single independent variable are significantly different for Tukey's HSD test ($p < 0.05$).*

Microbiota analyses were performed with qPCR for beneficial bacteria, *Lactobacillales*, *Bifidobacteriaceae*, *Clostridium* group IV, *Bifidobacterium longum*, *Akkermansia muciniphila*, *Faecalibacterium prausnitzii*, BPP (*Bacteroides-Prevotella-Porphyromonas*) group, and for commensal opportunistic bacteria, *Enterobacteriaceae*, ATOP (*Atopobium - Collinsella – Eggerthella)* group, *Clostridium* group I, *Escherichia coli* (total), *E. coli* (potentially toxigenic), and *Desulfovibrio* spp. Only an extract of the most important microbial targets for the matrix under study will be shown. In particular, **Table 2A** shows the results obtained for the beneficial bacteria. At the endpoint (EP) only SA showed a significant increase of *Lactobacillales* while, as regards *Bifidobacteriaceae*, all formulations favored their grown, exept CNO2, which instead reduced their abundance. *Clostridium group IV* undergoes a significant decrease in almost all formulations exept SMA ($p < 0.05$). Considering opportunistic taxa (**Table 2B**), *Enterobacteriaceae* increased at EP following fermentation of all samples, although with SA did not significantly ($p > 0.05$). Finally, significant shifts were also observed for the *Desulfovibrio* genus, which increased for CNO2 and decreased for SA.

Table 2 *Quantification of beneficial bacteria (A) and commensal opportunistic bacteria (B) compared to the baseline. Cases with different letters or numbers or symbols within a single independent variable are significantly different for Tukey's HSD test (p <0.05).*

A

qPCR Target	Cells/mL	Log.(F/C)		MANOVA
Lactobacillales	**BL**	**T1**	**EP**	
CO	4.80E+06 ± 3.64E+05	0.56	0.82^{AB}	*0.681632*
SA	4.80E+06 ± 3.64E+05[b]	0.72^{ab}	2.13^{aA}	*0.001842*
SMA	4.80E+06 ± 3.64E+05	0.94	1.74^{A}	*0.165766*
CNO_2	4.80E+06 ± 3.64E+05	0.72	1.10^{AB}	*0.418668*
BC	4.80E+06 ± 3.64E+05[a]	0.44^{ab}	-1.34^{aB}	*0.048217*
		0.932608	*0.043956*	**P value**
Bifidobacteriaceae	**BL**	**T1**	**EP**	
CO	4.10E+08 ± 4.77E+07	-0.11^{B}	0.48^{AB}	*0.056606*
SA	4.10E+08 ± 4.77E+07[b]	0.08^{abAB}	1.04^{aA}	*0.003197*
SMA	4.10E+08 ± 4.77E+07[b]	1.01^{aA}	2.04^{aA}	*0.013858*
CNO_2	4.10E+08 ± 4.77E+07	-0.52^{aB}	-0.83^{aB}	*0.058814*
BC	4.10E+08 ± 4.77E+07[a]	-0.32^{aB}	-2.43^{C}	*0.046692*
		0.0003459	*0.000315*	**P value**
***Clostridium* Group IV**	**BL**	**T1**	**EP**	
CO	1.36E+08 ± 1.83E+07[a]	-1.44^{aB}	-1.38^{aB}	*0.000121*
SA	1.36E+08 ± 1.83E+07	0.11^{A}	-0.46^{bAB}	*0.054040*
SMA	1.36E+08 ± 1.83E+07	0.02^{A}	-0.02^{bA}	*0.902609*
CNO_2	1.36E+08 ± 1.83E+07[a]	-0.64^{bAB}	-1.19^{bB}	*0.000436*
BC	1.36E+08 ± 1.83E+07[a]	0.28^{abA}	-1.81^{aB}	*0.000024*
		0.000021	*0.000011*	**P value**
BPP group	**BL**	**T1**	**EP**	
CO	6.82E+09 ± 3.05E+08	-0.27	-0.29^{A}	*0.918863*
SA	6.82E+09 ± 3.05E+08	-0.13	-0.47^{B}	*0.512972*
SMA	6.82E+09 ± 3.05E+08[b]	0.03^{b}	1.16^{aA}	*0.005472*
CNO_2	6.82E+09 ± 3.05E+08[b]	-0.68^{b}	-2.23^{b}	*0.025051*
BC	6.82E+09 ± 3.05E+08[a]	-1.70^{b}	-2.54^{aC}	*0.009275*
		0.244794	*0.000006*	**P value**

B

qPCR Target	Cells/mL	Log.(F/C)		MANOVA
Enterobacteriaceae	**BL**	**T1**	**EP**	
CO	7.85E+07 ± 4.58E+05[b]	0.84^{abB}	2.56^{aA}	*0.049707*
SA	7.85E+07 ± 4.58E+05	0.56^{B}	0.70^{B}	*0.052006*
SMA	7.85E+07 ± 4.58E+05[b]	0.94^{abB}	1.21^{abA}	*0.005644*
CNO_2	7.85E+07 ± 4.58E+05[b]	1.09^{abA}	1.86^{abA}	*0.000008*
BC	7.85E+07 ± 4.58E+05[a]	2.32^{aA}	3.93^{aA}	*0.006603*
		0.000616	*0.000444*	**P value**
Gruppo ATOP	**BL**	**T1**	**EP**	
CO	5.29E+05 ± 1.09E+05[b]	0.16^{b}	1.21^{aA}	*0.024434*
SA	5.29E+05 ± 1.09E+05	0.08	0.21^{B}	*0.880294*
SMA	5.29E+05 ± 1.09E+05	0.27	0.40^{bAB}	*0.574153*
CNO_2	5.29E+05 ± 1.09E+05[b]	0.72^{abA}	1.07^{abAB}	*0.049402*
BC	5.29E+05 ± 1.09E+05[a]	0.28^{B}	1.91^{aA}	*0.042082*
		0.852626	*0.026102*	**P value**
***Clostridium* gruppo I**	**BL**	**T1**	**EP**	
CO	1.54E+04 ± 3.06E+03[b]	1.68^{a}	2.49^{aAB}	*0.000208*
SA	1.54E+04 ± 3.06E+03[b]	1.24^{a}	2.48^{aAB}	*0.000308*
SMA	1.54E+04 ± 3.06E+03	0.93	1.27^{ab}	*0.245233*
CNO_2	1.54E+04 ± 3.06E+03[b]	1.80^{a}	2.03^{aAB}	*0.022968*
BC	1.54E+04 ± 3.06E+03[b]	1.67^{a}	3.05^{aA}	*0.000087*
		0.453844	*0.018590*	**P value**
***Desulfovibrio* spp.**				
CO	1.35E+06 ± 1.59E+05	1.33^{A}	1.25^{A}	*0.090244*
SA	1.35E+06 ± 1.59E+05[b]	-0.12^{bC}	-1.12^{bC}	*0.000012*
SMA	1.35E+06 ± 1.59E+05	0.19^{aA}	0.21^{B}	*0.065024*
CNO_2	1.35E+06 ± 1.59E+05[b]	0.90^{abA}	1.77^{aA}	*0.000001*
BC	1.35E+06 ± 1.59E+05	0.13^{B}	0.65^{B}	*0.080410*
		0.000012	*0.000005*	**P value**

6.3 Lactose-free milk

In vitro colonic fermentation with MICODE was used to study the effect on gut microbiota of lactose intolerant subjects of lactose-containing (L) and lactose-free (LF) milk. A non-targeted Principal Component Analysis (PCA) was applied for a landscape description of the volatilome. The results of the PCA (**Figure 3**) allowed to discriminate the molecules that characterized the baseline (BL), the blank control (BC), the lactose-containing milk (L) and the lactose-free milk (LF) at the two time-points (16 and 24h). In particular, negative molecules such as 1H-Indole, 3-methyl and Phenol, 4-methyl were the molecules that described the baseline, denoting a certain unhealthy profile of gut microbiota. Although colonic fermentation of LF improved the metabolite profile as evidenced by some descriptors as beneficial molecules Butanoic acid and Heptanoic acid, the predominance was represented by molecules belonging to the class of aldehydes, including Benzaldehyde and Benzaldehyde, 4-methyl, as well as Indole. In contrast, the colonic fermentation of L was described by SCFA and MCFA, as Propanoic acid, Acetic acid, and Octanoic acid.

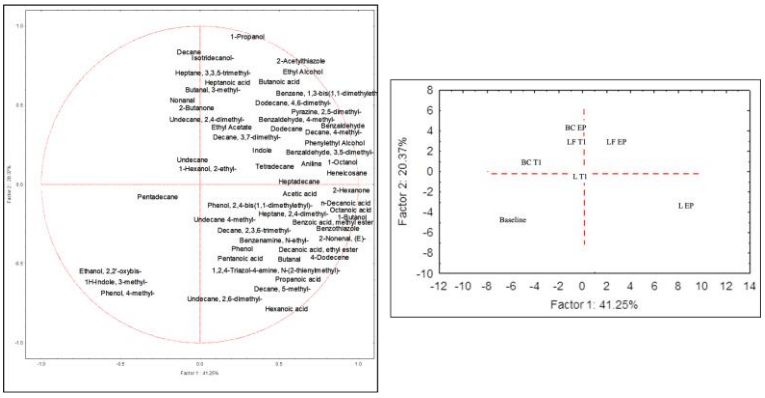

Figure 3 *Principal Component Analysis (PCA) of variables (**A**) and cases (**B**) on VOCs.*

Turning to the microbiota analyses, **Table 3** shows the results obtained from the qPCR of the main intestinal microbial groups. Both L and LF showed an increase of *Enterobacteriaceae*, although with L the increase was four-fould higer to that observed with LF, how confirmed by the sequencing results (**Table 4**). Both milk samples reduced the abundance of beneficial bacteria *Bifidobacteriaceae* and *Lactobacillales*.

Table 3 *qPCR abundance and Log2 Fold Change*

		Log2(F/C)	
Eubacteria	BL	T1	EP
L	2.08E+09 ± 7.68E+07	0.08	-0.28
LF	2.08E+09 ± 7.68E+07	-0.86	-0.31
BC	2.08E+09 ± 7.68E+07	-0.30	-0.43
Entero	BL	T1	EP
L	7.37E+05 ± 4.39E+04	4.07	6.14
LF	7.37E+05 ± 4.39E+04	2.01	1.40
BC	7.37E+05 ± 4.39E+04	3.34	7.67
Lactobacillales	BL	T1	EP
L	3.00E+05 ± 3.55E+04	-0.12	-0.28
LF	3.00E+05 ± 3.55E+04	-0.32	-1.27
BC	3.00E+05 ± 3.55E+04	-0.39	-0.64
Bifidobacteriaceae	BL	T1	EP
L	6.30E+05 ± 3.32E+04	-2.81	-2.34
LF	6.30E+05 ± 3.32E+04	-2.06	-1.93
BC	6.30E+05 ± 3.32E+04	-3.98	-2.59
Firmicutes	BL	T1	EP
L	1.60E+09 ± 8.40E+07	-1.65	-3.10
LF	1.60E+09 ± 8.40E+07	-3.83	-3.70
BC	1.60E+09 ± 8.40E+07	0.40	0.02
Bacteroidetes	BL	T1	EP
L	3.07E+08 ± 1.67E+07	-1.15	-1.71
LF	3.07E+08 ± 1.67E+07	-4.06	-4.11
BC	3.07E+08 ± 1.67E+07	-3.25	-3.38

**T1 = 18h; EP = Endpoint = 24h.*

Table 4 *Abundances and Changes of Family taxa by 16S r-DNA MiSeq Analyses* assessed on rapid-MICODE, the new trend of gut simulator!*

OTU ID#	% RQ				Log2(F/C) @ EP			ANOVA**
	BL Mean	L EP	LF EP	BC EP	ML	MLF	BC	P Value
Actinomycetaceae	0.009	0.006	0.004	0.024	-0.67	-1.20	1.34	0.21866
Bifidobacteriaceae	4.938	2.118	0.796	6.287	-1.22	-2.63	0.35	0.20230
Enterobacteriaceae	0.625	71.875	40.229	56.971	6.84	6.00	6.50	0.62593

**Sequencing of each sample was obtained from pooled DNA of two different experiments. The two experiments were performed with two sets of pools of colon microbiotas from three healthy lactose free certificated volunteers; # Constructed from biom file; **ANOVA for group comparison with 66 dependent variables from taxa at the family level. RQ = relative quantity; BL = Baseline; EP = Endpoint; L = Milk Lactose; LF = Milk Lactose Free.*

7. Conclusions and Future Perspectives

Food intolerances and dietary shift towards helthier foods and balanced or more appropriate diets, suggested to food industry to reformulate some foods of more concern to balance the diet basing on maximum or minimum levels of certain food components. The consequences of those changes on has not yet been elucidated. Indeed, the reduction or elimination of the respective toxic components or the inclusion of new ingredients may influence the gut microbiota. Despite the high technological quality of these new food products, it has not yet been clear whether their compositional characteristics are related to the perturbations of the intestinal microflora and their contribution to health. The case studies of the present work allowed us to define the effects of reformulated foods, foods without specific components, and new potential prebiotic ingredient on the human gut microbiota. In particular, a promising result was also obtained from the study of the prebiotic potential of hemp bran and its alcalase hydrolyzate, which showed a prebiotic potential comparable to that of FOS. The innovative salami formulations have been shown to promote general gut microbiota eubiosis by supporting the growth of beneficial microbial taxa, such as *Lactobacillales* and *Bifidobacteriaceae*, and by reducing negative microbial populations, such as *Enterobacteriaceae*. Finally, the study of the effect of lactose-free milk on the intestinal microbiota of lactose intolerant subjects has shown that the absence of lactose influences the composition and metabolism of the microbiota, reducing the abundance of beneficial microbial species, such as *Lactobacillales.*

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Optimization of microbrewing process for high quality gluten free beers production

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Industrial Doctorate

This Industrial PhD project focused on the optimization of a gluten free (GF) beer production by replacing 40% of barley malt with GF grains, taking into account both technological and sensory aspects. The experimental design was structured in three steps: 1) assessment of brewing attitudes of GF grains (sorghum, millet, buckwheat, quinoa and amaranth) on laboratory scale 2) technological optimization of grist composition and mashing conditions first on laboratory and then on pilot scale to further reduce gluten content 3) development of a gluten free beer on micro-industrial scale with the best sensory characteristics in terms of consumers acceptability.

Ottimizzazione della tecnologia di microbirrificazione per la produzione di birre gluten free di elevata qualità

Lo scopo del progetto di Dottorato Industriale è l'ottimizzazione del processo di produzione di birre gluten free (GF) mediante la sostituzione del 40% di malto d'orzo con cereali/pseudocereali GF, tenendo in considerazione aspetti tecnologici e sensoriali. Le attività sono state così strutturate: 1) valutazione delle attitudini alla birrificazione di sorgo, miglio, grano saraceno, quinoa e amaranto su scala laboratorio 2) ottimizzazione della formulazione e delle condizioni di birrificazione su scala laboratorio e pilota in modo da ridurre il contenuto in glutine 3) validazione su scala micro-industriale della birra GF con il più alto grado di accettabilità da parte del consumatore.

Key words: beer; beer adjuncts; consumers acceptability; gluten free; optimization; sensory analysis.

1. Introduction

Beer is traditionally produced with water, barley, hops and yeast. However, barley contains gluten, a protein fraction to which some persons are intolerant (Codex Alimentarius Commission, 2015). Even if during brewing process a natural gluten reduction is observed, final beer cannot be defined as "gluten free" (GF) since it still contains a gluten content above 20 ppm thus not considered safe for celiac people. Therefore, different strategies for GF beer production have been developed in the last years in order to satisfy the requirements of people with nutritional issues. About this topic, a comprehensive overview of the main approaches to produce GF beer has been published as result of the literature research carried out during the first year of this Industrial PhD project (Cela et al., 2020). GF beers on the market are mainly produced by using enzymatic or precipitation treatments. Both have some disadvantages: the first is not considered safe because the enzymatic degradation of gluten molecule leads to the development of small fragments, not accurately detected by the official method used to quantify gluten in fermented beverages – R5 competitive ELISA - with the risk of false negative results (Panda & Garber, 2019); the second requires additional operations to separate the clarified beer from precipitate thus increasing the cost of the entire brewing process (Watson et al., 2019). For these reasons, the use of non-gluten-containing grains in brewing could be a suitable approach in producing GF beer but still needs more-in-depth tools to optimize the brewing process. Based on these assumptions, the aim of this PhD project is the optimization of microbrewing process to obtain GF beers simply by acting on grist composition (by partial substituting barley malt with GF grains) and by controlling the main steps of brewing process with an impact on natural gluten reduction, such as wort boiling and cold maturation, without using enzymatic or precipitation treatments mainly adopted by big companies. This strategy could be interesting for microbreweries in order to produce craft GF beers that are still rare on the market. In Table 1 are reported the activities carried out to achieve the goal of this PhD project.

Table 1. *Experimental design of this Industrial PhD project.*
** ongoing activities ** will be conducted in the last semester of this PhD project*

Scale-up	Aim	Activities
Laboratory scale	Production of beer with 40% of unmalted GF grains as barley malt substitutes	• Selection of five GF grains according to literature review; • Assessment of brewing attitudes of sorghum, millet, buckwheat, quinoa and amaranth, used both in gelatinized and ungelatinized form; • Sensory analysis: overall liking and Check-All-That-Apply test.
Laboratory scale	Optimization of formulation by using Central Composite Design with the goal of maximizing: • extract content • overall liking	• Identification of the proper combination between sorghum and quinoa, which were considered the best-performing grains according to previous activities, and the optimum protein rest time; • Standard analysis on quality attributes of wort and beer samples; • Sensory analysis: overall liking and beer consumption behaviour.
Pilot scale* (Technology Campus Gent, Belgium)	Optimization of formulation with the highest extract content and overall liking on pilot scale	• Natural gluten reduction by implementing a: o longer boiling step; o longer cold maturation; • Comparison among the different approaches to reduce gluten content; • Physicochemical characterization, gluten quantification and consumer test on optimized pilot scale beers.
Industrial scale** (Birrificio 79 srl, Matera)	Development of a GF beer on micro-industrial scale with the best sensory characteristics in terms of consumers acceptability	• Production of optimized beer at the brewery "Birrificio 79 srl", industrial partner of this PhD project; • Sensory discrimination and consumer tests.

2. Materials and Methods

2.1 Assessment of brewing attitudes of unmalted GF grains

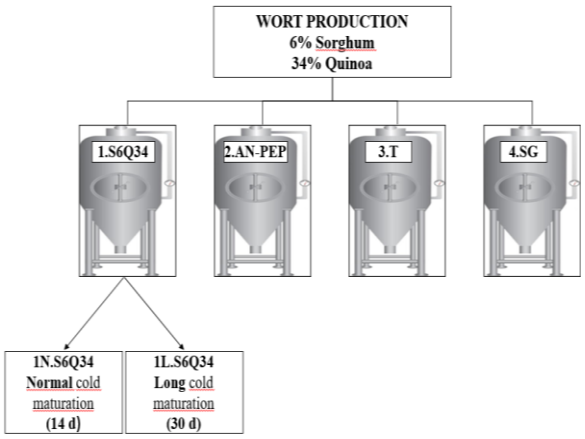
"American Pale Ale" beer from the brewery "Birrificio 79 srl" (Matera, Italy), industrial partner of this PhD project, was chosen as control sample (all-barley malt beer) since it was the one with the lowest gluten content (39±8 ppm). Five unmalted GF grains such as sorghum (SB), millet (MB), buckwheat (BB), quinoa (QB) and amaranth (AB), all of them provided by BMS Organic (Spoleto, Italy), were used as adjuncts (barley malt substitutes) in the maximum concentration of 40%, in compliance with the Beer Italian Legislation (Gazzetta Ufficiale della Repubblica Italiana, 1962). All mashing trials were performed in duplicate by using a 10-L pilot scale plant (Braumeister, Speidel, Ofterdingen, Germany), at Laboratory of University of Basilicata. GF grains were used both in gelatinized and ungelatinized form in order to evaluate the influence of gelatinization step on physicochemical and sensory characteristics of wort and beer samples.

2.2 Optimization of grist composition and mashing conditions to formulate GF beer

Among the five GF grains used in the first activity, only sorghum and quinoa were selected for the subsequent activity because they showed suitable brewing attitudes (in terms of extract, Free Amino Nitrogen, alcohol content, foam stability) and the highest sensory score (in terms of consumers acceptability). Central Composite Design (CCD) was performed to optimize the grist composition and mashing conditions to formulate GF beer with minimal experimental trials, taking into account both technological and sensory aspects. Sorghum:quinoa ratio (expressed as % of sorghum) (X_1) and time of protein rest (X_2) were selected as independent variables. The aim was to individuate the proper combination between sorghum and quinoa useful in maximizing both response variables: 1) extract content (Y_1) of wort so that during sparging it will be possible adding more water to reach the aimed extract and simultaneously obtaining a dilution effect on gluten content; 2) overall liking (Y_2). Time of protein rest was chosen as independent variable to evaluate its impact on gluten content since peptidase activity could promote gluten reduction in the following steps due to an enhanced protein breakdown (Watson et al., 2019).

2.3 Optimization of pilot scale GF beer

Among the eleven experimental mashing trials performed according to CCD, the formulation with the highest overall liking and extract content, consisting in 6% of sorghum and 34% of quinoa and brewed by performing 20 min of protein rest, was subsequently optimized in the 5hL pilot brewery of KU Leuven, Technology Campus Gent (Belgium). Since gluten content in final beer not only depends on grist composition but also on brewing steps, pilot scale production was performed with the aim to implement technological advances and to better control all of the brewing step that could promote a natural gluten reduction. Considering these assumptions, it was decided to extend: 1) time of boiling step, because it could enhances protein coagulation by glycosylation, formation of proteins-polyphenols complexes and denaturation (Kerpes et al., 2017); 2) time of cold maturation, in order to achieve an enhanced protein sedimentation.



The strategy used in this PhD project, by acting only on grist composition and managing some brewing step, was compared with enzymatic and precipitation treatments, to evaluate if it allowed to reduce gluten content below 20 ppm thus obtaining the "gluten free" statement. For this purpose, the pilot scale wort, brewed with 6% of sorghum and 34% of quinoa, was split into 4 fermenters. Each of them pointed to a different treatment, as showed in Figure 1. The first fermenter was further split to investigate the influence of a longer cold maturation (30 days) on naturally reduction of gluten content. RIDASCREEN competitive R5-ELISA (Art. No. R7021, RBiopharm, Darmstadt, Germany) will be performed on final beers to quantify the gluten content.

Figure 1. *Experimental design used to compare the impact of the different approaches to produce GF beer both on quality attributes of beer and on gluten reduction. 1.S6Q34= untreated pilot scale wort; 2.AN-PEP= enzymatic treatment performed by adding Brewers Clarex®; 3.T= precipitation treatment by using tannic acid (Brewtan® B); 4.SG= precipitation treatment with the use of silica gel (Daraclar® 7500).*

2.4 Physicochemical and sensory characterization of wort and beer samples and statistical analysis

The following standard wort and beer analyses were carried out on each sample according to EBC-methods (European Brewery Convention, 2007): extract (°P), free amino nitrogen (FAN) (mg/L), pH, colour (EBC-unit), alcohol (% v/v), total nitrogen (mg/L); foam stability (sec); bitterness (BU). As regard sensory analysis, all beer samples were assessed by a panel of untrained non-celiac consumers. They were asked to taste the beers and to express their overall liking by using the nine-point hedonic scale. Consumers were also asked to describe the sensory profile of beer using the Check-All-That-Apply (CATA) questionnaire. Data were analysed by one-way ANOVA. Significant differences between means were computed by Tukey's HSD (Honestly Significantly Different) test at a significance level of 0.05.

3. Results and Discussion

3.1 Assessment of brewing attitudes of unmalted gluten free grains

As showed in Table 2, partial replacement of barley malt with 40% of unmalted GF grains led to a significant decrease ($p<0.05$) in extract and alcohol content compared than all-barley malt sample (PR), except for QB. To improve the extract yield, a pre-gelatinization step was carried out. It was observed a significant increase in extract content (°P) in SB, MB and AB wort samples ($p<0.05$) but not in BB and QB (Fig.2). All wort samples brewed with unmalted GF grains showed a FAN content significantly lower than PR ($p<0.05$), but still in an acceptable range for a good fermentation performance (>130mg/L). As regard sensory analysis results, SB and QB were considered the most preferred by 66 untrained non-celiac consumers, receiving an acceptability score of 6.83 and 7.05, respectively, on a nine-point hedonic scale. Since all samples could be defined as "beer", because the extract content in all wort samples was > 10.5°P and alcohol content was > 3.5 %v/v (Gazzetta Ufficiale della Repubblica Italiana, 1962), as well as beers brewed with ungelatinized grains had nonetheless suitable sensory characteristics, in the following activities pre-gelatinization step was bypassed, with a view to more environmentally and economically sustainable time-saving process. However, all beer samples had still a gluten content above 20 ppm, detected by qualitative test kit AgraStrip® Gluten G12™ (Romer Labs, Inc., Union, MO). Therefore, the subsequent activities were carried out to further reduce gluten in final beers. Results in more detail regarding this first activity are reported in Cela et al. (2022).

Sample	Extract (°P)	FAN (mg/L)	Alcohol (%v/v)	Gluten (qualitative	Overall liking
PR	13.71±0.35^{a}	322±11^{a}	5.63±0.14^{a}	> 20 ppm	-
SB	11.91±0.15^{b}	178±17^{d}	4.73±0.14^{bc}	> 20 ppm	6.83±0.15^{ab}
MB	11.91±0.37^{b}	150±3^{e}	4.87±0.14^{b}	> 20 ppm	6.47±0.17^{b}
BB	11.31±0.33^{c}	221±33^{b}	4.38±0.13^{d}	> 20 ppm	6.44±0.16^{b}
QB	13.80±0.15^{a}	196±20^{c}	5.49±0.10^{a}	> 20 ppm	7.05±0.15^{a}
AB	11.03±0.29^{c}	175±18^{d}	4.60±0.14^{e}	> 20 ppm	5.85±0.23^{c}

Table 2. *Physicochemical properties of wort and beer samples. n= 2 technological repetitions. Values in the same column followed by a different letter are statistically different ($p<0.05$), following pairwise comparison by Tukey's HSD test. PR= all barley malt; SB= 40% unmalted sorghum; MB= 40% unmalted millet; BB= 40% unmalted buckwheat; QB= 40% unmalted quinoa; AB= 40% unmalted*

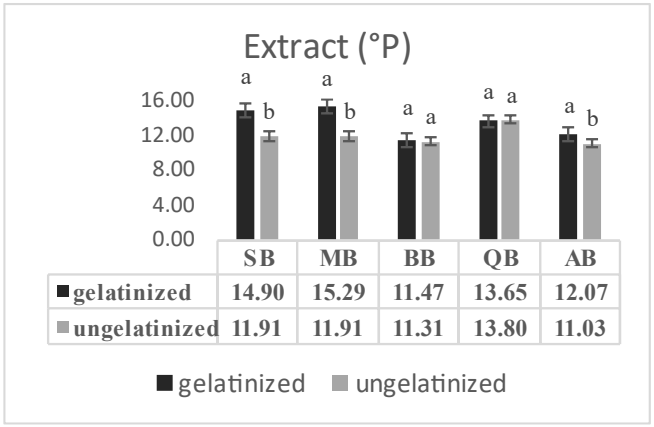


Figure 2. *Extract content of samples brewed with ungelatinized and gelatinized gluten free grains. SB = sorghum; MB = millet;BB = buckwheat; QB =quinoa; AB = amaranth. Different letters indicate significantly different values (p < 0.05) according to Tukey's HSD test.*

3.2 Optimization of grist composition and mashing conditions to formulate GF beer

Eleven mashing trials were randomly performed according to CCD. A significant negative Pearson correlation between sorghum concentration (%) and extract content (%w/w) was observed (r= -0.759, p<0.01). The higher the % of unmalted sorghum used in mashing the lower the extract content of final wort. Protein rest is suggested when higher % of unmalted grains are used in brewing, as in this study, but it is also performed aiming at activate proteolytic enzymes in order to break down high molecular weight proteins surrounding the starch, as for sorghum. Therefore, it could promote the enzymatic conversion of starch during mashing. Nevertheless, this study did not confirm this statement since no significant effect (p>0.05) of protein rest time on extract content was observed. There was no significant correlation between overall liking of final beers and both sorghum concentration and time of protein rest (p>0.05). Sensory acceptability depends on personal preferences of the consumers therefore also psychological and sociological conditions or consumption behavior may cause the different overall liking score (Granato et al., 2010). This is the reason why sensory data are difficult to fit in a mathematical model, mainly when panel is formed by untrained consumers. Scatter plots between independent and response variables used in this activity are presented in Figure 3.

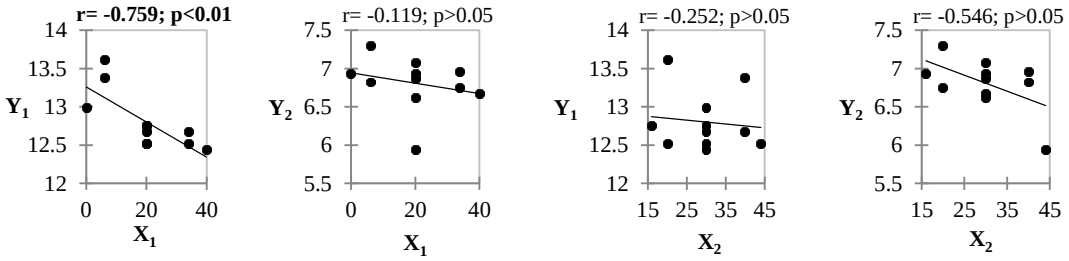


Figure 3. *Scatter plots relationship between independent and response variables and Pearson correlation coefficient (r). X_1= sorghum concentration (%); X_2= protein rest time; Y_1=extract content (% w/w); Y_2= overall liking*

Runs	Sample ID	Independent variables		Responses	
		X_1	X_2	Y_1	Y_2
1	**S6PR20**	6	20	**13.61^{a}**	**7.30^{a}**
2	S34PR20	34	20	12.52^{cd}	6.76^{bc}
3	S6PR40	6	40	13.38^{ab}	6.82^{bc}
4	S34PR40	34	40	12.67^{cd}	6.96^{abc}
5	S0PR30	0	30	12.99^{bc}	6.93^{abc}
6	S40PR30	40	30	12.44^{d}	6.68^{bc}
7	S20PR16	20	16	12.75^{cd}	6.93^{abc}
8	S20PR44	20	44	12.52^{cd}	5.94^{d}
9	S20PR30	20	30	12.52^{cd}	6.87^{bc}
10	S20PR30	20	30	12.75^{cd}	6.62^{c}
11	S20PR30	20	30	12.67^{cd}	7.08^{ab}

Table 3. *Experimental design and values of response.*
X_1= Sorghum concentration (%); X_2= Time of protein rest (min); Y_1= Extract (%w/w); Y_2= Overall liking. Different letters in the same column represent statistical different results according to the Tukey HSD test (P< 0.05).

As reported in Table 3, brewing with 6% of sorghum and 34% of quinoa and by performing 20 min of protein rest, allowed to maximize extract content and overall liking. However, this formulation did not fit in the "gluten free" statement because it showed a gluten content above 20 ppm.

3.3 Optimization of pilot scale GF beer

According to the boiling trial performed on laboratory scale by using a temperature-controlled mash bath (LB-8, Lochner, Bayreuth, Germany), no significant difference (p>0.05) emerged between the hot break precipitated after 60 and 90 min of boiling. Taking into account the result of this trial, wort boiling during pilot scale production lasted 60 min also because long boiling time implies some disadvantages: it negatively influences foam retention and involves high evaporation rate and high energy consumption, not suited for a sustainable process.

Table 4. *Standard quality attributes of pilot scale beer samples. Data are expressed as mean ± standard deviation (n=4). Values in the same column followed by a different letter are statistically different (p<0.05), following pairwise comparison by Tukey's HSD test. 1.S6Q34= untreated beer sample; 2.AN-PEP=enzymatic treatment; 3.T=precipitation treatment by using tannins; 4.SG= precipitation treatment by using silica gel.*

Sample	Apparent Extract (%w/w)	Apparent Degree of Fermentation (%)	Alcohol (%v/v)	pH	Colour (EBC-U)	Foam stability (sec)	Bitterness (IBU)
1.S6Q34	3.36±0.01^{b}	74.58±0.08^{b}	5.30±0.01^{a}	4.29±0.00^{c}	28.04±1.38^{a}	135±3.67^{c}	34.52±0.58^{c}
2.AN-PEP	3.47±0.00^{ab}	73.18±0.01^{c}	5.08±0.00^{c}	4.38±0.00^{a}	24.95±0.07^{b}	212±0.82^{a}	36.48±0.67^{b}
3.T	3.54±0.00^{a}	72.39±0.00^{c}	4.98±0.00^{d}	4.39±0.01^{a}	21.20±0.06^{c}	210±2.04^{a}	38.82±0.91^{a}
4.SG	3.11±0.18^{c}	75.97±1.06^{a}	5.27±0.00^{b}	4.35±0.00^{b}	23.94±0.41^{b}	163±2.04^{b}	38.26±1.02^{a}

Preliminary results of pilot scale samples are reported in Table 4. Further physicochemical analyses and gluten quantitative measurement are currently ongoing. In addition, consumer test will be performed to explore the consumers acceptability of optimized beer.

4. Conclusions and future perspectives

The aim of this Industrial PhD project is the optimization of microbrewing process for GF beers production by using GF grains, taking into account both technological and sensory aspects. PhD activities were carried out in a scale-up approach (from laboratory to micro-industrial scale) to demonstrate the industrial application of this research work. The laboratory scale activities outlined that brewing with 40% of unmalted GF grains resulted in an acceptable final product from physicochemical and sensorial point of view. In particular, beer brewed with 6% of sorghum and 34% of quinoa received the best sensory score in terms of consumers acceptability showing that those aforementioned GF grains are promising as brewing adjuncts since they did not adversely affect the main brewing parameters and the sensory satisfaction of final beers. The pilot scale (5hL) activities at Technology Campus Gent in Belgium are currently ongoing. Quantitative gluten measurement on pilot scale beers will confirm or decline the hypothesis that it is possible naturally reduce gluten content below 20 ppm simply by acting on grist composition and by carefully controlling brew parameters, without the need for enzymatic or precipitation treatments. In addition, a consumer test will be performed to explore the consumers acceptability of the beers. Furthermore, the optimized experimental beer production will be validated on micro-industrial scale, at "Birrificio 79 srl", industrial partner of this PhD project. A conclusive sensory analysis will suggest if the unconventional beer brewed with GF grains can be appreciated by traditional beer consumers as well, thus suggesting a new competitive landscape for beer market.

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Leavened baked goods for improving the functionality

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Ph.D. thesis is divided into two main activities. The main activity aimed to investigate the *in vitro* digestibility of baked goods. In particular, how cereal flours, starters, enzymes, and process parameters may affect the *in vitro* digestibility of sourdough and baker's yeast bread. As part of the thesis the second activity, focused on protein digestibility and related compounds that can be positively affect the *in vitro* digestibility of different breads.

Prodotti da Forno Fermentati per Migliorare la Funzionalità

La tesi di dottorato è suddivisa in due attività principali. L'attività principale ha mirato a studiare la digeribilità *in vitro* dei prodotti da forno. In particolare, è stato valutato come le farine di cereali, gli starter, gli enzimi e i parametri di processo influenzano la digeribilità *in vitro* del pane prodotto con lievito di birra e lievito madre. In secondo luogo, l'attenzione è stata posta sulla digeribilità delle proteine e i suoi composti correlati che possono influire positivamente sulla digeribilità *in vitro* dei diversi pani prodotti.

Key words: digestibility; sourdough breads; IVPD (*in vitro* protein digestibility); pGI (predicted glycemic index); ammino acids; nutritional indexes.

1. Introduction

This oral communication reports the main results of the following four activities directed to:

A1) Experimental design and production of a wide range of sourdough and baker's yeast breads.
A2) Biochemical and nutritional characterization of the sourdough and baker's yeast breads.
A3) Selection of the factors having the most effect on the *in vitro* digestibility of sourdough breads.
A4) Investigation on peptides, amino acid profile and related digestible protein quality indices of the best conditions.

Baked goods represent a staple food of human diet. Since many years, breads and other baked goods underwent investigation to improve their sensory, rheology and shelf-life attributes (Arora et al., 2021). Nowadays, the global population has shifted its focus toward the consumption of foods containing health and nutritional claims, for the prevention of disease and the maintenance of well-being state (Graça et al., 2021). Digestibility represents a new target to improve health features of fermented baked goods, such as bread, where protein and starch digestibility represent a key role (Liu et al., 2017; Rizzello et al., 2019). Fermented baked goods digestibility is predominantly related to raw materials and manufacturing factors. In particular, the proteins hydrolysis that occurs during bread production represents one of the key agents in the digestive process, mainly depending on the amino acid balance and quantity (Maurya and Kushwaha, 2019). Many studies highlighted that bread digestibility may be positively influenced by long fermented sourdough and its microbial composition (Demirkesen-Bicak et al., 2021; Rizzello et al., 2019). As far as we know, no studies are yet available to show how a wide range of factors affect bread digestibility. This work aimed at assessing the *in vitro* bread digestibility, as nutritional marker, of different experimental bread. In particular, the activity focused on the identification and investigation of the main drivers affecting the sourdough bread digestibility.

2. Experimental Procedures

Forty-six different breads were manufactured with different raw materials/enzymes (flour, fungal proteases, LAB cytoplasmic extracts, and gluten), type of sourdough (fresh or commercial liquid or dry), strains of lactic acid bacteria and yeasts, and time and temperature of fermentation. Breads were firstly evaluated for their acidic parameters, total free amino acids (TFAA) value, *in vitro* protein digestibility (IVPD) and predicted glycemic Index (pGI) to screen the best conditions. The analysis on the total FAA and IVPD allowed a deeper evaluation of bread digestibility. Most digestible breads were analysed for complete amino acid profile and related digestible protein quality indices (Chemical Score, Essential Amino Acids Index, Biological Value, Protein Efficiency Ratio). Furthermore, protein and peptide patterns of bread's dough were used to determine the level of protein hydrolysis through RP-HPLC-UV analysis and Tricine-sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE).

3. Materials and Methods

3.1 Screening of factors potentially affecting the *in vitro* digestibility of sourdough breads.
This work was based on a previous work in which authors demonstrated that sourdough fermented bread is more digestible compare with baker's yeast bread (Rizzello et al., 2019). Based on this, factors presumptively influencing *in vitro* digestibility grouped on categories such as raw materials, enzymes, lactic acid bacteria and yeasts strains and processing conditions.

3.2 Sourdough propagation.
Six different flours (soft wheat, SW; durum wheat, DW; whole wheat, WW; spelt, S; rye, R; and oat, O) were used for making sourdoughs according to a traditional protocol (Rizzello et al., 2019). Sourdoughs were prepared with cell suspensions of *L. plantarum* CR1, *F. rossiae* CR5 and *S. cerevisiae* E10 (initial cell density of ca. 7.0 Log cfu/g and ca. 6.0 Log cfu/g for lactic acid bacteria and yeast, respectively) and fermented for 12 h at 30 °C. Sourdoughs were mature after two back slopping steps. Mature sourdoughs (20%, w/w) were used for further fermentation for 6 h and again for 6 h or 24 h at 30 °C to produce type I sourdoughs S_6 and S_{24}, respectively. Different sourdoughs were produced with soft wheat flour and lactic acid bacteria strains previously selected based on high and low peptidase activity (P) and fermentation quotient (FQ), in addition to the above starters. The maltase negative yeast *Kazachstania* sp. was inoculated in soft wheat flour instead of *S. cerevisiae* E10 to produce sourdough K.

3.3 Bread Making.
Forty-six different baker's yeast (BB) and/or sourdough (SB) breads (Dough yield; DY=160) combinations were manufactured. Baker's yeast bread (BB) was made mixing flour (62.5% w/w), water (37.5% w/w) and 1.5% (w/w) of baker's yeast. The fermentation lasted 1.5 h at 30 °C.
The sourdough bread consisted of 20% (w/w) selected sourdough (S_6 and S_{24}), which was mixed with flour (46.7% w/w) and water (23.3% w/w), and further incubated for 4 h at 30 °C. Breads were baked at 220 °C for 30 min. For each condition one baker's yeast bread (BB), one sourdough bread produced with S_6 (SB_6), and one sourdough bread produced with S_{24} (SB_{24}) were manufactured. For ready to use-commercial sourdough, dried (5% w/w) and liquid (20% w/w) were added to produce dough and fermented for 1.5 hours or for 4 hours. The three control breads (SW-BB, SW-SB_6, SW-SB_{24}) were compared with the different thesis.

3.4 Breads characterization.
Apart from a preliminary investigation (pH and TTA), the forty-six experimental breads were evaluated for their biochemical and nutritional characteristics. The water/salt-soluble extract of breads (WSE) were used to determine the amount of total FAA (Doi et al., 1981). The *in vitro* protein digestibility (IVPD) of breads was determined as described by Akeson and Stahmann (1964). A procedure mimicking the *in vivo* digestion of starch was carried out to evaluate the starch hydrolysis rate and *in vitro* starch hydrolysis index (HI) (de Angelis et al., 2009). The pGI, determining the hydrolis index (HI), was evaluate according to Capriles and Arêas (2013).

3.5 Selection of the best conditions. The selection of the most promising conditions was carried out in two steps. The first (Q1) and third (Q3) quartile was calculated on the dataset (total FAA, IVPD and pGI). Firstly, the selection of breads considered values higher than 3rd quartile for total FAA and IVPD, and lower than 1st quartile for pGI. Secondly, within the first group of observation selected (n=19), data from total FAA and IVPD variables has been standardized to select breads with the higher protein digestibility score.

3.6 Investigation on protein hydrolysis. Selected doughs were characterized for the water/salt soluble protein and peptide profiles. RP-HPLC analysis was carried out on WSE and proteins and peptides were detected by measuring UV absorbance at 214 nm (Yu et al., 2013). Tricine-sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), was carried out according to the method of (Schägger, 2006).

3.7 Individual amino acids profile and nutritional indexes. The individual amino acids profiles of the six selected breads were analyzed by a Biochrom 30+ series Amino Acid Analyzer as described by (Rizzello et al., 2014). In order to evaluate nutritional indexes FAA profile was determined in the hydrolysate. Chemical Score (CS), which estimates the amount of protein required to provide the minimal EAA pattern as re-defined by FAO in 2007, indicates the CS of the most limiting EAA that is present in the test protein. EAAI, BV, PER and NI were calculated according to the models developed by Oser (1959).

3.8 Statistical analysis. All statistical analyses were performed in Statistica 8.0 (StatSoft Inc., Tulsa, USA) and XLSTAT® software (version 2020.5.1.1052, Addinsoft, New York). Data were subjected to Principal Component Analysis (PCA), percentile calculation and to one-way ANOVA; comparison of sample means was obtained by Tukey's procedure with a 95% confidence interval ($p < 0.05$).

4. Results and Discussion

Since long time, both tradition and empirical observations concur on the higher digestibility of sourdough baked goods compared to faster processes using chemical leavening or baker's yeast. Recently, has been demonstrated through an *in vivo* challenge that the sourdough bread promotes a faster oro-cecal transit, a higher absorption of free amino acids in the blood and decreases the post-prandial glycemia (Rizzello et al., 2019). Based on these results, we designated a wide range of factors that potentially affect *in vitro* bread digestibility with a focus on protein hydrolysis.

4.1 Bread making with different conditions.

All baker`s yeast breads made with different flours (SW, WW, DW, S, R and O) showed values of pH significantly ($p < 0.05$) higher than those breads made with the same flours but fermented with sourdough. Contrarily, the highest acidification was found in breads produced with the sourdough fermented for 24 h (S_{24}). Overall, bread produced with longer fermented sourdough (S_{24}) showed the best values in term of IVPD. Breads produced with soft wheat breads were used as references and compared with breads belong to each category (flours, LAB and yeast strains, cytoplasmic extract, proteases, commercial sourdough, and temperature). The lowest concentration of total FAA was found in SW-BB (89 ± 10), but the use of S_{24} with soft wheat flours increase the release of total FAA in SW-SB_{24} (586 ± 13 mg/kg). Rye and spelt flours together with S_{24} seemed to have the potential to increase the content of total FAA (676 ± 6 and 652 ± 8 mg/kg, respectively). The higher IVPD% values were found on S-SB_{24}, R-SB_{24} and WW-SB_{24}, made with spelt, rye and whole wheat flours (79.5 ± 0.4, 78.9 ± 0.4 and 79.0 ± 0.5 %, respectively). The use of oat flour showed a lower pGI (55.4 ± 0.4) compared with SW-SB_{24} (58.7 ± 0.5) but did not have a strong effect on protein digestibility, while as found for the IVPD % values S-SB_{24}, R-SB_{24} and WW-SB_{24} showed the lowest pGI among all breads produced (53.5 ± 0.4, 53.5 ± 0.4 and 53.5 ± 0.4, respectively). The use of lactic acid bacteria strains with high peptidase activity caused an increase of the accumulation of total FAA. P(+)-SB_{24} was the bread with the highest content of total FAA (603 ± 8 mg/kg) and IVPD (79.9 ± 0.7) and lowest pGI (61.4 ± 0.1) among breads produced with sourdough with supplementary lactic acid bacteria strains [FQ(+), FQ(-), P(+), P(-)]. The values of IVPD for FQ(+)-SB_{24} was 3.2 % higher compared to FQ(-)-SB_{24}, but the addition of LAB with different FQ did not affect the pGI. The addition of the cytoplasmic extract in soft wheat bread (CEB-BB) led to an increase of total FAA and to an IVPD value of 72.8 ± 0.3% compared with the control bread (SW-BB). The addition of fungal proteases E1 and E2 (*Aspergillus oryzae* and *A.niger)*, reflected a release of total FAA when soft wheat dough was added with protease E2 compared to E1-SB_{24} (612 ± 9 and 646 ± 7). The values of IVPD and pGI for E1-SB_{24} and E2-SB_{24} did not statistically ($p > 0.05$) differ (79.0 ± 0.5 % and 63.0 ± 0.4; and 78.3 ± 0.4 % and 62.3 ± 0.9, respectively). Among bread produce with sourdough with high and low temperature, the highest values of total FAA and IVPD were observed for bread 37°C-SB_{24} (total FAA of 672 ± 24 mg/kg and IVPD of 80.1 ± 0.26 %).

4.2 Principal component analysis.

The Principal Component Analysis (PCA) was applied on the overall dataset. PC1 and PC2 explained, respectively, 69.57 and 19.89 % of the total variance. The PCA divided the samples in three groups. The variable of pH and pGI contribute majorly to discriminate baker's yeast bread, whereas sourdough breads, made with sourdough with longer fermentation time (S_{24}) are strongly correlated with protein variables (total FAA and IVPD). An intermediate group was formed by breads fermented with S_6 (Figure 1).

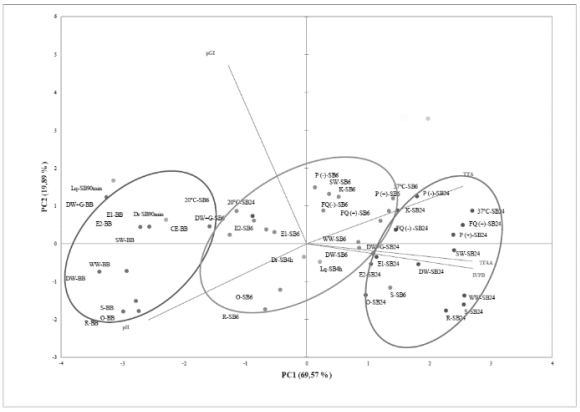

Figure 1. *Principal component analysis (PCA) on the biochemical and nutritional characteristics of forty-six breads. Biplot of PC1 (69.57%) vs. PC2 (19.89%) includes variables such as pH, total treatable acidity (TTA), total free amino acid (FAA), in vitro protein digestibility (IVPD) and predicted glycemic index (pGI). Baker's yeast bread, BB. Sourdough S6 bread, SB6. Sourdough S24 bread, SB24.*

4.3 Selection of the most promising conditions.

The overall data set was subjected to the percentile calculation (Figure 2). Breads placed above the 75th percentile of total FAA (threshold, 571.33 mg/kg) and IVPD (threshold 76.91%) dataset, and below the 25th percentile of pGI values (threshold 58.80) were selected (n=19). Among these group three breads made with different flours (WW-SB_{24}, S-SB_{24} and R-SB_{24}) and three breads made with the other conditions (E2-SB_{24}, P(+)-SB_{24} and 37°C-

SB_{24}) were selected for the highest total FAA and IVPD (equally weighted variables). The results of total FAA (mg/kg), IVPD % and pGI of the selected breads are reported in Table 1.

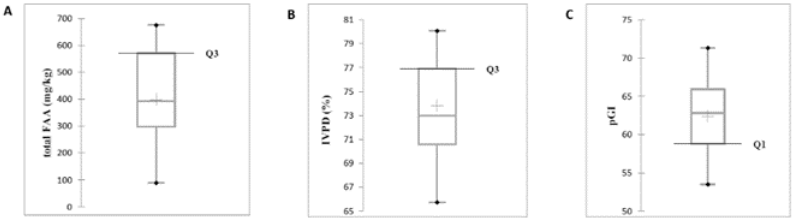

Figure 2. *Box plot of the sorted values of total FAA (A), IVPD (B), pGI (C). Q1, first quartile; Q3, third quartile. Selection of breads: total FAA values, x > Q3 (571.33 mg/kg) ; IVPD, x > Q3 (76.91%) ; pGI, x < Q1(58.80).*

Table 1. *Biochemical and nutritional characteristics of the best performing breads; TFAA, total free amino acids; IVPD, in vitro protein digestibility; pGI, predicted glycemic index.*

Bread	TFAA (mg/kg)	IVPD %	pGI
WW-SB24	586 ± 13	79.0 ± 0.5	53.6 ± 0.5
S-SB24	652 ± 8	79.5 ± 0.4	53.5 ± 0.4
R-SB24	676 ± 6	78.9 ± 0.4	53.5 ± 0.4
E2-SB24	646 ± 7	78.3 ± 0.4	62.3 ± 0.9
P (+) -SB24	603 ± 8	79.9 ± 0.7	61.4 ± 0.1
37°C-SB24	672 ± 24	80.1 ± 0.3	64.5 ± 0.5

4.4 Protein derivatives and nutritional indexes.

Samples (WSE of doughs before baking) were analyzed through a reverse-phase chromatography to investigate proteins and peptides profiles (MW less than 20 kDa). SW-BB showed the lowest total area under the curve (143.80 mAU*min) compare with the sourdough breads produced with the same flour (SW). The highest total area was found for S-SB_{24}, E2- SB_{24}, P (+)-SB_{24} and 37°C-SB_{24}. The Tricine-SDS-PAGE of albumin/globulin fractions showed the presence of polypeptides ranged from ca. 6.5 to 66 kDa. Overall, the profiles of doughs fermented with baker`s yeast and sourdoughs did not differ, apart from less intense profile found in dough made with rye flour (R-SB_{24}). The use of whole wheat flour showed the highest content of essential amino acids (Thr, Val, Met, Ile, Leu, Phe, Lys, Trp) among all breads selected (Table 2). Apart from WW-SB_{24}, a higher significant ($p < 0.05$) amount of essential amino acids was found for R-SB_{24} (Thr, Val, Lys), S-SB_{24} (Trp), E2-SB_{24} (Met, Ile, Leu, Phe). The use of whole wheat and E2 fungal protease (50 ppm, w/w) increase the amount of GABA compared to soft wheat baker's yeast bread (215.05 and 147.89 mg/kg, respectively). The highest chemical score was found in E2-SB_{24} (43.1 ± 1.2%).

Table 2. *Concentration of essential free amino acids (mg/kg) and GABA of selected breads. NF means not found. $^{a-e}$Values in the same row with different superscript letters differ significantly ($p < 0.05$) based on one-way ANOVA (Tuckey-Kramer). The data are the means of three independent analysis ± standard deviations (n =3).*

	SW-BB	SW-SB_6	SW-SB_{24}	WW-SB_{24}	R-SB_{24}	S-SB_{24}	E2-SB_{24}	37°C-SB_{24}	P(+)-SB_{24}
Thr	10.49±0.10^{i}	13.67±0.07^{h}	18.31±0.13^{g}	70.62±0.52^{a}	52.15±0.38^{b}	40.59±0.30^{d}	43.11±0.32^{c}	22.67±0.16^{f}	37.02±0.27^{e}
Val	25.70±0.80^{g}	14.12±8.90^{h}	59.41±1.85^{f}	190.47±5.96^{a}	131.81±4.12^{b}	101.56±3.17^{d}	111.61±3.49^{c}	60.56±1.89^{f}	76.20±2.38^{e}
Met	4.26±0.08^{h}	5.16±0.10^{g}	17.24±0.36^{f}	72.88±1.52^{a}	46.36±0.97^{c}	41.13±0.86^{d}	41.79±0.87^{d}	29.98±0.62^{e}	60.55±1.26^{b}
Ile	20.62±0.35^{h}	23.01±0.39^{g}	46.51±0.79^{f}	124.58±2.12^{a}	82.54±1.40^{c}	79.84±1.36^{c}	72.77±1.24^{d}	54.43±0.92^{e}	91.17±1.55^{b}
Leu	14.57±0.42^{g}	28.75±0.83^{f}	103.95±3.01^{e}	348.40±10.10^{a}	210.72±6.11^{c}	201.26±5.84^{c}	175.98±5.10^{d}	99.04±2.87^{e}	223.83±6.49^{b}
Phe	12.11±0.19^{g}	14.26±0.23^{f}	54.26±4.89^{e}	172.95±2.84^{a}	99.63±1.64^{b}	96.87±1.59^{c}	93.55±1.54^{d}	51.50±5.84^{e}	101.31±1.66^{b}
Lys	15.08±2.11^{e}	13.12±1.84^{e}	44.48±6.23^{d}	194.45±27.27^{a}	106.50±14.93^{b}	104.64±14.67^{b}	96.05±13.47^{b}	45.15±6.33^{d}	72.37±10.14^{c}
Trp	57.91±0.11^{f}	43.80±0.08^{g}	61.97±0.12^{e}	188.58±0.36^{a}	24.43±0.04^{h}	125.29±0.24^{b}	109.64±0.21^{c}	72.86±0.14^{d}	72.54±0.14^{d}
GABA	40.98±5.43^{c}	43.71±1.97^{e}	42.78 ± 1.93^{e}	256.03±11.58^{a}	105.54±4.77^{d}	116.58±5.27^{cd}	188.87±8.54^{b}	110.31±4.99^{cd}	118.81±5.37^{c}

The same was found for the EAA index (74.4 ± 0.8) and BV (69.4 ± 0.9), while SW-BB had the lowest values of both indexes (EAA index, 50.8 ± 1.3; BV, 43.7 ± 0.6). Among the other breads, WW-SB_{24}, R-SB_{24}, S-SB_{24}, and P(+)-SB_{24} showed the higher EAA index but they not statistically differ among them, and R-SB_{24}, S-SB_{24} showed the higher BV. SW-BB had the significant ($p<0.05$) highest value of PER (28.3 ± 0.8), while R-SB_{24} and SW-SB_{24} showed the lowest value. The highest Nutritional Index was found for E2-SB_{24} (6.5 ± 0.2). SW-SB_{24}, S-SB_{24} and 37°C-SB_{24} had almost similar values of NI, which statistically ($p < 0.05$) differed compared to WW-SB_{24} and R-SB_{24}. Nutritional indexes values of selected breads are reported in Figure 3.

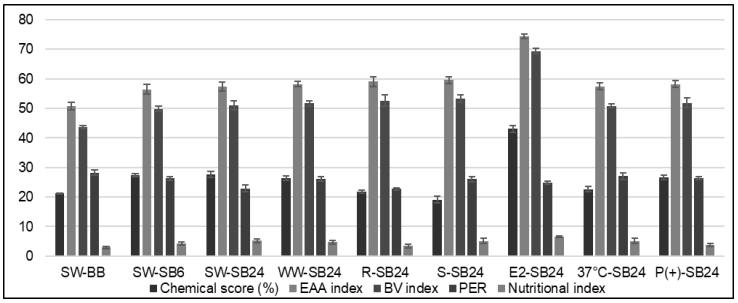

Figure 3. *Bar chart of nutritional indexes of selected breads including chemical score (CS), essential amino acid index (EAA), biological value (BV) and protein efficiency ratio (PER).*

5. Conclusion and future prospective

We believed to have used the most efficient approach to investigate the potential effect of raw material and processing parameters to evaluate *in vitro* bread digestibility. The preliminary screening among all the factors considered, allowed us to have an overview about the correlation between acidic conditions, total FAA, IVPD% and pGI and different use of raw materials and processing parameters. These findings showed a greater digestibility of sourdough breads produced with whole wheat, spelt and rye flours. In addition, the use of LAB strains selected for high peptidase activity, the addition of fungal proteases and fermentation of sourdough at the optimum temperature at 37°C yielded breads with improved digestibility and quality of protein nutritional indexes. In this work an *in vitro* approach was necessary due to the multitude of factors screened. Therefore, further investigations are required to substantiate that these highly digestible and nutraceutical products could be readily accepted by consumers. Nevertheless, our findings are a valuable starting point for optimizing and enhancing the technological processes, and for an *in vivo* challenge.

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Application of Next Generation Sequencing for the characterization of microbial hazard in Italian dairy and meat food productions realized in small-scale plants

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The PhD thesis is aimed at investigating the prevalence and characterization of foodborne bacteria collected from selected artisanal food products. Two ready-to-eat productions (soft cheese and salami) and their environmental sites were characterized. Whole genome sequencing combined with bioinformatics approaches were further implemented to most of the detected bacteria, to trace the contamination source and elucidate genetic traits of concern. Salami and cheese did not present any health risk regarding foodborne pathogens, but *Listeria monocytogenes*, *Staphylococcus aureus* and *Enterobacteriaceae* were present in the environment. *Klebsiella* spp. reported antimicrobial resistance and virulence genes, suggesting an increasing surveillance.

Applicazione del Sequenziamento di Nuova Generazione per la caratterizzazione del rischio microbico in produzioni alimentari di origine lattiero-casearia e carnea appartenenti alla filiera artigianale italiana

La tesi di dottorato ha lo scopo di indagare la prevalenza e la caratterizzazione di batteri di origine alimentare isolati da alimenti artigianali. Sono stati analizzati un formaggio a pasta molle e un salame insieme al relativo ambiente di produzione. Il sequenziamento genomico combinato con approcci bioinformatici è stato utilizzato sulle specie microbiche prevalenti, al fine di tracciarne la fonte di contaminazione e studiarne i tratti genetici. Salame e formaggio non presentavano batteri patogeni, ma nell'ambiente erano presenti *Listeria monocytogenes*, *Staphylococcus aureus* ed *Enterobacteriaceae*, quali *Klebsiella* spp. che ha riportato geni di resistenza e virulenza, suggerendo una crescente sorveglianza.

Key words: artisanal food chain, foodborne bacteria, Whole Genome Sequencing, bioinformatics analyses

1. Introduction

According to the PhD thesis main purpose, this oral communication illustrates the results obtained from the following activities:

1) collection of samples from food matrices and environmental sites along a defined production period encompassing different batches;
2) application of cultured-based and molecular methods for the detection of foodborne bacteria (*Enterobacteriaceae, Listeria Monocytogenes and Staphylococcus aureus*);
3) DNA extraction and Whole Genome Sequencing of most prevalent bacteria;
4) application of advanced bioinformatic genotyping tools to describe key features of proven clinical importance (e.g., antimicrobial resistance and virulence) and predict the strains relatedness for assessing the contamination sources along each production chain.

2. Microbial safety of dairy and meat fermented artisanal foods

Artisanal foods have become increasingly popular in recent years and quickly turned into a fast-growing niche market among the food enterprise sectors. Several aspects could be addressed behind their wider expansion. Firstly, their growing support to social and environmental issues, such as sustainability, traceability, and the commitment to everything local (Cirne *et al.*, 2019). Moreover, since these productions are likely to be realized with local raw ingredients, they are perceived as more genuine and with higher quality ingredients compared to industrial ones, thus rising consumers' appreciation and attraction. Nevertheless, artisanal foods are more exposed to contamination events compared to industrial ones due to the lack of a full automation in production processes and the challenges in monitoring environmental parameters. In such scenario, is highly recommended to control the microbial quality of these productions and their environment to minimise safety risks for the consumers. Among traditional products, soft cheeses are consumed and appreciated worldwide thanks to their delightful sensorial characteristics and valuable nutritional properties. Nevertheless, they have been frequently vehicle of foodborne pathogens transmitted along the processing plant (cheese-making, ripening and storage), resulting in direct or cross-contamination events. For instance, *Staphylococcus aureus* and *Listeria monocytogenes* could be disseminated through the processing plant via floor drains, workers' hands and cheese-contact surfaces. Moreover, Salmonella represents a pathogen of concern in cheeses production (Possas *et al*., 2021). Other bacteria commonly found in cheese are represented by coliforms, members of the family *Enterobacteriaceae*, which are generally

used as indicators for microbiological conditions of food or their processing environments (Gelbíčová *et al*., 2021; Martin *et al*., 2016). Among them, Shiga toxin-producing *E. coli* (STEC) have been associated with outbreaks of strong evidence related to cheese consumption in 2018 (ECDC, 2018). *Enterobacter* and *Citrobacter* could be also found in milk and cheese environments (Trmčić *et al*., 2016). Moreover, *Klebsiella pneumoniae* and *Klebsiella oxytoca* strains have found to be persistent between the production environment and the personnel in a processed cheese plant (Gelbíčová *et al*., 2021). Another popular and widespread traditional product is represented by dry fermented sausages made of pork (so-called "salami"). Typically, they are realized with traditional methods and some of them are produced from local pig breeds, conferring unique organoleptic sensory profiles. The safety of these products is pursued by a combination of hurdles such as ingredients (e.g., NaCl or species) and physicochemical changes (e.g., drying phase) which promote an antagonistic environment for pathogens survival (Vignolo *et al*., 2010). Nevertheless, poor hygienic conditions could contribute to the spread of foodborne pathogens, especially when no starter cultures are added. *Salmonella* is frequently associated to outbreaks linked to the consumption of fermented sausages and other hazards could be also represented by *L. monocytogenes*, especially for its wide survival in processing plants, *S. aureus* and *E. coli* O157:H7 (Patarata *et al*., 2020). *Enterobacteriaceae* could also be found in the sausage mass because of contamination occurred either during slaughtering procedures, being commensal of the gastrointestinal tract of animals, or from utensils used during processing (Fernández-López *et al*., 2008). To support the compliance with current microbiological criteria, such as Regulation (EC) No. 2073/2005, it is crucial to evaluate and control hazards in each food operation for ensuring the safety of the food supply, especially in those products that do not undergo thermal treatments before consumption.

3. Whole Genome Sequencing for food safety investigations

The combination of NGS and bioinformatics is revolutionising public health surveillance of bacterial pathogens and food microbiology. Whole Genome Sequencing (WGS) represents one of the foremost NGS applications in food microbiology. In the public health sector, microbial typing and characterisation methods traditionally applied in the microbiology laboratory such as serotyping, virulence profiling and AMR determination have been increasingly replaced by WGS. This technology has been increasingly expanded to the food industry, aimed at improving the quality and safety of food products. For instance, when a pathogen or spoilage contamination event occurred, WGS could be implemented for tracing the source of microbial contamination along the food chain. By investigating single nucleotide polymorphism along the entire genome, WGS shows a higher discriminatory power compared to previous molecular subtyping methods. In such scenario, this technology could help to (I) identify sources of infection and the transmission route, (II) distinguish between new and recurrent introduction of an organism into the production environment, (III) evaluate the efficacy of cleaning/disinfection procedures (Jagadeesan *et al*., 2019). Furthermore, WGS enables to predict genetic traits such as strains' virulence or AMR, as well as the location of these determinants (chromosome or mobile genetic elements) which could be also addressed to prevent AMR spread and consumer exposure through food products in a One Health perspective. These applications made this technology ideally suited for use in national and international surveillance systems in support of food safety and public health (Brown *et al*., 2019).

4. Materials and Methods

A total of six batches belonging to selected artisanal food RTE productions between January 2020 and May 2021 in small-scale enterprises located in Norther Italy were analysed. The productions corresponded to a soft cheese made from pasteurized cow milk and a salami produced from an Italian swine autochthonous breed with no addition of starter cultures. Food samples, including raw materials and final products, and environmental swabs were taken at each key processing stage. After collection, samples were investigated for *Listeria monocytogenes* (ISO 11290-1:2017), *Salmonella spp*. (ISO 6579-1:2017), *Staphylococcus aureus* (ISO 6888-1/A1:2004), *E. coli* VTEC (ISO 16649:2018). Moreover, *Enterobacteriaceae* were detected by enriching 25 g of food samples or environmental swabs in 225 mL of Buffer Peptone Water (BPW) and incubated for 24 h at 37 °C. BPW pre-enriched cultures were then streaked on MacConkey agar and incubated for 24 h at 37 °C. Suspected colonies were submitted to biochemical test (RapID™ ONE System and RapID™ STAPH PLUS System, Thermo Scientific) and PCR (Wesley *et al*., 2002; Perelle *et al*., 2004; Chander *et al*., 2011; Saraiva *et al*., 2018) for pathogen confirmation. Only for the most prevalent species, antibiotic susceptibility was carried out using the Sensititre™ EUVSEC ready to use plates (Thermo Scientific, USA) testing against 14 antibiotics. Their DNA was further extracted for sequencing purpose using the MagAttract HMW DNA Kit (Qiagen, Hilden, Germany) and libraries were prepared using the TruSeq DNA sample Prep Kit (Illumina, Milan, Italy). The whole genome of selected isolates was paired-end sequenced (250 bp) using the MiSeq platform (Illumina). Quality of raw reads was checked, and *de novo* assembly was carried out with the assembling pipeline fq2dna v21.06 (https://gitlab.pasteur.fr/GIPhy/fq2dna). Taxonomy of strains was confirmed with ReferenceSeeker v1.7.3 (https://github.com/oschwengers/referenceseeker) and Kleborate v2.0.0 (https://github.com/katholt/Kleborate). Contigs were then submitted to specific genotyping tools such as Kleborate to predict the presence of key AMR loci along with virulence factors. The location (chromosomal or plasmid) of genes was assessed by plasmid typing

and reconstruction with MOB-suite v3.0.1 (https://github.com/phac-nml/mob-suite). AMR and virulence patterns were further characterized by comparison with the same target gene retrieved from public sequences, to speculate on hypothetical inter-connection between the artisanal food and other settings (clinical, environmental or in-house). Moreover, pangenome analyses were carried out with Panaroo v1.2.3 (https://github.com/gtonkinhill/panaroo) and IQ-Tree v2.0.6 (https://github.com/Cibiv/IQ-TREE), and phylogenomic reconstructions were performed based on core gene alignments to assess the genetic relatedness of strains along with sequence type assignment (MLST v2.19.0, https://github.com/tseemann/mlst). Free software environment for statistical computing and graphics (R v4.1.2, https://cran.r-project.org/) as well as web-based tool (https://itol.embl.de/, https://jameshadfield.github.io/phandango/#/, https://app.rawgraphs.io/) were used for plotting data and for their interactive visualization.

5. Results and Discussion

5.1 Occurrence of pathogens across the artisanal productions

A total of 1170 samples were overall collected and tested over the 14 months period. *L. monocytogenes* (n=4) was identified only in water drainage channels located in the area dedicated to the drying phase of salami, but no transmission in the food products was detected. Whilst no evidence for food contamination has been provided, these finding confirm *L. monocytogenes* ability to grow and survive in the food production environment and suggest the importance to maintain the areas free from this pathogen, especially in those where there is a high-risk of cross-contamination. VTEC and *Salmonella* weren't detected either in cheese or salami. Regarding *S. aureus*, overall n=6 samples have been confirmed as positive in cheese (final product) and salami (raw material-meat, processing environment, salami during ripening). An occurrence of 80% has been recently described for *S. aureus* isolated from Italian artisanal cheeses, although outbreaks due to consumption of contaminated cheese are rarely reported (Johler *et al*., 2015). The absence of *S. aureus* at the end of the salami ripening doesn't address this pathogen of concern for food consumption, although improvements in hygienic procedures of environmental sites are suggested to achieve pathogens eradication. Other detected bacteria belonged to *Enterobacteriaceae* family, with the majority confirmed as *Klebsiella pneumoniae* and *K. oxytoca* (n=75), followed by *Enterobacter cloacae* (n=56), *Citrobacter freundii* (n=51) and *Hafnia halvei* (n=26). *E. cloacae*, *C. freundii* and *H. alvei* were detected in raw materials and environment of salami and cheese, with the latter showing strains also during the final product storage. In literature, cheese has been described as vehicle of *E. cloacae*, as well as *H. alvei* and *C. freundii* during cheese processing and ripening (Maifreni *et al*., 2013; Tabla *et al*., 2018). Moreover, *Citrobacter* spp. and *H. alvei* have been already found at the start of pork mince fermentation processes (Charmpi *et al*., 2020). Regarding *Klebsiella* spp., representing the highest number of *Enterobacteriaceae*, previous surveys showed that this genus tends to be present in traditional cheese and fermented sausages worldwide. For instance, EU and not-EU traditionally processed cheeses (György & Laslo, 2021; Ogbolu *et al.*, 2014) reported strains of *K. pneumoniae* and *K. michiganensis.* Likewise, *Klebsiella* spp. has been isolated in Europe before the ripening process of pork sausages (Roig-Sagues *et al*., 1996) and in fermented sausages produced without starter addition (Barbieri *et al*., 2021). Considering the higher number of strains distributed in salami and cheese compared to other species, genotyping of collected *Klebsiella* spp. has been carried out by WGS. Whilst not being commonly associated to foodborne illnesses, *Klebsiella* species represent a significant public health problem which has been a focal point over the recent years, due to their role in contributing to the spread of antimicrobial resistance as well as their association not only with nosocomial but also, in some cases, to food related infections (Hu *et al*., 2021). Thus, an increasing number of studies are needed to assess the hazard of *Klebsiella* spp. in food and related environment, especially from Italian artisanal sites for which few investigations are available so far.

5.2 Antimicrobial susceptibility testing

Antimicrobial susceptibility was tested for 73 *Klebsiella* spp., since two strains didn't show plate's growth when being resuscitated from storage at −80°C. MIC results showed all isolates resistant to sulfamethoxazole and 72 of 73 (99%) also to ampicillin.

5.3 WGS and sequence-based taxonomic assignment

A total of 75 *Klebsiella* spp. were sequenced and raw reads of 250 bp were *de novo* assembled into 75 draft genomes. Firstly, strains species previously assessed with biochemical and molecular testing were confirmed by sequence-based taxonomic assignment against selected databases (https://www.ncbi.nlm.nih.gov/refseq). Results showed that identified *K. pneumoniae* and *K. oxytoca* belonged to closely related species distributed withing three complexes, such as *K. pneumoniae* (KpSC), *K. oxytoca* (KoSC) and *K. ornithinolytica* (KornSC). KpSC gathered 17 strains encompassing *K. pneumoniae* sensu stricto (n=16) and *K. variicola* subsp. *variicola* (n=1), KoSC comprised 38 genomes with *K. oxytoca* sensu stricto (n=11), *K. michiganensis* (n=15), *K. pasteurii* (n=5) and *K. grimontii* (n=7) and KornSC was composed by *K. ornithinolytica* (n=13) and *K. planticola* (n=5). Two isolates were identified as *Citrobacter* spp., hence a total of 73 *Klebsiella* species were confirmed, with a 6% overall prevalence among cheese and salami. KpSC and *K. ornithinolytica* were mainly found in salami (94% and 100% of strains respectively), whereas KoSC and *K. planticola* in cheese (respectively 68% and 100%). These results confirm the WGS as the most powerful approach and gold-standard providing reliable taxonomic species

identification for *Klebsiella* spp..

5.4 Pangenome analyses

Regarding the pangenome inference, a total of 19365 gene clusters were identified among the *Klebsiella* spp. population, among which a higher proportion of accessory content was assessed, representing the 85% of all genes. Thus, pangenome was represented by a huge gene variation, underling the heterogenicity of this bacterial population.

5.5 Identification of AMR and virulence loci

Antimicrobial resistance (AMR) gene screening were resistant to penicillins related to the presence of the chromosomally encoded β-lactamases SHV and LEN in KpSC, which correlates with ampicillin MIC results. Moreover, all KoSC showed ESBL related genes *bla*$OXY_{1\text{-}2\text{-}4\text{-}5\text{-}6}$ coding for β-lactamases resistance genes, showing like KpSC a correlation with ampicillin resistance. Aminoglycosides resistance associated genes (*str*AB, *aph*3-Ia, *aph*(3')-Ia) were also detected in KoSC resulting in 8 out of 38 isolates recovered from both food productions and sulfonamides (*sul*2) associated to one *K. pasteurii* strain isolated from cheese. For KornSC, whilst ampicillin resistance was spread among all isolates, no ampicillin resistance determinant genes were identified. Similarly, the phenotypic resistance to sulfamethoxazole was confirmed in all SC but one. However only *K.pasteurii* harboured the *sul*2 gene. Probably other mechanisms such as the overexpression of multidrug efflux pumps might be associated to ampicillin and sulfometoxazole resistance in these isolates. Overall, few antimicrobial resistances were addressed to *Klebsiella* spp., except for detected ESBL genes in both artisanal productions. In cheese, ESBL genes were found in both environmental and stored cheese samples, persisting for all batches, whereas for salami they were mainly detected in raw materials. Beside the identification of AMR, virulence loci concerning siderophores systems were detected. All *K. pneumoniae* harboured acquired aerobactin (*iuc*) alone or in combination with yersiniabactin (*ybt*), with the latter present in 26 out of 38 KoSC genomes and all *K. ornithinolytica*. The *iuc* and/or *ybt* genes have been predominantly observed in isoltes collected from raw materials and environmental samples taken from first processing steps of salami production, suggesting this stage as critical for the spread of these determinants. In cheese, the *iuc* gene was found in one isolate collected in a final product sample during storage. Interestingly, the aerobactin system was predicted in a single lineage (*iuc3*) carried by the conjugative IncFIB/IncFII plasmid which was shared by all samples in high sequence similarity (>98% of average nucleotide identity). To gain insight into the transmission dynamics of acquired *iuc3* locus, a comparison was carried out including *iuc3* sequences hosted by public genomes of *K. pneumoniae* collected from livestock pig samples and hospitalized patients in a close area (Pavia city, Italy) in 2017 and 2018. Public and food (salami and cheese) *K. pneumoniae* were selected based on plasmid replicon type and pMLST, in order to compare the same plasmid profiles. Clustering alignments showed a high similarity between plasmid and *iuc3* sequences reflected by average nucleotide identity >97%, suggesting possible horizontal transfer across human, livestock and food.

5.6 Allele calling and strains phylogeny

The maximum likelihood (ML) phylogenetic tree built on concatenated core genes displayed three distinct major clades according to species complex as well as several minor sub-clades gathering taxonomic species and sequence types (ST). None of the KornSC genomes were assigned to specific ST, suggesting that the MLST scheme originally built on *K. pneumoniae* is not suitable for this SC. MLST typing showed that ST4242 and ST3254 resulted the mainly assigned for KpSC, with the same ST shared by isolates collected from different samples origins or batches of the salami factory. For instance, ST3254 was found in n=8 genomes from raw materials and environment taken from the same batch, whereas ST4242 was found in n=6 genomes from raw material samples distributed among different batches. The same trend was reported for KoSC, with *K. oxytoca* (ST36 and ST18), *K. pasteurii* (ST387 and ST386), *K. grimontii* (ST377 and ST378) and *K. michiganensis* (ST183) reported STs gathering n=2/n=7, n=2, n=3 and n=2 genomes respectively across the same or different samples origin (environment and stored cheese) or across more than one batch. These results suggested the occurrence of closely related *Klebsiella* spp. which persisted in the environmental sites over 14 months and contaminated raw materials and final products.

6. Conclusions and Future Perspectives

From a safety standpoint, salami did not present any health risk regarding foodborne pathogens (*L. monocytogenes, S. aureus, Salmonella* and VTEC) as well as *Klebsiella* spp.. It is suitable that the combination of autochthonous microflora and environmental conditions after casing may partially explain the reduction and disappearance of some microbial groups at the end of production. For cheese, *S. aureus* were isolated from samples of stored product, however it's not possible to make speculation on public health hazard since few data were collected. Moreover, the surface contamination with *L. monocytogenes, S. aureus* or *Enterobacteriaceae* indicated insufficient cleaning and disinfection procedures in both processing units, thus suggesting their improvement to minimize the risk of cross-contamination events. Finally, the WGS applied to *Klebsiella* spp. indicated a persistence of strains in both artisanal food chains. The public health implications addressed to these populations

should not be underestimated, due to the potential transmission of antimicrobial-resistant bacteria to humans or transferring of AMR and/or virulence determinants to other colonizing bacteria.

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Valorization of olive oil extraction by-products through functional bread making

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In the last years, olive pomace, a by-product of olive oil extraction, has shown great interest. The aim of the work is to valorize the olive pomace of two Sardinian olive cultivars, Bosana and Semidana, by incorporating them into the formulation of functional baked products to improve their nutritional value. The olive pomace was characterized by macro-composition analysis, while, in order to optimize the bread formulations, the flour and the freeze-dried pomace were subjected to rheological analyses. The flour of type 00 has been substituted by the freeze-dried pomace of the two varieties, in the percentages of 1%, 2%, 3% and 5% (w/w).

Valorizzazione dei sottoprodotti dell'estrazione dell'olio di oliva mediante la produzione di prodotti da forno funzionali

Negli ultimi anni, il patè di oliva, sottoprodotto dell'industria olearia, ha registrato un interesse crescente nella comunità scientifica. Lo scopo del lavoro è la valorizzazione del patè di oliva di due cultivar di olive sarde, Bosana e Semidana, mediante la loro inclusione nella formulazione di prodotti da forno funzionali con l'obiettivo di aumentare il loro valore nutrizionale. Il patè di oliva è stato caratterizzato mediante analisi di macro-composizione, mentre per ottimizzare le formulazioni del pane, la farina e il patè liofilizzato sono state sottoposte ad analisi reologiche, mediante farinografo e reofermentometro. La farina di tipo 00 è stata sostituita dal patè liofilizzato delle due varietà, nelle percentuali di 1%, 2%, 3% e 5% (p/p).

Key words: olive pomace; olive oil by-products; flour; bakery products; nutraceuticals.

1. Introduction

Olive oil extraction results in several by-products consisting of olive leaves and branches, olive-mill wastewater (OMWW), in addition to vegetation water, and the remaining solid product known as olive pomace (Alu'datt, et al., 2008). Yearly, 2,881,500 tons of olive pomace are generated worldwide making it the most important waste by-product (Lammi, et al., 2018). The total composition of Olive Pomace consists of a combination of around 20% of olive husk and pulp, 15% crushed olive stone, and olive-mill wastewater with a moisture content of around 65% (Chebaibi, et al., 2019). These elements are characterized by a high content of indigestible fibers mainly cellulose, hemicellulose, and lignin, as well as polyphenols, mainly tyrosol, hydroxytyrosol, decarboxymethyl oleuropein aglycone, and luteolin, and tannins known as antinutritive compounds, and in addition to proteins and fatty acids, mainly oleic acid (Dal Bosco, et al., 2012). Two main reasons lie behind the urge to discover alternative uses for olive pomace rather than disposing of it. The first one, is the negative impact it imposes on the environment, being considered the main pollutant in the Mediterranean region affecting mostly the characteristics of ground water and soil quality, due to its long period of degradation, high contents in organic acids and lipids, high salinity, and acidic pH (Dal Bosco, et al., 2007 & Lammi et al., 2019). The second one is the benefits that olive pomace imposes on human health, being a major source of polyphenols and other functional elements, which help in the prevention or even cure of diseases like cognitive diseases, cardiovascular diseases, cancers, diabetes, autoimmune diseases, and high blood lipid profile (Cioffi, et al., 2010 & Palmieri, et al., 2012). Being the olive pomace as an underestimated food matrix and being unaware of all the benefits it may provide, the first part of the Ph.D. project was dedicated to the physicochemical characterization of the pomace obtained from the milling of different cultivar of olive cultivated in Sardinia, Italy, in order to be added in the second part of the Ph.D. thesis as a functional ingredient to improve the nutritional and sensory quality of bakery products, mainly bread.
This oral communication reports the main results of the following three activities directed to:
A1) determine the macro-composition of olives, olive oil, and olive pomace;
A2) determine the rheological properties of the dough with different substitution percentage;
A3) bread baking trials.

2. Materials and Methods

2.1 Sampling

Olive oil and pomace samples of two typical Sardinian cultivars Bosana and Semidana were collected at Accademia Olearia (Alghero, Italy) during the olive oil harvest 2021-2022 crop season. Olives were milled through a two-phase centrifugal extractor Leopard DMF (Pieralisi) able to produce a pomace made up of wet pulp without stones. Olive pomace samples were immediately stored at -20°C until the analysis. Olives and olive oil samples

were immediately analyzed.

2.2 Characterization of olive and olive oil

The olives were pitted, homogenized, and analyzed for dry matter (DM) (AOAC, 2000), oil content (O) with Randall method (ISO 659:2009) total soluble solids content (TSSC) (Migliorini, 2011), total polyphenols (TP) (Singleton and Rossi, 1965) and the color attributes were determined with a colorimeter (Minolta CR-300 equipped with a measuring head CR300), using the CIELAB (L^*, a^*, b^*) color system. Bosana olives reported the follow parameters: DM% 43.51 ± 0.08; O/DM% 32.78 ± 0.38; TSSC/DM% 7.96 ± 0.04; TP (g gallic acid/100g fresh olive) 9.8 ± 0.3; L^* 74.88 ± 30.31, a^* 29.11 ± 22.71, b^* -21.58 ± 18.81. Semidana olives reported the follow parameters: DM% 38.93 ± 0.03; O/DM% 73.18 ± 1.68; TSSC/DM% 10.62 ± 0.04; TP (g gallic acid/100g fresh olive) 8.7 ± 0.2; L^* 79.40 ± 22.73, a^* 17.14 ± 24.73, b^* -9.72 ± 19.76.

Olive oils were analyzed for free acidity (FA), peroxide value (PV) and UV spectrophotometric indices (K232, K270, and ΔK) (Commission Regulation (EEC) n. 2568/91), total polyphenols (TP) (Caponio et al., 2018), chlorophylls content (TC) (Pokorny et al., 1995), and the color attributes were determined with a colorimeter (Minolta CR-300 equipped with a measuring head CR300), using the CIELAB (L^*, a^*, b^*) color system. Bosana olive oil shown the follow parameters: FA% 0.33 ± 0.02, PV (m_{eq} O_2/kg) 8.83 ± 0.42, TP (mg GAE/kg) 512.7 ± 9.8, TC (mg/kg pheophytin a) 0.08 ± 0.00, K232 2.13, K270 0.14, ΔK 0.015, L^* 98.95 ± 0.26, a^* -0.76 ± 0.22, b^* 2.81 ± 0.93. Semidana olive oil shown the follow parameters: FA% 0.23 ± 0.01, PV (m_{eq} O_2/kg) 9.60 ± 1.00, TP (mg GAE/kg) 352.6 ± 4.6, TC (mg/kg pheophytin a) 0.10 ± 0.00, K232 1.81, K270 0.11, ΔK -0.003, L^* 97.04 ± 0.08, a^* 0.35 ± 0.18, b^* 1.50 ± 0.20.

2.3 Characterization of olive pomace

The dry matter content was measured after drying the olive pomace in an oven at 105 °C to constant weight (ISO 662:1998). The fat content was analyzed gravimetrically by the Randall method with SER158 Solvent AutoExtractor (Velp Scientific) (AOAC 2003:06). The total nitrogen was determined using the Kjeldahl method and applying a conversion factor of 6.25 to determine the protein content (AOC 960.52). The ashes content was estimated by a muffle furnace at 550 °C (AOAC 923.03). The total dietary fibers were determined according to AACC 32-05.01 and AOAC 985.29. An aliquot of each sample was freeze-dried (FD) (8L -50°C series, Labconco), and the color attributes were determined in each powdered sample with a colorimeter (Minolta CR-300 equipped with a measuring head CR300), using the CIELAB (L^*, a^*, b^*) color system. All measurements were done in triplicate. Data were analyzed by t-test using the software Statgraphics Centurion 18 (Statgraphics Technologies, Inc.).

2.4 Dough characterization

The percentage of water was added to the dough after subjecting it to the analysis of consistency and stability through the farinograph. The fermentation properties of the dough including dough development, and gas release were tested using a F3 Rheofermentometer (Chopin, Paris, France). The studied doughs in addition to the control were also those containing different percentages (1%, 2%, 3%, and 5%) of each of both freeze-dried olive pomace of Bosana and Semidana. Measurements were done in duplicate. Data were analysed with one-way ANOVA using Statgraphics.

2.5 Bread-making

The control dough (CTRL) was made of flour 00, 2% (w/w) of yeast, 1.8% (w/w) of salt, 54% (w/w) of water with kneading for 8 minutes. Substituting pomace doughs were made with the same formulation of the CTRL dough with the variation of water based on cultivar and the percentage of substitution of the pomace; Bosana 1% (water: 54%), Bosana 2% (53.5%), Bosana 3% (53%), Bosana 5% (51.8%), Semidana 1% (54.5%), Semidana 2% (54.2%), Semidana 3% (53.5%), Semidana 5% (53%).

The first mass leavening took 30 minutes at 30 °C and 85% humidity. The second leavening took place after inserting 250 g of dough into bread molds (11.5 x 7.5 x 6.5 cm) in the same conditions to reach up to double the volume. The bread was baked at 200 °C for 35 minutes in a preheated static electric oven.

3. Results and Discussion

3.1 Olive pomace characterization

The proximate composition and color parameters of olive pomace of the two cultivars Bosana and Semidana are shown in **Table 1**. The macro-composition of raw olive pomace showed significant differences between both cultivars in all analyses. Bosana had significantly higher dry matter (25.36%), fat (17.57%), and ash (5.29%) contents, whereas Semidana had a significantly greater protein content, compared to that of the Bosana. Caponio et al., (2022) reported the similar dry matter and protein contents of freeze-dried olive pomace, whereas remarkable differences were seen in the total dietary fiber/DM resulting in 20.10% compared to 61.1-66.7% observed throughout this study.

Table 1 *Macro-composition and color analyses of freeze-dried (FD) olive pomace of cultivars Bosana, and Semidana.*

Pomace variety	Dry Matter % (DM) raw pomace	Fat/DM % raw pomace	Protein/DM % raw pomace	Ash/DM % raw pomace	DM% FD pomace	Total Dietary Fiber/DM % FD pomace
Bosana	25.36^{a} ± 0.22	17.57^{a} ± 0.16	1.80^{a} ± 0.40	5.29^{a} ± 1.05	98.49 ± 0.07	61.1^{a} ± 0.99
Semidana	19.67^{b} ± 1.30	12.5^{b} ± 1.22	4.24^{b} ± 0.06	4.29^{b} ± 0.33	98.31 ± 0.46	66.7^{b} ± 0.52

Pomace variety	L^*	a^*	b^*
Bosana	98.17 ± 0.92	0.33 ± 0.19	-2.45^{a} ± 1.46
Semidana	98.68 ± 5.46	-0.11 ± 1.19	1.88^{b} ± 2.38

Different letters within each column mean significant differences according to the t-test (p-value <0.05).

Concerning the freeze-dried olive pomace, significant differences were only seen in the total dietary fiber, with the Semidana having the higher content of 66.7%, in addition to nitrogen and protein contents of 0.98% and 6.14% in the Bosana, respectively.
These differences may be due to the cultivar itself or to the harvesting period and relative yield.
For the color attributes, the L^* and a^* parameters did not show any significant differences between the two cultivars, while the b^* parameter was significantly different between the freeze-dried pomace of both Bosana and Semidana.

3.2 Characterization of the dough

Table 2 *Macro-composition and color analyses of freeze-dried olive pomace of cultivars Bosana, and Semidana.*

Sample	Dough development time (min:sec)	Consistency (FE)	Added water % (w/w)	Stability (min:sec)
CTRL	17:40.0	502.0	54.0	2:00.0
Bosana				
1%	1:25.0	500.0	54.0	2:01.5
2%	1:28.0	494.5	53.5	1:32.5
3%	1:12.0	497.5	53.0	1:17.0
5%	1:14.0	493.0	51.8	1:10.0
Semidana				
1%	1:30.5	498.0	54.5	2:39.0
2%	1:19.5	497.5	54.2	2:02.5
3%	1:26.0	494.5	53.5	1:54.0
5%	1:19.0	487.5	53.0	1:27.0

Table 2 summarizes the data of dough development time, consistency, added water, and stability of the control dough and the doughs obtained through the substitution of the flour with olive pomace Bosana and Semidana at different percentages to optimize the formulation of the bread.

The dough fermentation properties as measured by the rheofermentometer are shown in **Table 3**. Upon fermentation, yeast produces gas that spreads into the dough and increases the number of air bubbles. The withheld gas in the dough is the ratio between gas production and gas retention. On the other hand, a lower (Hm-h)/Hm indicates greater dough stability, therefore, a greater gas retention ability (Farbo et al., 2019). Increasing pomace percentages affected somehow negatively the formation of the dough.
The height (Hm) of all samples fortified with the two olive pomace cultivars at all percentages of use (1%, 2%, 3%, and 5%) showed a significant decrease compared to the control. The gas retention expressed by Vr/Vt has significantly increased with the addition of the pomace regardless of the cultivar and the percentage of use; it highlights the greater ability of the samples implemented with the olive pomace of both cultivars to retain gas during the leavening process, as it is significantly higher in doughs containing pomace compared to the control (p<0.05). The retention coefficient is associated with the stretching dough's capacity and the quality of the gluten protein network (Farbo et al., 2019).
These results mean that increasing pomace percentages weakens the gluten network, and thus, a poorer capacity to leaven and retain gas. However, apart from the control, the best consistency, viscosity, and stability of the dough were found to be the one implemented with 1% of pomace of the Semidana cultivar. The latter showed a greater water absorption capacity. Increasing percentages of olive pomace make the dough lose its consistency.

Table 3 *Effect of olive pomace percentages (1%, 2%, 3%, 5%) of Bosana and Semidana on the dough by rheofermentometer.*

Sample	Dough Development							Gas Behavior				
	Hm (mm)			(Hm-h)/Hm (%)			T1(h)	T'1 (h)	(CR) Vr/Vt: (%)			
CTRL	51.10^{a}	±	0.85	5.50^{b,c}	±	2.69	01:47:15	01:54:45	90.90^{c}	±	0.71
Bosana												
1%	31.95^{c}	±	0.07	1.00^{d}	±	0.14	02:35:15	02:16:30	95.90^{b}	±	3.25
2%	23.75^{d}	±	0.35	3.65^{c}	±	0.07	02:39:00	02:27:45	98.80^{a}	±	0.00
3%	13.00^{e}	±	3.96	8.40^{a}	±	0.42	02:24:00	02:26:15	99.10^{a}	±	0.14
5%	12.55^{e}	±	0.07	6.05^{b}	±	1.34	01:46:30	02:30:45	99.20^{a}	±	0.14
Semidana												
1%	41.40^{b}	±	0.57	0.65^{d}	±	0.92	02:48:00	02:23:00	98.85^{a}	±	0.07
2%	32.10^{c}	±	1.13	0.30^{d}	±	0.28	02:57:45	02:30:45	98.95^{a}	±	0.21
3%	25.10^{d}	±	6.90	0.50^{d}	±	0.25	02:53:00	02:35:30	99.16^{a}	±	0.21
5%	19.50^{d,e}	±	0.28	0.80^{d}	±	0.85	02:51:45	02:38:15	99.20^{a}	±	0.14

Hm is the maximum dough development height; (Hm-h)/Hm represents the decrease in dough height at the end of the analysis of 3h; T1 is the time at which the dough reaches the maximum height. In gas behavior, T'1 is the time of maximum gas production; (CR) Vr/Vt is the percentage of has withheld at the end of the analysis of 3h (Farbo et al., 2019). Different letters within each column mean significant differences according to one-way ANOVA and Duncan MRT (*p*-value <0.05).

4. Conclusions and Future Perspectives

In this part of the Ph.D. project, we studied the behavior and rheological characteristics of dough made with flour of type 00, and supplemented with four percentages (1%, 2%, 3%, and 5%) of freeze-dried olive pomace of two Sardinians olive cultivars Bosana and Semidana.

Macro-compositions of raw pomace of both cultivars were significantly different from each other, as for the freeze-dried pomace, Semidana had significantly higher total dietary fiber content, whereas, Bosana had a significantly greater content of nitrogen and protein.

The rheological analyses showed that increasing percentages of pomace make the dough lose its stability and its gas retention capacity, thus making it harder for the dough to leaven in a gluten-free-like network.

The use of freeze-dried olive pomace as a potential partial substitute for flour; however, seems like a promising approach due to the high nutraceuticals it contains.

Olive pomace is supposed to improve the nutritional value of the bread for the fibers, protein, and phenolic contents. This is to be studied in the upcoming part of the Ph.D., in addition to the sensory properties of the newly formulated bread.

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Development and investigation of functional foods conceived for specific categories of consumers and produced with selected strains isolated from healthy vaginal environment and human breast milk

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This PhD thesis is aimed to the development of functional foods designed for specific types of consumers, such as women and infants with the specific aims to increase such category well-being throughout a functional food strategy. For this, *Lactobacillus* and *Bifidobacterium* strains, isolated from healthy vaginal environment and human breast milk, characterized for their functional and technological aptitude, were employed for the production of several fermented foods.

Sviluppo e studio di alimenti funzionali ideati per specifiche categorie di consumatori e prodotti con ceppi selezionati isolati da ambiente vaginale e latte materno

Questa tesi di dottorato ha riguardato lo sviluppo e lo studio di alimenti funzionali concepiti per specifiche categorie di consumatori, come donne e bambini. Più in particolare, cercando di sviluppare una strategia alimentare innovativa con lo scopo di preservare o aumentare il benessere di specifiche categorie di consumatori (donne e neonati), ceppi di *Lactobacillus* e *Bifidobacterium*, isolati dall' ambiente vaginale di donne sane e dal latte materno e caratterizzati da un punto di vista funzionale/tecnologico, sono stati impiegati per la produzione di diversi alimenti fermentati.

Key words: Functional foods, Probiotics, Breast milk, Female microbiome

1. Introduction

In accordance with the PhD thesis project previously described, this oral communication reports the main results of the following five activities directed to:
A.1) Investigation on metabolic and probiotic characterization of vaginal lactobacilli isolated from healthy vaginal environment for their potential inclusion in foods.
A.2) Evaluation of the potential of microencapsulation by *spray-drying*, performed on selected vaginal lactobacilli, already characterized for their technological and functional properties, using soy milk as carrier.
A.3) Use of encapsulated and unencapsulated probiotic vaginal lactobacilli to produce functional fermented soy-milks designed for the well-being of female gender.
A.4) Investigation on the effects of the formulated soymilk products, containing the vaginal lactobacilli, encapsulated or not, on the vaginal microbiome of donors potentially more prone to vaginal infections, such as postmenopausal women, using a fecal model systems.
A.5) Isolation of *Lactobacillus* and *Bifidobacterium* spp. strains by human breast milk and their further investigation as probiotic candidates also including the study of selected technological features assuming their inclusion for the development of functional food product for infants.

2. Materials and Methods

2.1 Investigation on metabolic and probiotic characterization of vaginal lactobacilli isolated from healthy vaginal environment their potential inclusion in foods.

Selected vaginal *Lactobacillus* strains, already characterized for their antimicrobial activity against several uro-genital and gastro-intestinal pathogens (Parolin *et al.*, 2015) were also investigated for selected technological features of interest for food sector (Siroli *et al.*, 2017). However, the use of selected microbial strains claimed as probiotics, also in food preparations, cannot avoid their deep functional and metabolic characterization. For this, some properties such as fermentation ability in different substrates, and the ability to maintain high viability in food matrix during refrigerated storage were also tested for their further application as functional cultures in food products. Consequently, in order to dissect their metabolic potential, a Biolog phenotype microarray analysis was applied (D'alessandro *et al.*, 2021a). Furthermore, the strains were studied for their hydrophobicity, auto-aggregation and the ability to adhere to a human intestinal cell line such as Caco-2 cells. Indeed, the ability to survive a simulated digestion process when inoculated in milk and stored at 4◦C was observed (D'alessandro *et al.*, 2021a).

2.2 Microencapsulation by *spray-drying* on functional vaginal lactobacilli

After the probiotic and metabolic characterization, the most performing vaginal strains were subjected to *spray-drying* process using a Mini Spray-Dryer, a laboratory scale spray-dryer equipped with a single fluid nozzle. Regarding process conditions different inlet air temperatures (150 and 110°C) with corresponding outlet temperatures of 85 and 70 °C were tested (D'Alessandro *et al.*, 2021b). The residual viability of spray-dried samples was determined by plate count method. The moisture content was determined by weight loss after drying 2 g powder at 103 °C for 3 h, while water activities were measured by using a water activity meter. The morphology of the powder material wall and the encapsulate were observed using a scanning electron microscope (Hitachi S-510) with an accelerated voltage of 15kV. In addition to evaluate the resistance over time of these encapsulated strains stored at room temperature, at + 4 ° C and -20 ° C, several samplings were carried out, more specifically after 7, 14, 30, 90 and 365 days of storage. (D'Alessandro *et al.*, 2021b). In order to evaluate the resistance of these encapsulated strains of the passage through stomach and duodenum, the method proposed by (Vinderola *et al.*, 2011) with certain modifications was performed.

2.3 Development of fermented soy-milks made with commercial starters and unencapsulated and encapsulated functional vaginal lactobacilli as co-starters

The production of fermented milks was carried out in lab conditions. Commercial soy-milk was splitted in 100 mL containers and each one was inoculated with starter culture (*L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus*) at level of 6 log CFU/mL while the functional strains, encapsulated or not, were inoculated at level of at least 7 log CFU/mL. The inoculated milks were incubated at 42 °C until the reaching of pH 4.6. After that, the fermented milks were stored at 4 °C for 60 days**.** The reduction of pH during fermentation and its evolution in fermented milks during refrigerated storage was checked. The resistance of probiotic strains to simulated gastrointestinal digestion was performed after 30 days of refrigerated storage, according to the method proposed by Vinderola *et al.*, 2011. In addition, also the potential antagonistic activity showed by these formulated soy-milk products against selected gastrointestinal pathogens during the storage was assessed. As regard the sensory properties of these formulated products, their volatile compounds were investigated using a GC–MS combined with a solid phase micro extraction (SPME) according to (Patrignani *et al.*, 2016).

2.4 Investigation on the effects of the formulated soymilk products containing functional vaginal lactobacilli on the vaginal microbiome of postmenopausal women, using a fecal model systems

Formulated soy-milk products were submitted to an in vitro simulated digestion according to the INFOGEST protocol (Brodkorb *et al.*, 2019). Digested products were then added into a faecal culture model system. More specifically, independent batch fermentations were performed at pH-uncontrolled in a carbohydrate-free basal medium (CFBM) (Al-Tamimi *et al.*, 2006) with feces from different post-menopausal women as human donors. Briefly, CFBM was prepared and reduced overnight in an anaerobic chamber one day before the sample processing. On the day of the assay, fresh fecal samples, were added (10% v/v) to the reduced CFBM and then were distributed into 100 mL bottles of the ANKOMRF system (ANKOM Technology, USA). An overnight incubation in anaerobic conditions was performed at 37 ◦C prior to the addition of the digested soy-milk products in order to allow microbiota to stabilize in the culture medium. A set of independent fermentations were performed with feces from each donor, using glucose as no prebiotic positive control at a final concentration of 0.3% (v/v). Fermentations were carried out under anaerobic conditions at 37 ◦C during 48 h. The cumulative gas produced along the different fermentations was monitored in real-time by using the ANKOM RF system. The pH of cultures was determined with a pH-meter and was considered as an indicator of the progression of fermentation. The analysis of short-chain fatty acids was performed by gas chromatography in the fecal culture supernatants in order to determine the molar concentrations of three main compounds: acetate, propionate and butyrate. The remaining branched short-chain fatty acids, namely isobutyrate and isovalerate, were also quantified.

2.5 Isolation of *Lactobacillus* and *Bifidobacterium* spp. strains by human breast milk and their further investigation as probiotic candidates also including the study of selected technological features assuming their inclusion for the development of functional food product for infants.

As regards to the collection of human breast milk, 30 mothers attending M. Bufalini Hospital in Cesena (Italy) have donated the samples, with a written informed consent in accordance with the Ethics Committee of the University of Bologna (Prot. n. 16617, 26/01/2021) and the Ethics Committee of the hospital Maurizio Bufalini (Prot. n.1523, 12/05/2021). Sample collection and plating were performed according to Zacarías *et al.*, 2011. The taxonomic identity of isolates presenting typical lactobacilli or bifidobacteria morphology was investigated by amplifying, sequencing and comparing their 16S rRNA gene. After the identification of 10 *Lactiplantibacillus*, 4 *Lactobacillus* and 2 *Bifidobacterium* strains to evaluate their potential antagonistic activity against pathogenic and spoilage species frequently associated to food products and gastrointestinal infections, the method reported by Siroli *et al.*, 2017 was applied with some modifications. Moreover, the anti-biofilm activity against pathogenic species and the antibiotic susceptibility were determined. Furthermore, other functional parameters such as hydrophobicity, auto-aggregation, adhesion to intestinal cell lines (Caco-2 cells), and resistance to simulated digestion process were evaluated too. As regards to their technological aptitude, the fermentation kinetics and

viability in milk were observed, as well as their release of aroma compounds using a GC–MS combined with a solid phase micro extraction (SPME) according to (Siroli *et al.*, 2017).

3. Results and Discussion

3.1 Investigation on probiotic and metabolic characterization of vaginal lactobacilli isolated from healthy vaginal environment for their potential inclusion in foods.

Biolog phenotype microarray analysis performed on vaginal strains could be considered a useful starting point to characterize several strains and to individuate their specific metabolic properties to be considered for their inclusion in food matrix. First of all, all the strains were characterized by a strong catabolic activity against α-D-glucose, D-fructose, maltose, and D-mannose. Furthermore, the majority of the strains were also able to use gentiobiose, maltotriose, D-raffinose, stachyose, and sucrose. As regards the ability to use n-acetyl-D-glucosamine, shown by all the strains, this quality is of potential interest as this compound is the monomer unit of chitin, the second most abundant carbohydrate after cellulose. On the other hand, most of these selected vaginal lactobacilli were also able to catobolize D-cellobiose and α-ketobutyric acid. Thus, the data obtained in this research suggest that some strains could be exploited for the formulation of symbiotic functional foods in which probiotics and prebiotics (deriving from the metabolic activities of added probiotics) are simultaneously present. Additionally, the ability to catabolize α-ketobutyric acid is significant because this compound is the precursor of 3-hydroxy-4,5-dimethyl-2(5H)- furanone, an aromatic compound responsible for the burnt, sugar, and curry flavor in dairy products. Regarding the growth kinetics on α-D-lactose, D-galactose, and D-trehalose, almost all strains showed low efficiency of use. As regards the functional parameters high values in terms of hydrophobicity and auto-aggregation (over 70%) were recorded for most of the vaginal lactobacilli considered in this study, even compared to *L. rhamnosus* GG, a commercial probiotic strain used as reference. In this context, this connection is positively recognized; in fact, strains gifted with a hydrophobic cell surface and strong auto-aggregation ability could have a greater chance for adhesion to human cells. However, most of these considered were characterized by medium adhesion to a model of intestinal epithelial cells. Despite of this, after the simulated digestion process the decrease in cells viability was limited for all the strains, showing therefore a good resistance.

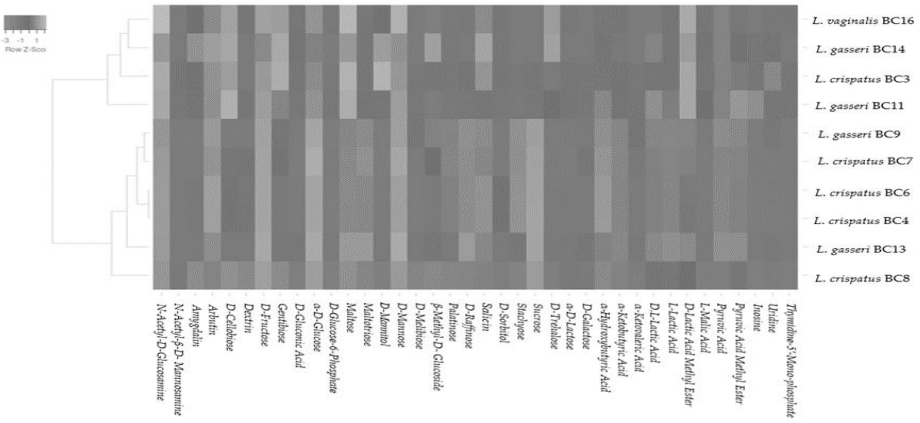

Figure 1 Heat map showing the metabolic differences among selected functional vaginal strains where light grey is referring to high catabolic activity and black to low activity.

3.2 Microencapsulation by *spray-drying* on functional vaginal lactobacilli

The optimized *spray-drying* conditions (110°C, 70 °C as *inlet* and *outlet* temperatures) and the choice of soy milk as carrier were able to produce bacterial microcapsules gifted with high viability during the storage and after the simulated digestive process (Figure 2). In addition, the microcapsules resulted not only with a good strain viability but also with suitable morphology (regular spherical shape and 6 μm average diameter), good technological features in terms of water activity and moisture content for food application.

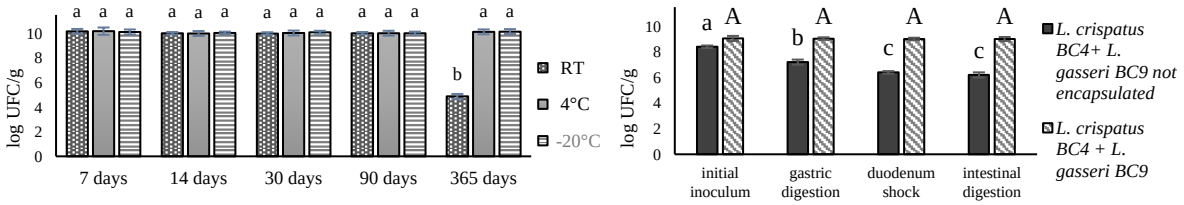

Figure 2 Cell loads (log CFU/g) *L. crispatus* BC4 + *L. gasseri* BC9 powders during the storage and after the simulated stomach-duodenum passage. Results are shown as average ± SD. Samples with different letters are significantly different ($p < 0.05$)

3.3 Development of fermented soy-milks made with commercial starters and unencapsulated and encapsulated functional vaginal lactobacilli as co-starters

Consequently, with the perspective of use encapsulated powders in food formulations to obtain novel functional foods, the encapsulated lactobacilli were then inoculated as adjuncts to produce fermented soy-milks. During the manufacturing of the food products, all the samples, containing the starter cultures and the probiotic adjuncts (encapsulated or not), reached the pH 4.6 in 7 h at 42 °C. The data (Figure 3) showed a small decrease in starter cultures cell loads, also considering the progress of the storage. However, after 60 d of refrigerated storage, the cell load of *L. crispatus* + *L. gasseri* was higher in soy-milk yoghurt where the probiotic strains were added in encapsulated form. The performances of microencapsulation in maintaining strain viability are also demonstrated during the simulated digestive passage performed after 30 d of product storage. Moreover, especially the product with the encapsulated strains showed a remarkable antagonistic activity *vs* gastrointestinal pathogens (Figure 4).

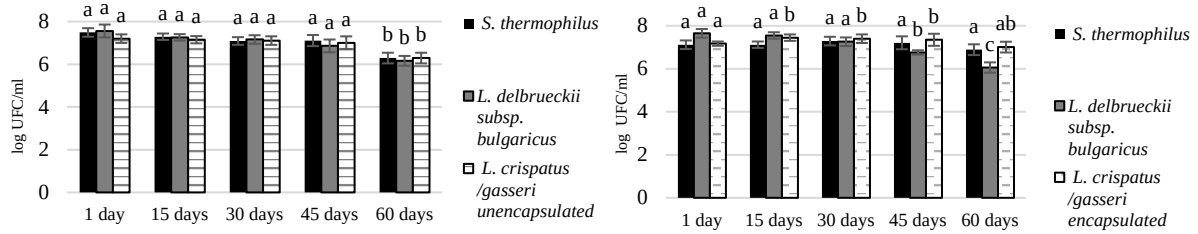

Figure 3 Cell viability of starters (*S. thermophilus*, *L. delbrueckii* subsp. *bulgaricus*) and encapsulated or not probiotic lactobacilli (co-starters) in fermented soy-milks during 60 d of storage. Results are shown as average ± SD. Samples with different letters are significantly different ($p < 0.05$).

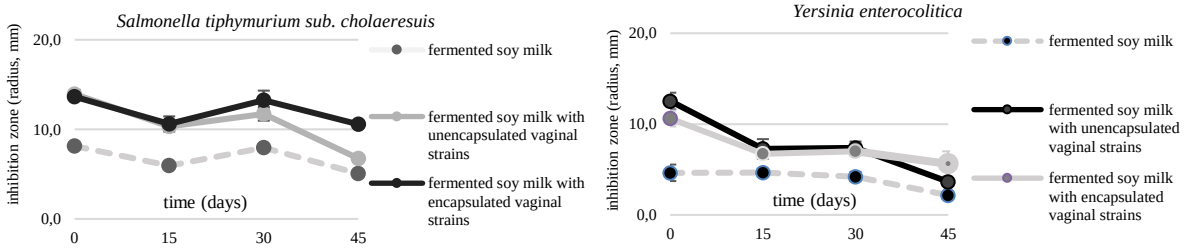

Figure 4 Antagonistic activity of fermented soy-milk products against gastro-intestinal pathogens.

3.4 Investigation on the effects of the formulated soymilk products containing functional vaginal lactobacilli on the vaginal microbiome of postmenopausal women, using a fecal model systems

Formulated soymilk products were subjected to INFOGEST protocol to simulate the gastro-duodenal digestion. To procede with the colonic fermentation, these digested products were then added into a faecal culture model system using faeces of post-menopausal women as donors. In fact, this specific group of donors is generally potential more prone to vaginal infections. This approach was useful to obtain information on gas production in real time. More specifically, each donor's postmenopausal microbiota is generally able to utilize the carbohydrates provided in the soy product and it appears that the presence of the vaginal strains cannot influence this behavior. In addition, during the colonic fermentation, to observe the metabolic activity of the vaginal strains and postmenopausal microbiome, several samples were collected for the determination of the short chain fatty acid (SCFA) and differences on the production of SCFA were observed depending on the type of formulated soymilk product tested.

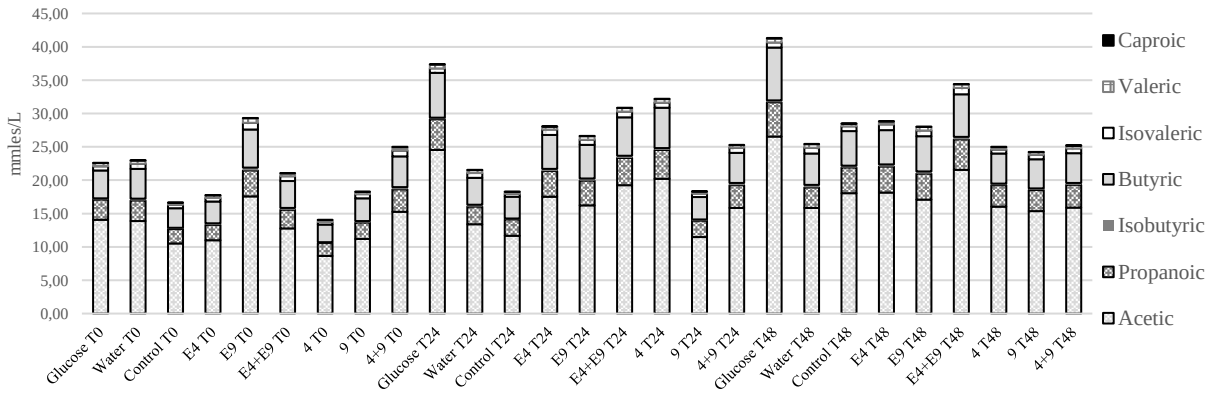

Figure 5 Determination of the SCFA (mM) observed during the colonic fermentation (0, 24, 48 h) of the digested formulated soy-products. The presented graph is referred to one of the selected donors recruited for this study.

3.5 Isolation of *Lactobacillus* and *Bifidobacterium* spp. strains by human breast milk and their further investigation as probiotic candidates also including the study of selected technological features assuming their inclusion for the development of functional food product for infants

In recent years, human breast milk has proved to be an interesting source for obtaining new and specific probiotic strains, including lactic acid bacteria and bifidobacteria, also for infants with the aim of promoting their correct immunological and intestinal microbiota development. Furthermore, these genera are commonly used as starters and co-starters in the production of several dairy products, exhibiting good viability in low pH products such as fermented milk. In this context our data clearly a remarkable aptitude to adhere to intestinal cells, even higher than *L. rhamnosus* GG, a recognized probiotic strain for the majority of our strains. In this sense, it's important to report how the ability of a candidate probiotic to adhere to gut mucosal surface contributes to the microbial persistence in a specific environment. This behaviour resembles hydrophobicity and auto-aggregation features of breast milk strains herein analysed, underlining their high potential as probiotics. In addition, all tested strains showed a remarkable inhibitory activity against pathogens of food interest as well as intestinal pathogens. More specifically, the majority of the lactobacilli produced halos of inhibitions ranging between 6 and 10 mm toward *L. monocytogenes* SCOTT A, *L. innocua* ATCC 51742, *S. enteritidis* MB1409, *S. enteritidis* E5, *E. faecium* BC104, *E. coli* 555 and *S. aureus* DSM 20231. All the isolated strains turned to be highly effective also towards intestinal pathogens, especially against *Y. enterocolitica*. The antimicrobial activity was also analyzed in terms of anti-biofilm effect, since, from a clinical point of view, the establishment of a biofilm by a pathogenic species increases antibiotic resistance and makes its eradication challenging. Thus, the ability of a beneficial strain to interfere with pathogen adherence and biofilm formation represents an advantage. Also in this case, all breast milk isolated strains showed the capability to inhibit the formation of biofilm by pathogens, in particular *L. plantarum* strains were very effective towards the tested pathogens. Moreover, in order to also investigate their technological potential, the strains fermentation kinetics and viability in pasteurized whole milk clearly indicated the unsuitability as fermentation starters for the majority of the strains, due to their slow fermentation kinetics. Nevertheless, especially for some strains, the maintenance of high viability in pasteurized milk has been highlighted, also during the refrigerated storage (> 6.5 log CFU/g), indicating their potential suitability as adjunct cultures in dairy products such as fermented milks. Their potential role as co-starters was confirmed especially for the lactic acid bacteria since the volatilome of milks inoculated with these selected lactobacilli/bifidobacteria showed a release of diacetyl, acetoin, and acetaldehyde, that could positively contribute to the specific flavour of dairy products. Instead, bifidobacteria strains were characterized by the highest production of short chain organic acids such as acetic acid and hexanoic acid. In conclusion the attained data could represent an important contribution to better understanding the proper application of the studied strains, also considering their final use.

4. Conclusions and Future Perspectives

The research performed in these 3 years has allowed to select from different environments microbial strains gifted of tailored functionalities to develop functional products conceived for specific consumers. The results obtained in model system are very promising and the future perspective is to set-up *in vivo* trials to confirm the potentialities of the functional products on post-menopausal women and infants in order to strength the idea of functional products as food strategy to increase human well-being.

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Impact of high dynamic pressure treatments on the physicochemical properties and technological functionality of pea proteins

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The aim of this PhD thesis is to characterize pea proteins at different levels and subject them to high dynamic pressures (HDP) pre-treatments at different intensities, to induce structural changes and study the effect on the technological and functional properties of pea proteins. Analyses were carried out at the structural (circular dichroism, SDS-PAGE, thermal properties and total free sulfhydryl groups) and at the technological-functional level (TEAC, TPC, solubility, WHC, OHC, interfacial tension and dilatation rheology, emulsifying and gelling capacity). HDPs can induce structural changes that can allow a modulation of pea proteins technological properties and an optimized exploitation in different food systems.

Impatto dei trattamenti ad alte pressioni dinamiche sulle proprietà fisico-chimiche e sulla funzionalità tecnologica delle proteine di pisello

Lo scopo di questa tesi di dottorato è caratterizzare le proteine di pisello a diversi livelli e sottoporle a pretrattamenti ad alte pressioni dinamiche (HDP) a diverse intensità, per indurre cambiamenti strutturali e comprendere come influenzano l'espressione delle loro proprietà tecnologiche e funzionali. Le analisi sono state effettuate a livello strutturale (dicroismo circolare, SDS-PAGE, proprietà termiche e gruppi solfidrilici totali liberi) e tecnologico-funzionale (TEAC, TPC, solubilità, WHC, OHC, tensione interfacciale e reologia dilatazionale, capacità emulsionante e gelificante). Le HDP possono indurre cambiamenti strutturali che consentono una modulazione delle proprietà tecnologiche delle proteine del pisello e un loro utilizzo ottimizzato in diversi sistemi alimentari.

Key words: pea proteins, high dynamic pressures, molecular characterization, technological properties, emulsions, gels

1. Introduction

In recent years, the trend of consumers to embrace healthier lifestyles has become increasingly widespread, with particular attention to the food consumed. For this reason, the demand for foods that are as natural as possible, vegan and / or vegetarian, plant-based or meat analogues, has also grown. In this context, the study and research on plant proteins as well as on replacing animal protein with innovative sources have increased while trying to provide the final food products with a standardized and high quality. Among plant proteins, pea proteins (extracted from the seed of *Pisum sativum* L.) have found great consensus in research and in food industry, thanks to their low production cost, ease of production and high nutritional level, as they contain a great amount of lysine (Lu et al., 2020). Pea proteins are mostly globular proteins, and the three major storage ones are legumin, vicilin and convicilin, firmly linked together. These characteristics prevent proteins from expressing technological properties at their best, such as the emulsifying one (since most of the hydrophobic groups are shielded inside the structure), or solubility, because of the scarce solubility of globular proteins, thus affecting all other properties. To this regard, the application of innovative and green technologies can help in the modification and modulation of the techno-functionality of such proteins. Among them, high dynamic pressure homogenization (HDP) is a technology of high potential which induces modification of plant-derived protein structures to steer their functionalities (Dumay et al., 2013). Thanks to mechanical forces and cavitation phenomena, HDP can indeed reduce the size of plant-derived protein particles and induce structural changes, affecting important functional properties for food formulation like solubility, emulsifying, gelling, and foaming capacity. In this context, HDP technique has already been exploited as pre-treatment to modify structures and improve the technological properties of faba bean (Yang et al., 2018), soy (Zhao et al., 2018), lentil (Saricaoglu, 2020) and pea proteins (Melchior et al. 2021). The application of this technology as a pre-treatment on proteins, results in, among others, an increase in solubility, emulsifying, foaming and oil holding capacity and, in general, leads to an improvement in the structural, functional and rheological properties.
Then, the aim of this PhD thesis is to study the application of HDPs pre-treatment to induce structural modification and modulate the techno-functional properties of pea proteins for the formulation of innovative plant-based food products. In the present work, a selection of results focusing on solubility, water and oil holding capacity, surface properties and emulsifying activity is presented.

2. Experimental Procedure

Based on these assumptions, this PhD project is essentially made of two phases: the first which involved the study and characterization of pea proteins (IP) at the structural, chemical-physical, and technological level. The second phase was based on the application of high dynamic pressures at different intensities (600 and 1000 bar), and the characterization of the modified proteins (IP600 and IP1000), in order to evaluate how these pre-treatments influenced the proteins at the different levels taken into account. The study of pea proteins at a structural level was carried out with circular dichroism and SDS-PAGE; then, thermal properties were evaluated by DSC to identify the denaturation temperature and, at last, the total free sulfhydryl groups assay was carried out, as an additional element to understand the extent of the structural changes made by HDPs. Regarding functional and technological characterization, TEAC and TPC, solubility, water and oil holding capacity, interfacial tension and rheology, emulsifying and gelling capacity were analysed; the properties and stability of the emulsions and gels formulated with the modified proteins were evaluated as well.

3. Materials and Methods

3.1 Materials

The pea protein isolate (IP) was obtained following the method described by Kornet et al. (2020), based on isoelectric precipitation: frozen peas were mixed, and the obtained paste was dried at 70°C for ca 6h, grinded and passed through a sieve of 100µm. Then the obtained flour was mixed in water at pH 8 for 2h and then centrifuged; the supernatant was adjusted to pH 4 and mixed for 1h. The pellet was collected, re-dispersed in water at pH 7 and mixed for other 2h. At last, the suspension was freeze dried and collected for use.

3.2 Methods

Molecular characterization

The circular dichroism spectra were recorded by a Jasco J-810 Spectropolarimeter (Jasco Corporation, Tokyo, Japan) at 25 ◦C, in the spectral range of 190–700 nm, and at 1 mg/mL of protein concentration. For SDS-PAGE, samples were characterized in the presence (reducing conditions) or absence (non-reducing conditions) of 2-mercaptoethanol. The determination of total free sulfhydryl group was carried out following the method described by Peng et al (2016), by using Ellman's reagent. Thermal properties were investigated using a DSC (Pyris 8500, PerkinElmer, Shelton, USA): 5µL of a 10% (w/v) IP, IP600 and IP1000 solutions were placed in a 50µl closed pan and an empty stainless-steel pan was used as reference. Samples were heated from 10 °C to 105 °C at 10 °C/min and nitrogen was used as carrier gas.

Functional and technological properties characterization

The general antioxidant activity of the samples was determined by means of the Trolox Equivalent Antioxidant Capacity (TEAC) method using ABTS assay and the Total Phenolic Content (TPC) method, using Folin-Ciocalteu reagent. Solubility was evaluated by using the method described by Kornet et al. (2020), at 1.0% (w/v) of pea protein concentration. Water and oil holding capacity were assessed following the method described by Fuentes-Alventosa et al. (2008). Interfacial tension between protein solutions (0.001% - 0.005% - 0.01% w/v) dispersed in PBS (50mM, pH 6.5) and sunflower oil was measured after 30 min of equilibration time using an Attension Sigma 700/701 tensiometer (Biolin Scientific Oy, Espoo, Finland), equipped with a Du Noüy platinum ring (diameter: 120.39 mm). Pre-treatments were carried out on IP using a high-pressure homogenizer (Panda Plus 2000, GEA Niro Soavi, Parma, Italy) equipped with a heat exchanger, at two different pressure intensities for 5 cycles, namely 600 bars (IP600) and 1000 bars (IP1000). Emulsions were formulated by dispersing IP-IP600-IP1000 (0.05%-1.0% w/v) in PBS (50mM, pH 6.5) and 10% of sunflower oil. Solutions were pre-homogenized for 1 min and then emulsified using the high-pressure homogenizer with 150 bars, for 10 cycles. Emulsifying capacity was evaluated by measuring particle size and distribution of oil-in-water model emulsions using a laser diffraction particle size analyser (Mastersizer 3000; Malvern, Worcestershire, UK). The stability of emulsions was also evaluated after 7 days of storage at 4°C. Analyses were carried out in triplicate on different batches and ANOVA and Tukey's test were performed to define significant differences at the 0.005 significance level. OriginPro 2016 software (OriginLab Corporation, Northampton, MA, USA.) was used for data analysis and modelling.

4. Results and Discussions

4.1 Molecular characterization

SDS-PAGE analysis in both reducing and non-reducing conditions was carried out to understand the structural composition and relative abundance of the three different proteins and how they were affected by the applied HDP pre-treatments; the results obtained were also used to calculate the pea proteins molecular weights. In non-reducing conditions, starting from a relative percentage intensity band equal to 3.1% for convicilin and 7.5% for vicilin, it was found that both treatments decreased the amount of such subunits. Instead, it was noted that, from an initial 6.0%, legumin increased, especially with the 600 bars pre-treatment. On the other hand, in reducing conditions, the percentage of convicilin decreases with both treatments compared to the control (3.3%). Moreover, under the same conditions, disulphide bonds of legumin were broken, allowing the identification of the two alpha and beta subunits: the presence of alpha legumin increased from 8.3% to 9.6% for 600 bars and to 9.5% for 1000 bar, while

that of the beta subunit decreased with respect to IP, whose starting relative percentage was 8.5%. The proteins were then submitted to DSC analyses, to determine the denaturation temperature and the enthalpy of the transition, as well as to the quantification of the total free sulfhydryl groups; both results are shown in Table 1. IP showed a denaturation temperature in line with what previously reported in literature (Sun and Arntfield, 2011) and a decrease of this value was observed in the treated samples, corresponding to the increase in the applied pressure, with a consequent decrease also in the energy necessary for the phenomenon to occur, reflecting the unfolding of the protein with the HDP pre-treatments. This behaviour is also confirmed by the content of total free sulfhydryl groups, according to which a significant decrease in the treated proteins was observed due to the oxidation (and / or probably even conversion) of the sulfhydryl groups into disulphide bonds (Peng et al., 2016). In all the parameters considered, however, no significant differences were observed between the two pressures applied.

Table 1 *Denaturation temperature, ΔH and total free -SH groups of native and modified IP. Different letters within the same column are significantly different ($p < 0.05$).*

	T_{ONSET} (°C)	ΔH (J/g)	µM SH/ g protein
IP	91.49 ± 0.55 [a]	5.66 ± 2.06 [a]	1.47 ± 0.08 [a]
IP600	82.76 ± 1.07 [b]	1.76 ± 0.07 [b]	0.32 ± 0.06 [b]
IP1000	80.93 ± 2.12 [b]	2.82 ± 1.62 [a, b]	0.39 ± 0.02 [b]

4.2 Functional and technological characterization

The HDP treatments induced significant structural and molecular changes in the treated proteins, that might have in turn been reflected in their functional and technological properties. As a first step, the pea proteins were thus assessed for their antioxidative properties through two different assays: the TEAC method to evaluate the antiradical activity and the TPC to assess the reducing activity; results are reported in Fig. 1, for both control and treated samples. As far as the TEAC results are concerned, IP value was very similar to that obtained in literature on untreated pea proteins (Wang et al., 2017); the HDP impaired such properties causing a significant decrease of TEAC values, which was more consistent in the IP600 samples. A similar trend is also evident in the total phenolic content. In literature, the antioxidant properties were closely linked to the molecular structure, molecular weight, amino acid sequence of the protein and the presence of specific amino acids like cysteine and aromatic ones (Žilić et al., 2012); moreover, it was proven that proteins subjected to treatments such as electron beam irradiation, heat and enzymatic treatment were reported to increase their antioxidant activity. To the best of our knowledge, the effect of high-pressure treatments on TEAC and TPC of plant proteins has not been investigated yet and thus no data for comparison are available.

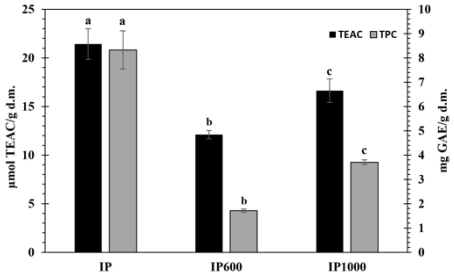

Figure 1 *TEAC (black) and TPC (grey) of native and modified IP. Different letters indicate statistically significant differences ($p < 0.05$.).*

Table 2 *Solubility, WHC and OHC of native and modified pea proteins. Different letters on the same rows indicates statistically significant differences ($p < 0.005$).*

	IP	IP600	IP1000
Solubility (%)			
pH 2	41.15 ± 3.74 [a]	96.20 ± 3.64 [b]	93.09 ± 2.16 [b]
pH 4	34.16 ± 3.29 [a]	66.33 ± 0.91 [b]	58.26 ± 1.46 [c]
pH 7	87.05 ± 4.93 [a]	97.69 ± 2.30 [b]	81.68 ± 1.27 [a, c]
pH 10	88.68 ± 4.14 [a]	98.52 ± 1.71 [b]	92.72 ± 1.59 [a, b]
WHC (g H_2O / g prot.)	1.48 ± 0.01 [a]	1.51 ± 0.01 [a]	2.92 ± 0.01 [a]
OHC (g oil / g prot.)	1.72 ± 0.01 [a]	0.92 ± 0.01 [b]	2.70 ± 0.01 [c]

In Table 2 the results regarding solubility at different pH values, WHC and OHC are reported. Solubility is one of the technological properties that is influenced the most by the pre-treatments: the treatment significantly increased the solubility of IP (only with a slight decrease of it at 1000 bars), especially at acidic pH and in particular at the isoelectric point, which for pea protein is 4.5. This aspect is interesting for a possible use of the modified protein for acid food formulations. There are several works in literature that confirm this behaviour of proteins subjected to high dynamic pressures: the treatment decreases the particle size of native protein, leading to an increase in the surface area of the molecules in contact with the aqueous environment, thus increasing water-protein interactions (Yu et al., 2018). Furthermore, high pressures can dissociate insoluble protein aggregates into soluble ones, but this does not happen with the higher intensity pressures (in this case 1000 bar), which instead could cause the formation of aggregates, reducing the solubility (Chen et al., 2016; Melchior et al., 2022), as can also be seen from the results showed in Table 2. While the water holding capacity was not affected by either of the two treatments at high dynamic pressures, the oil holding capacity, on the other hand, increased significantly with the pre-treatment at higher intensity, while it decreased with the pre-treatment at 600 bars. Theoretically, with the increase of the applied pressure the protein should unfold and expose the hydrophobic groups previously hidden inside the structure, but this only happens with the treatment at 1000 bars. The lower OHC at 600 bars may be due to the fact that the treatment certainly caused conformational changes, but perhaps induced a different exposure of the hydrophobic groups compared to the 1000 bars treatment. This could also explain why the application of high pressures increased the solubility but not the WHC consequently: the induction of conformational changes at different levels could lead to a different exposure of the hydrophilic groups in aqueous environment (or a change in the water-protein interactions), which is then reflected in divergent results for the technological properties studied. Similar trends for WHC and OHC have been obtained in other works (Yu et al., 2018) and certainly these aspects need a more in deep characterization to better understand the changes made to the protein by the treatments.
Interfacial tension was evaluated to investigate the adsorption behaviour of proteins at the oil-water interface: in all the samples the interfacial tension decreased as the protein concentration increased, but no significant differences are noted between samples, except for the lowest concentration at 0.001% w/v. Therefore, despite the structural modification, the HDP treatment had no effect on the interfacial properties of the system. However interfacial rheology measurements showed that pre-treatment at 1000 bars significantly increased the elasticity of the protein at the interface, probably due to the unfolding of the globular protein, which in this case managed to better adapt to the oil-water interface. After the adsorption behaviour, the emulsifying properties were studied. To this aim, emulsification curves were prepared for each sample by testing different pea proteins concentrations, keeping equal the homogenization procedure. In the experimental conditions used, for the three pea proteins samples stable emulsions were formed at 1.0% (w/v) protein concentration, whose droplets size distributions after preparation and after 7 days of storage at 4°C are shown in Fig. 2 (a, b). All emulsions exhibited a bimodal distribution, with a population of smaller droplets centred around 1 µm and a population of larger particles centred on 8-9 µm. IP resulted in a $D_{4;3}$ value of 5.77±0.01 µm and a shift of the dimensional range towards smaller dimensions was observed in the emulsion formulated with IP600 (with a $D_{4;3}$ value of 4.95±0.02 µm) while with the emulsion formulated with IP1000 there was a shift on coarser populations, which showed a $D_{4;3}$ of 6.61±0.04 µm.
Upon storage, the emulsion formulated with IP600 resulted the most stable as, contrarily to IP and IP1000, its droplet size distribution did not change significantly; oppositely, IP and IP1000 droplet size distribution moved towards large diameters and wider distributions were also observed, especially in IP1000 samples, which showed a population of larger particles centred mostly on 100 µm. Such a different behaviour could be due to a change in the charge of the adsorbed protein layers with an increase of the electrostatic repulsive forces among the oil droplets in the IP600 samples, preventing aggregation and flocculation phenomena and providing the systems with improved physical stability (Yu et al., 2018). Moreover, the higher stability of IP600 could be related to lower vicilin/legumin ratio (as assessed from the band/line intensity quantification), that is even reported in literature as a key factor for higher stability of emulsions (Pedrosa et al., 2020).

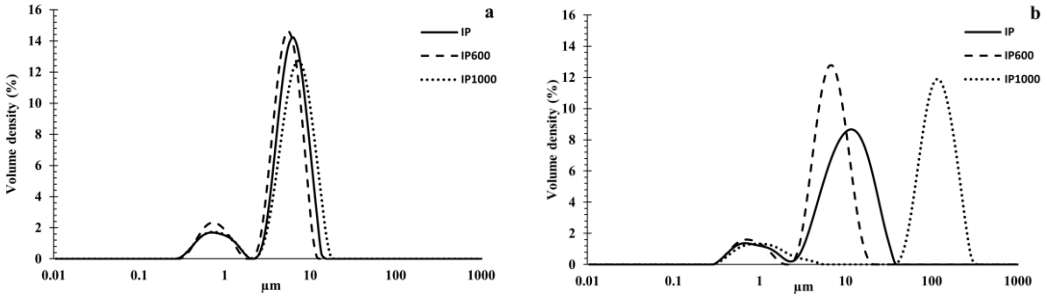

Figure 2 *Droplet size distributions of emulsions formulated with 1.0% (w/v) of protein suspensions, 1 min after the preparation (a) and after 7 days of storage at 4°C. Continuous black lines refers to IP, dashed to IP600 and dotted to IP1000.*

5. Conclusions and Future Perspectives

In the present work, HDPs have modified the ratio between the main proteins, such as legumin, vicilin and convicilin, influencing above all the solubility, OHC and emulsifying properties, depending on the pressure used. Taking this into account, pre-treatments with high dynamic pressures can be considered a green technology useful for modulating technological functionalities of pea proteins at different levels for targeted exploitation in different food systems, depending on the technological property to be improved or modified. A more in-depth characterization of the behaviour of native and modified pea proteins at the oil-water interface, of their emulsifying ability as well as of their gelling capacity is currently ongoing.

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Role of typical foods from Abruzzo region in reducing oxidative, inflammatory and metabolic stress in frail elderly people and/or affected by degenerative diseases

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In the present PhD thesis, it was evaluated if foods from Abruzzo region (legumes, garlic, almonds) could: 1) have antioxidant capacity *in vitro* and in an intestinal cellular model; 2) modulate the inflammatory response of immune system cells. These foods were analysed in a form suitable for consumption and considering the digestive process. Moreover, properties of legumes were compared to those of edible insects, novel protein-rich foods with low ecological impact. The final purpose is the inclusion of traditional foods in the diet of elderly people affected by aging-related diseases, to modulate oxidative and inflammatory risk factors.

Ruolo di alimenti tipici della regione Abruzzo nella riduzione dello stress ossidativo, infiammatorio e metabolico in anziani fragili e/o affetti da malattie degenerative

Nella presente tesi di dottorato è stato valutato se alimenti di origine abruzzese (legumi, aglio, mandorle) possano: 1) avere capacità antiossidante *in vitro* e in modello cellulare intestinale; 2) influenzare la risposta infiammatoria di cellule del sistema immune. Tali alimenti sono stati analizzati in forma idonea al consumo e considerando il processo digestivo. Inoltre, le proprietà dei legumi sono state confrontate con quelle degli insetti edibili, fonti proteiche innovative a basso impatto ecologico. Lo scopo ultimo è l'inserimento degli alimenti tipici nella dieta di anziani affetti da patologie legate all'invecchiamento, per modularne fattori di rischio infiammatori e ossidativi.

Key words: antioxidant, anti-inflammatory, functional food, legumes, garlic, almonds, elderly, Abruzzo.

1. Introduction

Aging leads to the development of "homeostenosis" defined as the alteration of homeostasis with a reduction of physiological resources, lower resistance to stress and a decline of immune function in presence of oxidative and inflammatory stress; the perturbation of the homeostasis is related to the onset and development of various pathologies, as infectious and degenerative diseases. A balanced diet, with frequent consumption of plant-based foods, can have a role in the maintenance of the homeostasis and in the prevention and/or in reduction of age-related diseases. Almonds, garlic and four legumes, all from Abruzzo region, were selected basing on their functional properties. In fact, legumes have shown antioxidant capacity *in vitro* (Zhang & Chang, 2019; Rybiński et al., 2018), but also - in the case of lentils - the ability to improve inflammation in animal models (Martínez et al., 2016), while chickpeas can modulate lipid metabolism (Martínez et al., 2016). In addition, a diet based on legumes had a better effect than a common hospital diet in preventing insulin resistance and decreasing blood pressure during bed rest (Gao et al., 2019). Garlic can be consumed with legumes and it had antioxidant, anti-inflammatory and immunomodulatory effects, showing a promising impact against pathologies associated with the low-grade inflammatory state (Quesada et a., 2020). Finally, the introduction of almonds in a balanced diet has brought positive effects on risk factors for CVD, oxidative stress and inflammation in patients with type 2 diabetes (Liu et al., 2013) and modulating cholesterol (Damasceno et al., 2011).
This oral communication reports the main results of activities directed to:

1) Select suitable foods and preparation techniques for consumption, as well as proper extraction and *in vitro* digestion methods.
2) Assess the antioxidant capacity *in vitro* and on an intestinal cellular model by adding legume extracts/digested samples, and comparing them to edible insect as an alternative ecologic protein source.
3) Asses the modulation of the inflammatory response in cells of immune system by the digested foods.
4) Design a clinical study investigating the *in vivo* effect of the selected traditional foods on elderly people, select eligible subjects for the clinical study and evaluate their anthropometric and nutritional status.

This project was developed as a collaboration between University of Teramo (TE, Italy), Biochemistry and Cellular Biology Institute (*Istituto di Biochimica e Biologia Cellulare* – IBBC) of National Research Council (Consiglio Nazionale delle Ricerche - CNR) in Naples (NA, Italy) and the Alfa Polaris nursing home in Avezzano (AQ, Italy).

2. Materials and Methods

Six food products from Abruzzo region were selected: four legumes - chickpeas, grass peas, lentils and peas var. *Roveja* – almonds and red garlic. These foods have been identified among the ones potentially contributing to the

reduction of oxidative, inflammatory and metabolic stress. Four edible insects, dried and ready-to-eat, were selected for comparison: grasshoppers, giant worms, mealworms and silkworms. The other foods were analysed after being prepared for human consumption: the almonds were shelled but not peeled, the red garlic was peeled and legumes were soaked and boiled with common domestic procedures. The food thus prepared was subjected to water extraction, according to Di Mattia et al. (2019) and/or *in vitro* digestion, following the harmonized INFOGEST protocol (Brodkorb et al., 2019). Antioxidant capacity was determined *in vitro* using Ferring Reducing Antioxidant Power (FRAP) assay (Di Mattia et al., 2019) and in a cellular model of intestinal oxidative stress: normal colon mucosa cells (NCM460) were treated with a combination of the radical inducing molecule 2,2′-azobis (2-amidinopropane) dihydrochloride (ABAP) and aqueous extracts/digesta of legumes/insects; the ROS production was monitored through the fluorescence emitted by 2′,7′ dichlorofluorescein (DCF) probe. As regards effect on immune system, digesta of all foods were investigated for their ability to induce production of pro-inflammatory cytokines as TNF-α and IL-6 and anti-inflammatory cytokine IL-10 in macrophages obtained from healthy human donors; cytokines were quantified through ELISA kit. Foods that induced an inflammatory response were tested for their ability to induce an immune memory response: human monocytes were treated with food digesta and/or with pro-inflammatory lipopolysaccharide (LPS) and then, after a resting period, subjected to a second stimulus, homologous or crossed. Finally, the ability of digested foods to influence cytokine production induced by treatment with LPS in human macrophages or THP-1 cell line was evaluated. Selection of suitable elderly subjects was carried out; inclusion criteria were the presence of arterial hypertension and/or cognitive disorders related to aging; exclusion criteria were age under 65 and presence of neoplasms and/or cognitive or neurological disorders not related to aging. Measurement of anthropometric parameters on them was carried out by bioelectrical impedance analysis, using an Bioimpedancemeter InBody 120.

3. Results and Discussion

3.1 *In vitro* antioxidant capacity

Legumes showed FRAP values ranging from 0.05 to 0.17 mmol Fe2+/100 g d.m. for aqueous extracts and from 0.19 to 0.40 mmol Fe2+/100 g d.m. for digesta, being the lowest value of chickpeas and the highest from lentils. Edible insects showed higher absolute values than pulses: from 1.90 (mealworms) to 17.89 (silkworms) mmol Fe2+/100 g d.m. for aqueous extracts and 1.65 and 13.0 mmol Fe2+/100 g d.m. (giant worms and silkworms, respectively) for digesta. Aqueous extracts of silkworm showed the highest reducing ability among all the samples, while chickpeas showed the lower FRAP value. The digestion process did not induce significant variations between FRAP values of digesta respect to the aqueous extract of the same insect, with the exception of silkworm, showing a reduction of about 25%. Conversely, digesta of all legumes showed a significantly higher reducing ability respect to the corresponding aqueous extract. Overall, edible insects showed superior reducing activity respect to legumes.

3.2 Antioxidant capacity on cellular models

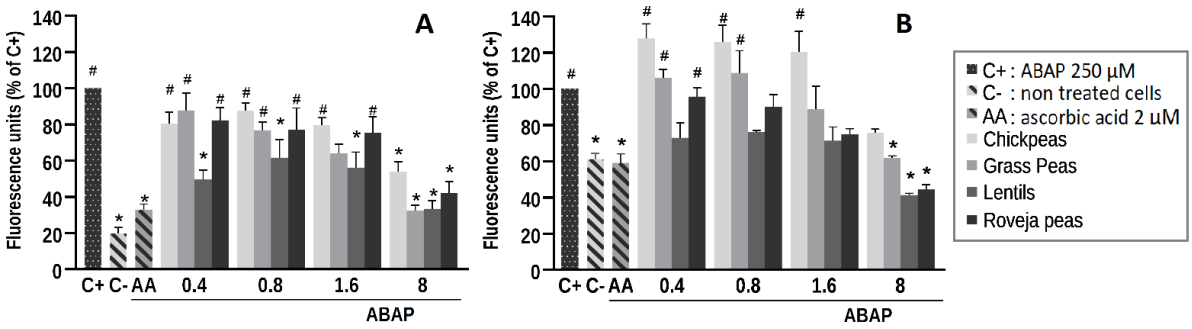

Figure 1 - *Effect of aqueous extracts (A) or digesta (B) of legumes on ABAP-induced intracellular ROS production in NCM460 cells. Values are expressed as percentage of fluorescence respect to the positive control (C+). Each column represents the mean ± SEM (n=3). Concentrations expressed as g d.m./L. *p<0.05 versus C+; # p<0.05 versus AA, according to one-way analysis of variance (ANOVA) followed by Bonferroni's post hoc analysis.*

Figure 1 shows the intracellular ROS production values of NCM460 cells after treatment with four concentrations (0.4, 0.8, 1.6, 8 g d.m./L) of aqueous extracts (fig. 1A) or digesta (fig. 1B) of legumes in the presence of ABAP. Aqueous extracts of all the legumes at the highest concentration (8 g d.m./L) showed a significant antioxidant capacity, while lentils were significantly effective at all the tested concentrations. Conversely, digesta of lentils showed a significant antioxidant capacity only at the higher concentration, together with *Roveja* peas and grass peas. Comparing results obtained by the same legume after aqueous extraction or digestion, only chickpeas at 0.4, 0.8 and 1.6 g d.m./L were significantly different: aqueous extracts reduced ROS production more than digesta.

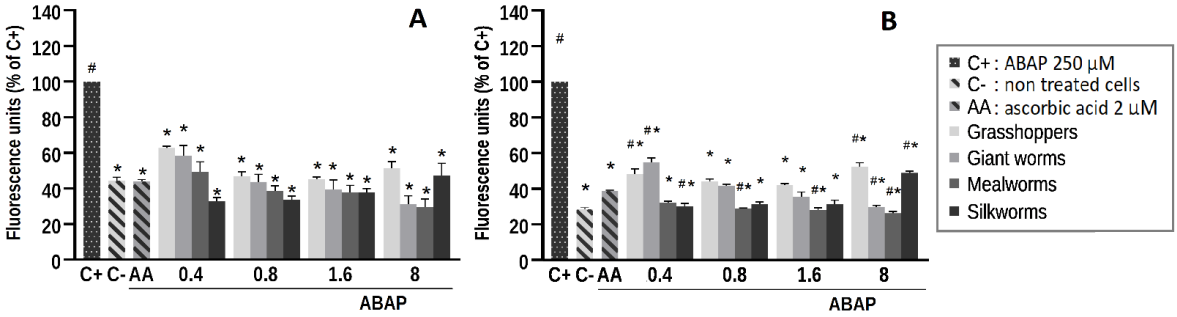

Figure 2 – *Effect of aqueous extracts (A) or digesta (B) of edible insects on ABAP-induced intracellular ROS production in NCM460 cells. Values are expressed as percentage of fluorescence respect to the positive control (C+). Each column represents the mean ± SEM (n=3). Concentrations are expressed as g d.m./L. * p<0.05 versus C+; # p<0.05 versus AA, according to one-way analysis of variance (ANOVA) followed by Bonferroni's post hoc analysis.*

All insects, both as aqueous extracts (fig. 2A) or digesta (fig. 2B) at the tested concentrations, were able to significantly reduce the ROS production induced by the ABAP treatment in NCM460 cells. Comparing results of each insect at the same concentration, there is no significant difference between aqueous extract and digesta; the only exceptions were mealworms and grasshoppers tested at 0.4 g d.m./L, whose antioxidant capacity is significantly higher after digestion. Comparing these results, it can be noted that edible insects modulated the chemically induced oxidative stress at all the concentrations, while legumes showed this ability mainly at the higher concentrations.

3.3 Induction of cytokine production in macrophages

The digesta of all foods were tested at concentrations 10 and 100 µg/mL on macrophages, obtained by *in vitro* differentiation of monocytes isolated from the blood of healthy donors, to evaluate whether they were able to influence inflammatory response. Of all the 10 digested foods tested, only *Roveja* peas, chickpeas, mealworms and almonds induced the activation of macrophages, measured as the production of pro inflammatory cytokines TNF-α and IL-6. Moreover, the production of anti-inflammatory cytokine IL-10 induced by these four foods was evaluated.

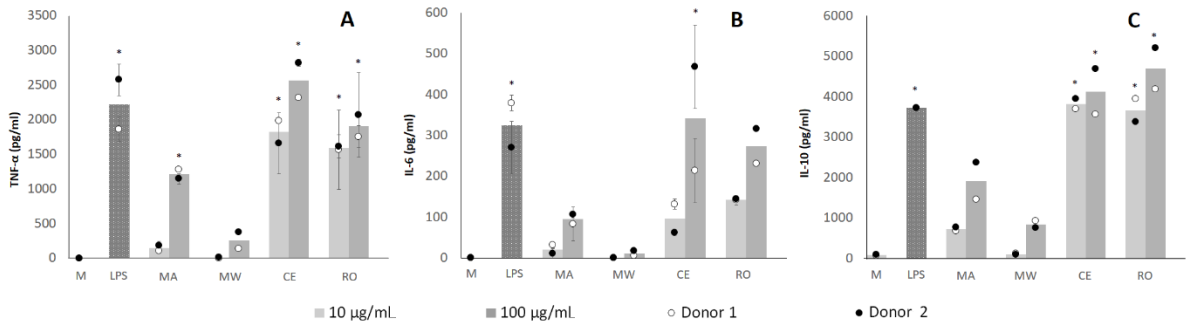

Figure 3 – *Production of pro-inflammatory cytokines TNF-α (A) and IL-6 (B), and anti-inflammatory IL-10 (C) in human macrophages induced by 24h of treatment with 10 or 100 µg/mL of food digesta. LPS: 10 ng/mL. M, untreated cells; LPS, lipopolysaccharide; MA, almonds; MW, mealworms; CE, chickpeas; RO, Roveja. The diagrams show the mean of the two donors. *, p <0.05 vs M according to non-parametric One-Way ANOVA test, followed by Bonferroni's t-test.*

Figure 3 shows the levels of TNF-α, IL-6 and IL-10 induced in macrophages of two healthy donors; macrophages treated with digested chickpeas and *Roveja*, showed values comparable to those obtained for treatment with 10 ng/mL of lipopolysaccharide (LPS). Foods that did not induce cytokine production were tested for their ability to reduce the TNF-α production induced by a treatment with LPS. Food digesta were tested at 10 or 100 µg/mL vs LPS 5 ng/mL in macrophages from human donors and vs 0.1 o 10 ng/mL of LPS in macrophages obtained by the differentiation of monocytes from THP-1 cell line. None of the selected food showed the ability to counteract TNF-α production induced by the LPS treatment at the tested concentrations.

3.4 Induction of immune memory in human monocytes

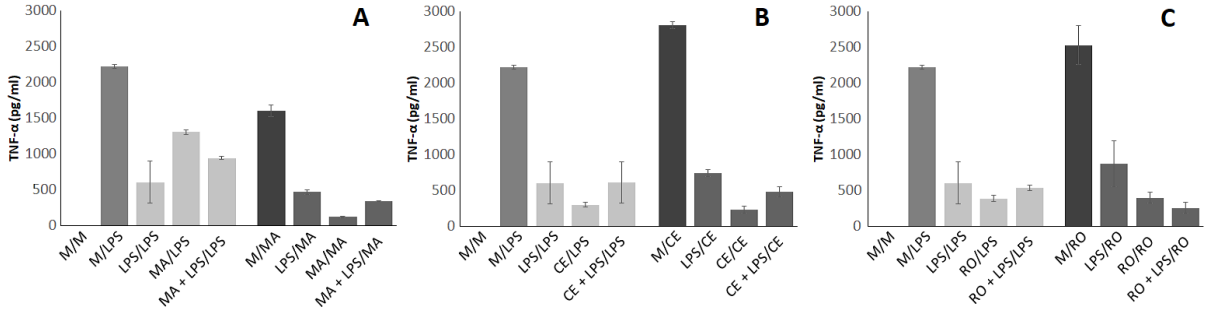

Figure 4 – *Production of TNF-α in human monocytes treated for 24h with 100 µg/mL of food digesta or 10 ng/mL or LPS, seven days after a previous treatment with the corresponding food and/or LPS for 24h. M, no treatment; LPS, lipopolysaccharide; MA, almonds; MW, mealworms; CE, chickpeas; RO, Roveja.*

The foods able to induce cytokine production in macrophages were investigated to their ability to stimulate innate immune memory, i.e., a change in the reactivity in innate immune cells previously exposed to various stimuli, in monocytes isolated from healthy donors; LPS is able to induce innate memory. As shown in figure 4, cells that were subjected to a treatment with digesta, LPS or a combination of both showed a lower TNF-α production when treated for a second time with LPS or the corresponding digesta respect to cells that were stimulated for the first time. It implies that almonds, chickpeas and *Roveja* could induce innate memory in human monocytes. The potential ability of the two legumes and of almonds to stimulate immune memory is particularly interesting because studies on effect of foods on immune memory are innovative; therefore, more evidences involving further donors needs to be acquired in order to confirm this result.

3.5 Observational study

A preliminary screening led to the selection of 30 elderly patients of the nursing home "Alfa Polaris", potentially eligible for the development of the clinical study. The Bioimpedance meter, used to collect anthropometric measures, requires the ability of the patient to stand still for the time of the measurement: unfortunately, half of the subjects were not able to do it. Therefore, data of 15 subjects were recorded. Thirteen of the selected subjects were women and two were men; 8/15 were aged between 80 and 89 years and 7/15 between 90 and 99 years; 6 subjects were affected by pathologies related to arterial hypertension, 5 suffered from cognitive decline due to aging and 4 had both types of pathologies.

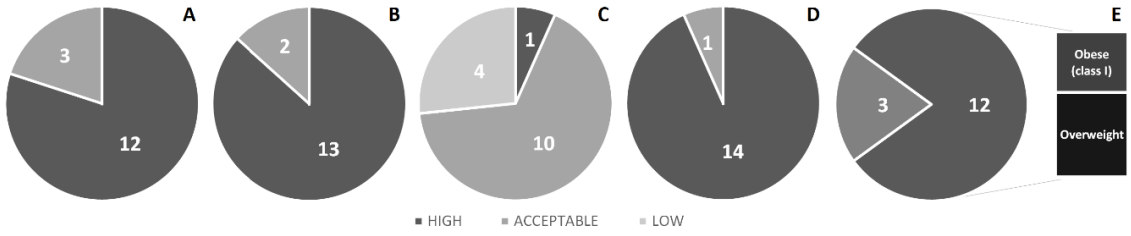

Figure 5 – *Number of subjects that showed a weight (A), body fat mass (B), skeletal muscle mass (C), waist-hip ratio (D) and body mass index (E) higher, acceptable or lower respect to ideal values.*

The instrument measures various parameters and the results are reported with the respective ranges of acceptability for each patient; among these, the parameters and indexes significant for this study were weight, body fat mass, skeletal muscle mass, waist-hip ratio and BMI. In figure 5 are reported the number of subjects that showed a higher, acceptable or lower value of each parameter respect to their range of acceptability. The majority (12/15) of the subjects showed a body weight higher than the specific range reported for each patient (fig. 5A). Specifically, based on the BMI results (fig. 5E), 7 subjects were overweight and 5 were obese of class 1. As expected, fat mass contributes more than skeletal muscle mass to the excess of weight: the 12 subjects also had higher fat mass (fig. 5B), while the lean mass was higher only for one subject (fig. 5C), thus confirming the evaluation given by the BMI. Moreover, 13/15 subjects had a waist-hip ratio above the upper limits (fig. 5D); this index is the ratio between the waist and hips circumferences and it is used as a risk indicator of the development of serious pathological conditions. As regards blood pressure, most of the subjects (11/15) showed normal values for systolic pressure, while all diastolic blood pressure values were acceptable. In conclusion, the elderly subjects selected and evaluated during the observational study are eligible to be involved in the clinical study following the established protocol.

3.6 In vivo study protocol

A 3-month *in vivo* pilot study on the available subjects has been planned. Selected traditional foods will be suggested for consumption to eligible hosts of the nursing home; as regard legumes, they will replace portions already provided or other protein sources to reach 3 portion/week, as suggested by guidelines for elderly people. The effects of the dietary changes will be assessed through the measurement of the anthropometric parameters, blood pressure and analyses, in order to obtain a profile of markers useful for evaluating the redox, metabolic and inflammatory state of the subjects. These analyses are: blood count, leukocyte count and formula, glycemia, insulin, C reactive protein, total-, HDL-and LDL-cholesterol, triglycerides, uric acid, total proteins. Results of all analyses will be collected before the study and at the end of every month of treatment. Selected markers will be analysed to establish if the regular consumption of selected food can have a beneficial effect on the subjects.

4. Conclusions and Future Perspectives

The antioxidant capacity of legumes and edible insects, both as aqueous extracts or after *in vitro* digestion, were assessed and compared: unlike edible insects, effective also at lower concentrations, legumes showed the ability to modulate chemically induced oxidative stress mainly at the highest concentration tested. However, lentils, grass peas and Roveja peas maintained this property after *in vitro* digestion, making these products promising for the *in vivo* study.

None of the investigated foods was able to reduce LPS induced TNF-α production in the applied conditions; however, the ability showed by chickpeas, peas var. *Roveja* and almonds to stimulate immune memory is particularly interesting, since studies on effect of foods on immune memory are innovative.

A clinical study on elderly people was designed, aiming to investigate the *in vivo* effect of the selected traditional foods on redox, metabolic and inflammatory state of the subjects through a profile of related markers. Eligible subjects for the clinical study were selected and their anthropometric and nutritional status were assessed.

As regards future perspectives, the ability of chickpeas, *Roveja* peas and almond to stimulate immune memory will be assessed on additional donors; moreover, selected foods will be further evaluated for anti-inflammatory properties, testing their effect on inflammatory response of a more complex model, i.e. a co-culture of intestinal and immune cells. Furthermore, antioxidant capacity of almonds and garlic will be assessed both in vitro and in the selected cellular model. The designed clinical study will be carried out on the eligible elderly subjects to establish if the regular consumption of selected food can have a beneficial effect on their redox, metabolic and inflammatory state.

5. Legend

AA, ascorbic acid; ABAP, 2,2′-azobis (2-amidinopropane) dihydrochloride; BMI, body mass index; CE, chickpeas; DCF, 2′,7′ dichlorofluorescein; d.m., dry matter; ELISA, enzyme-linked immunosorbent assay; IL, interleukin; LPS, lipopolysaccharide; MA, almonds; MW, mealworms; RO, Roveja; ROS, reactive oxygen species. TNF-α, tumour necrosis factor α.

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Spotlight on the meta community of the south Tyrolean cheese microbiome

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Tutor: Prof. Marco Gobbetti

This PhD project dealt with the use of omic sciences, more specifically with metagenomics, combining culture-dependent and culture-independent microbiological methods, to study and elucidate not only the microbiota, but also and mainly the complex microbiome of cheeses produced in the Alto-Adige's region throughout the manufacturing and ripening process.

Studio sulla metà comunità del microbioma del formaggio altoatesino

Questo progetto di dottorato ha affrontato l'uso delle scienze omiche, in particolare della metagenomica, combinando metodi microbiologici dipendenti dalla cultura e indipendenti dalla cultura, per studiare e chiarire non solo il microbiota, ma anche e principalmente il microbioma complesso dei formaggi prodotti nell'Alto- La regione dell'Adige durante tutto il processo di lavorazione e stagionatura..

Key words: Next.-generating-sequencing; 16S; metagenomics; dairy.

1. Introduction

Microorganisms naturally present in raw milk and in the manufacturing environment use milk as a growth substrate during the cheesemaking process, and activate their own metabolic pathway, interacting with other microbial populations. Therefore, the entire process of manufacturing dairy products, as well as the ripening of these products, is governed by microbial interactions, which are affected by the manufacturing technology applied (e.g., time, temperature, quality of raw milk, etc.), impacting on the physical and chemical composition of cheese whether positive and therefore desired or negative (Fox et al., 2004; Gobbetti et al. 2018).

Despite the extensive knowledge about the microbiological diversity of milk (e.g., native lactic acid bacteria, starter culture, non-starter lactic acid bacteria, adjunct cultures, other spoilage microorganisms) the cheese-making process, and more specifically cheese, has always been based and designed on the use of standardized starter cultures, and with the assumption that these cultures act alone and with an exclusive role in the fermentation and ripening of these products. However, nowadays this concept has become quite outdated since there is an increasing knowledge of the complex microbiome that governs fermented food matrices such as cheese (Afshari et al., 2018; Vrieze et al. 2018).

The influence of external factors on the microbiome of fermented dairy products, as well as their influence during the ripening of these products where the change of the microbial growth kinetics observed, has been discussed as an important factor in the generation of sensory attributes (Marco et al., 2017; Jonalla et al., 2018). In summary, high microbial diversity in milk tends to generate a high number of sensory descriptors of interest in cheeses, and such diversity can change both in different sections of the cheese (e.g., core vs. rind) and over the storage time. For example, according to Montel et al. (2014) microbiological biodiversity decreased inside the cheeses, where a small number of lactic acid bacteria species are numerically dominant, but remain relatively high on the cheese surface, where numerous species of bacteria, yeasts and molds reside.

Therefore, a better understanding of stochastic (e.g., intra and inter biodiversity, growth kinetics), deterministic (e.g., biotic and abiotic factors) and temporal drivers is necessary in the current scientific scenario in order to allow the manipulation of the entire process of manufacturing cheeses, whether artisanal or industrial level (Shafquat et al., 2014). To illustrate this scenario, a study by Wolfe et al. (2015) on the drivers affecting the cheese microbiota biodiversity showed that when defining the interactions and the functionality of the cheese microbiome, this complex ecosystem may be successfully reproduced in vitro, representing a potential breakthrough for the dairy products industry.

Knowing the importance of a greater understanding of this topic for both for science and for the industry, several authors have been studying the complex microbiota of cheeses in recent years, either through traditional or molecular methods. The use of advanced high-throughput sequencing (HTS) techniques combined with the total DNA sequencing of cheese samples and the use of multi-omic platforms has revolutionized the concept of fermented food microbiome and reframed the concept of cheesebiome (Jonalla et al., 2018; Raes & Bork, 2008).

Parallel to this, additional studies have been proposed in recent years in order not only to isolate and identify the microbiota of fermented products but also to define the functionality of this microbiome, its metabolic level, the influence of different external factors on this complex microbiome and also the role of sub-dominant cultures within that microbiome. Thus, a study that brings together all these parameters presents itself as something innovative and with great potential both for the scientific area and for the industry.

In conclusion, we can discuss the use of metabolic networks for the generation of flavors. The basic aim of this project is to structure and stabilize the societal organization of the cheesebiome, intended as supra-entity

characterized by a supra-genome, and introducing a new paradigm that ensures resilience. To demonstrate the effectiveness, we intend to reconstruct metabolic networking for flavor development, as a primary cheese attribute. Specific to each cheese variety, flavor results from many physical-chemical and biological factors of influence, as well as hypothetical pathways that synthesize various chemical compounds (Stefanovic et al. 2017; Wolfe et al. 2014). As made available from the cheesebiome, the web of enzymatic reactions represents the metabolic portfolio required to obtain desired sensory characteristics. Discovering basic science fundaments, we can reconstruct, control and expand these pathways linking supra-genome, supra-phenotype and flavor genesis. This will allow the exploitation of typical/traditional cheeses, mainly from the South Tyrol area, used as a model system.
Said that, in accordance with the PhD thesis project previously decribed, this oral communication reports the main results of the following activities aimed to:

(i) Collecting samples from three traditional dairy farms in Alto Adige area producing raw cow's milk cheeses.
(ii) Structuring and defining the societal organization of the cheesebiome.
(iii) Identifying the subdominant populations, showing resilience during the ripening process.
(iv) Creating the first Alto Adige's dairy biobank;
(v) Defining the metagenetics of Alto Adige's dairy samples.
(vi) Drawing the genome-scale metabolic catalogues (GSMCs) targeting cheese ecosystems in order to identify all metabolic reactions and their associations based on merging information about gene functions, biochemical reactions, and the theoretical background.

2. Materials and Methods

A total of 18 samples of raw milk, natural starter, curd, cheese after 1, 2 and 3 months ripening were collected from three dairies from Alto Adige: Caseificio Scuola Agricola di Salern, Caseificio Enghlorn and Caseificio Malga Drassbergalm. The natural starters were produced in loco using cow's raw milk or cheese whey, and cheese ripening was carried out according with the traditional procedure of each dairy.
After collection, all samples were immediately processed for the enumeration of total aerobic and anaerobic bacteria, mesophilic cocci, thermophilic cocci, mesophilic bacilli, enterococci, coliforms, Staphylococcus-Micrococcus, Propionibacterium, yeasts and molds by using selective media. Cell number of bifidobacteria in milk samples was also estimated. Total aerobic and anaerobic bacteria were cultivated onto PCA at 30°C for 24 h and Wilkins Chalgreen at 37°C for 48 h, respectively. Mesophilic and thermophilic cocci onto M17 agar at 30°C for 24 h and 45°C for 48 h, respectively. Mesophilic bacilli onto MRS agar at 30°C for 48 h, enterococci onto Slanetz agar at 37°C for 24 h, coliforms onto VRBGA at 37°C for 24 h, Staphylococcus-Micrococcus onto Baird Parker agar at 30°C for 48 h. Where Propionibacterium onto YELA at 37°C in anaerobiosis for 1 week, yeasts and molds onto Potato Dextrose Agar for 72 h at 25°C, and bifidobacterial onto Bifidobacterium agar at 37°C for 72 h in anaerobic conditions.
Total DNA extraction of about 1200 isolates and amplification of a16S fragment were carried out. The process of identification was performed by using the BLAST (Basic Local Alignment Search Tool) tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) from the National Center for Biotechnology Information.

3. Results and Discussion

3.1. Sampling and culture dependent methods (selective plate count)

Cell number of all microbial groups are shown in Table 1. Propionibacteria have not been detected in any 10 g of sample. There were no significant differences between the 3 dairies, having shown the same general behavior, so the table below reports only the results obtained for the Salern dairy.
A growth in the population of aerobic microorganisms and a stabilization of these were observed in the first month of maturation of the cheeses, however, an increase in the population of anaerobics was observed until the second month of maturation and then a stabilization of this class of microorganisms. This greater growth of late anaerobic microorganisms may be due to the gradual and slow decrease of the redox potential in the interior of the cheeses during the maturation process, in which the cheese mass becomes more compact and more isolated from the external environment due to the formation of the external crust of the cheese. (Fox et al. 2004)
Among the samples, the cheeses from the dairy of Salern, the only samples produced with starter culture fermented milk starter-culture type and not with fermented whey starter-culture like the others, showed the highest microbial count peak in the second month of maturation for total anaerobic and mesophilic bacilli. This may be due to the fact that bacillus can develop better and without competition from thermophilic bacteria commonly present in fermented whey, increasing their initial bacterial load at the beginning of the cheese ripening process and maintaining high levels until the end of the second maturation month (Walstra et al., 2005).

Table 1. *Cell counts of milk, natural starter, curd and cheese at different time of ripening (1, 2 and 3 months) from Alto Adige's Salern dairy enumerated on selective agar media.*

Salern	***MILK*** *Log CFU/mL*	***STARTER*** *Log CFU/mL*	***CURD*** *Log CFU/g*	***1 month*** *Log CFU/g*	***2 months*** *Log CFU/g*	***3 months*** *Log CFU/g*
Total aerobic	4.0 ± 0.95^{a}	3.1 ± 0.07^{a}	4.3 ± 0.03^{a}	7.2 ± 0.01^{b}	7.1 ± 0.13^{b}	7.2 ± 0.03^{b}
Total anaerobic	3.0 ± 0.02^{a}	6.7 ± 0.00^{b}	5.8 ± 0.07^{c}	7.3 ± 0.07^{d}	8.4 ± 0.12^{e}	8.3 ± 0.22^{e}
Mesophilic cocci	3.6 ± 0.02^{a}	4.9 ± 0.03^{b}	4.2 ± 0.07^{c}	7.2 ± 0.06^{d}	7.1 ± 0.09^{d}	7.0 ± 0.02^{d}
Thermophilic cocci	3.0 ± 0.05^{a}	6.9 ± 0.04^{b}	8.0 ± 0.06^{c}	7.1 ± 0.12^{d}	7.3 ± 0.04^{d}	7.3 ± 0.03^{d}
Mesophilic bacilli	3.7 ± 0.10^{a}	2.5 ± 0.10^{b}	4.9 ± 0.07^{c}	7.2 ± 0.06^{d}	8.8 ± 0.01^{e}	8.2 ± 0.00^{f}
Enterococci	1.7 ± 0.49^{a}	3.2 ± 0.11^{b}	3.8 ± 0.03^{b}	6.5 ± 0.04^{c}	6.9 ± 0.03^{c}	7.8 ± 0.06^{c}
Coliforms	0.9 ± 0.21^{a}	1.6 ± 0.06^{b}	1.8 ± 0.06^{b}	6.4 ± 0.09^{c}	3.0 ± 0.07^{d}	4.4 ± 0.01^{e}
Staphylococcus	3.4 ± 0.04^{a}	4.6 ± 0.19^{b}	5.2 ± 0.09^{c}	4.5 ± 0.01^{b}	5.0 ± 0.03^{c}	6.6 ± 0.01^{d}
Yeasts and molds	4.6 ± 0.03^{a}	3.6 ± 0.00^{b}	4.9 ± 0.04^{c}	3.5 ± 0.02^{d}	4.7 ± 0.02^{e}	5.8 ± 0.04^{f}
Bifidobacteria	*1.2 ± 0.09*	*N/A*	*N/A*	*N/A*	*N/A*	*N/A*

3.2. Culture independent methods (metagenomics)

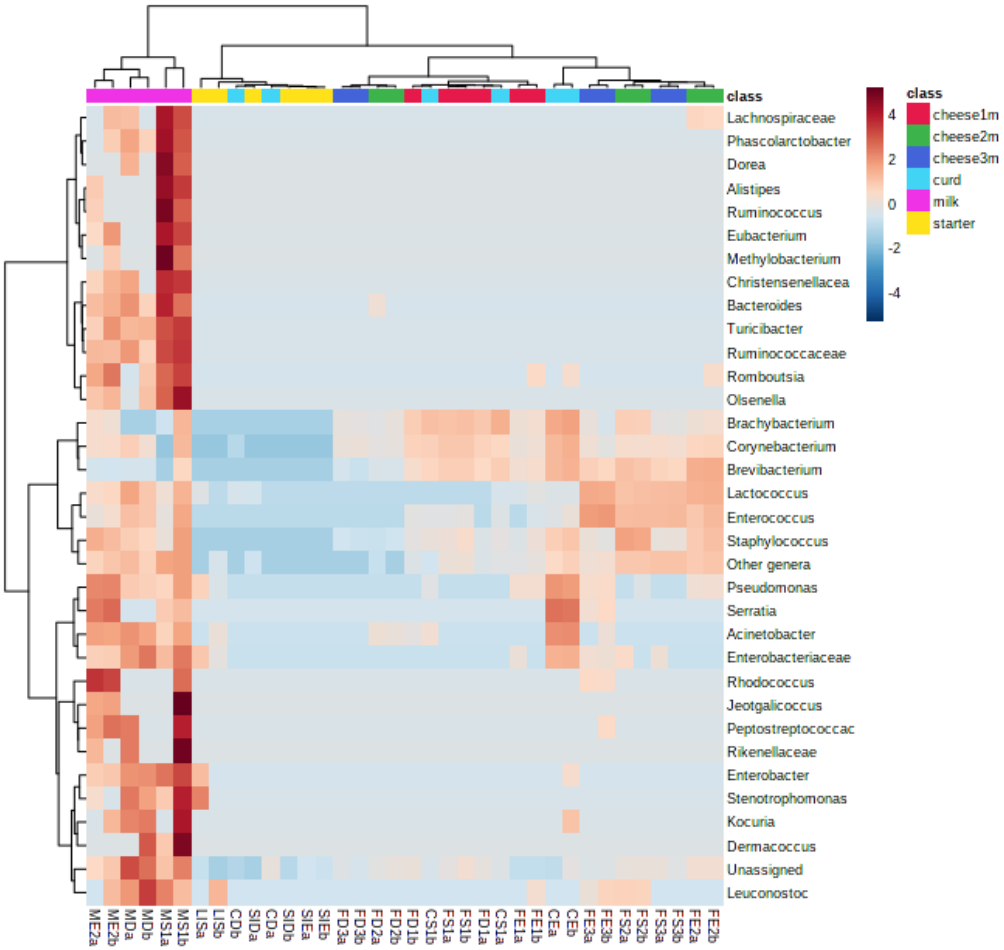

Figure 1. *Heat-map of the Miseq-Illumina 16s analysis results, illustrating the relative abundance of the 34 genera of bacteria with significant difference between samples (ANOVA $p<0.05$).The color key defines the percentage relative abundance in the samples. Only operational taxonomic units (OTU) with an incidence above 0.05% in at least one sample are shown. MS: Salern Milk, ME: Englhorn Milk, MD: Dressbergalm Milk, LIS: Salern Milk Starter, SIE: Englhorn Whey starter, SID: Dressbergalm Whey Starter, CS: Salern Curd, CE: Englhorn Curd, CD: Dressbergalm Curd, FS: Salern Cheese, FE: Engelhorn Cheese, FD: Dressbergalm Cheese; 1-2-3: 1-, 2-, and 3-months ripening; a-b: replicate.*

A total of 46 species of bacteria were identified, including Streptococcus thermophilus (24%), Lactococcus lactis subsp. lactis (19%), Enterococcus faecalis (16%), Lacticaseibacillus paracasei (14%), and Lactiplantibacillus plantarum (6%). The 16S and ITS Illumina-MiSeq analysis showed that raw milk samples had a higher biodiversity

(with 148 and 123 taxonomic groups at genus level in 16S and ITS, respectively), number of sequences analysed before and after filtering, observed operation taxonomic units (OTUs), Chao1 and Shannon indices than starters, curd and cheese samples (Table 2). Lactobacillus and Streptococcus among bacteria, and Debaryomyces and Nectriaceae among yeasts dominated starter, curd and cheese samples (Figure 1). Other species, rarely found in dairy products, were also identified such as *Acinetobacter johnsonii*, *Chryseobacterium aquaticum*, *Citrobacter amalonaticus*, *Klebsiella oxytoca*, *Escherichia coli*, *Hafnia paralvei*, *Serratia liquefaciens*, and *Corynebacterium flavescens*.

Table 2. *Number of sequences analysed before and after filtering, observed operation taxonomic units (OTUs), Chao1 and Shannon indices for 16S rRNA and ITS amplification from milk, starter, curd and cheese samples*.*

	16S					ITS				
Sample ID	**Total reads (n)**	**Non-chimeric reads (n)**	**Chao1**	**Observed OTUs**	**Shannon**	**Total reads (n)**	**Non-chimeric reads (n)**	**Chao1**	**Observed OTUs**	**Shannon**
MS1a	159506	101685	458	446	7.15	161739	83692	641	639	7.28
MS1b	108626	70522	826	812	7.14	142634	95232	69	68	5.30
LISa	149434	107346	32	27	0.13	148952	53470	53	53	4.46
LISb	344817	246056	35	25	0.09	119692	62785	59	58	5.02
CS1a	144607	102092	61	51	0.30	95257	83491	24	23	0.33
CS1b	116272	81790	48	42	0.27	93383	80596	33	32	0.37
FS1a	135033	89111	95	78	1.78	82569	73226	16	16	0.10
FS1b	125635	79856	77	64	1.72	109382	97001	12	12	0.10
FS2a	141594	96991	147	133	2.31	152097	135230	14	14	0.10
FS2b	129620	90495	144	133	2.13	127189	113012	12	12	0.04
FS3a	132310	89291	146	138	2.35	235506	186672	20	19	1.36
FS3b	137700	92339	142	136	2.26	125134	94442	20	20	1.44
ME2a	97206	64482	137	130	3.12	152278	76321	63	63	3.78
ME2b	115137	78488	202	187	3.39	165112	85683	79	78	3.51
SIEa	122533	85530	47	42	1.51	161077	69036	35	35	3.72
SIEb	98373	66343	42	38	1.34	184073	89468	39	39	3.57
CEa	116115	76024	119	98	3.08	123612	79556	20	20	1.31
CEb	127540	84799	150	135	3.02	107307	69944	24	24	1.31
FE1a	124975	84901	60	50	1.01	98945	80823	18	18	1.14
FE1b	113187	74772	76	60	1.14	91041	74128	18	18	1.17
FE2a	107869	71013	135	123	2.26	111024	90866	20	18	1.10
FE2b	122796	80440	151	131	2.55	84420	68645	15	15	1.17
FE3a	125773	84841	140	129	2.01	83993	69107	22	22	1.45
FE3b	117694	76063	154	134	2.19	124853	100878	32	30	1.46
MDa	51578	23951	206	206	3.81	53693	27734	9	9	1.88
MDb	62759	36192	168	166	2.57	130129	64991	37	36	4.09
SIDa	89106	50375	58	51	1.81	135642	104866	17	17	1.26
SIDb	72113	47361	61	53	1.68	117363	83614	23	23	1.20
CDa	59057	18986	26	26	1.51	134700	73928	29	28	1.94
CDb	124902	82595	66	54	1.71	147495	102464	23	23	1.79
FD1a	88210	58842	78	64	1.73	74429	66348	13	13	0.08
FD1b	81353	52062	60	55	1.68	129787	115499	15	14	0.07
FD2a	124679	85013	86	69	1.72	113180	94952	20	18	1.18
FD2b	115424	79248	83	69	1.71	132959	111952	25	25	1.13
FD3a	101013	65721	65	59	1.69	85969	76052	21	21	0.64
FD3b	107104	71281	66	55	1.62	99971	87004	23	23	0.52

**MS: Salern Milk, ME: Englhorn Milk, MD: Dressbergalm Milk, LIS: Salern Milk Starter, SIE: Englhorn Whey starter, SID: Dressbergalm Whey Starter, CS: Salern Curd, CE: Englhorn Curd, CD: Dressbergalm Curd, FS: Salern Cheese, FE: Engelhorn Cheese, FD: Dressbergalm Cheese;*
1-2-3: 1-, 2-, and 3-months ripening; a-b: replicate.

As corroborated by Figure 1 and by the high observed OTU for milk samples in table 2, we can see that there is a significantly greater diversity in the microbiota present in the milk samples than in the other samples, creating a cluster of differentiation of these samples from the others. According to Fox et al. (2004) and Robinson (2002), it is to be expected a very diverse microbiota in milk, which during the cheese production process, acidification, fermentation and maturation is reduced due to a natural selection due to biotic and abiotic factors such as reduced redox potential, scarcity of substrate, interspecies competition, among other factors.
Such data lead us to believe that a more in-depth study of the microbiota and microbiome through the correlated use of the omic sciences of proteomics and metabolomics can clarify the effects of this kinetics of microbial diversity in the cheese production process and in the characterization of these specific cheeses.
Thus, gas chromatography analyzes for the identification and quantification of volatile compounds and organic acids, analysis of the profile of soluble and insoluble peptides at pH 4.6 through FPLC-Akta and UREA-PAGE respectively, and the analysis of transcriptome through sequencing of Total mRNA from the samples for the definition of gene expression and design of metabolic pathways are in the final stages of statistical analysis and will soon bring further clarification to the data presented here.

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Utilization of citral as antimicrobial on the mutants of Σ1278b library of *Saccharomyces cerevisiae*

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Citral showed antimicrobial effect toward yeast. In order to study this function, MIC was determined on the wild type strain of Saccharomyces cerevisiae Σ1278b. This concentration was tested later on the whole bank, consequently, to select 2 mutants for the study: DeltaSFL1 and DeltaSNF1. As a result, part of the mutants was resistant to the component, specifically DeltaSFL1 showed higher resistance toward the MIC and the subMIC of the component comparing to the wild type and the DeltaSNF1 strains.

Utilizzo del citrale come antimicrobico sui mutanti della biblioteca Σ1278b di *Saccharomyces cerevisiae*

Citral ha mostrato un effetto antimicrobico nei confronti del lievito. Per studiare questa funzione, la MIC è stata determinata sul ceppo wild type di Saccharomyces cerevisiae Σ1278b. Questa concentrazione è stata testata in seguito su tutta la banca, di conseguenza, per selezionare 2 mutanti per lo studio: DeltaSFL1 e DeltaSNF1. Di conseguenza, parte dei mutanti era resistente al componente, in particolare DeltaSFL1 ha mostrato una maggiore resistenza verso il MIC e il subMIC del componente rispetto al wild type e ai ceppi DeltaSNF1.

Keywords: Citral, antimicrobial, mutants, Saccharomyces cerevisiae, DeltaSNF1, DeltaSFL1.

1. Introduction

Citral exhibits antimicrobial activity towards Saccharomyces cerevisiae and other yeast (Fancello et al., 2016). Previous studies showed this component great potential in inhibiting and eradicating biofilms formed by foodborne pathogenic microorganisms (Espina et al., 2017). The S. cerevisiae library Σ1278b contains circa 5,000 haploids that each hold a single deletion in a non-essential gene. (Dowell et al., 2010; C. Boone, personal communication, 2015). With these mutants, screening was effectuated to determine the principal action of citral as anti-microbial. As a first step, MIC was determined on the wild type strain, to test later the screening and the selection of mutants specifically chosen for the study: DeltaSNF1 and DeltaSFL1. Sub MIC for the selected mutants was defined later. Sfl1 known as a particular transcriptional repressor, interacts specifically with Tpk2, preventing its transcriptional repression of FLO11. Losing function of Sfl1 greatly stimulates pseudohyphal growth and increases FLO11.

SNF1 (sucrose non-fermenting protein kinase) is required for yeast growth on sucrose and has important functions in transcription of genes repressed by glucose related to growth on non-glucose carbon sources besides other biological process. Same as mammalian AMPK, yeast SNF1 also consists of three subunits, the catalytic subunit (Snf1), and the non-catalytic b and c subunits. (Hardie et al.,1998). In addition, glucose depletion prompts FLO11 via the AMP kinase homolog Snf1p by the activation of the transcriptional repressors Nrg1p and Nrg2p (Kuchin et al. 2002;Van De Velde and Thevelein 2008). FLO11 is a glycerol phosphoinositol-anchored cell surface protein promoting mother-daughter cell adhesion leading cells to bind to and invade growth substrates.

Table 1 *Genes as described in Saccharomyces GENOME DATABASE.*

Gene name	ORF name	Function
SNF1	YDR477W	Regulation of pseudohyphal and filamentous growth in response to starvation and carbohydrate metabolic process.
SFL1	YOR140W	Transcriptional repressor and activator; involved in repression of flocculation-related genes, and activation of stress responsive genes.

2. Materials and methods

For the screening, firstly, the minimal inhibitory concentration (MIC) was determined as the lowest EO concentration of Citral (3,7-Dimethyl-2,6-octadienal, Geranial and neral mixture), inhibiting visible growth of the tested microorganism. EO stock solution was first prepared with a concentration of 20 µl/ml. Stock solution was

then diluted with sterile water and 1% DMSO, giving a succession of concentrations ranging from 0.039 to 20 µl/ml (Fancello et al.,2016). Overnight cultures were used to prepare microbial inoculation for the test, reaching an OD of 0.2. Aliquots of 100 µL of diluted inoculation at desired cell concentration were added to each well in the 96-well micro-dilution polystyrene plate already containing 100 µL of the EO dilution. The plates were then incubated at 30°C for 24 h. DMSO alone (at 1% concentration) was used as negative control, DMSO and sterile water as positive control. Each experiment was repeated twice.
The mutants were used from the master plates kept at -20°C, by adding YPD broth, G418 (Gold Biotechnology protocol reference), in 96 well microplates and replicating with a pin replicator, incubated for 24-48 hr till growth. secondly, the following reagents were mixed and filled with a volume of around 25 ml in petri plates: 0.1% of DMSO, YPD agar, in addition to the appropriate concentration of citral (MIC).
Once the prepared mixture was solidified, the replication from the preculture mutants was performed on the prepared mixture in a parallel way. Each plate containing the mutants was repeated twice.
Regarding the MIC and subMIC of the chosen mutants, an initial OD of 0.02 was settled to prepare microbial inoculation used for the test, employing synthetic medium with the appropriate amino acids and nucleotides as mentioned in the reference of Andersen et al.2014.
Aliquots of 100 µL of diluted inoculation at desired cell concentration were added to each well in the 96-well micro-dilution polystyrene plate already containing 100 µL of the EO dilution. The plates were then incubated at 30°C for 24 h.
Saccharomyces GENOME DATABASE (SGD) was used to identify gene ontology (GO) representing molecular functions, cellular locations, and biological process. Each gene name is abbreviated correspondently to the name description of the cellular location.

3. Results and discussion

With the absence of turbidity, the MIC was determined as for wild type strain 0.156 µl/ml. DeltaSFL1 showed a higher resistance toward citral with a MIC of 0.0781 and a subMIC of 0.039, while DeltaSNF1 and WT had the same resistance with a MIC of 0.156 and a subMIC of 0.0781. (see figures).

Table 2 *MIC and subMIC of the 3 studied strains with synthetic medium*

Strain	MIC (µl/ml)	SubMIC (µl/ml)
Wild type	0.156	0.0781
DeltaSNF1	0.156	0.0781
DeltaSFL1	0.0781	0.039

Overall, the bank tested, 533 mutants with the exact MIC, resisted to the citral where growth was observed, these mutants were selected for the study, focusing on DeltaSFL1 and DeltaSNF1.
Previous studies showed great potential of citral as an inhibitor and eradicator of biofilms formed by foodborne pathogenic microorganisms (Espina, Berdejo, Alfonso, García-Gonzalo, & Pagán, 2017), in addition to decreasing the permeability barrier of plasma membrane and the intracellular ATP pool while increasing of the extracellular ATP. Scaricot et al.,2021 showed that S. cerevisiae cells treated with citral (2.0 mM) exhibited a mitochondrial hyperpolarization that can be responsible for the increase of intracellular ROS. In the DeltaSFL1 strain, the gene is deleted where no Phosphorylation (P) by Tpk2 is present meaning that Flo8 is promoted for binding to FLO11 instead of SFL1 and with the presence of citral the function of FLO11 could be depleted. For the DeltaSNF1 strain with the presence of citral, glycogen is low accumulated due to the absence of the gene, making it hard for activation of glucose repressed gene and repression of glucose induced genes.

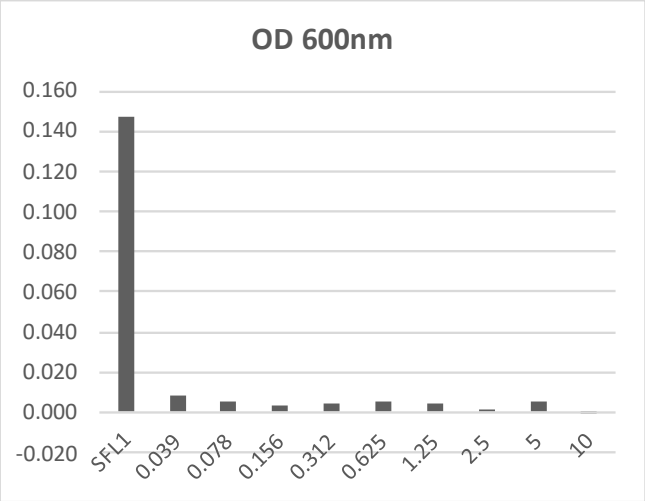

Figure 1 *Optical density of citral different concentration for the DeltaSFL1 strain with synthetic medium*

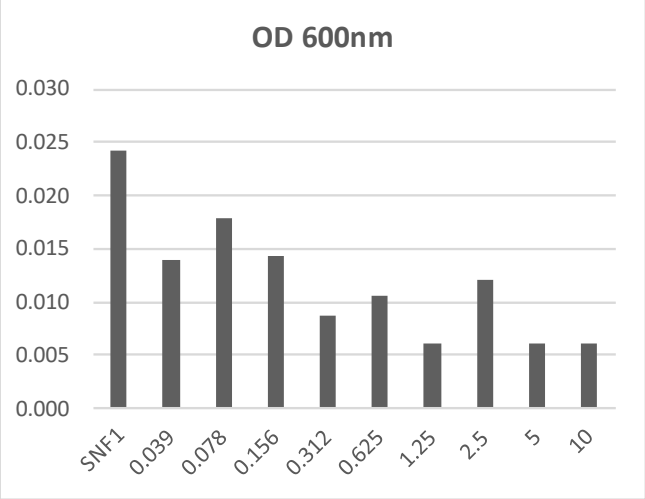

Figure 2 *Optical density of citral different concentration for the DeltaSNF1 strain with synthetic medium*

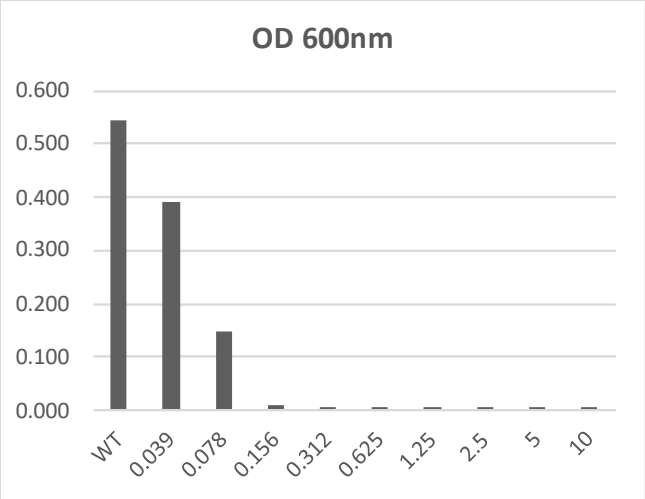

Figure 3 *Optical density of citral different concentration for the Wild Type strain with synthetic medium*

4. Conclusions and future perspectives

Citral showed anti-microbial effect on the mutants of Σ1278b. DeltaSFL1 and DeltaSNF1 are considered two interesting mutants to analyse since they were resistant to the component, meaning that the knockout of the genes is involved in the function; the non-function gives the resistance to the yeast to the citral. These mutants could be studied further depending on their specific role with correspondent conditions in the yeast and these results could be used as a model for eradicating biofilm formation, in addition to the study of other phenotype involved in different pathways of the yeast.

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The complexity of protein network in foods: insight in the protein structure in cereal products

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The macroscopic properties of food matrices and their characteristics are defined by the molecular events that involve their structure and by interactions among macromolecules in response to technological processes. This PhD work focuses on studying the molecular events responsible of the development of a protein network in cereal matrices: i.e., formation of the gluten network. Research activities are meant to shed-light on the (sometimes underrated) high level of complexity of the gluten network and to understand the connections between processes and molecular events or properties that provide a basis for gluten development.

La complessità dei network proteici negli alimenti: visione della struttura proteica nei prodotti cerealicoli

Le proprietà macroscopiche delle matrici alimentari e le loro caratteristiche sono definite dagli eventi molecolari che coinvolgono la struttura e le interazioni tra le macromolecole in risposta ad una trasformazione tecnologica. La presente tesi di dottorato è focalizzata sullo studio degli eventi molecolari responsabili dello sviluppo di un network proteico: i.e, la formazione del network glutinico. Le attività di ricerca sono volte a mettere in luce l'alto livello di complessità (a volte sottovalutato) del network glutinico e comprendere la correlazione tra il processo e gli eventi molecolari o le proprietà che sono alla base dello sviluppo del glutine.

Key words: protein network; gluten; cysteine thiols; fluorescent probe; nanoparticles.

1. Introduction

In accordance with previous research descriptions (Emide, 2021; 2020), the present work involves the following activities:
A1) labelling and mapping of protein thiols. The development of a protocol for the fluorescent labelling and mapping of *Triticum* spp. proteins thiols, in both non-destructuring (saline buffer) and destructuring (urea or surfactants) conditions;
A2) labelling accessible thiols with gold nanoparticles (AuNPs). Geometrical and spatial features of the network (i.e. mesh size of the network) will be investigated according to the physical accessibility to AuNPs, by exploiting their intrinsic reactivity toward thiols.
A3) from model to real systems. The application of the protocol developed in A1 on different raw materials (wheat lines or cultivars) and model systems (such as doughs prepared in a farinograph under controlled conditions, or model bread).

2. State of the art

Gluten is commonly defines as “the rubbery mass that is possible obtain by washing a wheat dough under a stream of running water in order to remove starch and the water-soluble components” (Wieser, 2007). The gluten network originates by the interaction among the insoluble storage proteins: gliadins and glutenins. From a physiological point of view, that’s proteins are deposited in the starchy endosperm inside the kernel (Peter R. Shewry et al., 2002) and represent the main nitrogen reserve for the germinating embryo (Bietz & Simpson, 1992). Gluten proteins account for 80% of wheat protein and are the largest contributor to wheat quality (D’Ovidio & Masci, 2004). Investigation of the structure of gluten proteins and of the resulting network, represent a big challenge for protein chemistry. A model of the geometrical structure of the gluten network was proposed by Belton (1999): the “loops” and “trains” model represent a milestone in the investigation focused on understanding the viscoelastic properties of the gluten network that is crucial for a lot of peculiar features of the foodstuff, like gas retention during dough proofing and in the final development of porous structure in baked products (Aguilera, 2019).
Many studies have addressed the possible correlation between protein and baking performance of different wheat cultivar (Bean et al., 1998). However, most of the approaches have some intrinsically tricky aspect, as quantification and identification of proteins extracted are paramount in every proteomics work. Although the sheer size and number of studies regarding wheat gluten, only a few addressed the structure/function relationship at the base of the molecular characteristics of raw materials, dough and final product.

3. Experimental approaches

In this PhD thesis the experimental approach consists in a step-by-step investigation, with a progressive increase in resolving power of the techniques and in the complexity of the system. The starting point was the characterization of the matrices under scrutiny (flour/semolina, dough and bread) with approaches largely described in literatures, i.e. differential solubility, quantification of accessible thiols (Iametti et al., 2006) and fluorescence-based approaches (Bonomi et al., 2004), to obtain a description of the system. Structural investigation was deepened by a thiolomic approach based on the fluorescent labelling of cysteine thiols. Rheological studies were conducted at side of the biochemical investigations of the matrices in order to compare and integrate the information from both the approach to fully characterize the matrices and better understand the molecular events at the base of protein structural evolutions and the formation of the network.

4. Materials and Methods

Three different studies, aimed at shedding light on different aspects of gluten formation, were conducted by using the fluorescent labelling protocol set-up during the first two years of the PhD (Emide, 2021; 2020). Each study required different raw materials. (1) Time-course characterization of model doughs, prepared by using two masterbatches of flour and semolina from *Triticum* spp. (courtesy of Molino Quaglia, Vighizzolo d'Este, Padova, Italy). (2) Characterization of a model bread, made by using a commercial flour purchased in the local supermarket. (3) Comparative description of three wheat near-isogenic lines, mutated to obtain different amylose contents (courtesy of Professor Francesco Sestili, DAFNE, University of Tuscia).
Characterization of the protein pattern of the different systems was obtained by suspending the samples in a saline buffer (50 mM sodium phosphate, 0.1M NaCl, pH 7.0), and performing the protein separation in SDS- and 2D-PAGE. Protein identification was achieved by comparing the Coomassie Brilliant Blue stained gels with the available literature (see, for example: Žilić et al., 2011; Dupont et al., 2008). Differential solubility studies and the titration of accessible thiols by 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) was performed according to Iametti et al. (2006) to obtain an overall description of the protein organization in the samples/system under scrutiny.
Protein labelling was realized by suspending the sample in saline buffer (50 mM sodium phosphate, 0.1M NaCl, pH 7.0), in the absence or in presence of different destructuring agents (urea or SDS, tested at various concentrations). The fluorescent probe 5-(iodoacetamido)fluorescein (5-IAF) was used for thiols labelling. The labeled proteins were separated by SDS-PAGE and 2D-PAGE. Fluorescent images of the gels were acquired by a VersaDoc™ Image Analysis System (Bio-Rad). Then, the gels were stained by traditional Coomassie Brilliant Blue to obtain a total protein map. The fluorescent image and the Coomassie stained gels were compared and analyzed through the ImageLab software Version 6.0.1 (Bio-Rad).

5. Results and Discussion

5.1 Set-up of the fluorescent labelling protocol and thiolomic approach

Common characterizations based on protein quantification can only provide limited information about the entire gluten system. The evolution of the protein structure and formation of intermolecular interactions drive the change from storage proteins to gluten, and play a key role for the characteristics – and quality – of the products. From this vision comes the necessity of a method suitable to perform three-dimensional investigation about the protein structure in complex systems, to investigate and understanding the structure-function relationship in food systems. The rationale of fluorescent labelling had been extensively described by Iametti et al. (2013) and Emide (2021; 2020). Although the method is described in literature (Gorman, 1987; Toseland, 2013), here a thiolomic approach was developed to investigate in detail protein structural characteristics and their geometrical features in the raw matrices as well as in the function of the transformation process. The thiolomic approach can be very useful for ruling out or highlighting differences in apparently similar matrices, such as different lines of flour or semolina. The combination of the thiolomics results and of common biochemical characterizations (e.g. differential solubility and DTNB titration), can therefore provide detailed information on the whole system.
Main goal of the thiolomic approach is to evaluate the hierarchy of the thiols accessibility and distribution in the protein structure. For these reason the labelling of gluten proteins with the fluorescent probe 5-IAF was performed under different destructuring condition obtained by using 4M urea (a non-polar chaotrope) and 2% (w/v) sodium dodecyl sulphate (SDS; an anionic surfactant). Thiols accessibility (i.e. fluorescent labelled proteins) is increased by the presence of urea and SDS, able to weak hydrophobic interactions. Interesting, urea and SDS show the same ability in solubilizing gluten proteins, but different properties as for allowing thiol accessibility (Figure 1).
Different concentrations of chaotrope and surfactant have been tested to find the best condition to obtain the solubilization and the labelling of thiols. We found 2M urea to be the lowest chaotrope concentration allowing full accessibility of thiols. In parallel, 2M urea is able to solubilize the totality of the gliadins and most of the glutenins, but increasing the concentration up to 3M improve the solubilization of the high-molecular weights glutenin fraction (HMW-GS). In the case of SDS, 0.2% w/v (i.e. the critical micellar concentration – CMC – at 20°C) is the threshold to promote protein solubilization, even if 0.5% SDS is needed to solubilize the totally of the gluten proteins. Moreover, increasing the temperature up to 60°C does not resulted in an increase of thiols accessibility.

At least, both chaotropes resulted effective in solubilizing gluten proteins. Nevertheless, SDS-PAGE and fluorescence imaging show differences in thiol accessibility, that is much higher in urea than in SDS. The developed thiolomics procedure comprises several phases in which the different classes of protein are eventually separated according to their solubility. We found necessary to remove albumins and globulins in a first step, after herein called "dealbuminization". Although albumins and globulins represent only the 15-20% of the total wheat proteins, they are rich in thiols. Thus, the dealbuminization step allow to obtain a clear and clean separation of the gluten proteins, that remain in the insoluble fraction after removal of albumins and globulins,.

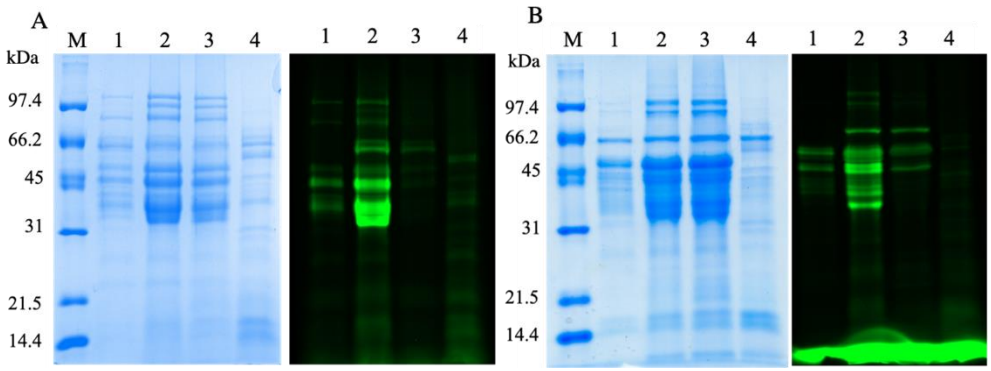

Figure 1 *Flour (panel A) and semolina (panel B) proteins labelled with 5-IAF. The protein map is blue-stained on the left, whereas the fluorescent images is green-staines on the right. M) molecular weight markers; 1) "gluten fraction" labelled under non-destructuring conditions; 2) "gluten fraction" labelled in urea 4M; 3) "gluten-fraction" labelled in SDS 2%; 4) "non-gluten fraction" labelled in non-destructuring conditions.*

5.2 Studying the three-dimensional evolution of protein network: the dough formation

Thiols-disulfide exchange represents a crucial step of the technological transformations of cereal products. Studying the geometrical evolution of the protein network represents a key point to obtain reliable evidences about the structure/function relationship of proteins and of the whole network. Of the flour and semolina used as reference, 50g were kneaded in a farinograph. During the kneading, samples were collected before and at the full development of the gluten, as well as in the overmix. Samples were lyophilized and subjected to solubility studied and thiolomic investigations (Figure 2).

Differential solubility (Figure 2, panel A) represent a simple tool to obtain preliminary information about the nature of the interactions that stabilize the protein aggregates. No changes in the solubility were observed at the different kneading times in all the tested condition (saline buffer, in presence of 4M urea and 50mM DTT when required) no major differences was shown by flour samples. Results described the expected behavior of the flour: a low solubility in saline buffer, where are more soluble the non-gluten proteins (albumin and globulin); but with the addition of the chaotrope and the reducent are observed in higher solubility of all the other proteins/aggregates. No major differences were observed in presence of urea in the flour and at 1 minute of kneading, while at the full development of the gluten (7 minute) is observed a decrement of the value. Interesting, the results obtained with the addition of the reducent don't show major differences from the raw flour and at all the kneading times. Semolina had proven to have the same behavior (data not shown).

The fluorescent labelling protocol was performed as previously described (Figure 2, panel B). No major differences were observed in the flour when samples were labeled in the absence of denaturant. On the contrary, semolina (data not presented) showed little differences in the region between 45 and 66.2 kDa, mainly attributable to gliadins, and around 97.4 kDa, mainly attributable to high molecular weight glutenin (HMW-GS), where a band is no longer observed after 30 min kneading.

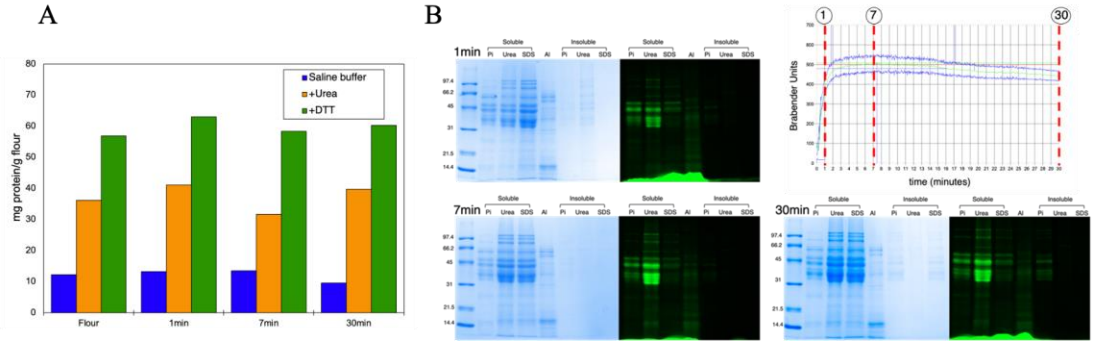

Figure 2 *Panel A) Differential solubility of the dough made with the farinograph. Panel B) thilomic characterization of the dough at the different kneading time.*

From the results is observed an apparent negligible role of thiols-disulfide reaction in the development of the gluten network under the tested conditions. Future study will be aimed at extend the rheological investigation also in function the role of the temperature, by producing standard dough in apparatus that involve also a heating step.

5.3 Studying the three-dimensional evolution in a product: the bread case

The effect of baking on the protein network was tested on a bakery product: a model bread made by using a commercial flour in a home bread maker. An aliquot of dough was collected before the baking step. The dough and the final bread were lyophilized prior to perform the analysis.
The thiolomic approach reported in Figure 3 (Panel A) allows to show some differences of thiol accessibility in the gliadin fraction (45 to 66.2 kDa). In detail, a band at about 66.2 kDa looks to be less fluorescent in the bread compared to the flour and the dough. All this information cannot be provided by the classical biochemical characterization (i.e. solubility and titration of thiols accessibility), showing all the power and the high level of resolution of our approach. Results clearly show that the baking step plays a major role on the evolution of the protein-protein interaction in the network, pushing up disulphide interactions.
The results about the differential solubility are reported in Figure 3 (panel b): they show a fundamental role of the disulphide interactions in the stabilization of the proteins aggregate in the bread. Whereas the protein network in the dough seem to be stabilized prevalently by hydrophobic interactions, as figure out from the previous results, after the baking process the intermolecular disulphide interactions significantly increase resulting in the protein network characteristics of the bread. The effect of the formulations (i.e. the addition of other ingredients like lipid and simple sugar) and the impact of technological transformations are currently under scrutiny.

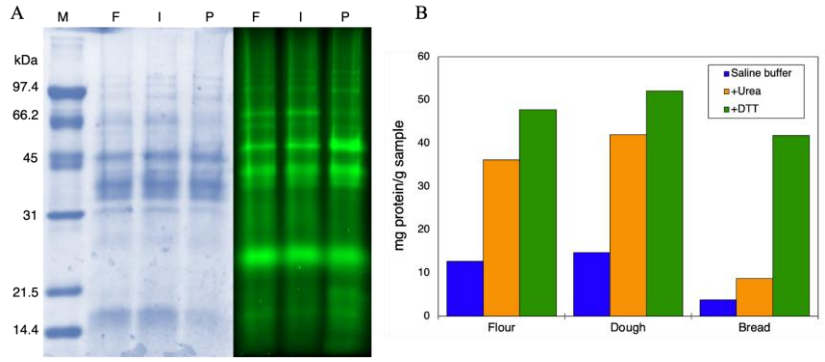

Figure 3 *Panel A) Thiolomic approach applied on flour (1), dough (2) and bread (3) labelled with 5-IAF. The protein map is blue-stained on the left, whereas the fluorescent images is green-staines on the right. Panel B) Differential solubility of the flour, urea and bread samples.*

5.4 Thiolomic description of cereal system: structural difference

The thiolomic approach was applied to rule out differences in three near-isogenic lines of wheat, muted to increase (amilostarch) or to virtually annul (waxy) the content of amylose (Emide 2021). SDS- and 2D-PAGE, combined with the thiolomic characterization (adequate for the specific sample under scrutiny), proved to be a fundamental tool to better understand the role of the starch fraction on the technological properties of virtually identical gluten proteins. Extending these studies on doughs obtained under various operating conditions and on the final products is expected to improve the current view of the molecular basis of protein behavior in these systems. Ongoing collaborative efforts will also help to clarify to what extent this type of molecular-based information may also be useful to breeders and farmers.

6. Conclusions and Future Perspectives

A central point of the work is the geometrical description of the cereal systems: the evolution of the characteristics of the matrices from the dry state of the flour/semolina until the final product (a model bread). The main concept stressed out is that the protein content is not the only important parameter to evaluate the features of protein network. Therefore, for a deep knowledge of the protein network and its impact on the characteristics of finished products, is of paramount importance to study at molecular level the interactions among proteins and the other components of the matrices (e.g. starch, lipids, etc.).
New methodologies are required to study gluten proteins overcoming the difficulties related to the intrinsic physical and chemical properties of the storage wheat protein (insoluble, intrinsically disordered and inserted in a complex matrix). The thiolomic approach had proven to be a very powerful tool in the study of the molecular events at the base of the development of the gluten network, understanding also the role of the technological transformations. The presented results are referred only to the development and application of the fluorescent

labelling protocol. As a future perspective, the fluorescent labelling will be enhanced by exploiting the reactivity of AuNPs with thiols. AuNPs make it possible to gain information on the "geometrical" accessibility of thiols in proteins that are involved in the formation of the network, and use of AuNPs of different size (from 5 to 200 nm) should allow to analyze the accessibility of thiols as a function of the compactness and of the geometrical features of the protein network. Proteins "fished" by AuNPs have been identified - in preliminary feasibility studies - by mass spectrometry after removal of non-covalently absorbed proteins and release of fragments by proteomics-grade trypsin (Marengo et al., 2019).
The overall results will be a complete characterization of the protein network under the molecular and geometrical point of view. These descriptions are of paramount importance in order to understand and explain the macroscopic characteristics of the finished products in terms of the molecular events that affect the structural properties of the network and of the food products.

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Processing and innovation in the Neapolitan pizza manufacturing

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This PhD thesis was aimed at filling a gap in the scientific knowledge of the phenomena that occur during the cooking phase of the Neapolitan pizza in the traditional wood oven. The activities carried out concerned the collection of data useful for modelling the physical processes that occur during the cooking of Neapolitan pizza.

La Pizza Napoletana: processi ed innovazioni

Questo lavoro di tesi ha avuto come obbiettivo quello di colmare una lacuna sulla conoscenza scientifica dei fenomeni che intervengono durante la fase di cottura della pizza napoletana in un tradizionale forno a legna. Le attività svolte hanno riguardato la raccolta di dati utili per la modellazione dei processi fisici che avvengono durante la cottura della pizza napoletana.

Key words: Neapolitan pizza, wood-fired oven, thermal profile, water vapor transport rate, baking

1. Introduction

In accordance with the PhD thesis project previously described (financial support of MIUR: PRIN 2017-2017SFTX3Y) this oral communication reports the main results of the following four activities concerning:
A1) Start-up procedure and evaluation of the wood-fired pizza oven;
A2) Mapping of the thermal profile of pizza sample during cooking;
A3) Water vapor transport rate of pizza samples as a function of cooking time;
A4) Evaluation of the degree of browning-burning during cooking time.

2. The Neapolitan pizza

Neapolitan pizza is one of the most popular products of Italian gastronomy. Its spread around the world has led to the development of numerous variants of the original technology, adapting the process to different consumer tastes and processing techniques compatible with regulations in force in various regions and countries. Although different, the different ways the preparation of the pizza is based on a few steps: the preparation of the dough and its leavening, the lamination of the dough obtained, the garnish and cooking in wood-fired oven. The way in which these operations performed distinguish the Neapolitan pizza from the others version.
To protect the art of making pizza at "Neapolitan style", the European Commission Regulation no. 97/2010 (EC, 2010) entered the name Pizza Napoletana in the register of traditional specialities guaranteed (TSG) of Class 2.3 (Confectionery, bread, pastry, cakes, biscuits, and other baker's wares) to define and thus preserve its original characteristics, and in 2017, the United Nations Education, Scientific and Cultural Organization (UNESCO) inscribed the art of the Neapolitan pizza maker (Pizzaiuolo) on the Representative List of the Intangible Cultural Heritage of Humanity (UNESCO, 2017).

3. Pizza Baking

The production specification requires only the use of wood-fired ovens for cooking Neapolitan pizza. Such ovens generally consist of a base of tuff and fire brick covered by a circular cooking floor over which is built a dome made of refractory materials to minimize heat dispersion. Their geometric dimensions allow the temperature of the cooking floor and vault to be kept at about 430 °C and 485 °C, guaranteeing the Neapolitan pizza cooking speed and typical character characterized by a raised rim with very thin crust and irregular cooking, soft to the cut, with the typical flavor of well-cooked bread, and a central part finely alveolar soft, elastic, easily foldable with possible sporadic bubbles, more or less scorched, in the parts not covered by the garnish.
The heat transfer during the cooking process of a wood-burning oven involves several mechanisms of heat transport at the same time. During the start-up phase, the combustion of the wood in the rear part of the oven allows the transfer of heat to the refractory bricks which are brought to the operating temperature. Heat is transmitted from the flame to the bricks essentially through two mechanisms: radiation and conduction.
During operation, combustion is slowed down and regulated to balance the heat dispersed in the environment and that absorbed by the pizza during cooking in order to maintain the temperature profile inside the oven constant over time. As regards the heat supplied by the oven to the pizza being cooked, it is transferred by conduction through the contact surface between the floor and the pizza, while by radiation and natural convection to the parts of the pizza not in direct contact with the oven floor.

The thermal power transmitted by conduction from the floor to the pizza, depends on the temperature difference between the floor and the base of the pizza, as well as on the thermal properties of the dough.
The power transmitted by radiation from the top of the oven to the top surface of the pizza will depend on the geometric characteristics of the oven, the properties (emissivity) of the construction materials, the geometry and characteristics (emissivity) of the surface of the pizza, as well as the temperatures of the top surface of the oven and the surface of the pizza. Finally, the heat transmitted by convection will depend on the temperatures of the surface of the pizza and the surrounding air and on the convective transmission coefficient which depends on the properties of the air in contact with the exposed surface of the pizza.
All these mechanisms evolve in transitory conditions since the temperatures of the pizza, the floor and the air in contact with the surface change significantly during cooking.
From this brief analysis the cooking process is not linked to the way in which the total energy provided to the oven but rather to the temperature profile that is established in the oven during the cooking of the pizza.
The use of wood-fired ovens is, on one side, a prerequisite for assuring the main sensory characteristics of the Neapolitan pizza, on other side, it is the Achilles' heel of this food product because the wood burning is a significant source of air pollutants (carbon monoxide, polycyclic aromatic hydrocarbons, sulfur dioxide, nitrogen oxide, black carbon, and particulate matter, PM).
In fact, the use of the wood-fired oven has been banned in many cities and countries, and in these circumstances, the Associazione Verace Pizza Napoletana would allow the use of an alternative oven, such as the so-called Scugnizzo Napoletano electric oven (Izzo Forni, Naples, Italy), since this oven succeeded in a series of physical and sensory tests. Nevertheless, many traditionalists and especially the members of another opposing Association (Associazione Pizzaioli Napoletani) were skeptical about this type of oven and disapprove its use because it did not meet the general requirements (Falciano et al., 2022_a).
An adequate modeling of the heat transmission phenomena that govern the rapid cooking of pizzas could generate the creation of types of ovens capable of provide the same thermal power transmitted by traditional wood-fired ovens with a lower environmental impact, with less production of combustion fumes and more in compliance with the safety standards which in some territories prohibit the use of this type of oven.

4. Experimental Plan

In this PhD thesis an experimental procedure was set up by carrying out the following tests in sequence, that is: study of the start-up procedure of a newly built wood-fired pizza oven, evaluation of the preheating time necessary for cooking the pizza samples, mapping of the thermal profile of the pizza samples during the cooking phase in pseudo-stationary oven conditions , study of the mass transfer dynamics of the pizza samples as a function of the cooking time, evaluation of the degree of browning-burning of the pizza samples during the cooking time.

5. Materials and Methods

An oven was used which had the oven chamber can be approximated to a cylinder, having diameter and height of 90 cm and 20 cm, respectively, surmounted by an oblate ellipsoidal vault of the same height and had a semicircular open mouth, its diameter and height being equal to 44 and 22 cm, respectively (Fornace Saputo Srl, Casapulla, Caserta, Italy). A thermal imaging camera was used for temperature monitoring (FLIR E95 42 °, FLIR System OU, Estonia). The pizza dough was prepared as described by Falciano et al. (2022_b) and were cooked for 80-100 s. Image analysis of the samples was performed using the electronic eye (visual analyzer 400 IRIS, Alpha M.O.S., Toulouse, France).

6. Results and Discussion

6.1 Start-up procedure of the wood-fired pizza oven

A correct start-up procedure is aimed at controlling the intensity of the thermal reactions that occur during the firing of the refractory bricks installed inside the wood-fired pizza oven in the studio. In clayey materials, these reactions can be endothermic (due to the process of dehydration, change in the crystalline phase or destruction of the reticular structure) or exothermic (due to oxidation or the formation of a new crystalline phase) (Grim and Johns, 1951). The lattice water loss from clay mineral components can be abrupt, so the rate of heating must be controlled to limit structural change and cause little or no brick cracking.
In this case, as suggested by the manufacturer of the oven, the oven was cooked at a rate of 1 kg of wood every 20 min for 1 hour on the first day, for 2 hours on the second day, for 4 hours on the third day and for about 8 hours on the fourth. Subsequently this procedure was repeated in order to evaluate the time required for preheating the oven for cooking the pizza.
Fig. 1 shows the temporal trend of the temperatures of the vault of the oven (T_V) and of the floor (T_{FL}) during the cooking procedures. A strong increase in both temperatures can be noted as a consequence of the heat released by the combustion of the logs. Furthermore, since the heating time during each phase was lengthened from 1 h to about 8 h, the initial values of T_V and T_{FL} tended to increase progressively thanks to the low thermal dispersibility of the insulated walls of the furnace. Furthermore, from the results we can deduce that 6 hours of preheating are

ideal for cooking pizza, therefore all the cooking tests of the samples were carried out after 6 hours of preheating (Falciano et al., 2022$_c$).

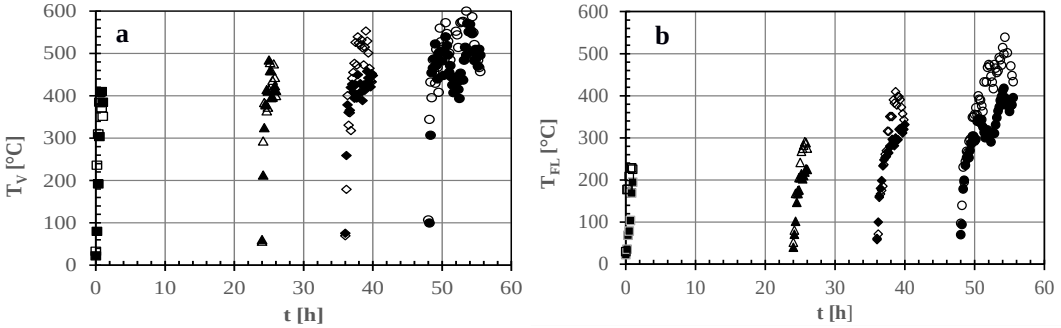

Figure 1 *Time (t) course of the oven vault (T_V: **a**) and floor (T_{FL}: **b**) temperatures as measured using a thermal imaging camera during the first start-up procedure (closed symbols) and the repeated one a week later (open symbols): ■, □, day 1; ▲,△, day 2; ◆, ◇, day 3; ●, ○, day 4.*

6.2 Mapping of the thermal profile during cooking

Variables in heat distribution are the cause of differences in pizza quality. On the one hand, there are technical variables, such as the design of the oven, the materials used, etc. and on the other hand there are process variables such as temperature and cooking time which must be synchronized to obtain the desired quality, and it is assumed that the process is carefully controlled in order to meet these requirements (Manhiça, 2014), since the uneven temperature distribution and random perturbations in the oven often lead the dough to undergo inconsistent heat treatment (Wong et al., 2007). The cooking of the samples was performed under almost stationary oven conditions after 6 hours of preheating and a floor temperature of approximately 442 °C.

Table 1 *Values (mean ± sd) of six repeated baking tests performed in a wood-fired pizza oven fed with 3 kg/h of oak logs using five different pizza samples: effect of time (t) on the temperature of the oven floor exposed to fire (T_{FL}) or shielded by the pizza sample (T_{FLbp}), temperatures of the pizza rim (T_{SR}), upper (T_{SU}) and lower (T_{SL}) areas.*

t (s)	T_{FL} (°C)	TFLbp (°C)	T_{SR} (°C)		T_{SU} (°C)		T_{SL} (°C)
Pizza not garnished (A)							
0	442 ± 9 ^{a}	442 ± 9 ^{a}	21 ± 0.1 ^{a}		21 ± 0.1 ^{a}		21 ± 0.1 ^{a}
20	441 ± 7 ^{a}	363 ±10 ^{b}	80 ± 3 ^{b}		103 ± 2 ^{b}		84 ± 2 ^{b}
40	436 ±11 ^{a}	348 ± 5 ^{b}	116 ± 3 ^{c}		138 ± 7 ^{c}		97 ± 2 ^{c}
60	435 ± 7 ^{a}	332 ± 7 ^{c}	130 ± 6 ^{d}		157 ± 6 ^{d}		102 ± 2 ^{d}
80	432 ± 9 ^{a}	325 ± 5 ^{c}	148 ± 9 ^{e}		182 ± 9 ^{e}		106 ± 3 ^{d}
				Tomato area	**Oil area**	**Mozzarella area**	
Pizza garnished with 70 g tomato puree (B)							
0	443 ± 8 ^{a}	440 ± 7 ^{a}	21 ± 0.1 ^{a}	21 ± 0.1 ^{a}			21 ± 0.1 ^{a}
20	442 ± 7 ^{a}	339 ±10 ^{b}	83 ± 2 ^{b}	59 ± 2 ^{b}			75 ± 2 ^{b}
40	439 ± 7 ^{a}	328 ± 6 ^{b}	113 ± 4 ^{c}	71 ± 2 ^{c}			92± 3 ^{c}
60	438 ± 8 ^{a}	320 ±10 ^{b,c}	124 ± 3 ^{d}	76 ± 2 ^{d}			96 ± 2 ^{c}
80	436 ± 6 ^{a}	304 ± 5 ^{c}	136 ± 3 ^{e}	81 ± 2 ^{e}			101 ± 2 ^{d}
Pizza garnished with 30 g sunflower oil (C)							
0	446 ± 5 ^{a}	448 ± 7 ^{a}	21 ± 0.1 ^{a}		21 ± 0.1 ^{a}		21 ± 0.1 ^{a}
20	443 ± 6 ^{a}	351 ±11 ^{b}	86 ± 3 ^{b}		100 ± 3 ^{b}		81 ± 2 ^{b}
40	441 ± 7 ^{a}	342 ± 9 ^{b}	116 ± 7 ^{c}		128 ± 6 ^{c}		93± 5 ^{c}
60	439 ±11 ^{a}	327 ± 7 ^{c}	149 ± 7 ^{d}		148 ± 5 ^{d}		101 ± 3 ^{d}
80	434 ± 8 ^{a}	314 ± 7 ^{b,c}	169 ± 9 ^{e}		156 ± 4 ^{d}		105 ± 2 ^{d}
Pizza garnished with 70 g tomato puree and 30 g sunflower oil (D)							
0	440 ± 7 ^{a}	438 ±10 ^{a}	21 ± 0.1 ^{a}	21 ± 0.1 ^{a}	21 ± 0.1 ^{a}		21 ± 0.1 ^{a}
20	438 ± 5 ^{a}	332 ±12 ^{b}	88 ± 3 ^{b}	61 ± 3 ^{b}	89 ± 5 ^{b}		74 ± 3 ^{b}
40	437 ± 7 ^{a}	318 ± 5 ^{b,c}	115 ± 5 ^{c}	73 ± 2 ^{c}	100 ± 4 ^{c}		87 ± 2 ^{c}
60	437 ± 6 ^{a}	313 ± 7 ^{b,c}	128 ± 5 ^{d}	79 ± 2 ^{d}	103 ± 2 ^{c}		93 ± 2 ^{d}
80	436 ± 6 ^{a}	309 ± 7 ^{c}	141 ± 2 ^{e}	84 ± 2 ^{e}	106 ± 2 ^{c}		102 ± 2 ^{e}
Pizza garnished with 70 g tomato puree, 30 g sunflower oil and 80 g mozzarella cheese (E)							
0	442 ± 9 ^{a}	437 ±12 ^{a}	21 ± 0.1 ^{a}	21 ± 0.1 ^{a}	21 ± 0.1 ^{a}	15 ± 0.1 ^{a}	21 ± 0.1 ^{a}
40	439 ± 4 ^{a}	336 ±10 ^{b}	98 ± 3 ^{b}	63 ± 2 ^{b}	92 ± 4 ^{b}	51 ± 2 ^{b}	74 ± 2 ^{b}
60	438 ± 7 ^{a}	325 ± 6 ^{b,c}	113 ± 3 ^{c}	73 ± 2 ^{c}	98 ± 3 ^{c}	55 ± 2 ^{c}	86 ± 2 ^{c}
80	436 ± 6 ^{a}	314 ± 7 ^{b,c}	130 ± 5 ^{d}	77 ± 3 ^{d}	101 ± 2 ^{c}	59 ± 1 ^{d}	92 ± 2 ^{d}
100	436 ± 5 ^{a}	307 ± 6 ^{c}	155 ± 5 ^{e}	87 ± 2 ^{e}	110 ± 32 ^{d}	67 ± 2 ^{e}	106 ± 3 ^{e}

The table 1 show the mapping of the samples temperatures during the cooking phase detected using the FLIR thermal imaging camera, and for each sample the following points were detected: floor exposed to fire (T_{FL}) or shielded by the pizza sample (T_{FLbp}), temperatures of the pizza rim (T_{SR}), upper (T_{SU}) and lower (T_{SL}) areas.
During the cooking of the samples, the wood-fired oven behaved in almost quasi-steady state conditions, its floor temperature showing no statistically significant variation around 437 ± 5 °C. The temperature of the upper central areas of the samples of pizza topped with sunflower oil tended to the boiling point of sunflower oil at room pressure, while those seasoned with tomato increased to a value well below the boiling point of water, that is 82-84 °C. The lower areas of each sample in direct contact with the oven floor has rapidly reached a temperature higher (105-106 °C) or lower (101-102 °C) than the boiling point of water depending on its lower or higher moisture content, respectively. Sample E with the same cooking time (80 s) compared to the other samples, has a lower oil and tomato temperature due to the presence of mozzarella which slows down the transfer dynamics, in fact the finally cooking was at 100 s.

6.3 Determination of the water vapor transport rate

The water vapor transport properties change according to the quantity of water contained in the sample, the size, the nature of the sample itself and its constituents and are directly influenced by changes in temperature and heating rate during the cooking process (Manhiça, 2014).
Figure 2 shows the water evaporation rate (%) of 5 pizza samples during the cooking at different times.
The sample C, garnished with sunflower oil, shows greater weight loss than all other samples at the end of cooking. This result is explained by the physical characteristics of sunflower oil which has a low specific heat and reaches higher temperatures during cooking, has a high thermal conductivity, favoring a fast transmission of heat to the pizza dough and a rapid evaporation of the water.
The lowest values are found in sample E garnished with tomato, sunflower oil and mozzarella cheese, followed by samples B and D, whose thermal conductivity properties are strongly influenced by the amount of water present in the garnish ingredients, which is the main constituent, slowing the transmission of heat to the pizza dough with a consequent slowdown in the transport of water vapor.
The evaporation rate of sample A has a value among the previous ones, as it is not influenced by other topping materials, and depends exclusively on the properties of the dough itself, which has a specific heat greater than oil and lower than tomato and mozzarella cheese.

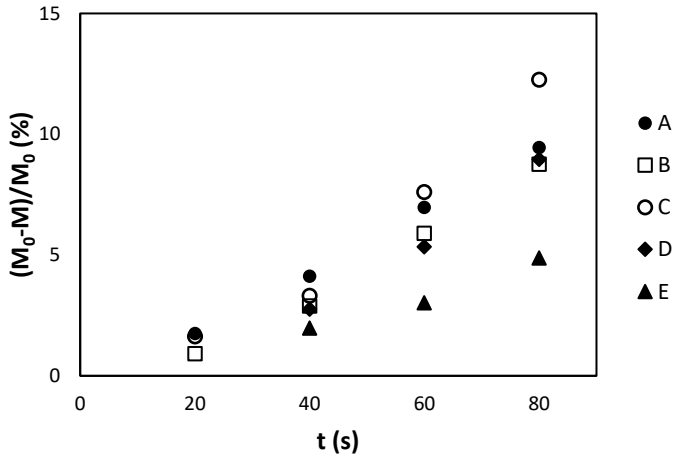

Figure 2 *Water vapor transport rate (%) of pizza samples cooked in a wood-oven as a function of time: A, pizza not garnished (●); B, pizza garnished with 70 g tomato puree (□); C, pizza garnished with 30 g sunflower oil (○); D, pizza garnished with 70 g tomato puree and 30 g sunflower oil (◆); E, pizza garnished with 70 g tomato puree, 30 g sunflower oil and 80 g mozzarella cheese (▲). Each value is expressed as mean ± SD (n = 6).*

6.4 Image analysis: browning/burning

Image analysis was evaluated using the IRIS electronic eye, a machine vision technology that transforms images into digital images. Images are processed as a spectrum of colors on a maximum scale of 4096 colors, with the area of each significant color marked as a percentage. This approach is very useful for detecting the color change of a product related to the kinetic development of phenomena that occur during the phases of a process, such as cooking. By evaluating the individual areas representing browning and burning, it was possible to associate the individual colors with clusters in order to tabulate the degree of browning and burning. Table 2 shows the percentage evolution of the two color groups, referring respectively to the browning and burning of the upper and lower areas of the pizza samples. The degree of browning-burning of the lower areas has very low percentages compared to the upper areas. Sample A shows a higher degree of browning-burning since the analyzed surface is greater. Samples B and D have a slightly higher degree of browning-burning than samples C, this may be due to the more uniform distribution of the tomato during the filling phase, which allows a more homogeneous heat transfer along the entire surface of the rim. At the same time (80 s) samples E has a lower degree of browning / burning than the other samples, probably due to mozzarella slowing down the transfer of heat and mass. The degree of browning and burning of the lower areas is higher in samples B, D and E, which being of higher mass, favor greater adhesion to the hob thus allowing a more homogeneous heat conduction along the surface.

Table 2 *Evolution of the degree of browning / burning (%) of the upper and lower area of the pizza samples during cooking as a function of time: A, pizza not garnished; B, pizza garnished with 70 g tomato puree; C, pizza garnished with 30 g sunflower oil; D, pizza garnished with 70 g tomato puree and 30 g sunflower oil; E, pizza garnished with 70 g tomato puree, 30 g sunflower oil and 80 g mozzarella cheese. Each value is expressed as mean ± SD (n = 6).*

t s)	Browning area (%)					Burning area (%)				
	A	B	C	D	E	A	B	C	D	E
	upper areas of the pizza									
20	0,01±0,0	2,46±1,0	0,00±0,0			0,00±0,0	0,13±0,2	0,00±0,0		
40	0,26±0,2	3,14±1,2	0,07±0,1	1,92±0,3	0,08±0,1	0,03±0,1	0,54±0,3	0,00±0,0	0,00±0,0	0,00±0,0
60	4,70±1,0	8,15±2,0	2,05±1,5	4,26±0,5	0,28±0,1	0,38±0,1	1,65±0,8	0,79±1,7	0,09±0,1	0,17±0,0
80	26,16±5	10,98±2	8,61±1,6	10,67±5	2,25±0,1	7,85±6,4	2,92±0,1	1,38±1,1	3,16±2,0	0.62±0,1
100					7,31±0,3					3,95±0,3
	lower areas of the pizza									
20	0,00±0,0	0,32±0,3	0,09±0,1			0,00±0,0	0,04±0,0	0,00±0,0		
40	0,03±0,0	1,04±0,4	0,20±0,3	1,28±0,9	0,13±0,0	0,00±0,0	0,13±0,1	0,00±0,0	0,05±0,0	0,04±0,0
60	0,06±0,1	4,72±1,7	0,11±0,5	1,37±1,4	0,40±0,0	0,00±0,0	0,56±0,1	0,00±0,0	0,07±0,0	0,14±0,0
80	1,39±1,2	7,61±1,2	0,32±0,2	5,91±1,0	1,65±0,1	0,01±0,0	0,87±0,4	0,00±0,0	0,37±0,2	0,80±0,1
100					2,81±0,1					0,94±0,1

7. Conclusions and Future Perspectives

In this work the heat and mass transfer dynamics of a wood-fired pizza oven on a pilot scale such as those commonly used in Neapolitan pizzerias in Italy were evaluated, thanks to the aid of a FLIR thermal imaging camera. First, its start-up procedure was performed, and it was investigated how the furnace can be put in pseudo-stationary conditions. Secondly, a mapping of the temperatures of five differently pizza samples was made during cooking, in pseudo-stationary oven conditions and the mass of evaporated water was quantized.

The results of the investigation show that the dynamics of heat and mass transfer are influenced by the mass and physico-chemical properties of the ingredients used for the garnish, significantly affecting the cooking process. The oil used for the gasket, for example, being by its nature a good conductor, accelerates the heat exchanges with the surrounding materials, thus speeding up the evaporation of water during cooking. On the contrary, tomato or mozzarella, being an aqueous matrix, require more heat for evaporation and, under the same cooking conditions, it slows down the process. Finally, the degree of browning and burning was assessed by analyzing the lower and upper areas of the pizza samples.

The results show that the chromatic evolution is related to the physico-chemical properties of the sample, to the mass of the sample and to the distribution of heat. Knowledge of the dynamics of heat and mass transfer during the pizza cooking phase can be of significant importance for the modeling and development of new cooking technologies.

8. Nomenclature

T_{FL}, temperature oven floor exposed to fire (°C); T_{FLbp}, temperature oven floor shielded by the pizza (°C); T_{SR}, temperature of the pizza rim (°C); T_{SU}, temperature upper pizza areas (°C); T_{SL}, temperature lower pizza areas (°C); T_v, temperature oven vault (C°); M_0, initial weight (g); M, final weight (g).

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Isolation and characterization of new strains of *Akkermansia muciniphila* with a focus on antibiotic-resistance phenotypic and genotypic traits

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Akkermansia muciniphila is a commensal bacterium commonly found in healthy gut microbiota. The type strain of the species, ATCC BAA-835 has recently been approved as novel food. Despite its recognized health benefits, according to EFSA (2020), this species is not recommended for QPS status due to safety concern, mainly related to the ability to degrade mucin and to the presence of several antimicrobial resistance genes found in the 39 analyzed genomes. However, information on the antimicrobials susceptibility profile to this species is still lacking. The aim of the present study was the isolation and identification of new strains of *A. muciniphila*, to characterize their AMR profile, integrating phenotypic and *in silico* methods.

Isolamento e caratterizzazione di nuovi ceppi della specie *Akkermansia muciniphila* con focus sui tratti fenotipici e genotipici di antibiotico-resistenza

Akkermansia muciniphila è un batterio commensale che si trova comunemente nel microbiota intestinale sano. Il ceppo tipo della specie, ATCC BAA-835, è stato recentemente approvato come nuovo alimento. Nonostante i suoi riconosciuti benefici per la salute, secondo l'EFSA (2020), questa specie non è raccomandabile per lo stato QPS a causa di problemi di sicurezza, principalmente legati alla capacità di degradare la mucina e alla presenza di diversi geni di resistenza agli antimicrobici trovati nei 39 genomi analizzati. Tuttavia, mancano ancora informazioni sul profilo di suscettibilità agli antimicrobici di questa specie. Lo scopo del presente studio è stato l'isolamento e l'identificazione di nuovi ceppi di *A. muciniphila*, per caratterizzarne il profilo AMR, integrando metodi fenotipici e *in silico*.

1. Introduction

Akkermansia muciniphila is a commensal bacterium that accounts for 1-5% of human intestinal microbiota. The presence of this bacterium is commonly associated with a healthier status in humans. The type strain, *A. muciniphila* MucT (=DSM 22959; =ATCC BAA-835), has been extensively studied and is considered a next-generation beneficial bacterium (Cani et al. 2022). Although this species has not yet been granted qualified presumption of safety (QPS) status (EFSA 2020), recently the EFSA panel has declared that pasteurized *A. muciniphila* strain MucT can be considered safe as a novel food, opening the door to its commercialization as a food supplement (EFSA 2021). *A. muciniphila,* along with other health-associated gut bacteria, represent a new possible weapon against diet-related disorders and inflammation. However, these microorganisms are posing new challenges. A crucial aspect is the correct assessment of their safety. In Europe, information on antimicrobial resistance for bacteria deliberately introduced into the food chain is of paramount importance to declare a microorganism safe for human and animal consumption. For this purpose, phenotypic testing, together with the search of the whole genome for the presence of known antimicrobial resistance genes, should be performed (EFSA 2018). Nevertheless, until now these approaches have not yet been widely implemented for this new class of microorganisms. There is a lack of clear and univocal protocol for the evaluation of the antibiotic susceptibility of these species and indications for microbiological cut-off values to be used to distinguish resistant strains from susceptible strains. With the introduction of *A. muciniphila* in the food chain, the evaluation of the antimicrobial susceptibility of this bacterium becomes fundamental to not spreading anti-drug resistances in the gut bacterial population. However, only a few studies have described the antibiotic resistance profile of *A. muciniphila* through phenotypic tests, while most of the available information derives from genomic data. In this context, the aim of the present study was to characterize the AMR profile of newly isolated strains of *A. muciniphila*, by integrating phenotypic and *in silico* methods. The results of our study provide new insight into the antibiotic-resistance phenotypic and genotypic traits of this new promising species.

2. Materials and Methods

2.1 Bacterial strains and culture conditions

A. muciniphila DSM 22959 has been purchased by DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany). The other 5 strains (*A. muciniphila* Sap1, AmaP1, Vtp7, Rcp22, Amup9), were isolated from healthy donors (Filardi 2021). All strains of *A. muciniphila* were grown at 37 °C in an anaerobic chamber (N2:H2:CO2 90:5:5) for 48 h in yeast medium broth (YM). YM contained (l-1): 0.45 g KH2PO4; 0.45 g K2HPO4; 0.9 g (NH4)2SO4; 0.9 g NaCl; 0.1 g MgSO4; 0.1 g CaCl2; 4 g NaHCO3; 1.9 ml CH3COOH (35 mM); 1 g L-Cysteine HCl monohydrate; 2.5 g yeast peptone; 5 g yeast extract and 2.5 g glucose and, it has been further

supplemented with 6 g L-threonine and 2.8 g N-acetylglucosamine. Antibiotic sensitivity was determined using Iso-Sensitest broth (Oxoid) supplemented with (l-1): 0.5 g L-Cysteine HCl monohydrate, 0.01 g Hemin, 0.01 g Vitamin K1, 6 g L-threonine and 2.8 g N-acetylglucosamine. *Escherichia coli* Nissle 1917 (serotype O6:K5:H1) was isolated from the probiotic product ECN (Cadigroup Farmaceutici) in Luria-Bertani (LB) agar plates. For the determination of the minimum inhibitory concentrations (MICs) of selected antimicrobials, *E. coli* Nissle 1917 was cultivated in cation-adjusted Muller-Hilton broth (caMHB) or sIST broth, under aerobic conditions at 37 °C for 24h.

2.2 Whole-genome sequencing and genomic analysis

The genomes of the new *A. muciniphila* isolates were sequenced by Illumina NovaSeq™ 6000 sequencing system (GENEWIZ). *De novo* assembling was performed by the assembly toolkit SPAdes 3.14.1 (Bankevich et al. 2012). The software fastANI (Jain et al. 2018) was used to compare by alignment-free computation of whole-genome Average Nucleotide Identity, the similarity between our draft genomes with 188 *Akkermansia* sp. complete genomes available on http://cmprod1.cibio.unitn.it/akkermansia_genomes/fna/ (Karcher et al. 2021). The CheckM software was used for assessing the quality (completeness and contamination) of the assemblies (Parks et al. 2015). The pangenome analyses was performed using roary (Page et al. 2015). Resistance Gene Identifier (RGI) on Comprehensive Antibiotic Resistance Database (CARD) (Alcock et al. 2020), was used to predict the resistome of our strains based on homology and SNP models. The Mobile Element Finder Tool (Johansson et al. 2020) was used to predict mobile genetic elements (MGEs) in our genomes by aligning the assembled contiguous sequences to reference sequences of previously known elements.

2.3 Total cell count and viability assessment by flow cytometry

Total cell counting and viability assessment of the bacterial cultures were performed by flow cytometry (FC), following the ISO 19344 procedure (2015), with some modifications. Briefly, bacterial samples were diluted (approximately 1×10^6 cells ml-1) in filtered phosphate-buffered saline (PBS) pH 7.4 to maintain an events rate in the flow lower than 2000 events s-1. The samples were stained with 0.1 µM SYTO™ 24 (Thermo Scientific) and 0.2 µM propidium iodide (PI; Sigma) and incubated in the dark at 37 °C for 15 min. FC analysis was performed with a C6 Plus flow cytometer (BD Biosciences, Milan Italy) with thresholds FSC-H 1000 and SSC-H 1000. All parameters were collected as logarithmic signals. Green (SYTO™ 24) and red (PI) fluorescence were detected in the FL1 (excitation 488 nm, emission filter 530/30) and FL3 (excitation 488 nm, emission filter 670 LP) channels, respectively. Electronic gates on the SYTO24/PI density plot were used to select and measure the total bacterial concentration (events ml-1), active fluorescent unit (AFU), and non-active fluorescent unit (nAFu), as described in ISO 19344 (2015).

2.4 Antimicrobial agents and MICs determination

According to EFSA 2018, for Gram-negative bacteria, the antimicrobials to be tested and the cut-off to be considered should be those for *Enterobacteriaceae*. Therefore, in this work the antimicrobials used were: ampicillin, gentamicin, kanamycin, streptomycin, tetracycline, ciprofloxacin, colistin, fosfomycin, sulfamethoxazole. MICs were determined by the standard macrodilution broth method in 24-wells plates, using serial two-fold dilutions of antimicrobials with a bacterial inoculum density of $5x10^5$ AFU ml-1, in a final volume of 2 ml. The growth controls contained only inoculated broth without antimicrobials, negative controls comprised media that was not inoculated. When ethanol and dimethyl sulfoxide (DMSO) were used as diluents of antimicrobials, their effect on cell growth was checked. In both cases, no inhibition of bacterial growth was seen. Incubation took place in an anaerobic chamber (N2:H2:CO2 90:5:5) at 37 °C for 48 h. *E. coli* Nissle was cultivated in aerobic conditions for 24h at 37 °C. The MIC is the lowest concentration of antimicrobial where no visible growth is measured in the wells. MICs were determined at least in duplicates.

2.5 Ciprofloxacin susceptibility assay and efflux pump inhibitors activity

Susceptibility of *A. muciniphila* strains to ciprofloxacin was also evaluated in the presence of two Efflux Pump Inhibitors (EPIs), carbonyl cyanide 3-chlorophenylhydrazone (CCCP; Merck) or phenylalanine-arginine beta-naphthylamide (PaβN; Merck), to determine the contribution of efflux pumps to ciprofloxacin sensitivity. The concentrations of the EPIs used (CCCP 80 µM; PaβN 10 µM) were decided based on the determination of their MICs. CCCP was soluble in the growth medium with DMSO (1% v/v). DMSO alone was used as a control, and it did not affect cells growth. MICs were determined as mentioned above.

3. Results and discussion

3.1 Genome sequences, species classification, and pan-genome analysis

In our study, we isolated 5 new strains of the species *A. muciniphila*, whose genomes were sequenced (Filardi 2021). *De novo* assembly of the genomic data, revealed varying genome sizes ranging from 2.65 to 3.42 Mbp. The G+C contents of the genomes ranged from 54.9 to 55.8% and the number of unique protein-coding genes varied from 2098 to 2750. According to the recent literature (Guo et al. 2017, Karcher et al. 2021), *Akkermansia* species cannot be identified by 16S rRNA analysis alone. To unambiguously classify the new strains at species level, the

whole-genome average nucleotide identity values between the newly sequenced genomes and the *A. muciniphila* type strain genome were calculated using fastANI (Jain et al. 2018). ANI values in the range of 95–96% were considered as a limit for the definition of a new species (Rosello-Mora and Amann 2015). Our five strains exhibited ANI scores ranging between 98.5 and 99%, except for AMuP9 (97.4%), and were therefore classified as belonging to the *A. muciniphila* species. Comparative genomic analysis of the newly isolated (5) and publicly available (188) *Akkermansia* genomes, identified an open pangenome of 19738 genes, including 212 core genes (shared between the 99% and 100% of the strains), 34 soft-core genes (between 95% and 99%), 4618 shell genes (between 15% and 94%), and 14874 cloud genes (less than 15% of the strains). The coding sequence alignment of the genomes (n=193) performed during the pan-genome analyses allowed to categorize our strains in the candidate *Akkermansia* sp. described by Karcher et al. (2021). The results based on CDS presence and absence (shell genes only selected n=4618) revealed the existence of three different clades, corresponding to *A. muciniphila* species and the new putative species proposed by Karcher et al.(2021), namely SGB9228 and SGB9223. Our five strains are all grouped under *A. muciniphila* species (Figure 1). Furthermore, the core-gene alignment analyses confirmed the previous result showing a clusterization of our isolates in the cluster of the *A. muciniphila* type strain.

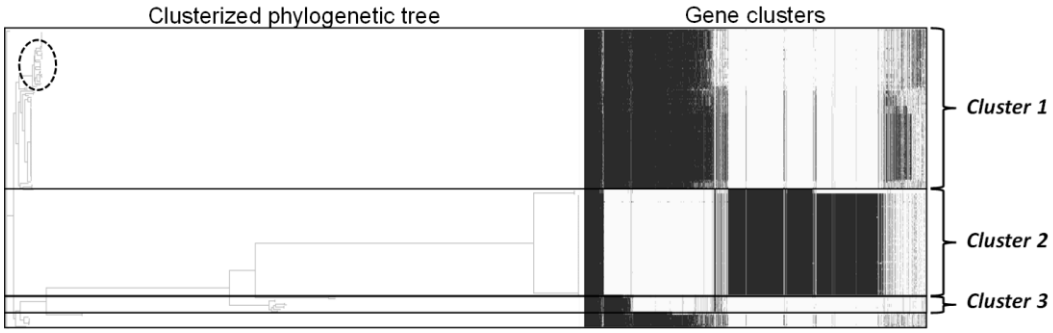

Figure 1. Phylogenetic tree of the 193 *Akkermansia* sp. genomes. The shell genes were used for the clusterization based on gene presence (black) or absence. The genomes are clusterized into three groups corresponding to *Akkermansia* sp. (cluster 1, 113 genomes), SGB9223 (cluster 2, 69 genomes), SGB9228 (cluster 3, 11 genomes). The dashed oval highlights the position of our five genomes.

3.2 Antimicrobial resistance genes and mobile DNA elements

Genome sequences of newly isolated *A. muciniphila* strains, were examined for the presence of ARGs and compared to the type strain. Our genome analysis revealed that all the strains carry potential ARGs. All strains were characterized by the presence of the gene *adeF*. This gene encodes a membrane fusion protein belonging to the resistance-nodulation-cell division (RND) efflux pump system AdeFGH (Leus et al. 2018), potentially involved in resistance to fluoroquinolones and tetracycline. However, since only this single gene was found in our strains, we can speculate that the complete AdeFGH operon, coding for a functioning efflux system, is absent or at least different from that found in pathogenic strains. The gene *aph(6)-Id*, associated with resistance to aminoglycosides, specifically with the inactivation of streptomycin, and the gene *sul2*, determinants of sulfonamide resistance, were specifically found only in the genomes of two *A. muciniphila* strains, Vtp7 and Amap1. The latter also carries the *tetW* gene, which confers resistance to tetracycline. Our *A. muciniphila* strains were also screened for the presence of MGEs, possibly involved in the ARGs transfer. One insertion sequence (IS), ISAmu1, as in the type strain, has been also identified in the genomes of *A. muciniphila* Sap1 and Amap1, however, it is not located in the vicinity of ARGs. In the genome of the Amap1 strain, also a putative transposon (Tn6205) has been found. In this strain, the AMR genes *aph(6)-Id* and *sul2* can be both associated with the MGE, being located in the transposon sequence (*aph(6)-Id*) or in its vicinity (*sul2*), posing some risk of genetic transferability that should be further analyzed.

3.3 Antibiotic sensitivity profiles

A. muciniphila strains were also tested for their antibiotic susceptibility, according to the EFSA document 2018. The results are summarized in Table 1. For antibiotic susceptibility testing, the culture medium used must allow the growth of the strains under assessment and not interfere with antimicrobials. IST is the nutrient medium recommended by the British Society for Antimicrobial Chemotherapy. However, for specific bacteria, other formulations may be required (EFSA 2018). In our study, a modified version of IST (sIST) was used to allow the growth of *A. muciniphila*. To verify that the added components did not interfere with antibiotics, we used *E. coli Nissle* as a control strain, which is the only Gram-negative probiotic bacterium, belonging to the *Enterobacteriaceae* family. MICs determined in sIST were compared with those received from parallel determinations in caMHB, another conventional susceptibility test medium. MICs determined in the two media were compatible, as such, we can state that the sIST components did not interfere with the phenotypic test.

All *A. muciniphila* strains resulted susceptible to ampicillin, tetracycline, colistin, fosfomycin, and sulfamethoxazole. Only one strain harboring *tetW* gene showed reduced sensitivity to tetracycline, but all *A. muciniphila* strains showed low sensitivity to ciprofloxacin and aminoglycosides. Interestingly, all strains present

adeF gene encoding a subunit of the resistance-nodulation-cell division (RND) efflux pump system and potentially involved in the resistance to ciprofloxacin. Conversely, only two strains showed a possible genetic determinant for resistance to aminoglycosides (*aph(6)-Id*), indicating that poor sensitivity to this class of antibiotics could be due to a more general intrinsic mechanism. Our results are in line with the document EFSA 2021, where it is reported that *A. muciniphila* BAA-835 presented high resistance levels to aminoglycosides, vancomycin, and ciprofloxacin similarly to other *A. muciniphila* strains tested.

3.4 Role of efflux pump inhibitors on ciprofloxacin reduced susceptibility

Since all *A. muciniphila* strains studied in this work possess a gene (*adeF*) encoding a subunit belonging to the AdeFGH efflux pump system and showed low sensitivity to ciprofloxacin; we wanted to investigate the involvement of efflux pumps in the reduced susceptibility to ciprofloxacin. To this aim, we performed combined experiments, in which MICs for ciprofloxacin were determined with and without EPIs. We selected two EPIs, CCCP and PaβN, that differ in terms of their mechanisms of action. CCCP is a strong inhibitor of RND efflux pumps, being able to interfere with their energy source, mainly acting as a proton motive force dissipator (AlMatar et al. 2021). PAβN is a broad-spectrum efflux pump inhibitor, acting as a competitive inhibitor, preventing efflux of the antibiotics by binding the substrate-binding pocket of the efflux pumps and impairing the antibiotic bond at its affinity site (Jamshidi et al. 2017). In presence of EPIs, the MICs of ciprofloxacin were equal to or 2-fold lower than the MIC values determined without EPIs (Table 1). These slight variations in MICs represent the normal standard deviation of MIC dilution tests. Thus, no evidence of active drug efflux was observed in ciprofloxacin.

Table 1. *MICs (mg l^{-1}) of tested antibiotics for* Akkermansia muciniphila *strains*

A. muciniphila	**AMP**	**GEN**	**KAN**	**STR**	**TET**	**COL**	**FOSF**	**SULF**	**CIPR**	**CIPR + CCCP**	**CIPR + PaβN**
DSM 22959	4	**256**	**>512**	**256**	< 2	< 2	< 4	< 4	**128-256**	**128**	**64-128**
Sap1	4	**256**	**>512**	**256**	< 2	< 2	4	< 4	**128**	**128**	**128**
Amap1	4	**128**	**>512**	**512**	**64**	< 2	4	< 4	**128**	**128**	**128**
Vtp7	4	**128**	**>512**	**256**	< 2	< 2	< 4	< 4	**64-128**	**64**	**64**
Rcp22	4	**256**	**>512**	**512**	< 2	< 2	< 4	< 4	**128-256**	**128**	**128**
Amup9	2	**128**	**512**	**256**	< 2	< 2	< 4	< 4	**128**	**64**	**64-128**
E. coli Nissle 1917	8	2	8	16	8	0.5	64	32	< 0.25	nd	nd
E. coli Nissle 1917 caMHB	4-8	2	4-8	8	4	0.5	32	16	< 0.25	nd	nd
***Enterobacteriaceae* (EFSA 2018)**	**8**	**2**	**8**	**16**	**8**	**2**	**8**	**256**	**0.06**	**nd**	**nd**

AMP, Ampicillin; **GEN**, Gentamicin; **KAN**, Kanamycin; **STR**, Streptomycin; **TET**, Tetracycline; **COL,** Colistin; **FOSF,** Fosfomycin; **SULF**, Sulfamethoxazole; **CIPR**, Ciprofloxacin; **CCCP,** carbonyl cyanide 3-chlorophenylhydrazone; **PaβN,** phenylalanine-arginine beta-naphthylamide**; CaMHB**, cation adjusted Muller Hilton broth. **nd,** not determined.

4. Conclusion

Our study provided new data on the phenotypic and genotypic antimicrobial resistance profile of *A. muciniphila*, showing a similar level of susceptibility within the species. Indeed, all the strains analyzed, including the type strain, showed a low susceptibility to ciprofloxacin and aminoglycosides. Poor sensitivity to these classes of antibiotics, being not related to any ARGs of concern, could be due to a general intrinsic mechanism. Only one of the strains showed traits of concern carrying three ARGs, one conferring resistance to tetracycline (t*etW*) and, two associated with a MGE. We also wanted to investigate the involvement of efflux pumps activity in reduced susceptibility to ciprofloxacin, as in all the genomes was found the *adeF* gene, encoding an RND efflux pump system (AdeFGH). According to our data, no evidence of active drug efflux was observed in ciprofloxacin. Even if *adeF* is consistently found in the *A. muciniphila* species genomes, we can hypothesize that the complete AdeFGH operon, which codes for a functioning efflux system, is absent or at least different from that found in pathogenic strains. In conclusion, further studies on a larger number of strains are needed to better assess antibiotic resistance in this species.

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Promotion of nutrition knowledge and sustainability of dietary behaviors in different student populations

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This PhD thesis aims at identifying and developing effective educational interventions to raise knowledge and awareness about healthy and sustainable nutrition in young adults, and eventually shifting dietary behaviors accordingly, by customizing the intervention for different student populations.

Promozione della conoscenza nutrizionale e della sostenibilità dei comportamenti alimentari in diverse popolazioni di studenti

Questa tesi di dottorato mira a identificare e sviluppare progetti educativi efficaci, atti ad aumentare la conoscenza e la consapevolezza dei giovani adulti verso un'alimentazione sana e sostenibile, ed eventualmente modificare i comportamenti alimentari, personalizzando l'intervento per diverse popolazioni di studenti.

Key words: healthy and sustainable diets; education intervention; nutrition knowledge; university students; chefs.

1. Introduction

In line with the PhD thesis project described above, this oral communication reports the main findings of the following four research activities:
1) A systematic review investigating the adherence to dietary guidelines among nationally representative cohorts of young populations living in Europe;
2) Lifestyle outcomes of two representative samples of Italian and American university students in a study applying healthy and sustainable nudges to improve university students' food choices;
3) The effect of a menu reordering intervention to encourage plant-based dietary choices at dining services at the University of California, Los Angeles (UCLA);
4) Assessment of nutrition and environmental knowledge in apprentice chefs before and after an education intervention.

2. Research Studies

2.1 Adherence to dietary guidelines among nationally representative cohorts of young populations living in Europe: a systematic review

Purpose and methods. This systematic review aimed to investigate the current compliance with dietary guidelines and/or nutrition recommendations, at national or international level, among nationally representative cohorts of European young populations (i.e., children, adolescents, and young adults). Specifically, the quality of diet has been evaluated in terms of food groups and/or dietary energy content and macronutrients intake. Literature search was made from their inception to February 2022 through three different electronic databases: PubMed, Scopus, and Web of Science. To obtain an updated picture of the current and actual dietary habits of young European populations (2–35 years), only articles written in English for which data collection was carried out from 2010 onwards were included.
Results. Starting from a total of 8693 documents, 29 papers were selected and included for data extraction. The included studies were conducted across 14 countries: Spain (n = 7), United Kingdom (n = 5),), Netherlands (n = 3), Poland (n = 2), Cyprus (n = 2), Sweden (n = 1), Ireland (n = 1), Balearic Islands (n=1), France (n = 1), Greece (n = 1), Serbia (n = 1), Norway (n = 1), Germany (n = 1), Denmark (n = 1), Croatia (n = 1). Several documents reported data for different age groups; in these cases, only results for 2–35-year-old participants have been included. Most of the studies focused on the dietary habits of children (n=12) and adolescents (n=17), whereas fewer data were found for the populations of young adults (n=7). In general, adolescents' dietary habits were characterized by low intakes of fruit and vegetables, starchy foods, milk and milk and yogurt, cheese, fish, eggs, legumes, nuts, and seasoning fats, and excessive intakes of meat and meat products, sweets, and sugar-sweetened beverages (SSBs). Intakes of fast food, alcoholic and non-alcoholic beverages were reported in a few studies and were mostly in line with recommendations. Also, the habit of having breakfast was investigated in only three studies, showing medium to high adherence to this recommendation. In almost all included studies, both children's and adolescents' diets resulted in excessive intakes of protein, short fatty acids (SFAs), sugars, and sodium; in contrast, fiber intake was nearly insufficient. At the same time, carbohydrate and fat intakes varied between low and high adherence to recommendations in children, whereas in adolescents the intakes of carbohydrate and fat were on average adequate and poor, respectively. In addition, a medium adherence to Mediterranean Diet (MD)

in Greek and Croatian children and adolescents was reported, and two other diet quality scores were used in Norwegian and Swedish adolescents showing medium adherence to dietary guidelines and nutritional recommendations. Dietary habits of young adults have been reported in few studies. Food groups intake was reported only for Dutch and Spanish populations, showing poor consumption of fruit and vegetables, starchy foods, legumes, nuts, cooking fats, milk, and dairy products in almost all young adults. Spanish young adults also had inadequate intake of fish, eggs and olive oil, excessive intakes of meat and meat products and good adherence to recommendations for sweets, SSBs, and alcoholic beverages. On the other hand, Dutch had excessive intakes of SSBs, fast food, and alcoholic beverages, whereas the intakes of cheese, meat and meat products, fish, and eggs were different between genders, with females showing on average better intakes. In addition, for non-alcoholic drinks the results were in contrast in the Dutch populations. Considering the nutritional profile of the young adults' diet, results are limited and referred only to Dutch subjects, who showed adequate intake of carbohydrates, low intake of fiber, excessive intake of SFAs, and mixed results for protein and sodium. For the latter, females showed better intake. Finally, adherence to MD was analysed for Spanish and Croatian, who showed on average a poor and medium level of adherence, respectively.

Table 1 *Anthropometric and socio-demographic data, adherence to MD, eating behaviour and physical activity by country.*

Variables	US students (n=1510)	IT students (n=1454)	*p*-value
Gender			<0.001 [a]
Females	890 (59)	875 (60)	
Males	568 (38)	567 (39)	
Not-binary/third gender or prefer not to say	52 (3)	12 (1)	
Age (years)	21.0 (19.0-22.0)	22.0 (20.0-23.0)	<0.001 [b]
BMI (kg/m^2)	25.1 (21.5-30.4)	22.2 (20.1-24.5)	<0.001 [b]
BMI category			<0.001 [a]
<18.5 (underweight)	103 (7)	150 (10)	
18.5–24.9 (normal weight)	647 (43)	998 (69)	
25.0–29.9 (overweight)	351 (23)	599 (17)	
30.0–34.9 (obesity)	409 (27)	467 (4)	
Financial situation			<0.001 [a]
Not enough to get by	102 (7)	118 (8)	
Just enough to get by	502 (33)	425 (29)	
Only have to worry about money for fun and extras	620 (41)	492 (34)	
Never have to worry about money	224 (15)	263 (18)	
I prefer not to answer	62 (4)	156 (11)	
MD score (on a 0-12-point scale)	5.0 (3.0-7.0)	6.0 (4.0-8.0)	<0.001 [b]
Adherence to the MD			<0.001 [a]
Poor (≤ 3 points)	507 (34)	252 (17)	
Average (4-7 points)	710 (47)	799 (55)	
Good (≥ 8 points)	293 (19)	403 (28)	
Dietary pattern			<0.001 [a]
Omnivore	1161 (77)	1302 (90)	
Vegetarian	76 (5)	38 (3)	
Vegan	28 (2)	16 (1)	
Flexitarian	156 (10)	66 (5)	
Pescatarian	31 (2)	20 (1)	
Raw foodism	5 (0)	3 (0)	
Fruitarian	43 (3)	6 (0)	
Others not specified	10 (1)	3 (0)	
Canteen attendance			<0.001 [a]
Never/rarely	453 (30)	824 (57)	
Less than once per week	219 (15)	191 (13)	
Once-twice per week	275 (18)	221 (15)	
3-4 times per week	282 (19)	138 (9)	
5-6 times per week	132 (9)	54 (4)	
Once per day or more	149 (10)	26 (2)	
MVPA recommendation			0.627 [a]
Met	783 (52)	741 (51)	
Not met	727 (48)	713 (49)	
Walking at least 30 min per day			<0.001 [a]
Met	284 (19)	392 (27)	
Not met	1226 (81)	1062 (73)	

Data are reported as absolute number (%) or as median (25th-75th percetile). [a] Chi-square test. [b] Nonparametric Mann–Whitney test for independent samples. MD: Mediterranean Diet. MVPA: Moderate to Vigorous Physical Activity.

2.2 Using healthy and sustainable nudge to improve university students' food choices: lifestyle outcomes of two representative samples of Italian and American university students

Purpose and methods. This cross-sectional analysis is part of an intervention study aimed to evaluate the effectiveness of nudge to promote healthy and sustainable food choices among university students in the context of online preorder system simulating students' choices for lunch at the canteen. This study was conducted on two populations of Italian and American university students from 18 to 24 years old recruited through a marketing agency (Qualtrics, Provo, UT) in May 2022. The gender distribution, and the geographic area of residence for both countries were considered to obtain two representative samples of the university student population living in Italy and US. A self-administered online survey was carried out aiming to assess and compare the eating habits of Italian and American university students. The primary outcome of this cross-sectional analysis was the assessment of adherence to the Mediterranean Diet (MD) by using the validated KIDMED questionnaire (Serra-Majem et al., 2004). Also, the subjects were asked to indicate the dietary pattern that mostly represented them (e.g., omnivore, vegetarian diet, etc..) and their usual attendance of the university canteen. Socio-demographic and anthropometric information was self-reported. Heigh and weight were used to calculate the body mass index and estimate the weight status through WHO's standard cutoffs (WHO, 2010). In addition, the students' compliance with World Health Organization (WHO) guidelines on physical activity (WHO, 2020.) was assessed through the validated Nordic Physical Activity Questionnaire-short version (Danquah et al., 2018). An additional question adapted from the validated IPAQ (IPAQ, 2005.) was added to assess whether participants had an adequate walking time (≥ 30 mins a day for ≥5 days per week).

Results. A total of 1964 students correctly completed the survey. The descriptive analysis of the main characteristics of the sample population is shown in Table 1. In both samples, the majority were females (US 59%, IT 60%) with a mean age of 21.0 and 22.0 years for US and Italy, respectively. The distribution among BMI categories was significantly different between the two countries (p<0.001), with most Italian students reporting normal body weight (69%) and, in contrast, a higher proportion of American students was overweight (23% *vs.* 17%) or obese (27% *vs.* 4%). Furthermore, a significant association was found between country and level of adherence (p<0.001). Despite both samples had a medium adherence to the MD, Italian students showed significantly higher scores (p<0.001). In addition, different adopted dietary patterns were observed (p<0.001). In both the countries, most students (US 77% *vs* IT 90%) reported themselves as omnivorous, however American students were found to be more prone to alternative patterns, such as vegetarian, vegan, pescatarian and fruitarian. In addition, a significant association was also found for canteen attendance (p<0.001). Specifically, the American students reported eating more regularly in the canteen, whereas the majority (57%) of Italian students indicated never or rarely attending the canteen. Finally, only half of the American and Italian students met the Moderate to Vigorous Physical Activity recommendation, with no significant difference between the two countries, whereas a significant association was found for the habit of walking for at least 30 minutes (p<0.001).

2.3 The effect of a menu reordering intervention to encourage plant-based dietary choices at UCLA dining services

Purpose and methods. This intervention study aimed at encouraging plant-based food choices by re-ordering the menu according to the carbon footprint (g CO_2 equivalents) of foods. The project was conducted during the fall quarter 2021 at The Study at Hedrick, a UCLA dining service. This cafeteria provides breakfast, lunch, and dinner to UCLA students on a meal plan, which includes a prepaid number of meals and the ability to choose from a variety of foods and beverages without being influenced by price. Students could order their meals on-site, using the self-service kiosks, or remotely through a pre-order platform; in all cases, the menu configuration was the same, and the different menu options (e.g., pizza, salads, skillets, bagels, and sandwiches) were customizable by choosing from different ingredients. Normally, the ingredients are in alphabetical order, but during this intervention study the order was modified twice. For the first 5 weeks, the food items were listed in descending order, from the most to the lowest environmentally impactful. Subsequently, for the following 5 weeks, the order was reversed, and the foods were sorted in ascending order, from the food with the lowest to the one with the highest carbon footprint, on average. Similarly, the drinks offering, which included beverages (i.e., water, hot drinks, and soda), fruit juices, milk, and soy milk, were reordered as the food options according to the same criteria. During the first and second 5 weeks of the intervention, all sales data were recorded through the centralized MyMicros web-delivered reporting platform and exported to an Excel worksheet as the total quantity sold during the two considered periods. Sales data for each ingredient or beverage were divided between animal- and plant-based foods and summed according to food category (e.g., beef, pork, legumes, vegetables, etc.).

Results. A total of 389,302 and 400,409 items were selected respectively during the first and the second data collection periods. As shown in Table 2, a significant association between ordered menus and sales was found both considering their distribution between foods of animal and plant origin (p<0.001) and among food categories (p=0.003).

Table 2 *Distribution of food choices by food typology and food category during the two data collections.*

	Sales		
Food category	**1st data collection** Highest carbon footprint items before	**2nd data collection** Lowest carbon footprint items before	***p*-value**
Animal-based	204,148 (52.4)	201,587 (50.3)	<0.001
Plant-based	185,154 (47.6)	198,822 (49.7)	
Beef	7,208 (1.9)	7,304 (1.8)	0.003
Pork	50,361 (12.9)	48,849 (12.2)	
Poultry	42,388 (10.9)	40,585 (10.1)	
Fish	2,645 (0.7)	2,864 (0.7)	
Cheese	73,523 (18.9)	68,805 (17.2)	
Eggs	11,536 (3.0)	14,624 (3.7)	
Milk	16,487 (4.2)	18,556 (4.6)	
Soy milk	7,656 (2.0)	7,802 (1.9)	
Beverages	67,418 (17.3)	62,425 (15.6)	
Fruit juices	18,522 (4.8)	23,099 (5.8)	
Cereals	1124 (0.3)	1,768 (0.4)	
Fruit	31,098 (8.0)	32,956 (8.2)	
Vegetables	42,992 (11.0)	49,400 (12.3)	
Legumes	4,840 (1.2)	7,794 (1.9)	
Nuts	3,529 (0.9)	5,037 (1.3)	
Vegan cheese	5,178 (1.3)	5,654 (1.4)	
Vegan sausages	2,797 (0.7)	2,887 (0.7)	

Data are reported as frequency (% of total sales over 5 weeks). § Chi-square test.

Overall, results show that students were more likely to choose plant-based options when these were placed at the top of the menu. Among animal-based foods, the largest decreases were in the pork (-0.7%), poultry (-0.8%), and cheese options (-1.7%), whereas milk (+0.4%) and eggs (+0.7%) increased, while fish and beef were comparable between the two data collection periods. On the other hand, vegetables (+1.3%), fruit juices (+1.0%), legumes (+0.7%), and nuts (+0.4%) were the plant-based foods for which the largest increase was recorded during the second data collection, whereas beverages decreased, and sales of soy milk, cereals, fruits, vegan cheese, and sausages remained fairly stable compared to the first data collection.

2.4 Assessment of nutrition and environmental knowledge in apprentice chefs before and after an education intervention

Purpose and methods. The aim of this ongoing study is to evaluate changes in nutrition knowledge and learning outcomes about food environmental impact in a sample of apprentice chefs enrolled at the International School of Italian Cuisine (ALMA) in Parma before and after an educational intervention. All students registered for the Corso Superiore di Cucina Italiana provided by ALMA were invited to participate to the study and represented the intervention group. To evaluate changes in students' knowledge about nutrition and food sustainability, two online questionnaires were administered twice to each student, at the beginning (T0) and at the end of the intervention (T1). A Nutrition Knowledge questionnaire already validated for Italian university students (Rosi et al., 2020) and an original Food Sustainability questionnaire, specifically developed for the study (validation ongoing), were used to assess the learning outcomes. Lectures addressing energy and nutrient content of food as well as food and diet impact on human health and environment were given to students. At the same time, also students attending other courses provided by ALMA, and not undergoing to the training plan, were invited to participate as control group and filled out the same two online questionnaires at the beginning and at the end of their course.

Results. Until now, a total of 388 apprentice chefs (mean age 21.0 (19.0-24.0) years, 22% females) completed the study and were included in the preliminary analysis, 133 students for the control group and 255 for the intervention group. As reported in Table 3, a significant improvement (p<0.001) of learning outcomes has been found in the intervention group, both for nutrition and food sustainability knowledge. On the contrary no significant changes have been found in the control group. Moreover, at T1 both the nutrition and food sustainability knowledge were significantly higher (p<0.001) in the intervention group, confirming the effectiveness of the educational intervention. Students' nutrition knowledge of the intervention group was higher not only at T1, as expected, but also at T0 (p<0.001), suggesting that at baseline the two groups were not comparable in terms of knowledge. This heterogeneity could be due to the different samples size of the two groups. It can be assumed that the enlistment of new students for the control group will be crucial to make comparable the intervention and the control group.

Table 3 *Within and between group comparisons of knowledge scores obtained at time 0 (T0) and time 1 (T1).*

		T0			T1			
Group	**Section**	**Median**	**IQR**	**Range (min-max)**	**Median**	**IQR**	**Range (min-max)**	***p*-value***
Control	NK	63.0	57.0-69.0	43.0-79.0	64.0	59.0-68.5	43.0-79.0	0.099
Intervention		67.0	60.0-71.0	38.0-83.0	72.0	67.0-76.0	41.0-83.0	<0.001
***p*-value#**		<0.001			<0.001			
Control	FSK	12.0	11.0-14.0	1.0-19.0	13.0	11.0-14.5	7.0-19.0	0.229
Intervention		13.0	11.0-14.0	4.0-19.0	16.0	14.0-17.0	4.0-22.0	<0.001
***p*-value#**		0.798			<0.001			

NK, nutrition knowledge score (0-90); FSK, food sustainability knowledge score (0-23); IQR, interquartile range. *Non-parametric Wilcoxon test for paired samples. # Non-parametric Mann-Whitney U test for independent samples.

3. General Conclusions And Future Perspectives

To summarize, the systematic review shows that young European populations have eating habits that are poorly aligned with dietary guidelines and nutritional recommendations, and increasingly resembling Western-type diets characterized by a poor consumption of vegetables, fruit, grains, legumes, and nuts, and an excessive consumption of meat and meat products, sweets and SSBs. Moreover, a limited number of nutritional surveys has been addressed to nationally representative samples in the last decade in Europe, therefore an accurate and reliable information on current eating habits for that geographical area is prevented. In particular, the few data on young adult populations highlights the knowledge gap related to this period of life characterized by greater independence and central to root healthy dietary habits that can persist into adulthood. Understanding the dietary habits of young adult populations is fundamental to develop effective nutrition education projects able to improve their eating behaviors and overall well-being. To address this issue, a survey on two representative samples of American and Italian university students was conducted to obtain a picture of their current food habits. The preliminary results have confirmed a higher adherence to the MD in Italian students compared to American ones, but a progressively moving away from traditional Mediterranean pattern was confirmed. The analysis on nudges effectiveness is still ongoing but findings from another nudging intervention carried out at UCLA dining services are promising. Sale data recorded during the nudge intervention highlighted a positive impact of menu re-ordering to rise plant-based choices and decrease consumption of animal-based foods. However, the choice of beef options was slightly reduced, pointing out the need to integrate menu re-ordering with other more effective actions. In this context, the optimisation of the catering service should involve different levels besides food communication (e.g., food selection, food preparation, food communication) to improve the quality of the food supplied considering first food safety and health-enhancing criteria. Due to the key role of cooks in promoting healthy and sustainable eating behaviours within the general population, it is important to provide future chefs with proper practical tools to make them able to prepare nutritionally balanced and culturally acceptable meals, as well as able to mitigate climate change. To pursue this objective, a prospective study was conducted to assess the nutrition knowledge and awareness on food sustainability in a sample of apprentice cooks enrolled at the International School of Italian Cuisine (ALMA) where an educational intervention has been provided. Based on the preliminary results and considering increasing the sample size of control group, we are expecting to confirm the positive findings about the effectiveness of educational intervention in increasing chefs' nutritional and environmental knowledge.

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Shelf-life estimation as a strategic tool for the eco-design of a sustainable food packaging

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This PhD thesis dealt with the research and inclusion of *indirect environmental effects* into food packaging Life Cycle Assessment (LCA) studies with the greater scope to define eco-design approaches in the field. This research focused on the interrelationship between food packaging properties and performances, shelf-life estimation, and potential reduction of food waste. The last part of the research project investigated the possibility to develop a simplified tool for food-packaging eco-design coupled with a predictive tool for shelf-life estimation.

La stima della shelf-life come mezzo strategico per l'eco-design di packaging alimentari sostenibili

Questa tesi di dottorato ha affrontato la ricerca e l'inclusione degli effetti ambientali indiretti negli studi di Life Cycle Assessment (LCA) degli imballaggi alimentari con il maggiore scopo di definire approcci di eco-design nel campo. Questa ricerca si è concentrata sull'interrelazione tra le proprietà e le prestazioni degli imballaggi alimentari, la stima della durata di conservazione e la potenziale riduzione degli sprechi alimentari. L'ultima parte del progetto di ricerca ha esaminato la possibilità di sviluppare uno strumento semplificato per l'ecodesign degli imballaggi alimentari abbinato a uno strumento predittivo per la stima della durata di conservazione.

Key words: sustainability, shelf life, food packaging, Life Cycle Assessment methodology, food waste

1. Project Overview

According to the Ph.D. research plan described in the previous years (Frigerio, 2020), and consequently to the results presented during the second year (Frigerio, 2021), this oral communication reports the detailed results related to the methodological choices for shelf-life inclusion in food packaging Life Cycle Assessment study.
The main scope of the Ph.D. project is to develop a holistic approach for evaluating the sustainability of food packaging solutions. Moreover, the last part of the research project investigated the possibility to develop a simplified tool for food-packaging eco-design coupled with a predictive tool for shelf-life estimation. This communication will focus on these aspects:

1) Relevance and current practice of shelf-life inclusion as an *indirect effect* directly in LCA studies for food-packaging systems.
2) Comparison of different Functional Units (F.U.) for shelf-life functionality by means of a case study on fresh packaged raspberries
3) Future perspective in the field

2. Relevance of shelf-life in eco-design approaches

The role of shelf-life assessment in the development and selection of both food and packaging solutions has always been crucial, especially when sustainability decisions must be made by food business operators. Over the last decades, mostly due to the current sustainability requirements, eco-design principles and sustainable development strategies have gained greater importance in the food packaging field. In Europe, the Circular Economy Action Plan, as part of the European Green Deal, along with EU's plastics strategy and recent updates on packaging and packaging waste on plastics and packaging solutions, highlighted the value of using these materials more sustainably. Such political pressure has led to the necessity of re-designing packaging solutions. In the case of food packaging, the search for more sustainable solutions is mainly associated with physical modification or substitution of materials, by preferring other raw materials' sources (e.g., recycled and/or bio-based plastics) or disposal options (e.g., compostable materials). However, in accordance with eco-design principles, all properties of food packaging must be considered to provide a full picture of its sustainability (Pauer et al., 2019). Long seen as an additional environmental burden, food packaging has recently been redeemed as a beneficial player in food-packaging environmental assessments due to its properties related to food protection and convenience (Wikström et al., 2018). Exploiting the potential shelf life of food products, thanks to innovative and improved packaging solutions, could be an effective strategy for enhancing the sustainability of food-packaging systems from both an economic and environmental point of view (Wohner et al., 2019). For these reasons, a correct estimation of shelf-life is a critical point for effective strategies towards both food packaging eco-design and food waste reduction. Moreover, different eco-design strategies should be applied for different food products as the gain in environmental benefits could vary. For these reasons, the development and selection of eco-designed food packaging solutions must

consider a multitude of factors, requiring a deep knowledge of the systems under study.

3. Current methodological practice for *indirect effects* inclusion in LCA studies

Life Cycle Assessment (LCA) is a methodology used to validate eco-design strategies for food packaging solutions. An effective implementation of eco-design principles must rest on a balanced consideration of all relevant attributes and functions of packaging solutions. Shelf-life inclusion into LCA studies permits to account for the so-called *indirect environmental effects* of food packaging solutions. Indirect effects refer to the environmental benefits coming from specific packaging properties that could reduce the probability of food waste at different levels (Lindh et al., 2016). However, even if an increasing number of food-packaging LCA studies are considering shelf-life and its environmental effects, harmonization among different LCA methodological approaches has not yet been reached. Such ambiguity could lead incomparable results and confusion among practitioners. The importance of a well-defined Functional Unit for unequivocal shelf-life inclusion into food-packaging LCA it is fundamental for reaching standardization in this field. Among the recent food-packaging LCA that consider shelf-life effects on environmental results, different functional units have been proposed. Shelf-life could be either be expressed as an inherent characteristic of the food-packaging system, or as a pre-defined timespan. The study proposed by Zhang et al. (2015) is a great example of the *"inherent"* approach. The authors compared, through LCA methodology, four packaging alternatives for fresh beef. The functional unit was defined as "the delivering 1 kg fresh beef to the retail gate and displaying it until the end of shelf life", which lead to the modelling of four specific scenarios per each packaging system as a direct function of its performances. Concerning the *"explicit"* approach, Gutierrez et al. (2017) proposed a comparative LCA for cheesecake packaging alternatives considering a pre-defined timespan of 28 days. Such difference results not only in a lack of clarity and consistency among practitioners, but also in difficult result interpretation.

4. Comparison of methodological choices for shelf-life accounting in LCA studies: a case study

The case of packaged fresh raspberries was used to investigate the impact of functional unit definition on the results of comparative LCA studies. A comparison of three food-packaging systems was carried out to evaluate the environmental profiles derived by direct and indirect effects of the product's life cycles. Two functional units, that differently express the shelf-life functionality, were implemented in the study. This study is intended to help foster the harmonization of methodological approaches for food packaging Life Cycle Assessment studies.

4.1. Materials and methods

A comparative environmental analysis of three different packaging solutions for packaged fresh red raspberries (*Rubus ideaeus* L., 1753) was performed by means of Life Cycle Assessment methodology. Three packaging solutions (Table 1) were considered in this study: Traditional (I), Passive (II), and Active solution (III). The study considered both packaging and raspberries life cycle with a "cradle-to-grave" approach. The end-of-life scenario was modelled based on European (EU28+NO/CH) plastic packaging waste management shares. ISO 14040: and ISO 14044:2021 requirements were followed in this case study. SimaPro v 9.3.0.2 software (PRé Sustainability, Amersfoort, The Netherlands) and Ecoinvent v 3.8 (*cut-off* allocation criteria; geographical area: Europe) were used to model the systems. ReCiPe 2016 v1.1 midpoint, hierarchist methodology (Radboud University, NL; Norwegian University of Science and Technology, NO; Pré Sustainability, NL) was used for impacts assessment.

Table 1 *Details of packaging solutions under study*

Coded name	Characteristics	Packaging system	Expected shelf-life (days)
Traditional	In air	PET tray (14 x 9.5 x 5 cm)	4
Passive	Passive atmosphere	PET tray (14 x 9.5 x 5 cm); LDPE masterbag (34 x 25.5 cm; thickness of 25 µm)	6
Active	Active sachets	PET tray (14 x 9.5 x 5 cm); LDPE masterbag (30 x 35 cm; thickness of 25 µm); active devices	11

4.2. Goal and scope definition

The following functional units were selected: "One pack containing 250g of fresh raspberries", referred as *"Inherent functional unit"* and "250g of packaged fresh raspberries over a 11-day timespan", defined as *"Explicit functional unit"*. The implementation of the two functional units calls for two different LCA approaches.

4.3. Mathematical modelling of potential food waste

Reference flows were modelled based on the relative functional units. For the inherent approach, the reference flows account for all inputs and outputs linked to the life cycle of one pack of fresh raspberries (250 g). In the case of the explicit approach, the flows required by each system are modelled to fulfil the demand of the product on the market for 11 days. For this reason, 2.7 traditional solutions, 1.8 passive solutions and 1 active solution life cycles are needed to fulfil the requested timespan. The determination of the relationship between shelf life and food loss & waste probability (FLWP) was based on a probability chart first proposed by Conte et al. (2015). This approach is based on two main assumptions: FLW probability is 100% when shelf-life is null (I); FLW probability is 0 when shelf-life is infinite (II). The maximum expected shelf-life for raspberries (11 days) corresponds to the minimum avoidable waste for raspberries, equal to 2.5% (FAO FLW Database). As the relationship between FLWP and shelf-life is described by quality decay over time, a first-order kinetic curve was used to estimate it (Figure 1). The PFLW for the expected shelf-lives could be retrieved by the fitting equation:

$$y = e^{-0,335x} \quad (1)$$

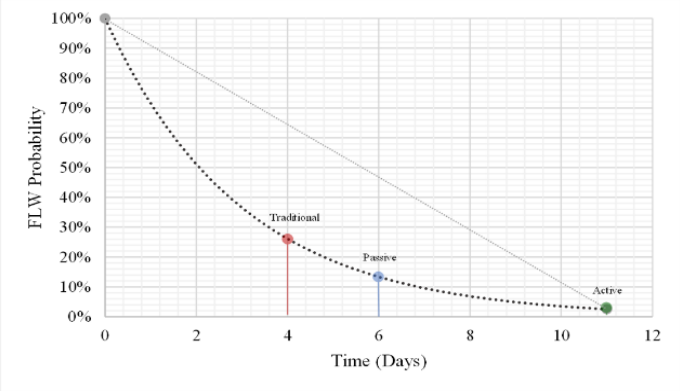

Figure 1 *Food loss and waste probability chart for packaged fresh raspberries.*

Different PFWL values depending on expected shelf-lives were calculated and summarized in Table 2. For the Explicit F.U., as the defined timespan is the same for all packaging systems, the PFLW estimated for 11 days was used as the reference value (PFWL=2.5%). In this case, the three systems show different PFLW values due to the different number of life cycles necessary to fulfil the Explicit Functional Unit.

Table 2 *Probability of Food Loss and Waste as a function of different shelf-lives and functional units.*

Food-packaging system	Shelf-life for Inherent F.U.	PFLW for Inherent F.U.	Time span for Explicit F.U.	PFLW for Explicit F.U.
Traditional	4 days	26.2 %	11 days	2.5*2.7 = 6.8 %
Passive	6 days	13.4 %	11 days	2.5*1.83 = 4.6 %
Active	11 days	2.5 %	11 days	2.5*1 = 2.5 %

4.4. Main results

The hotspot analysis highlighted the relevance of the impacts deriving from food production, which includes also the produced mass related to food waste. However, not in all the calculated impact categories such factor is as important, and attention should be given also to packaging production. In fact, raspberry production represents the main source of environmental impacts for Land Use (98.5 %), followed by Water Use (97.4 %). While, for Stratospheric Ozone Depletion packaging production is the most impactful factor with a responsibility of 79.9 %.

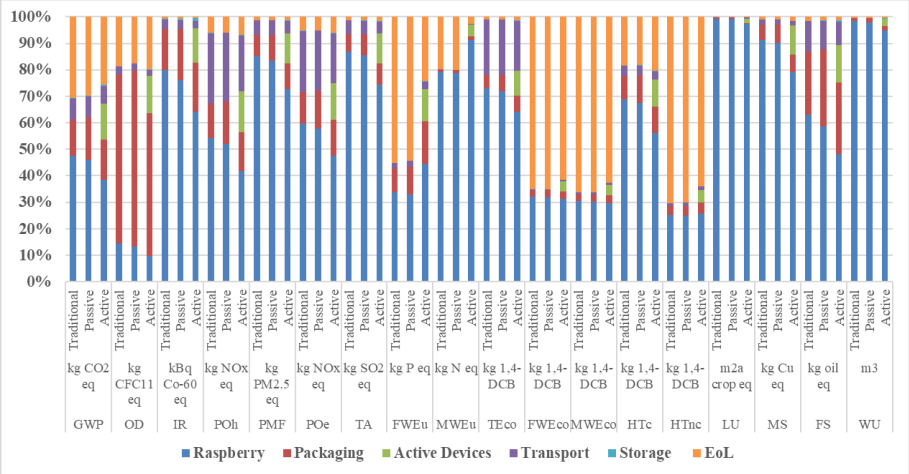

Figure 2 *Hotspot results coming from both F.U. The different contributing factors are highlighted in different colour, showing their relative contribution to the impacts of each impact categories.*

Characterization results obtained from the two functional units were graphically represented in the same figure to show the differences in the results (Figure 3). For Inherent F.U. (bars, Figure 3), active solution represents the worst scenario in 10 impact categories out of 18. Such results are accountable to packaging production and end-of-life, including active devices production and disposal. On the other hand, traditional solution shows the highest impacts in the impact categories that are mainly associated with an increased raspberry production as a consequence of a shorter shelf-life (4 days equal to 26,2% potential food waste). Passive solution resulted in the most balanced solution, representing the right compromise between shelf-life length and packaging inputs.
Explicit F.U. (symbols, Figure 3), due to different modelling choices, shows different trends. Traditional system (squares) represents the worst case for all the impact categories analysed. This is accountable for the greater quantities of inputs and outputs modelled to fulfil the Explicit F.U. (2.7 life cycle to fulfil the 11-day timespan). On the other hand, Active system (triangles) represents the best available solution for the required timespan. Passive solution (circles) represents the averaged solution. However, in this latter case, the overall best solution is active packaging system since it results in less environmental impacts in all impact categories.

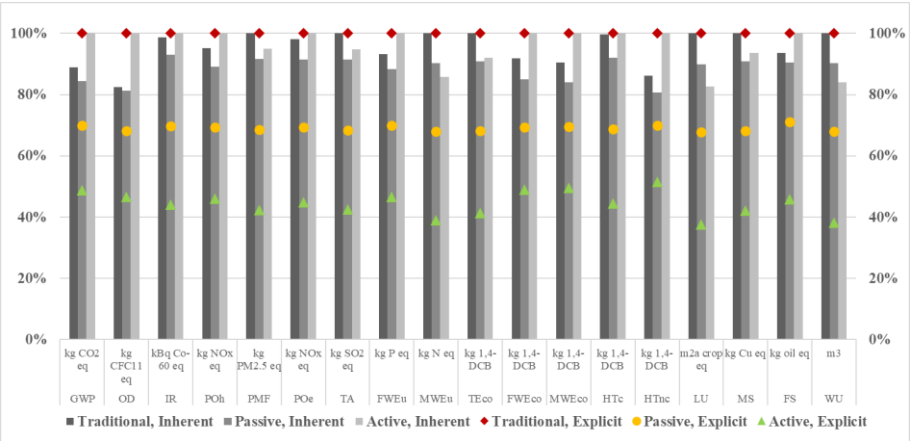

Figure 3 *Characterization results coming from Inherent F.U. (bars) and Explicit F.U. (symbols). Differences within and between the*

4.5. Discussion

Contrary to other LCA studies, that distinguished the two approaches depending on whether shelf-life was considered, this study included shelf-life as a fundamental part of the functional unit. It was demonstrated that depending on how the shelf-life is considered within the definition of functional unit in LCA studies, both results and methodological approaches changes consequently. In both cases, hotspot analysis suggests that food production is the main responsible for environmental impacts in the food-packaging life cycles. Such results confirm the principle that reducing food waste could mitigate the overall environmental profile of food-packaging systems. However, characterization and comparison results change depending on the defined functional unit. For

this reason, different purposes and scopes are answered by the two proposed functional units. Inherent F.U. permits the discussion on potentialities and issues related to different packaging solutions. Such approach could be useful for strategic decision-making processes in screening multiple packaging solutions by providing useful insights. On the other hand, Explicit F.U. includes the modelling of consequences on production, logistic and retail due to a stated performance requirement (i.e., 11-day timespan). Explicit F.U. should be implemented when strategic logistic and retailer choices must be met as it permits to discriminate packaging solutions based on their ability to fulfil such requirements.

5. Conclusion and Future perspective

The relevant role of shelf-life coupled with packaging performances and characteristics for an effective sustainability assessment of packaging environmental performances was studied. Moreover, the investigation of methodological approaches to directly include *indirect effects* into LCA studies was performed with the greater scope to harmonize current practices. It could be concluded that the search for sustainable food packaging should rely on different aspects of food-packaging life cycles. The balance between packaging inputs and protection should be assessed and interpretated to obtain reliable results when dealing with food packaging eco-design. The implementation of such concepts through LCA methodology requires expertise in modelling in interpretation phases. For such reasons, the development of an LCA-based simplified tool that could thoroughly calculate the environmental profile of packaging solutions is necessary to foster eco-design principles. Such tools should assist in validating the usage benefit of new packaging solutions and selecting the best packaging solution to reduce impacts coming both from food losses and packaging inputs. Mathematical simulation approaches coupling environmental impact assessment and prediction of gas transfer between foodstuff, packaging material, and environment are promising tools in the field (i.e., for Modified Atmosphere Packaging solutions) (Guillard et al., 2018). More research should be done in this direction to pave the way to a new definition of packaging sustainability and offer an innovative instrument for sustainable strategic choices within the food packaging field.

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Use of *Lactiplantibacillus* strains and yeasts for the production of fermented table olives and extra virgin olive oil

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This PhD thesis was focused on the characterization and selection of lactic acid bacteria (LAB) belonged to the *Lactiplantibacillus* group, and oil-born yeasts to be used as starter and/or adjunct cultures to improve the organoleptic and nutritional quality of extra virgin olive (EVOO) oil and fermented table olives. The PhD project will provide further information on the metabolism of phenolic compounds and the stress response mechanisms in LAB and yeasts, and on the effect of these cultures on the quality of the products.

Utilizzo di ceppi appartenenti al genere *Lactiplantibacillus* e lieviti per la produzione di olive fermentate e olio extravergine di oliva

La tesi di Dottorato è finalizzata alla caratterizzazione e selezione di batteri lattici appartenenti al gruppo *Lactiplantibacillus* e di lieviti da usare come colture starter e/o aggiuntive per migliorare la qualità organolettica e nutrizionale dell'olio extravergine di oliva e delle olive da tavola fermentate. Questo progetto fornirà nuove informazioni sul metabolismo dei composti fenolici e sui meccanismi di risposta allo stress nei batteri lattici e lieviti, nonché sull'effetto di questi microrganismi sulle proprietà di olio e olive fermentate.

Key words: *Lactiplantibacillus*; yeasts; starter cultures; extra virgin olive oil; fermented table olives.

1. Introduction

In accordance with the PhD thesis project, this oral communication reports the main results of the following activities:

a) production of fermented table olives (processed as Gaeta olives PDO) by using *Lactiplantibacillus pentosus* O17 as starter culture;
b) evaluation of yeast survival in extra virgin olive oils (EVOOs; *Leccino* and *Coratina* cultivars) and their effect on the analytical indices of EVOOs.

2. State of the Art

Extra virgin olive oil (EVOO) and table olives are traditional products of Mediterranean area obtained, respectively, from mechanical extraction and microbial fermentation of olive tree fruits (*Olea europea* L.). Fresh drupes, being characterised by a high phenolic compounds content (including the bitter secoiridoid oleuropein), cannot be immediately consumed after harvest and, therefore, the production of olive oil and the microbial fermentation are strategies to remove bitterness.

The microbiota of EVOO and fermented olives partially reflect that of olive fruits, and is affected by several factors including cultivars, harvest timing, geographical area, fruit maturation, production processes.

The microbiota of olive oil mainly consists of yeasts and, to a lesser extent, of bacteria and moulds, which are mostly entrapped in micro-drops of vegetation water (Zullo and Ciafardini, 2022). As widely demonstrated, members of *Candida oleophila*, *C. diddensiae*, *C. norvegica*, *C. parapsilosis*, *C. adriatica*, *C. temnochilae*, *C. dendronema*, *C. boidinii*, *Nakazawaea molendini-olei*, *N. wickerhamii*, *Saccharomyces cerevisiae*, *Kuraishia capsulate* and *Yamadazyma terventina* have been frequently isolated from fresh and stored olive oils (Mari et al., 2016; Guerrini et al., 2019; Zullo and Ciafardini, 2020; Zullo et al., 2021). On the contrary, few data on the occurrence of bacteria and moulds are available (Ciafardini and Zullo, 2002; Pizzolante et al., 2018; Santona et al., 2018; Fancelo et al., 2020).

The microbiota of fermented olives includes lactic acid bacteria (LAB; mainly belonging to the species *Lactiplantibacillus plantarum* and *Lpb. pentosus*) and yeasts (e.g. *Candida boidinii*, *C. diddendiae*, *C. olivae*, *Wickerhamomyces anomalus*, *Debaryomyces hansenii*, *Pichia galeiformis*, *P. membranifaciens*); members of *Clostridium* spp., *Pseudomonas* spp., *Staphylococcus* spp., *Enterobacteriaceae* and moulds are found on fermented olives of poor quality (Bonatsou et al., 2017).

Yeasts and LAB may positively or negatively affect the physicochemical and sensory features of olive products, based on the presence of specific enzymatic activities (Perpetuini et al., 2020; Zullo and Ciafardini, 2022). ß-glucosidases and esterases (involved, respectively, in the degradation of oleuropein and its derivative oleuropein aglycons) lead the reduction of bitter taste and the improvement of antioxidant (hydroxytyrosol production) features (Perpetuini et al., 2020; Zullo and Ciafardini, 2022). On the other hand, phenoloxidases and peroxidases (enzymatic oxidation of polar phenols; Zullo et al., 2020), pectinases (pectin metabolism with release of methyl

and ethyl free fatty acid esters; Zullo and Ciafardini, 2022) and lipases (hydrolysis of triglycerides with the release of free fatty acids; Ciafardini et al., 2006a; 2006b) negatively affect the quality of EVOO. Moreover, yeasts can produce metabolites related to positive or negative attributes of olive oils that affect the quality of this product (Zullo et al., 2013; Guerrini et al., 2019).
During the fermentation processes, LAB promote the brine acidification through the production of lactic acid from fermentable sugars, improving the microbiological stability and shelf-life of olives; yeasts, on the other hand, produce metabolites related to positive attributes and enhance the growth of LAB through the degradation of phenolic compounds (Bonatsou et al., 2017; Perpetuini et al., 2020).
Some productions are still craft-based, driven by the autochthonous olive microbiota, and result in a final product of variable quality. The use of appropriate LAB and/or yeast starter cultures, then, may reduce the development of spoilage microorganisms and leads to a more controlled and stable fermentation process. Moreover, the use of selected and characterized olives-borne LAB and/or yeasts, may improve the organoleptic and nutritional properties of products (Bonatsou et al., 2017; Perpetuini et al., 2020).

3. Materials and Methods

3.1. Production of fermented table olives

Black olives of *Itrana* cultivar were hand-harvested at the stage of full maturity (March 2022) in olive orchards located in Formia (Lazio, Italy). Olives without mechanical or insect damage were washed with tap water, put in sterile vessels (olive:water, 3:2) and processed as *Gaeta Olives* (production regulations PDO) with some modifications as described in Figure 1. *Lpb. pentosus* O17, previously selected for phenolic compound tolerance, oleuropein degradation and hydroxytyrosol formation, biofilm formation, high radical scavenging activity, resistance to simulated gastro-intestinal trait and to the combinations of NaCl ≤ 8% and pH ≥ 4.0–4.5 (Giavalisco, 2021; Zotta et al., 2022) was used as starter.
Lpb. pentosus O17 were cultivated in WMB (Zotta et al., 2012) supplemented with 4% (w/v) NaCl (WMB+N) for the adaptation to saline environment (30°C, 24h), and standardized to a final population of 10^6 cfu/g in olives.
O17 strain was used to inoculate the trial B and C, while the trial A (spontaneous fermentation) was used as control. The pilot-scale fermentations were performed in triplicate.
Fermentations were monitored at regular time intervals (up to 6 months) through the determination of pH, salt concentration, total phenol content (on both olives and brines) and titratable acidity (on brines). Microbiological analyses were carried out for both brines and olives. LAB was detected on mMRS (Ricciardi et al., 2015) with 100 mg/L cicloeximide and 15 mg/L nalidixic acid (mMRS+C+AN), 30°C for 48 h; yeasts and moulds on Glucose-Yeast Extract-Agar (GYEA) with 100 mg/L chloramphenicol, 30°C for 48 h and for 5 days, respectively; *Enterobacteriaceae* on VRBGA, 24 h at 30°C; total bacterial count on Gelatin PeptoneAgar (AG), 30°C for 48 h; halophilic LAB, yeasts and other bacteria on mMRS+C+AN, GYEA and AG supplemented with 6% (w/v) NaCl, respectively.

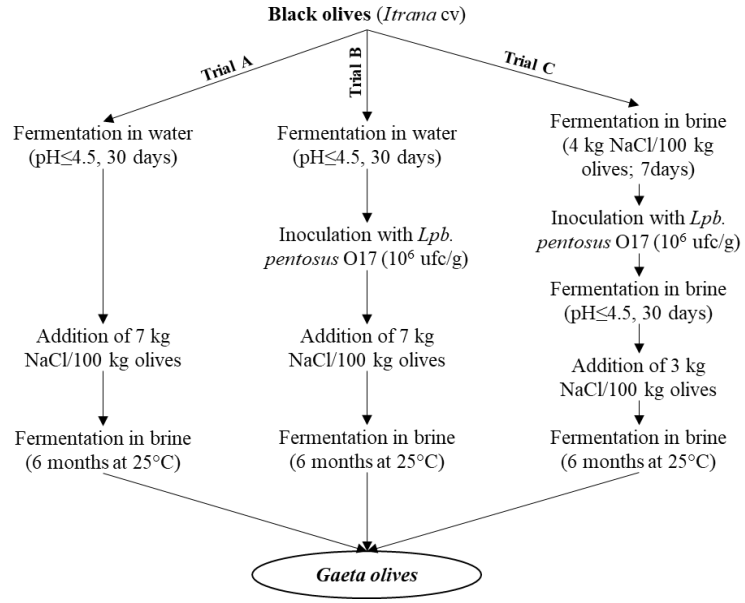

Figure 1. *Flowchart for the production of Gaeta-like olives.*

3.2. Extra virgin olive oil

3.2.1. Survival of yeasts in EVOO

Seventeen oil-born yeasts previously characterized (Giavalisco, 2021) for enzymatic activities, for the ability to grow in presence of different sugars, at low temperatures, high NaCl concentrations, low pH, for oleuropein

hydrolysis and for OMW survival, were inoculated (final population 10^6 ufc/mL) in 3-months old EVOOs from *Leccino* and *Coratina* cv, as described by Zullo et al. (2013). Uninoculated EVOOs (U-EVOO) and U-EVOOs containing 0.5 mL of sterile water (the water content used to prepare the cell suspension; UW-EVOO) were used as controls. All sample were incubated in dark, for 6 months at 20°C and, at 1-month intervals, survival of yeasts was evaluated by plate counting on MYPG agar (28°C, 3 days of incubation).

3.2.2. Effect of yeast inoculum on the analytical indices of EVOO

Free fatty acids, peroxide values, UV spectrophotometric indices (K_{232}, K_{270}, extinction coefficients K_{266} and K_{274} were also measured and used for estimating ΔK) and total phenol content were evaluated for uninoculated (controls) and inoculated olive oil samples at the beginning of trials (t0) and after 3 and 6 months (t3, t6) of storage. All parameters were determined in triplicate, according to the European Community's 2568/91 Regulation (EC, 1991) and following amendments (European Community's 2019/1604).

4. Results and Discussion

4.1. Production of fermented table olives

The production of Gaeta-like olives, based on spontaneous fermentation (as from PDO disciplinary; trial A), was compared with two fermentative processes (trails B and C) driven by *Lpb. pentosus* O17, used as a starter culture. This communication includes the main results obtained after 3 months of fermentation.

The washing step of fresh drupes, before incubation, was effective in reducing the initial microbial population (LAB < 9 cfu/g, yeasts 2.0×10^2 cfu/g, moulds < 9 cfu/g and *Enterobacteriaceae* 6.0×10^4 cfu/g).

After 30 days of incubation, *Enterobacteriaceae* were reduced (< 9 ufc/mL) in both olives and brines of all trials; while *Lpb. pentosus* O17 was detected at a level of 10^6 cfu/mL in brines and 10^5 cfu/g on olive surface in trials B and C, as demonstrated by the colonies morphology (light-blue colonies with dark-blue centres) on mMRS+C+AN. The addition of NaCl (7 kg NaCl/100 kg olives; after 25 days of fermentation; trial B) reduced the viability of *Lpb. pentosus* O17 and no colony attributable to the strains was detected on mMRS+C+AN (after 40 days). On the other hand, competition with yeast population may have affected the development of *Lpb. pentosus* O17; after 3 months of fermentation, in fact, yeasts increased up to 10^4 cfu/g to the olive surface and up to 10^6 cfu/mL in brines, for all trials. Moulds were not detected. The presence of pathogenic microorganisms (*Salmonella* spp. and *Listeria monocytogenes*) will be evaluated at the end of fermentation process (6 month).

The presence of starter culture boosted the pH decrease in brines (up to pH 4.5 after 25 days; trials B and C) compared to the spontaneous fermentation (pH 4.5 after 35 days; trail A); however, the addition of NaCl slowed the acidification, probability because of inhibition of starter culture. NaCl content in brine decreased during fermentation due to the partial diffusion into olives; on the other hand, the total phenolic content in olives decreased because of partial diffusion in brine and/or to yeast metabolism. The extraction of total DNA from both olives and brines, for metataxonomic studies, is in progress. Microbiological and physicochemical analyses carried out in the first step of fermentations (3 months) revealed that the use of starter culture promoted a faster acidification due to the more efficient consumption of soluble sugars, and reduced enterobacteria in a shorter period compared to spontaneous fermentation.

However, the further analyses to be carried out until the final product is obtained (6 months of fermentation) will allow to clarify the potential effect of *Lpb. pentosus* O17 on the quality of Gaeta-like olives.

4.2. Extra virgin olive oil

4.2.1. Survival of yeasts in EVOO

The three-month-old olive oils (*Leccino* and *Coratina* cv) were characterized for analytical indices and classified as EVOOs. Yeast viability decreased during EVOO storage, although the level of survival was strain-dependent. The greater reduction of yeast population, however, was measured after 1 month of storage. Generally, except for *Schwanniomyces polymorphus* (2.6 log cycle reduction), a high level of survival (about 1.4 log cycles reduction up to 6 months) was observed for all yeasts in both EVOOs (although they had a different total phenol content; Zotta et al., 2022), confirming that the oil microbiota consists mainly of yeasts. In the controls U-EVOO and UW-EVOO no growth was detected during the storage.

Many authors have already shown the persistence of different species (*C. parapsilosis*, C. *adriatica*, *C. diddensiae*, *N. wickerhamii*, *N. molendiniolei*, *Y. terventina*) in olive oils after storage (ranging from 3 up to 24 months; Ciafardini and Zullo, 2002; Ciafardini et al., 2013; Zullo et al., 2013; Guerrini et al., 2019). However, the survival of yeasts in olive oils is strongly conditioned by the oil composition, physical treatments (e.g., filtration), high phenols and low water contents (Ciafardini and Zullo, 2022; Zullo and Ciafardini, 2022).

4.2.2. Effect of yeast inoculum on the analytical indices of EVOO

The effect of yeasts on the analytical indices was evaluated after 3 (t3) and 6 (t6) months of storage. No difference was found between U-EVOOs and UW-EVOOs, indicating that the addition of water did not affect the analyzed parameters.

Although the yeast strains did not show any lipolytic activity in *in-vitro* assays (Giavalisco, 2021), different acidity values (expressed as % of oleic acid) were detected in EVOOs during storage. Acidity increased more in the second period (3-6 months) of storage compared to the first one (0-3 months), for both *Leccino* and *Coratina* EVOOs.
After 6-months storage, only the *Leccino*-EVOO inoculated with *L. fermentati* Y26 (0.818%) and *N. wickerhamii* Y18 (0.885%) slightly exceeded the acidity limit of EVOO (≤ 0.800%). The other stains seemed to have a protective effect (FFA reduction up to 57%) compared to the U-EVOO. The reduction in FFA was less evident for inoculated *Coratina*-EVOO. Nevertheless, the *Coratina*-U-EVOO exceeded (0.870%) the limit fixed for EVOO classification.
At the end of storage, the peroxide values (expressed as milliequivalent of active oxygen per kg of olive oil, mEq O_2/kg oil) as well as the values of K_{232}, exceeded those defined for EVOO (≤ 20.0 mEq O_2/kg oil and ≤ 2.5, respectively). The extinction coefficients K_{270} and ΔK, linked to secondary oxidation with consequent formation of aldehydes and ketones, overcame the EVOO limits ($K_{270} \leq 0.22$; $\Delta K \leq 0.01$), depending on the inoculated strain.
During the 6-months storage, total phenol content (TPC) decreased differently according to the strain used. The highest reduction was detected in the first period (0-3 months) of storage, from 10% (*L. fermentati* Y26) to 24% (*N. molendiniolei* Y7 and Y28) in *Leccino*-EVOO and from 12% (*C. boidinii* Y6 and *N. wickerhamii* Y18) to 46% (*S. polymorphus* Y1) in *Coratina*-EVOO.
The results suggested that the presence of certain yeast species could damage the EVOO quality as they negatively affect the peroxide values and the analytical indices associated with the primary and secondary oxidation of lipids. In contrast with these results, some authors shown that the presence of some strains belonging to *N. wickerhamii*, *Y. terventina*, *N. molendini-olei* (Guerrini et al., 2019) and *C. diddensiae* (Zullo et al., 2013) did not impair the analytical indices of oil remained commercially classified as EVOOs after the storage period.
Other authors (Ciafardini et al., 2015; Zullo et al., 2018a; 2018b; Zullo and Ciafardini, 2022) found that some yeasts negatively affected the organoleptic properties of olive oil.
Given the significant survival of yeasts in olive oil samples, the control of their development and their contamination during the production process is of crucial importance to guarantee the quality of final product. Moreover, the use of undamaged drupes, colonized by a "healthy" microbiota may contribute to obtain a high-quality oil.

5. Conclusions and Future Perspectives

The microbiota of olive fruits and the changes that occur during the production of oil and fermented olives strongly affect the final products. Yeasts are certainly the microbial group that mostly survives in both oil and table olives and, therefore, strategies for their control are needed to reach high-quality products.
The use of properly selected starter cultures may be an efficient strategy for the production of fermented olives, while for the production of olive oil the control of fruit quality as well as process and storage conditions are fundamental.
Furthermore, further studies on the evolution of olive oil and fermented olives microbiota, could be useful to better control and tune the production processes of these products.

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Flour rheological properties assessed through empirical and fundamental methods

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Fundamental rheology is a useful tool for studying viscoelastic and microstructure properties of cereal flour dough. This technique can be combined with empirical methods to have complementary information on the characteristics of these systems. In this study, the fundamental rheological analysis of dough made through a rheometer for an in-depth characterization was set up and the outputs were correlated to the results of the farinograph and alveograph analysis. Dough studied through fundamental rheology were prepared from flours with different extraction degree, and were hydrated according to the output of the farinograph analysis.

Proprietà reologiche di sfarinati valutate attraverso metodi empirici e fondamentali

La reologia fondamentale rappresenta uno strumento utile per studiare le proprietà viscoelastiche e microstrutturali degli impasti a base di farine di cereali. Questa tecnica, combinata con i metodi empirici, fornisce informazioni complementari sulle caratteristiche di tali sistemi. In questo studio è stato messo a punto un protocollo per la caratterizzazione degli impasti mediante l'utilizzo di un reometro, i cui outputs sono stati correlati con i risultati ottenuti mediante analisi farinografica e alveografica. Gli impasti studiati con la reologia fondamentale sono stati ottenuti da farine con diverso tasso di estrazione idratate in base ai risultati dell'analisi farinografica.

Key words: cereals, wheat, dough, empirical rheology, fundamental rheology, viscoelastic properties.

1. Introduction

This study reports the main results regarding the assessment of the technological aptitude of dough by means of empirical methods (farinograph and alveograph) and fundamental rheology (rheometer). Various authors studied dough and flour properties using both fundamental and empirical rheology measurements (Janssen Van Vliet et al., 1996; Watanabe Bell et al., 1998; Kenny Wehrle et al., 1999; Ronda Pérez-Quirce et al., 2013; Sanchez Puppo et al., 2014), however, a correlation between the parameters from the two methods has not yet been reported. To validate this type of investigation, dough from different types of flours were studied.

2. Materials and Methods

Commercial wheat flours (flour type: 00, 0, 1, 2, 0 Manitoba indicated as type 0 M and whole grain indicated as WG) and semolina (conventional, S, and whole grain, S-WG) were purchased from a local store. Proximate composition of samples was evaluated using Official Methods. Moisture and ash content were determined according to ICC standard 109/1 and 104/1 (ICC, 1995), respectively; protein and lipids were determined following the methods of the Official Journal of Italian Republic n. 186/1994; fiber content was determined using enzymatic essay K-TDFR-100A/K-TDFR-200A 04/17 kit (Megazyme, Ireland) and carbohydrates were calculated by difference.

Farinograph and alveograph analysis was carried out according to AACC International Methods 54-20 and 54-30, respectively. Farinograph main indices were water absorption, dough development time and dough stability, while tenacity and elasticity were determined through alveograph analysis.

For fundamental rheological analysis, dough were prepared through farinograph kneader according to their optimum water absorption and development time. Fundamental rheological tests were carried out through a rheometer (Haake MARS III-Thermo Scientific) equipped with a 20 mm parallel plate probe working with 2 mm gap distance, except for temperature sweep measurements where the gap distance was fixed at 1 mm to ensure a homogenous heat dispersion within the sample. Samples were checked through creep-recovery and oscillatory amplitude, frequency and temperature sweep tests. For rheological testing each replicate represents a separately mixed dough.

SPSS software (version 22.0, IBM SPSS Statistics, Armonk, NY, USA) was used for statistical analysis. Data were evaluated by the analysis of variance by ANOVA, multiple comparisons using Scheffè's post hoc test and bivariate correlations with two-tailed significance for Pearson coefficient quantification. All numerical results are averages of 3 independent replicates values are represented as the mean ± standard deviation.

3. Results and Discussion

Proximate analysis and main outputs of the fundamental and empirical rheology are summed up in Table 1.

Table 1 *Proximate analysis and rheological data outcomes. WA = water absorption; DDT = dough development time; SD = dough stability; P = tenacity; L = elasticity; G' LVE = amplitude sweep consistency value; K = frequency sweep consistency value; n = frequency sweep stability; T Gel = gelatinization temperature; Δ Log G' = gelatinization process rate; J_{max} = maximum deformation; J_r = recovery ability.*

	Type 00	Type 0	Type 1	Type 2	WG	Type 0 M	S	WG-S
Proximate analysis								
Moisture (%)	13.3 ^{a} ± 0.02	13.3 ^{a} ± 0.03	12.3 ^{b} ± 0.01	13.4 ± 0.01	12.3 ^{b} ± 0.02	12.6 ± 0.01	12.5 ± 0.01	11.0 ± 0.01
Fat (% fw)	0.80 ± 0.03	1.30 ± 0.02	1.00 ± 0.03	1.40 ± 0.04	1.90 ± 0.02	1.10 ± 0.02	1.50 ± 0.03	2.80 ± 0.02
Carbohydrate (% fw)	73.00 ± 0.03	72.00 ± 0.03	69.00 ± 0.05	65.00 ± 0.05	62.00 ^{a} ± 0.02	70.00 ± 0.04	68.00 ± 0.04	62.00 ^{a} ± 0.05
Protein (% N*5.70 fw)	10.00 ± 0.01	10.40 ± 0.02	11.50 ± 0.03	12.00 ± 0.01	12.30 ± 0.01	13.00 ± 0.02	14.00 ± 0.02	15.00 ± 0.01
Ash (% fw)	0.32 ± 0.02	0.41 ^{a} ± 0.01	0.70 ^{b} ± 0.03	0.71 ^{b} ± 0.02	1.56 ^{c} ± 0.04	0.44 ^{a} ± 0.03	0.80 ± 0.03	1.55 ^{c} ± 0.06
Fiber (% fw)	1.80 ^{a} ± 0.01	2.00 ± 0.02	4.00 ± 0.02	7.15 ± 0.01	9.70 ± 0.02	1.80 ^{a} ± 0.03	2.90 ± 0.04	8.00 ± 0.05
Empirical rheology								
WA (%)	53.70 ± 0.10	54.10 ± 0.20	57.00 ± 0.10	57.40 ± 0.10	66.90 ± 0.10	59.50 ± 0.20	64.10 ± 0.10	67.00 ± 0.20
DDT (min)	1.70 ± 0.10	1.80 ^{a} ± 0.20	1.80 ^{a} ± 0.30	1.90 ^{ab} ± 0.20	8.10 ± 0.10	2.10 ^{b} ± 0.10	5.50 ^{a} ± 0.10	5.60 ^{a} ± 0.20
DS (min)	8.30 ± 0.10	8.80 ± 0.15	10.10 ± 0.20	12.30 ± 0.10	13.40 ± 0.10	18.70 ± 0.25	17.90 ^{a} ± 0.30	18.10 ^{a} ± 0.20
P (mm H_2O)	69.00 ± 2.10	72.90 ± 1.50	78.00 ± 3.14	162.0 ± 1.40	200.0 ± 1.20	65.00 ± 2.40	137.0 ± 1.97	140.0 ± 2.30
L (mm)	80.00 ± 0.70	77.00 ± 0.80	55.70 ± 0.95	30.50 ± 0.60	21.7 ± 0.85	118.0 ± 0.90	85.00 ± 1.10	44.00 ± 0.95
P/L (-)	0.86 ^{a} ± 0.20	0.93 ^{a} ± 0.15	1.40 ± 0.25	5.31 ± 0.10	9.21 ± 0.15	0.55 ± 0.10	1.61 ± 0.20	3.18 ± 0.25
Fundamental rheology								
G' LVE (10^4 Pa)	1.11 ^{a} ± 0.12	1.21 ^{ab} ± 0.10	1.34 ^{b} ± 0.14	1.80 ± 0.18	2.01 ± 0.10	1.01 ^{a} ± 0.10	1.18 ^{ab} ± 0.15	2.40 ± 0.20
K (10^4 Pa s^n)	1.24 ^{a} ± 0.10	1.25 ^{a} ± 0.07	1.29 ^{a} ± 0.08	1.78 ± 0.12	1.97 ± 0.08	1.11 ± 0.09	1.29 ^{a} ± 0.06	2.81 ± 0.08
n (-)	0.14 ^{a} ± 0.03	0.15 ^{a} ± 0.01	0.16 ^{ab} ± 0.02	0.17 ^{b} ± 0.02	0.19 ^{bc} ± 0.01	0.16 ^{ab} ± 0.02	0.19 ^{bc} ± 0.02	0.22 ^{c} ± 0.03
T Gel (° C)	56.40 ± 0.50	58.30 ^{a} ± 0.40	58.90 ^{a} ± 0.50	60.50 ± 0.70	63.50 ± 0.50	57.70 ± 0.40	55.80 ± 0.50	63.00 ± 0.60
Δ Log G' (Pa)	0.90 ^{a} ± 0.02	0.88 ^{a} ± 0.03	0.82 ± 0.01	0.70 ^{a} ± 0.01	0.67 ± 0.02	0.85 ± 0.01	0.79 ± 0.02	0.70 ^{a} ± 0.03
J_{max} (10^{-4} 1/Pa)	11.10 ± 0.09	10.55 ± 0.07	9.30 ± 0.11	8.15 ± 0.10	7.24 ± 0.08	15.71 ± 0.14	9.53 ± 0.10	3.92 ± 0.06
J_r (%)	62.8 ^{a} ± 0.10	62.3 ^{a} ± 0.15	58.2 ± 0.18	56.8 ^{b} ± 0.13	55.5 ± 0.10	62.6 ^{a} ± 0.14	56.9 ^{b} ± 0.09	47.9 ±0.12

Proximate composition (Table 1) shows that, as expected, protein, ash and fiber content increase with the extraction degree. From the farinograph analysis outcomes (Table 1) it emerges that samples from less refined flours required more water (WA) and time (DDT) for complete dough development and had higher stability (DS) compared to samples obtained from more refined flours. This was mainly due to the higher amount of proteins and fiber characterizing less refined flours. Moreover, considering the outcomes from alveograph analysis (Table 1), tenacity (P) values increased with the extraction degree, while elasticity (L) showed an opposite trend. As a consequence, P/L ratio representing the elastic resistance and extensibility balance of dough increased with the extraction degree. The detected trends were in agreement with the available literature data and can be related, again, to the higher levels of fiber and its interactions with the proteins (Mirsaeedghazi et al., 2008).

Figure 1 shows an example of fundamental rheology tests carried out. Numerical outcomes of fundamental rheology are reported in Table 1. Amplitude sweep test (Figure 1 A) is performed by deforming the sample at a fixed frequency and measuring the sample response in order to individuate the linear viscoelastic range (LVE) wherein both elastic (G') and viscous (G'') moduli are not influenced by the applied deformation (Lynch Dal Bello et al., 2009). LVE of the dough studied corresponded to a zone of small deformation ($\gamma < 10^{-3}$) in all the samples. After this deformation limit, the moduli changed with the applied strain thus entering the non-linear region (NL). However, no significant differences were detected in the LVE of the different dough. The phase angle values (Tanδ) given by G''/G', can assume values close to 0 for solid-like materials, equal to 1 when G'=G'' and higher than 1 for liquid like material (Mezger, 2020). As reported in Figure 1A Tanδ is smaller than 1 demonstrating the predominant solid-like behavior of dough. Moreover, the maximum value of G' modulus (in LVE) expresses dough consistency. G' was found increasing with extraction degree, an aspect that was in agreement with farinograph dough stability (DS) values because the higher is the overall consistency, the lower is dough's breakdown. The consistency data were also in agreement with alveograph tenacity (P) and elasticity (L) outcomes because higher values led to less elastic and thus stronger dough.

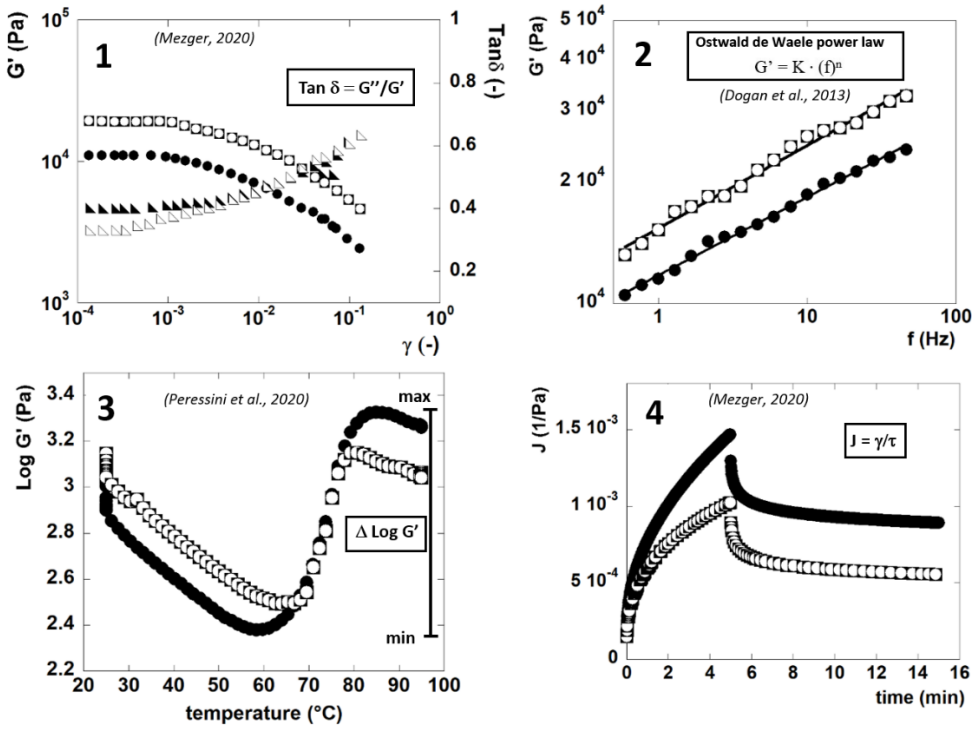

Figure 1 *Fundamental rheology tests; A) amplitude sweep; B) frequency sweep; C) temperature sweep; D) creep-recovery. Full back dots = Type 00 flour; empty white dots = WG flour. In the amplitude sweep, triangles refer to Tan δ values.*

Frequency sweep test (Figure 1 B) conducted fixing the deformation level within the LVE allowed the study of the dependence of G' and G" moduli with the oscillation frequency, obtaining information on the stability of the system. G' modulus response was fitted to Ostwald de Waele power model (Dogan et al., 2013):

$$G' = K \cdot (f)^n \qquad (1)$$

K represents the consistency of the material while n (-) represents the dependence with the oscillation frequency, expressing the stability of the system. Correlation coefficients of fitting, R, ranged between 0.93 and 0.99, thus indicating the good attendance of the interpolation. As expected, both *K* coefficients and *n* values were in agreement with amplitude sweep results and farinograph outcomes. Temperature sweep test (Figure 1 C) allowed the assessment of starch gelatinization process measuring the deformation response of the sample to the temperature increase (Peressini, 2001). Particularly, the process rate was quantified by means of Δ Log G' values (Peressini et al., 2020). G' trend with temperature was properly described in literature (Angioloni & Dalla Rosa, 2005) and, particularly, gelatinization temperature resulted to increase with the extraction degree, probably due to the increasing in fiber and protein content related to the binding effect of the available dough water, thus hindering starch granules hydration process which is the basis of the gelatinization of starch. As a consequence, the process rate resulted to be lower. Finally, creep and recovery test (Figure 1 D) was conducted under high stress conditions exceeding the LVE, thus simulating productive process conditions, for example a baking process (Peressini, 2017). The main indices for the description of the samples' behaviour in non-linear (NL) range of deformation were the maximum deformation (J_{max}) and the recovery ability (J_r), both expressed as creep compliance given by the ratio between strain and stress ($J = \gamma/\tau$) (Steffe, 1996). However, this test confirmed dough viscoelastic character evidencing its intermediate behaviour between elastic materials that recover their shape completely and viscous ones that remain deformed and the results showed that less refined samples had lower maximum deformation but also lower recovery ability.
The statistical analysis of correlations are summed up in Table 2 wherein the significant correlations (*p-value* < 0.05) were highlighted by cell coloring.

Table 2 *Statistical analysis of correlations.*

Proximate composition and empirical rheology		**WA**	**DDT**	**DS**	**P**	**L**	**P/L**
Moisture	Pearson Correlation	-.746	-.549	-.545	-.175	.172	.015
	Significance	.054	.202	.206	.708	.712	.975
Fat	Pearson Correlation	.814	.702	.605	.646	-.358	.428
	Significance	.026	.079	.150	.117	.430	.338
Carbohydrate	Pearson Correlation	-.802	-.728	-.365	-.856	.753	-.764
	Significance	.030	.063	.421	.014	.051	.045
Protein	Pearson Correlation	.802	.534	.908	.335	.008	.019
	Significance	.030	.0.049	.005	.462	.987	.968
Ash	Pearson Correlation	.887	.862	.332	.767	-.683	.647
	Significance	.008	.013	.046	.051	.091	.116
Fiber	Pearson Correlation	.631	.651	.058	.870	-.901	.882
	Significance	.129	.113	.901	.011	.006	.009

Proximate composition and fundamental rheology		**G' LVE**	**K**	**n**	**T Gel**	**Δ Log G'**	**J_{max}**	**J_r**
Moisture	Pearson Correlation	-.600	-.695	-.807	-.528	.395	.551	.784
	Significance	.154	.083	.028	.223	.380	.200	.037
Fat	Pearson Correlation	.774	.850	.669	.620	-.699	-.623	-.786
	Significance	.041	.015	.051	.137	.081	.135	.036
Carbohydrate	Pearson Correlation	-.720	-.651	-.850	-.796	.976	.770	.856
	Significance	.003	.015	.054	.006	.001	.053	.064
Protein	Pearson Correlation	.460	.562	.880	.310	-.494	-.374	-.715
	Significance	.299	.189	.009	.498	.259	.408	.071
Ash	Pearson Correlation	.885	.851	.876	.857	-.848	-.800	-.867
	Significance	.008	.015	.010	.014	.016	.031	.012
Fiber	Pearson Correlation	.926	.813	.667	.945	-.951	-.790	-.750
	Significance	.003	.026	.102	.001	.001	.035	.049

Empirical rheology and fundamental rheology		**G' LVE**	**K**	**n**	**T Gel**	**Δ Log G'**	**J_{max}**	**J_r**
WA	Pearson Correlation	.643	.662	.918	.592	-.707	-.550	-.748
	Significance	.120	.105	.004	.161	.076	.201	.049
DDT	Pearson Correlation	.576	.550	.762	.554	-.668	-.549	-.605
	Significance	.176	.201	.046	.197	.101	.202	.150
DS	Pearson Correlation	.161	.274	.649	.097	-.271	.021	-.382
	Significance	.730	.552	.011	.836	.556	.964	.398
P	Pearson Correlation	.723	.605	.645	.680	-.918	-.675	-.612
	Significance	.047	.015	.118	.043	.004	.096	.144
L	Pearson Correlation	-.809	-.661	-.424	-.789	.809	.826	.622
	Significance	.028	.010	.343	.035	.027	.022	.136
P/L	Pearson Correlation	.660	.485	.366	.758	-.845	-.514	-.390
	Significance	.010	.027	.420	.048	.017	.238	.387

Statistical analysis of correlations confirmed the previous presented trends of empirical rheology outcomes with extraction degree. Particularly, protein and ash resulted positively correlated to farinograph outcomes while fiber was correlated to alveograph analysis. As expected, the higher was the protein content, the higher was dough stability (DS), while in the case of alveograph outcomes, they resulted to be strongly correlated with fiber content that increased tenacity (P) and decreased elasticity (L). Proximate analysis components were correlated also to rheometer outcomes. In fact, considering fundamental rheology, frequency sweep stability index (n) coming from Ostwald de Waele power law model (Equation 1) resulted to be positively correlated with protein content, confirming farinograph stability results. This was again in agreement with the expected data, because the higher is the protein content and the higher is the gluten matrix formation. Moreover, higher fiber contents led to higher consistency of dough (G' LVE and K) but also to higher gelatinization temperatures (T Gel) and lower gelatinization degree (Δ Log G'), dough recovery ability (J_r) and maximum deformation (J_{max}). On the other hand, carbohydrate levels resulted to be correlated with consistency values and temperature sweep outcomes with opposite trend. Furthermore, considering the main aim of this investigation, the most important correlations were those between empirical and fundamental rheology. Particularly, both alveograph and farinograph analysis can be correlated with rheometer results. Consistency values (G' LVE and K) were positively correlated with dough tenacity (P) and negatively correlated with its elasticity (L) because the higher is the strength of the former and the lower it can be extended. Moreover, P values were positively and negatively correlated with T Gel and Δ Log G', respectively, while L values showed a negative correlation. This latter resulted to be positively correlated with J_{max} values. Obviously, configuration ratio P/L reflects P and L correlation. The negative correlation between P and Δ

Log G' can be explained considering the higher amounts of fiber leading to higher P values. In fact, as previous discussed, fiber and proteins conferring higher P values are able to bind the available dough water hindering starch hydration and swelling process. Finally, all farinograph indices resulted to be positively correlated with n stability values.

4. Conclusions and future perspectives

The adopted approach for dough characterization in relation with raw material extraction degree showed a good correlation between empirical and fundamental rheology within LVE, demonstrating its reliability and usefulness. Farinograph and alveograph analysis indicated that higher protein contents were related to higher stability, while higher amounts of fiber led to higher tenacity and lower elasticity of dough. However, dough viscoelastic properties can be in-depth studied through fundamental rheology methods. Particularly, the predominant solid-like character of dough was confirmed and fundamental rheology consistency results were in agreement with alveograph tenacity and elasticity results. Farinograph stability outcomes were also confirmed by frequency sweep data and the overall stability seemed to be mainly influenced by protein content. Starch gelatinization process started in a temperature range between 55-65 °C in all the samples. Gelatinization profiles were similar but gelatinization temperature increased with the extraction degree. Non-linear behaviour of dough showed that part of the deformation generated by a loading stress phase was not recovered because of its viscoelastic character. Moreover, the increasing in the extraction degree seemed to decrease dough recovery ability. Finally, on the basis of the statistical analysis of correlations between empirical and fundamental rheology, it can be affirmed that the combination of the two approaches can allow the achievement of complementary informations for an in-depth rheological characterization of dough. In this way, it is possible to accurately investigate the effect of every process variable on dough properties, also in a very small-scale. Moreover, considering that empirical methods require some adjustments to the official protocols, in some cases and with particular samples (i.e. high amylose wheat flour but also some whole grain flours) (De Arcangelis et al., 2021), analytical limits and thus incorrect outcomes, the integration of fundamental rheology could be a tool to overcome these limits. However, starting from these results, the study of the rheological characteristics of dough but also isolated starch and non-starch polysaccharides can be considered. Moreover, the effectiveness of the combined approach for the prediction of functional properties and technological aptitude of flours and dough and its applicability on final products should be checked.

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Innovative techniques to encapsulate food-grade bioactives

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This work aims to investigate novel encapsulation techniques for food-grade bioactive formulations. An innovative encapsulation technique, called Particle from Gas-Saturated Solution technique, was used to encapsulate bioactive compounds into different food-grade wall materials. To this purpose linseed oil was encapsulated with or without the addition of ether an extract of supercritical carbon dioxide (CO_2) extracted carrot pomace or pure β-carotene. The chemical-physical characterization, as well as the oxidative stability of the produced microparticles was investigated. The results highlighted the efficiency of the encapsulation technique and the ability of carrot pomace extract and β-carotene to retard the oxidation of linseed oil.

Tecniche innovative per l'incapsulazione di bioattivi alimentari

Questa tesi di dottorato comprende lo studio di tecniche innovative per incapsulare composti bioattivi da utilizzare nell'ambito alimentare. Una tecnica innovativa, chiamata "Particle from Gas Saturated Solutions", è stata applicata per incapsulare composti bioattivi in matrici alimentari. Per questo studio olio di lino è stato incapsulato con o senza l'aggiunta di β-carotene o di un estratto ottenuto attraverso l'estrazione con l'anidride carbonica supercritica dagli scarti della lavorazione delle carote. Le polveri ottenute sono state caratterizzate con analisi chimico-fisiche. Inoltre, è stata analizzata la stabilità ossidativa dei campioni. I risultati hanno evidenziato le potenzialità d'incapsulamento della tecnica e la capacità dei bioattivi di ritardare l'ossidazione dell'olio incapsulato.

Key words: carrot pomace; β-carotene, antioxidant capacity; particles from gas saturated solutions; oxidative stability.

1. Introduction

According to the PhD project previously described (Klettenhammer 2021), this oral communication reports the main results of the following activities directed to:
A1) Supercritical CO_2-extraction of bioactive compounds from carrot pomace as natural antioxidants to enhance the oxidative stability of linseed oil encapsulated in sunflower wax by Particle from Gas-Saturated Solution technique (PGSS) technique.
A2) Production of solid-lipid particles by PGSS technique loaded with linseed oil and increasing concentrations of β-carotene.

2. Microencapsulation

In recent years a growing consumer awareness for healthy and nutritious food was recognized. Therefore, the demand for natural and functional food products is constantly increasing. Bioactives became thereby of major interest, because they have beside their nutritional function also an additional health benefit. Nethertheless, bioactives are very sensitive and prone to degradation. Consequently, they must be protected to maintain their positive effect (Biesalski *et al.*, 2009). Innovative encapsulation techniques based on supercritical CO_2 demonstrated to be a promising solution, due to their mild operating conditions and their sustainable and safe approach. Especially, the PGSS technique is a favorable technology to encapsulate lipophilic bioactives (Nissim Garti and D. Julian McClements, 2013; Temelli, 2018). In the first part of this PhD project, a natural bioactive-rich extract was recovered from carrot pomace with a supercritical CO_2-extraction using ethanol (14% w/w) as co-solvent. Finally, this extract was encapsulated with linseed oil in sunflower wax by the PGSS technique to study the protective effect of the extract against the oxidation of encapsulated linseed oil. In the second study instead, linseed oil was encapsulated in glycerol stearate and the oxidation of linseed oil was extended by β-carotene as antioxidant.

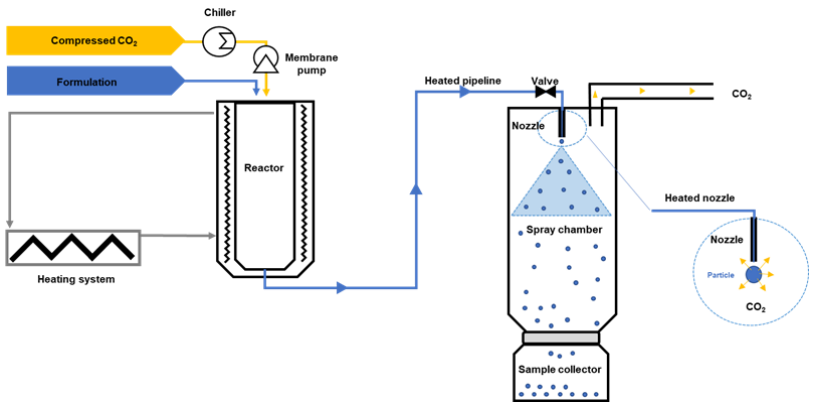

Figure 1*: Setup of the PGSS system used for encapsulation.*

3. Experimental Procedure

In this PhD thesis three main experimental procedures were pursued, that are: i) Extraction of carrot pomace, a by-product from the carrot juice production, by supercritical carbon dioxide with the use of ethanol as co-solvent; ii) Encapsulation of linseed oil in sunflower wax by PGSS technique by different process conditions and retarding the oxidation of linseed oil by the extract gained in the previously mentioned step; iii) Encapsulation of linseed oil in glycerol stearate by PGSS technique and retarding the oxidation of linseed oil by β-carotene. This study included a profound analysis of the degradation of linseed oil and β-carotene during the oxidation.

4. Material and Methods

For the preparation of the carrot pomace, fresh carrots were extracted using a juice extractor equipped with rotating blades (Kenwood juice extractor, Bolzano, Italy). The carrot pomace was then dried using a freeze-dryer (Epsilon 2-6D LSC plus, Martin Christ, Osterode am Harz, Germany) and milled with a hammer mill reaching a particle size lower than 0.8 mm. The dried and milled carrot pomace was extracted using a supercritical CO_2-extraction system (Superfluidi s.r.l., Padova, Italy). The extractions were carried out at 30 MPa, 60 °C with a solvent flow rate of 2 L/h for 120 min and with the presence of ethanol (14% w/w) as co-solvent (de Andrade Lima, Charalampopoulos and Chatzifragkou, 2018; Klettenhammer *et al.*, 2022). The characterization of the carrot pomace extract comprised the determination of the total phenolic content of carrot pomace extract according to the Folin–Ciocalteu method (Singleton, Rossi and Jr, 1965), the total carotenoid content of carrot pomace extract which was determined by spectrophotometrical analysis (Hiranvarachat and Devahastin, 2014) and the characterization of carotenoids in the extracts by liquid chromatography coupled with high-resolution mass spectrometry. The antioxidant activity of the carrot pomace extract was assessed by the 2,2-Diphenyl-1-picrylhydrazyl assay (Brand-Williams, Cuvelier and Berset, 1995) and also carried out by the Ferric Reducing Antioxidant Power method (Benzie and Strain, 1996). For the encapsulation with the PGSS technology, carrot pomace extract was mixed with linseed oil in concentration ranging from 0.8 to 16 mg/g. Subsequently, the oil enriched with the extracts (1 g) was mixed with sunflower wax (5 g). The particle formation was performed at 55°C for glycerol stearate and 65 °C for sunflower wax, a saturation time of 30 min and pressure from 10 to 30 MPa. After 30 min saturation time, the outlet valve of the reactor was opened, and the supercritical mixture was sprayed trough a nozzle of 600 µm into a cyclone chamber. The encapsulation efficiency was defined as the total amount of encapsulated linseed oil and carrot pomace extract divided by the total amount of oil (Ndayishimiye *et al.*, 2020). For the physical characterization of the particles the particle size distribution was analyzed by an automatized sieving system (Retsch GmbH, Verder Scientific, Germany) and by a light scattering technique using a laser diffraction Mastersizer Hydro 3000 MU (Malvern Instruments Ltd., Malvern, Worcestershire, UK). Bulk and tapped density as well as the flowability were also measured (Jinapong, Suphantharika and Jamnong, 2008; Fernandes, Borges and Botrel, 2014). The morphology of the microcapsules was analyzed using a Scanning Electron Microscopy system (PhenomTM ProX, Eindhoven, The Netherlands). The oxidative stability of the produced ingredients was assessed by isothermal calorimetry (Thermal Activity Monitor, Model 421 TAM III, TA Instruments, USA) at 40 °C, oxygen measurements were performed with a Fibox 4-trace fiber-optic oxygen meter (PreSens GmbH, Regensburg, Germany), peroxide value analysis accordingly to IDF Standards (*International IDF Standards International Dairy Federation, 74A:1991, Sec.*, no date), the determination of the change of the fatty acids profile by Gas Chromatography with a Flame Ionization Detector using a chromatograph (Thermo Scientific TRACE 1300, Milan, Italy) as well as nuclear magnetic resonance measurement using a Jeol JNM-ECZ400R/M3 spectrometer (Jeol Ltd., Tokyo, Japan). The determination of β-carotene degradation products was assessed by liquid chromatography coupled with high-resolution mass spectrometry using a Thermo Scientific Q-Exactive high resolution mass spectrometer (MS) coupled to an Ultimate 3000 ultra-high pressure liquid chromatography system with a 4-channel detector.

5. Results and Discussion

5.1. Supercritical CO_2-extraction of bioactive compounds from carrot pomace as natural antioxidants to enhance the oxidative stability of linseed oil encapsulated in sunflower wax by PGSS technique.

The extraction with supercritical CO_2 from carrot pomace allowed to obtain an extraction yield equal to 4.6 ± 0.3 % (w/w). The determination of the oxidative stability, performed by isothermal calorimetry, demonstrated that pure linseed oil was oxidizing within 5 h at 40 °C (as indicated by the onset time of Fig. 2A). The encapsulated linseed oil at different pressures (10, 20 and 30 MPa) demonstrated an increased shelf life . The encapsulation at 10 MPa of pure linseed oil without the carrot pomace extract extended the oxidative stability up to five times more. Interestingly, the microcapsules produced at 10 MPa showed a better oxidative stability. They reported an oxidative stability two times longer compared to those produced at 30 MPa. Indeed, higher pressures led to microcapsules with smaller sizes and consequently higher surface areas exposed to air becoming more prone to oxidation. In a second step, microparticles were produced at 10 MPa and increasing amounts of carrot pomace extract were added to the formulation (Fig. 2B). The samples reported an improved oxidative stability up to 43 ± 3 h with the addition of 16 mg / g oil compared to the encapsulated oil at the same setting without carrot pomace extract (28 ± 2 h). To summarize, it was demonstrated that microcapsules prepared at 10 MPa reported the highest oxidative stability, which was furthermore improved up to 15 h more with the addition of carrot pomace extract (16 mg/g of oil).

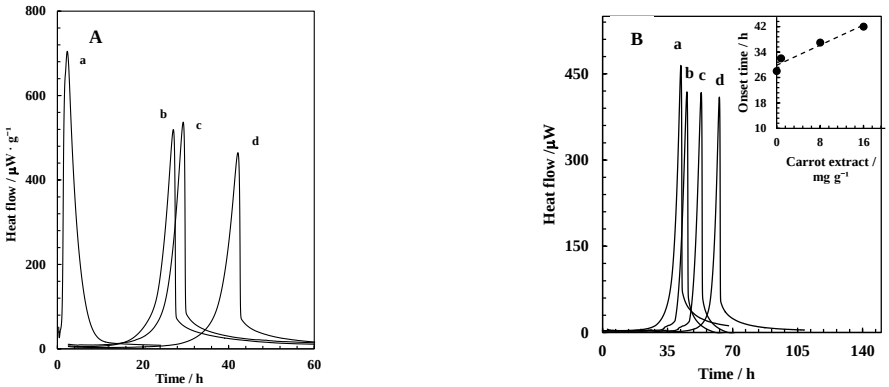

Figure 2: A) *Effect of PGSS encapsulation on the oxidative stability of linseed oil as a function of the processing pressures: a, linseed oil; b, linseed oil encapsulated at 30 MPa; c, linseed oil encapsulated at 20 MPa; d, linseed oil encapsulated at 10 MPa;* **B)** *Heat flow traces of linseed oil with increasing CPE amount encapsulated by PGSS at 10 MPa: (a), no extract; (b), 0.8 mg/g; (c), 8 mg/g; (d), 16 mg/g.*

The antioxidant properties of the CPE depended mainly on its content of phenols and carotenoids. The amount of total phenols was about 1.4 mg Gallic Acid Equivalent / g dry sample as determined by the Folin–Ciocalteu assay. Instead, the total carotenoids content was equal to 0.2 mg / g dry sample. These quantities and the values from the DPPH assay (2.5 mg Trolox Equivalent Activity / g of dry sample) explained the strong antioxidant power of the carrot pomace extract. The main carotenoids identified in the extract by high-resolution mass spectrometry in positive ionization mode were β-carotene (46.3%) followed by α-carotene (38.5%), lutein (8.7%), antheraxanthin (4.5%) and violaxanthin with 1.2% of the total carotenoids content. Some small size peaks were also detected and identified as zeaxanthin (0.4%) and alloxanthin (0.4%). The obtained microparticles were composed of linseed oil, CPE and sunflower wax as wall material. The pressure significantly affected chemical-physical properties of microcapsules. An increase from 10 to 30 MPa decreased the oil encapsulation efficiency from 91.68% to 86.23% (w/w). Moreover, at higher pressures the bulk and tapped densities reported the lowest values, while the Carr index increased from 12% (10 MPa) up to 25% (30 MPa).

5.2. Production of solid-lipid particles by PGSS technique loaded with linseed oil and increasing concentrations of β-carotene.

In this study solid-lipid microparticles were produced by PGSS technique encapsulating linseed oil in glycerol stearate as wall material. The results demonstrated the possibility to produce microparticles with a five times higher oxidative stability compared to the bulk oil as confirmed by the measurement of peroxides formation and oxygen consumption during an oxidation study performed at 40 °C. The obtained microparticles reported a porous and not homogeneous morphology with sizes ranging from 6.44 to 58 μm (Fig. 3). The oxidative stability of the particles was furthermore improved by increasing concentrations of β-carotene. A concentration of 1.6 mg β-carotene / g oil extended the oxidative stability of the particles up to 2.4 times, compared to particles without the addition of β-carotene. The analysis of oximetry traces showed that the increase of the induction times was linearly dependent on the amount of added antioxidant. Based on these findings, the study showed promising results for the use of PGSS as protecting encapsulation technique of oils rich in unsaturated fatty acids. Microcapsules formulated with β-carotene (1.6 mg/g of oil) were also analyzed by HPLC-HRMS to determine the degradation of β-carotene after

the oxidation. The compounds detected in the not oxidized sample were β-carotene, 3-OH-β-apo-11-carotenal and the 3-OH-β-apo-carotenone (Tab. 1). After microcapsules oxidation, β-carotene disappeared completely and both apo-carotenals significantly increased (n=3, $p < 0.05$). Moreover, 5,6-Epoxy-β-ionone was also detected after microparticles oxidation.

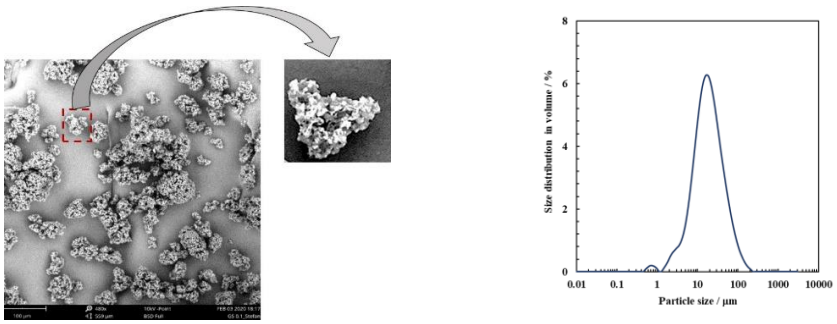

Figure 3: *Scanning electron microscope images and particle size distribution of linseed oil microparticles encapsulated by PGSS using glycerol stearate as wall material*

Table 1: *Detected compounds (β-carotene and its oxidation products) by HPLC-HRMS in microcapsules of linseed oil formulated with β-carotene (1.6 mg/g of oil).*

Compound	Molecular formula	Theoretical mass (m/z)	Acquired mass (m/z)	Area before oxidation (%)	Area after oxidation (%)
ß-carotene	$C_{40}H_{54}$	536.4376	536.4376	1684410±84220	n.d.
5,6-Epoxy-β-ionone	$C_{13}H_{20}O_2$	209.1536	209.1536	n.d.	116341±5817
3-OH-β-apo-11-carotenal	$C_{15}H_{22}O_2$	235.1692	235.1692	22820±1141	746182±37309
3-OH-β-apo-carotenone	$C_{18}H_{26}O_2$	275.2005	275.2005	76547±3828	1487730±74385

6. Conclusion and Future Perspectives

This PhD thesis highlighted the potential of PGSS as novel technique for the encapsulation of bioactive compounds. It also showed that bioactive compounds from carrot pomace were successfully extracted by supercritical CO_2-extraction and the determined antioxidant capacity of the obtained extracts highlighted the value of valorized by-products from vegetable products. The results demonstrated also the possibility to use two different wall materials for the PGSS process and the ability to obtain microcapsules with carrot pomace extract and β-carotene as natural antioxidants to retard the oxidation of microencapsulated linseed oil.

7. Nomenclature

PGSS, Particle from Gas-Saturated Solution technique; MPa, Megapascal; CPE, carrot pomace extract; HPLC-HRMS, High Performance Liquid Chromatography coupled with High-Resolution Mass Spectrometry.

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Development of innovative methods for the multiple analysis of allergens in processed foods

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This industrial doctoral thesis aimed to develop a multi-target method, based on ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS), capable to detect allergenic traces of different species of nuts and peanuts in a single analysis in complex matrices such as bakery products. Allergy to nuts and peanuts represents a global relevant problem, especially due to the risk of their ingestion as hidden allergens, as a result of cross-contamination between production lines at industrial level occurring during food manufacturing. Mass spectrometry methods have a very high potential for the identification and quantification of allergenic ingredients at part-per-million (ppm) levels through the detection of peptide markers arising from the artificial digestion of their proteins.

Sviluppo di metodi innovativi per l'analisi multipla di allergeni in alimenti processati

Questa tesi di dottorato industriale ha avuto l'obiettivo di sviluppare un metodo multi-target, basato sulla spettrometria di massa tandem accoppiata alla cromatografia liquida ad ultra alta prestazione (UHPLC-MS/MS), in grado di rilevare tracce allergeniche di diverse specie di frutta a guscio e arachidi in un'unica analisi in matrici complesse come i prodotti da forno. L'allergia alla frutta a guscio e alle arachidi rappresenta un problema di rilevanza mondiale, soprattutto a causa del rischio di una loro ingestione come allergeni occulti, a causa di fenomeni di contaminazione incrociata nelle linee di produzione a livello industriale, che si potrebbero verificare durante la produzione alimentare. I metodi di spettrometria di massa hanno un potenziale molto elevato per l'identificazione e la quantificazione degli ingredienti allergenici a livelli di parti per milione (ppm) attraverso la rilevazione di markers peptidici derivanti dalla digestione artificiale delle loro proteine.

Keywords: Tree nut allergy; peanut allergy; hidden allergens; LC–MS/MS analysis; nut specific peptide markers

1. Introduction

In accordance with the GANTT plan of the industrial doctoral thesis project, the research work was divided into the activities summarized below:

1) literature search for the selection of unique peptide markers for allergenic proteins deriving from nuts (almond, hazelnut, walnut, cashew, pistachio) and peanuts and assessment of the selected markers and related MS/MS transitions stability by high resolution mass spectrometry in raw and toasted nuts and peanuts;
2) production in the laboratory of bakery products (biscuit) including or not allergenic ingredients at different levels of concentration and development of rapid extraction and pre-enrichment methods of their protein component, followed by artificial digestion by trypsin and LC-MS/MS analysis of marker peptides with a high-resolution mass spectrometer. In-house validation of the method and study of proteins chemical modifications induced by heat treatments. Transfer of the method to an industrial analytical laboratory during the 6-months research stay at the Barilla Group's productive plant in Parma;
3) comparison between the performance related to different allergen extraction/enrichment strategies and assessment of the best marker peptides for allergenic ingredients quantification by liquid chromatography-triple quadrupole mass spectrometry, during the 6-months research stay at the CER groupe (Marloie, Belgium).

After the selection of the marker peptides for each nut species under study and for peanuts, their detection in raw and roasted matrices through the use of high-resolution mass spectrometry was assessed, after the optimization of sample preparation steps. This study allowed the selection of thermostable peptides, on which the subsequent analysis of allergenic ingredients in an in-house made biscuit, chosen as a representative bakery product, was focused. In fact, an incurred biscuit was prepared in the laboratories of CNR-ISPA in Bari by contaminating the flour with allergenic ingredients and then proceeding to a typical production protocol. Through the preliminary study of this model matrix, the impact of the thermal and technological treatments was verified by studying the stability of the marker peptides selected in the previous activity. To select rapid protein extraction methods, two extraction modes were set and compared: i) ultrasound-assisted extraction using an immersion probe (Pilolli et al., 2017; Pilolli et al. 2018); ii) incubation at room temperature under vigorous shaking, followed by sonication (Planque et al. 2016; Planque et al 2017). After the internal validation of the developed method, with calibration of peptide responses performed using incurred biscuit matrices including different levels of allergenic ingredients, and the study of the peptide chemical modifications, the analytical method was transferred to the research laboratories of the Barilla Group's plant in Parma. Here, fortified matrices were prepared through the addition of

allergenic ingredients at two levels. The two levels of inclusion were designed with the aim of having: i) a matrix including a high content of single allergens that would allow the accurate identification of the marker peptides in the chromatographic traces and ii) a matrix with a reduced allergen content, in order to verify if the developed method was sufficiently sensitive for the detection of allergens at low concentrations, as it could happen in a real case. The fast and sensitive multi-target method described so far was developed using a state-of-the-art high-resolution mass spectrometer equipped with an orbital trap analyzer based on the Orbitrap technology and able to perform also MS/MS analyses. During the subsequent research period, carried out at the CER Groupe in Belgium, the method was adapted to a low resolution mass spectrometer capable of MS/MS acquisitions, namely, an instrument equipped with a triple quadrupole mass analyzer, to evaluate if similar analytical performances could be achieved also using a less expensive and certainly more widespread instrumentation.

2. Experimental Procedure

The LC-MS/MS analytical method was developed, in the first steps, on raw and toasted allergenic ingredients and subsequently perfected for the analysis of allergen-free or of incurred biscuits, chosen as a complex matrix representative of baked products. The allergen-free biscuit and biscuits incurred at different levels of allergenic ingredients, produced in the laboratory at the CNR-ISPA and then in the pilot plants of the Barilla Group, were analyzed according to the following protocol.
Protein extraction from all mentioned matrices was performed using a 200 mM Tris-HCl buffer (pH = 9.2) containing 5 M urea and the resulting extracts were sonicated, centrifuged, and filtered through membranes of cellulose acetate with a porosity of 5 µm. The extracts were purified using SEC columns and subsequently subjected to artificial digestion with trypsin for 16 hours; a mixture of isotopically labelled peptides, used as internal standards, was added to the digestate. The next steps were centrifugation, further filtration through a 0.45 µm regenerated cellulose (RC) filter and purification on a C18 SPE column. The collected fraction was dried under a current of nitrogen and suspended in 100 µL of 0.1% formic acid in acetonitrile / water (90/10, v / v solution). The next step was the LC-MS and MS/MS analysis. The latter was based on an Ultimate 3000 liquid chromatograph coupled to a quadrupole-Orbitrap hybrid mass spectrometer (Q-Exactive Plus, Thermo-Fisher) available in the laboratories of both CNR-ISPA and the Barilla company. The tryptic peptide mixture was separated using, respectively, an Aeris Peptide chromatographic column (150 x 2.1 mm, packed with 3.6 µm particles and characterized by a XB-C18 stationary phase) and an Acquity UPLC Peptide BEH C18 column (150 x 2.1 mm, packed with 1.7 µm particles with pores diameter exceeding 300 Å). In both cases, 20 µL of each sample were injected and separations were performed using a water/acetonitrile elution gradient (see below). At the CER Groupe in Belgium the tryptic digestates were analyzed by a Water Xevo TQ-S mass spectrometer, equipped with a triple quadrupole mass analyzer. As before, 20 µL of each sample were injected and the peptides were separated using an Acquity UPLC Peptide BEH C18 (2.1mm x 150 mm, packed with 1.7 µm particles with pores diameter exceeding 130 Å) chromatographic column. A water/acetonitrile elution gradient was adopted also in this case (see below).

3. Materials and Methods

Raw materials used and preparation of the incurred biscuit
Almond, pistachio, hazelnut, walnut, peanut and cashew were purchased from Besana, Milan, Italy.
In order to evaluate the stability of the potential marker peptides selected for the 6 allergenic ingredients in a complex matrix and to evaluate the impact of the technological treatments, a biscuit was produced and analyzed in-house, adding the 6 allergens during the dough preparation and before cooking. This recipe was used for the preparation: 402.4 g of flour, 1 g of salt, 2 g of bicarbonate, 180 g of sugar, 90 g of extra virgin olive oil, 160 g of water, 6.01 g of egg powder, 6.01 g of skimmed milk powder, 6.02 g of toasted peanuts, 6.16 g of toasted hazelnuts, 6.02 g of toasted pistachio, 6.02 g of toasted almonds, 6.16 g of toasted cashews and 6.24 g of toasted walnuts. The dough was divided into discs with a diameter of about 5.5 cm and a thickness of about 1 cm and baked in the oven for 20 min at a temperature of 200°C. At the end of cooking, the concentration of each individual allergenic ingredient was recalculated considering the loss in water due to heat treatment: the final concentration of each allergen was 7677.64 mg/kg (ppm). Following the same procedure, a sample of "allergen-free" biscuit was produced, simply by substituting the flour for the quantities of added allergens. A simulated contamination at a 1000 ppm level was obtained by mixing appropriate quantities of allergen-free and incurred biscuit; lower levels of contamination were created using the 1000 ppm matrix.
The LC and MS conditions used for the two types of high- and low-resolution mass spectrometers are listed below.

Chromatographic conditions for LC-MS analyses based on the Q-Exactive Plus, Thermo-Fisher spectrometer
Two solvents were used for the chromatographic separation of the tryptic peptides, namely water + 0.1% Formic Acid (A) and Acetonitrile + 0.1% Formic Acid (B), working at a flow of 200 µL/min. The column temperature was maintained at 30°C during the entire chromatographic run.
MS/MS conditions for the Q-Exactive Plus Thermo-Fisher spectrometer
Analyses were performed in positive polarity using two acquisition modes: the first corresponds to the **Full-MS /**

dd-MS2 mode, consisting of two acquisition events. The first was a Full-MS event, based on the following settings: microscan 1, resolution 70 k, AGC target 10^6, maximum injection time 30 ms, scan range 200-2000 m/z; the dd-MS2 event was based on the following settings: microscan 1, resolution 17.5 k, AGC target 10^5, maximum injection time 60 ms, loop count 5, TopN 5, isolation window 2.0 m/z, isolation offset 0.4 m/z, stepped collision energy 27, 30; dd settings: minimum AGC target 5.10 × 10, intensity threshold: 8.3 × 102, charge exclusion 4-8, > 8, peptide match preferred, exclude isotopes on, dynamic exclusion 15 s. The second acquisition mode was a **Full-MS / AIF** one, achieved by setting the following parameters for the Full MS event: microscan 1, resolution 140 k, AGC target 10^6, maximum injection time 200 ms, scan range 300-2000 m/z, and the following parameters for the AIF event: microscan 1, resolution 70 k, AGC target 10^6, maximum injection time 200 ms, (N) CE / stepped nce: 27.30, scan range 250-2000 m/ .

Chromatographic conditions for LC-MS analyses based on the Xevo TQS spectrometer

Two solvents were used for the chromatographic separation of the tryptic peptides, namely water + 0.1% Formic Acid (A) and Acetonitrile + 0.1% Formic Acid (B), working at a flow of 200 µL/min The duration of the chromatographic run was 26 minutes. The temperature of the column was maintained at 50°C during the entire chromatographic run.

MS/MS conditions for the Xevo TQS spectrometer

The detection of peptides related to allergenic proteins by ***the Xevo TQS spectrometer*** was carried out using electrospray ionization in positive polarity and the "monitoring of multiple reactions" (MRM) mode, i.e., the detection of characteristic fragments of the ions of the peptides.

The MS method used had the following settings: ESI source in positive mode (ES +) with desolvation temperature of 500°C, Capillary voltage: 2.5 kV, Pressure in the collision cell: approx. 2×10^{-3} mbar and Source temperature at 150°C.

4. Results and Discussion

4.1. Identification of marker peptides with the UHPLC-HRMS / MS method in raw and toasted nuts

Once the sequences of the main marker peptides for allergenic proteins of each nut species and of peanuts were collected from the literature, the m/z ratios corresponding to their mono-, bi- and tri-charged ions were calculated, considering the possibility of multiple ionization in the HESI interface of the mass spectrometer. Subsequently, ion current extraction for each m/z ratio corresponding to the selected peptides was performed, thus obtaining eXtracted Ion Current (XIC) chromatograms. An example of this type of data processing is represented in the upper part of Figure 1, which shows the XIC trace relating to the peptide sequence GNLDFVQPPR, belonging to the allergenic protein Pru du 6 (Prunin 1) of almond, whose mono-charged ion was detected with an m/z ratio equal to 1142.59500. The lower part of the figure shows the expansion of the very high-resolution mass spectrum obtained through spectral averaging in the time interval of the chromatographic peak, which highlights the peaks of the isotopic pattern of the precursor ion, with the spacing equal to one m/z unit, as expected for a mono-charged ion.

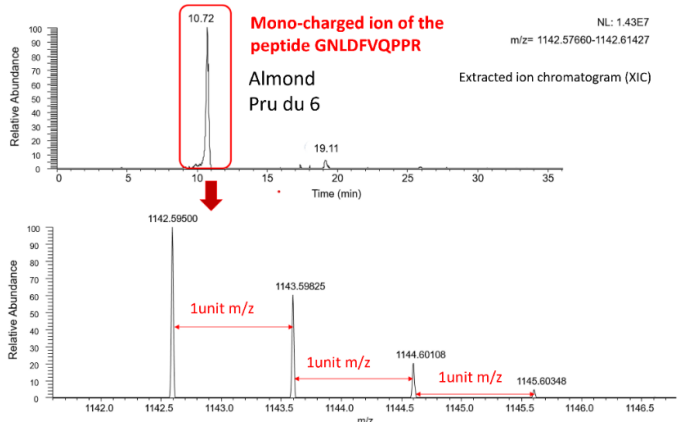

Figure 1 *Above) XIC chromatogram relating to the mono-charged ion of one of the tryptic peptide sequences associated with almond allergenic protein Pru du 6; below) expansion of the high-resolution mass spectrum averaged under the peptide chromatographic peak, that highlights the isotopic pattern of the ion.*

4.2. Incurred cookies analysis and in-house validation of the analytical method

Following the selection of the thermostable peptides which returned a high signal intensity, the study continued on the biscuit matrix. Samples of cookies contaminated with each of the allergenic ingredients at levels of 10, 20, 50, 100 and 200 ppm were used to obtain calibration lines and then to calculate the limits of detection and quantification (LOD and LOQ). The LOD and LOQ for each ingredient were calculated as 3 and 10 times the standard deviation on the intercept of the corresponding calibration line divided by its slope, respectively. All calibration lines obtained had a R^2 value better than 0.997- 0.999, depending on the case, thus suggesting an excellent linearity of the response. Furthermore, the developed method allowed to reach LOD values between 2.4 and 4.8 ppm and LOQ values between 8 and 15.9 ppm, which are very promising in terms of detection/quantification of traces of the allergenic ingredients of interest in processed foods. To evaluate the

precision of the developed method, a further experiment was carried out by preparing a sample of incurred biscuit matrix at a fixed concentration of 50 ppm for each allergenic ingredient. The repeatability of the method was validated as intra-day and inter-day precision of the analytical method (percentage coefficient of variation in the peak areas at a fixed concentration, CV%). Specifically, on days 1, 2 and 3 the CV% was studied by analysing the tryptic digest of the 50 ppm biscuit injected 5 times. For most of the peptides the intraday CV% was found to be less than 17%, therefore almost all the peptides showed an acceptable response variability, considering the absence of an internal standard in this specific case. The evaluation of the CV% was, in fact, based on the absolute XIC areas collected for each selected peptide. After calculating the intraday CV% for each of the 3 days, the data acquired on different days were averaged providing an inter-day CV% between 2 and 22%. Overall, the variability of the responses was low, thus indicating a good precision of the method. For the sake of example, Table 1 shows CV% data referred to a single peptide for each allergenic ingredient, used to evaluate the repeatability of the method.

Table 1 *Method precision study by evaluating intra-day and inter-day analytical repeatability for a series of marker peptides of allergenic ingredients in incurred biscuits at 50 ppm.*

INGRED.	SELECTED PEPTIDES	Intra-day evaluation			Inter-day evaluation
		DAY 1	DAY 2	DAY 3	DAYS 1,2,3
		CV%	CV%	CV%	CV%
Peanut	SPDIYNPQAGSLK	1.6	1.7	1.6	2.1
Almond	TEENAFINTLAGR	3.1	3.9	0.7	3.2
Hazelnut	ALPDDVLANAFQISR	6.4	3.2	8.7	6.1
Wanut	ADIYTEEAGR	5.6	2.3	1.2	6.4
Cashew	ELYETASELPR	1.1	0.5	0.5	3.5
Pistachio	FEWISFK	2.1	1.7	1.3	4.1

After the transfer of the method to the Barilla company laboratory and after the production of the incurred biscuit in a company pilot plant, the same evaluations described so far were carried out on the instrumental repeatability and on the reproducibility of the analytical protocol and, at the same time, of the production process, through the analysis in duplicate and in two acquisition modes (Full MS-AIF and Full MS-ddScan) of biscuits (finished product), doughs (semi-finished product) and flours (raw materials).To evaluate the instrumental repeatability and reproducibility of the preparation protocol, the CVs% were calculated for each of the pairs of injection replicates of each tryptic digest and, secondly, the obtained values were averaged until a single CV% value was obtained relative to each peptide (see Table 2). Specifying that peak areas were not normalized on any internal standard also in this case, the values obtained showed good repeatability (values between 3 and 7% for test 1, between 2 and 8% for test 2).

Table 2. *Example of the instrumental repeatability evaluation made for each marker peptide on the different biscuit matrices analyzed. The case of two tryptic peptides deriving from almond is reported in the table.*

INGRED.	SELECTED PEPTIDES	Injection replicates	Biscuit (Full/MS-AIF) TEST 1 (low allergenic protein content) Rep 1, Rep 2 CV%	Average CV%	TEST 2 (high allergenic protein content) Rep1, Rep 2 CV%	Average CV%
Almond	TEENAFINTLAGR	A bis	2	3	1	2
		A	3		2	
		B bis	4		2	
		B	4		2	
	ADIFSPR	A bis	3	4	3	2
		A	4		3	
		B bis	5		1	
		B	3		3	

For the evaluation of the extraction method, the CV% were calculated for the two replicates of the same sample (biscuit A, biscuit A-bis; biscuit B, biscuit B-bis; dough A, dough A-bis; dough B, dough B-bis) for test 1 (low allergenic protein content) and for test 2 (high level of allergen inclusion). For most of the peptides, a variability of less than 17% was obtained in both tests and for all the matrices analyzed (biscuit, dough and flour), therefore it was considered acceptable, considering the absence of an internal standard. From the evaluation of the reproducibility of the production process, a CV% value between 11 and 19% for test 1 and 6 and 13% for test 2 emerged for the nuts, indicating that the production process was rather standardized.

4.3. Method transfer to a low resolution mass spectrometer based on a triple quadrupole analyzer

During the research period spent at the CER Groupe in Belgium the analytical method developed previously was transferred to a low-resolution triple quadrupole mass spectrometer, in order to evaluate its analytical performances. The transfer of the method required several tests, aiming primarily at optimizing the collisional energy (through MRM methods) for each selected marker peptide, in order to have an instrumental response as high as possible. The same incurred biscuit matrix produced previously at different contamination levels (2.5, 5, 10, 20, 50 100 ppm) was analyzed in the CER laboratories, along with the allergen-free biscuit. The analyses allowed the construction of the calibration lines and the consequent calculation of LOD and LOQ values. The resulting values could then be compared with those previously obtained using the high-resolution instrumentation, as exemplified in Table 3, in which the best LOD/LOQ values obtained in each case are compared.

Table 3. *Comparison of the LOD and LOQ values obtained for each allergenic ingredient in a biscuit matrix using a high resolution or a low-resolution mass spectrometer.*

	Low-resolution MS		**High-resolution MS**	
SPECIES	**LOQ (ppm)**	**LOD (ppm)**	**LOQ (ppm)**	**LOD (ppm)**
Peanut	8	2.4	8	2.4
Almond	4	1.2	7.6	2.3
Hazelnut	6.1	1.8	15.3	4.6
Walnut	8	2.4	17	5
Cashew	4.8	1.4	11	3.3
Pistachio	4.7	1.4	15.9	4.8

5. Conclusions and Future Perspectives

The research work performed during this PhD thesis shows that traces of nuts and peanuts hidden in baked products as a result of cross-contamination occurring in production plants, posing a potential risk for allergic consumers, can be detected and quantified down to ppm levels, with a multi-analyte approach, through the LC-MS/MS analysis of peptides generated upon the artificial tryptic digestion of their allergenic proteins. It was proved that this level of analytical performance can be reached both by high-resolution and by low-resolution MS instrumentation and can be easily achieved also in industrial laboratories, thus opening interesting perspectives in terms of applicability of the method for baked products control in a large range of laboratories. The actual quantification of hidden allergens might lead to a better use of precautionary allergen labelling, limiting this approach only to products in which a realistic threat exists for allergenic consumers. Finally, it is reasonable to foresee that approaches similar to the one developed for nuts and peanuts in baked products will be applied also to other relevant allergenic ingredients and food matrices in the next future.

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Monitoring and management of chemical and physical wine parameters by using different tank materials into the winemaking process

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The activities performed for the PhD project consisted in evaluating the impact of different tank materials for the wine aging. To this aim in the first phase of the study, a Sangiovese red wine was monitored for the qualitative parameters during 12 months of aging in different tank materials. The materials involved were raw earthenware amphora, raw and coated concrete, stainless steel, new and used oak barrels. In the second phase, the data obtained were used for (i) modeling the kinetics of monitored parameters and (ii) evaluate the impact of different tank material on wine quality parameters. Then, materials involved into the red wine aging were characterized for their physical properties and their Oxygen Transmission Rate (OTR).

Monitoraggio e gestione dei parametri chimico-fisici di un vino rosso mediante l'utilizzo di vasi vinari di diverso materiale nel processo di vinificazione

La presente tesi di dottorato ha avuto come obiettivo la valutazione dell'impatto di vasi vinari di diverso materiale sulla maturazione di un vino. A tal fine, nella prima fase dello studio, un vino rosso Sangiovese è stato monitorato per i parametri qualitativi durante 12 mesi di affinamento in contenitore di diverso materiale. I materiali coinvolti erano la terracotta grezza, il cemento grezzo e rivestito, l'acciaio inossidabile, il legno di rovere. Nella seconda fase, i dati ottenuti sono stati utilizzati per (i) modellare la cinetica dei parametri monitorati e (ii) valutare l'impatto di diversi materiali del serbatoio sui parametri di qualità del vino. Infine, i materiali coinvolti nell'invecchiamento del vino rosso sono stati caratterizzati per le loro proprietà fisiche e il loro tasso di trasmissione dell'ossigeno (OTR).

Key words: red wine aging, alternative new materials, kinetics modelling, sensory evaluation, oxygen transmission rate.

1. Introduction

This oral communication reports the main results of the following four activities directed to:

A1) The monitoring of the main chemical and physical red wine parameters in order to model their kinetics during one-year aging (Guerrini et al., 2021)
A2) The study of the impact of different tank materials on wine quality and the combined effect with the bottle aging (Maioli et al., 2022)
A3) The physical characterization of the different materials involved into the aging test and the determination of their Oxygen transmission rate (OTR) (Maioli et al. 2022).

2. Red wine aging and the material of the tank

Wine aging is a fundamental phase for obtaining a stable product. Many physical−chemical reactions take place during this phase that changes the wine chemical structure and sensory profile. During red wine aging, several factors, such as kind of tank material, dissolved oxygen, and phenolic composition, are involved in the evolution and stabilization of wine. In particular, the choice of the aging tank affects the final wine characteristics since it modulates the oxygen permeation and the release of compounds such as tannins or elementals. Nowadays, winemakers have a wide range of different kinds of tanks available for the wine aging phase. Traditionally, the oak barrel is considered one of the best tanks to improve wine sensory complexity and stability of color but wine, beyond wood and stainless steel, can be aged also in other materials such as concrete or amphorae according to the wine characteristics and the need of market differentiation and distinction. The aging tank choice, however, should be made with awareness of the specific influence of the tank material on the physical−chemical characteristics of wine, according to varietal characteristics and oenological goal, in order to achieve a defined sensory profile and wine style. It is known, for example, that every kind of tank material is characterized by a specific oxygen permeability (OTR) and this affects the formation/degradation of compounds with important consequences on wine aging.

3. Materials and Methods

3.1 Wine and materials involved

The wine used for the experiment was a Sangiovese from 2018 harvest. After completing the malolactic fermentation, it was centrifuged at 0 NTU and racked in different tank materials, including: raw earthenware amphorae (AM), coated (CC) and uncoated concrete (CR), stainless steel (SS), used (TO) and new oak barrel (TN). The wine used as reference were stored in 1 L glass bottle (GB). All the different tanks were placed in an underground cellar at the Cantina Sociale Colli Fiorentini Valvirginio cooperative winery (Montespertoli, Florence, Tuscany), where the temperature ranged between 15 and 22 °C and relative humidity was ~ 80% over the year. Wines were analysed monthly to model their parameter kinetics and at the three different time of aging (_6, _12_6+6). At six months of aging part of every wine obtained were bottled in order to evaluate also the combined effect of different tanks and the bottle aging (_6+6). The experiment was set on industrial scale, with the volume of each tank of 5 hL and in triplicate.

3.2 Wine chemical and physical parameters monitored

During the 12 months of aging, in order to model the evolution kinetic, wines were monthly sampled and analyzed for several qualitative parameters. In particular, standard parameters (alcohol, acidity, pH), color indices (color intensity - CI, Hue, total phenol index - TPI); acetaldehyde, HPLC-DAD phenolic profile (monomeric anthocyanins and polymeric pigments), redox potential – ORP and dissolved oxygen - DO.
Wines were also chemically analyzed at 6 and 12 months of aging in different tank materials and after 6 months of bottle aging after 6 months in different tanks. In particular, wines were characterized for their phenolic and volatile profile, the elemental content and the tartaric stability.

3.3 Sensory evaluation

A sensory evaluation was performed at different time of aging: 6 and 12 months of aging in different tank materials and after 6 months of aging in different tanks plus 6 months of bottle aging. Sensory analyses were carried out following the quantitative descriptive analysis method (QDA) (Gacula 2008) by a panel of 13 trained judges (8 males and 5 females) after 6 months of aging and 9 trained judges (3 males and 6 females) after 12 months, recruited from students, staff, and friends of DAGRI Department in Firenze. The panel that evaluated the wines after 6 months first tasted and described the taste and tactile descriptors of the samples The samples (30 mL) were poured at room temperature (around 19 °C) and presented under red light to mask little color differences, in standard tasting glasses (ISO-3591, 1977).

3.4 Modelling

All the data collected during the one-year aging were processed to model their evolution, with the tank material (M), storage time (t) and temperature (T) as factors. Stainless steel (SS) was chosen as the reference material. Kinetic models of the collected data were performed as described in the literature when available, otherwise a polynomial curve was adopted to obtain a good phenomenological fit. In the case of polynomial fitting, the tested independent variables were M, t, T and their interactions (M × t × T). Moreover, t and T were tested at the first, second and third polynomial degree. The variables and interactions were tested for significance with an ANOVA test, and the chosen threshold was $p<0.05$. According to the principle of model parsimony, all not-significant variables were dropped in the residual and the models were consequently simplified. R software (V. 4.0.0) was used for all the statistical analyses.

3.5 Materials characterization

The materials used for the manufacturing of the aging tanks were also analyzed. In particular earthenware raw amphora, raw and epoxy-coated concrete samples were characterized for their physical property and their Oxygen Transmission Rate. The procedure and the tool used is described in literature (Nevares & del Alamo-Sanza 2021; del Alamo-Sanza et al. 2017).

3.6 Statistics

The chemical and sensory data of the wines were analyzed by multifactor analyses of variance (MANOVA) applying an LSD, least significant difference test, with 95% significance level, and frequency distribution, analyzed by the Chisquare test, was performed using Statgraphics Centurion (Ver.XV, StatPoint Technologies, Warrenton, VA). Tank material and replicates were considered as factors for both the chemical and the sensory analysis. Principal component analysis (PCA) was carried out using the software XLSTAT 2020.5.1.

4. Results and Discussion

4.1 Kinetic modelling of wine aging parameters

This study highlighted the redox phenomena tendencies occurring in red wines during one-year aging in tanks of different materials. Redox potential and polymeric pigments kinetics during one year aging and relative model equation are showed in figure 1a and 1b. These tendencies were related to a series of operating conditions and chemical-physical phenomena such as: (1) the oxygenation level of the wines at the beginning of aging; (2) the level of oxygen permeability and impregnation of the tank materials; (3) the amounts of molecules released by the tanks; (4) the wine temperature; and (5) the oxygen consumption rate caused by the different components in the

wines. For all the different tank materials, the synergic or antagonistic action of the operating conditions and chemical–physical phenomena resulted in a common trend of the physical and chemical parameters relating to the redox state of the wines. In the first approximatively 100 days of aging there was a rapid consumption of oxygen (i.e., decrease in DO) and a rapid decrease in the redox potential, which could be correlated to reactions with rapid oxygen consumption such as the oxidation of polyphenols. From ~ day 150 up to the end of aging, the oxygen level increased slightly, while the redox potential increased, reaching a similar value to the beginning of aging. Rapid oxygen consumption reactions were replaced by slow oxygen consumption (i.e., anthocyanin–tannin ethylene bridge polymerization reactions), which, as well as the wine temperature, were probably responsible for increasing the redox potential. In the wines, the polymerization reaction mediated by acetaldehyde seemed to be activated when the redox potential increased, again after reaching the lowest level during the one-year aging, demonstrating that a defined level of redox potential is needed to trigger the reaction. Moreover, all the reactions that occurred in the wines during aging caused a general trend of variation in the wine color, which was similar for all the tanks. The experimental data were modeled, and the kinetic models were able to describe the differences between the wine samples aged in the different tank materials. The stainless steel and epoxy-coated concrete tanks were the least suited to allowing a variation in the redox state of the wines, which was exactly the opposite of the oak barrels; the behavior of the raw earthenware amphorae and uncoated concrete tanks, on the other hand, was somewhere in-between, but tended to be more similar to the oak barrels. The level of oxygen permeability and impregnation of the tanks and the degree of substances released by the tank materials could explain the well-known greater redox attitude of oak barrels compared to stainless steel and epoxy-coated concrete tanks, which were the most chemically inert. The behavior of the raw earthenware amphorae and the uncoated concrete tanks appears interesting and deserves specific studies to investigate all the above-mentioned phenomena in relation to the chemical composition and production process of the materials.

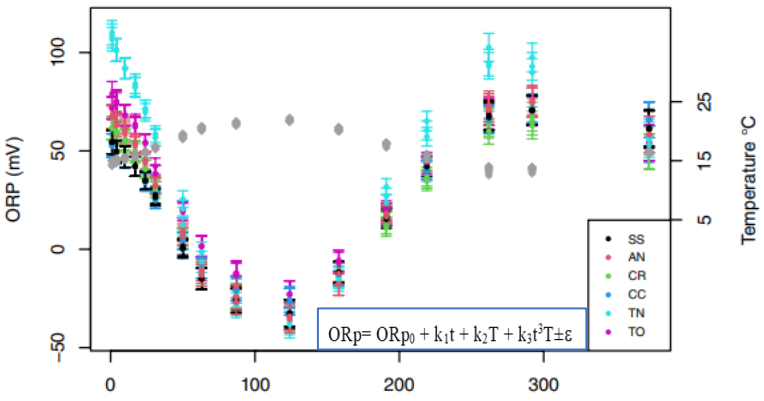

Figure 1a. *Kinetic models of the redox potential (ORP—mV) and temperature (°C—grey diamonds) measured in the different tanks during the one-year aging (Error bars represent the standard error; SS: stainless steel tank, AN: raw earthenware amphora, CR: uncoated concrete tank, CC: coated concrete tank, TN: new oak barrel, TO: used oak barrel).*

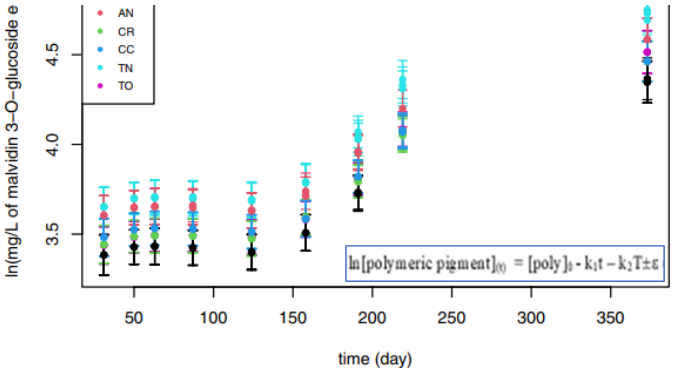

Figure 1b. *Kinetic models of the polymeric pigments (mg/L of malvidin 3-O-glucoside eq.) measured in the different tanks during the one-year aging (Error bars represent the standard error; SS: stainless steel tank, AN: raw earthenware amphora, CR: uncoated concrete tank, CC: coated concrete tank, TN: new oak barrel, TO: used oak barrel).*

4.2 Chemicals of wines aged in different tank materials and sensory analysis

In the present work, a simultaneous comparison of most of the types of containers currently used in the cellars was carried out. Therefore, new-alternative and traditional tank materials have been compared during the aging at the industrial scale of a single variety red wine and the impact on the chemical and sensory characteristics of the wine has been evaluated. In figure 2 is reported the distribution (PCA - score & loadings) of all wines aged in different tanks according to their chemical parameters at the three different time of sampling. The results allowed to highlight that earthenware raw amphorae AM and uncoated concrete CR promoted the wine color stabilization similarly to the new oak barrel already after 6 months aging. This aspect was confirmed by the wine composition after 6 months of bottle aging as they showed the highest content in polymeric pigments, even when compared to the same wines aged for 12 months in the same tanks. Moreover, earthenware raw amphorae and uncoated concrete released in wines a relevant amount of elementals causing an acidity decrease, affecting also the sensory profile. In particular, the main released elementals were sodium, copper and iron in both of wines aged in earthenware raw amphora and uncoated concrete (Maioli et al. 2022). The bottle aging, combined with different tank materials,

enhanced the complexity of the wine volatile profile thanks to the reductive status inside the bottle that seemed to promote the varietal precursors hydrolysis (Maioli et al. 2022).
Sensory analysis (Figure 3) showed that, as expected, the TN_6 wine was related to all the woody attributes (spicy, wood, vanilla, and butter). The TO_6 wine was related to a lesser extent to the woody attributes and also to berry jam and cherry odor that were in turn also related to the AM_6 wine. The SS_6, CC_6 and CR_6 could not be directly related to any of these attributes. The GB_6 wine was related to reductive odor and vegetal flavor attributes. Twelve months aging (_12) were more similar between them with respect to the 6 months aging (_6 + 6) wines. The wine AM and SS wines were similar in both the aging time modality (_6 + 6 and _12), while the CR wines showed the highest differences between the two kinds of aging. The CC_6 + 6 and CR_6 + 6 appeared to be similar and associated to the floral flavor, floral odor, sweetness attributes, and to a lesser extent to acidity, while the SS_6 + 6 and the AM_6 + 6 to the berry jam odor. Interestingly, the results of the sensory evaluation were similar to the distribution of wines according to the aforementioned chemical composition. In fact, the distribution of these wines underlined that the bottle aging affected the perceived quality of wines and showed that the _6 + 6 wines were perceived significantly more different as compared to the _12 months wines.

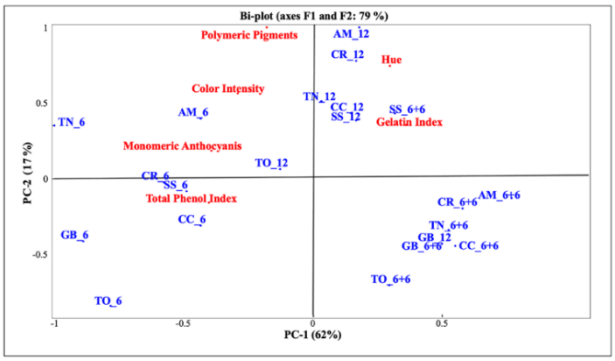

Figure 2. *Principal component analysis (PCA): scores and loadings plot of the polyphenol compounds, color indices, and gelatin index measured in the Sangiovese red wine during aging (_6: 6 months aging in tank; _12: 12 months aging in tank; _6 + 6: 6 months aging in tank plus 6 months of bottle aging; TO: used oak barrel; TN: new oak barrel; CR: uncoated concrete; CC: epoxy coated concrete; SS: stainless steel; AM: raw earthenware amphorae; GB: glass bottle with wine bottled at the beginning of the experiment and used as a reference).*

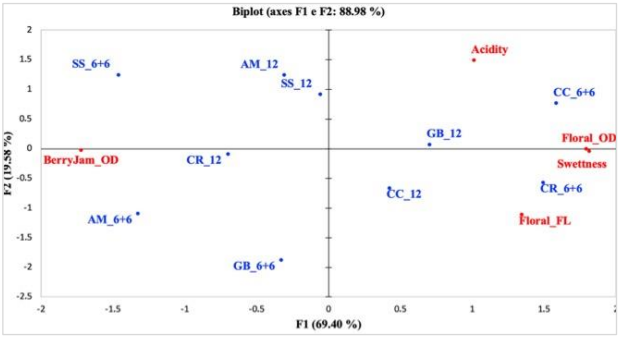

Figure 3. *Principal component analysis (PCA): scores and loadings plot of the significative sensory attributes evaluated in the Sangiovese red wine at 12 months aging (_12: 12 months aging in tank; _6 + 6: 6 months aging in tank plus 6 months of bottle aging; CR: uncoated concrete; CC: epoxy coated concrete; SS: stainless steel; AM: raw earthenware amphorae; GB: glass bottle with wine bottled at the beginning of the experiment and used as a reference).*

4.3 Materials characterization and OTR

All materials were first characterized by their physical properties, including thickness, dry weight, density, and water absorption. All these properties are intrinsic to the material and the manufacturing process. The epoxy-coated concrete was the only materials that did not show water absorption and it was impermeable to oxygen in all the tests carried out. This type of material, due to its internal epoxy-coating, does not allow any exchange, including water absorption, according to the results found in the literature (Nevares & del Alamo-Sanza, 2021). The other types of materials, on the other hand, were permeable to oxygen in all the conditions analysis carried out (dry, humid and model wine contact). When the material was in dry condition, the OTR values of the materials differed mainly as a function of density. The densest materials had lower OTR while with lower densities higher OTR was observed (Maioli et al., 2022).

5. Conclusions and Future Perspectives

These findings could be useful for winemakers since the tank material represents an important choice in the wine production process as a function of the oenological aim and definition of the wine style. In fact, in order to obtain a color stability of the red wine, materials such uncoated concrete or earthenware raw amphorae could be a good alternative to wood when peculiarity of the varietal character needs to be preserved without conferring to the wine the volatile compounds that are typical of the toasted oak.
Further research will be necessary in order to highlight the elementals release trend and the oxygen permeation dynamic during time and, similarly to the study available in the literature for oak barrel, according to the subsequent re-fill of the tank.

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Bioetholgy of a promising parasitoid associated with fig pests

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Activities of the PhD project are described. First, the overview of the literature concerning the genus *Sclerodermus*, a quasi-social parasitoids. Secondly, the study of intraspecific competition (between individuals of the same species) in *S. brevicornis*. Finally, choice behaviour was analysed in relation to the degree of kinship between the parasitoids and the host on which they were reared (target or substitution). Females usually cooperate with each other, but kinship relationships and the rearing host may influence their behaviour.

Bioetologia di un promettente parassitoide associato agli insetti dannosi per le piante di fico

Alcune attività del progetto di dottorato sono descritte. Come prima cosa il riassunto della bibliografia riguardante il genere *Sclerodermus*, parassitoidi quasi sociali. Secondariamente lo studio della competizione intraspecifica (tra individui della stessa specie) in *S. brevicornis*. In ultimo è stato analizzato il comportamento di scelta in relazione al grado di parentela tra i parassitoidi e all'ospite su cui erano allevati (target o di sostituzione). Le femmine infatti solitamente cooperano tra di loro, ma le relazioni di parentela e l'ospite di allevamento possono influenzarne il comportamento.

Key words: Parasitoids, bioethology, behavior, choice test, *Sclerodermus* sp., sociality.

1. Introduction

In accordance with the PhD thesis project previously described (Malabusini, 2020), and also considering the preliminary data discussed in Malabusini (2021), this essay improved preliminary results of the main activities:
A1) Update the State-of Art, comparing data from different contribution all over the year.
A2) Intraspecific competition studies: this aspect was tested on *S. brevicornis* reared on *Psacothea hilaris hilaris* (A2.1), in order to evaluate the effect of the overexploitation of the host on *S. brevicornis* performances.
A3) The influence of kinship degree and of the rearing host on parasitoid performance and behaviour.

2. Insects causing fruit losses

Insect pests destroy approximately 14% of all potential food production globally and it is estimate that fruit losses from pests and pathogens accounts for at least 3 million tonnes of productions to the EU fruit industry (EU, 2018). Historically, pest control in orchards has been based on the use of broad-spectrum pesticides. However, these have caused a number of problems: the reduction of beneficial organisms, pesticide resistance, the threat to people's health, and food safety endangered by pesticide residues (Damos *et al*., 2015). For all these reasons, the EU increased the restrictions on the use of plant protection products in recent years, and therefore biological control is once again a viable option (Ghosh, 2011).
The yellow-spotted longhorn beetle *Psacothea hilaris hilaris* (Pascoe) (Coleoptera, Cerambycidae, Lamiinae, Lamiini) represents one of the exotic wood boring beetles imported in the Mediterranean area during the last decades (Jucker *et al*., 2006; Cocquempot and Lindelöw, 2010) associated to fruit plants belonging to the Moraceae family, as *Ficus* spp. and *Morus* spp. The most serious damages, that usually lead to the death of the tree, are caused by the larvae that develop and feed into the trunk and branches (Lupi *et al*., 2013). Xylophagous insects are among the most difficult plant pests to control, as they spend most of their life cycle inside wood of living plants; but there are some parasitoid species that, thanks to their morphology, are able to find the larvae inside the tunnels. In fact, in China species of the genus *Sclerodermus* are already used for this purpose in biological control programs (Yang *et al.*, 2014; Lupi *et al*., 2017).

3. Bioethology of parasitoids

In Italy, in association with *P. h. hilaris* was found *Sclerodermus brevicornis* (Lupi *et al*., 2017), a quasi-social parasitoid. The females share the oviposition site and take care of the offsprings in group until their emergence (Tang *et al*., 2014). This aspect allows females to attack and complete development on big-size hosts, that otherwise would have not been attacked by a solitary small female (Tang *et al.*, 2014; Liu *et al*., 2021). In order to optimise the use of biocontrol agents and, consequently, mass rearing, the study of their bioethology is fundamental. Deepening knowledge of their life cycles, their sex ratio, the host parasitisation methods and their behavioural strategies can contribute to the efficiency of their action in the field.

The study of behaviour must consider that the interactions become more intense as the degree of sociality increases. Indeed, recent research on parasitoid biology has demonstrated that some aspects of insect behaviour can be affected by the degree of kinship (Abdi *et al*, 2020a). Cooperation between two *S. brevicornis* foundress, during the paralysis of the host, occurs especially when the two females are relatives (Abdi *et al*., 2020b).
To ensure the effectiveness of biological control, it is also crucial to conduct studies on less studied aspects, including the mass production of organisms (Maurya, 2020).
It is known that mass rearing systems are affected by the host used to produce new offsprings (Abdi *et al.*, 2020). Since rearing *Sclerodermus* sp. on its natural hosts is time consuming and physically demanding, it is necessary to find a factitious host with lower production costs and that is easier to rear (Vincent *et al*., 2021). *Corcyra cephalonica* was successfully used for the rearing of *S. brevicornis* (Abdi *et al*., 2020), but the influence on parasitoid behaviour of the host from which they emerged, has not yet been studied.

4. Materials and Methods

A1) Concerning the analysis of the literature, a database was created using different platforms [Web of Knowledge, Scopus, Google Scholar, Explora/Minerva (UNIMI Platform), Research Gate] and papers collected but not available online, until the end of 2021. The research was not restricted by date and language, and different key words, regarding *Sclerodermus* species were used. Each scientific contribution found was first classified with: authors, the species, year of publishing, and type of contribution (paper, book). To organize and easily identify the type of research developed by each contribution, we chose 10 topic-words able to define the main topic and other 17 as sub-topics. From each paper, data relating to the biology of parasitoids, both raw and average data, were extrapolated, collected and analyzed.
A2) To force the competition a single larva of *P. h. hilaris* (0.47-0.52 g) has been put in contact with an increasing number of foundresses (n=10, 25, 35, 45, 55 foundress/victim), collected from the same brood (N (larvae x foundress number) =21), as explained in Malabusini (2021). The samples, under controlled condition, have been checked once per day, taking note of the parasitisation, the presence of eggs, larvae, adults and the number of offsprings.
A3) To evaluate the effect of kinship and rearing host on choice behaviour, choice tests (Figure 1) were set up under controlled conditions and checked as explained for kinship choice test in Malabusini (2021). Choice test to assess the effect of rearing host on the behaviour consisted in a three-sector Petri dish with one larva of *P. h. hilaris* (0.25 ± 0.004 g) and one larva of *C. cephalonica* (0.039 ± 0.008g) and four *S. brevicornis* females coming from different rearing system (4 from *P. h. hilaris*, 4 from *C. cephalonica*, 2 from *P. h. hilaris* + 2 from *C. cephalonica*). The choice tests have been checked three times a day, taking note of the movements of the parasitoids according to their origin and also to the biological parameter associated to parasitisation (egg laying, egg hatching, larval presence, pupae and offspring number).

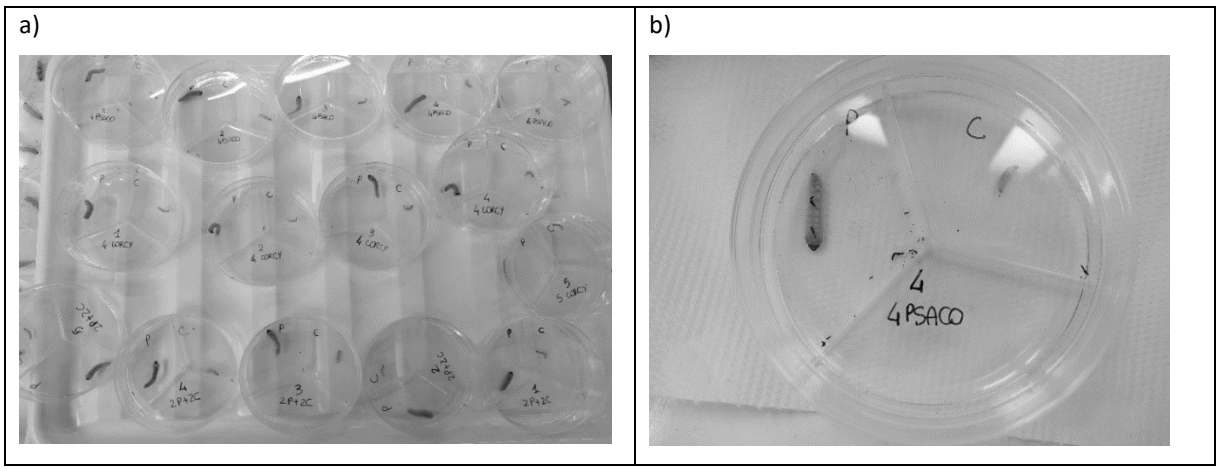

Figure 1. *(a) Example of a Chioce-tests group; (b) Example of a single Choice-test in which the two different hosts can be seen* (P. h. hilaris *on left and* C. caphalonica *on right)*

5. Results and Discussion

5.1. Analysis of the literature

255 articles from 127 journals and 6 books were surveyed; conference presentations and proceeding were excluded from the search as they were difficult to find. Considering the 27 topic-words all together, the most used topic-words were “parasitism rate”, “behaviour” and “biological control”, respectively 10%, 10% and 9%, immediately followed by “biology”, “development”, and “systematic”. Analyzing all the different contributes (N=255), in relation with their main topic (the first topic-words assigned), the 12% (N=31) of them regarding medical and health-related work (most of them (N=17, 55%) referred to *S. domesticus* specie), 18% (N=47) of them to

biological control and overall, the 60% (N=154) of them prevalently regarding the bioethology of *Sclerodermus* sp.

There are 80 recognized species of *Sclerodermus* in the world, among them, *S. guani*, *S. domesticus*, *S sichuanensis* and *S. pupariae*, cover most of the papers respectively 35%(N=101), 12% (n=33), 10% (N=27) and 8% (N=24) of the cases. Only for the 46% (N=11) of the number of species cited, there are some information in journals about their bioethology.

Looking at the number of publications (Figure 2) from 1809 to the present days, it is possible to observe that while the mean number, until 2000, is 1.8 ± 0.15 publications per year with a maximum of four, from five-year-period 2001-2005 these number have started to increase with some fluctuation, reaching a peak of 13 mean of publications in the period 2016-2020. Considering the main topics covered by the different publications over the years, up to 1969, almost all the publications dealt with biology, systematics and medical issues. Since 1970, biocontrol has been discussed, and gradually all topics have been covered, with an increase in the number of papers dealing with development and behaviour since the 2000s.

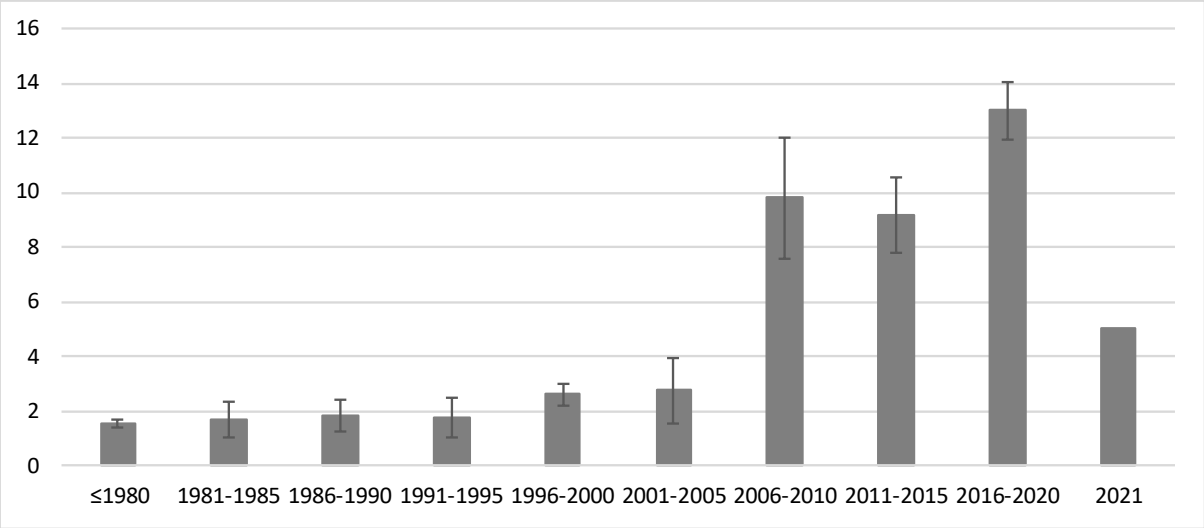

Figure 2. *Histogram of the mean number of publications per five years. "≤ 1980" represents the mean from 1809 to 1980. For 2021 the number represents the number of publications in 2021.*

5.2 Intraspecific competition

As we explained in Malabusini (2021) in *P. h. hilaris* test, the failure rate increased with the foundress number (R^2=0.9844). The highest number of failures (71%) was found in tests with 55 foundresses/host. We also found that there is a significantly curvilinear correlation between number of offspring and numbers of foundresses (log-linear regression including a quadratic term: Foundresses: F (1,103) = 14.42, p < 0.001, %Dev = 11.25; Foundresses^2: F1,102 = 6.19, p = 0.014, %Dev = 4.83). When foundress numbers were 10 and 25, they produced almost 100 offspring, but this number declined to around 15 as foundress numbers further increased. Considering only the 51 groups of foundresses that produced adult brood sizes, it ranged between six and 235 (mean ± SD = 115.59 ± 59.82), but there was a curvilinear relationship between brood size and foundress number (Foundresses: F1,49 = 5.63, p = 0.02, %Dev = 1.45; Foundresses^2: F (1,48) = 9.89, p = 0.003, %Dev = 2.56). In contrast, as usual, the per foundress production (offspring/foundress group size) declined significantly, as the number of foundresses increased (F1,103 = 81.24, p < 0.001, %Dev = 2.60), from 8 offspring to 0.5 offspring. The time took from the contact with the host and the emergence of adult offspring varied between 24 to 58 days and also within each developmental stage. It was marginally not significantly influenced by foundress number (cohort survival analysis, with replicates in which no adult offspring were produced treated as censors: G4 = 8.76, p = 0.067, n = 105). Analyzing all the timings of successive stages of brood production, it was observed a progressive decline in sample sizes, due to brood production failures at previous stages.

5.3 Kinship and rearing host influence

As shown in Figure 3, in 11 out of 14 trials (78.6%) carried out with 4 *S. brevicornis* females from breeding on *P.h. hilaris* (4Psacothea), the females oviposit on both larvae (PC); also in the "4 Corcyra" trials the percentage (58.3%, 7 out of 12 trials) of oviposition on both larvae was higher. In contrast, the host choice for oviposition by *S. brevicornis* in the "2Corcyra+2Psacothea" trials were evenly distributed, with no clear preference.

Despite this, it appears that, in each choice test and on each larva, neither the timing of development (on *P. h. hilaris*: F(2,19)=1.63, p=0.22) on *C. cephalonica*: F(2,23)=1.23, p=0.31), nor the number of eggs laid (40.33± 3.50; F(2,37)= 0.10, p>0.05) or adults emerged (24.3±3.39; F(2,37)=1.72, p=0.19) was influenced by the different groups created with parasitoids from different rearing system.

However, differences in behaviour were obtained; it seems that *S. brevicornis* from rearing on *P. h. hilaris* preferred to move between the two host larvae ($F(2,157)=3.63$, $p<0.05$), exploring both. Remaining, however, like those from *C. cephalonica* longer on the larvae of the host target.
The analysis on the tests with 2 couple of *S. brevicornis* from the two different rearing host (2Corcyra+2Psachotea) confirmed what was observed for the choice tests analyzing the degree of relatedness explained in Malabusini (2021). Females of *S. brevicornis* prefer to be alone or in the company of a non-kinship (in this case also not from the same rearing system) rather than in the company of relatives ($F(4,107)=10.09$, $p<0.001$).

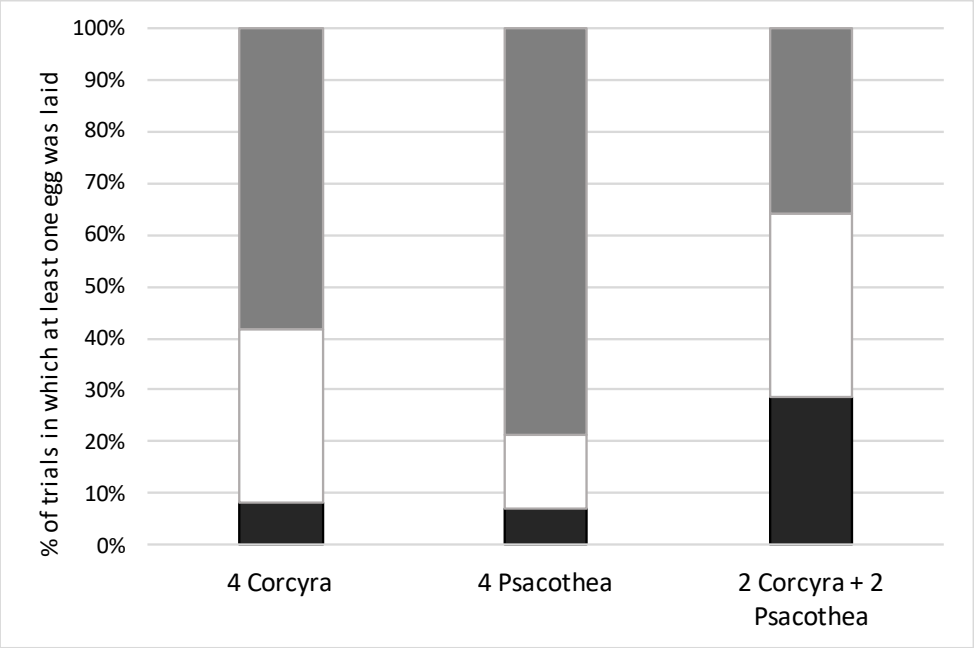

Figure 3. *Percentage of tests in which females of* S. brevicornis *oviposit only on* C. cephalonica *(Black), only on* P. h. hilaris *(White), or on both larvae (Grey), divided by the type of test [4 parasitoids from rearing on* C. cephalonica *(4 Corcyra); 4* S. brevicornis *females rear on* P. h. hilaris *(4 Psacothea); 2 parasitoids from rearing on* C. cephalonica *and 2 from rearing on* P. h. hilaris *(2Corcyra+2Psacothea)].*

6. Conclusion and future perspective

The analysis of literature shows that interest in these parasitoids has increased considerably in recent years; and also, the interest in their bioethology, also due to the recent interest in biological control and quality mass rearing. *S. brevicornis* is quasi-social, consequently females usually cooperate, but, when the number of parasitoids on the same host is too high, the percentage of failure in obtaining new offsprings increases, as the number of offspring per foundress decreases. The rearing host fortunately does not seem to influence the performance of the parasitoid, however, both kinship and rearing host can influence the choice behavior.
It was found that *S. brevicornis* females reared on the target host made more movements between victims than those reared on the factitious one. This behavioural difference may be a consequence of the different rearing host: usually parasitoids reared on the factitious host are morphologically smaller than those reared on the target host and probably feel intimidated by the larger larva (Abdi *et al.*, 2020b; Tang *et al.*, 2014; Wei *et al.*, 2017). Parasitoids with larger size possess more physiological and behavioural advantages, as they are able to subdue larger and high-quality hosts, compared to smaller specimens, and also the host-parasitoid interactions tend to increase with parasitoid size (Godfray, 1994). These aspects could explain the greater propensity for movement of *S. brevicornis* from *P. h. hilaris* rearing.
Each *Sclerodermus brevicornis* female, even the ones reared on *C. cephalonica*, has been found to stay longer on the *P. h. hilaris* larva, this is probably due to the greater palatability of *P. h. hilaris*, the larger size and the fact that they are their natural host. This result seems to confirm that, regardless of the rearing host, *S. brevicornis*, even if released in the field, would remain 'faithful' in their choice of target host.

The reproductive strategies implemented, and consequently the associated behaviour, are based on the kinship of the foundresses and the size of the host larva (Abdi *et al.*, 2020b). If, as in this case, the host larva is large (considering *P. h. hilaris*), and the foundresses are not 'sisters', the tendency is to wait, behaving selfishly, and then to take advantage of the already paralyzed host to oviposit its brood, as suggested by Abdi *et al.* (2020b). The difference, highlighted in these tests, is that *S. brevicornis*, faced with a choice, preferred to separate from its 'sisters' in order to gain access to both hosts, as also explained in Malabusini (2021).

In conclusion, these results once again underline the complexity of the behaviour of these parasitoids (Tang *et al.*, 2014). Therefore, following an in-depth knowledge of bioethology, the biological control of the longhorn beetle *P. h. hilaris* in Italy could be feasible in the field by the release of *S. brevicornis*, which has been found to maintain an almost complete specificity of action for the target host, even when reared on the factitious one, *C. cephalonica*. Furthermore, the origin of *S. brevicornis* did not affect the development time and brood size in the laboratory. However, it will be useful to deepen the knowledge about the behaviour of this parasitoid by creating increasingly complex systems to get as close as possible to the situation we would find in field, which is difficult to investigate.

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The ability of *Streptococcus thermophilus* ST870 to modulate urease activity in healthy subjects' fecal samples depends on the biomass production process

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The main aim of the PhD project is the optimization of the fermentation and post-fermentation processes for probiotics production to implement their stability and their fitness during the gastrointestinal tract transit in human subjects. Here it was evaluated the recovery of viable *S. thermophilus* ST870 after lyophilization with two different cryoprotectants, cryo-A and cryo-B. The recovery of *S. thermophilus* ST870 viable cells was not affected by using different cryoprotectants. Most importantly, *S. thermophilus* ST870/cryo-A maintained a high level of urease activity, and it was able to significantly reduce stool urease activity in the subjects after one week of administration.

La capacità di *Streptococcus thermophilus* ST870 di modulare l'attività ureasica nei campioni fecali di soggetti sani dipende dal processo di produzione della biomassa

Lo scopo di questo dottorato di ricerca è l'ottimizzazione dei processi di fermentazione e post-fermentazione per la produzione dei probiotici con elevata stabilità e resistenza al passaggio gastrointestinale in soggetti umani. Qui è stato valutato il recupero di cellule vitali di *S. thermophilus* ST870 dopo liofilizzazione con due crioprotettori, cryo-A e cryo-B. Il recupero delle cellule vitali di *S. thermophilus* ST870 non è stato influenzato dall'uso di diversi crioprotettori. Ancora più importante, *S. thermophilus* ST870/cryo-A ha mantenuto un alto livello di attività ureasica, ed ha ridotto significativamente il contenuto di attività ureasica fecale nei soggetti dopo una settimana di somministrazione.

Keywords: cryoprotectant; probiotic; urea; urease activity; interventional study.

1. Introduction

One of the main PhD project activities (Martinović, 2020; Martinović, 2021) was focused on the optimization of post-fermentation conditions for improving the gastrointestinal transit (GIT) of *S. thermophilus* ST870 in humans by using previously developed strain-specific molecular tool (Martinović, 2021). Specific objectives of this activity included: (i) quantification of *S. thermophilus* ST870 DNA in stool samples using previously developed strain-specific qPCR assay (Martinović, 2021); (ii) evaluation of cryo-A or cryo-B interference with *S. thermophilus* ST870 recovery after the GIT; (iii) evaluation of subjects' stool frequency, consistency, and safety after the administration of *S. thermophilus* ST870 biomass produced with cryo-A and cryo-B; (iv) effect of the administered *S. thermophilus* ST870 biomass produced with cryo-A and cryo-B on the level of urease activity in stool samples.

2. Materials and methods

2.1 Preparation of freeze-dried *S. thermophilus* ST870/cryo-A and cryo-B

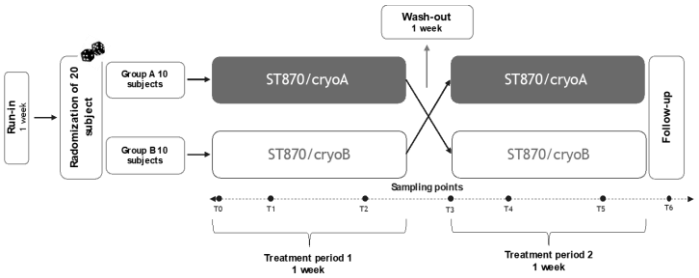
Freeze-dried *S. thermophilus* ST870 biomasses have been prepared by Sacco Srl using a single fermentation batch. At the end of the fermentation process, *S. thermophilus* ST870 biomass was collected and concentrated by centrifugation, and splitted into two aliquots. One aliquot was supplemented with the cryoprotectant A (cryo-A) and the second aliquot was supplemented with the cryoprotectant B (cryo-B). Both the aliquots were subsequently freeze-dried and packaged in 1 g sachets. Before starting the intervention study, the viability of *S. thermophilus* ST870/cryo-A and cryo-B was quantified by flow cytometry according to the protocol described in Mora and coauthors (2019).

2.2 *In vivo* interventional study design and protocol

The study was a randomized cross-over (Figure 1) with 20 healthy (non-diseased) adult volunteers of both sexes (60 % females, 40 % males), aged from 23 to 57 years (average age 33 ± 11). Exclusion criteria were people with the abnormality of the gastrointestinal tract (i.e., inflammatory bowel diseases such as Crohn's disease, ulcerative colitis), pregnancy, metabolic diseases, primary or secondary immunodeficiency, antibiotics intake within 1 month before the screening visit, subjects hypersensitive or allergic to any study product's ingredient and subjects participated in other clinical trials in the past 3 months.

During the first visit (T0) each volunteer signed a consent document and received the general information about the entire procedure. The study consisted of a run-in phase (1 week) during which the volunteers followed their

conventional diet with a ban of probiotic-fermented milks, traditional yogurt, fermented mozzarella, fresh cheeses (e.g., stracchino and crescenza), probiotic, prebiotic, and symbiotic foods and supplements. During this period volunteers were instructed on how to take the products in the treatment phase. Volunteers received the questionnaire to evaluate the intensity of symptoms (e.g abdominal pain and bloating), stool frequency, and consistency by using the Bristol stool scale.



Prior to starting with the treatments subjects were randomized in 2 groups of 10 people. Afterwards, in the first treatment phase which involved the consumption of one sachet per day (1 g) containing $1x10^{11}$ FU/g of lyophilized biomass of *S. thermophilus* ST870/cryo-A or cryo-B (Figure 1).

***Figure 1**: In vivo study design and time sampling*

The sachet product was reconstituted in drinkable water just before intake, under fasting conditions, in the morning before breakfast, or alternatively in the evening at least 2 hours after the last meal of the day, before going to bed. Following the one week of treatment, volunteers underwent one week of a wash-out, identical to the run-in period. After the wash-out, the volunteers began the second treatment phase lasting one week, which involved taking one sachet per day in the presence of cryo-B (for those who had taken the probiotic in the presence of cryo-A- during the first treatment phase), or cryo-A (for those who had taken the probiotic in the presence of cryo-B during the first treatment phase) (Figure 1). Volunteers delivered the completed questionnaire at the end of the follow-up period. The study protocol was approved by the Research Ethics Committee of the University of Milan (opinion no. 52/21, May 2021).

2.3 Sample collection and analysis

Each stool specimen (at least 2 g) was collected in sterile containers, stored at 4°C, and delivered to the laboratory within 24 h. To verify the viable recovery of the *S. thermophilus* ST870 strain after human GIT, the collected fecal samples were immediately subjected to the analysis. The fecal samples, 1g were diluted in Maximum Recovery Diluent (Scharlau, Italy), homogenized in a sterile stomacher bag, serially diluted, and plated on a modified milk-based medium (Della Scala *et al*., 2019). After incubation (37°C for 48 h) the biomass was collected, then DNA extraction was performed with QIAsymphony® SP (Qiagen, Hilden, Germany) automated system by using QIAsymphony® kit. For total DNA counts 0.25g of the fecal sample was weighted, resuspended in the first QIAsymphony® kit solution, and extracted as described before. For all q-PCR analysis the reaction mixture was prepared as recommended by the manufacturer. The amplification was carried out using SYBER® Green chemistry with the following thermal program: initial hold at 95°C for 3 min, followed by 37 cycles of 95°C for 20 s, 62 °C for 25 s, and 72 °C for 5 s. Data analysis was performed using the CFX Maestro Software (Bio-Rad, California, US).

Urease activity in the fecal sample was determined using a spectrophotometric assay based on pH increase due to the urea hydrolysis and consecutive ammonia release. The assay carried out was based on the phenol red assay described by Lanyi (1987) with some modifications already described in Della Scala *et al.* (2019). To this aim the specimens were subjected to total protein extraction using Precellys® 24 Beadbeater (Bertin, France) and urease assay was carried out under standardized protein concentration. Urease activity was performed in 96 well plate at 37 °C and kinetics was monitored every 15 min using a Biotek EON spectrophotometer (BioTek Instruments, Inc., CA, US). The results were expressed as maximum velocity (maxV) as mO.D.$_{555\ nm}$/min). Analogously, but starting from 1 g of freeze-dried biomass the urease activity was measured in cryo-A and cryo-B sachets.

2.4 Statistical analysis

Statistical analyses were performed by Prism-GraphPad software, version 8.4.3. All data were checked for normality and homoscedasticity and then parametric or non-parametric statistics was applied. The following statistical elaborations were performed to identify significant differences between treatments: Mann-Whitney, t-test. When appropriate, a post-hoc tests were performed. Significance was set at $p < 0.05$.

3. Results and discussion

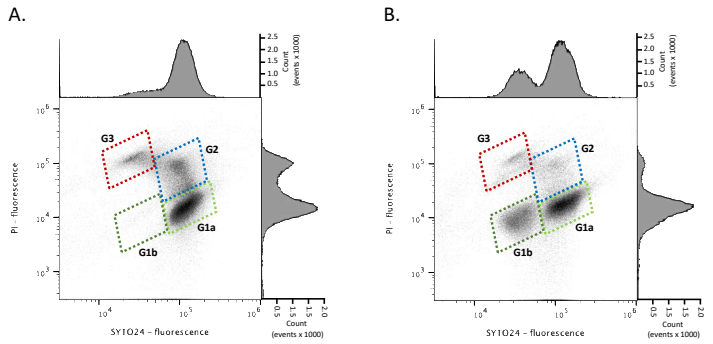
3.1 Quantification of *S. thermophilus* ST870 cell viability and urease activity in cryo-A and cryo-B sachets

Quantification of cell viability in *S. thermophilus* ST870 cryo-A and cryo-B sachets was carried out as described in ISO 19344 IDF 232 (2015) and in Mora *et al*. (2019). Cryo-A and cryo-B biomasses showed the same number of viable cells (Table 1) but significantly different amounts of $4.8x10^9$, $1.7x10^{10}$ FU/g damaged and $8.5x10^9$, $3.3x10^{10}$ of dead cells for cryo-A and cryo-B, respectively.

***Table 1**. Quantification of live, dead, and damaged cells in S. thermophilus ST870 cryo-A and cryo-B*

Product	G1a	G1b	G1a+G1b	G2	G3	Total
cryo-A (T0)	$1.6x10^9±1x10^8$	$1.0x10^{11}±2x10^9$	$1.03x10^{11}±3x10^9$	$3.8x10^{10}±1x10^9$	$1.4x10^{10}±2x10^9$	$1.52x10^{11}±2x10^9$
cryo-A (T6)	$1.1x10^9±2x10^8$	$9.5x10^{10}±1x10^9$	$9.6x10^{10}±3x10^9$	$5.9x10^{10}±1x10^9$	$1.1x10^{10}±2x10^9$	$1.66x10^{11}±6x10^9$
cryo-B (T0)	$3.7x10^{10}±1x10^9$	$6.9x10^{10}±1x10^9$	$1.10x10^{11}±2x10^9$	$7.5x10^9±1x10^8$	$3.5x10^9±2x10^8$	$1.21x10^{11}±2x10^9$
cryo-B (T6)	$3.6x10^{10}±1x10^9$	$6.9x10^{10}±2x10^9$	$1.10x10^{11}±4x10^9$	$1.4x10^{10}±2x10^9$	$2.6x10^9±4x10^8$	$1.27x10^1±6x10^9$

G1a, G1b, G2, G3: cytometric populations of viable cells. The results are expressed as fluorescence units (FU/g)



Interestingly, the use of cryo-A or cryo-B had a different effect on the cytometric populations of viable cells, based on Syto24 fluorescence. Using cryo-A, only one main population of live cells, the G1a was detectable (Figure 2A), whereas the use of cryo-B determined the formation of an additional population of live cells, the G1b, which was characterized by a lower level of Syto24 fluorescence (Figure 2B).

***Figure 2**: Cytometric populations of viable cells*

3.2 Total *S. thermophilus* ST870 DNA counts in healthy adults' fecal samples

The *S. thermophilus* ST870 DNA was quantified by using a previously developed qPCR strain-specific protocol (Martinović, 2021). *S. thermophilus* ST870 counts ranged from 3.59 to 8.15, and from 4.57 to 8.22 $\log_{10}$ cells per gram of feces in subjects that consumed *S. thermophilus* ST870 prepared with the cryo-A and cryo-B, respectively (Figure 3A). The higher percentage of *S. thermophilus* ST870 detection for both the time collection points, T1/T4 and T2/T5, was measured for those subjects that consumed *S. thermophilus* ST870 prepared with the cryoprotectant A (cryo-A T1/T4: 90, cryo-A T2/T5:100%; cryo-B T1/T4: 80, cryo-B T2/T5:95%). However, there were no significant statistical differences (p=0.420; p=0.967) for the same sampling point times between subjects that consumed *S. thermophilus* ST870 prepared with the two different cryoprotectants (cryo-A T1/T4 vs cryo-B T1/T4; cryo-A T2/T5 vs cryo-B T2/T5) (Figure 3A). Furthermore, statistics showed no significant difference (p=0.987) between the total detected amount of *S. thermophilus* ST870/cryo-A and cryo-B (Figure 3B).

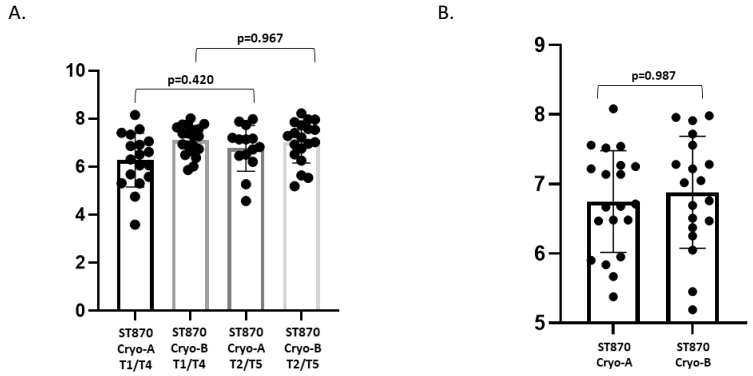


***Figure 3**: Total DNA counts with individual subject values per sampling point (A), and averages of cryo-A and cryo-B treatments (B). Mann-Whitney test, p<0.05. Dots represent individual subjects' DNA counts (log_{10}cells per gram of feces)*

3.3 Recovery of viable *S. thermophilus* ST870 in the fecal sample and its persistence

For recovery of viable ST870 we used a milk-based medium (Della Scala *et al*., 2019) combined with the developed strain-specific qPCR ST870 protocol (Martinović, 2021). At baseline (T0), no viable ST870 cells were present in the analyzed fecal samples. This was expected considering that the subjects were instructed not to consume any food or probiotic supplements possibly containing *S. thermophilus* strains. We were able to recover ST870 strain in 90 % of the subjects, demonstrating that ST870 can survive human GIT when consumed as lyophilized biomass irrespectively of the cryoprotectant used. As most of studies in humans (Martinović *et al*., 2020) were performed by using not accurate methods no reliable data are available on the *in vivo* survival of other *S. thermophilus* strains. Moreover, the GIT transit is a strain-depended probiotic trait, and the recovery of viable cells depends on the molecular and/or culture-based methods used (Martinović *et al*., 2020).

In the first-time collection points (T1 and T4) *S. thermophilus* ST870 was recovered in 55 and 45 % of the subjects that consumed the microbial biomass prepared with cryo-A and cryo-B, respectively. The recovery of live *S. thermophilus* ST870 increased to 75 % of the subjects for both treatments in the second time collection point. Intra-individually, in 10 subjects the recovery was higher when they consumed the cryo-A biomass, in 7 subjects the recovery was higher when consumed the cryo-B biomass. The recovery was absent in two subjects during the

whole study period and one subject showed low recovery at the end of the study period (subject 16, Ct 29.6). On average, there were no significant statistical differences (p=0.889; p=0.133) for the same sampling point times between different treatments (cryo-A T1/T4 vs cryo-B T1/T4; cryo-A T2/T5 vs cryo-B T2/T5) (Figure 4A). Moreover, no statistical difference between the averages of cryo-A and cryo-B treatments (Figure 4B).

The recovery of viable *S. thermophilus* ST870 from fecal samples collected, at the end of the wash-out period (T3), i.e., 4 to 5 days from the last product intake, no one subject was positive for the recovery of strain ST870. On the contrary during the follow-up (T6, 2-3 days after the second treatment) *S. thermophilus* ST870 was detected in 50% of the subjects, thus highlighting the persistence of ST870 strain in human gut for a maximum of 2-3 days after the last product intake.

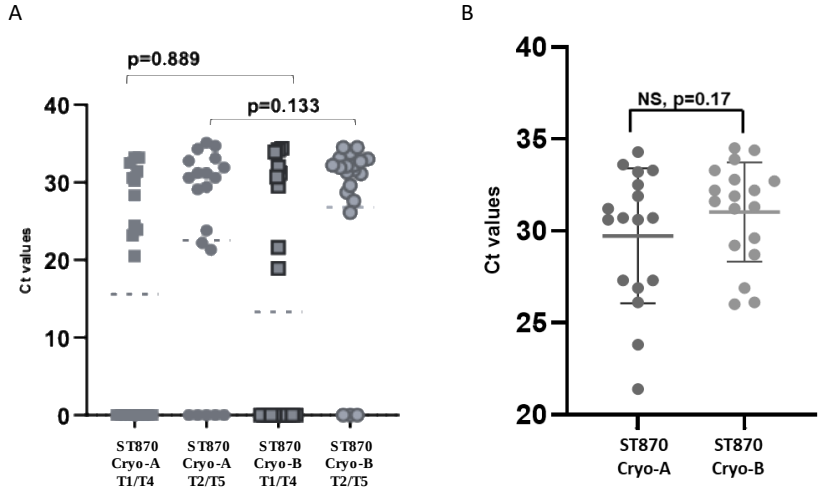


Figure 4*. Ct values of viable DNA counts with individual subject values per sampling point (A), and log difference between averages of cryoA (S) and cryoB (M) treatments (B). Mann-Whitney test, p<0.05. Dots represent individual subjects' Ct values.*

3.4 Defecation frequency and stool consistency:

Upon probiotic treatment no relevant changes were observed for defecation frequency and consistency. The most subjects reported one stool evacuation per day during the entire study duration, with inconsiderable changes in defecation frequency when the ST870 was lyophilized by using cryo-A or cryo-B cryoprotectants. Stool consistency did not significantly change (p=0.124) during the study period, with score 4 (like a smooth soft sausage or snake). Already satisfactory parameters of digestive parameters and relative short probiotic administration period could be explanations for not considerable changes.

3.5 Urease activity in the fecal sample

The samples collected during run-in (Baseline, B), and the second time collection points from both treatments (Cryo-A T2/T5, Cryo-B T2/T5) were analyzed. We observed modulation of urease activity in a stool sample due to *S. thermophilus* ST870 consumption. In 14 subjects, a decrease in urease activity was observed after administration of *S. thermophilus* ST870/cryo-A compering to the cryo-B, and in 6 subjects a decrease in urease activity after consumption of *S. thermophilus* ST870/cryo-B compering to cryo-A (Figure 5). The differences showed to be statistically significant in 13 subjects, out of which urease activity significantly decreased in 10 subjects in the case of *S. thermophilus* ST870/cryo-A compering to *S. thermophilus* ST870/cryo-B. Relative to the baseline, a decrease in the urease activity was observed in 16 subjects when *S. thermophilus* ST870/cryo-A was administered, and in 10 subjects when *S. thermophilus* ST870/cryo-B was used. On average, *S. thermophilus* ST870/cryo-A showed to be significantly better for decreasing initial urease activity in the stool sample, p=0.022 (Figure 5). The decrease of stool urease activity in IBDs patients after consumption of the commercial probiotic supplement containing *Bifidobacterium longum*, *B. infantis*, *B. breve*, *Lactobacillus acidophilus*, *L. delbrueckii* subsp. *bulgaricus*, *Lactocaseibacillus casei*, *Lactiplantibacillus plantarum*, and *S. thermophilus* strains were also reported in the study of Brigidi and coauthors (2001). Furthermore, modulation of urea hydrolysis by beneficial urease positive *Lactobacillus reuteri* 100-23 strain was reported in the murine stomach (Wilson *et al*., 2014). Inoculation of a conventional murine host with commensal *Escherichia coli* engineered to express urease led to dysbiosis of the gut microbiota, resulting in a predominance of *Proteobacteria* species and it was associated with a worsening of immune-mediated colitis in these animals (Ni *et al*., 2017). Moreover, in IBD patients, it was reported that intestinal inflammation leads to the growth suppression of indigenous beneficial urease positive bacteria such as *Blautia* and *Ruminococcus* spp., and niche replacement by urase positive harmful bacteria (Ni *et al*., 2017; Ryvchin *et al*., 2021). Based on our data and on the available literature, we could hypothesize that the administration of beneficial urease positive bacteria, such as *S. thermophilus* could led to higher urea consumption in the upper part of the gut, thereby determining a strong reduction of urea in the large intestine, and a consequent decrease of harmful fecal urease-positive bacteria, such as *Proteus mirabilis*, *Klebsiella pneumoniae*.

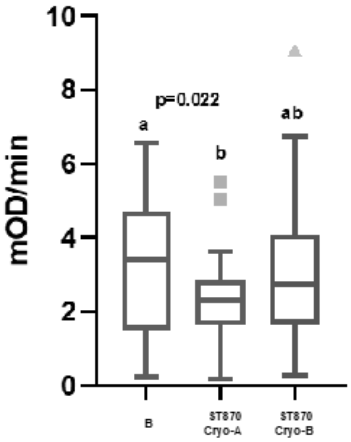


***Figure 5**. Boxplots, urease activity mV (mOD/min) averages at baseline (B), cryo-A and cryo-B. Fridman test showed significant difference between baseline and treatment periods (p=0.019), with Dunn's post hock test showing significant difference between baseline and sucrose treatment (p=0.022). B: baseline; U.A: urease activity; nd: non detected; ↓ decrease of urease activity. Data are the means of four replicates ± standard deviation. A vs B statistically elaborated using t-test (p<0.05)*

4. Conclusion

This study represents the first evidence that links *S. thermophilus* to a specific probiotic mechanism, *i.e.* the reduction of urease activity in fecal samples. Moreover, this study highlight, how the manufacturing conditions could play a role influencing the molecular composition and functionality *in vivo* as recently underlined for other probiotics (Duboux *et al.*, 2021). Clinical studies on IBD subjects are necessary to verify if the administration *S. thermophilus* ST870/cryo-A could be considered therapeutic for IBD patients.

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Application of metabolites secreted by plant growth-promoting bacteria to selected crops and evaluation of nutritional quality thereof

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The present Ph.D. research project falls within the framework of identifying new technologies for sustainable agriculture to satisfy the emerging demand for healthy food. It specifically falls within bio-based products such as microbial plant biostimulants which have emerged as a promising alternative to agrochemicals and a new frontier of investigation. In detail, the role played by selected strains of the *Enterobacter* and *Pantoea* group in the production of novel plant postbiotics was investigated. Possible effects on food quality are also considered. A multidisciplinary approach comprising microbiology, genetics, metabolomics, and nutrition was applied.

Applicazione di metaboliti prodotti da batteri promotori della crescita delle piante ad una selezione di colture e valutazione del loro effetto sulla qualità dei frutti

Il progetto di dottorato si inserisce nell'ambito della ricerca di nuove tecnologie per un'agricoltura ecosostenibile in grado di soddisfare la crescente richiesta di cibo sano e sostenibile. In particolare, ha lo scopo di i) studiare e definire le potenzialità di ceppi selezionati di microrganismi promotori della crescita delle piante, appartenenti al gruppo *Enterobacter* e *Pantoea*, nella produzione di bio-stimolanti da applicare a colture orticole o arboree, e ii) valutare la qualità nutrizionale delle produzioni. Il progetto propone un approccio multidisciplinare che comprende la microbiologia, genetica, metabolomica e nutrizione. Lo studio punta a fornire le basi per un'applicazione dei biostimolanti su larga scala.

Keywords: Plant Growth-Promoting Rhizobacteria; Indole-3-Acetic Acid; Microbial biostimulants; Sustainability; Metabolomics

1. Introduction

The intensive use of synthetic chemical fertilizers and pesticides has long guaranteed high yields but determined, on the other hand, environmental problems, ranging from groundwater contamination and soil quality degradation to biodiversity reduction. Effective and sustainable alternatives to synthetic chemicals have been searched, so that agriculture can manage the dual challenge of achieving food security while promoting sustainable food production systems. Over the last decades, significant interest has been shown in using plant biostimulants, which have emerged as a promising alternative to agrochemicals and have become a new frontier of investigation.

Plant bio-stimulants are products "stimulating plant nutrition processes independently of the product's nutrient content with the sole aim of improving one or more of the following characteristics of the plant or the plant *holobiont*: i) nutrient use efficiency, ii) tolerance to abiotic stress, iii) quality traits, iv) availability of confined nutrients in soil or rhizosphere" (European Union, 2019). Regulation (EU) 2019/1009 distinguishes between microbial and non-microbial, and the term "microbial plant biostimulants" can be applied, per law, only to Arbuscular Mycorrhizal Fungi (AMF) and Plant Growth-Promoting Rhizobacteria (PGPR) belonging to the three taxonomic groups of *Azotobacter*, *Azospirillum*, and *Rhizobium* (European Union, 2019).

Within the scientific community, several genera (e.g., *Pseudomonas*, *Bacillus*, *Pedobacter*, *Pantoea*, *Luteibacter*, *Acinetobacter*, *Lysobacter*, and *Enterobacter*) have been however investigated for their plant growth-promoting (PGP) traits and ability to produce indole-3-acetic acid (IAA) (Leontidou *et al.*, 2020). This compound is the most common plant hormone of the auxin class, which regulates various plant growth processes. IAA and other auxin-related compounds are produced by plants and microorganisms (Keswani *et al.*, 2020).

Identifying the major bacterial producers of IAA and other auxin-related compounds have become pivotal in identifying paths for sustainable agriculture. Hence, more and more PGPR strains have been studied because the production of bacterial auxins has proved, so far, to vary from strain to strain, and is affected by different factors, among which strain growth conditions and precursor availability, such as tryptophan (Duca *et al.*, 2014).

In accordance with the Ph.D. thesis project, this oral communication reports the main results of the research activities directed to:

A1) optimize the growth conditions of selected PGPR strains for high production of metabolites;
A2) understand the effect of metabolites application on tomato seedlings and *P.communis* cv DarGazi plantlets;
A3) acquire knowledge on the genomic features of *P.agglomerans* strain C1;
A4) understand the exometabolome of *P.agglomerans* strain C1.

2. Materials and Methods

2.1. Optimization of growth conditions for optimal production of metabolites by selected PGPR strains of the *Enterobacter* and *Pantoea* group

The two *P. agglomerans* strains used in this study were DSM3493 and C1. DSM3493 is the type-strain of the *P. agglomerans* genus; *P. agglomerans* strain C1 was previously isolated from the phyllosphere of lettuce (*Lactuca sativa* L.) plants treated with vegetal-derived protein hydrolysates (Luziatelli *et al.*, 2016). *Enterobacter* sp. strain P-36 was originally isolated by a commercial mycorrhizal inoculum and described as a strong producer of IAA (Agnolucci *et al.*, 2019).

Different growth media (i.e., vegetal peptone-yeast extract (VY) medium, Lennox broth (LB), Yeast Extract Sucrose (YES) broth, the saline M9) and conditions were investigated as reported in the literature (Luziatelli, Ficca, *et al.*, 2020; Luziatelli, Gatti, *et al.*, 2020; Luziatelli *et al.*, 2021). Strain growth was investigated in Erlenmeyer flasks and strain P-36 was grown in a 2-L stirred-tank fermenter, as well. Briefly, seed cultures were pre-activated and used to inoculate 25mL of Trp (4mM) amended medium. Cultures were grown in 250mL Erlenmeyer flasks, in triplicate, in an INFORS HT Multitron incubator at 180 rpm and 30°C. After 24-hour growth, 10mL of each culture was recovered for indole auxin quantification by Salkwoski's reagent. Growing conditions were optimized by Response Surface Methodology (RSM).

For the optimization of the indole auxin production by strain P-36, batch fermentation was carried out in a 2-L stirred tank fermenter connected to an Applikon ADI 1020 Bio Controller (Applikon Biotechnology, Delft, NL, USA). The fermenter was inoculated with an appropriate volume of an LB culture (OD_{600}: ~6.3) grown overnight in shake flasks at 180 rpm and 30 °C to have an initial cell density of 0.5 or 1×10^9 cells/mL (corresponding to a predicted initial OD_{600} of 0.2 or 0.4).

2.2. Application of metabolites to tomato seedlings and *P. communis* cv DarGazi plantlets

As regards the application of *P. agglomerans* strains metabolites to tomato seedlings, seedlings were cut to 1 cm from the collar, dipped into a solution of strain metabolites or commercial fertilizer (quick dip method), and placed in transparent polypropylene microboxes with filtering covers containing wetted quartziferous sand. Microboxes were sealed with parafilm® to ensure relative humidity close to saturation. Growth chamber parameters were kept constant. After seven days, roots were recovered and rinsed with distilled water. For root morphology determination, three cuttings per experimental unit were selected and the number of adventitious roots were counted manually. Entire roots were scanned to determine the root morphological traits by WinRHIZO.

The effect of microbial metabolites on plantlets of *P. communis* cv DarGazi was also investigated. In detail, shoots in the proliferation medium were sub-cultured at week intervals under a 16-hour light photoperiod using white fluorescent lamps at constant temperature (23±1°C). For adventitious rooting experiments, 20 mm long micro-cuttings, excised from proliferated shoot clusters, were used. Microcuttings were sub-cultured under the same conditions as shoots. The rooting medium was supplemented with i) exogenous auxin, or ii) IBA, or iii) a quantity of medium rich in *P. agglomerans* strain metabolites to achieve a final auxin content in the rooting medium of 0.2, 0.4 mg L^{-1}, and 1 mg L^{-1}, respectively. Plantlets treated with exogenous auxins (IBA) were used as control. Plantlets were sampled seven days after treatments (DAT) and 14 DAT. Control IBA was sampled at 35 DAT, as well, to compare with each other the rooted samples.

2.3. Genome sequencing of P. agglomerans strain C1

The genomic features of strain C1 were determined according to the protocol specified in (Luziatelli, Ficca, *et al.*, 2020; Luziatelli, Ficca, *et al.*, 2020). Briefly, the genome was sequenced by Illumina MiSeq technology. Genomic DNA was first extracted by PureLink Genomic DNA Mini Kit and quantified by Qubit dsDNA HS Assay Kit. Genome sequencing was performed, and run statistics were determined by CLC Genomics Workbench 12. The phylogenetic tree was built from user-selected genomes by the FastTree method using the Phylogenetic Tree Building Service available at the Patric website (www.patricbrc.org). Gene prediction analysis and functional annotation of the genome were performed by Rapid Annotation by Subsystems Technology (RAST).

2.4. Analysis of the exometabolome of *P. agglomerans* C1 strain

For untargeted metabolomics, the analysis was carried out by Quadrupole Time-of-Flight Liquid Chromatography-Mass Spectroscopy (Agilent 6550 Q-TOF coupled with an Agilent 1290 UHPLC). As to targeted metabolomics, MS and MS/MS acquisition were carried out following the FiehnLab HILIC protocol, as described in (Luziatelli, Ficca, *et al.*, 2020).

3. Results and Discussion

3.1. Optimization of PGPR strains growth conditions for optimal production of metabolites

The formulation of the different media was varied by i) changing growth conditions (i.e., growth medium volume, temperature, rotation speed); ii) adding the IAA precursor, tryptophan (Trp), or otherwise; iii) substituting it with other amino acids, e.g., glycine; and iv) varying other factors, such as the carbon source concentration, the pre-inoculum volume, or the initial optical density (OD). It emerged that Trp addition is mandatory for IAA production.

Glycine is not a valuable alternative. Higher carbon source concentration, pre-inoculum volume, and OD do not positively affect IAA production. Optimization by RSM showed that 25 mL medium inoculum, 180 rpm and 30 °C were the optimal growth conditions (Figure 1). The optimal conditions were applied to all experiments with the different growth media.

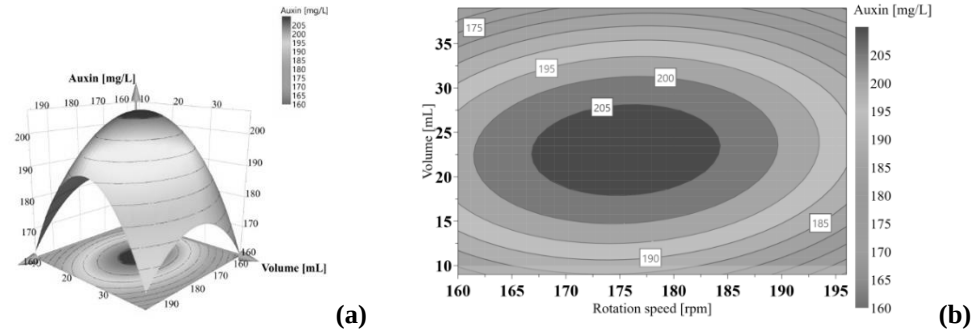

Figure 1 **(a)** *Response surface plots (3D, left) and* **(b)** *contour plots (2D, right) of auxin production as a function of significant interactions between factors i.e., rotation speed and volume.*

Investigation of auxin yield upon strain C1 growth in the four media (i.e., YES, M9, LB, and VY medium) showed that LB and VY media allow the highest IAA production (Figure 2).

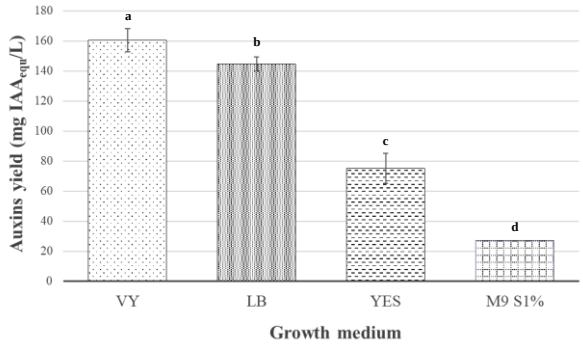

Figure 2 *Effect of growth medium on the indole auxin production yield by P. agglomerans C1 strain. Differences in letters indicate that the values are significantly different (p<0.05).*

The ability to produce IAA was also investigated in *Enterobacter* strain P-36. Upon growth in VY, LB, and YES medium amended with Trp, at varying fermentation time, it emerged that the highest auxin yield occurred in VY and LB medium (Figure 3), and the highest auxin/IAA level was obtained when strain P-36 was grown for 72 hours. The use of the VY medium determined a two-fold increase compared to the animal-based peptone (LB). Some differences, however, emerged between the two strains. When they were grown in Erlenmeyer flasks, the highest indole auxin yield was obtained upon 24-hour growth for strain C1 and upon 72-hour growth for strain P-36. Moreover, the auxin yield was by far higher for strain P-36 than for strain C1.

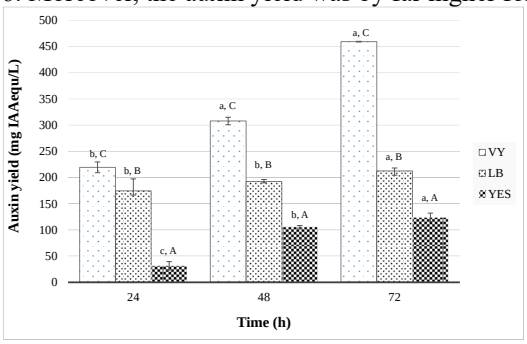

Figure 3 *Effect of growth medium and incubation time on the indole auxin production yield by the Enterobacter sp. P-36 strain. Results are means±SD of three independent experiments. Differences in letters indicate that the values are significantly different (p<0.05). Lower-case (a,b,c) letters are referred to values of the same series (growth medium), while capital (A,B,C) letters indicate statistically significant differences among values of different series (same incubation time)*

The *Enterobacter* strain P-36 was also cultivated in a 2-L fermenter under batch operating mode with VY medium amended with Trp. With respect to the growth in flasks, the highest indole auxin production was obtained after 48-hour growth in the fermenter. In addition, when the initial inoculum was varied, a similar auxin production was obtained with reduced fermentation time, that is, after 24 hours. Upon growth in the VY medium, auxin production was two-fold higher than in LB.

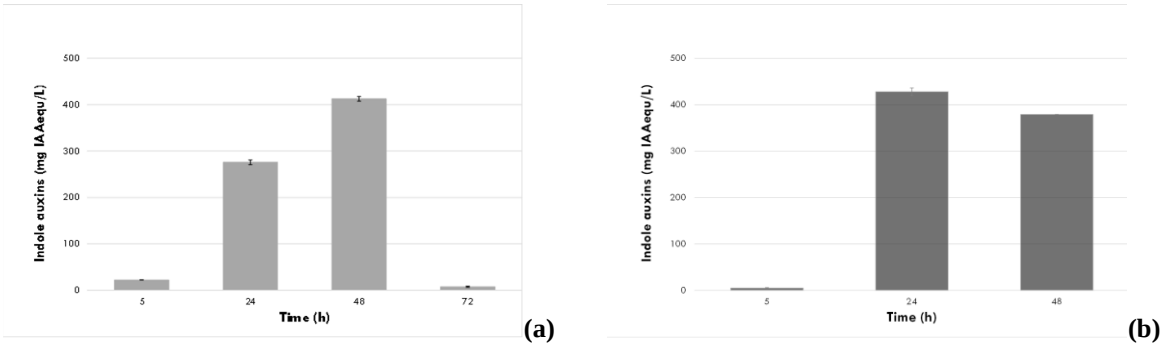

Figure 4 *Auxin accumulation profile during batch cultures in VY+Trp medium inoculated at* ***(a)*** *0.5 x 10^9 cell/mL and* ***(b)*** *1.0 x 10^9 cell/mL of Enterobacter sp. P-36 strain. The error bars represent standard errors of the means (n=3). Differences in letters indicate that the values are significantly different (p<0.05).*

The *Enterobacter* sp. strain P-36 was also cultivated in a 2-L fermenter under batch operating mode. Under optimal conditions, a similar auxin production was obtained with reduced fermentation time (Luziatelli *et al.*, 2021).

3.2. Application of metabolites from *Pantoea* strains to tomato seedlings and *P. communis* cv DarGazi plantlets

Rooting test on tomato cuttings was performed with five different treatments: i-ii) metabolites by *P. agglomerans* strain C1 and DSM3493; iii) LB; iv) LB+Trp; v) standard fertilizer. Treatment with strain C1 metabolites was more efficient than metabolites by another strain (Figure 5). Treatment with LB gave non-homogenous results among the different cuttings, while treatment with the standard fertilizer gave poor results.

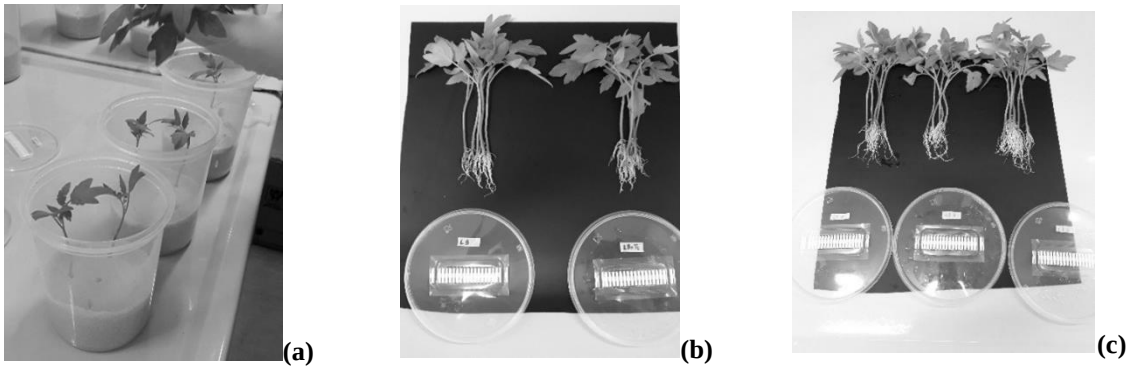

Figure 5 *Application of P. agglomerans strains' metabolites to tomato seedlings.* ***(a)*** *Transparent polypropylene microboxes containing wetted quartziferous sand and tomato seedling;* ***(b)*** *Tomato seedlings treated with LB medium;* ***(c)*** *Tomato seedlings treated with metabolites by P. agglomerans C1 strain.*

3.3. Analysis of metabolites produced by *Pantoea* strain C1 (genetics and metabolomics approaches)

The comparative genome analysis of strain C1 focused on PGP traits and heavy metal resistance showed 99% similarity between strain C1 and other *P. agglomerans* strains (Luziatelli *et al.*, 2019). Functional analysis of the strain C1 genome showed several genes contributing directly or indirectly to PGP and biocontrol activities. Genes encoding key enzymes involved in IAA synthesis and secretion were identified. Two operons likely involved in the biosynthesis of spermidine, a class of compounds correlated with lateral root development, pathogen resistance, and alleviation of oxidative, osmotic, and acidic stresses, were also found. Regarding the indirect mechanisms of PG promotion, genes encoding enzymes involved in the synthesis of volatile organic compounds were identified (Luziatelli, Ficca, *et al.*, 2020).

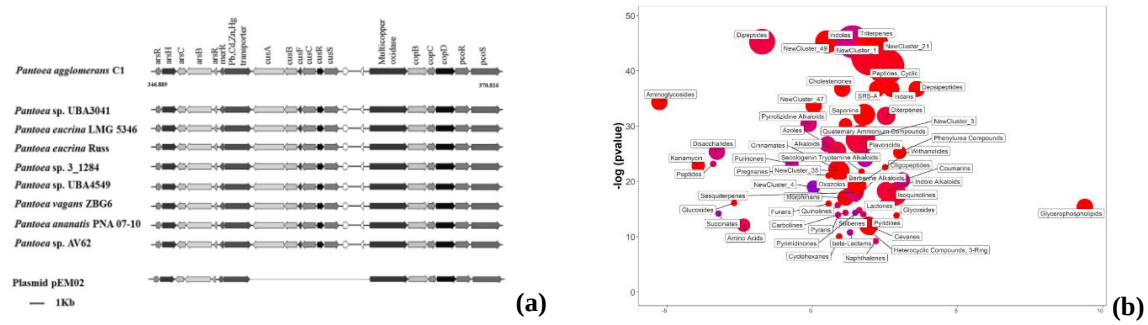

Figure 6 ***(a)*** *Organization of the heavy metal gene cluster of strain C1 and comparison with other Pantoea genomes that have the same 19-gene cluster*; ***(b)*** *ChemRICH analysis of exometabolome of C1 strain grown on YES versus YEG medium. Red clusters are associated with higher outcomes;violet ones are associated with lower outcomes.*

Characterization of strain C1 exometabolome by UHPLC-ESI-Q-TOF-MS analysis showed that the growth medium affects the metabolites produced by the strain. Strain growth in the YES+Trp medium determined an increase in IAA, indole-3-carbinol, quinoline, cyclohexane derivatives, and a significant decrease of IAA precursors (i.e., indole-3-acrylic acid, indole-3-acetaldehyde, indole-3-acetamide, indole-3-carboxylic acid). YES medium stimulated the production of other metabolites, such as compounds with antimicrobial activity (methyl hexadecanoic acid, furanomycin, and 2-hydroxyethylclavam).

4. Conclusions and Future Perspectives

PBs represent a new frontier of investigation to assure food security. So far, the European legislation acknowledges and regulates some PBs; however, it is necessary to foster research activities to identify novel strains or select those that have shown to have more efficient PGP traits and be more promising in bioactivity and bio-control. More investigations are also required to fully understand the specific effects on the morphological traits of plants and fruits. The PGPR strains investigated in this Ph.D. project proved to be good auxin producers and enhancers of the rooting system. As a final step, the nutritional and antioxidant profile of a selected crop treated with metabolites from the selected PGPR strains will be analyzed, to contribute to bridging the gap between agriculture and nutrition.

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Optimization of rapid analytical protocols for monitoring the contamination with hydrocarbons of petrogenic origin in the olive oil supply chain

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One of the aims of the current PhD project was to develop, optimize and validate rapid and solvent saving sample preparation protocols aimed at the removal of endogenous intereferences from olive oil, in order to perform an accurate and high sensitivity determination of mineral oil hydrocarbons (MOH) contaminants. These were then applied for the analysis of samples taken along the olive oil supply chain in order to identify the main sources of contamination.

Ottimizzazione di protocolli analitici rapidi per il monitoraggio della contaminazione con idrucarburi di origine petrogenica nella filiera dell'olio di oliva

Uno degli obiettivi del presente progetto di dottorato è stato quello di sviluppare, ottimizzare e validare protocolli di preparazione del campione rapidi e a basso consumo di solvente rivolti alla rimozione di interferenti endogeni presenti nell'olio di oliva, allo scopo di poter determinare in modo accurato e sensibile gli di oli minerali (MOH). Questi sono poi stati utilizzati per l'analisi di campioni prelevati lungo la filiera dell'olio di oliva allo scopo di identificare le possibili fonti di contaminazione.

Keywords: mineral oil hydrocarbons; contaminants; olive oil; sample preparation; monitoring; sources.

1. Introduction

In accordance with the PhD thesis project previously described (Menegoz Ursol, 2021), this oral communication reports the main results of the following activities:
A1) optimization and validation of a sample preparation protocol involving a microwave assisted saponification (MAS) followed by epoxidation, aimed at the removal of triglycerides and of endogenous olefins in olive oil, for the determination of mineral oil aromatic hydrocarbons (MOAH) up to 0.5 mg/kg;
A2) organization of a sampling involving all the steps of the olive oil supply chain, considering different olive groves and mills distributed throughout the Italian territory, and analysis of the different samples exploiting the sample preparation protocol reported in A1.

2. State of the art

Mineral oil hydrocarbons (MOH) are environmental and processing contaminants of petrogenic origin, which consist of complex mixtures of thousands of hydrocarbon isomers. These compounds can be divided into mineral oil saturated hydrocarbons (MOSH) and aromatic hydrocarbons (MOAH), based on their chemical structure and toxicological relevance. MOSH includes paraffins (linear and branched alkanes) and predominantly alkylated naphthenes (cyclic alkanes), while MOAH includes mono- or polyaromatic compounds with an alkylation degree greater than 98% (Bratinova and Hoekstra, 2019), which distinguishes them from the polycyclic aromatic hydrocarbons (PAH). Although data are still controversial, the latest evidence have proven MOSH to accumulate in human organs and tissues based on their structure and molecular weight, generating inflammatory states and volume increase in the site of accumulation, while MOAH to carry out carcinogenic, genotoxic and mutagenic actions, with particular reference to polyaromatic species with 3–7 rings (EFSA, 2012), which therefore are of greater concern for consumer safety.
According to the recent guidance by the Joint Research Centre (JRC) (Bratinova and Hoekstra, 2019), the instrumental analysis is based on on-line high performance liquid chromatography HP (LC)-gas chromatography (GC), coupled to flame ionization detector (FID), firstly described by Biedermann *et al.* (2009), and currently considered the reference method for MOH analysis. The use of this particular configuration derives from the fact that, due to their different toxicology, MOSH and MOAH need to be quantified separately. Their separation is obtained with the LC, while the actual chromatographic determination of the two individual fractions is performed by the GC. Due to the high amounts of isomers present, the GC is not able to separate every single compound belonging to the mixture of hydrocarbons and for this reason they appear in the chromatographic trace as humps of unresolved peaks. Although vegetable oils can be analyzed directly, reaching the required sensitivity can be tricky if the instrumental determination is not preceded by suitable sample preparation aimed at the removal of both triglycerides and endogenous compounds, the latter behaving as interfering compounds. In particular, even

though on one hand oil can be directly injected into the instrument, on the other the column capacity (2 mm × 250 mm silica gel HPLC column) towards the triglycerides is just of 20 mg, significantly limiting the attainable sensitivity. Moreover, the presence of biogenic compounds, like *n*-alkanes in the MOSH fraction and squalene (or other kind of olefins) in the MOAH fraction, disturbs the interpretation of the chromatograms and requires their removal to achieve reliable quantifications. As solutions to these analytical criticalities, several protocols have been proposed in the literature, exploiting either the saponification (Guinda *et al.*, 1996; Koprivnjak *et al.*, 1997) or the elution through fat retainers (Biedermann *et al.*, 2009; Zurfluh *et al.*, 2014) for the elimination of triglycerides and the epoxidation (Biedermann *et al.*, 2009; Nestola and Schmidt, 2017) and the elution through Alox (Fiselier *et al.*, 2009; Moret *et al.*, 2011) to remove olefins and endogenous *n*-alkanes, respectively. However, these protocols have often shown to be solvent and time consuming, and to require significant sample handling, with the risk of introducing contamination.

About the occurrence, the presence of these contaminants in vegetable oils is widespread due to the marked affinity for fatty matrices, given the non-polar character conferred by their chemical structure, and to the high level of mechanization reached for the handling and processing of the raw material. Indeed, the evaluation of the presence of MOH in vegetable oils has already been a matter of study, and data on occurrence as well as assumptions about possible sources of contamination has already been described in the literature (Moret *et al.*, 2009; EFSA, 2012; Brühl, 2016; Purcaro *et al.*, 2016), e.g. environmental pollution, lubricating oils, pesticides etc. Despite this, a specific study along the olive oil production chain, to assess the incidence and the extent of each source, was lacking.

For this reason, the aims of this project were to optimize and validate rapid, highly sensitive and solvent saving methods for MOSH and MOAH quantification in olive oils, and to exploit these methods to analyze samples resulting from different samplings along the entire olive oil supply chain. This was intended to try to identify the critical steps of the supply chain where the contamination occurs and, where possible, to precisely define the source, with the purpose to allow the implementation of strategies aimed at minimizing the contamination in this matrix. As reported above, this paper is focused on activity A1 and A2. About A2, the description will be focused on the first part of the supply chain, which turned out to be the most critical, i.e. the harvesting operation.

3. Materials and methods

For activity A1, two different extra virgin olive oils (EVOOs) were fortified at different levels using two different mineral oils (Gravex and motor oil) and subjected to the analytical protocol to be validated, involving a MAS and an epoxidation. Briefly, 1 g of fortified oil was saponified at 120 °C for 20 min with 10 mL of 1.5 N methanolic potassium hydroxide (KOH), in presence of 10 mL of *n*-hexane, using MAS. A double wash of the hexane phase using milliQ water and methanol, and a 30 min storage at -18 °C between the two washes, were applied. The hexane phase was then evaporated to a volume of 700 µL and epoxidation took place following the Nestola and Schmidt protocol (2017). Recovery, repeatability and LOQ, with a particular focus on the MOAH fraction, were evaluated.

For activity A2, 15 different olive samples were hand-picked directly from the trees in various olive groves located in different Italian regions. From the same olive groves, other 17 samples were collected after the harvesting operations (2 of them were in double since they were harvested using two different harvesting methods and were considered separately), taking them from the containers usually used for their transportation to the mill (e.g. plastic bins, trailers etc.). Olive oil was physically extracted from the olive samples using an Abencor system, a small laboratory plant for the milling of olives, in order to resemble the milling occurring in a real plant and obtain comparable data.

4. Results and discussion

4.1 Validation of the MOAH protocol

Two EVOOs were spiked at different levels with two different mineral oils (figure 1A) and quantitative recoveries as well as good repeatability (RSD% always below 20% for both of them), were obtained for all the fortification levels (2.0-40.7 mg/kg for MOSH and 0.5-9.9 mg/kg for MOAH), even at concentrations of added MOAH close to the LOQ (0.5 mg/kg for the total hump, 0.2 mg/kg for each single C-fraction). MOAH recovery data are reported in table 1. Also linearity was confirmed in all the tested range of concentrations by the coefficients of determination (R2) always above 0.998. This range was chosen in order to cover the range of contaminations usually found in this type of oil. The validated method, also applied for MOSH determination (C-fraction LOQ: 0.5 mg/kg, total hump LOQ: 1.0 mg/kg), gave excellent results despite the presence of endogenous *n*-alkanes, since by finding a compromise between sensitivity and resolution it was possible to avoid the signal overload due to their presence and to directly evaluate the MOH contamination even without their removal.

Table 1 *Recovery and RSD at different fortification levels for each internal standard.*

Sample	Type of mineral oil	Number of replicates	MOAH added (mg/kg)	Recovery % (mean)				RSD (%)			
				5B	1-MN	2-MN	TBB	5B	1-MN	2-MN	TBB
EVOO1	motor oil	6	**0.5**	96.4	104.7	105.8	81.9	9.4	9.4	9.2	9.0
		4	**1.0**	99.2	105.2	106.0	84.8	5.1	3.5	3.3	4.4
		4	**1.5**	96.6	102.9	103.8	83.4	8.1	7.0	6.6	7.6
		4	**2.0**	97.3	104.3	105.2	83.8	4.8	2.6	2.2	5.2
		4	**4.9**	100.7	106.7	107.4	87.2	2.4	3.0	3.3	1.8
		4	**9.9**	102.4	109.8	110.4	87.8	2.6	4.7	5.0	1.7
EVOO2	Gravex	6	**0.8**	93.4	99.7	101.7	79.4	5.0	4.2	4.8	5.0
		6	**1.4**	94.6	102.4	102.6	79.8	2.2	2.7	2.9	2.6
		6	**2.8**	100.6	106.0	108.0	83.3	5.8	5.7	6.1	4.4
MEAN RECOVERY*				**97.9**	**104.6**	**105.7**	**83.5**				

**all replicates at different spiking levels*

Different recoveries were observed for the different internal standards (table 1 and figure 1B). This turned out to depend on their different partition between the aqueous/alcoholic phase and the *n*-hexane phase (already visible in step 2 when adding the KOH solution, before the MAS procedure), and on the fact that part of the *n*-hexane phase remained in the aqueous/alcoholic phase, concentrating the standards in the organic solvent (this effect is well visible in step 3 for TBB). This required the introduction of correction factors for the recovery, to be applied to the analytical data based on the standard used to perform the quantification.

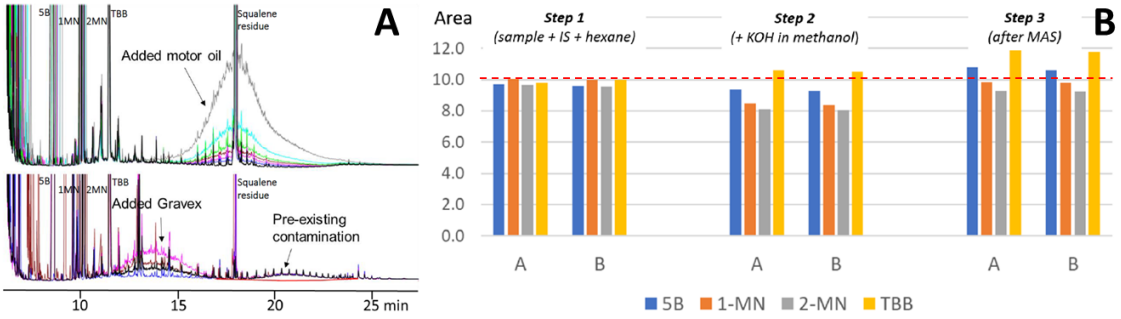

Figure 1 *(A) Overlay of the LC-GC-FID traces of the MOAH fractions of the two fortified EVOOs. The overlay of chromatograms starts from the unspiked matrix and the humps with increasing area refer to the different fortification levels reported in table 1. (B) Behaviour of the internal standards of the MOAH fraction in different steps of the MAS procedure.*

In conclusion, a rapid and solvent-saving method, based on MAS followed by epoxidation and LC-GC-FID, was optimized and validated for high-sensitivity determination of MOAH in EVOO.

4.2 Monitoring along the supply chain

As reported in paragraph 3, different olive samples were sent to our laboratory from different producers operating in the olive oil supply chain. The investigation had the purpose of tracing the contamination, starting from the trees up to the oil extracted at the mill, into a fairly large number of different Italian production realities, located in various conditions of potential contamination. However, this contribution is specifically focused on the harvesting phase, which turned out to be the most critical step. In addition to the olives collected from the trees and from the containers for their transport to the mill (after the harvesting operations), in some cases it was also possible to obtain samples of grease/lubricating oil/hydraulic oil used for harvesting operations, with the purpose of comparing their chromatographic profile and the MOSH/MOAH ratio, with that possibly found in the olives.
Figure 2 shows MOSH and MOAH levels of samples collected directly from the tree and after the harvesting operations. At this point of the supply chain, two possible sources of contamination were taken into consideration: environmental contamination (evaluated as closeness to possible source of contamination such as urban areas, industrial sites and vehicular traffic, etc.) and phytosanitary/fertilizing treatments.
Except for two samples (TB4 and I2), 13 out of the 15 hand-picked olives samples showed MOSH ranging from <LOQ to 2.7 mg/kg, in line with the background levels normally found in olives collected at this point of the supply chain (Menegoz Ursol *et al.*, 2022). Only in one case (SI1) a MOAH contamination of 1.1 mg/kg was highlighted, even though the origin remained unknown. More in detail, 7 out of 8 different samples collected very close to roads with medium-high traffic (<700 m) or urban areas (<3 km) (TB5, T1, IB1, IB2, IB3 and I1) showed contamination levels below the LOQ for MOSH, and only T2 showed levels a little bit higher (1.6 mg/kg). On the contrary, 3 out of 6 samples located more distant from possible contamination sources (TB1, IB4 and SI1) reported levels between 1.6 and 2.7 mg/kg. Again, the other 3 did not show contamination above the LOQ (TB2, TB3,

TIB1). Based on these data, no correlation was found between the levels of contamination and the position of the olive grove with respect to potential sources. No correlation was found even with respect to the carrying out of phytosanitary/fertilizing treatments, as no substantial differences were highlighted among samples from olive groves which underwent or not treatments, or between samples from conventional and biological farming. Finally, for the two samples where the contamination was significantly higher, the explanation was found in the possible contamination occurred during sample collection/manipulation or, in the context of the treatments, related to the addition of mineral oil-based products to the atomizer to allow a better dispersion/adhesion of the active principle to the plants (a not declared practice), or to a leak of lubricating oil from its mechanical components (e.g. the pump). Therefore, based on these data, no particular criticalities were highlighted in relation to the presence of mineral oils in the olives collected from the trees.

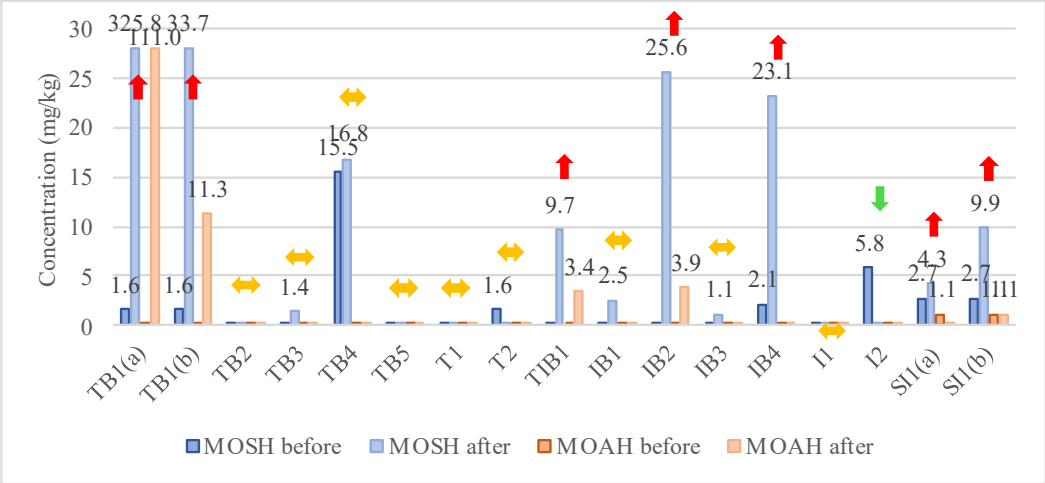

Figure 2 *Comparison of MOH concentrations in the oils extracted from olives sampled before (hand-picked from the trees) and after the harvesting operations. Absence of data labels indicates levels below the LOQ (1.0 mg/kg for MOSH and 0.5 mg/kg for MOAH).*

On the contrary, a significant increase in contamination was witnessed for 7 samples out of 17 due to the harvesting operations, highlighting their significant contribution on the final contamination (figure 2). For almost all of them it was possible to clearly identify the source thanks to the comparison of the profile of the contamination with those of the lubricants used in the machinery. An example of source identification for sample IB4 is now reported. For IB4 sampling, whose olives were harvested with a straddle harvester, the correspondence was found in the grease used to lubricate the mechanical parts of the latter. In fact, as visible in figure 3, the *n*-$C_{21\text{-}50}$ distribution, centered on *n*-C_{33}, matched the contamination found in the olives. Moreover, the absence of MOAH (just below the LOQ), in presence of a significant MOSH contamination, fitted with the classification of this grease as a food-grade lubricant (refined to remove/minimize the aromatic fraction, and in the specific case containing 2% of MOAH). For confirmation, also the oil from the hydraulic circuit of the same machinery was sampled, which however showed a molecular weight distribution located at earlier retention times, i.e. centered on *n*-C_{28} and covering the range *n*-$C_{17\text{-}44}$, and therefore not matching with the contamination into the olives. The source was therefore determined unambiguously.
According to the same reasoning, the source was also identified for almost all the other samples reporting a significant increase in MOH contamination.

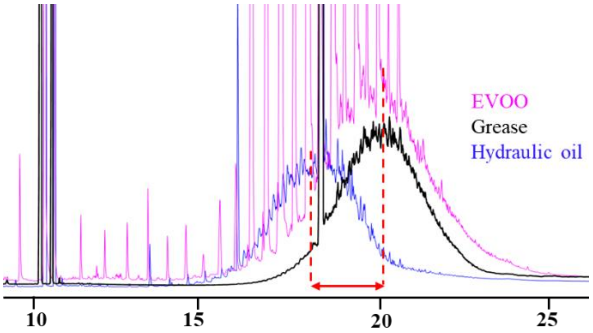

Figure 3 *Overlay of chromatograms of the mineral oils used in the harvesting machinery and the contaminations found in the olives sampled after the harvesting operations for sampling IB4.*

However, as evident from the bar diagram (figure 2), a wide number of samples did not report any significant increase in the contamination level, excluding problems in the harvesting step.

In conclusion, what was found to be different for these samples, compared to the others, was the lower level of mechanization in the harvesting phase. The contamination preferably occurred on olives harvested with big machinery, such as trunk shakers, olive harvesters or similar, rather than with smaller equipments like hand-held combs. As a direct consequence, the type of cultivation also appeared to be a discriminating factor. Indeed, olives from olive groves where the production density is high, and where the age and the size of the trees allow it, as in the case of intensive or super-intensive cultivation, are more suitable for mechanized harvesting (Lo Bianco *et al*., 2021) and therefore more prone to be contaminated by mineral oils. Anyway, the harvesting resulted to be a step to be kept under control in order to minimize contamination on the finished product.

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Exploitation of unconventional plant matrices and agri-food waste through biotechnological process

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This PhD thesis is focused on the development of biotechnological protocols for the valorization of unconventional ingredients and food by-products. Firstly, a clean-label gluten-free bread was set up by fortifying maize and rice flours with selected protein-rich and naturally hydrocolloids-containing flours, and a type-II sourdough. Secondly, the inclusion of hemp flour was evaluated in two different clean-label formulations: gluten-free bread and yogurt-like, evaluating the optimal bioprocessing conditions to obtain high nutritional, functional, structural, and sensorial properties. Finally, the use of wasted bread as substrate for the cultivation of *Haloferax mediterranei* aiming at production of bioplastic was set up.

Processi biotecnologici per la valorizzazione di matrici vegetali non convenzionali e scarti dell'industria agroalimentare

Questa tesi di dottorato descrive protocolli biotecnologici per la valorizzazione di ingredienti alternativi e sottoprodotti dell'industria alimentare. In primo luogo, è stata messa a punto la formulazione di un pane senza glutine *clean-label* fortificando mais e riso con sfarinati naturalmente ricchi di proteine e idrocolloidi, e un lievito naturale di tipo II. In secondo luogo, è stato valorizzato lo sfarinato di canapa in due diverse formulazioni *clean-label*, pane senza glutine e *yogurt-like*, valutando le condizioni ottimali del bioprocesso per ottenere elevate proprietà nutrizionali, funzionali, strutturali e sensoriali. Infine, è stato messo a punto un protocollo biotecnologico per valorizzare scarti di pane per la produzione di bioplastica tramite utilizzo di *Haloferax mediterranei*.

Key words: Gluten free, clean-label, plant-based, biotechnological protocols, bioplastic.

1. Introduction

In accordance with the PhD thesis project, this oral communication reports the main results of the following four activities directed to:
A1) Design of a "clean-label" gluten-free bread to meet consumers demand;
A2) Hemp flour and sourdough biotechnology for high-quality gluten free bread;
A3) Design of a plant-based yogurt like including hemp flour - formulation and characterization of a new recipe;
A4) Exploitation of wasted bread as substrate for bioplastic (PHBV) production by using *Haloferax mediterranei* and sea water.

2. Materials and Method

2.1 Design of a "clean-label" gluten-free bread to meet consumers demand

The formulation of the clean label GF bread was designed by evaluating the impact of different protein-rich flours and naturally hydrocolloids-containing flours on volume increase of dough during leavening, alveolus percentage, and specific volume of loaf. The biochemical characteristics of a type-II sourdough fermented by the exo-polysaccharides (EPS) producer *Weissella cibaria* P9 were evaluated through the analysis of organic acid, peptides, and free amino acids (FAA) concentrations. In details, organic acids were determined by Megazyme kits K-DLATE and K-ACET. Peptides concentration was determined by the o-phtaldialdehyde (OPA) method, while FAA were analyzed by a Biochrom 30+ series Amino Acid Analyzer (Biochrom Ltd). Moreover, the content of soluble dextran was determined by an enzyme-assisted method using a mixture of dextranase (Sigma-Aldrich) and α-glucosidase (Megazyme). The final formulation of bread was selected by 8 trained panelists and then characterized for its proximal composition and nutritional characteristics.

2.2 Hemp flour and sourdough biotechnology for high-quality gluten free bread

The inclusion of hemp flour in clean label GF bread was evaluated to increase the nutritional and functional properties. Flours characterized by a high content of simple sugars (chestnut, quinoa, and carob pulp) were used in combination with hemp flour in order to evaluate the higher production of exo-polysaccharides (EPS) by using three different EPS-producing strains of lactic bacteria at inoculation densities of 6 or 7 log cfu/g, and fermentation temperatures of 25 or 30 °C. Sourdoughs fermented according to the best conditions tested were subsequently characterized for their sugars, phytic acid, and organic acids contents by using Megazyme kits, and single and total free amino acids content by using Biochrom 30+ series Amino Acid Analyzer (Biochrom Ltd).
Three bread including maize and rice flours and fortified with chestnut and hemp flours previously fermented by *Leuconostoc mesenteroides* 12MM1, or *Weissella cibaria* P9, or *Leuconostoc pseudomesenteroides* 20193 were

produced. Doughs before baking were evaluated for fructose, glucose, maltose, and asparagine contents while the acrylamide content was evaluated after baking. Bread were characterized for their macro- and micro- nutrient composition according to official methods, Megazyme kits, and internal validated methods by Food Safety Lab (Corato, Italy). The texture was evaluated by using FRTS-100N Texture Analyzer (Imada). The sensory analysis of the final recipes was performed by a trained panel group composed of ten assessors.

2.3 Design of a plant-based yogurt like including hemp flour: design and characterization of a new recipe

A new formulation of plant-based yogurt-like similar to the conventional yogurt in terms of textural and sensory properties, with increased nutritional characteristics, and without using additives was set up. The effects of different flour concentration (rice and hemp flours) on fermentation performances and on the final viscosity of the product were evaluated using a pooled starter of three lactic acid bacteria Therefore, *Lactiplantibacillus plantarum* 18S9 and *Leuconostoc mesenteroides* 12MM1 (previously isolated and selected for increasing the antioxidant properties of hemp flour (Pontonio et al., 2020), and *Leuconostoc pseudomesenteroides* DSM20193 (widely characterized as EPS-producer) (Galli et al., 2020) were used. The functional and nutritional properties were analyzed after the fermentation in terms of antioxidant activity (evaluated as the scavenging activity towards the $ABTS^+$ radical), and macro- and micro- nutrient composition as described in 3.3. The organoleptic properties were evaluated before and after fermentation. To evaluate the possibility to improve the organoleptic characteristics and meet the consumer's appreciably one YL flavored with vanilla powder and agave syrup was added to the sensorial characterization. Moreover, the shelf life of the product in terms of microbial spoilage and changes of the viscosity and pH was evaluated every 10 days up to 30 days.

2.4 Exploitation of wasted bread as substrate for bioplastic (PHBV) production by using *Haloferax mediterranei* and sea water

Wasted bread was used as substrate for bioplastic production. The best ratio of wasted bread in water for obtaining the homogenate was evaluated by the amount of carbohydrates released without enzymes and by using pepsin (Sigma-Aldrich, 3 mg/g of bread), α-amylase (Sigma-Aldrich, 5 mg/g of bread) and Veron PS (AB enzymes, different concentrations). Instead of minerals supplement for *Haloferax mediterranei* DSM1411 growth microfiltered sea water was used. Therefore, the halophilic archea growth was defined spectrophotometrically in substrates obtained i) by the 6 h-pepsin treatment, ii) by the 200g/100kg Veron PS treatment, iii) without enzymatic treatment. The substrates were added with microfiltered sea water (40:60) and 160g/L of NaCl. The pH was adjusted to 7.2 with 1M ammonia solution. After fermentation of 72 h at 37 °C, microbial biomass, raw and purified PHBV were evaluated. The cell dry matter (CDM) resuspended in deionized water for lysis was washed with deionized water (20 mL/g) and centrifuged (with supernatant removal) for 8 times, instead of the separation in $CHCl_3$:H_2O. This sample (rPHBV), the sample obtained after purification with ethanol from rPHBV (aPHBV), and the sample obtained by using the conventional extraction and purification as described by Raho and colleagues (2020) (pPHBV) were then characterized. The PHBV purity, the hydroxyvalerate (HV) contents, the FT-IR spectra in attenuated total reflection mode, and the thermal response of the biopolymers by thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC) were evaluated.

3. Results and discussion

3.1 Design of a "clean-label" gluten-free bread to meet consumers demand

The influence of the fortification of the rice-corn blend with protein-rich and structuring flours on the technological properties of dough and bread showed, among protein-rich flours, highest volume increase of dough (+33.3 ± 1.6 %) and the highest specific volume of bread (1.27 ± 0.03 ml/g) when quinoa flour was used with good results also when teff and lentil flours were used. Regarding the structuring flours, all (singly or mixed) positively affected the specific volume of bread, however psyllium, hydrated chia and the mixture 4:1:1 (psyllium:hydrated chia:flaxseed) corresponded to the best results in terms of volume increment of dough, alveolus percentage of bread slice and specific volume of bread, compared to the others. In details, the mix led to the best (i) volume increase (+34.7 ± 1.6 %), (ii) alveolus percentage (26.4 ± 3.3 %), and (iii) specific volume of bread (1.42 ± 0.02 mL/g). According to these results, quinoa, teff, or lentil flours, and the mix of psyllium (4% wt/wt), hydrated chia (1% wt/wt) and flaxseed (1% wt/wt) were selected for further analysis.

Chestnut and quinoa flours were used as sucrose sources to promote the synthesis of EPS by *W. cibaria* P9 during sourdough fermentation. Their total sugars content corresponded to 26.6% and 6.0%, respectively, while sucrose concentration was 15.22 ± 0.27% (of dry matter) in chestnut and 2.95 ± 0.12% (of dry matter) in quinoa. The shortest lag time was found in rice:corn control sourdough, sourdough with 20% of chestnut, and sourdough with 10% of both chestnut and quinoa (circa 1.81 h). The acidification kinetics showed the shortest lag time in the previously mentioned fortified sourdough (1.04 ± 0.05 and 0.84 ± 0.05 h, respectively). The highest pH was found when 20% of chestnut was included in the formulation (4.28 ± 0.05). As expected, the sucrose provided with quinoa and chestnut flours supplementation led to a higher content of EPS after fermentation and the highest amount was detected in 20% chestnut-containing sourdough (0.65 ± 0.06 g/kg). Overall, the use of sourdough significantly modified the bread flavor profile. Panelists perceived higher values of elasticity, saltiness, color of crust and crumb, acidic smell and flavor when sourdough was included in the formulation. Moreover, the inclusion of protein-rich flours resulted in an increase in saltiness, crust and crumb color, but a decrease in acidity. When teff was used, the highest levels of salty and grassy tastes (5.2 ± 0.4 and 4.2 ± 0.7) were detected. According to

sensorial and textural properties, the formulation including quinoa flour, chestnut (20%) sourdough, and the mix of structuring flours was used to make bread. The experimental bread was characterized by higher moisture (43.6 ± 2.4%) and carbohydrates (47.0 ± 1.7 g/100 g) content compared to the reference dataset, with only 0.6 ± 0.2 g/100 g represented by sugar (Table 1).

Table 1 *Proximate composition and nutritional evaluation of the experimental clean-label GF bread. Results were compared to a reference dataset referring to commercial gluten free breads available in the Italian market (Rizzello et al., 2016)*

	Experimental bread	Reference*
Energy (Kj)	962 ± 45	1032
Moisture (%)	43.6 ± 2.4	37.0
Total carbohydrates (g/100g)	47.0 ± 1.7	45.9
Sugar (g/100g)	0.6 ± 0.2	1.9
Dietary fiber (g/100g)	1.7 ± 0.3	8.8
Total lipids (g/100g)	1.8 ± 0.4	3.8
Saturated lipids (g/100g)	0.3 ± 0.1	1.0
Protein (g/100g)	5.0 ± 0.2	2.7
Salt (g/100g)	0.1 ± 0.1	1.6
In vitro protein digestibility (%)	76.9 ± 2.7	21.17
Predicted glycaemic index	85.0 ± 3.1	81.5

*Data corresponds to the median values of the distribution of the results obtained for all the commercial products

The amount of lipids was very low (1.8 ± 0.4 g/100 g), and only 0.3 ± 0.1 g/100 g was represented by saturated ones. The protein content of bread was 5.0 ± 0.2 g/100 g representing 8.7% of the total calories provided by bread. This value represents a protein content two-fold higher than the reference value characterizing commercial products (Rizzello et al., 2016) The IVPD of the experimental bread was circa 77%, while a median value of circa 21% characterized commercial GF products. Moreover, the predicted glycemic index, which was calculated evaluating starch hydrolysis kinetics during 3 h of mimicked digestion, was 85 ± 3.1.

3.2 Hemp flour and sourdough biotechnology for high-quality gluten free bread

Overall, the use of chestnut flour led to higher production of EPS, while higher inclusion of hemp flour showed lower EPS production. Therefore, the chestnut/hemp (70/30 w/w) combination was used for the subsequent fermentation tests with different temperatures (25 and 30 °C) and inoculation density (6 or 7 log10 cfu/g). The lowest EPS production was detected with fermentation parameters set at 30°C and inoculum density of 7 log10 cfu/g, while the highest with 25 °C and inoculum of 6 log10 cfu/g. Among different lactic acid bacteria, *Weissella cibaria* P9 produces more EPS with the maximum value of 7.85 ± 2.1 mg/kg. Moreover, dough fermentation conducted with the latter temperature and inoculum density led to lower decrease of pH (4.43 ± 0.34) and lower increase of TTA (16.0 ± 3.2 mL of NaOH 0.1 M, respectively).

The sourdough characterization showed differences in composition depending on LAB strain used for fermentation. After fermentation, lower pH (4.17 ± 0.19) and higher TTA (18.20 ± 0.07) was found in the sourdough fermented by *Leuconostoc mesenteroides* 12MM1 (s12MM1). The lactic acid production during fermentation ranged from 68.47 ± 0.26 to 124.75 ± 1.58 mmol/kg. Sourdough fermented by *Weissella cibaria* P9 (sP9) showed the lower production of lactic acid (68.47 ± 0.26 mmol/kg) and the greater production of acetic acid (25.76 ± 0.12 mmol/kg), which led to the lower fermentation quotient (2.66). Interestingly, the concentration of glucose and fructose increased at the end of fermentation and sP9 was characterized by the highest concentration for both (26.84 ± 1.07 and 39.91 ± 1.33 g/kg, respectively). These values corresponded to concentrations 4.5 and 7.7 times higher than that found in the unfermented dough (5.95 ± 0.32 and 5.25 ± 0.30 g/kg, respectively). The concentration of free amino acids, already present at high concentrations in the dough before fermentation (1.059 g/100g of dry weight) increased during fermentation from 6 (s12MM1) to 20% (sP9). As expected, the phytic acid decreased in sourdoughs and the lowest concentration was detected in sP9 (0.39 ± 0.05 g/kg) which corresponds at the 33% of the original quantity. The inclusion of hemp flour in bread formulation led to a general increase of all acrylamide precursors contents evaluated before baking. Maltose was the reducing sugar with higher concentration in all formulation ranging from 0.95 ± 0.07 to 9.16 ± 0.61 g/kg with the lowest value in bread without the inclusion of hemp and chestnut flours (dough with corn and rice flours, dCR). Significant differences ($p < 0.05$) were found among dough with the inclusion of sourdough (ds-). Only the dsP9 was found with a decreased content of maltose, from 8.39 ± 0.39 to 6.54 ± 0.61 g/kg. Glucose and fructose, which increased with the fortification of unfermented chestnut and hemp flours from 0.14 ± 0.04 to 2.16 ± 0.18 g/kg and from 0.02 ± 0.01 to 2.06 ± 0.11 g/kg, respectively, decreased in all ds- samples. After baking, the acrylamide content in bread (b-) was evaluated. The lowest concentration wad detected in bCR (17.32 ± 4.00 µg/kg) while the highest one in bsP9 (36.74 ± 8.84 µg/kg). Not significant differences were found among bCT, bs12MM1, and bsDSM20193.

The fermentation of the mixture of chestnuts and hemp flours led to a greater perception of acidic smell and taste, while a slightly increase of the presence of alveolus in bread slice and higher elasticity was evaluated by assessors. The textural analysis showed a general decrease in springiness, cohesiveness, and chewiness of the fortified bread. Conversely, sourdough inclusion led to the increase of hardness in bs12MM1 and bsDSM20193 and chewiness in bsDSM20193 (24.55 ± 0.23 N).

The fortification of the bread (b-) with the mixture of chestnut and hemp flours led to an increase in ashes, total carbohydrates, proteins, fat, and fiber. The total fiber content was higher than 6 g/100g in all fortified bread, the

lower limit for considering them "high in fiber" according to the Regulation EC n. 1924/2006. Calcium, iron, phosphorus, magnesium, potassium, sodium, and zinc increased in fortified bread, mainly for the iron content showing the maximum concentration in bsP9 (12.6 ± 0.5 mg/kg). In addition, the magnesium content in all fortified breads was found higher than 56.25 mg/100g, constituting a significant amount of nutrient as defined in Annex XIII of EU Regulation n. 1169/2011. Calcium markedly increased in fortified bread from 2.4 to 2.9 times. Vitamins B1 (from 2.2 ± 0.2 to 2.4 ± 0.2 mg/kg), B3 (from 5.8 ± 0.3 to 7.4 ± 0.4 mg/kg) and B9 (from 0.169 ± 0.021 to 0.219 ± 0.024 mg/kg) significantly increased in all fortified bread. Furthermore, a significant increase in vitamin B3 (from +10 to + 28%) and B9 (from +5 to + 30%) was found in samples fortified with fermented doughs compared to bread fortified with unfermented flours.

3.3 Design of a plant-based yogurt like including hemp flour: formulation and characterization of a new recipe

The optimal inclusion of rice and hemp flour in the formulation was selected according to the viscosity, pH and TTA values. After gelatinization, the viscosity measured between 6.2 ± 0.3 and 8.3 ± 0.4 Pa × s with no statistically significant changes in the pH of the individual samples within the product group. Similarly, even after fermentation, there were no variations among the yogurt like products in terms of pH, which ranged between 4.73 ± 0.09 and 4.82 ± 0.22. In contrast, a modest increase in TTA was found with 30% of the total flours. The viscosity of fermented products dropped significantly in recipes with greater quantities of hemp flour and when 30% of total flour was used, the viscosity of the yogurt like was lowered by 48%. According to these results, the combination of rice and hemp flour (23.4 % w/w and 6.6% w/w, respectively) in tap water was used to set up the production protocol. The fermentation of 16 hours led to a significant increase in the cell density of the lactic bacteria (9.10 ± 0.23 log10 cfu/g). Probably due to matrix acidification and microbial competition, yeast and enterobacteria showed a significant reduction while molds were not detected. To confirm the aptitude of the inoculated LAB to ferment the matrix, the kinetics of growth and acidification were evaluated. In the case of growth, the latency period was 0.97 ± 0.21 while the μmax and A values were 0.76 ± 0.10 and 2.41 ± 0.14, respectively. Similarly, acidification kinetics showed results consistent with microbial growth with latency period value of 0.98 ± 0.18, and Vmax and A values of 0.26 ± 0.04 and 2.42 ± 0.08, respectively. As expected, at the end of fermentation an increase in lactic acid and acetic acid was detected with a fermentation quotient of 3.26. Unexpectedly, the quantity of free amino acids decreased at the end of the fermentation (-38%). Among all the amino acids, the main decreases were found for asparagine and glutamic acid. The biochemical and nutritional characterization was performed before and after fermentation demonstrating the possibility for the product to be claimed as a source of protein and fiber. Moreover, a decrease of in vitro glycemic index (54.97 ± 3.75), and an increase of mineral and vitamins contents (calcium, iron, and vit. B3), as well as antioxidant properties (+ 10%) was detected in the fermented product. The shelf-life evaluation demonstrated the high viability of lactic acid bacteria also after 30 days of storage under refrigerated condition (> 8.65 ± 0.35 log10 cfu/g), without the growth of spoilage microorganism (yeast, mold, and enterobacteria). No change in viscosity was found after 10 days of storage, while slightly decrease after 30 days was detected. Finally, when agave syrup (5% w/w) and vanilla powder (0.5% w/w) were added to the plant-based yogurt like, the acidic smell and taste, bitterness, and astringency were attenuated (Figure 1) confirming that the addition of flavorings to YL can improve their sensorial characteristics (Montemurro et al., 2021).

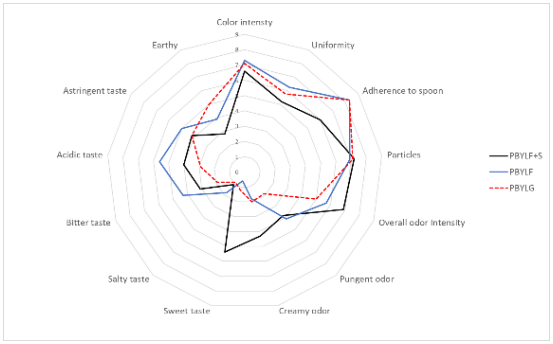

Figure 1 *Sensory evaluation of YL before (PBYLG) and after fermentation (PBYLF). The latter wasevaluated also with the inclusion of 5% (w/w) agave syrup and 0.5% (w/w) of vanilla powder.*

3.4 Exploitation of wasted bread as substrate for bioplastic (PHBV) production by using *Haloferax mediterranei* and sea water

The preparation of the substrate for the growth and production of PHBV involved a first phase of assessment of the percentage of use of bread in the hydrolysate demonstrating that the use of 150 g/L of bread resulted in higher carbohydrate concentration and extraction yield (32.1 ± 1.2 and 30.5%, respectively). As expected, the use of enzymes led to a significant increase in extraction yields, corresponding to 50% when pepsin and α-amylase were used. Therefore, considering the needs in minerals content for *Hfx. mediterranei* growth and PHBV production, and its source of isolation, the bread hydrolysate was diluted with microfiltered sea water in different ratio. The evaluation of the growth of *Hfx. mediterranei* was conducted by using absorbance at 600 nm. A stunted growth of the microorganism was observed in wasted bread substrate supplemented with lower than 40% seawater (data not

shown), while mixtures containing 45, 60 and 75% (v/v) seawater corresponded to relevant increases in *Hfx. mediterranei* cell density. Pepsin addition resulted in a significantly greater microbial cell density compared to the substrate obtained without enzyme treatment. Particularly, the 6h pepsin treatment (P6) greatly improves absorbance (2.028 ± 0.092). Cell densities were much lower when Veron PS was utilized and the concentration of 200 g/100 kg (PS200) resulted in the greatest microbial growth. Overall, incubation for 72 hours and the usage of 60 percent seawater produced the best results of all the conditions examined. Therefore, cell dry matter and PHBV production were evaluated in the wasted bread homogenate P6, PS200, and NE. All the condition included the 60% of microfiltered sea water and the fermentation for 72 hours. The highest CDM was found for the substrate obtained with Veron PS (2.92 ± 0.41 g/L), while the lowest value was observed in the substrate obtained after 6 hours of pepsin treatment (2.17 ± 0.24 g/L). Surprisingly, a high bioplastic amount was extracted from the substrate produced without enzymes (0.510 ± 0.028 g/L), and only a slightly higher amount characterized the substrate obtained with the pepsin treatment (0.526 ± 0.051 g/L) with a yield of 19.76% and 23.96%, respectively. The substrate treated with Veron PS corresponded to the lowest polymer yield (11.81%).
According to these results, wasted bread substrate obtained without enzymes addition was employed for fermentation at 3L-bioreactor level. Under this condition, the CDM weight at the end of the process was 6.37 ± 0.92 g/L while the polymer collected (with the conventional extraction) corresponded to 1.529 ± 0.241 g/L. Purification with ethanol led to the recovery of 1.293 ± 0.216 g/L of the polymer (pPHBV). Similar values were found when PHBV was recovered from CDM using repeated washing (rPHBV, 1.487 ± 0.182 g/L) and then precipitated by adding ethanol (aPHBV, 1.187 ± 0.198 g/L).

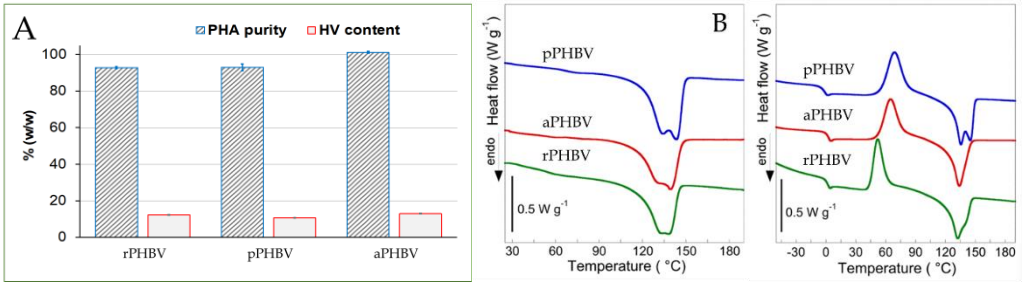

Figure 2 *(A) Cell dry matter (CDM) and PHBV production by Hfx. mediterranei DSM1411 in different wasted bread derived substrates produced using pepsin for 6 hours (P6), Veron PS at the concentration of 200g/100kg (PS200) and without enzymes (NE) and diluted in the ratio 40:60 v/v/ with microfiltered sea water, (B) DSC thermograms of PHBV produced in the final formulation of wasted bread/seawater substrate according to three different extraction and purification procedures.*

Based on GC analysis, all samples were found to be characterized by a high PHBV content with a purity grade ranging from approximately 93% (w/w) to 100% (w/w), respectively found for pPHBV/rPHBV and aPHBV. The HV content ranged from 10.78 ± 0.10 to 12.95 ± 0.04 % and significant higher purity and HV content were found in aPHBV (Figure 2a). The spectra of the samples by FT-IR spectroscopy were similar among all samples.
The TGA curves of the analyzed samples showed a constant initial slight weight decrease up to about 210 °C, presumably due to the decomposition of non-polymer biomass fraction or of impurities coming from the extraction process. Moreover, the differential scanning calorimetry (DSC) allowed to evaluate the thermal behavior of PHBV samples which showed the melting in the 100-150 °C temperature range and a double endothermic peak (Figure 2b). The second peak Is usually found in PHBV due to the recrystallization after the first melt in more ordered morphology. Besides a lightly lower glass transition temperature (Tg), pPHBV displayed a higher crystallinity respect with the other two samples.

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Development of edible coating functionalized with hydroxyapatite, complexed with bioactive compounds for the shelf-life extension of food products

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This PhD thesis is about the development of an edible coating functionalized with hydroxyapatite complexed with bioactive compounds with the aim to extend the shelf life of food products. In the first phase of the project, quercetin glycoside compounds (QUE) have been selected as a bioactive compound to be loaded into hydroxyapatite crystals (HA), used as carrier; then the chemical-physical characterization of HA-QUE complexes has been carried out. An alginate-based coating was developed and optimized, and QUE kinetics release was studied. Finally, its effectiveness as an active coating able to extend the cold storage time of two types of fresh food products (chicken fillets and cut papaya) was evaluated. During the last year, lactoferrin (lacto) was added to HA-QUE complexes, and through a synergy study, the optimum concentrations of both bioactive compounds were determined. The stability of the HA-lacto-QUE complexes was evaluated, as well as the kinetics release of both compounds. The effectiveness of alginate-based coating loaded with HA-lacto-QUE complexes on fresh pork meat has been evaluated. Data elaboration is in progress.

Sviluppo di rivestimento edibile funzionalizzato con idrossiapatite, complessato con composti bioattivi per l'estensione della shelf-life di prodotti alimentari

Questa tesi di dottorato riguarda lo sviluppo di un rivestimento edibile funzionalizzato con idrossiapatite complessata con composti bioattivi con l'obiettivo di prolungare la durata di conservazione dei prodotti alimentari. Nella prima fase del progetto, i composti glicosidici della quercitina (QUE) sono stati selezionati come composti bioattivi da caricare in cristalli di idrossiapatite (HA), utilizzati come carrier; quindi, è stata effettuata la caratterizzazione chimico-fisica dei complessi HA-QUE. È stato sviluppato e ottimizzato un rivestimento a base di alginato ed è stato studiato la cinetica di rilascio della QUE. Infine, è stata valutata la sua efficacia come rivestimento attivo in grado di prolungare il tempo di conservazione a freddo di due tipologie di prodotti alimentari freschi (filetti di pollo e tagliata di papaia). Nel corso dell'ultimo anno, la lattoferrina (lacto) è stata aggiunta ai complessi HA-QUE e, attraverso uno studio di sinergia, sono state determinate le concentrazioni ottimali di entrambi i composti bioattivi. È stata valutata la stabilità dei complessi HA-lacto-QUE, nonché il rilascio cinetico di entrambi i composti. È stata valutata l'efficacia del rivestimento a base di alginato caricato con complessi HA-lacto-QUE su carne suina fresca. L'elaborazione dei dati è in corso

Key words: *Edible coating, quercetin, lactoferrin, hydroxyapatite, layer-by-layer technique, shelf life.*

1. Introduction

The new packaging strategy to preserve quality and extend the shelf life of food products is represented by edible films and coatings obtained by natural biopolymers which can be consumed as a part of the product or after their removal [1]. Edible coatings are developed directly on the food surface unlike edible films that are before produced and then used as wrapping materials. In addition, the incorporation of active substances, such as antioxidant and antimicrobial compounds, is an effective way to further enhance the preservation performances of the edible coatings. On the basis of the above, the activities carried out during these 3 years of PhD had the main objective the development of active edible coatings able to preserve the quality and safety of food products. Respecting the gant plan, the main activities concerned:

1. The chemical-physical characterization of bioactive compounds quercetin glycoside compounds (QUE) and lactoferrin (lacto) free and adsorbed into hydroxyapatite crystals (HA), and the antimicrobial activity of both compounds.
2. Release study of bioactive compounds
3. Development of active edible coatings containing hydroxyapatite/bioactive compounds complexes
4. Application of active alginate-based coating for shelf-life extension of fresh meat products and fresh-cut fruits.

2. Experimental Section

2.1 Quercetin glycoside compounds free and adsorbed in hydroxyapatite

The HA-QUE complexes were prepared by gently mixing HA with different QUE concentration at 37°C for 24

h. The absorption capacity of HA crystals with different concentrations of QUE was evaluated, as well as the morphology of HA and HA-QUE complexes through SEM and Z potential analysis. Minimal Inhibitory Concentration (MIC) of free QUE and HA-QUE complexes versus *Pseudomonas fluorescens*, *Salmonella thyphimurium* and *Listeria monocytogenes* was evaluated.

2.2 Development of active edible coatings containing hydroxyapatite and quercetin glycoside compounds
During the PhD first year, different concentrations of sodium alginate (1%, 1.5% and 2%) and the cross-linker calcium chloride (0.75%, 1%, 2%) were tested to develop the edible coating by the layer-by-layer (LbL) electrostatic deposition technique. Edible coatings were then characterized by colour and thickness according to [2]. Coatings were then loaded with different concentration in free QUE and HA-QUE complexes, for the evaluation of QUE release profile and the measurement of water vapour permeability.

2.3 Application of active edible coatings on fresh chicken fillets and fresh-cut papaya
Alginate edible coatings enriched with QUE and HA/QUE complex were evaluated as effective active coatings able to slow down the spoilage of fresh chicken fillets during the cold storage. The microbiological parameters (Total viable count, Psychotropics bacteria count, *Pseudomonas spp*. and Enterobacteriaceae), were measured for 11 days at the cold storage at 6°C, as well as the total volatile basic nitrogen, the texture, colour and sensory parameters. Alginate-based coatings activated with HA/QUE complexes were also employed for the shelf-life extension of fresh-cut papaya, by monitoring microbiological parameters (Total viable count, lactic acid bacteria, yeasts and moulds, headspace gas composition, ABTS, DPPH radical-scavenging assays, total carotenoids, sugars and vitamin C content for 14 days at 6°C.

2.4 Duplex complex with HA: introduction of Lactoferrin
A further aim of this research project was to enhance the antimicrobial effect of the HA-QUE complex by introducing lacto as an additional natural antimicrobial. The single complexes HA-QUE and HA-lacto at 200, 100 and 50 ppm were prepared as reported by Malvano et al.,2021[2]. Subsequently the single complexes were centrifuged and evaporated under vacuum for 2 h, then QUE was added to the HA-lacto complex and lacto to the HA-QUE complex, always with the three concentration levels (200–100–50 ppm); finally, the complexes were mixed for 24 h at 37°C and their antimicrobial activity was evaluated. The additive or synergistic interaction of each complex was calculated using the statistical approach [3]. It was possible to estimate the synergistic coefficient Csyn (synergistic coefficient). Moreover, the fractional inhibitory concentration (FIC index) was used to confirm the effect of tested combinations. By means SEM images, the morphology of the double complexes was evaluated, and the formation of the bond through the FT-IR spectrum was confirmed by comparing with spectra of single compound and single bond with HA.

2.5 Development of alginate-based coating charged with the double complex and HA. Application on meat product.
HA-lacto-QUE complexes, at optimized concentration of 100 ppm were loaded into alginate-based coatings and a release study was carried out. The effectiveness of alginate-based coating loaded with HA-lacto-QUE complexes on fresh pork meat has been evaluated. Data elaboration is in progress

3.Results and Discussion

3.1 MIC and Physical chemical characterization of HA-QUE and HA-lacto-QUE complexes
The MIC value of HA-QUE complex against *Pseudomonas fluorescens* was 500 ppm, while against *Salmonella Typhymurium* and *Lysteria monocytogenes* at 500 ppm the complex HA-QUE showed 79.81% and 51.94% of antimicrobial activity respectively. The 100% of inhibition for the same bacteria was obtained only with the double complex HA–lacto–QUE at 100 ppm. Infact the complex HA-QUE-lacto at same concentration showed only 40% of inhibition against *Pseudomonas fluorescens, 84% e 20%* against *Salmonella Typhymurium* and *Lysteria monocytogenes* respectively. The combination of HA–lacto–Que at 100 ppm showing the best synergistic Csyn values (Csyn= 6.75 ± 2.67), confirmed by FIC value of 0.4 according to FIC index interpretation of ≤0.5 that suggests the synergistic interaction.
SEM images of HA and all complexes with HA showed μm particle size. As reported previously by Fulgione et al.,2016 [4] HA nanocrystal aggregated spontaneously in micrometric clusters probably justified by their zeta-potential values close to - 0.0505 mV.
The FT-IR spectrums of HA-QUE and HA-lacto-QUE confirmed the presence of QUE showing the characteristic peaks of QUE at 3248 ^{cm}–1 (stretching O-H), 1670 cm^{-1} (C–O stretching) and 1500 cm^{-1} (C–C stretching). The spectral region of the lacto has been confirmed, and the peaks correspond to α-helix (1658-1650 cm-1), β-sheet (1640-1615 cm-1), β-turn (1700-1660 cm-1).
In the figure 1a, the release behavior of QUE from coatings loaded with HA/QUE and HA-lacto-QUE complexes was reported.
The coated loaded with HA/QUE complexes starts releasing before than coating loaded with HA-lacto-QUE ones.

Moreover, the kinetic release of both coatings doesn't shown significant differences, so the presence of lacto doesn't seem to affect it.
The release behavior of lacto from coatings loaded with HA/ lacto and HA-lacto-QUE complexes was reported in the figure 1b. Also in this case, the active compound is released before when it is adsorbed alone into HA crystals than when it is adsorbed with QUE. However, the presence of QUE seems to influence the release of lacto that is faster in the case of HA-lacto-QUE complexes.
As regards the coatings loaded with HA-lacto-QUE complexes, the release of lacto, as well as the achievement of equilibrium, occurs before than the QUE release.
This is probably due to the difference in the solubility of both active compounds into aqueous medium and to the different interactions that the single active compound has with HA structure.

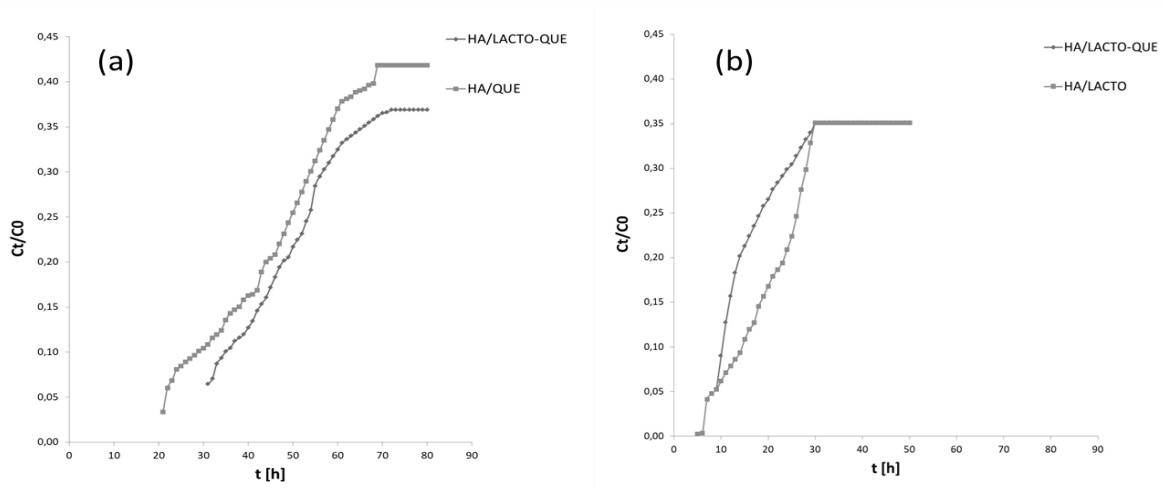

Figure 1: *(a) QUE release in edible coating loaded with HA/QUE complexes and HA-lacto-QUE complexes, (b) lacto release in edible coating loaded with HA/lacto complexes and HA-lacto-QUE complexes.*

3.2 Effect of alginate edible coating enriched with HA-QUE and HA-lacto-QUE during the cold storage of fresh chicken/pork fillets and cut-papaya.

The developed alginate edible coatings loaded with QUE and HA/QUE complex were used to extend the shelf-life of fresh chicken fillets. Through this first application of the active coating, the increased antimicrobial action of the QUE complexed to HA was mainly considered. Fresh chicken meat is highly perishable due to the rapid microbial growth of the *Pseudomonas* species that represent the most common spoilage bacteria recorded in poultry meat.

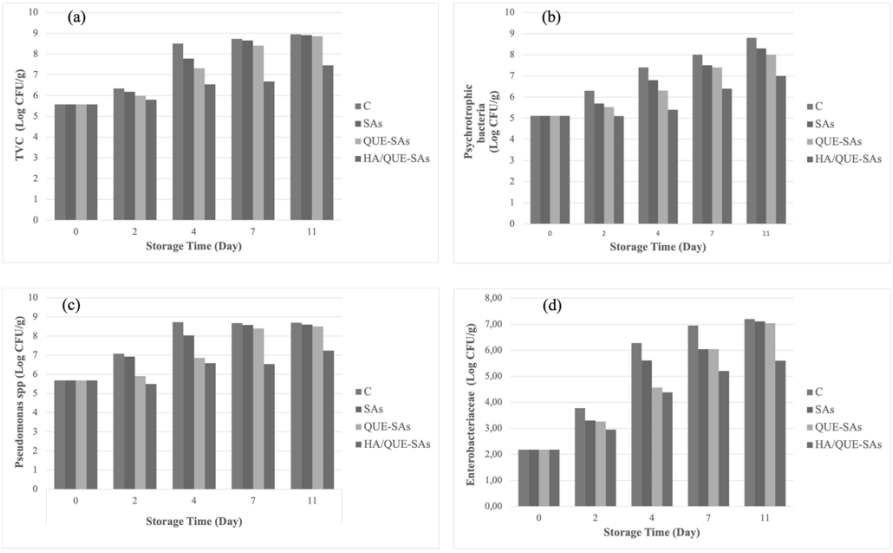

Figure 2: *Changes in total viable bacterial count (TVC) (a), Psychrotrophic bacterial (b) Pseudomonas spp (c) and Enterobacteriaceae (d) of chicken fillet samples during the storage period*.

As expected, the growth of all microbial parameters was observed as the storage time increased (figure 2). For each storage day, lower values were observed for QUE-based coating and HA/QUE-based coating in comparison with control samples and alginate-based coating. In view of the value of 7-8 log CFU/g, which is the recognized

and accepted limit of TVC value for fresh poultry meat, in HA/QUE- based coating samples this limit was reached at 11 days that is 3 days later compared to the control, alginate-based coating and QUE- based coating samples. Moreover, the comparison among the coated samples pointed out a positive effect of HA to slow down the changes in hardness during the storage time of 11 days as well as the total volatile basic nitrogen. Finally, the outcomes of colour, odour and taste evaluation both in raw and cooked fillets pointed out that the coating with HA/QUE was able to preserve the sensory attributes of fresh poultry meat until the 11th day of storage. All data were shown in detail in Malvano et al.,2021 [6].
In the second application, the capability of alginate-based coatings loaded with HA/QUE to extend the cold storage of fresh-cut papaya was evaluated.
Fresh-cut papaya shows a high nutritional interest as it is an excellent source of carotenoids, nutritional compounds that act as natural antioxidants, protecting cellular components from oxidative damage. The alginate-based coating showed a positive effect in maintaining the total carotenoids content of the fresh-cut papaya, as shown in figure 3. The lowest losses in total carotenoids observed for QUE and HA/QUE, recorded in all storage days, could be explained by the presence of QUE which, acting as a reducing agent, protect carotenoids from oxidative degradation and thus preserve their content in the fruits [7].

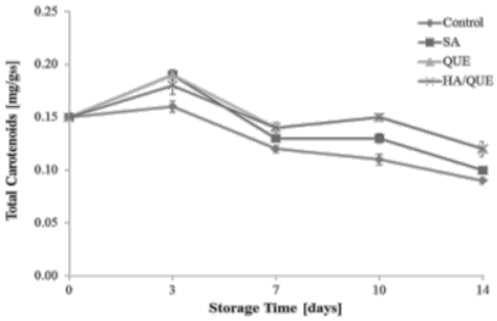

Figure 3: *Change in total carotenoids content*

The percentage change in antioxidant activity for of all samples was evaluated through both DPPH and ABTS radical-scavenging assays. ABTS results showed the same trend of DPPH ones: a lower degradation of antioxidant compounds in coated fruits with QUE was reached in all days of the storage period, with an antioxidant activity significantly ($p<0.05$) higher for HA/QUE samples than the other ones after 14 days of storage.
These results confirmed the effectiveness of the alginate-based edible coating enriched with HA/QUE complexes to preserve antioxidant compounds of fresh-cut papaya during the entire storage period.
The physical characterization of alginate-based coatings with and without active compounds showed how the presence of HA crystals allowed an increase of the film thickness as well as a decrease of water vapor transmission.
The microbial analysis carried out during the storage period pointed out the positive effect of HA charged with QUE to inhibit the growth of spoilage bacteria. At the end of cold storage, the total bacteria count in papaya samples covered with HA/QUE alginate coating was 4.8 log CFU/g significant lower ($p<0.05$) then 8.3 log CFU/g for uncoated samples. All data were shown in detail in Montone et al.,2022 [8].
With the aim of to evaluate the synergic effect between lacto and QUE, developed alginate-based coating was loaded with HA-lacto-QUE complexes and employed as edible coating for shelf-life extension of pork fillets. The investigated microbiological parameters pointed out the efficacy of HA-lacto-QUE alginate-based coatings to inhibits the growth of spoilage bacteria. In view of the value of 7-8 log CFU/g, which is the recognized and accepted limit of TVC value for fresh meat [9], in samples covered with HA-lacto-QUE based coating this limit was reached at 14 days that is 4 days later compared to the control and the samples covered with only lacto/QUE based coating samples. Study is in progress.

4.Conclusions and Future Perspective

Consumers in Europe are increasingly looking for sustainable and environmentally friendly products and packaging is an essential component of this. According to a 2021 study by the FMCG Packaging Observatory, "in the last 6 months, 14% of Italians have stopped buying products due to a packaging that did not present elements of sustainability and more than half of the respondents said that it could do so in the near future" in the case of food packaging, the new frontier is represented by edible packaging. Literally, packaging that not only protects food, prolonging its shelf-life, but that can be consumed with it. There are, however, a number of problems, which limit the growth of edible coatings, such as problems of contamination of raw materials during storage, design considerations, investments and replacement costs of competing technologies. To overcome these uncertainties, long-term laboratory- and pilot-scale experiments are needed to assess the performance and reliability of these preservation systems.

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Formulation and processing strategies for obtaining bakery products tailored to the elderly's needs

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Healthy dietary patterns during the entire lifespan, and especially in the elderly, are considered key factors to promoting active healthy ageing. Nowadays, elderly subjects (aged > 60 years) represent worldwide the faster growing segment of the population. The number of the elderly is predicted to increase up to 2 billion by 2050 (WHO, 2002) and tailored foods have to be designed to meet their nutritional needs and sensorial expectation. The overall aim of this PhD project is to design foods tailored for the needs of the elderly in order to contribute to the concept of active and healthy ageing.

Strategie di formulazione e processo per ottenere prodotti da forno su misura per i bisogni degli anziani

Sane abitudini alimentari durante la vita, e durante la vecchiaia, sono considerate fattori chiave per promuovere un invecchiamento in salute. Attualmente, i soggetti anziani (con età > 60 anni) rappresentano a livello mondiale una fascia di popolazione in crescita. Si prevede che il numero di anziani aumenterà a 2 miliardi entro il 2050 (WHO, 2002) ed è necessario sviluppare alimenti che incontrino i loro bisogni nutrizionali e le loro aspettative sensoriali. L'obiettivo generale di questo progetto di dottorato è progettare alimenti su misura per i bisogni nutrizionali degli anziani al fine di contribuire al concetto di invecchiamento attivo e sano.

Key words: active ageing, *in vitro* digestion, bread, age-tailored foods, food design.

1. Introduction

The elderly are becoming a large proportion of the world's population in developed countries and the health care costs for this growing age group will likewise increase (WHO, 2002). In this context, adequate nutrition is one of the key elements in a health promotion strategy. However, available food products in the market may not be suitable for the elderly in terms of sensory characteristics, nutritional properties, and cost. In fact, the elderly people have specific food preferences, and, in this regard, food appearance and texture play a key role in food choice and perception, which in turn may affect further digestion and nutrient absorption (Satusap et al., 2014). It is known that sensory performance, eating capability and oral processing can be altered in the elderly due to denture status, lower chewing and swallowing efficiency, and loss of chemosensory sensations. The main age-related changes in the oral processing are decreases in bite force, the number of sensory receptors, which leads to an increase in sensory thresholds, saliva secretions, and motor activity of the tongue and masticatory muscles. Also, during ageing food digestion and nutrient absorption can be compromised due to the altered gastrointestinal functions, *e.g.* food transit and digestion rate, enzyme output, and gut microbiota composition (Rémond et al., 2015), and consequently, the food digestibility and nutrient release rate can change. These age-related factors could compromise the ability of individuals to attain the required nutritional needs. It is a matter of fact that the elderly are prone to have an unbalanced diet in terms of both energy content and nutrient composition (Agarwal et al., 2013). Alteration of carbohydrate digestibility in the elderly is responsible for an increased risk of type 2 diabetes (Rémond et al., 2015). In addition, an inadequate protein intake may be responsible for body protein loss, muscle mass reduction, and sarcopenia-related issues, such as frailty, disability, and falls (Agarwal et al., 2013). The Italian population's reference intake (PRI) of protein is 0.9 g/kg body weight per day for adults, but, in order to prevent protein malnutrition, the suggested dietary target (SDT) for the elderly is 1.1 g/kg per day (SINU, 2014). Based on these considerations, there is a need to design age-tailored foods to promote the well-being and health of the elderly (Calligaris et al., 2022). To be suitable for the elderly a food should overcome physiological limitations, which affect taste and aroma perceptions, mastication, and digestion (Rémond et al., 2015). *In vitro* digestion models represent a useful tool to screen food matrices behaviour during digestion under specific gastrointestinal (GI) conditions. However, to date, a standardized protocol to assess food behaviour during digestion under elderly conditions has not been developed yet (Mackie et al., 2020). The aim of this PhD project was to design foods tailored for the needs of the elderly population in order to contribute to the concept of active and healthy ageing.

2. Experimental plan

As already pointed out, foods for the elderly should provide desirable sensory properties, balanced distribution of macronutrients and micronutrients, adequate fatty acid ratio and protein quality, low glycaemic index, and must be affordable (Calligaris et al., 2022). Among all bakery products, bread is one of the oldest staple foods worldwide and a traditional product for the elderly diet.

The aim of this PhD thesis was to design a new bakery product aimed at the elderly, by means of the application of technological and nutritional approaches. Firstly, the desirable sensory properties that an ideal bread targeting elderly consumers should have were identified by means of Check-All-That-Apply (CATA) approach, which allows to capture consumers' perception of food products. Then, in the light of sensory preferences of the elderly, a functional bread, *i.e.* protein enriched bread, tailored to old-aged consumers were designed. To this purpose, pea proteins were selected, based on nutritional needs and the current interest in enlarging the use of plant proteins in foods. Finally, oral processing performance, GI and colonic digestibility in the elderly of the new product were assessed. In order to study the digestion fate of nutrients, an *in vitro* digestion method mimicking elderly GI conditions was developed. The method was first tested to study and discriminate protein and carbohydrate digestibility between the adult and elderly conditions. In accordance with the purpose of this PhD thesis project, here we discussed the main results of activities aimed at:

1) identifying the desirable sensory and mechanical properties of a bread targeting elderly consumers, and understanding whether the products currently present in the Italian market meet their expectations;
2) developing a protein-rich bread intended to meet the nutritional and sensory requirements of the elderly and to evaluate the *in vitro* protein digestibility;
3) comparing protein and carbohydrates *in vitro* digestibility under adult and elderly GI conditions.

3. Experimental activity

3.1 Bread for the elderly: identifying the desirable sensory properties

In order to design a bread targeting elderly consumers, it is essential to understand the desirable sensory properties it should have. The aim of this study was to define the desirable sensory and mechanical properties of a bread intended for the elderly and to understand whether the products currently present in the Italian market meet their expectations (Moretton et al., *submitted*).

3.1.1 Materials and methods

Eleven samples of commercial breads having different formulations and manufacturing processes (*e.g.,* wheat sandwich, wheat burger, rye, Sardinia, Tuscany, bruschetta bread, and durum wheat loaf) were assessed for their physical properties (moisture content, texture and colour). A pilot test was conducted to develop a free listing questionnaire of bread attributes considering appearance, odour, taste, flavour, texture and chewing properties. To identify drivers of liking and possible directions for product reformulation, 76 healthy elderly (60–75 years old) evaluated the liking and the sensory characteristics of samples. Initially, subjects were asked to score their liking using a Visual Analogue Scale (VAS), (rated 0 "extremely disliked", rated 10 "extremely liked"). Subsequently, using a CATA method, subjects were asked to complete a questionnaire format comprising 36 attributes related to sensory characteristics of bread and to defined all the appropriate terms to describe their ideal bread.

3.1.2 Results and discussion

The mean liking scores of the tested Italian commercial bread were low (Table 1), with more than half of the products remaining below the acceptability, probably due to the fact that samples presented are generally consumed and appreciated after being toasted or with spreads. In this regard, chewy and saltless descriptors corresponded to poor elderly's satisfaction, whereas the ideal product should have homemade appearance, odour and flavour of whole bread, crusty and crumbly texture, salty taste and "easy-to-eat" properties (data not shown).

Among samples tested, the ideal bread was positioned between a traditional Italian bread with a crispy texture, and a rustic loaf with homemade appearance. These results seem to be related to the physiological changes of the elderly: the reduction of the sensory perception during ageing could lead to prefer salty products with more intense odour and flavour, and a crunchy texture.

Table 1 *Mean liking scores and standard error of the mean (SEM) of samples.*

Sample	Overall liking scores
A	5.3 ± 0.2^{ab}
B	5.1 ± 0.3^{bc}
C	5.4 ± 0.3^{ab}
D	4.5 ± 0.3^{bc}
E	4.0 ± 0.2^{c}
F	5.0 ± 0.3^{bc}
G	4.4 ± 0.2^{bc}
H	4.8 ± 0.3^{bc}
I	6.4 ± 0.3^{a}
J	3.8 ± 0.2^{c}
K	4.2 ± 0.2^{bc}

Different letters indicate significant differences according to Tukey's Honest Significant Difference post hoc test.

Moreover, acquired results pointed out that sensory evaluations overlap well with texture and colour parameters, and moisture content of samples (data not shown). Overall, the results of this study allowed to identify the desirable sensory properties of bread for the elderly population, suitably enriched in nutrients (*e.g.* proteins). Also, it is mandatory to put effort into developing a bread perceived as salty but with low salt content to reduce the risk of salt-related diseases.

3.2 Bread for the elderly: formulation, processing and digestibility

To meet the sensory as well as nutritional requirements of the elderly, pea protein-rich bread was developed, and the *in vitro* protein digestibility was evaluated. We chose pea proteins because they are cheap, sustainable, well-balanced in terms of amino acid profile and do not present an allergenic character.

3.2.1 Material and method

The basic formulation of white bread consisted of 60.5% wheat flour, 30% water, 5.9% sunflower oil, 2% sucrose, 1% dried yeast and 0.6% sodium chloride. Protein enriched breads were formulated by substituting 5 and 10% of wheat flour with pea protein concentrate (80% pea proteins), which was subjected or not to high pressure homogenization (HPH). In particular, 5% pea protein dispersion was subjected to 70 MPa x 3 passes (Panda Plus 2000; GEA Niro Soavi, Parma, Italy). The dough was fermented at 35 °C and 80% RH for 1 h, and baked at 160 °C for 35 min with RH varying from 80 to ~5% in a professional oven. Bread loaf was left cooling at 20 °C and moisture content, aw, colour, and firmness were evaluated. Three batches were produced for each formulation.

3.2.2 Results and discussion

Table 2 shows the effects of flour substitution with pea protein concentrate on the physical and physical chemical parameters of breads.

Table 2 *Moisture, aw, colour, and firmness of white bread, and protein enriched breads obtained by substituting 5% and 10% of wheat flour with pea protein concentrate.*

			White	5%	10%
Moisture (%)		Crumb	35.7 ± 0.4^{a}	35.6 ± 1.1^{a}	33.2 ± 0.7^{b}
		Crust	7.0 ± 0.9^{a}	6.8 ± 1.4^{a}	8.0 ± 1.5^{a}
aw		Crumb	0.92 ± 0.00^{b}	0.93 ± 0.00^{ab}	0.94 ± 0.00^{a}
		Crust	0.57 ± 0.01^{b}	0.59 ± 0.06^{b}	0.71 ± 0.04^{a}
Colour	**L***	Crumb	72.6 ± 0.9^{a}	71.2 ± 3.3^{a}	68.6 ± 2.6^{b}
		Crust	60.2 ± 4.9^{a}	56.4 ± 4.3^{b}	55.3 ± 5.1^{b}
	Hue angle	Crumb	92.6 ± 1.2^{a}	89.0 ± 0.4^{b}	85.2 ± 1.7^{c}
		Crust	69.4 ± 4.5^{a}	65.6 ± 3.1^{b}	64.1 ± 3.5^{b}
Firmness (N)		Crumb	1.2 ± 0.1^{c}	1.7 ± 0.3^{b}	3.1 ± 0.5^{a}

Data points Means ± SD; $^{a, b, c}$ in the same row means indicated by different letters are significantly different ($p < 0.05$).

Moisture content was only slightly affected by the wheat flour substitution with pea protein concentrate, pointing out that temperature and cooking time mostly influenced this parameter. Moreover, 10% flour substitution with protein concentrate gave both crumb and crust bread with the highest aw values, due to the lower efficiency of proteins to bind water compared to carbohydrates of wheat flour. As expected, the addition of protein concentrate significantly decreased L* and hue angle values of both protein enriched samples, as compared to white bread. Crumb firmness significantly increased with the increase in protein concentrate level added to the bread, exhibiting up to 3 times higher values as respect to the white bread. Similar results were found for the breads enriched with HPH treated pea protein concentrate (data not shown).
Previous results showed that the HPH treatment was able to increase the protein digestibility of plant proteins (Melchior et al., 2022), which is generally reported to be lower than that of animal proteins. HPH increasing effect on protein digestibility has been attributed to protein unfolding which favours the activity of digestive enzymes. At present, oral processing performance, GI and colonic digestibility of protein enriched breads are under study, by means of *in vitro* digestion methods mimicking adult and elderly GI conditions. The former is represented by the INFOGEST standardized protocol, while the method simulating the GI conditions of the elderly was set up during my PhD period, as reported in Chapter 3.3.

3.3 Comparison of protein and carbohydrates *in vitro* digestibility under adult and elderly conditions

In vitro digestion models represent a useful tool to screen food matrices behaviour during digestion under specific GI conditions. However, to date, a standardized protocol to investigate food behaviour during digestion in the elderly has not been developed yet (Mackie et al., 2020). The aim of this study was to set up an *in vitro* digestion method mimicking elderly GI conditions and use it to compare *in vitro* digestibility of nutrients in adult and elderly conditions (Melchior et al., *submitted*). Milk whey, wheat gluten, pea and rice proteins were used as model systems, and wheat and whole wheat breads were considered as complex food matrices.

3.3.1 Materials and methods

Whey, wheat gluten, pea, and rice proteins, wheat and whole wheat breads were subjected to static *in vitro* digestion protocols simulating adult and elderly gastrointestinal conditions. In the former case, the INFOGEST protocol proposed by Brodkorb et al. (2019) for adults was used. An *in vitro* static digestion method mimicking the elderly GI conditions was set up (Melchior et al., *submitted*), based on *in vivo* data available in the literature about the GI tract and nutrient digestibility of the elderly and information taken from dynamic *in vitro* digestion studies (Shani-Levi and Lesmes, 2014). Table 3 shows the parameters of *in vitro* digestion systems simulating adult and elderly GI conditions.

Table 3 *Parameters of in vitro digestion systems simulating adult and elderly GI conditions.*

Digestive phase	Conditions	Adult	Elderly
Oral	Salivary α-amylase activity (U/mL)	75	150
	KCl in simulated salivary fluids (mmol/L)	15.1	30.1
	Rate (rpm)	15	7
Gastric	pH	3.0	4.5
	Pepsin activity (U/mL)	2000	1500
	Phase duration (h)	2	3
	Rate (rpm)	15	7
Intestinal	pH	7.0	6.6
	Pancreatin activity (U/mL)	100	46
	$CaCl_2(H_2O)_2$ (0.3 M) (v/v)	0.001	0.002
	Phase duration (h)	2	3
	Rate (rpm)	15	7

Protein digestibility was assessed after gastric and intestinal phases by bicinchoninic acid assay (BCA) while starch digestibility was determined by measuring glucose concentration during the intestinal phase by HPLC. Results are the mean of triplicate *in vitro* digestions of each sample.

3.3.2 Results and discussion

Digestibility of whey, gluten, pea and rice proteins upon gastric and intestinal phases of the *in vitro* digestion under systems simulating both adult and aged gastrointestinal conditions is shown in Figure 1.

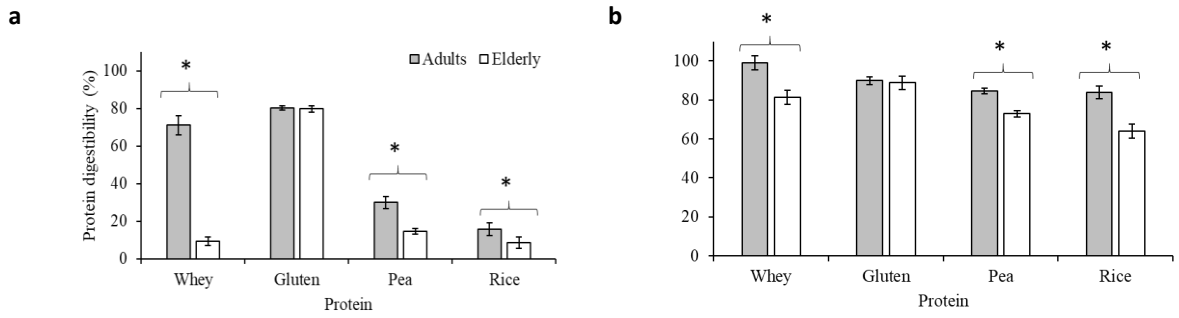

Figure 1 *Protein digestibility of whey, gluten, pea and rice protein at the end of gastric (a) and intestinal (b) phases under adult and elderly conditions. *: indicate statistically significant ($p < 0.05$) difference between adult and elderly GI conditions.*

At the end of the adult gastric phase (Figure 1a), a high protein digestibility was observed for whey (71.1%) and gluten (80.3%), while pea and rice proteins exhibited lower digestion degrees. Under the elderly conditions, the digestibility profile of gluten during the gastric phase was similar to that under the adult conditions, whereas the digestibility of whey, pea, and rice proteins was lower, accounting for 9.5%, 14.5%, and 8.7%, respectively. During the intestinal phase (Figure 1b), an overall digestibility reduction was detected under the elderly conditions in comparison with the adult ones in the case of whey, pea and rice proteins, while no differences were found for gluten. Acquired results showed that the elderly GI conditions deeply affected proteolysis leading to a general reduction of protein digestibility in comparison to the adult model. The proteolysis extent depended on the protein source with whey and rice proteins showing about 20% reduction using the model mimicking the elderly gut, followed by pea proteins (about 10% reduction) and gluten (about 4% reduction).

To investigate in depth the digestion pathway under the adult and elderly GI conditions, the protein and carbohydrates digestibilities of wheat and whole wheat breads were also analysed (Table 4). At the end of gastric phases, no differences in protein digestibility (Table 4) were observed under the adult and elderly conditions among bread types, confirming that gluten digestibility was not affected by physiological alteration (Melchior et al., *submitted*). The protein digestion mostly occurred during the intestinal phase and it was affected by the gastrointestinal conditions applied and by the formulation.

Table 4 *Protein digestibility at the end of gastric and intestinal phases, and carbohydrate digestibility during the intestinal phase, of wheat and whole wheat bread under adult and elderly conditions.*

Simulated GI conditions	**Protein digestibility** (%)			**Carbohydrate digestibility** (mg glucose/g dry matter)		
	Phase	Wheat bread	Whole wheat bread	Time (min)	Wheat bread	Whole wheat bread
Adult	Gastric	25.7 ± 2.6	21.4 ± 1.5	0	12.2 ± 0.9	5.1 ± 0.2
				20	617.5 ± 60.4	533.4 ± 106.4
	Intestinal	54.1 ± 3.6*	45.1 ± 3.1*	60	599.8 ± 32.0	646.5 ± 31.8*
				90	592.8 ± 48.8	709.3 ± 20.4*
				120	631.7 ± 43.7	627.8 ± 3.4*
Elderly	Gastric	20.7 ± 1.4	21.2 ± 1.6	0	26.2 ± 2.9*	8.3 ± 0.9*
				20	672.2 ± 16.4	452.0 ± 1.0
	Intestinal	39.3 ± 1.2	38.9 ± 2.3	60	623.9 ± 4.0	458.2 ± 6.7
				90	598.1 ± 2.6	455.5 ± 19.1
				120	613.0 ± 9.6	439.2 ± 5.0
				180	605.9 ± 6.0	451.8 ± 17.0

*: indicate statistically significant ($p < 0.05$) difference between adult and elderly GI conditions for wheat and whole wheat bread.

Proteins were more effectively digested under adult conditions as compared to elderly ones and the food matrix structure influenced the nutrient release. In particular, the presence of fibre would have altered the activity of proteolytic enzymes (Reynaud et al., 2020). Regarding carbohydrates digestibility under the adult conditions, a sharp increase in glucose concentration was observed during the first 20 min in both bread types, and glucose concentration further increased during the intestinal digestion of whole wheat bread, reaching a peak at 90 min, whereas wheat bread did not release additional glucose up to the end of the intestinal digestion. Under the elderly conditions, after 20 min a significantly lower glucose release was observed for whole wheat bread compared to the wheat one. This value was even significantly lower than that observed under adult conditions for the same bread type. Moreover, significant differences in glucose release among all bread types were observed throughout the whole course of intestinal digestion under elderly conditions. Differences detected in carbohydrate digestibility can be attributed to the GI alterations occurring during ageing, probably due to the lower enzyme concentration, as well as to the chemical composition and physical properties of bread (Hiolle et al., 2020). To conclude, the developed *in vitro* digestion method simulating the GI conditions of the elderly was able to discriminate protein and carbohydrate digestibility as respect to the INFOGEST protocol for adults. Moreover, the obtained results may contribute to a better understanding of food digestibility under different GI conditions and its dependence on physiological and formulation factors, and ultimately would help to design age-tailored foods.

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Sustainable approaches to winemaking and wine aging

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This PhD thesis research project is aimed to test innovative and eco-friendly technologies for the ageing and stabilisation of wine. Both the use of innovative coadjuvants such as toasted vine-shoot chips and the adoption of eco-friendly technologies such as ultrasound were tested in order to improve and differentiate the sensorial quality of wine.

Keywords: wine aging, vine-shoots chips, eco-friendly technologies, ultrasound, sensorial quality

Approcci sostenibili per la vinificazione e l'invecchiamento del vino

Questo progetto di ricerca ha come obiettivo quello di testare tecnologie innovative ed ecocompatibili nelle fasi di invecchiamento e stabilizzazione di un vino. Sia l'uso di coadiuvanti innovativi come i chips di tralci di vite tostati, sia l'adozione di tecnologie ecocompatibili come gli ultrasuoni, sono stati sperimentati al fine di migliorare e differenziare la qualità sensoriale del vino.

1. Introduction

The aim of this communication is to report the main results of three key activities of this PhD project. In particular, the activities are outlined as follows:
A1. production of Primitivo vine-shoot chips and their volatile characterization;
A2. use of vine-shoot chips in Nero di Troia winemaking process;
A3. application of toasted vine-shoot chips and ultrasound treatment in the ageing of Primitivo wine.
In a recent review, Ma *et al.* (2022) divided wine ageing processes into two categories: traditional ageing techniques (e.g. oak barrel) and artificial ageing techniques (e.g. wood products or ultrasonic field). Among these, the use of pieces of oak wood is widely spread in winemaking, in order to enrich wine sensory profile with wood compounds. Recently, the use of various types of wood chips from different botanical species (such as chestnut and acacia) was tested to improve the quality of wine (Lisanti *et al.*, 2021). Furthermore, Cebrián-Tarancón *et al.* (2022) have shown the possibility of using vine-shoot chips during the winemaking and ageing processes, in order to positively contribute to phenolic and volatile composition of wine. Vine-shoots are viticulture wastes generated during vine annual pruning, often burnt causing significant environmental problems. In order to minimize this problem, phenolic extracts have been prepared from vine-shoots and tested in winemaking to reduce the dosage of SO_2 (Cruz *et al.*, 2018), being a source of antioxidant compounds such as stilbenes (Noviello *et al.*, 2022). Differently, their use as an alternative to oak chips is correlated to the phenolic and volatile composition of the toasted vine-shoot chips (Cebrián-Tarancón *et al.*, 2018). Also ultrasound, one of the most promising environment friendly and low-cost technology, has been tested as an artificial ageing techniques to accelerate the ageing process of wine, improving its quality (Ma *et al.*, 2022). In this perspective the great part of activities of the PhD project are focused. In fact, to the best of our knowledge vine-shoots of typical Italian varieties have not been studied yet as well as their potential use as an alternative to oak chips to differentiate the sensorial profile of Italian wines. Therefore, vine-shoot chips were used in winemaking and wine ageing. Moreover, the use of ultrasound was evaluated to improve and accelerate this process.

2. Materials and methods

2.1 Production of Primitivo vine-shoots chips and their volatile composition

Vine-shoots of Primitivo cultivar were pruned randomly in a vineyard located in Laterza (Puglia, Italy) and stored at dark and room temperature (18 ± 3 °C) for six months. After this time, vine-shoots were divided into two fractions: 1) the first was ground by a hammer miller to a particle size around 2-20 mm long and subjected to a toasting process at 180 °C for 45 minutes (Cebrián-Tarancón *et al.*, 2018), using a thermostatic oven (TFC 120 Forced air Oven, ArgoLab); 2) the second one was boiled for 10 minutes, dried overnight at 45 °C, then ground and toasted under the same conditions. Volatile compounds of natural (P-C), toasted (P-T), boiled and toasted (P-BT) vine-shoots chips and oak chips (OC) were determined by SPME/GC-MS according to Cardinale *et al.* (2021), with some modification.

2.2 Use of vine-shoots chips in Nero di Troia winemaking process

The experimentation involved the addition of 12 g/L (Cebrián-Tarancón *et al.*, 2019) of toasted vine-shoots, boiled and toasted vine-shoots (see section 2.1) and oak chips (OC, for comparison) in two different steps of Nero di Troia winemaking: i) before fermentation/maceration (BF-T, BF-BT, BF-OC); ii) after fermentation/fining in

wine, for 35 days (AF-T, AF-BT, AF-OC). A control sample without chips (C), was included. Total polyphenolic content (TPC, as mg/L of gallic acid equivalents), flavonoids (F, as mg/L of (+)-catechin), anthocyanins (A, as mg/L of malvidin-3-glucoside), flavans reactive to vanillin (FRV, as mg/L of (+)-catechin), proanthocyanidins (P, as mg/L of cyanidin chloride) were determined (Gambacorta *et al.*, 2011). The colour indices (CI, colour intensity; T, tonality) were assessed according to the Glories procedure (1984). Antioxidant activity was performed by ABTS assays (Difonzo *et al.*, 2017). All these parameters were determined using an UV–visible spectrophotometer (ThermoFisher Scientific, Rodano, Italy). Volatile compounds were determined by SPME/GC-MS, according to Cardinale *et al.* (2021), with some modification.

2.3 Application of toasted vine-shoot chips and ultrasound treatment in the ageing of Primitivo wine

Toasted vine-shoots chips of Primitivo cultivar were obtained as described in section 2.1. Then, 50 L of Primitivo wine was divided into three parts: 1) wine without any treatment (C, control); 2) wine with 10 g/L of toasted vine-shoot chips (I, infusion); 3) wine with toasted vine-shoot chips and subjected to ultrasound treatment (U+I, ultrasound + infusion): toasted vine-shoot chips (10 g/L) were added to 500 mL of wine in a beaker and sonicated for 30 minutes with a ultrasound system (Bandelin electronic, Berlin, Germany) at a frequency of 20 kHz and power of 150 W in a thermostatic bath (20 ± 5 °C). Each treatment was carried out in triplicate. After each storage period considered (7, 14, 21 and 28 days), the chips were removed by filtration in samples I and U+I and all wines (C, I and U+I) were analysed simultaneously in triplicate. The wines were subjected to the same characterisation described in section 2.2.

2.4 Statistical analysis

Principal components analysis (PCA) were performed for the data of activity A1. All the data about phenolic composition and colour indices (activity A2) were subjected to statistical analysis one-way ANOVA followed by Tukey's HSD test at α=0.05. Two-way analysis of variance (ANOVA) with interactions was performed on the analytical data of the activity A3. A clustering analysis with the construction of a polar heat map was used to analyse the volatile composition of the wines (activities A2 and A3). Statistical analysis was performed using Minitab Statistical Software (Minitab Inc., State College, PA, USA) and Origin 2021 (OriginLab, Northampton, MA, USA).

3. Results and discussion

3.1 Volatile composition of Primitivo vine-shoots and oak chips

In order to distinguish differences of volatile profiles of Primitivo vine-shoots samples and oak chips, PCA (Principal Component Analysis) was used. Fig. 1 showed the total contribution of first two PCs was 87.4 % of PC1 59.7% and PC2 27.7%. The samples OC, P-C, P-T and P-BT were well separated, indicating that differences of volatile profiles were presented. In fact, the toasted (P-T) and boiled and toasted (P-BT) vine-shoots, were located on the positive side of the axis of the PC1 and were associated with a greater abundance of lactones such as butyrolactone (caramel aroma) and different furanic compounds (e.g. furfural, 5-hydroxymethylfurfural, 2-furanmethanol or 2-furancarboxaldehyde, 5-methyl-). These compounds derived from the degradation of wood polysaccharides (cellulose and hemicellulose) which could lead to almond and toasted aroma in the wine (Jordão *et al.*, 2022). Most of the volatile compounds found in toasted vine-shoot chips have also been identified in different toasted vine-shoots (Cebrián-Tarancón *et al.*, 2018). Conversely, oak chips (OC) and control vine-shoots (P-C) were located in the negative axis of the first component. The first one showed a greater amount of alcohols (e.g. 1-pentanol, 1-hexanol), the second correlated well with compounds such as eugenol or isobutyl acetate. On the other hand, OC were located on the negative axis of the PC2 component and differently from all vine-shoots sample that were located on the positive axis.

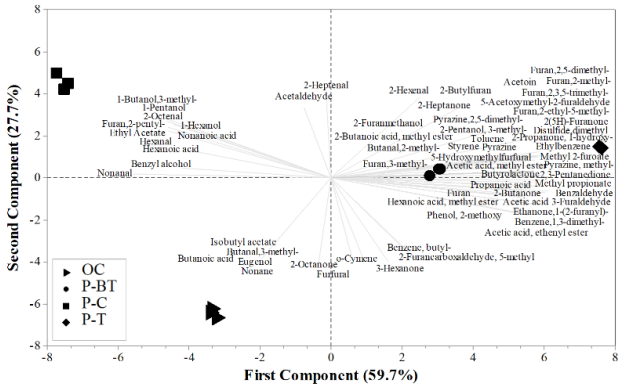

Figure 1 *Biplot of the principal component analysis (PCA) carried out on the main characteristic volatile profiles of OC (oak chips), P-BT (boiled and toasted vine-shoots chips), P-T (toasted vine-shoots chips) and P-C (natural vine-shoots chips).*

3.2 Use of vine-shoots chips in Nero di Troia winemaking process

Table 1 shows the phenolic parameters of Nero di Troia wines obtained by applying vine-shoot and oak chips treatments during aging. The addition of chips increased TPC, antioxidant activity (ABTS), A, F, FRV. These results are in agreement with Baiano and De Gianni (2016), who reported that the addition of oak chips increased the concentration of A, anthocyanins sensitive to SO_2 and FRV in Nero di Troia wines. In particular, the addiction of vine-shoots chips caused a statistically significant increase of TPC compared to the control (+20-30%, approximately). About the colour indices, BF-T, AF-T and AF-BT showed lower CI. Instead, wines in which chips have been added during infusion presented the highest T values, typical of aged wines.

Table 1 *Chemical characteristics, phenolic composition and colour indices of Nero di Troia wines.*

	C	BF-T	BF-BT	BF-OC	AF-T	AF-BT	AF-OC
TPC	*1039±14e	1252±42d	1374±20b	1329±10c	1225±48d	1360±4b	2074±13a
ABTS	9737±511c	11201±629b	11927±382b	11511±71b	12058±472ab	11404±321b	13261±588a
A	101±6c	113±5c	146±5a	142±5a	124±1ab	122±2b	145±5a
F	931±8b	998±14ab	1094±45a	1027±38ab	1036.69±18.80b	1013±91ab	1093±20a
FRV	621±4d	649±6bc	671±0.3a	635±8.05cd	665±14ab	638±12bcd	672±3a
P	1138±16bcd	1099±4cd	1206±46b	1089±42d	1182±45bc	1116±13cd	1309±17a
FRV/P	0.54±0.00bc	0.59±0.00a	0.55±0.02ab	0.58±0.02a	0.56±0.01ab	0.57±0.02ab	0.51±0.01c
CI	0.42±0.01c	0.39±0.00d	0.47±0.00a	0.45±0.00b	0.35±0.00e	0.39±0.01d	0.43±0.01c
T	0.78±0.02c	0.80±0.01c	0.78±0.00c	0.79±0.00c	0.95±0.00a	0.86±0.01b	0.84±0.01d

*Different letters in the same rows indicate significant differences among treatments (Tukey HSD test, $p < 0.05$). Abbreviations: C, control wine; BF-T, BF-BT, BF-OC, respectively wines obtained with the addition of toasted, boiled and toasted vine-shoots and oak chips before fermentation; AF-T, AF-BT, AF-OC, respectively wines obtained with the addition of toasted, boiled and toasted vine-shoots and oak chips after fermentation; TPC, total polyphenolic content (mg/L); ABTS, antioxidant activity by ABTS assay (µmol/L); F, flavonoids (mg/L); A, anthocyanins (mg/L); FRV, flavans reactive to vanillin (mg/L); P, proanthocyanidins (mg/L); CI, colour intensity; T, tonality.

Fig. 2 shows the polar heatmap with a circular dendogram which allows to characterize the volatile patterns of Nero di Troia wines. Many of these compounds have been found in wines treated with oak and vine-shoots chips or aged in barrels (Cebrián-Tarancón *et al.*, 2022; Călugăr *et al.*, 2020). Overall, both the time of addition of the chips and the type of chips affected the volatile composition of the wines obtained. The AF-T and AF-BT wines were characterised by the higher presence of esters such as ethyl acetate and ethyl octanoate (fruity and brandy notes) and the BF-BT by compounds such as phenylethyl alcohol (floral and rose notes); benzyl alcohol (chocolate and tobacco notes); linalool (floral and citrus notes).

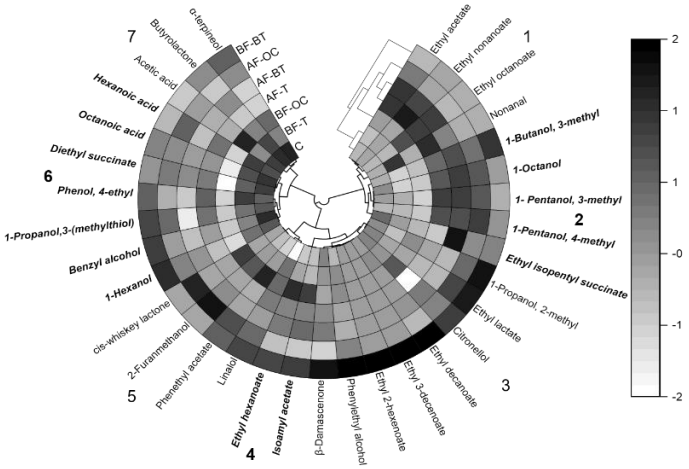

Figure 2 *Polar heatmap with a circular dendrogram deriving from a hierarchical cluster analysis of the volatile profiles of the Nero di Troia wines. The numbers from 1 to 7 indicate the different clusters. For sample codes, see section 2.2.*

3.3 Application of toasted vine-shoot chips and ultrasound treatment in the ageing of Primitivo wine

Table 2 shows the effect of treatments and storage time on the phenolic composition of wines. Both treatment and storage time influenced the phenolic parameters analysed. All the treatments reduced the concentration of A, F, FRV and P. Furthermore, both treatments caused a reduction of CI ($p \leq 0.001$) and an increase of T ($p \leq 0.001$). In particular, wine U+I presented the highest value of T, followed by wine I and C. It is well known that during aging period the colour of wine change. In fact, during the treatment with wood chips a decrease of colour intensity occurs together with a colour tonality increase (Jordão *et al.*, 2019). Storage time resulted in a decrease in TPC, A and F after 28 days. Differently, antioxidant activity increased. This reduction in phenolic content during bottle storage is generally attributed to polymerisation, oxidation and complexation reactions. No influence of time was observed for FRV, P and T.

Table 2 *Phenolic characteristics of Primitivo wines as a function of treatments (C, control; I, infusion; UI, ultrasound and infusion) and time of storage (7,14,21,28 days).*

	F	A	FRV	P	FRV/P	TPC	ABTS	CI	T
Tr									
C	*2566±90a	332±15a	1607±73a	2896±143a	0.56±0.04a	2803±86a	17808±736a	0.96±0.04a	0.67±0.01c
I	2440±101b	264±25b	1410±57b	2524±107b	0.56±0.04a	2677±186ab	16924±957b	0.87±0.06b	0.76±0.02b
U+I	2407±108b	251±27b	1377±44b	2672±285b	0.55±0.09a	2628±153b	16721±708b	0.89±0.06b	0.79±0.03a
Sig.	**	***	***	***	ns	*	**	***	***
Tm									
7	2536±90a	307±31a	1463±110a	2609±163a	0.56±0.03a	2851±162a	16087±832c	0.97±0.02a	0.74±0.04a
14	2438±53ab	284±37ab	1484±99a	2581±231a	0.61±0.07a	2710±147ab	17062±339b	0.85±0.05c	0.72±0.05a
21	2544±123a	287±39ab	1396±94a	2793±277a	0.51±0.03b	2640±103b	17900±571a	0.88±0.04bc	0.73±0.06a
28	2367±109b	251±47b	1507±146a	2806±280a	0.54±0.07ab	2610±129b	17556±681ab	0.91±0.06ab	0.77±0.07a
Sig.	**	*	ns	ns	**	**	***	***	ns
Int.	ns	*	ns	**	***	*	***	***	***

*In column, data followed by different letters indicate statistically significant differences at $p < 0.05$. *Significance (Sig)*: ns, *, ** and ***, not significant or significant at $p \leq 0.05$, $p \leq 0.01$ or $p \leq 0.001$, respectively. Abbreviations: Tr, treatments; Tm, time; TPC, total polyphenolic content (mg/L); ABTS, antioxidant activity by ABTS assay (μmol/L); F, flavonoids (mg/L); A, anthocyanins (mg/L); FRV, flavans reactive to vanillin (mg/L); P, proanthocyanidins (mg/L); CI, colour intensity; T, tonality.

The Tr*Tm interaction was significant for TPC, A, ABTS, P, FRV/P, CI and T as shown in Fig. 3. After 7 days, the wine I presented the highest value of TPC (Fig. 3a), which decreased after 28 days of storage with a similar value to the wine U+I. Moreover, C wine showed higher values of A during storage, compared to the other samples with chips (Fig. 3b). Regard to antioxidant activity increased after 28 days and C wine showed the highest value (Fig 3c). Concerning P (Fig. 3d), their content in the U+I wine increased during storage: compared to the other two samples, P had the lowest value at 7 days and the highest at 28 days. The impact on FRV/P ratio was different (Fig. 3e): the wine C at 7 days and after 28 days had the highest value of FRV/P, similar to I wine. On the contrary, U+I wine presented the lowest value after 28 days, indicative of a wine with less astringency, similar to what occurs during the ageing process in barrel. It is well known that a low FRV/P ratio resulted in a predisposition to chromatic and tannic stabilization of wines (Suriano *et al.*, 2015). Finally, the Fig. 3f confirmed that CI decreased in the wines I and U+I during storage, indicating a more rapid evolution of colour. Moreover, the samples with chips presented a higher value of T (Fig. 3g) than the control, which increased during storage, especially for U+I wine.

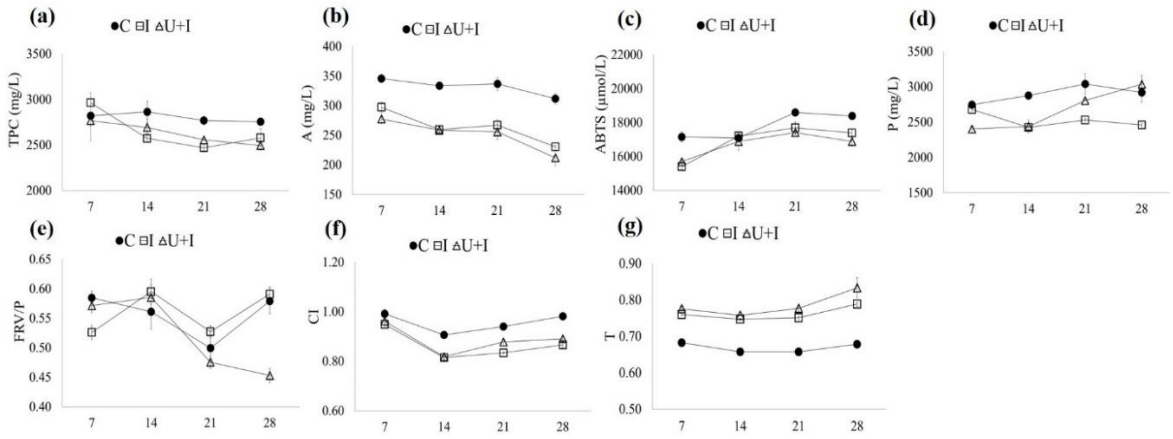

Figure 3 *Interaction between time of storage and treatment on phenolic parameters such as: (a) total polyphenolic content (TPC); (b) anthocyanins (A); antioxidant activity by ABTS assey (c); (d) proanthocyanidins (P); (e) ratio of flavans reactive to vanillin and proanthocyanidins (FRV/P); (f) colour intensity (CI); (g) tonality (T).*

A total of 50 volatile compounds were identified in the wines. Alcohols and esters were the most abundant classes of compounds. In fact, wines were clusterized in four homogeneous groups and compounds in seven groups (Fig. 4). Certainly, visible changes in volatile compounds correlated with the treatments and the time of storage were observed, suggesting that the wines treated with vine-shoots chips had different profiles compered to the control. In general, the first three clusters characterized the wines I and U+I and included acohols, esters and aldehydes. Among these, particular attention was given to compounds such as furfural, 2-furancarboxaldehyde, 5-methyl-, benzaldehyde due to their potential positive impact on wine aroma properties and because they are specific compounds released from wood (Dumitriu *et al.*, 2019). The fourth clusters of volatiles characterized the wines I28 and I14 for compund such as octanal and nonanal. All these compounds contribute to wine aroma with fruity, floral, spicy, wood and toasted. Overall, the last three clusters contained compounds such as acetic acid, ethyl acetate or β-Damascenone particularly presented in the control wine.

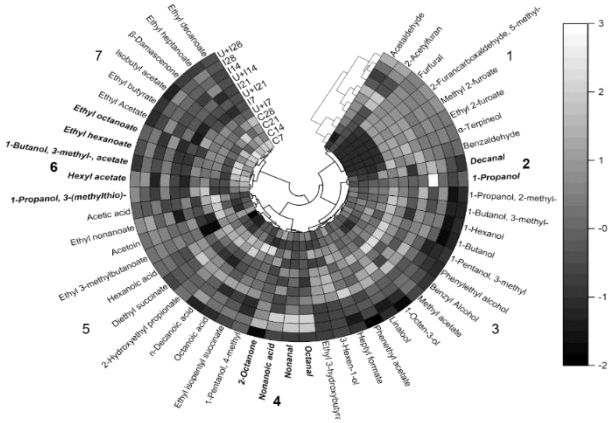

Figure 4 *Polar heatmap with a circular dendrogram deriving from a hierarchical cluster analysis of the volatile profiles of the Primitivo wines. The numbers from 1 to 7 indicate the different clusters. For sample codes, see section 2.3.*

4. Conclusions

During this PhD project, the possibility of using sustainable approaches in Italian wine production was tested. For the first time, the volatile composition of natural and treated vine-shoot chips of the Primitivo cultivar was evaluated and compared to oak chips. This characterization pointed out the interesting potential of these wastes, which could be used as a new oenological coadjutant. Indeed, the addiction of these chips during winemaking and aging of Nero di Troia caused an increase of the global phenolic fraction and a modification of wine color and volatile composition. Moreover, the addition of toasted vine-shoot chips and ultrasound treatment accelerated the Primitivo wine's colour stabilisation process and imparted distinctive aroma compounds. Therefore, these processes could be contribute to obtaining high-quality wines using alterantive and sustanable aging methods.

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Design and validation of healthy leavened bakery products: Focus on chemical-physical and sensory properties

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This PhD thesis deals with the development and characterization of bakery products enriched with functional ingredients to modify their carbohydrate digestibility. The experimental procedure was based on the validation of their health-promoting efficacy and the determination of important acceptance parameters such as texture and flavor profile.

Progettazione e validazione di prodotti da forno lievitati con proprietà salutistiche : focus su proprietà chimico-fisiche e sensoriali

Questa tesi di dottorato si occupa dello sviluppo e della caratterizzazione di prodotti da forno arricchiti con ingredienti funzionali per modificarne la digeribilità dei carboidrati. La procedura sperimentale si è basata sulla validazione della loro efficacia salutistica e sulla determinazione di importanti parametri di accettazione come la consistenza e il profilo aromatico.

Key words: Bakery products; protein hydrolysates; textural properties; volatile compounds.

1. Introduction

This oral communication reports the results of the research carried out during the 6 months mobility period at AgroParisTech in Paris (France) under the supervision of Prof. Barbara Rega. The aim of this study was to investigate the effects of adding different protein hydrolysates in a muffin recipe in terms of reactivity potential and physico-chemical implications on product quality by organizing the research approach in three main steps:
A1) Formulation of muffin prototypes with 4 different types of protein hydrolysates (casein, pea, rice and soybean) added at 2different addition levels (5%, 12.5% by weight) to partially replace wheat flour. A Control muffin (0% hydrolysate addition) was also prepared for comparison.
A2) Evaluation of the quality determinants linked to texture by rheological and physical determination of batters and baked prototypes.
A3) Evaluation of the quality determinants linked to chemical reactivity and odor potential of the new ingredients re by the analysis of the VOC profiles by GC-MS and isotopic dilution on samples of the baked prototypes.

2. Physical properties

Wheat (Triticum aestivum L.) is the traditional cereal used for baked products. However, in new approaches, wheat flour is increasingly partially replaced by functional compounds and, in the specific case of this PhD thesis, by hydrolyzed proteins from various sources. The main textural parameters associated with spongy baked product, such as muffins are the elastic consistency of the batter and the softness of the crumb. The mixing of the batter was performed in a two-step process, in which eggs, sugar, water and oil are beaten to a thick foam, and then flour, proteins hydrolyzed in different percentages and baking powder are carefully folded in. After that, the batter was poured into the cups and baked. Accordingly, muffin batter can be defined as a complex emulsion of fat in water in which the mixture of eggs, sugar, and water is the continuous phase, while air bubbles are the discontinuous phase in which flour particles are dispersed (Martínez-Cervera *et al.* 2012) and break down into smaller cells during whipping. This process step also promotes the diffusion and accumulation of surfactant compounds (proteins and lipids) at the air-water interface, which stabilize the gaseous cells by forming a continuous viscoelastic film on the bubble surface, creating a thermodynamically favorable situation. The final step in the production process is baking. During baking, the temperature of the products increases with a gradient from the core (up to 100 °C) to the surface (up to the set temperature, e.g. 180 °C). Then, the addition of baking powder promotes gas formation during baking, which leads to an increase in internal pressure. All this stimulates the growth of gas cells due to the expansion of incorporated air, evaporation of water, and decrease in gas solubility (Godefroidt *et al.*, 2019). As the water evaporates, the product is subsequently dehydrated, resulting in a sample weight loss of typically 5-14% (Gómez *et al.*, 2012). Taking into account this moisture loss, muffin expansion can be determined as the ratio between the final cake volume and the initial batter volume, which typically ranges from 1.5 to 1.8 for wheat. This value can be affected by both the initial viscosity and the density of the batter, which significantly affects its ability to hold air bubbles. In general, the addition of exogenous protein may compete with egg protein in settling at the water-air interface and destabilize the batter. In addition, as the protein content increases instead of flour, the percentage of starch, whose gelatinization is important for structuring the crumb,

would gradually decrease. To date, no relationship has been established between batter density and cake volume (de la Hera *et al.*, 2012).

3. Reactivity and odor potential

During the processing of bakery products, many reactions can occur that are promoted by the energy of the thermomechanical steps (beating and baking); the most important are Maillard reaction, and caramelization. Volatile organic compounds, commonly abbreviated as VOCs, include a variety of low molecular mass compounds (< 300 Da) belonging to different chemical families, including alcohols, aldehydes, ketones, acids, terpenes, and sulfur, nitrogen, and furan compounds (Jeleń and Gracka, 2017). Due to their high volatility, they can be transmitted to the olfactory receptors in the nasal cavity by orthonasal or retronasal inhaled air (Reineccius and Peterson., 2013). The Maillard reaction is an important chemical pathway in which, as reported by Parliment (1999), volatile odors can be produced during heat processing of raw materials or foods such as cakes, breads, coffee, and meat due to heat-induced nutrient degradation. The reaction shows the interaction between reducing sugars, such as glucose and fructose, and -NH2-carrying compounds, such as amino acids, peptides, and proteins, and involves 3 steps:
1. the initial phase (without coloration or odor), in which a reducing sugar is nucleophilically bound to the carbonyl function by an amine compound (imine formation). This so-called Schiff base can be rearranged via 1,2-eneaminols into an aminoketone (Amadori compound) or an aminoaldose (Heyns compound).
2. Intermediate phase with development of reaction intermediates (with slight initial coloration and considerable aroma). The main products of this phase include furan compounds such as HMF (or hydroxymethylfurfural), which has a low molecular weight and absorbs in the UV; Strecker aldehydes, which are aromatic molecules and in turn precursors of many aromatic compounds whose reaction mechanism starts with oxidative decarboxylation and deamination of amino acids by an α-dicarbonyl intermediate, e.g. deoxyosone, pyruvaldehyde formed by sugars
3. The final stage involves reactions such as aldol condensation and aldehyde-amine condensation and leads to the formation of melanoidins by condensation of intermediates via several pathways. The result is a dark brown to black color.
In addition to the Maillard reaction, heat-induced degradation of sugars can occur via another reaction pathway, caramelization, in which the major markers α-dicarbonyl compounds, especially deoxyosones, are formed by enolization and dehydration of sugars but without the participation of amine groups (Belitz *et al.*, 2009). These are the same intermediates discussed for the Maillard reaction. Therefore, the hypothesis of this study is that the introduction of hydrolyzed proteins involves a change in the chemical composition of the processed ingredients and may affect the reactivity of the food and thus the quality. Therefore, the objective of this study was to measure the above-mentioned main characteristics of texture and the reactivity of VOCs produced both during the whipping of batters and during baking.

4. Experimental procedure

In this part of the thesis, an experimental procedure has been developed, which includes two main steps.

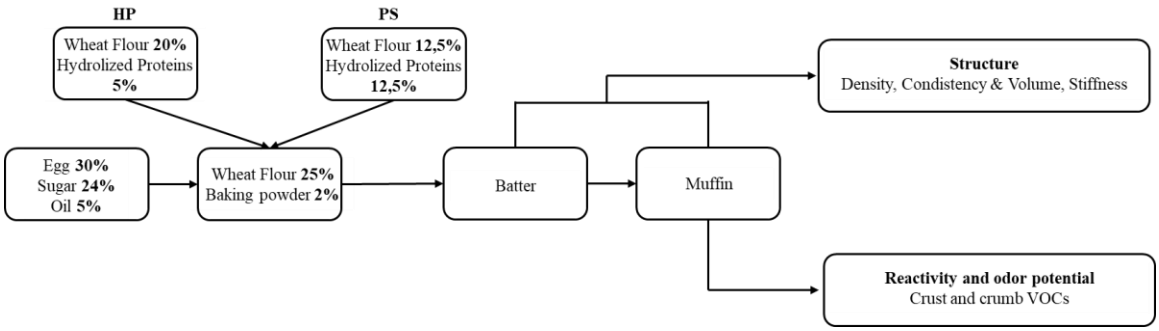

Figure 1 *Formulation and analysis strategy of study.*

1. in the first phase, batters and muffins enriched to 0, 5 and 12.5% by weight with different protein hydrolysates (casein, soybean, rice and pea) were prepared and then the physical properties were measured. In particular, the pH, dry matter, density and consistency of the batter were determined, while for the muffins the volume, density, textural properties and dry matter were measured.
2. In the second step, the complex profile of the volatile compounds (including odor and reaction markers) were characterized. The mains compounds analyzed in different compartments of the muffins (crust and crumb) to account for the different advancement degree of the thermal reactions occurring during baking.

5. Material and Method

In the first phase of the experimental design, the moisture content of both the batters and the muffins was measured in triplicate by differential weighing before and after drying at 105 °C for 24 hours. Batter density was determined in triplicate by slowly drawing batter into a 10-mL open-mouth syringe and calculating the ratio between the mass of the batter and the volume of the syringe previously defined with water. Batter consistency was determined using a TAHD+ texture analyzer with a 5-kg load cell (Stable Micro Systems, Surrey, UK) filled with 40 mL of batter at a speed of 2 mm/s until a distance of 20 mm was reached. The consistency was obtained as the average force calculated at the plateau of the [N]/[mm] curve. Muffin density was determined by calculating the mass-to-volume ratio. This latter was determined using a laser scanner (VolScan Profiler, Stable Micro Systems, Surrey, UK). These two measurements provide information on the amount of air retained during baking. Finally, the mechanical properties of the aerated muffin crumbs were analyzed by uniaxial compression test with a TAHD+ TEXTURE ANALYZER equipped with a 5 kg load cell (Stable Micro Systems, Surrey, UK). Muffin slices measuring 3 cm x 3 cm x 3 cm were compressed to 81% (maximum compression) of their original height at a rate of 2 mm/s using a 10 cm diameter aluminum probe. The crumb stiffness was expressed as Young's modulus corresponding to the slopes of the stress-strain curves during compression from 1% to 6%. Finally, volatile compounds were analyzed in triplicate by Head-Space Solid Phase Microextraction (HS-SPME) coupled to GCMS on 2 g of the ground and thawed muffin samples poured into 20mL transparent vials. To each sample two deuterated internal standards (furfural-d4 and 2-methylpyrazine-d6) were added at a final concentration of 20 mg/L to allow the quantification of the VOCs. Extraction parameters were as follow: DVB/CAR/PDMS fibre (50/30 µm, 2 cm, Supelco) incubation (14 min) and extraction (40 min) at 50 °C. The volatiles were separated on a DBwax capillary column (30 m×0.25 mm× 0.5 µm, Phenomenex, Aschaffenburg, Germany) using helium as the carrier gas (1.2 mL/min). The initial oven temperature of 40 °C (held for 40 min) was increased to 240 °C at 4 °C/min. Mass spectra were recorded in electron impact ionization mode at 70 eV using an ISQ single quadrupole mass spectrometer (Thermo Fischer Scientific). Data were acquired in both full scan (TIC, m/z 33-300) and SIM mode using specific m/z values for selected compounds.

6. Results and Discussion

6.1 Physical properties

Table 1 *Batter and Muffin characteristics. Different letters within a column indicate significantly different averages (p<0.05). Control = control formula, HP = high protein (12,5%) replacement level, PS = protein source (5%) replacement level in the formulas, Ca= Casein*

	Batter			Muffin			
	Density (g/cm^3)	**Dry matter (%)**	**Consistency (N)**	**Volume (cm3)**	**Density (g/cm^3)**	**Dry matter (%)**	**Stiffness crumb (Pa)**
Control	$0,89 \pm 0,02^{d}$	$93,95 \pm 0,04^{b,c}$	$0,10 \pm 0,00^{b,c}$	$92,83 \pm 0,76^{a,b}$	$0,31 \pm 0,00^{d,e}$	$97,39 \pm 0,07^{c}$	$21,33 \pm 0,006^{a}$
HP-Ca	$0,76 \pm 0,02^{d}$	$94,12 \pm 0,04^{b}$	$0,03 \pm 0,00^{d}$	$91,13 \pm 1,81^{b}$	$0,31 \pm 0,00^{c,d}$	$98,27 \pm 0,04^{a}$	$2,46 \pm 0,001^{b}$
HP-Rice	$1,05 \pm 0,01^{a}$	$92,13 \pm 0,03^{d}$	$0,08 \pm 0,01^{b,c}$	$82,03 \pm 2,05^{c}$	$0,35 \pm 0,01^{a}$	$97,63 \pm 0,07^{b}$	$6.71 \pm 0,008^{b}$
HP-Pea	$0,53 \pm 0,01^{d}$	$93,9 \pm 0,04^{a}$	$1,20 \pm 0,04^{a}$	$76,20 \pm 1,61^{d}$	$0,33 \pm 0,01^{b}$	$98,29 \pm 0,015^{a}$	$7,69 \pm 0,002^{b}$
HP-Soy	$0,96 \pm 0,01^{b,c}$	$93,27 \pm 0,03^{g}$	$0,11 \pm 0,00^{b,c}$	$83,73 \pm 0,75^{c}$	$0,33 \pm 0,00^{b}$	$97,71 \pm 0,05^{b}$	$2,82 \pm 0,000^{b}$
PS-Ca	$0,93 \pm 0,01^{c}$	$94,64 \pm 0,02^{e}$	$0,06 \pm 0,01^{c,d}$	$96,23 \pm 1,50^{a}$	$0,30 \pm 0,00^{d,e}$	$96,18 \pm 0,07^{d}$	$3,43 \pm 0,002^{b}$
PS-Rice	$0,98 \pm 0,01^{b}$	$93,95 \pm 0,04^{c,d}$	$0,08 \pm 0,00^{b,c}$	$91,20 \pm 2,52^{b}$	$0,32 \pm 0,01^{b,c}$	$95,94 \pm 0,03^{e}$	$2,64 \pm 0,001^{b}$
PS-Pea	$0,97 \pm 0,01^{b}$	$91,9 \pm 0,03^{d}$	$0,15 \pm 0,00^{b}$	$95,30 \pm 1,00^{a,b}$	$0,31 \pm 0,01^{c,d}$	$96,08 \pm 0,02^{d,e}$	$2,10 \pm 0,000^{b}$
PS-Soy	$0,96 \pm 0,00^{b,c}$	$92,13 \pm 0,03^{f}$	$0,08 \pm 0,00^{b,c}$	$96,17 \pm 1,19^{a}$	$0,30 \pm 0,00^{c,d,e}$	$95,48 \pm 0,06^{f}$	$2,51 \pm 0,001^{b}$

As mentioned above, muffin batter is considered a liquid foam consisting of a continuous aqueous phase containing proteins and in which lipids are emulsified, and a dispersed phase containing gas cells and starch granules. Therefore, texture is closely related to the physico-chemical properties of the batter and in particular those of proteins and starch. For this reason, it was particularly important to study the changes in the rheological properties of the batters and muffins as a function of the degree of flour replacement by different protein hydrolysates, provided that no other process changes were made. Table 1 shows the changes in chemical and physical properties caused by the introduction of the different protein hydrolysates at different percentages of substitution (5%= PS; 12.5%= HP) and relatively to the control (0%). The density of the batter is a very important parameter together to

the consistency of the batter and the volume of the muffins after baking. In general, the introduction of protein hydrolysates into the formulations increased the batter density for the PS and HP series, only in the case of HP-Casein and HP-Pea the density was significantly lower ($p < 0.05$) than in the Control and in all other formulations. The lowest density measured was that of HP-Pea with 0.53 g/cm^3. Regarding batter consistency, only HP-Casein and HP Pea differed significantly ($p < 0.05$). In particular, the consistency of HP-Casein decreased significantly, while the consistency of HP Pea was the highest (1.20 N). On the other hand, muffin density and volume, as well as crumb stiffness, are important parameters and indicators of the structural quality of muffins after baking. In this study, muffin density did not show the same large differences as dough density, which is especially important for HP prototypes with lower starch content, since gelatinization is important for crumb structure (Gómez *et al.*, 2012). In contrast, PS prototypes were significantly equal to the control in the volume parameter ($p > 0.05$). However, an opposite effect was observed for HP prototypes, except for HP-Casein, which were significantly lower ($p < 0.05$), especially HP-pea with 76.20 cm^3. Correlation analysis showed that batter density was negatively correlated with both batter consistency (Pearson correlation $p < 0.01$) and muffin volume (Pearson correlation $p < 0.05$), indicating a strong influence of both the different protein matrices introduced and the degree of flour substitution. Finally, crumb stiffness was not correlated with any of the described parameters and showed only a significant difference ($p < 0.05$) between the protein-enriched samples and the control

6.2 Reactivity and odor potential

Figure 2 shows an overview of the VOCs profiles extracted from muffin crusts (where thermal reactions are the most advanced). It is worth noting that the muffins containing hydrolysates are in general very different from the control in term of nature and amount of volatiles. The VOCs profiles of these muffins were particularly rich in markers of the Maillard and caramelization reactions. This confirms that baking triggers these reactions especially in the formulas containing hydrolysates, most probably for the higher content of active precursors (peptides and free amino acids) Figure 2, highlighted zone). Specifically, 77 volatile organic compounds were identified in crust and the crumb of the prepared samples, in the crumbs the profiles being different in terms of relative amounts (data not shown).

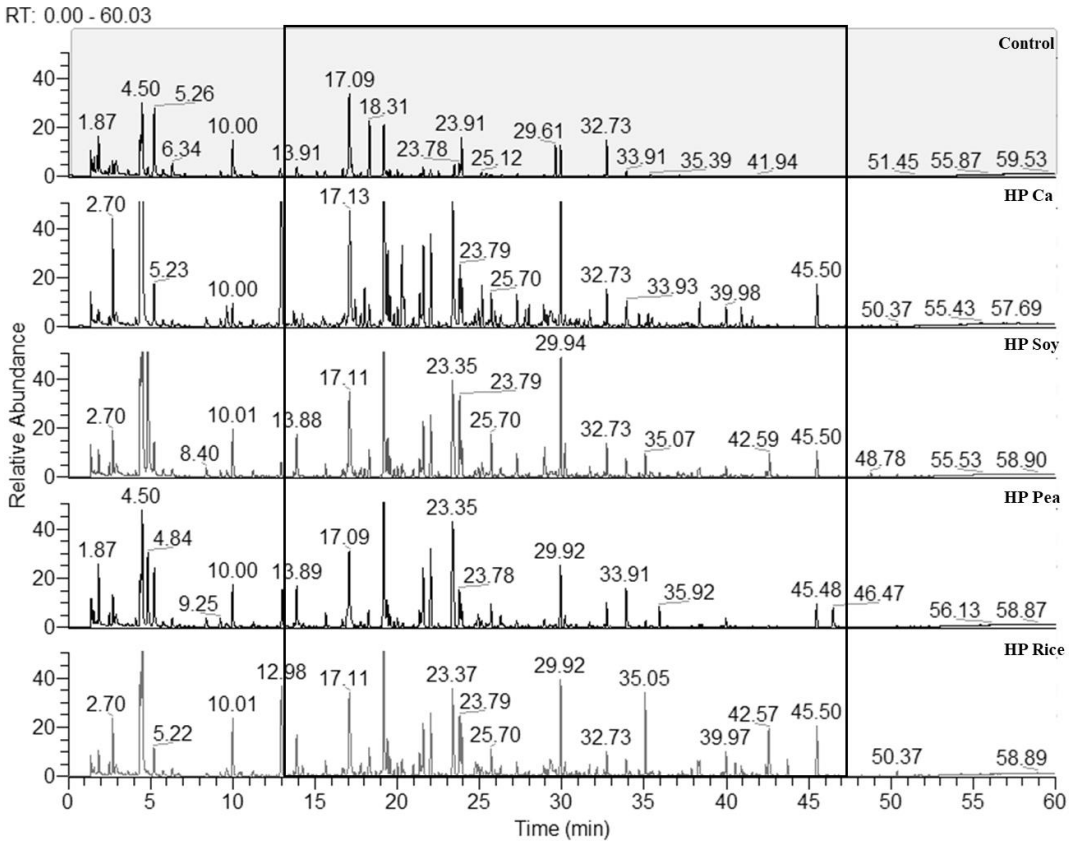

Figure 2 *HS-SPME-GCMS profiles of the volatiles compounds presents in the muffin crust: control (top), HP-rice and HP-pea (bottom), recorded in total ion current mode (TIC). In the square is highlighted the pyrazine and furanic compounds zone.*

The formation of pyrazines is largely explained by a condensation reaction between two α-aminocarbonyl compounds, which in turn are formed together with Strecker aldehydes by Strecker degradation from the reaction between α-dicarbonyl compounds and amino acids (Belitz *et al.*, 2009). These heterocyclic compounds are known for their roasty, nutty, and earthy odours and are very important contributors for the aroma of bakery products as

they have a very low odor-threshold value. In this study, the identified structures ranged from simple pyrazine to its single- or multiple-alkyl-substituted or amidated form. Among the 9 pyrazine compounds identified, the relative abundance of these compounds appeared to be the same in the different hydrolysate-based formulations. The main differences between HP-Pea and all other formulations were a higher occurrence of 2-methylpyrazine (RT: 17.20) and 2,5-ethylpyrazine (RT: 20.04 (hazelnut, cocoa powder odors). On the other side 2,6-dimethylpyrazine (RT: 19.42, roasted hazelnut, roasted meat odors) seems to be more abundant in HP-Casein. Strecker aldehydes are other very important odor active compounds. With a distinct malt and sweet aroma. Among them, 2-methylbutanal (RT: 4.44) and 3-methylbutanal (RT: 4.56), which are associated with oxidative decarboxylation of isoleucine and leucine (Belitz et al., 2009) were the most intense. Among the formulations tested, HP-Pea had the highest levels of these compounds. Other markers for Maillard and caramelization reactions include ketones: e.g., 2,3-pentanedione (sweet, buttery, fresh yoghurt), which was most abundant in the HP- Casein sample, 1-hydroxy butan-2-one (sweet, coffee, pyrazine), and butyrolactone (oily, creamy), which were most abundant in HP-Casein, followed by HP- Soybean and HP-Pea. In addition to ketones, several other products of the browning reaction were detected, including furanic compounds such as furfural (RT: 23.82), 5-methylfurfural (RT: 27.35), 2-furan methanol (RT: 30.00), and 5-(hydroxymethyl)furfural (RT: 50.41). These compounds were always found at higher concentrations in the hydrolytate-containing muffins than in the Control. Specifically, HP-Pea had the highest level of 5-methylfurfural (sweet, caramel, bread) but the lowest level of 5-(hydroxymethyl)furfural (herbal hay tobacco) compared to the other formulations. Other compounds identified include alcohols, e.g. 1-hexanol and pentanoic acid and lipid oxidation markers e.g., octanal (smells of citrus, floral, fatty, and soap) from the oxidation of oleic acid (C18:1), hexanal (smell green, herbaceous, leafy), 1-pentanol (tallowy, balsamic, fruity, sweet, similar to spindle), and 2-octental (hazelnut-like, greasy, and toasted) from the oxidation of linoleic acid (C18:2). Finally, the same analyses were performed on the crumb of each formulation, and all previously identified molecules were found. However, in all cases, the amounts were lower than for the crust samples, which could be explained by the slower advancement of the thermal reactions in the crumb (temperature up to 100°C and higher moisture content).

7. Conclusions

The addition of various protein hydrolysates to the formula of muffins, in partial replacement of wheat, resulted in interesting organoleptic and structural properties. From a physicochemical and structural point of view, the greatest differences in batter density and consistency, as well as in muffin volume after baking, were observed in the HP prototypes. In particular, HP-Pea showed the greatest discrepancy between these parameters, suggesting that in the formulation the type of hydrolysate chosen has as much influence as the degree of addition. In contrast, the analysis of the volatile profiles showed that the hydrolysate-containing muffins are particularly rich in thermal reaction markers, mostly originating from the Maillard reaction and caramelization, which are initiated during the baking of the batters. The largest amounts of these markers were detected in the crust of products with high protein content (HP) relatively to the Control and the PS samples. For these compounds, both the source of the protein hydrolysate (i.e. cereal, legumes, animal source) and the level of addition to the formula had an evident impact. Based on the odor descriptors reported in the literature, it is expected that the products containing hydrolysates could have a distinctive odor from the control and from each other (because of the different relative proportions of active aroma compounds present in the different prototypes). As an example, HP-Pea is particularly rich in compounds with pleasant aroma (e.g. Pyrazines). However, further sensory studies need to be conducted to better understand if these differences are really perceived and in which manner. In addition, for completeness, it would be necessary to identify the volatile compounds present in the distinctive row materials (hydrolysate powders) as well as in the batters to better understand the origin and the evolution of the reaction markers at each step of the processing.

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Adding unprecedent economic and social values to the side- and by-products of Mediterranean fruit and vegetables by reshaping them in novel source of nutrients and tailored food products mediated by 3D printing technology

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This PhD thesis is dedicated to the recovery and reshape of not edible parts and/or by-products of the Mediterranean fruits and vegetables as novel source of nutrients. The raw materials – from different fruit and vegetables, have been used to produce dried powders with modulated granulometry, chemical and physical properties. Such food powders have been used as nutritional and structuring ingredients to create innovate food products personalized for nutritional and sensory properties mediate by 3D Food printing technology. With this aim, 3D digital models have been created by using several CAD software aiming to optimize the printing movements. The drying curve and isotherm have been mathematically modelled. The particle size of the dried powder will be optimized to create printable food formulas intended for innovative 3D printed food. After determining the nutritional value and appropriate conditions for the 3D printer nozzle, the food powder will be mixed with different gels and made suitable for printing with the 3D printer.

Valorizzazione economica e sociale di scarti e sottoprodotti di origine vegetale dell'area del Mediterraneo attraverso il loro riutilizzo come nuove fonti di nutrienti per la produzione di alimenti personalizzati mediante l'impiego della tecnologia di 3D Food Printing

Le attività sperimentali sono dedicate alla valorizzazione degli scarti e/o sottoprodotti della filiera di produzione di alimenti ortofrutticoli attraverso il loro riutilizzo come ingredienti ricchi di nutrienti. In particolare, gli scarti sono stati impiegati per la produzione di polveri disidratate da usare per la produzione di innovativi prodotti alimentari personalizzati ottenuti mediante tecnologia di stampa 3D.

1. Introduction

3D Food Printing, 3DFP, is the only technique capable to translate digital images into tangible food products and this opens the way for innovative and intricated shapes and dimensions maximizing food's eye appeal, helping in differentiating or identifying food products, and improving the overall enjoyment of food consumption. However, 3DFP is well beyond the creation of food with fascinating visual aspects, with many additional benefits on the food chain, nutritional/healthy and sensory properties, satiety, consumer's behavior, and sustainability (Oral et al., 2021). For instance, personalized 3D printed food considers the production of foods with desired sensorial and nutritional properties offering many solutions to contribute to the better health status of consumers. In addition, at its core 3DFP has the process of dispensing/depositing small amounts of food material per unit of time offering the opportunity of dosing each ingredient with high accuracy to modulate the content of macro- and micronutrients according to the needs of each individual or small group of consumers. Finally, though, 3DP supports the decentralization of food manufacturing and the consumer-centric system of production allowing the manufacture of products close to the final customer (Gao et al., 2015; Chan et al., 2018). By reducing the tight dependence on the supply chain, it would be possible to increase the overall sustainability of the food system with a significant reduction in energy consumption and gas emission generated by transportation, and the amount of food waste or food loss at industrial or home level (Derossi et al. 2021a). Under this scenario, the re-use of by-products from the production of fruit and vegetables, after a first step of transition toward innovative powders

2. Experimental procedures

The external, not edible, leaves (for artichokes) and the skin (for eggplant and tomato) which are usually discarded as waste product, were employed for the drying treatments. Dehydration was performed separately for artichokes, eggplants and tomatoes at temperatures of 30°C, 40°C and 50°C. In the first 30 minutes of the drying process, three replicates were analyzed every 15 minutes. After the first 30 min, different sampling times have been considered keeping the total number of samples greater than 10 along till 7 h of dehydration. At each sampling time, weight changes, moisture content (AOAC, 2005) and water activity have been measured by water activity-meter (Decagon device). The obtained data were modelled by using the Page's model (Eq.1), by plotting the moisture ratio (MR) as a function time dehydration time. This allowed to reduce the effect of the variance of moisture content for fresh food (Jangam et al., 2010).

3. Material and methods

3.1 Raw materials

The raw materials – artichokes, eggplants and tomatoes - have been chosen among the most consumed of fruit and vegetables in Mediterranean Area as well as among those that more than others suffer from loss due to surplus-crops, unmarketable products, high amount of side- and by-products. The first list of potential raw food materials has been reviewed also by considering the nutritional and functional content of the whole products and their side- and by-products.

$$MR = \exp(-k*t) \quad \text{(Eq.1)}$$

Where MR is the dimensionless moisture ratio (Eq.2), Mt is moisture content at any time of drying (kg water/kg w.b matter), Mi is the initial moisture content (kg water/kg w.b) and Me is the equilibrium moisture content (kg water/ kg w.b. matter).

$$MR = (Mt-Me)/(Mi-Me) \quad \text{(Eq.2)}$$

Also, *k* (1/min) is the rate constants, *t* (min) and *n* is a fitting parameter (dimensionless). Furthermore, the moisture content as a function of water activity was modelled by using the GAB equation (Eq.3).

$$EMC = \frac{x_0 * C * K * a_w}{(1 - k * a_w) * (1 + (c-1) * k * a_w} \quad \textit{Eq. 3}$$

Where, X_0 is the moisture content in the monolayer, *C* is Guggenheim constant and *K* is Related to the heat of adsorption in the multilayer (Maroulis et al.,1988; Nguyen et al., 2020) . All fitting procedures have been performed by using the packages of STATISTICA.

3.2 Food powders and food-ink preparation

After dehydration the by-products from artichokes, eggplant and tomatoes have been submitted to grinding process by using semi-professional coffee grinder that allows to modulate the particle size. More specifically three different level of grinding have been used – fine, medium, and coarse – and the obtained powders have been studied by analysing the granulometric curve (Analysette 22, Fritsh) and by exploring the morphological properties of the solid particle after acquiring 2D X-ray microtomographic images (Skyscanner 1174, Bruker).
Food-inks have intended as new food formulas obtained in different modalities and served as two different levels of complexities: 1. Food-inks based on hydrocolloids enriched with innovative food powders; 2. Cereal-based food formula enriched with the novel food powders.

3.3 3D Computer Aided Design for printing movements optimization

Different procedures have been used to generate the G-codes aiming to optimize the printing movements avoiding possible inaccuracies of the food structure. More specifically, we used three different technical option for creating the 3D digital model and for the slicing step that allow to define the printing movements: 1. Tinkercad-CURA; 2. Rhinoceros-Grasshoper; 3. Full control G-code.

3.4 Chemical and physical analyses

3D printed structure were analyzed for their nutritional content, texture, microstructure properties, printability, and mastication work. All the analyses were performed by following the main international methodologies (Severini et al., 2016; Derossi et al., 2020; Derossi et al., 2022)

4. Results and Discussion

4.1. Dehydration of waste products and modelling

Dehydration curves well modelled by the Page's model which satisfactory fitted the experimental data obtaining correlation coefficients, r, greater than 0.98 for all the drying temperatures and for any kind of vegetables. Figure 1 shows representative images of the dehydration curve and the desorption isotherm for external leaves of artichokes submitted air-drying at 50°C for 7 h.
Also, after the mathematical description of the drying process, another series of experiments have been performed aiming to validate the estimated results. To do this, the drying times necessary to obtain a desired moisture content of 5 % and a water activity less than 0.4 has been estimated by using the results of table 1. Then, a new series of dehydration experiments were performed proving the high robustness of the mathematical modelling that allowed to obtain only slight discrepancies between estimated and experimental data (data not shown).

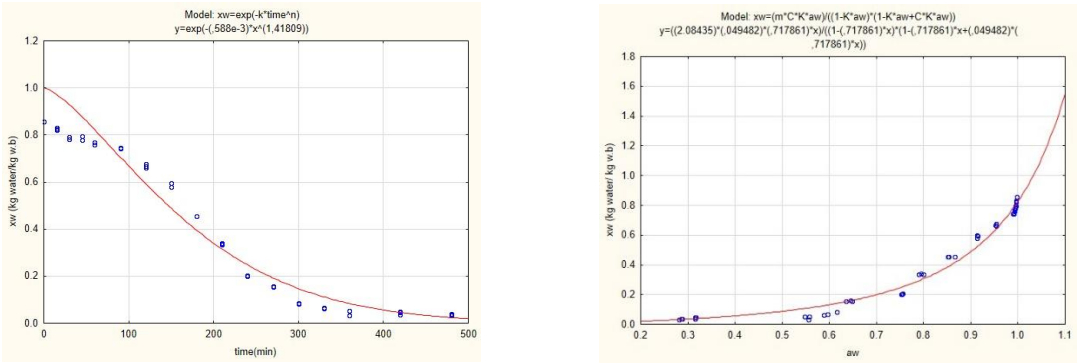

Figure 1 – Dehydration and isotherm curves for external not-edible artichokes leaves.

Table 1 – Estimated parameters regarding the dehydration treatment of external leaves, no edible, of artichokes.

Parameters	*Estimates*	*Standard error*	*p-value*	r^2
Dehydration temperature = 40°C				*0.985*
k (min^{-1})	*0.000588*	*0.00028*	*<0.01*	
n (dimensionless)	*1.41*	*0.0916*	*<0.01*	
Dehydration temperature = 50°C				*0.992*
k (min^{-1})	*0.000587*	*0.000270*	*<0.01*	
n(dimensionless)	*1.63*	*0.1014*	*<0.01*	
Dehydration temperature = 60°C				*0.992*
k (min^{-1})	*0.000551*	*0.000356*	*<0.01*	
n(dimensionless)	*1.83*	*0.161*	*<0.01*	

4.2 Improved accuracy of the printing movements

At first, ink-gels from different hydrocolloids (starch, pectin, xantan gum, etc.) have been used to study and optimize the printing movement of the 3D printer. The printing conditions such as printing speed, flow, layer height, nozzle size, retraction, retraction speed, etc., have been studied to avoid discrepancies between the 3D digital model and the edible structures. Figure 2 shows the digital models and the printed structures for two level of complexities. For the first time, complex structures have been created by using mathematical equations that can be merged to get unprecedented geometries. The figure proves the high accuracy of the printed model obtained by using the software Gcode full control that opens for the generation of new food product never thought before.

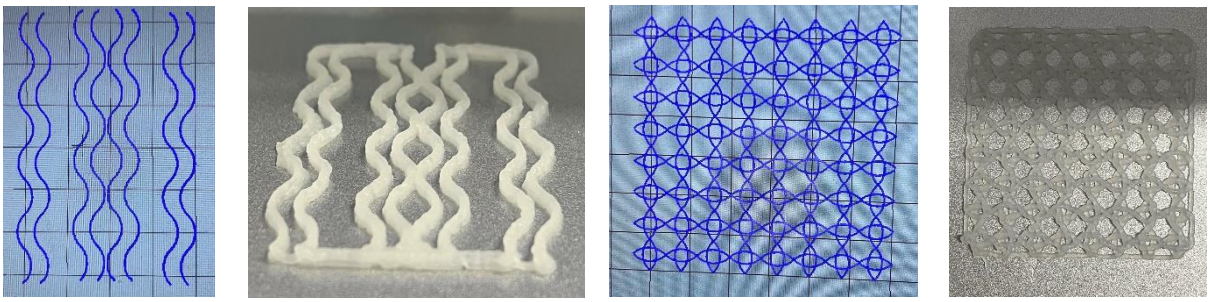

Figure 2 – Comparison between the 3D virtual models and printed structures obtained by using the Full control G-code.

4.3 Effect of 3D printing structure on baking time

Figure 3a shows different 3D models of the innovative food structures. It is worth to note that by modifying the infill level it was possible to reduce the baking time as proved by the rate constants, k, estimated by fitting the moisture content as a function of baking time. This prove that 3D Printing can be employed as prototyping method to define the best internal and external morphological features to reduce the time in line with the principles of a more sustainable food sectors.

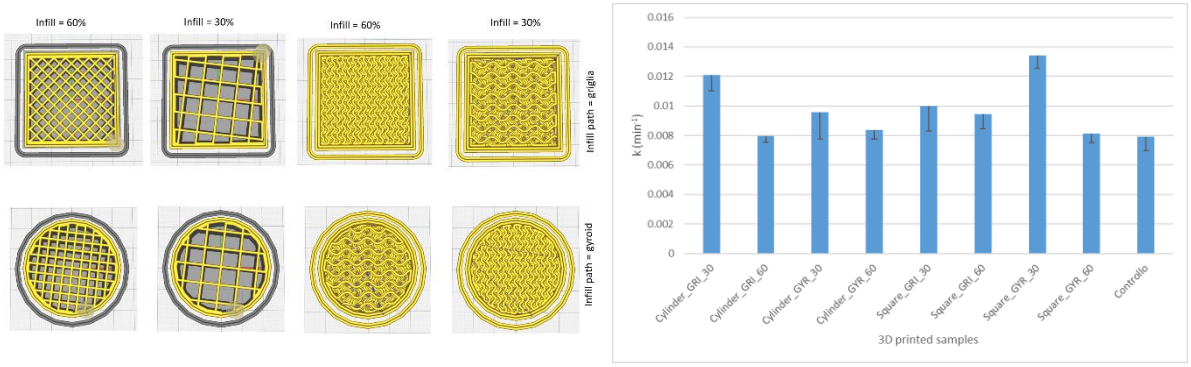

Figure 3 – Digital models obtained through the slicing software Cura by modifying the infill density for the parallel piped and cylinder geometric shape.

5. Conclusions, and future perspectives

The obtained results at the moment of writing this paper, show the potentials of 3D food printing for creating innovative food products, personalized for their sensory and nutritional properties. Creating innovative food formulas from waste food will help human beings to be efficient for planet earth and for daily nutritional requirements. For the first time, innovative methods to generate 3D virtual models and to control the printing movements have been explored. This software allows the creation of unprecedented different modelling structures avoiding the non-printing movements and creating a continuous printing path. These abilities made 3D printer more efficient and consumed less energy. Because using the ability of a 3D food printer, we are able to create different shapes and that will help with mastication problems. Due to the COVID-19 pandemic some research activities and analyses currently are being performing at the moment of writing this paper. The preliminary results shows that the food powders from waste and by-products of artichokes, eggplants and tomatoes allow not only to improve the printability and the structural stability of the 3D printed structures by extending the opportunity to create innovative food products with personalized/desired sensory properties, but also can increase the nutritional content of the final products.

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Functional food and *per os* microbial delivery system with a potential role in the prevention of diseases related to the human intestinal microbiota

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This PhD thesis concerned the study of a functional food enriched in components typically present in a Mediterranean diet (MD) and a *per os* delivery-system for microorganisms, respectively aimed to reduce the comorbidities in some diet-related diseases and to treat diseases having as background intestinal microbiota dysbiosis.

Alimenti funzionali e sistemi microbici di *delivery per os* con un potenziale ruolo nella prevenzione delle malattie connesse al microbiota intestinale umano

Il presente progetto di dottorato ha riguardato lo studio di un alimento funzionale arricchito in componenti tipicamente presenti nella dieta mediterranea (MD) e di un sistema *per os* per il transito di microrganismi fino al tratto intestinale, rispettivamente con l'obiettivo di ridurre la comorbidità in alcune patologie legate alla dieta e per il trattamento di malattie legate al microbiota intestinale umano.

Key words: Food microbiology; microbial ecology; functional foods; gut microbiota; microencapsulation; biosilica; gastrointestinal resistance; fecal microbiota transplantation (FMT).

1. Introduction

In accordance with the above-described PhD thesis project, this oral communication concerned the main results of the following four activities:

A1) administration of a food survey to evaluate the eating habits and the adherence to the MD in people aged between 30–60 years aiming to enroll 40 overweight healthy subjects located in Apulian region (Italy), suitable for a dietary intervention
A2) study and evaluation of the effect of the food intervention on the eating habits, anthropometric and biochemical parameters of the enrolled subjects
A3) set up of a microbeads silica-based system to allow the delivery at colon level of a fecal slurry
A4) evaluation of heat tolerance of fecal microbiota encapsulated into microbeads.

2. Pivotal role of Mediterranean diet in microbiota modulation and disease prevention

The maintenance of a well-being status in humans and the prevention of diseases associated to unbalanced eating habits, is strongly affected by macro- and micronutrient intakes. Among the dietary lifestyle, mostly widespread, Western diet is characterized by a high consumption of red and processed meat, refined cereals and sugars, and foods rich in saturated fats. Gut microbiota reflects this unhealthy food consumption through a reduction of the microbial diversity, loss of some bacterial patterns mainly having a health promoting role associated to an increase of pathogenic microorganisms, such as *Escherichia–Shigella*, as well as an increased abundance of not-beneficial *Clostridium* and *Bacteroides* (Marlow et al, 2013).
On the other hand, MD is characterized by a high daily intake of fruit and vegetables, extra virgin olive oil, unrefined cereals, a moderate consumption of fish and a low consumption of meat and dairy products. Some studies observed that the adoption of these healthy eating habits leads to a reduction of species belonging to Proteobacteria and to the family of *Bacillaceae*, known to contribute in ongoing dysbiosis status as well as inflammation (Rajilić-Stojanović et al. 2009; Marlow et al, 2013). In addition, subjects following a MD regimen, showed a higher faecal concentration of short-chain fatty acids and higher presence of bacterial species fermenting fibres than those with a low adherence to MD (De Filippis et al., 2016).
UNESCO recognizes MD as an intangible cultural heritage, which is beneficial for the prevention and treatments of many chronic diseases (Saul et al., 2012) and, in this way, microbiome-targeted dietary interventions based on MD constitutes a powerful tool for the prevention and treatment of different diseases (De Filippis et al., 2018). The use of well-defined dietary supplements represents a great opportunity to study the effect of specific dietary components on gut microbiota and its activities, and a way to investigate the possibility to integrate the diet with tailored nutrients able to ameliorate basic health parameters.

3. Potential of *per os* formulation biosilica based to modulate gut microbiota composition

The symbiotic crosstalk between host and gut microbiota can be altered by different factors such as antibiotic

exposures, diet, lifestyles, and many pathological states. Intestinal dysbiosis have been associated to gastrointestinal tract syndromes (Crohn's disease ulcerative colitis, irritable bowel syndrome) and syndromes related to other somatic districts such as diabetes, obesity, rheumatoid arthritis, nephropathy, metabolic syndrome, and *Clostridium difficile* infections (CDI) (Cénita et al., 2014). An impaired gut microbiota can be treated by probiotics as both preventive or curative purposes. However, among the most effective therapies in eradicating such pathologies there is the fecal microbiota transplantation (FMT). FMT defines the transferring process of fecal material, contained viable microbial cells of gut microbiota, from a healthy donor to a patient.
In this way it is possible to restore the microbial diversity and related functions at colon level. Currently, FMT consists in the infusion of liquid filtrated feces only in CDI patients even though it represents an emerging tool to treat a wide range of disorders. For the administration of fecal suspension, nasogastric or nasoduodenal tube, colonoscopy or enema are already used (Choi HH et al. 2016). Based on this background, the development/evaluation of a *per os* formulation able to deliver a fecal slurry was carried forward this PhD thesis.
Among the main challenges aiming to microorganism administration there is the gastrointestinal (GI) resistance, whether they have to be administrated by food or by drug formulation. In addition, probiotics marketability requires preserving the viability also during the storage processing and against thermal shock events.
Several protection systems have been developed to preserve the viability of microorganisms during digestion and long/short-term of storage, improving their delivery. Among innovative strategies, the encapsulation provides protection against adverse stressors. Usually, the encapsulation took into account the production of deposition layers or entrapping macro-matrices containing and protecting bacteria from the external environment (Rokka et al., 2015). Materials exploited for encapsulation undergo specific manufacture procedures (nuzzling extrusion, coacervation, bead fluidification, electro-spraying, spray-chilling) to produce micro- to millimeter bead-like shapes which physically entrap the microorganisms. However, these methods are time- and money-consuming. The promising material of biosilica extracted from *Coscinodiscus granii* diatom species produces a semi-spherical or molten globule containing structures able to contain cells. Subsequently, biosilica microcapsule structures can be also surrounded by carrier matrices with gastro-enteric protecting properties, such as polysaccharides or complex natural biopolymers (e.g., alginate, carrageenan, or chitosan). These innovative co-composed microencapsulation systems have an interesting potential to develop less invasive therapies for the fecal microbiota transplantation (FMT).

4. Materials and methods

4.1. Study design, volunteer enrolment, and dietary intervention

40 volunteers were enrolled after the administration of a Food-Frequency Questionnaire (FFQ) to assess the MD adherence score. Only volunteers with an adherence score less than or equal to 5 (Benítez-Arciniega et al., 2011), and a BMI between 25–29.9 Kg/m^2 were selected for this study. The dietary intervention was a 8-week, two-arms, double-blind, placebo-controlled, parallel study. Each subject followed some dietary instructions for a 2-week run-in period, in order to stabilize starting conditions. Subsequently, volunteers were equally (for age and gender) randomized in two groups. The control group consumed the placebo, while the intervention group introduced in the own daily diet a functional food enriched in MD components food products (Tab. 1). Energy intake (kcal/die) was comparable between treatments. The dietary compliance was assessed by self-recorded food diary covering the three days prior to the beginning of the trial and the three final days.
At the beginning and at the end of the food intervention (T0 and T8, respectively), the participants underwent to blood analyses (metabolic, inflammatory, oxidative status, total cholesterol, cholesterol, LDL, insulin, triglycerides, AST, ALT, GAMMA GT), and anthropometric measurements (height, weight, plica thickness, waist circumference). This further profiling was collected to study the potential role of this specific dietary intervention in ameliorating healthy status.

Table 1 *Nutritional analysis for 100g of functional food with the following ratio among the ingredients: (3) pomegranate, (3) walnuts, (2) moringa, and (2) broccoli.*

Compounds	%
Humidity	58.2 ± 1.8
Total fat	21.3 ± 0.64
Total saturated fatty acids	2.25 ± 0.23
Carbohydrates	4.5 ±1.4
Total dietary fibres	6.4 ± 2.2
Protein	8.4 ± 1.0
Total phenolic compounds	0,32 ± 0,01

4.2. Food diaries analysis

Food diary filled by volunteers were analysed using the WinFood® sofware. This nutritional software allows us to record the food consumption and nutritional status based on the Italian Food Composition Databases. The

consumption of 90 compounds among macronutrients and micronutrients was evaluated for each volunteer at T0 and T8. For each nutrient, the average of the consumption values related to the prior three days and the last three days was calculated. In addition, to make as complete as dietary anamnesis, daily caloric intake was also evaluated.

4.2.1. Experimental procedure for microorganisms microencapsulated test in stress conditions

In this PhD thesis, a four-step experimental procedure, aiming to evaluate efficiency of biosilica-based coating to preserve cell viability towards gastrointestinal digestion and stress tolerance test, was set up. This was carried out by performing in sequence the following independent steps: i) application of the density gradient for the isolation of the fecal microbiota as previously described by Hevia et al., 2015; ii) microencapsulation protocol based on three vacuum pump cycles in order to promoting the cells entry in biosilica shells; iii) gastrointestinal simulation assay (Fernandéz et al., 2013); and iv) viability evaluation of microbial cells by plate count method.

4.2.2. Gastrointestinal digestion, intestinal delivery, and microbial cell vitality evaluation

To characterize the biosilica materials and to evaluate its potential to be used as pharmaceutical formulations and its heat tolerance, expriments were performed at the following conditions: i) 50°C for 30 min; ii) 55°C for 3 min.

5. Results and Discussion

5.1 Nutritional evaluation of dietary intervention on average dietary intake

The nutritional evaluation (average daily energy and nutrient intake) allowed us to determine the effect of MD-functional food consumption on nutritional profiles of volunteers. Out of the 90 nutrients that were evaluated by using WinFood®, 12 showed a significant difference ($p < 0.05$) between T0 and T8 (Tab. 2). In particular, the daily intake of vegetal fibers and vegetal proteins resulted to be higher at T8 in the intervention group. In addition, the vitamin C intake was significantly higher in treated subjects at the end food treatment than baseline values. The reduced level of vitamin B6 (biotin) in the diet of intervention group could be justified by the fact that this vitamin of group B is more present in animal-derivate food (e.g., milk, cheese, egg yolk) than vegetables (Zempleni et al., 1999). Moreover, the intervention group showed a reduced consumption of animal proteins during the trial. Notably, the functional food administration influenced the calories intake based on the result that subjects belonging to the placebo arm showed a significant increase ($p < 0.05$) of the average caloric intake between T0 and T8. Oppositely, the caloric intake did not differ ($p > 0.05$) between T0 and T8 in intervention group. This trend could be the result of a constant consumption of a food with a high fiber content (about 5.6 g per 75 g) as well as it is also known that foods enriched in fibers promote the sense of satiety reducing the overall daily food intake (Clarck et a. 2013).

Table 2 *Mainly nutrients affected by the 8 weeks dietary intervention and evaluation of their alteration between the T0 and T8. Only the average values of parameters significantly altered were reported.*

	Superfood	Placebo	Δ (T8-T0)	Average Superfood		Average Placebo		Δ Average (T0-T8)	
Nutrients	***p* T0 *vs* T8**	***p* T0 *vs* T8**	***p* Δ SvsP**	**T0**	**T8**	**T0**	**T8**	**Placebo**	**Superfood**
Calories (Kcal/die)	0.248	0.018	0.528	1531	1652	1491	1693	202.6	120.5
Alpha-tocopherol (mg/die)	0.014	0.129	0.345	2.2	1.4				
Tot. fibres (g/die)	<0.01	0.001	0.497	13.2	18.3	14.8	19.0		
Phosphorus (mg/die)	0.238	0.112	0.049					92.1	-69.8
Lipid (g/die)	0.030	0.009	0.617	59.5	70.7	61.0	75.6		
Oligosaccharides (g/die)	0.887	0.025	0.209			51.7	59.8		
Tot. polyphenols (mg/die)	0.041	0.207	0.180	620.9	441.4				
Potassium (mg/die)	0.099	0.231	0.042					131.1	-192.1
Tot. proteins (g/die)	0.230	0.044	0.519			59.7	67.6		
Tot. animal proteins (g/die)	0.261	0.642	0.286	30.8	27.9	27.9	29.4		
Tot. Vegetal proteins (g/die)	<0.01	0.614	<0.01	12.9	18.9			0.5	6.0
Vitamin B8 (mg/die)	0.014	0.239	0.014	6.6	4.6			1.2	-1.9
Vitamin C (mg/die)	0.017	0.282	0.332	110.2	84.2				
Vitamin K (mg/die)	0.758	0.020	0.020			48.8	13.4	-35.4	2.4

5.2 Effects of dietary intervention on anthropometrics and biochemical parameters

The 8 weeks food intervention seems to have led to variations in some blood parameters and anthropometrics measures (Tab. 3). Moreover, in the intervention group it was registered a reduction of BMI at T8, compared to T0. On the other hand, the same reduction was not observed in placebo group. Generally, subjects who assumed

the functional food registered a decrease of plica thickness and an improvement of blood parameters connected to a healthy state: HDL-cholesterol level is higher in the intervention group at T8, than T0, while transaminases (ALT and GGT) levels are lower. These changes are not observed in placebo group, suggesting a strict correlation between the preservation of healthy state and the consumption of super food enriched in MD compounds.

Table 3. *Blood and anthropometrics parameters evaluated at the begging and at the end of trial and evaluation of their variation during the 8 weeks dietary intervention. Only the average values of parameters significantly altered were reported.*

	Superfood	Placebo	Δ (T8-T0)	Average Superfood		Average Placebo		Δ Average (T0-T8)	
Blood parameters	***p* T0*vs*T8**	***p* T0*vs* T8**	***p* Δ S*vs*P**	**T0**	**T8**	**T0**	**T8**	**Placebo**	**Superfood**
BMI (Kg/m^2)	0.041	0.334	0.444	27.2	27.0			-3.7	3.0
Suprailiac fold (cm)	0.001	0.879	0.010	25.5	22.7			-0.6	-14.3
Waist (cm)	0.004	0.065	<0.01	89.7	87.7			-3.3	-18.0
Weight (cm)	0.004	0.262	0.017	80.6	78.9			-3.7	3.0
Subscapularis fold (cm)	0.005	0.081	0.161	22.3	20.3				
Triceps (cm)	0.007	0.179	0.139	20.5	18.7				
Blood Parameters									
HDL-cholesterol (mg/dl)	0.056	0.005	0.001			53.4	49.8	-3.7	3.0
Non-HDL-cholesterol (mg/dl)	<0.01	0.868	0.002	158.2	141.6			-0.6	-14.3
Total cholesterol (mg/dl)	<0.01	0.357	0.006	212.2	194.3			-3.3	-18.0
LDL-cholesterol (mg/dl)	<0.01	0.192	<0.01	137.4	123.1			-3.7	3.0
Triglycerides (mg/dl)	0.002	0.895	0.140	109.7	89.0				
ALT (UI/L)	0.002	0.291	0.122	34.67	30.2				
GGT (UI/L)	0.013	0.138	0.849	30.2	28.5				

5.3 Advantages of co-composite system in improving gastrointestinal and heat tolerance

The cell viability was evaluated in two different growth condition, anaerobic and aerobic, at the end of the simulated gastrointestinal digestion. No significant differences were found between the two growth conditions for each sample ($p > 0.05$). The cell viability of the microencapsulated sample (Polymers+Biosilica) was significantly higher ($p < 0.05$) than those containing only cells without any kind of coting (Fig. 1). In addition, a third condition based on the exclusive use of polymers was also tested to evaluate the efficiency of the presence of the excipient (biosilica). The protective combined effect of biosilica with polymers is significantly higher than the exclusive use of polymers ($p < 0.05$).

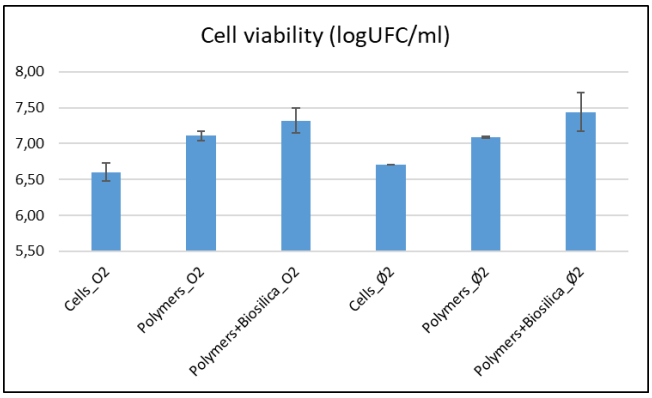

Figure 1 *Cell viability after gastrointestinal digestion. (Cells), cells without any kind of coating; (Polymers), cells coated with Shellac and chitosan polymers (1:1); (Polymers+Biosilica), cells encapsulated in biosilica shells and coated with polymers.*

The microbeads system silica-based seems to preserve the viability of cells after heat tolerance tests carried out as described in 4.4 (Fig. 2).

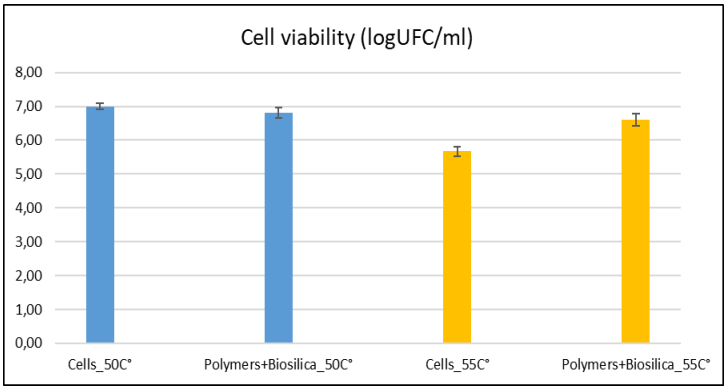

Figure 2 *Cell viability after heat tollerance tests carried out at 50 °C for 30 min and at 55 °C for 30 min. (Cells), cells without any kind of coating: (Polymers), cells coated with Shellac and chitosan polymers; (Polymers+Biosilica), cells encapsulated in biosilica shells and coated with polymers.*

6. Conclusions and Future Perspectives

The increased urbanization is driving the substitution of traditional diets with Western diets, which have higher refined food, fat content and meat consumption. The major consequence of this life style spread is the increased risk to develop metabolic disease (type II diabetes, coronary hearh disease, non-alcoholic fatty liver disease, and exc.). Scientific evidences have already reported how MD can bring benefits for the treatment of many types of diseases connected to gut dysbiosis. The development and the administration of foods enriched in typical MD components may allow the improvement of MD benefit accessibility, as this project have shown even though in a limited cohort of subjects

Thus, diet could have an important role in treatment and prevention of disease, however for the eradication and the treatment of other multifactorial diseases (e.g. Irritable bowel syndrome, recurrent Clostridium difficile infection, exc.), the development of successful therapies is still ongoing. In line with this, the current PhD project was also focused in developing a less invasive strategy for the FMT. The future prospective regarding the microencapsulated biosilica based system, is related to the possibility to scale up the process of biosilica production and optimize the microbiota extraction protocol, in order to start an experimental phase on murine subjects.

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Alternative antimicrobial strategies for the replacement of traditional preservatives and evaluation of the impact on the stability and safety of food products

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The PhD project is focused on the study of alternative antimicrobial strategies for foods, including biopreservation. Several natural preservatives and food products were studied, among which fresh potato gnocchi. The activities included analysis of food samples and ingredients, isolation of microorganisms, evaluation of sensitivity to biopreservatives, DNA extraction, Whole Genome Sequencing (WGS), and genomic analysis for species identification, molecular sub-typing, identification of virulence and antimicrobial resistance genes. Genes involved in the stress response induced by biopreservatives were assessed for future gene expression studies. *In situ* tests of gnocchi with biopreservatives in the formulation will be performed.

Strategie antimicrobiche alternative per la sostituzione di conservanti tradizionali e valutazione dell'impatto sulla stabilità e sicurezza degli alimenti

Il progetto di dottorato riguarda lo studio di strategie antimicrobiche alternative negli alimenti, tra cui la bioconservazione. Sono stati studiati diversi bioconservanti e matrici alimentari, tra cui gli gnocchi patate freschi. Le attività hanno incluso analisi di alimenti e ingredienti, isolamento dei microrganismi, valutazione della sensibilità a bioconservanti, estrazione del DNA, sequenziamento dell'intero genoma (WGS), e analisi genomiche volte all'identificazione della specie, sub-tipizzazione molecolare, identificazione di geni di virulenza e di antibiotico resistenza. Sono stati identificati geni coinvolti nella risposta allo stress, per futuri studi di espressione genica. Si eseguiranno test *in situ* su gnocchi, includendo i bioconservanti nella formulazione.

Key words: alternative antimicrobials; biopreservation; gnocchi; *Bacillus* spp.; genomic analysis; identification.

1. Introduction

In accordance with the PhD project previously described (Purgatorio, 2021), this oral communication focuses on the main results of the following activities:
(A1) sampling of raw materials and finished products, and isolation of microorganisms.
(A2) determination of antimicrobial activity of selected biopreservatives on isolated microorganisms.
(A3) DNA extraction intended for Whole Genome Sequencing (WGS) of isolated microorganisms.
(A4) genomic analysis of WGS data:

- quality check of raw WGS data, trimming, assembly, and annotation through ARIES (Advanced Research Infrastructure for Experimentations in genomicS) platform;
- species identification of selected bacteria, based on Ribosomal Multilocus Sequence Typing (rMLST) and confirmation through the analysis of the housekeeping genes *gyrB* (DNA gyrase β subunit), *rpoB* (RNA polymerase β subunit), and *tuf* (TU elongation factor);
- molecular sub-typing, through kSNPs (Single-Nucleotide Polymorphisms) and 7 loci-MLST (Multi-Locus Sequence Typing) analysis;
- identification of virulence genes (e.g., genes encoding for bacterial toxins, and bacteriocins) and antimicrobial resistance genes;

(A5) bibliographic research and selection of genes involved in the mechanisms of response to the stress induced by biopreservatives.

2. Biopreservation of fresh potato gnocchi

Gnocchi are a typical potato-based Italian product, which can be homemade or produced at industrial level and is generally considered similar to fresh pasta products. Nowadays, the active and frenetic lifestyle has led to an increase in the industrial production of gnocchi (Alessandrini et al., 2010). In particular, "ambient gnocchi" are becoming more and more popular because of their convenience. This specific type of industrial gnocchi is a pasteurized product, with acidifiers in the formulation, which can be stored at room temperature for several months. Stabilization techniques are essential for gnocchi, which are basically a very perishable product because of their high moisture content (up to 60%), high water activity (a_w 0.97-0.98), and the fact that they are formulated with raw materials, like potatoes and flours, which represent optimal substrates for the growth of many microorganisms (Cook and Johnson, 2009). However, in recent years, people are becoming increasingly aware of the environmental

and health problems that may result from an exaggerated use of synthetic additives in foods. Thus, food products with a "clean label" and a low environmental impact are highly demanded on the market. Therefore, consumers' interests have focused on the use of natural substances for preservation. Biopreservatives are known since ancient times for their bioactivities (antimicrobial, antioxidant, anti-inflammatory, etc.), because of their content in active molecules, such as terpenes, terpenoids, aliphatic, and phenolic components (Bakkali et al., 2008). Among them, essential oils (EOs), hydrolates, and plant extracts have been identified as natural antimicrobials. These substances are "Generally Recognized as Safe" (GRAS) (FDA, 2009), and so they are good candidates for food applications.

3. Experimental Procedure

This oral communication is focused on a part of the PhD project, aimed at identifying the microorganisms mainly responsible for the spoilage or safety of an important product for the Italian culinary tradition, which are gnocchi, if traditional preservatives (organic acids) are eliminated, or if modifications in the formulation are applied, in view of the replacement with natural preservatives. For this reason, molecular identification and characterization, and search of virulence factors and other types of genes, were carried out. In the present study, the results of *in vitro* tests with biopreservatives are reported, while *in situ* tests will be performed. As described in the introduction, the activities were: sampling of raw materials and gnocchi, isolation of microorganisms (A1), evaluation of sensitivity to several natural preservatives (A2), DNA extraction and WGS (A3), genomic analysis of WGS data (A4), and bibliographic research of genes involved in stress resistance (A5). This activity of the PhD project was developed as a collaboration between the University of Teramo (Teramo, Italy) and the Istituto Superiore di Sanità (Department of Veterinary Public Health and Food Safety - National Reference Centre for Botulism) (Rome, Italy).

4. Materials and Methods

(A1) Microbiological sampling was made on both raw materials (flours and spices) and on fresh gnocchi with acids (industrial gnocchi), without acids (elimination of both sorbic and lactic acid), or with only lactic acid (elimination of sorbic acid). All the gnocchi samples were packed in Modified Atmosphere Packaging (MAP), in plastic bags, and pasteurized. While the raw materials and the industrial gnocchi were analyzed only at time zero, the gnocchi in which the acids were completely or partially eliminated were tested up to 6 months, after storage in different conditions, including thermal abuse (30 °C, 37 °C and 55 °C), to mimic incorrect storage by consumers, and to evaluate the microorganisms responsible for the deterioration of the product, in optimal conditions for microbial growth. Microorganisms were isolated after the samplings.
(A2) EOs of *Origanum vulgare* var. *hirtum* thymol chemotype, *Thymus vulgaris* thymol chemotype, *Thymus vulgaris* p- cymen and thymol chemotype, *Ocimum basilicum* linalool chemotype, *Ocimum basilicum* geranial chemotype, *Syzygium aromaticum*, *Piper nigrum*, *Coridothymus capitatus* carvacrol chemotype were tested. *Coridothymus capitatus* hydrolate and two plant extracts based on phenolic compounds were also evaluated. Antimicrobial activity was assessed through the evaluation of Minimal Inhibitory Concentration (MIC) by microdilution (CLSI, 2011), against 17 isolated bacteria, presumptive belonging to the genus *Bacillus*, after observation of the morphology of the colony on specific culture media, microscopic observation, and Gram staining. *Bacillus* spp. was chosen for this activity because it was the major genus found in ambient gnocchi, at the same time involved in microbial spoilage.
(A3) The DNA of 54 isolates (chosen for diversity of morphology and matrix of origin), presumptively belonging to the genus *Bacillus*, including those tested for sensitivity to biopreservatives, were extracted using the Danagene Genomic DNA Kit (Danagen-Bioted S.L., Barcelona, Spain), with modifications. Genomic DNA quality was determined using NanoDrop spectrometer (Thermo Fisher Scientific, Wilmington, United States) and Qubit fluorometer (Thermo Fisher Scientific, Wilmington, United States), whilst integrity was assessed by agarose-gel electrophoresis. WGS was carried out on the Illumina Nextseq platform by BMR Genomics (BMR Genomics Srl, Padova, Italy) using paired-end sequencing protocol suggested by the producer.
(A4) Raw WGS data were processed through the ARIES platform (ARIES public Galaxy server – Istituto Superiore di Sanità) to obtain assembled and annotated genomes.
Isolates were identified at species level through the Ribosomal MLST tool of the database PubMLST (Public databases for molecular typing and microbial genome diversity) (Jolley et al., 2018). The assembly files obtained by running ARIES tools were used as input. Ribosomal MLST considers the variation of 53 genes encoding the bacterial ribosome protein subunits (*rps* genes), which are the ideal targets for a universal species identification because they are present in all bacteria and show variations useful to catalogue bacteria at all taxonomic levels (Jolley et al., 2012). Ribosomal MLST results were confirmed through the phylogenetic analysis of the housekeeping genes *gyrB*, *rpoB*, and *tuf*. As reported elsewhere (Caamaño-Antelo et al., 2015), these three housekeeping genes are suitable targets for the differential characterization of similar species, such as those of the genus *Bacillus*. Sequences of *gyB*, *rpoB*, and *tuf* genes were retrieved from each assembly and aligned with Clustal Omega (https://www.ebi.ac.uk/). Gene distances were calculated from alignments using the Jukes-Cantor method, whilst the dendrogram was generated using Neighbor-Joining method. Bootstrapping of 1000 replications was also performed. In addition, a concatenated gene tree was constructed with individual alignments in the following order: *gyrB*, *rpoB*, *tuf*. Sequences of standard strains (one for each species identified by Ribosomal MLST), retrieved

from publicly available genomes, were used to confirm species identification. *Levilactobacillus brevis* was used as outgroup.
To further investigate the genetic and clonally relatedness among the isolates, 7-loci MLST and k-SNP analysis were carried out. MLST was performed using the schemes adopted by PubMLST for *B. cereus* (*glp, gmk, ilv, pta, pur, pyc, tpi*) and *B. subtilis* (*glpF, ilvD, pta, purH, pycA, rpoD, tpiA*). kSNP3 (v3.0) with kmer size of 19 was run in ARIES using as input the aforementioned assembly files to draw a phylogenetic tree using Maximum Likelihood method. Isolates were grouped as *B. cereus* group and *B. subtilis.*
The identification of virulence and antimicrobial resistance genes was performed in ARIES running BLASTn tool and using as input file each assembly, searching the following genes: *ent*FM, *pipl*C, *nhe*ABC, *hbl*ACD, *sph, plc, Cyt*K, *bce*T, *pa, cya, lef, ba, gat*A, *met*G, *ces*ACDHPT, *dhb*ACEF, *spa*BCS, *spal, blt, psg*A, *mpr*F, *aad*K, *tmr*B (Brutscher et al., 2022; Deng et al., 2021; Sergeev et al., 2006;).

5. Results and Discussions

5.1 Sampling (A1)

Among raw materials, starch, semolina, and rice flour were the most contaminated, with aerobic mesophilic count of 10^3-10^4 cfu/g. Industrial ambient gnocchi were almost sterile, while for gnocchi without acids (completely or partially), and especially samples stored at 30 °C and 37 °C, the aerobic mesophilic count and *Bacillus* spp. count were up to 10^7 cfu/g, with the highest load at day 7. Loads were slightly higher for gnocchi without both acids. Moreover, starting from day 7, swelling for gas production in the packages of acid-free gnocchi stored at 30 °C and 37°C was observed. On the other hand, at the same times and temperatures, a phenomenon of slime and brown/reddish discoloration was observed on the surface of the gnocchi samples without sorbic acid and with only lactic acid. The swelling increased over time to a greater extent than the reddish colour, which instead did not vary significantly. 157 strains were isolated from the different samples. Among the isolates, there was a minor part of presumptive thermoduric lactic acid bacteria (LAB), and moulds, while the major part was made of presumptive *Bacillus* spp., and therefore the consequent analyses were carried on members of this group. In fact, these spore-forming bacteria are largely found in vegetable matrices in contact with ground, such as potatoes and grains (Del Torre et al., 2001).

5.2 Determination of the antimicrobial activity of biopreservatives (A2)

Origanum vulgare var. *hirtum* thymol chemotype, *Thymus vulgaris* thymol chemotype, *Thymus vulgaris* p- cymen and thymol chemotype and *Coridothymus capitatus* carvacrol chemotype EOs were the most effective. For the 17 tested *Bacillus* spp. strains, at 48 hours, MICs were around 0,3-1,3 µl/ml. The antimicrobial properties of these EOs are mainly due to their content in thymol and carvacrol, two active phenolic compounds (Pellegrini et al., 2018). On the other hand, the other EOs, the hydrolate, and the phenolic extracts, showed a lower activity, with higher MIC values.

5.3 DNA extraction and WGS (A3)

The obtained DNA was non-fragmented (verified by agarose gel electrophoresis), and pure (A260/A280: 1.8 - 2, A260/230: 2 - 2.2), and therefore suitable for WGS.

5.4 Genomic analysis (A4)

The 54 isolates tested by Ribosomal MLST belonged to the following species: *Bacillus subtilis* (34), *Bacillus cereus* (8), *Bacillus velezensis* (3), *Kosakonia cowanii* (3), *Bacillus thuringiensis* (2), *Bacillus atrophaeus* (1), *Bacillus paranthracis* (1), *Paenibacillus lutimineralis* (1), and *Pantotea agglomerans* (1). *Kosakonia cowanii* and *Pantotea agglomerans* belong to the *Enterobacteriaceae* family. *Enterobacteriaceae* members are widely found in soil, from which they can contaminate cereal products (Shaker et al., 2007). However, these bacteria were not found in the finished product, likely because inactivated by pasteurization. For these reasons, these isolates were not considered for further investigations. *Paenibacillus lutimineralis* belonging to the order *Bacillales*, was considered, for the purposes of this work, in the *B. cereus* group. Species identified by Ribosomal MLST were confirmed through phylogenetic analysis performed on *gyrB, rpoB*, and *tuf* genes, as reported in Figure 1A.
7-loci MLST provided some untypeable isolates because of the lack of genes included in the PubMLST.
The 16 *B. cereus* group isolates submitted to kSNP3 analysis were grouped into two clusters. Cluster A consisted of 11 isolates belonging to the species *B. cereus, B. paranthracis, B. thuringiensis* (*B. cereus* group), further grouped in 3 main subclusters (A1-A3). Six *B. cereus* isolates (24, 30, 35, 38, 39A, 72) resulted indistinguishable (subcluster A1), whilst isolates 4 and 11 clustered in the subclusters A3 and A2, respectively. Isolate 11 is the only *B. cereus* harboring genes encoding for the biosynthesis of cereulide. Cluster B consisted of 4 isolates. Three of them were *B. velezensis* and resulted indistinguishable, whilst the fourth belonged to *P. lutimineralis* (Figure 1B).
The 34 *B. subtilis* isolates submitted to kSNP3 analysis were grouped into 5 clusters. Cluster A consisted of 15 isolates and was further grouped in 2 subclusters (A1-A2). Isolates included in the subcluster A1 (40, 50, 58C, 58L, 59C, 60L, 70A, 70B, 78, 96) were undistinguishable. Cluster B and C consisted of 3 and 2 isolates, respectively. Cluster D consisted in 10 isolates. Nine of them were undistinguishable (Figure 1B).
BLASTn investigation on virulence and antimicrobial resistance genes provided the following main findings:

- *B. cereus* group (16 isolates). Twelve isolates harbored genes encoding for enterotoxin FM, non-hemolytic enterotoxin operon, phospholipase C, and hemolysin BL, with a coverage higher than 93% respect to the query. Ten isolates harbored genes encoding for cytotoxin K and enterotoxin T. Nine isolates harbored genes encoding for the major facilitator superfamily of antibiotic efflux pump, whilst seven carried the encoding for daptomycin resistance.
- *B. subtilis* (34 isolates). All isolates harbored the gene encoding for resistance to daptomycin and that encoding for major facilitator superfamily antibiotic efflux pump. Thirty-four isolates harbored genes encoding for resistance to tunicamycin and for defensin resistance. Thirty-three isolates harbored gene encoding for aminoglycosides. Three isolates harbored genes included in the operon involved in biosynthesis of the bacteriocin subtilin.

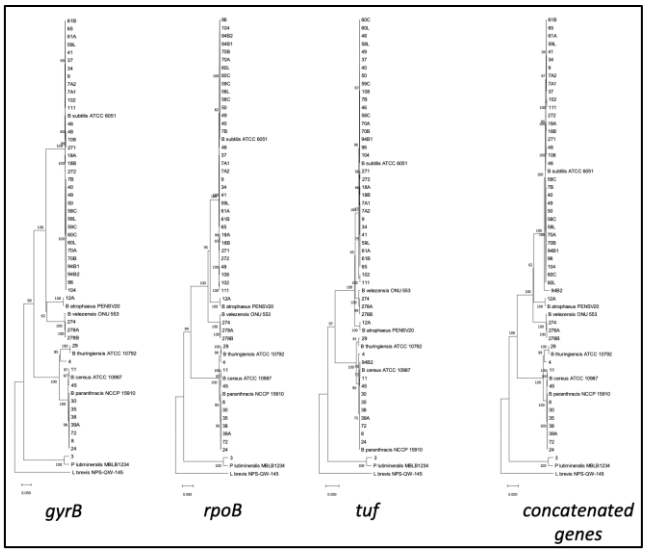

Figure 1A *Phylogenetic trees of gyrB, ropB, and tuf genes.*

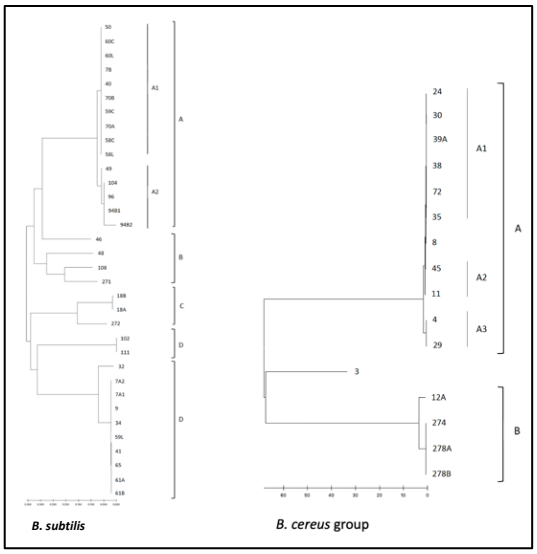

Figure 1B *Phylogenetic of kSNP3 analysis.*

Strains tested for MIC belonged to the species *B. subtilis* (10), *B. cereus* (4), *B. thuringiensis* (2), and *B. paranthracis* (1). The differences in MIC values between the different species were not significant. Regarding the origin of the isolates, we found *B. subtilis*, *B. velezensis*, *K. cowanii*, and *P. agglomerans* in the flours (starch, semolina, and rice flour) and spices (turmeric). *K. cowanii*, and *P. agglomerans*, as *Enterobacteriaceae*, cannot survive after pasteurization and do not represent a food safety concern for gnocchi. Conversely, *Bacillus* spp. can be a potential problem for both food spoilage and food safety. As spore-forming bacteria, they represent an alarm because of their heat resistance and tolerance to acids. *B. subtilis* was the mostly found, both in ambient gnocchi without acids and in those with only lactic acid, at various times and temperatures, including gnocchi with reddish discoloration on the surface. The ability of *B. subtilis* to produce red pigments has already been documented (Trivedi et al., 2017). Tolerance to lactic acid, exerted by *B. subtilis* strains, may be also related to their probiotic properties and to the capability of coexisting with LAB, favoring their development (Kimelman and Shemesh, 2019). Three of the *B. subtilis* isolates harbored the gene cluster involved in the subtilin biosynthesis and might exert probiotic properties. *B. cereus* was mostly isolated in gnocchi without both lactic and sorbic acid, at different times and temperatures of sampling, including swollen packages, and only in one case (isolate 24) in gnocchi with lactic acid. In fact, in these samples, the dominant microorganism was *B. subtilis*. Almost all the isolated *B. cereus* harbored genes encoding for virulence factors and might represent a risk for the consumer. *B. thuringiensis*, *B. antrophaeus*, *B. paranthracis*, and *P. lutimineralis* were sporadically isolated only in gnocchi without both sorbic and lactic acids, demonstrating the preservative properties exerted by lactic acid against these species.

5.5 Bibliographic research of genes involved in stress response (A5)

Stress conditions like exposition to sublethal concentrations of preservatives (such as biopreservatives), heat or acid stress, or starvation, lead to induction of general stress proteins. In *B. subtilis* and other related *Bacillus* spp. species, *sigB* gene is described as an alternative transcription factor activated by a variety of environmental stresses, including the stress imposed upon entry into the stationary growth phase. This gene seems capable of controlling the expression of more than 150 genes, including those of *sigB* operon (Hecker et al., 2007). *sigB* operon has similar gene clusters between different *Bacillus* spp. species, with some differences (e.g., three genes for *B. anthracis sigB* operon, and eight genes for *B. subtilis sigB* operon) (Fouet et al., 2000). Another molecular target affected in stressful conditions is the transcription factor *plcR*, involved in quorum sensing. In fact, as already demonstrated in *B. cereus*, the application of sub-lethal concentrations of biopreservatives can hinder the vital communication between bacteria, resulting in reduced expression of virulence factors (Jin et al, 2021). Further genes will be considered to better understand the molecular machinery involved in stress response, and the selected genes will be studied in depth to identify their arrangement (e.g., gene clusters, operons, and regulatory genes).

6. Conclusions and Future Perspectives

In conclusion, the microorganisms belonging to the genus *Bacillus* were those most involved in the spoilage of ambient gnocchi, especially in view of acids elimination. *B. subtilis* was found to be the dominant species, also in presence of lactic acid. *B. cereus* was also found, especially in the absence of both acids, and the strains showed to possess virulence genes, thus representing a potential danger to consumers. Therefore, the application of biopreservatives could be a sustainable alternative to organic acids, to preserve gnocchi. *In vitro* studies for the evaluation of the antimicrobial potential of natural substances against these bacteria gave promising results, especially for EOs rich in thymol and carvacrol, but *in situ* studies will be realized to investigate their real applicability to gnocchi, with particular attention to the sensory impact, since gnocchi have a neutral and delicate flavour. Moreover, future studies will be performed to investigate the molecular mechanisms that the isolated bacteria put in place when exposed to biopreservatives, starting from the findings of genes involved in stress response, and evaluating gene expression by real-time PCR.

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Optimization of cooking for food service: matching quality and nutritional requirement as drivers for development of innovative tools

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This doctoral thesis was carried out in collaboration with the company Electrolux Professional and concerned a kinetic study relevant to chemical, physical and nutritional changes occurring in chicken meat during cooking by using different oven cooking methods. The purpose of the study was to investigate the effect of different cooking methods on meat quality and digestibility in order to develop a predictive model to be applied in food service to optimize oven cooking processes.

Ottimizzazione della cottura per la ristorazione collettiva: combinazione di requisiti di qualità e nutrizionali come drivers per lo sviluppo di strumenti innovativi

Questa tesi di dottorato è stata svolta in collaborazione con l'azienda Electrolux Professional ed ha riguardato lo studio di modificazioni qualitative di tipo chimico, fisico e nutrizionale di carne di pollo durante processi di cottura in forno secondo diverse modalità. Lo scopo dello studio è stato quello di investigare l'effetto di metodi di cottura differenti sulla qualità della carne e la sua digeribilità allo scopo di sviluppare un modello predittivo finalizzato all'ottimizzazione dei processi di cottura in forni industriali utilizzati per il food service.

Key words: Kinetics; Modelling; Quality indices; Cooking process; Protein digestibility.

1. Aim of the work:

- Assessment of the evolution of several quality indices during chicken breast cooking according to the most common oven cooking methods in food service
- Kinetic modelling of the above-mentioned quality indices evolution as a function of cooking temperature and cooking methodology
- Computation of the relevant activation energy (Ea) and decimal reduction time (D_T)
- Assessment of *in vitro* digestion of cooked chicken breast as a function of cooking time and methodology

2. Introduction

In food service, meat is one of the most requested items and accounts for a significant percentage of the total cost of production. Oven cooking is the most frequently used cooking process for meat in commercial processing and food service operations because it facilitates a high volume of foodstuffs to be cooked at the same time in a controlled way.
An optimal meat cooking process should firstly ensure the safety of meat at the point of consumption through pathogen inactivation and also facilitate the development of desirable sensory attributes while maintaining a high nutritional value and the suitable technological performance e.g. in terms of cooking yield. During cooking, meat undergoes major chemical and physical changes which influence the final quality and consumer acceptability. Several parameters related to the oven such as methodology, temperature and cooking time can affect the final quality of cooked foods. Kinetic modelling of quality indices evolution of product during cooking represents a powerful tool for understanding their modifications during the thermal process predicting outcomes and permitting optimization of the cooking process (Ling et al., 2015).
Poultry meat is the most consumed meat in the world for its high nutritional quality, low religious barriers and cost effectiveness (OECD & FAO, 2021). While it is imperative to achieve a final internal cooked meat temperature of 75 °C, chicken is easily susceptible to drying and overcooking and for this reason it has been considered as a case study in the present project.

3. Materials and methods:

IQF chicken breast meat was thawed at 4 °C overnight the day before the analysis. Thawed chicken breasts of about same size around 310 ± 10 g were cooked individually in the centre of a vessel in an electric oven equipped with core temperature probe according to three different cooking methods and three different cooking temperatures. The tested cooking processes were:

- forced convection (FC): 150, 170, 190 °C for increasing times up to 38 min

- grill (G): 240, 260, 280 °C for increasing times up to 15 min
- sous vide (SV): 80, 95, 120 °C with 100% humidity for increasing times up to 35 min

Fillets were cooked for 15 different cooking times (5 cooking times for each temperature) within each cooking process and all experiments were replicated three times. At each cooking time, samples were cooled up to 4 °C and then weighed for cooking loss calculation, analysed for texture and colour measured. Shear force analysis was performed on 3x3x6 cm geometry samples derived from the internal part of whole cooked muscle using a Warner-Bratzler blade (Instron LTD, High Wycombe, UK) equipped with a 1 kN load cell. Colour data were collected using the L*a*b* colour space and expressed as chroma C*. Evolution of weight loss, shear force and chroma were studied according to kinetic modelling and the effect of temperature on reaction rates was studied through decimal reduction time (D_T) and activation energy (Ea) parameters.
Protein digestibility of cooked chicken breast was determined according to the INFOGEST *in vitro* static digestion method (Brodkorb et al. 2019). In particular, meat samples were cooked according to the three different methods for a cooking time matching with the optimal core temperature of 75 °C (USDA & FSIS, 2020) (25 min for FC, 13 min G, 25 min SV) and an overcooking time (35 min FC, 19 G, 35 min SV). Sulfhydryl content (Srinivasan et al., 1997), carbonyl content (Ganhão et al. 2010) and secondary structure by means of FT-IR spectroscopy (Alpha-P, Bruker Optics, Milan, Italy) were studied. Relationships between the different parameters were assessed by calculation of Pearson correlation coefficients.

4. Kinetic modelling:

Kinetics of quality attributes changes were elaborated to define the reaction order best describing the observed changes of the selected indicators as a function of cooking time. The best model was the zero order kinetic:

$$I = kt + I_0 \quad (1)$$

Where I is the selected indicator, I_0 is the value of the indicator at time zero and k is the zero order rate constant. Decimal reduction time (D_T), which is the interval of time required to reduce by an order of 10 the initial value of the indicator, was calculated considering I_0 as the value of the intercept, I as the decimal reduction of the indicator and k the zero order constant rate of the considered cooking trial at the defined temperature, as follows:

$$D_T = \frac{I - I_0}{k} \quad (2)$$

Thermal resistant constant (z-value), that is the temperature difference resulting in a decimal variation of D_T, was then calculated plotting D_T versus temperature and computed the inverse slope:

$$z = \frac{1}{slope} \quad (3)$$

The effect of temperature on the rate of quality indicators modifications was evaluated by means of the Arrhenius equation applying linear regression analysis:

$$lnk = lnk_0 - \frac{Ea}{R}\left(\frac{1}{T}\right) \quad (4)$$

where *k* is the apparent reaction rate, k_0 is the pre-exponential factor, R is the molar gas constant (8.31 J $mol^{-1}K^{-1}$) and *T* is the absolute temperature (K). *Ea* was extrapolated by linear regression analysis.

5. Results and discussion:

Cooking loss, shear force and colour were monitored over the different cooking methods at different temperatures. Zero order rate constants are reported in Table 1.
As shown in Table 1, a proportional increase of rate constants was observed according to process temperature, independently of the considered cooking method. The treatment characterized by the highest process temperature (G) showed the fastest quality indices evolution, followed by FC and SV. The temperature was able to accelerate all the physical and chemical changes within the meat, regardless of the cooking method applied.
Protein denaturation is one of the main phenomena happening in meat during the cooking process. Increasing temperatures cause denaturation and shrinkage of myofibrillar proteins in the range of 40-54 °C (myosin) and 66-73 °C (actin) and also solubilization and shrinkage of collagen in the range of 56–62 °C (Tornberg, 2005).

Table 1 *Zero order rate constants and relative coefficient of determination R^2 of cooking loss, texture and chroma evolution of chicken breast cooked according to different cooking methods at different temperatures and relative decimal reduction time (D_T)*

Cooking method	Quality index	T (°C)	k (index min^{-1})	R^2	D_T (min)
GRILL	Cooking loss	240	1.9782	0.99	18.20
		260	2.5195	0.99	14.29
		280	3.0729	0.99	11.72
	Shear Force	240	-1.0943	0.94	32.75
		260	-1.9292	0.99	28.45
		280	-3.3211	0.99	18.74
	Chroma	240	1.5258	0.97	28.08
		260	1.8933	0.99	22.55
		280	2.5486	0.99	13.74
FORCED CONVECTION	Cooking loss	150	0.7680	0.99	35.16
		170	1.0079	0.99	26.79
		190	1.2705	0.99	21.25
	Shear Force	150	-0.3765	0.99	101.19
		170	-0.5486	0.97	67.97
		190	-0.9121	0.99	53.22
	Chroma	150	0.4261	0.99	125.57
		170	0.8919	0.99	43.85
		190	1.0400	0.98	42.74
SOUS VIDE	Cooking loss	80	0.539	0.98	50.09
		95	0.8876	0.99	30.42
		120	1.1899	0.97	22.69
	Shear Force	80	-0.2958	0.96	108.39
		95	-0.7454	0.94	49.84
		120	-1.4568	0.97	38.51

*p-value of regression lines resulted in all cases statistically significant ($p<0.05$)

Results showed that cooking loss was time- and temperature-dependent and the reason is that the speed of the myofibrillar protein denaturation proportionally increased with time and temperature (Martens et al., 1982). These proteins hold most of the water retained within the muscle and when they get denatured the water is released, causing shrinkage and weight reduction (Tornberg, 2005). Shear force modification in meat is also linked to protein modifications. In particular, the tenderization of samples that was registered during cooking depended on denaturation and solubilization of connective tissue of proteins (Roldàn et al., 2015). Moreover, low temperature-slow cooking rates caused a lesser reduction in sarcomere length with respect to high temperature-fast cooking, with an improvement in tenderness (King et al, 2003). Maillard reaction dominated the colour changes: browning and crust formation were proportional to cooking temperature. For SV, chroma evolution was not considered because no colour changes were seen during cooking, due to the characteristic low temperatures and high humidity of this process (Roldàn et al., 2015).

D_T values are also shown in Table 1. D_T decreased at increasing process temperature for all the different cooking methods. G showed the lowest D_T values, followed by FC and SV. Among the quality indices, shear force always exhibited the highest D_T, with the highest value of 108 min reached at 80 °C for SV, while cooking loss was the lowest one, 11 min at 280 °C for G, resulting the most sensitive quality index to temperature changes.

z values were also evaluated. z values decreased according to thermal process temperature. They were higher for G (200–130 °C) followed by FC (180–85 °C) and SV (121–95 °C). In all cooking treatments, the cooking loss was the quality index characterized by the highest z value (~160 °C) followed by shear force (~130 °C) and cooking loss (~100 °C). In literature most of the z values are referred to as microbial destruction, whereas there are few z values related to quality attributes (Lund, 1975). To the best of our knowledge, no z values are reported in the literature regarding meat quality. The very high value of z found for meat in the present study could be attributed to the limiting phenomenon of water loss and evaporation and no real applicability can be found due to the limited cooking working temperatures.

To further demonstrate the dependency of quality index from temperature, activation energies (Ea) were calculated and results are reported in Table 2.

Table 2 *Activation energy (Ea) and relative coefficient of determination of cooking loss, texture and chroma evolution of chicken breast cooked according to different cooking methods at different cooking temperatures.*

Cooking method	Quality index	Ea (kJ mol^{-1})	R^2
GRILL	Cooking loss	25.99	0.99
	Shear Force	65.45	0.99
	Chroma	30.19	0.98
FORCED CONVECTION	Cooking loss	20.50	0.99
	Shear Force	35.91	0.95
	Chroma	24.79	0.93
SOUS VIDE	Cooking loss	22.15	0.86
	Shear Force	46.67	0.93

* p-value of regression lines resulted in all cases statistically significant ($p<0.05$)

As shown in Table 2, the Arrhenius model well described the temperature dependence of quality indices, showing high R^2. Shear force showed the highest value for all the treatments, with a maximum of 65.45 kJ mol^{-1} for G and they were in line with the ones reported in literature (Rabeler et al., 2018). Cooking loss showed instead the lowest values, 20.50 kJ mol^{-1} for FC. Similar Ea values were assessed by other authors on different food matrix regarding water transfer or drying activation energy to which meat cooking loss can be associated to. Cooking loss was confirmed to be the most sensitive indicator of temperature change that could be used as a quality index to be monitored to control a cooking process.
In order to investigate the impact of cooking on nutritional value, protein oxidation was analysed in terms of sulfhydryl and carbonyl groups (Table 3).

Table 3 *Sulfhydryl groups, carbonylic groups and content of secondary structures in chicken breast raw and cooked according to different cooking methods (FC, G, SV) for an optimal time (opt) and an overcooked time (over).*

Cooking treatment	Sulfhydryl groups (nmol/mg prot)	Carbonylic groups (nmol/mg prot)
Raw	25.86 ± 0.55^{b}	4.31 ± 1.18^{d}
FC optimal	50.45 ± 16.15^{ab}	11.62 ± 1.30^{c}
FC overcooking	52.54 ± 15.95^{ab}	17.31 ± 3.28^{ab}
G optimal	61.54 ± 14.51^{a}	14.69 ± 1.24^{bc}
G overcooking	62.55 ± 18.75^{a}	21.01 ± 4.06^{a}
SV optimal	57.99 ± 15.81^{a}	13.12 ± 2.66^{bc}
SV overcooking	49.97 ± 9.74^{ab}	13.97 ± 2.66^{bc}

[a-b] In the same column, means indicated with different letters are significantly different according to ANOVA and Tukey-HSD test ($p<0.05$)

The decrease of sulfhydryl groups with the increase of disulphide bonds, due to cysteine oxidation, is a common marker of protein oxidation (Zhang et al., 2013). Results showed that all cooked samples had higher sulfhydryl groups content than raw cooked ones and no particular differences were observed according to the cooking method applied or time. Probably, cooking helped the unfolding of protein with cysteine exposition and detection, but no oxidation occurred due to insufficient cooking time (Jiang et al., 2021). The increase in carbonyl groups content is considered another important protein oxidation marker: it is linked to the reaction between proteins and the aldehydes, as well as oxidization of side chains of amino acids (Zhang et al., 2013). Results showed that carbonyl groups content increased in cooked samples with respect to the raw ones. The effect of time on oxidation was seen on G overcooking treatment, with the highest amount of carbonylic group, followed by FC overcooking. No differences were seen in terms of cooking treatments. SV overcooking resulted in the same oxidation level of optimal cooking treatments. The vacuum packaging and low cooking temperatures, which were able to prevent intramuscular fat oxidation in comparison with meat cooking without vacuum can explain these results (Yin et al., 2020). Protein secondary structure modifications as affected by cooking method and time were also investigated (data not shown). During spectra elaboration, the band between 1650 and 1670 cm^{-1} were associated to α-helix structure and β-sheet were detected in the range of 1618–1640 cm^{-1} and 1670–1680 cm^{-1} respectively (Kong & Yu, 2007). Respect the raw meat, results showed that β-sheet structure amount significantly increased in FC and G (27%) for the optimal cooking time. However, the highest amounts were reached in overcooking and, in particular, in G, with an increase of about 40% respect to Raw and FC, about 35%. No differences were seen between Raw and SV. Regarding α-helix, the proportion seems to be lower for cooked samples with G overcooking presenting the lowest amount. The analysis indicated that during the cooking processing α-helix fractions turned into β-sheet and β-turns. Cooking process can break the hydrogen bonds between the amino hydrogen and carbonyl oxygen which is critical to the maintenance of α-helix structure. The intensity of heating rules the conversion of α-helix in other forms (Yin et al., 2020).

Protein digestibility was also studied according to gastric and intestinal phase digestion (data not shown). Two different times were studied for each cooking method, the optimal time and an overcooking time increased by 30% compared to the optimal one. Considering the effect of the cooking method for the optimal cooking time, no differences were seen between G and SV in terms of protein digestibility while FC seemed to decrease it by approximately 12% compared to G and SV in the gastric phase and 6% in the intestinal phase. Taking into account cooking time, results demonstrated that the overcooked condition reduced the protein digestibility for all the different cooking treatments both in the gastric and intestinal phases. In particular, in gastric phase, it was reduced by 34% for G and 23% for SV respect the optimal time and in intestinal phase it decreased of about 4% for FC and SV and 16% for G.

Thanks to Pearson correlation analysis, a negative correlation was found between carbonylic groups and protein digestibility in intestinal phase (r=-0.85, p-value=0.02). Proteins oxidation can hinder the enzymatic proteolysis and consequently reducing the amino acid release during digestion (Santé-Lhoutellier et al., 2008). Moreover, a different susceptibility to digestion has been associated to protein secondary structure. A very high significant negative correlation was found between β-sheet and protein digestibility in intestinal phase (r=-0.96, p-value=0.001). It seems that a higher proportion of β-sheet may partly cause a low access to gastrointestinal digestive enzymes, which may lead to lower protein nutritional value (Zhou et al., 2014). Results showed that the functionality of the total protein pool might be adversely affected as a consequence of stronger cooking conditions.

6. Conclusions and Future Perspectives

Cooking generates a series of complex chemical-physical modification, which can influence and compromise the final quality of meat if they are not properly controlled. A minimal loss of regulation of the cooking process e.g. prolonged times, can compromise important aspects of foodstuffs such as cooking loss and nutritional value. Proportionality of kinetic parameters indicates the possibility of modelling the cooking process. Identification and control of the most sensible quality index can drive the optimization of cooking through optimized cooking programs in cooking equipment or the use of innovative sensors monitoring the index and allowing a better management of the industrial cooking process.

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Biotechnological approaches to valorise alternative protein source, waste and by-products of food industries

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This PhD thesis aimed to advance the knowledge of tailor-made biotechnological processes to use waste, by-products and alternative protein sources for the production of innovative bakery products from sustainable and renewable sources.
The research activities were focused on two macro-topics. The first one concerned the production and characterization of cricket-based hydrolysates to be used as high protein ingredients for food formulations, especially bakery ones. The second topic was focused on the valorisation of by-products from the milling industry, throughout tailored biotechnological processes, to obtain pre-fermented ingredients to be reused in innovative food formulation.

Approcci biotecnologici per la valorizzazione di fonti proteiche alternative, scarti e sottoprodotti dell'industria alimentare

Questa tesi di dottorato ha avuto lo scopo di portare ad un avanzamento nella conoscenza di processi biotecnologici che permettono di sfruttare scarti, sottoprodotti e fonti proteiche alternative destinati alla produzione di prodotti da forno innovativi a partire da fonti sostenibili e rinnovabili.
Le attività di ricerca si sono concentrate su due macro-temi. Il primo ha riguardato la produzione e la caratterizzazione di idrolizzati a base di grillo da utilizzare come ingredienti ad alto contenuto proteico per formulazioni di prodotti da forno. Il secondo è stato incentrato sulla valorizzazione dei sottoprodotti dell'industria molitoria, attraverso processi biotecnologici, per ottenere prefermenti da riutilizzare come ingredienti in formulazioni alimentari innovative.

Key words: Yeast hydrolysates, Cricket powder, Sourdough, Innovative Bread, by-products, Milling industry

1. Introduction

This oral communication reports the main results regarding to:
(A1) Isolation of microorganisms from different food matrices and by-products of the milling industry. Identification and selection of microorganisms on the basis of their safety, technological and functional characteristics.
(A2) Development of formulations and biotechnological processes to obtain innovative ingredients to be used for the production of innovative foods studied in this project.
(A3) Characterization of the obtained innovative products, from a microbiological, technological, toxicological and functional point of view.

2. By-products and alternative protein source in food industries

The demographic increase, associated with the difficulty to finding essential raw materials and the need to reduce the environmental impact due to excessive industrialization, has made it necessary to identify and use alternative and sustainable resources. Approximately 88 million tons of food are wasted every year in Europe, with an estimated loss of 143 billion euros (according to FP7 FUSIONS project, 2016). These wastes represent a valid source of high added value compounds, but they are not widely used and represent a cost for industries. Their use could constitute an innovative and sustainable approach for the production of ingredients to be re-used in different industrial sectors, resulting in a reduction in the amount of waste, greater economic benefits, an improvement in environmental sustainability and the creation of interconnections between different sectors (Pleissner et al., 2016). Although these wastes have a high application potential, their use in food formulations is extremely limited due to a series of economic, social, and regulatory obstacles, as well as the lack of sustainable biotechnological approaches that allow a full exploitation of these resources. For these reasons, currently, the use of by-products is still limited and confined to the production of low added value compounds mainly intended for animal feed and production of biogas. The need to identify alternative sources of nutrition has also led to the identification of innovative protein sources that provide for the deficiency of animal origin protein sources that no longer meet the global demand. Different studies suggest that the consumption of edible insects (entomophagy) may represent a valid alternative to the consumption of the usual animal proteins (Stoops et al., 2016). In fact, among the potential benefits of the consumption of insects there is a high concentration of high nutritional values proteins and lipids, vitamins, fibers as well as the presence of bioavailable mineral microelements such as calcium, iron and zinc

(Rumpold and Schlüter, 2013). In addition to the nutritional benefits described by different authors, there are also ecological, economic and social ones. In fact, compared to livestock, farmed insects multiply much faster and with a higher food/protein conversion coefficient, require little space for reproduction and produce fewer greenhouse gas and ammonia emissions (Klunder et al., 2012). The increase in the consumption of edible insects can also represent a valid and sustainable solution to nutritional problems in developing countries (Klunder et al., 2012). Thanks to the many positive factors associated with the use of edible insects as a food source, in recent years, the idea of using them for industrial production attracted the attention of the media, research institutes and food industry operators. On the other hand, the microbiological evaluation of insect meal-based foods represents a fundamental activity to verify their suitability for safe human consumption. Moreover, for European consumers, with a rich culinary tradition, the consumption of insects is not well perceived and may only be possible if they are part of the formulations as power ingredients of familiar products such as bars, biscuits, bread and crackers.

3. Materials and Methods

The analyses carried out for the two macro-topics are similar, for this reason the materials and methods are described in a single chapter.

The cricket powder hydrolysate was produced according to Patrignani et al. (2020).

YPD (Oxoid, Basigstone, UK) and MRS (Oxoid, Basigstone, UK) added with maltose, were used for the growth of yeast and bacteria, respectively.

The pH values of the samples were determined by pH meter (BASIC 20, Crison, Modena, Italy).

Proteins were extracted according to the method proposed by Marco et al. (2007) with some modifications. The protein concentration of samples was determined using a commercial kit (2-D Quant Kit, GE HealthCare). Separation of proteins by 1-D electrophoresis was carried out on polyacrylamide gels as described by Laemmli (1970).

Lipid fractions were extracted from according to the method described by Boselli et al. (2001) with some modifications. Free fatty acids (FFAs) were obtained from the total lipid extracts using aminopropyl bonded sorbent columns (SPE-NH2) ISOLUTE (Biotage, UK). The total and free fatty acids methyl esters profiles analyses were carried out on an Agilent 6890 gas chromatograph (Agilent Technologies, Palo Alto, CA) coupled to an Agilent 5970 mass selective detector operating in electron impact mode (ionization voltage, 70 eV).

Volatile molecules analyses were performed using a GC-MS coupled with a solid phase microextraction (GC–MS-SPME) technique, according to Burns et al. (2008) with some modifications. The used fibre was SPME Carboxen/PDMS, 85 μm (Stalleflex Supelco, Bellefonte, PA, USA). The analysis was performed with an Agilent Technology 7890 N gas chromatograph, Network GC System combined with a Network Mass Selective detector HP 5975C mass spectrometer (Agilent Technologies, Palo Alto, CA, USA).

Biogenic Amines was analysed according to Gardini et al. (2013),

Chitin quantification was performed with filch test.

4. Results and Discussion

In order to provide a clearer description of the activities carried out and the main results achieved, the two macro-topics of the PhD project will be treated separately.

4.1 Production of innovative foods with high nutritional value

The first macro-topic concerned the production and characterization of cricket-based hydrolysates to be used as high protein ingredients for food formulations, especially bakery ones.

About that, the technological potential of two strains of *Yarrowia lipolytica* (PO11, RO24) and two of *Debaryomyces hansenii* (DB and SP6L12) were evaluated to develop in a matrix composed by cricket powder and water. The results obtained showed that all four yeast strains considered were able to develop in the starting matrix. The strain that most increased its cellular load was *Y. lipolytica* PO11 which reached values over 7 log CFU/g of product. Moreover, the data obtained showed that, after 72 hours from inoculation, the chitin content in the samples hydrolyzed with *Y. lipolytica* strains were lower than in the not inoculated control and in the samples hydrolyzed with *D. hansenii* strains. In particular, the greatest reduction of chitin was detected in the sample hydrolyzed by *Y. lipolytica* RO25 with a reduction of 28% (Table 1).

The analysis carried out by GC / MS highlighted peculiar fatty acid profiles in the obtained hydrolysates, in relation to the used strain. The presence of essential omega 3 and omega 6 fatty acids is of great importance. The amino acid profiles have also highlighted the importance of the starting strain. In fact, amino acids such as Histidine, Threonine, Leucine and Ornithine were detected, especially in the hydrolyzed by *Y. lipolytica*. The release of pleasant aromatic compounds, the increase in unsaturated fatty acids and the reduction of chitinized were an excellent starting point for obtaining innovative products based on cricket powder. The choice of a specific strain in the food sector was linked to the sensory, qualitative and nutritional characteristics that were wanted to impart to the final product, also taking into account the production process adopted.

Table 1 *Chitin content (g / g cricket powder) determined in No Hydrolysed cricket powder and in hydrolysed samples after 72 h from the inoculation with Y. lipolytica PO11, RO25 and D. hansenii DB and SP6L12.*

72h	*Chitin (g) / g cricket powder*		
No hydrolysed cricket	*0.823*	±	*0.010*
PO11 Cricket hydrolysate	*0.617*	±	*0.008*
RO25 Cricket hydrolysate	*0.59*	±	*0.009*
SP6L12 Cricket hydrolysate	*0.793*	±	*0.009*
DB Cricket hydrolysate	*0.693*	±	*0.010*

For this reason, on the base of the information obtained from the previously mentioned tests, the hydrolysate obtained from *Y. lipolytica* RO25, having the best characteristics, was used to produce a sourdough intended for the production of bread. Specifically, the sourdough obtained with the addition of 30% cricket powder hydrolysate from *Y. lipolytica* RO25, called "RO25 sourdough", was compared with a traditional sourdough, obtained with only wheat flour "Wheat sourdough" and a further control containing 30% of non-hydrolyzed cricket powder, obtained with the same method but without inoculation of yeasts, “Cricket sourdough”.
The obtained results showed that "RO25 sourdough" was characterized, compared to the two control samples, by a marked and peculiar profile in total proteins due to the well-known proteolytic capacities of *Y. lipolytica* (Figure 1). "RO25 sourdough" was also composed of a specific profile in free fatty acids, including arachidonic and linolenic acid having functional characteristics. In addition, it was also composed by a high concentration of C18:2, C18:1 and C16:1 which are considered aroma precursors. In fact, the presence of a high proteolytic activity and a high concentration of free fatty acids gave rise to a specific profile in volatile molecules. Through the GC/MS/SPME analysis it was possible to identify more than 60 molecules belonging to the classes of alcohols, aldehydes, ketones, acids, lactones and furanones that differentiate the sample obtained from *Y. lipolytica* RO25 compared to the control samples (Figure 2). Therefore, the results obtained showed the good potential of *Y. lipolytica* RO25 to produce hydrolysates to be used as a sourdough ingredient for the production of innovative bakery products with a high nutritional and functional value.
For this purpose, on the basis of the information described above, 75% of each sourdough obtained was used for the production of a dough and finally bread. In particular, the bread obtained from sourdough containing 30% cricket powder hydrolysed with *Y. lipolytica* RO25 "RO25 bread" was compared with the control obtained from sourdough containing only wheat flour "Wheat bread" and a second control obtained from sourdough made from cricket powder not hydrolysed with yeasts “Cricket bread”. The obtained data underlined the good characteristics of the bread made from cricket powder obtained from sourdough containing hydrolysate by *Y. lipolytica* RO25. In fact, this bread, as well as the starting hydrolysate and the sourdough subsequently, was characterized by a high concentration of polyunsaturated free fatty acids, protein fractions (albumins / globulin, prolamins and glutelin) and a low level of biogenic amines when compared with "Cricket bread". Indeed, the bread samples were characterized by the presence of biogenic amines (Table 2). However, the “RO25 Bread” sample showed a lower content level of cadaverine and tyramine than the “Cricket bread”, inally, the B.A.I. (biogenic amine index) was 2.9 and 5 for “RO25 Bread” and “Cricket bread” respectively.
Furthermore, the texture data show that the hardness values of the sample obtained from *Y. lipolytica* RO25 are not significantly different from those of “Wheat bread”.

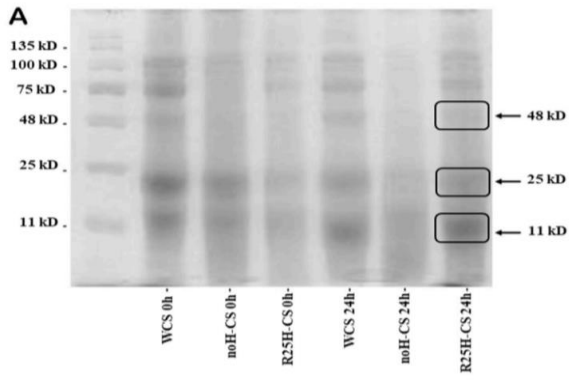

Figure 1 *Coomassie colored SDS-PAGE containing proteins extracted in reducing conditions of "Wheat sourdough" (WCS), "Cricket Sourdough" (noH-CS) and "RO25 sourdough" (RO25HCS), immediately after the second refreshment (0 h) and after 24 h of fermentation (24 h) at 25 ° C. Molecular weight markers in the first column and their values in kDa on the left.*

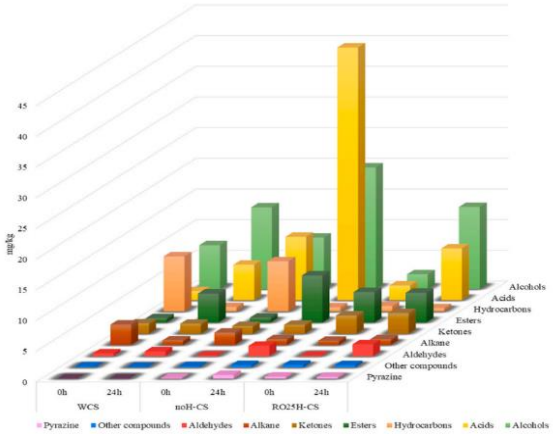

Figure 2 *Main classes of compounds (expressed in mg / kg) found in "Wheat sourdough" (WCS), "Cricket Sourdough" (noH-CS) and "RO25 sourdough" (RO25HCS), immediately after the second refreshment (0 h) and after 24 h of fermentation (24 h) at 25 ° C.*

Table 2 *Biogenic amine content in "Wheat Bread" (WCB), "Cricket Bread" (noH-CB) and "RO25 bread" (RO25HCS).*

Biogenic Amine		***WCB***	***RO25H-CB***	***noH-CB***
Cadaverine	*(mg/kg)*	*0.7±0.1*	*8.0±0.4*	*20±1*
Histamine	*(mg/kg)*	*<1**	*<1*	*<1*
Putrescine	*(mg/kg)*	*11.0±0.2*	*15.0±0.8*	*15.0±1.8*
Spermidine	*(mg/kg)*	*3.0±0.1*	*4.0±0.2*	*4.0±0.2*
Spermine	*(mg/kg)*	*2.7±0.1*	*3.0±0.1*	*2.0±0.1*
Tyramine	*(mg/kg)*	*30.0±0.2*	*17.0±0.9*	*28.0±1.4*
B.A.I. Index	*(mg/kg)*	*2.7±0.2*	*2.9±0.1*	*0.5±0.3*

Finally, the results obtained from sensory analysis underline the good opportunity of applying sourdough obtained from cricket flour hydrolyzed with *Y. lipolytica* RO25, as an ingredient for bread making.
In fact, the "RO25 bread" received positive evaluations for almost all the parameters considered. The results show that hydrolysed by *Y. lipolytica* RO25, compared with non-hydrolyzed cricket powder, can impart specific sensory and qualitative characteristics to the final product.
Finally, during the period abroad carried out in Germany at the Leibniz Institute for Agricultural Engineering and Bioeconomy (ATB) from January to May 2022, a process was set up with the aim of developing a bakery product, based on cricket powder, enriched with zinc, a trace element whose deficiency in the body can compromise the immune system. The enrichment process was carried out using microorganisms adapted to an environment with a high concentration of zinc sulphate and containing this micronutrient in their cell membranes. The enriched microorganisms were destined to produce sourdough and cricket powder hydrolysates to obtain a functional and high nutritional value baked product.
Furthermore, another studied important aspect concerns the allergenic potential of cricket powder and the possible effect that the subsequent processing steps, including fermentation and hydrolysis, may have on the allergenicity of the final baked product. This study was carried out in collaboration with Paul-Ehrlich-Institut (Germany) and the results will be available shortly.

4.2 Enhancement of by-products from the milling industry

The second macro-topic was focused on the enhancement of by-products from the milling industry, throughout tailored biotechnological processes, to obtain pre-fermented ingredients to be reused as ingredient in innovative food formulation.
In this regard, microbial strains were isolated from numerous mixtures obtained from the spontaneous fermentation of by-products of rye, durum and soft wheat processing. The pre-ferments were refreshed daily until the pH stabilization, then the final pH, the load of yeasts and lactic bacteria were assessed and finally the microbiological isolations were performed. Molecular methods were used to identify strains of yeasts and bacteria. Microbial sequencing data showed the presence, in the pre-ferments, of *Saccharomyces cerevisiae*, *Pichia kudriavzevii* and *Kazachstania unispora* for yeasts. In the case of lactic acid bacteria, the most commonly identified species were: *Pediococcus pentosaceus* (15 strains), *Leuconostoc mesenteroides* (4 strains), *Lactobacillus curvatus* (5 strains), *Lactobacillus brevis* (4 strains), *Lactobacillus plantarum* (7 strains), *Lactobacillus fermentum* (2 strains), *Lactobacillus paralimentarius* (4 strains), *Lactobacillus pentosus* (2 strains) and *Lactobacillus songhuajiangensis* (3 strains). The identified microbial strains were characterized, in vitro, for fermentation kinetics and the ability to produce volatile molecules, short-chain fatty acids (SCFA) and other natural antimicrobials and bioactive compounds. The strains of yeast and lactic acid bacteria (LAB) with the best characteristics were selected in order to obtain optimal microbial consortia for the production of pre-ferments to be used in the formulation of bakery products.
The next step was to select the best formulations for the preparation of preferments. Initially, several pre-ferments were produced using singly rye bran and soft and hard wheat by-products, fermented by a microbial consortium composed of *L. curvatus*, *L. mesenteroides* and *P. pentosaceus* as LAB and *S. cerevisiae* as yeast. The results obtained made it possible to select different formulations of by-products of the milling industry to be used for the optimization and development of the final pre-ferment to be used as ingredients in bread making. The formulations, and their water content, were based on fermentation kinetics (the best performance in terms of acidification speed and ratio between yeasts and LAB "optimal 1: 100"), the cohesion of the pre-ferment (which represent an important parameter), organoleptic acceptability (sensory characteristics), and the data relating to the composition of each ingredient used in terms of functional compounds (fiber content, fatty acid composition, antioxidant capacity, etc.).
The selected consortia were also tested on different bran mixtures and their metabolism was evaluated in terms of acidification, growth kinetics, volatile molecule profiles, SCFA and organoleptic acceptability. All the results obtained made it possible to define the optimal formulations of the pre-ferments and the most suitable microbial consortia.
Specifically, 4 mixtures of pre-ferment and 2 microbial consortia were the most interesting for producing pre-ferments as ingredients for bread making. Furthermore, the data obtained allowed to define the optimal level of inoculum (6 and 4 log CFU / g respectively for LAB and yeasts) of each microbial consortium tested.

The selected pre-fermented mixtures were used at 20 and 30% for the production of innovative breads. The results obtained underlined that all the tested preferences gave good performance to the final product. In fact, the percentage of preferment in the composition of the bread is directly proportional to the increase in antioxidant activities and total phenols, moreover, no negative effects were observed on the rheological parameters and, about the sensory aspect, the breads containing preferment highlighted an increase in gustatory intensity and overall acceptability.

5. Conclusions and Future Perspectives

It is widely known that by 2050 the world will be home to 9 billion people; to meet global demand it will be necessary to double food production. For this purpose, alternative protein sources and the enhancement of food by-products are two fundamental aspects to be taken into consideration.
The data showed in this PhD thesis highlighted the good potential of cricket powder hydrolysed by *Y. lipolytica* RO25 and of milling industry's by-products fermented with selected microbial consortia.
In fact, the cricket-based bread obtained from a cricket hydrolysed sourdough was characterized by the highest concentration of polyunsaturated free fatty acids and protein content. The health risks associated with the consumption of crickets were also investigated. The presence of biogenic amines is a risk factor for intoxications and also an indicator of food quality and the results showed a lowest BAI for "RO25 bread" suggesting its high overall quality with respect "Cricket bread". Moreover, the results of the allergenic potential of cricket powder and the subsequent steps will come shortly. Finally, sensory and texture analysis also showed promising results. These results demonstrated that the hydrolysates from *Y. lipolytica*, compared to the no hydrolysed cricket, were able to impart specific sensory and qualitative characteristics to the final product. These aspects make bread containing cricket powder hydrolysate a safe and highly nutritious functional food. On the other hand, reinforcing positive eating experiences may be crucial for repeat consumption and require a gradual familiarisation with the unique sensory properties of the cricket hydrolysed ingredient and the final baked product.
The results obtained from the tests with by-products from the milling industry were very promising. In fact, the bread samples obtained from by-products-based preferments content antioxidant and phenol compounds with functional features, and the sensory aspect makes this product attractive to the consumer. The use of by-products in food formulations has multiple economic, environmental and social advantages.
All the obtained results, applied on an industrial scale, could be a promising starting point to solve the food supply problems in the future. Obviously, a study concerning production costs and environmental impact must be further investigated.

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Heterologous expression of two novel antimicrobial peptides and investigation of their dedicated protease

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This doctoral thesis aims to elucidate advanced hypotheses regarding two new gene clusters attributed to the production of class IIa bacteriocins in *Lacticaseibacillus casei* strains. Despite the prediction of these molecules with bioinformatics systems, the *in vivo* production of these compounds in the target strains was not detected. However, we wanted to evaluate the actual possibility of expression and the real antimicrobial effect through heterologous expression involving exogenous and endogenous genes in the gene cluster under study. This approach made it possible to confirm its effectiveness

Espressione eterologa di due nuovi peptidi antimicrobici e studio della loro proteasi dedicata

Questa tesi di dottorato si rivolge alla delucidazione di ipotesi avanzate riguardanti a due nuovi cluster genici imputati alla produzione di batteriocine di classe IIa in ceppi di *Lacticaseibacillu casei*. Nonostante la predizione di queste molecole con sistemi bioinformatici, la produzione *in vivo* di tali composti non è stata rilevata nei ceppi in esame. Si è comunque voluto valutare l'effettiva possibilità di espressione e il reale effetto antimicrobico attraverso espressione eterologa coninvolgendo geni esogeni e endogeni al cluster genico sotto studio. Tale approccio ha consentito di confermarne l'efficacia.

Key words: bacteriocins, antimicrobial peptide, heterologous expression, fusion protein.

1. Introduction

Increasing the necessity to replace or reduce the usage of antibiotics, a group of proteinaceou compounds named bacteriocins, typically ribosomally synthesized, open a new panorama of applications in food preservation and human health.

Since bacteriocins were isolated from foods, such as meat and dairy products, which generally contain lactic acid bacteria, they have unknowingly been consumed for centuries. Lactic acid bacteria (LAB) are associated with food and the gastrointestinal tract of humans and animals; they are defined as "live microorganisms, which when consumed in adequate amounts, confer a health benefit to the host" (Dobson *et al.*, 2012; van Zyl, Deane and Dicks, 2020). Bacteriocins are among the new bioprotection strategies that have to be considered for fighting pathogens, possibly in combination with other technologies that enhance their antimicrobial effect (Caniça *et al.*, 2019). The effective production of these bacteriocins depends on several other associated proteins (Johnsen *et al.*, 2004; Fimland, Eijsink and Nissen-Meyer, 2002). Class IIa bacteriocin operons contain the precursor bacteriocin gene followed by an immunity gene, sometimes together with an accessory protein gene and finally an ATP-binding cassette (ABC) transporter gene. These peptides are expressed as precursor bacteriocins, meaning they have an N-terminal leader peptide fused to the core peptide. This plays an important role in secretion and cleavage recognition (Van Staden et al., 2019). Furthermore, it is responsible for keeping the peptide inactive inside the cell, preventing damage to cellular components. The leader peptide is cleaved upon secretion by a peptidase domain linked to the ABC transporter, which is responsible for the liberation of the mature peptide. The leader peptides from the class IIa bacteriocins have a common double glycine motif, after which cleavage occurs and the bacteriocins are activated. The cleavage-activation takes place natively as bacteriocins are secreted from the producer cell. The ABC transporter often contains an N-terminal protease domain, which belongs to the C39 class of cysteine proteases (Interpro: IPR005897) (Rice et al., 2014; Michiels et al., 2001) mediate from AMS/PCATs export peptides, where quorum signalling play a role. Bioinformatics studies have demonstrated that AMS/PCAT are widespread in bacterial genomes, both Gram-positive and Gram-negative, and have revealed the concomitant presence of AMS/PCAT ABC transporters and double-Gly leader peptide sequences (Beis and Rebuffat, 2019). Such transporters, would operate in the maturation and transport of a broad range of compounds, not only bacteriocins, such as microcins and other toxins (with or without posttranslational modifications), but also of a large panel of ribosomally synthesized and post-translationally modified peptides (RiPPs). Understanding that each bacteriocin requires a dedicated ABC transporter for secretion, investigation of the recognition mechanism has been partially debunked, showing a broad tolerance in recognizing the leader peptides with varying amino acid sequences and length. This indicated that possibly other bacteriocin ABC transporters require a non-strict structural conformation of the leader peptide rather than conserved amino acid residues for precursor recognition.

The production of bacteriocins is generally low in wild strains. In order to improve the production of bacteriocins, heterologous expression strategies for the production of *E. coli*, *Lactobacillus*, yeast cells, and chemical synthesis

have already demonstrated their effectiveness, opening a newer industrial interest for their applications (Sushida *et al.*, 2018). Many commercially available proteases can be non-specific and not cleave strictly between the leader peptide and the bacteriocin. Additionally, commercial proteases can be a limiting step in high scale heterologous protein production due to their costs. Therefore, cost-effective peptidases are essential for successfully up-scaling the heterologous expression. Identifying and optimising native proteases for industrial bacteriocin production can become the next step in their application and development. Bacteriocins produced by LAB offer various applications for the food, health and pharmaceutical industries. However, the high cost of bacteriocin purification from natural producers, or chemical synthesis, can limit their large-scale production. Bioinformatics mining and alternative heterologous expression can achieve high-level production of native bacteriocins and confirm in a relatively short term the antimicrobial proprieties of novel peptides with predicted characteristics. This study aimed to investigate novel bacteriocin operons *in silico* detected in *Lacticaseibacillus casei* named strains X and Y. Presented here is the development of fluorescent expression systems producing the predicted active peptide never described before. In addition, investigation of the proteases related to the secretion of these peptides was also an interest of the study.

2. Materials and Methods

2.1 Plasmid construction

To construct plasmids, pRSF Duet-1 and pACYC Duet-1 (Novagen, Inc., San Diego, CA, USA) were digested using both multiple cloning sites, and DNAs, extracted from an overnight MRS broth (Biolab, Merck, South Africa) culture of *Lacticaseibacillus casei* strains X and Y, were used as a template. Genomic DNA was extracted using the ZR Fungal/Bacterial DNA MiniPrep kit from Zymo research (USA, CA). PureYield plasmid extraction Miniprep kits were from Promega (USA, WI). Novel bacteriocins and dedicated ABC transporter genes were amplified from strains under study with genes of interest codifying a predicted novel bacteriocin (nucleotidic sequence and primer sets are currently under patenting process). Constructs were transformed into competent *E. coli* BL21 (DE3) (Novagen, Inc., San Diego, CA, USA) and plated onto Luria−Bertani (LB) agar (Biolab, Merck, South Africa) supplemented with kanamycin 50 μg/mL or chloramphenicol 25 μg/mL as selective antibiotics for pRSF- and pACYC-constructs, respectively. Plasmids were isolated and used for subsequent sequencing reactions (CAF, Stellenbosch) and cloning/transformations. After the correct construction vector was confirmed, single colonies were isolated and used in subsequent expression experiments. Several approaches were tested with commercial and non-commercial proteases using tagging bacteriocins with and without Green Fluorescent Protein (GFP) and Red Fluorescent Protein (RFP). All restriction enzymes, DNase, RNAse, Q5 polymerase and T4 ligase were from New England Biolabs (USA, MA). Vector design and tested in this study were: pRSF-GFP (control), pRSF-GFP-6Lbact (GFP fused with leaderless bacteriocin), pRSF-GFP-nis-bact6n (GFP fused with nisin leader-bacteriocin), pRSF-GFP-nis-bact3n (GFP fused with nisin leader-bacteriocin), pRSF-RFP-nisp- (RFP fused with truncate nisP protease). To investigate the bacteriocin ABC-transporter functionality, pACYC plasmid was used as a template. Constructs confirmed by sequencing were: pACYC- 6L C39X (GFP fused with nisin leaderless-bacteriocin and ABC-transporter from operon X) and pACYC- 6L C39Y (GFP fused with nisin leaderless-bacteriocin and ABC-transporter from operon Y).

2.2 Protein expression and purification

Overnight cultures of *E. coli* expressing bacteriocin and/or protease were used to inoculate (1.0%, v/v) 400 mL of Terrific broth supplemented with antibiotics and incubated at 37 °C under constant aeration until an OD_{600} nm of 0.6 was reached. Thio-B-Dgalactopyranoside (IPTG) Sigma-Aldrich (Milan, Italy) was added to the culture at 1 mM and it was incubated at 26 °C for 24 h. Cells were harvested (7025 g, 20 min at 4 °C), and the pellet resuspended in 40 mL start buffer (SB) supplemented with lysozyme 1 mg/mL Sigma-Aldrich (Milan, Italy), DNase 1 U/mL and RNase 6 U/mL New England Biolabs (Milan, Italy). The cell suspension was incubated on ice for 30 min and disrupted by sonication on ice (3 times at 70 % power output, 50 % pulses for 3 min). Lysed samples were centrifuged (15 870 xg, 60 min, 4 °C). The lysate cell-free supernatant was adjusted to a final imidazole concentration of 40 mM and loaded onto Ni-Sepharose 6 Fast Flow columns pre-equilibrated with SB40 (SB buffer containing 40 mM imidazole). Columns were washed with SB40 and His-tagged fusion proteins eluted using SB500 (SB buffer containing 500 mM imidazole). The eluent was diluted 1:50 with 50 mM Tris pH 8.3 buffer and passed through the DEAE Sepharose Fast Flow anion exchange with 200mM NaCl with 50 mM Tris pH 7.0. Fusion protein concentrations were determined using a BCA protein assay Sigma-Aldrich (Milan, Italy). The cleaved bacteriocin was diluted 1:6 with 75% Acetonitrile and incubated for 30 min under agitation (150 rpm) at 26 °C. The top layer containing the bacteriocin was spun at 3500 rpm for 4 min and then freeze-dried. Thereafter, a resuspension of concentrated bacteriocin in water suitable for HPLC was performed and loaded onto a Poroshell 120 EC-C18 HPLC column (120 Å, 4 μm, 4.6 mm × 150 mm, Agilent) and eluted with a linear gradient created with 0.1% (v/v) trifluoracetic acid (TFA) in analytically pure water (eluent A) and 0.1% (v/v) TFA in acetonitrile (eluent B). The flow rate was set at 1.3 mL/min, and the elution program utilized was as follows: 10% eluent A from 0 to 3 min (initial conditions), 3–30 min linear gradient from 10 to 90% eluent B. Separation was performed on an Agilent 1260 Infinity II LC system. Peaks detected were collected during elution and tested for antimicrobial activity using agar well diffusion assay.

2.3 Antimicrobial activity

Antimicrobial activity was tested by agar well diffusion assay. Briefly, overnight cultures of the strains to be tested for susceptibility to antimicrobial compounds were inoculated at approximately 1 x 10^7CFU/ml (indicator strains: *Listeria monocytogenes* EGD-e) into 45 ml of soft BHI agar (0.7% agar) (Biolab, Merck, South Africa). After agar plate solidification, a well was formed by the back side of a pipette tip, and then 100 µl of cleaved bacteriocin was added to the well. Plates were incubated at 30˚C overnight until clear visible zones were observed.

2.4 Scanning electron microscopy (SEM)

A single colony from a pure culture of *Listeria monocytogenes* EGD-e was inoculated in 10 ml of sterile BHI broth and incubated at 37 °C with shaking (120 rpm). After 24 hours of incubation, aliquots of 50 µl were added in 50 µl of sterile BHI broth (1:1). A coverslip treated with UV light for 30 min was used as support to prepare the sample for scanning electron microscopy. A final volume of 100 µl of diluted *Listeria monocytogenes* EGD-e culture (approximately 10^7 CFU/ml) was added to the coverslip top surface previously positioned in a sterile petri dish (35×15 mm). A total of 100 µl of purified peptide in 50 mM Tris and 200 mM NaCl buffer were added to the coverslip. Subsequently, the coverslips were incubated at 26 °C overnight for each treatment.

After this incubation period, the cells treated were fixed with 4 % paraformaldehyde (PFA) in PBS (pH 7.2) for 16 h at 4 °C. Coverslips were stained with 2 % OsO_4 for 30 mins, washed 3X with dH20, dehydrated in a graded ethanol series (20%, 50%, 70%, 90%, 100%, 5 min each) and sputter-coated with 50 nm Gold/Palladium. SEM was conducted using a ThermoFisher Apreo FESEM at a beam strength of 2 kV and a current of 20 nA.

3. Results and Discussion

The two stains of *Lacticaseibacillus casei* X and Y shared a unique operon structure different from other bacteriocins already described. However, the main difference among them was that a different ABC transporter containing a C39-like motif indicated the same processing bacteriocin machinery. Interestingly, the proteolytic domain in both operon, intermembrane helix component, and ATPase were missing in one operon. The illustration in **Figure 1** highlighting the differences between the operon organization and the ABC transporter.

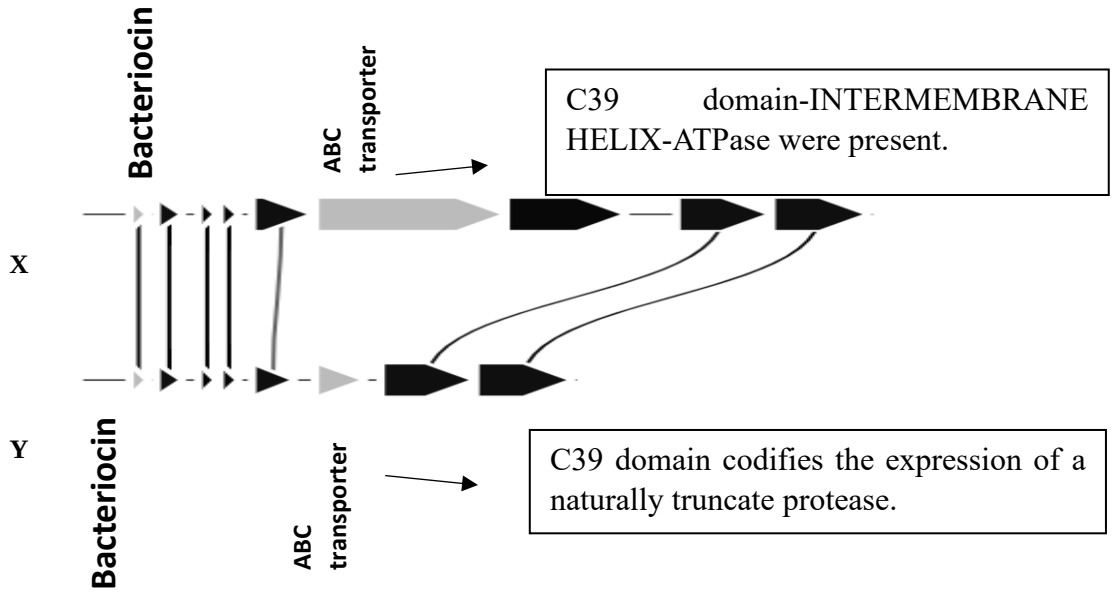


Figure 1: Bacteriocins operon organization witch differ in the present of a full ABC trasporter. In both cases C39 protease domain share the same aminoacidic sequence. X and Y indicate the operon structure found in Lacticaseibacillus casei X and Y in this study.

A broad typology of structural genes were already described for different bacteriocins, which can share a high similarity and mode of regulation. On the other hand, bacteriocins' mechanism of secretion and activation can be a complex topic not fully understood. Pereira *et al.* described that bacteriocin-containing leader and ABC transporter are the fundamental genes required for bacteriocin production (Mesa-Pereira *et al.*, 2017). This findings are essential to optimize bacteriocin production without using an exogenous protease, a significant cost voice in large-scale heterologous expression. The present study showed that the two novel bacteriocins predicted by bioinformatic tools and belonging to class IIa, were not *in vivo* expressed by the *Lacticaseibacillus casei* strains X and Y. Molecular approaches of gene fusion and heterologous expression were applied to confirm the antimicrobial proprieties with NisP truncate protease present in the nisin operon (Montalbán-López *et al.*, 2018). This is, to our

knowledge, the first description of the heterologous production of class IIa bacteriocin with a replaced leader using a lantipeptide protease. A fusion protein GFP – Bacteriocin and truncate NisP-Mcherry after purification were added to allow the releasement of the bacteriocin to explicate the antimicrobial activities tested. A summary displaying the effective bactericidal proprieties of the heterologously expressed peptides is shown in **Figure 2**.

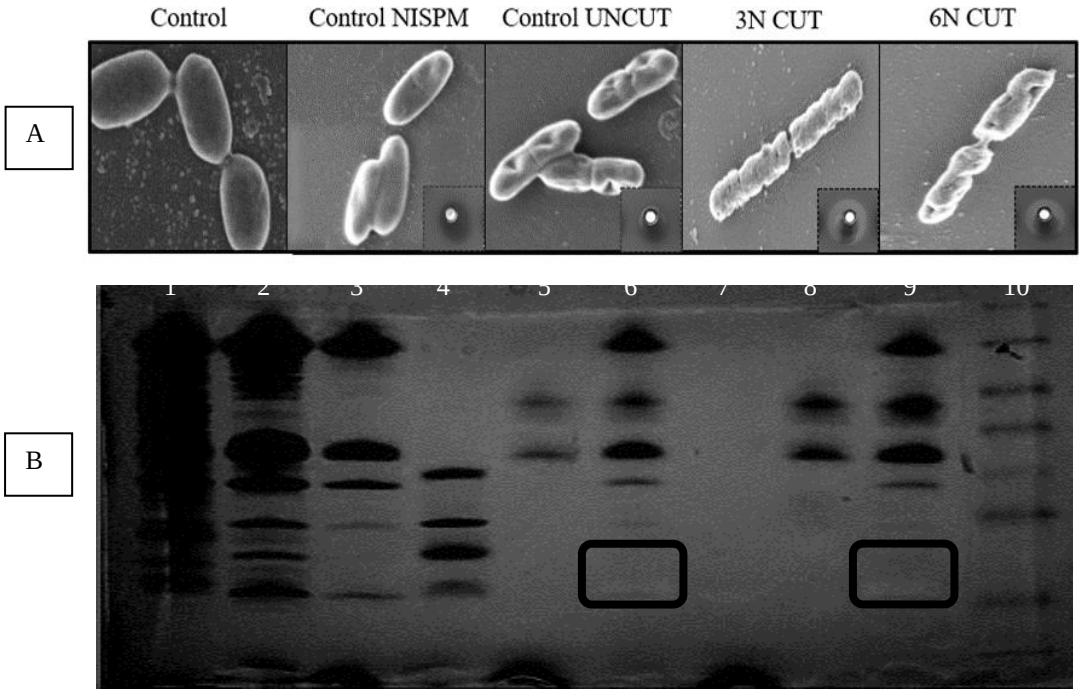
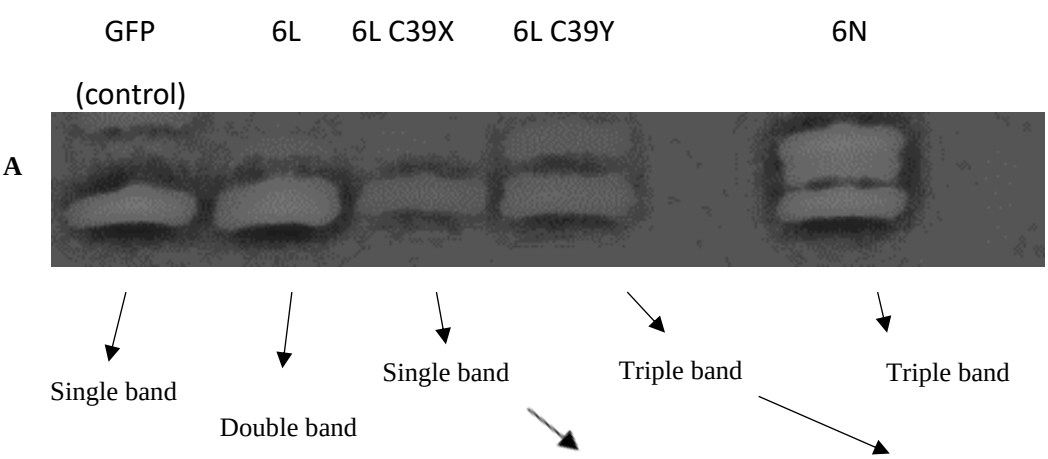


Figure 2:

A: *Scanning electron microscope (SEM) image showing the cellular membrane poration after the treatment with the antimicrobial peptide inducing destabilisation of the biological activity and causing cellular death. Inhibition halo is also show using agar well spot plate techniques. 6N and 3N were the novel bacteriocin.*

B: *Overlayed SDS PAGE showing the soluble fractions after bacteriocin cleavage reactions gels highlighedt the confirmation of antibacterial activity against Listeria monocytogenes EDG-e, the target strain used for the overlay. LANES: 1. Lysis of E. coli BL21 producing NispMcherry; 2. Elution histag purified NispMcherry; 3. NispMcherry after ion exchange purification; 4. GE Healthcare Rainbow™ Molecular; 5. 3N uncut; 6. 3N cut with NispMcherry; 7. Empty; 8 6N uncut; 9. Cut with NispMcherry; 10. PageRuler Unstained Low Range Protein Ladder.*

The proteolytic and bacteriocin secretion functionality of the novel bacteriocin ABC-transporter using leaderless-bacteriocins were also investigated. No cleavage of purified proteases was detected under non-denaturing and denaturing protein purification conditions, suggesting that misfolding can occur if these proteases are not positioned correctly in the cell membrane. However, by adding the plasmid construct in MCS2 independently to the long and short gene transcribing the Bacteriocin ABC transporter, we could determine bacteriocin production in *E. coli* directly secreted outside the cell. This result was obtained only for the ABC transporter containing C39 domain-intermembrane helix-ATPase, suggesting all these proteins were necessary for the correct bacteriocins maturation and secretion. Proteolytic activities were also detected by integrating GFP genes fused with leaderless bacteriocins. **Figure 3** reports the bacteriocin secretion from *E.coli*, and SDS PAGE showed the difference in GFP dimerization, which was influenced by the presence of fused bacteriocin.



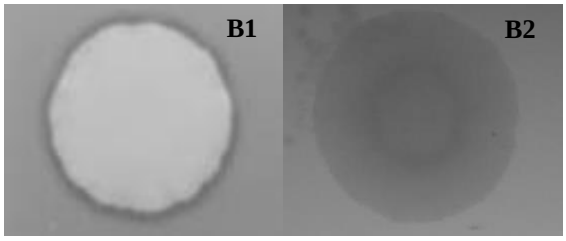


***Figure 3**:*
***A**- Comparison of GPF-bacteriocin from E.coli BL21.Co-espression of leaderness bacteriocin with a ABC – transporter from operon X was confirm to be able to cleave the bacteriocin (single band).*
***B1**- Overlayed with L.monocytogenes E.coli BL21 colony co-espressing leaderness bacteriocin with a ABC – transporter from operon X showing bacteriocin secrition.*

B2- Overlayed with L.monocytogenes E.coli BL21 colony co-espressing leaderness bacteriocin with a ABC – transporter from operon Y showing no bacteriocin secrition.

4. Conclusions and Future Perspectives

The presented results suggested that this system could be useful for expressing new bacteriocins *in silico* described, opening the door to the heterologous expression and harnessing the potential of new bacteriocins in the future. Confirmation of two novel bacteriocins with unique operon organization was first detected and their potential applications as bio preservatives against Gram (+) pathogens bacteria, such as *Listeria monocytogenes*, was proved. A novel approach in terms of bacteriocins production is one of the challenges required for implementing their application in food biopreservation. More investigation is required concerning the mode of regulation of these novel bacteriocins operon. A proven limitation is the presence of a naturally truncate ABC – transporter unable to process and secrete the bacteriocin in the native strains.

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What is the best time to harvest red grapes cv. Nebbiolo destined to withering? A three-years study

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This three-year study (vintages 2019-2021) dealt with the evaluation of the combined influence of different ripeness levels and withering rates on the standard chemical composition and phenolic profile of Nebbiolo winegrapes.

Quale è il momento migliore per raccogliere le uve destinate all'appassimento? Uno studio triennale

Questo studio triennale (vendemmia 2019-2021) ha riguardato la valutazione della combinazione di diversi livelli di maturazione delle uve e diverse durate del processo di appassimento sulla composizione chimica e sul profilo fenolico di uve Nebbiolo.

Key words: Postharvest withered grapes; phenolic compounds; withering process; reinforced wines; special wines.

1. Introduction

Sforzato di Valtellina DOCG ("Denominazione di Origine Controllata e Garantita" protected designation of origin) is a reinforced wine with a great traditional value produced in the Valtellina alpine valley (Lombardy, Italy) with partially withered red grapes of *Vitis vinifera* (L.) cv. Nebbiolo (Nicoletti et al., 2013).
Grape skins and seeds contain several classes of phenolic compounds which are strictly associated with red wine quality (Harrison et al., 2018), and the modifications occurring during withering have a great role in characterizing the wines produced with this technique. As previously demonstrated, the grape ripeness degree at harvest and the withering process strongly influences the physicochemical characteristics of grapes, affecting their mechanical properties, must composition and the content of secondary metabolites (Mencarelli et al., 2013; Poni et al., 2018; Rolle et al., 2013). Although the importance of these two variables have been extensively studied separately in recent years (Delgrado Cuzmar et al., 2018; Kontoudakis et al., 2009; Sanmartin et al., 2021), their combined effect still needs to be better understood.
The aim of this research is to assess the combined influence of different ripeness levels and withering rates on the standard chemical composition, mechanical properties, and phenolic profile of winegrapes in order to provide new insights and approaches to the management of withering, looking for the valorization of grape potentialities. The present study is part of a wider project aimed at the valorization of Sforzato di Valtellina DOCG special product.

2. Experimental Procedure

During this three-vintages study (harvest years 2019-2021) three binomials have been assessed:

(i) early harvest/long withering (EL)
(ii) medium-term harvest/medium-term withering (MM)
(iii) late harvest/short withering (LS).

Grape samples of cv. Nebbiolo (*Vitis vinifera* L.) were harvested from two vineyards located at the two opposite ends of the vine growing area in the east-west oriented valley -Valtellina upper (vineyard code A) and lower valley (B)- at three different ripeness levels following the soluble solid content (°Brix) and placed in single-layer plastic crates into a typical 'fruttaio' post-harvest dehydration room, following the wine type DOCG designation guidelines. As reported by the wine type guidelines (Mipaaf, 2014), the withering lasted until the 1st of December. Consequently, depending on the harvest

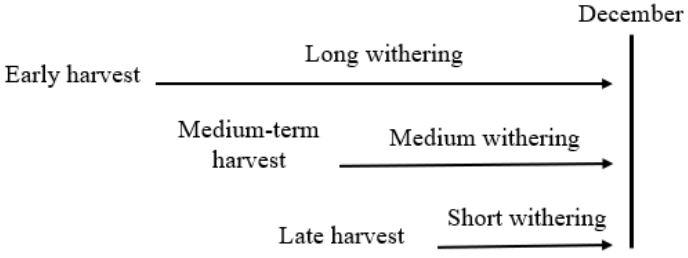

Figure 1 *Experimental plan: the three binomials.*

date, the withering period had different length. A schematic view of the experimental plan is shown in Figure 1. Grape must composition, mechanical parameters, and extractable phenolic profiles of grape skins and seeds were studied before and after the withering process.

3. Materials and Methods

Grape samples were collected in Valtellina (Lombardy, Northern Italy) and subjected to the experimental plan described in the next section. The evaluation of the standard parameters (°Brix, pH, total acidity) was carried out according to OIV methods (OIV, 2016). Reducing sugars were evaluated using a HPLC system equipped with a refractive index detector (Giordano et al., 2009). Phenolic compounds extractions from skins and seeds were performed separately following the method of Di Stefano and Cravero (1991) with slight modifications. Extractable total phenolic compounds (IPT) and anthocyanins (AT) were determined by spectrophotometric methods (Petrozziello et al., 2018). Mechanical properties (i.e. skin hardness and thickness) were evaluated following the method described by Río Segade et al. (2008). Statistical analysis was done using the R statistic software version 3.6.2 (R Foundation for Statistical Computing, Vienna, Austria). To identify the significant differences among the variables studied by one-way analysis of variance (ANOVA), the Tukey HSD post hoc test at $p<0.05$ was used.

4. Climate conditions

The weather conditions of the three consecutive harvest years studied (2019-2020) were very different from each other (Fig. 2).

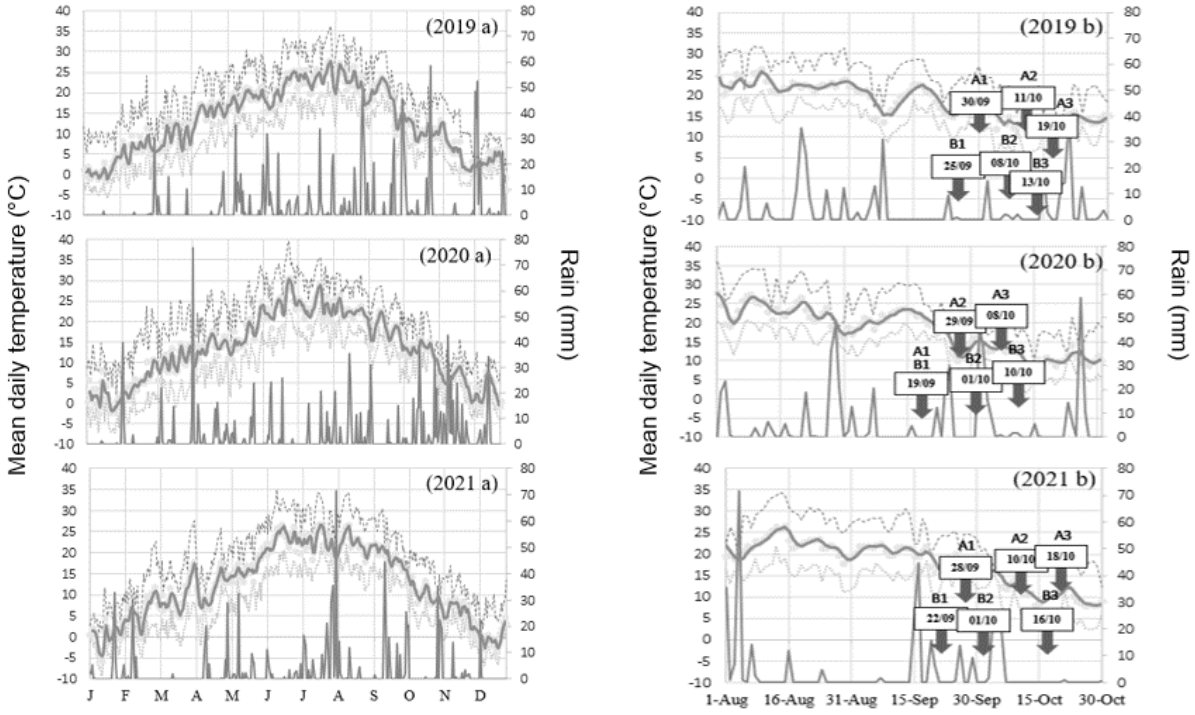

Figure 2 *Minimim (·····), maximum (·····) and average (—) daily temperature and rainfall (—) of the three consecutive harvest years studied (2019a, 2020a, 2021a), harvest times and weather conditions of the months near the harvest (2019b, 2020b, 2021b). The meteorological data were provided by ARPA Lombardia (Regional Agency for Environmental Protection of Lombardy, Italy).*

In general, the weather data recorded in the period near to the harvest time (Fig.2, b) show that 2019 was the hottest of the three years considered, 2020 was the wettest and 2021 resulted the driest and coolest harvest year.

5. Results and Discussion

5.1 Grape must composition

The must composition of fresh and withered grapes for the harvest 2019, 2020, and 2021 is reported in Figures 3 and 4. As Figure 3 shows, the longer is the withering process time, the greater is the percentage increase of sugars in withered grapes with respect to the fresh ones.

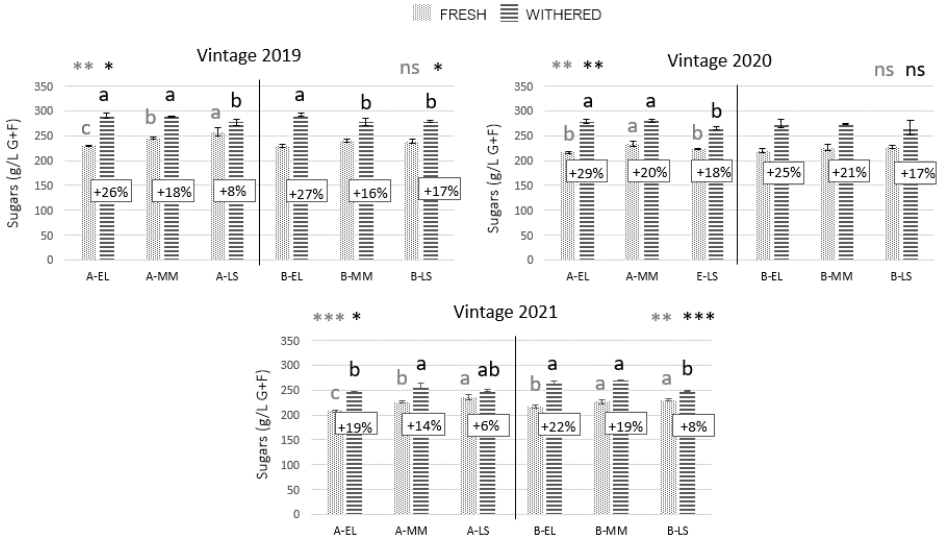

Figure 3 *Reducing sugars content (g/L) in fresh and withered grape musts, vintages 2019-2021.*

As can be seen in Fig. 4, the concentration effect balances the losses of acidity (expressed in g/L as tartaric acid) detected in withered grapes compared with the fresh ones. Consequently, at the end of the withering process, for both the vineyard studied during the three years of experiments, the EL thesis showed the higher sugar contents and total acidity values.

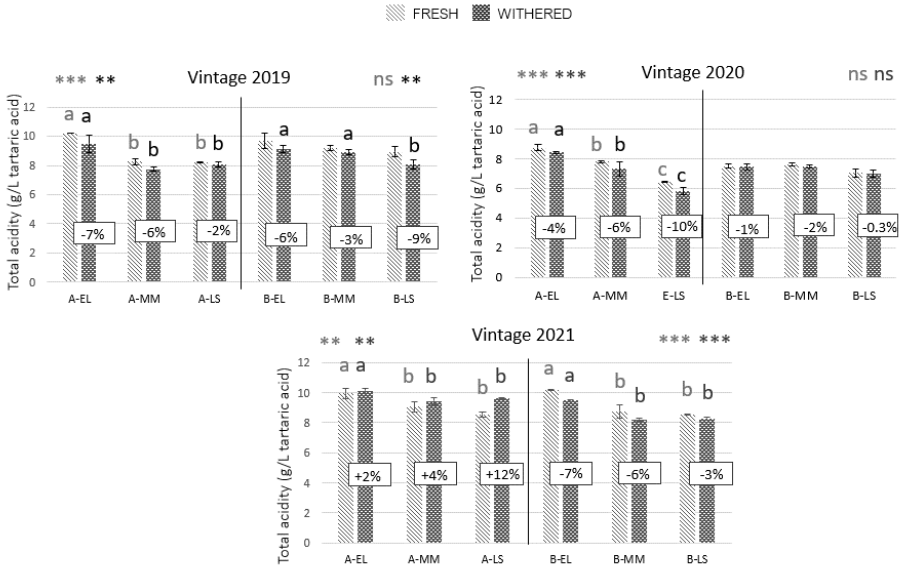

Figure 4 *Total acidity (g/L as tartaric acid) in fresh and withered grape musts, vintages 2019-2021.*

5.2 Phenolic compounds and mechanical properties

The content of extractable polyphenols in seeds seemed to show a decreasing trend by leaving the grapes on the plant longer, whereas this impact increased considerably after withering with respect to fresh samples, due to the concentration effect (Fig. 5). This tendency has been observed for all the three years of experiments, but the differences were not always statistically significant due to the sample variability, which was higher in the 2021 vintage, the driest of the three years studied, confirming the greater variability in the composition of grapes observed in dry years (Ramos et al., 2015).

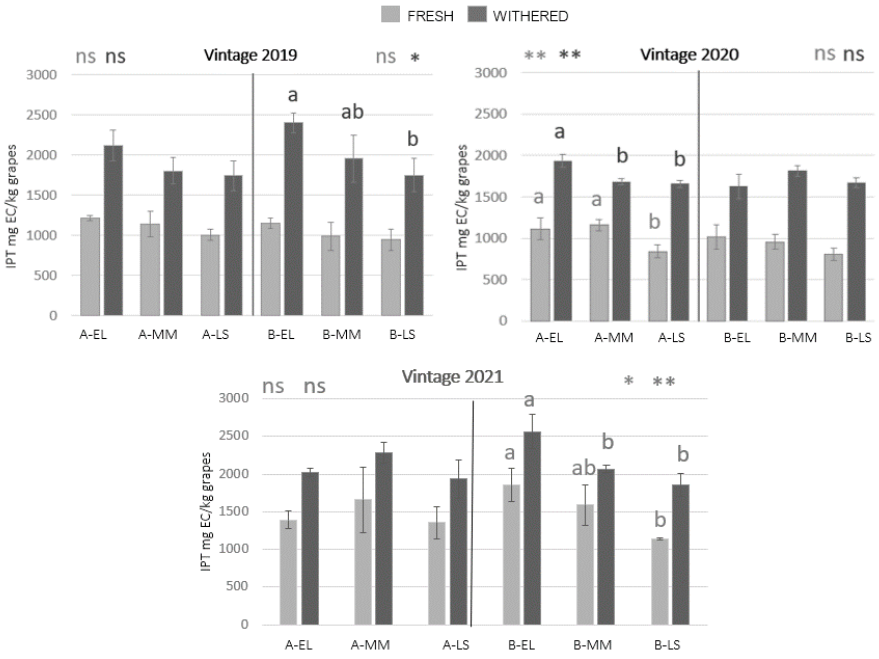

Figure 5 *Seeds extractable phenolic compounds (mg epicatechin/kg of grapes).*

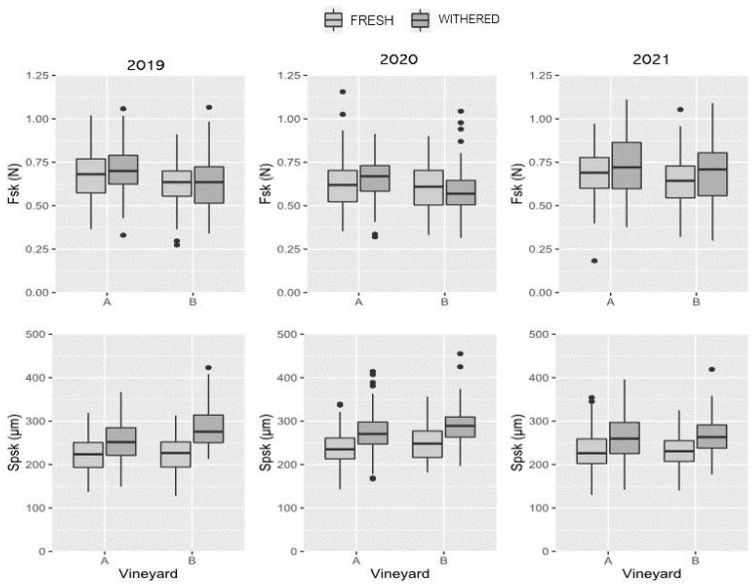

Figure 6 *Skins mechanical properties: berry skin maximum break force (Fsk) and skin thickness (Spsk).*

The skin phenolic compounds were less influenced by harvest period, but their concentrations on grape weight increased after withering (data not shown). During the first two years of experiments, skin extractable anthocyanins experienced a distinct trend for the two vineyards assessed: their concentration increased in withered samples from the upper-valley vineyard and decreased in those from the lower-valley. The grapes mechanical properties may have influenced this aspect, as previously demonstrated (Rolle et al., 2011). In 2021 this tendency has been less remarkable than the previous vintages probably due to the higher variability observed for the skin break force (Fsk) as shown in Fig. 6.

6. Conclusions and Future Perspectives

In conclusion, harvest time and withering length can be modulated according to the desired oenological objective, searching for the valorisation of grape potentialities. In general, early/medium harvest and long/medium withering gave the higher phenolic contents, particularly for seeds polyphenols, although the vineyard location and the weather conditions of the year influenced the withered grape phenolic characteristics. Therefore, the choice to anticipate the harvest time for grapes destined to withering process could be interesting in view of performing long wine ageing, starting from grapes characterized by more acidity and phenolics compounds, as well as for practical and grape's health reasons. The great weather differences among the three vintages studied allowed to highlight the common trends in very different situations. However, for the same reason, further studies are needed to better clarify the impact of the climate conditions of the year on the combined effect of the two variables studied.

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Use of response surface methodology to investigate the effect of partial substitution of sodium chloride with *Salicornia ramosissima* powder in wheat dough and bread

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This PhD thesis dealt with the investigation of the partial substitution of sodium chloride (NaCl) with the glasswort plant (*Salicornia ramosissima*) in wheat dough and bread and its impact on the rheological and technological properties respectively. Response surface methodology (RSM) was applied to obtain the optimal combination of savory enhancer amount and NaCl/*Salicornia* substitution ratio. Results showed that 1.8% of the first and 68% of the later is the most convenient composition. The use of Salicornia as a potential substitute of NaCl seems like a promising approach. However, such substitution is limited to a specific range (from 50% to 70%).

Salicornia ramosissima: può essere una valida alternativa al cloruro di sodio? Un caso studio con l'ultilizzo della metodologia delle superficie di risposta

Questa tesi di dottorato ha lo scopo di valutare l'effetto della sostituzione parziale e totale del cloruro di sodio con la Salicornia sulle proprietà reologiche degli impasti e sulle proprietà tecnologiche dei pani di farina di frumento tenero riformulando la quantità di sale (0,6, 1,2 e 1,8%) e il rapporto di sostituzione NaCl/Salicornia (0, 50 e 100%) mediante l'impiego della Metodologia della Superficie di Risposta (RSM). L'applicazione della funzione di desiderabilità ha consentito di evidenziare che i livelli ottimali delle due variabili indipendenti oggetto di studio erano pari all' 1,8% di quantità di sale e al 68% di sostituzione del sale con la Salicornia. L'uso della Salicornia come potenziale sostituito del sale sembra promettente. Tuttavia, tale sostituzione è limitata ad un intervallo specifico (da 50 a 70%).

Key words: Sodium intake reduction; *Salicornia ramosissima*; Response Surface Methodology and Optimization.

1. Introduction

The actual challenge is to achieve lower levels of sodium chloride that light the current health care needs in a timely manner while maintaining the microbiological safety and the quality of the product. As recently summarized in the review by Codină et al., (2021), several approaches have been proposed to reduce sodium intake in the daily diet, including the use of different chloride salts, flavor enhancers, or new technologies such as encapsulation. Recently, several edible plants are arousing interest after being identified to be rich with healthy component that may readily be integrated into the food sector. *Salicornia*, (commonly known as glasswort and sea beans), is an halophyte fiber rich plant with a valuable source of essential minerals, proteins, antioxidant compounds and vitamin A, in addition to its salty taste (Barreira et al., 2017). To the best of our knowledge, this is the first attempt to reduce sodium chloride in a baked product such as bread using *Salicornia*.
This oral communication reports the main results of the following conducted activities:
A1) Dough rheological properties measurement;
A2) Bread technological properties measurement;
A3) Response Surface Methodology optimization method or Desirability.

2. Materials and Methods

2.1 Materials

A commercial common wheat flour (Triticum aestivum) type 00 of 14% moisture, 11.6% protein, 3.8% fiber, 1.5% lipids, and 0.10% ash was provided by SIMEC Spa (Santa Giusta, OR, Italy). Fresh compressed yeast and sodium chloride were purchased from a local supermarket. *Salicornia ramosissima* powder was provided by the Salina Greens company (Alcochete, Setúbal, Portugal).

2.2 Dough rheological analysis

The rheological assessment of doughs was performed by using empirical and fundamental methods.

- The behavior of the dough during development and mixing was tested with the help of Farinograph-TS (model 827507, Brabender, Duisburg, Germany) equipped with a mixer S 300 N according to the standard procedure AACC, with slight modifications. The following farinograph parameters were determined: water absorption (WA, %, amount of water required to give a consistency of 500±20 Brabender Units (BU)), dough development time (DDT, min, time to reach maximum consistency), dough stability, (ST, min, time

to maintain the maximum dough consistency), and mixing tolerance index (MTI, BU, decrease in dough consistency at 5 min after the maximum peak).

- The F3 Rheofermentometer (Chopin, Villeneuve-La-Garenne, France) was used to measure dough characteristics during proofing. The parameters obtained from the Rheofermentometer curves included: the maximum height achieved by the dough (Hm, mm), the time required to obtain the maximum dough rise (T1, min), the dough height at the end of the test (h, mm), the decrease in the dough volume at the end of the test ((Hm-H)/Hm), %), the total quantity of gas produced (VTOT, mL), the volume of the gas retained in the dough at the end of the test (VRET mL), and the gas retention coefficient calculated as VRET/VT (%).
- Frequency sweep tests were carried out using a rotational rheometer (model MCR-92, Anton Paar GmbH, Inc., Graz, Austria) equipped with both a Peltier-temperature-control system and a parallel-plate geometry (50 mm diameter, 1 mm gap). Frequency sweep measurements were performed in the range of 0.1–10 Hz at constant strain (0.01%), which fall within the linear viscoelastic region idendified with a preliminary strain sweep test at a constant frequency of 10 Hz and a strain varying from 0.001 to 100 s-1. The storage modulus (G′), loss modulus (G″), and loss tangent (tan δ) were recorded as functions of frequency.

2.3 Bread measurement

Bread technological parameters were evaluated though the subsequent methods:

- Bread loaves volume was measured following the AACC 10-05.01 rapeseeds displacement method (AACC Standard 10-05.01). The specific volume was calculated as bread volume (mL) over bread weight (g).
- Evaluation of bread textural properties was carried out by means of texture profile analysis (TPA) on 20 mm wide slices using a texture analyzer (Model TA-XT plus, Stable Micro Systems, Surrey, UK) equipped with a 36 mm diameter cylindrical probe. From the resulting force-time curves, the following parameters were obtained: hardness, cohesiveness, springiness, resilience, gumminess, and chewiness.
- The crust and crumb color measurements were taken instrumentally on the day of baking by using a tristimulus colorimeter (Minolta CR-300, Konica Minolta Sensing, Osaka, Japan) equipped with a measuring head CR-300. The results were expressed in accordance to the CIELab measuring system (measurement area ø = 8 mm, illuminant D65) and the parameters acquired were L (brightness; analyzed sample was black when L = 0 or white if L = 100), a* (-a* means greenness and +a* redness), and b*(– b means blue; + b means yellow).

2.4 Experimental design

A response surface methodology (RSM) with a 2^3 face centered factorial Central Composite Design (CCD) was setup and analyzed using Design Expert Software Version 10 (State-Ease Corporation, Minneapolis, MN, USA) to investigate the influence of two applied independent factors, i.e., savory enhancer amount (X1) (0.6, 1.2 and 1.8 %) and NaCl/Salicornia ratio (X2) (0, 50 and 100 %), on the techno-functional parameters (dependent variables) of wheat dough and bread. The CCD experimental matrix consisted of 13 runs with five additional center points replicated to ensure reproducibility as well as to assess the experimental error of the model. Design Expert Software was also used to find the optimum levels of the independent variables by means of the desirability function. This optimization technique is considered an efficient method to optimize multiple responses simultaneously with optimal factor settings. The approach proposed by Derringer and Suich (1980) is to transform each response into an individual desirability index “dn”, which value varies between 0 and 1: 0 corresponds to a completely undesired response, while 1 to a response that reached its target. The individual desirability functions are forwardly combined into one composite response, which gives the total desirability function “D” (0<D<1).

3. Results and Discussion

3.1 Dough rheological analysis

3.1.1 Dough mixing properties

Analysis of variance and fit statistics showed that the selected quadratic models were significant and effectively represented the experimental data for some mixing properties, such as WA, DDT, and ST, as evidenced by the fitting levels of R^2 and Adj-R^2 that varied from 0.84 to 1.00 and from 0.75 to 0.99 (Table 1).

According to our findings, we stated that when the sodium chloride is partially or totally replaced with the *Salicornia* powder (with the exception of the samples prepared with NaCl/*Salicornia* ratio of 50% at the lowest level) the WA rises with a more noticeable effect as the amount of the savory enhancer used increases. Such an increase was possibly owed to the hygroscopic nature, in addition to the high fiber content of the *Salicornia* powder. Almeida et al (2010) found similar results for the WA of wheat flour doughs enhanced with dietary fibers of various origin.

NaCl replacement with *Salicornia* powder in the dough formula significantly prolonged DDT (up to 13.8 minutes at the center points), particularly at the middle levels of both factors. A possible justification could be that the richness in fibers of *Salicornia* powder altered the hydration of the flour components, along with the gluten structure formation, due to its ability to form hydrogen bonds with water (Gómez et al., 2003).

Dough processing behavior is also described by ST and the MTI. We distinguished that the use of Salicornia powder in combination with NaCl did not weaken dough stability, in fact, it improved its tolerance to mixing, as shown by the low MTI values recorded (9 BU at the center points), unrelatedly to the amount of flavor enhancer used. In contrast, the exclusive addition of *Salicornia* powder reduced dough stability, that decreased proportionally as the incremented amount, causing a weaker protein network with minor resistance to mechanical damage. The ability of NaCl to increase the strength of the dough has been reported by several authors (Beck et al., 2012; Codină et al., 2021). However, this capability of salt could be altered by both the presence and concentration in the dough formulation of other types of ions (Jekle et al., 2019). For instance, the bivalent cations Ca^{2+} and Mg^{2+}, introduced with *Salicornia* at the highest substitution levels.

3.1.2 Small deformation rheological measurements

Small deformation oscillatory frequency sweeps and strain amplitude sweeps were executed to study the effects of the two independent factors on the rheological properties of the formed wheat flour doughs. Regression models were showed to be highly significant for both the storage modulus (R^2: 0.92 and Adj-R^2: 0.86) and loss modulus (R^2: 0.81 and Adj-R^2: 0.71) (table1). In all the samples, the values of G′ were higher than those of G″, which confirm the typical elastic-like behavior of wheat flour doughs. The increasing level of savory enhancer leads to an intensification of the elastic modulus, but with a more pronounced effect at intermediate levels of NaCl/*Salicornia* substitution. So far, in the reported literature, the effect of sodium chloride on the viscoelastic properties of wheat flour doughs has led to contrasting results. Indeed, while some authors (Salvador et al., 2006; McCann and Day, 2013) have reported small and significant decreases in the values of the dynamic moduli of doughs at incremented sodium chloride addition, others detected a positive effect on the elastic component alone, without impacting the viscous component (Lynch et al., 2008).

3.1.3 Rheofermentometer analysis

From all the dependent variables analyzed through Rheofermentometer parameters only three are considerably affected ($p < 0.001$) by the levels of NaCl substitution in wheat flour. Regression models for T_1, h, and (Hm-H)/Hm values showed a significant effect with a coefficient of determination R^2 varying between 0.81 and 0.86 and Adj-R^2 ranging from 0.77 and 0.83 (table 1). In detail, the addition of swelling amounts of salt extended the time to reach the maximum dough development (T_1), with a prominent effect when NaCl was added separately. The dough height at the end of the test (h) rose to its maximum with 0% of substitution and 1.8% of savory enhancer amount, while the gradual addition of *Salicornia* flattened sensibly the final dough height. Yet, the maximum decrease in the dough volume at the end of the test ((Hm–h)/Hm ratio) has been detected at a high NaCl/*Salicornia* substitution level (40-100%) and the lowest amount of savory enhancer. On the contrary, the two independent variables had no influence on all parameters related to carbon dioxide production and the ability of the doughs to retain the gas produced.

Table 1. *Regression coefficients of predicted polynomial models for dough properties*

	Constant	X_1	X_2	X_1X_2	X_1^2	X_2^2	Lack Of fit	R^2	Adj-R^2
Mixing properties									
WA	53.53	-0.14	-0.02^{***}	0.01*	-	0.0001^{*}	-	0.88	0.82
DDT	-19.14	35.1^{*}	0.29	-	-12.59^{*}	-0.002^{**}	2.94^{ns}	0.84	0.75
ST	19	0.08**	0.08^{***}	-0.012^{*}	-	-0.001^{***}	12.15	1.00	0.99
MTI	22.67	-	-0.60^{***}	-	-	0.008^{***}	14.75	0.77	0.72
Viscoelastic properties									
G′	12165	10350^{***}	28.01	46.51^{*}	-3572	-0.77^{*}	0.44^{ns}	0.92	0.86
G″	6170	604^{**}	20.97	16.53	-	-0.39^{**}	7.29	0.81	0.71
Leavening properties									
h	2.70	13.00^{***}	-0.04	-	-	-	1.78^{ns}	0.86	0.83
(Hm-H)/Hm	57.29	38.10^{***}	0.12^{*}	-	-27.13^{*}	-	3.51^{ns}	0.86	0.82
T_1	79.54	26.94^{***}	-0.17^{*}	-	-	-	1.64^{ns}	0.81	0.77

ns: not significant ($p < 0.05$); * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.
X_1: Savory enhancer amount; X_2: NaCl/*Salicornia* ratio.

3.2 Bread measurement

3.2.1 Specific volume

One of the most important characteristics to assess bread quality is the specific volume. For instance, soft and well-developed bread loaves are distinguished with a high specific volume. The impact of the gradual substitution of salt with *Salicornia* powder resulted highly significant ($p < 0.001$) with the following coefficients R^2: 0.97 and Adj-R^2: 0.95 (table2). This parameter tends to increase when NaCl/*Salicornia* ratio exceeds the 40% independently of the savory enhancer amount. These results were in fact expected knowing that lowering salt content induce a higher specific volume due to a better dough fermentation and a greater gas production. Similar findings were observed in other articles that treated the similar topics (Pasqualone et al., 2019; Beck et al.,2012).

3.2.2 Textural properties

The consumer acceptance of bread relies on several factors, most critically its texture that could be evaluated in various ways, for instance texture profile analyses (TPA). By dint of the regression models, we were able to assess the significant variance of hardness ($p < 0.01$), gumminess, and chewiness ($p < 0.05$) with R^2 ranging between 0.78 and 0.80 and Adj-R^2 equal to 0.7, 0.65 and 0.63, respectively (table2). Together, they attain the maximum with 0% of NaCl/*Salicornia* substitution and over 1% of savory enhancer amount. In sum, bread hardness, gumminess and chewiness are likely to be more perceived with the expansion of salt quantities. In accordance with these findings, Fayaz et al (2021) mentioned that the increase of chewiness is correlated with a strong dough gluten network which in turn is attributed to NaCl. On the contrary, Lynch et al., (2009) stated that no significant change was detected for bread hardness with different salt levels and that only bread with 0% salt was significantly harder.

3.2.3 Color parameters

The brown color of bread is a valued characteristic required for a better quality. The given color is proved to be associated with the Maillard reaction (reaction between free reducing sugars and proteins) formed during heating processes. Both crust and crumb colors were evaluated; for the first, only b* (yellowness) parameter was found to be significantly affected by the NaCl/Salicornia ratio (R^2: 0.80 and Adj-R^2: 0.60) (table2). Nevertheless, for the later, all color parameters L* (brightness). a* (redness). and b* (yellowness) showed high significant model (R^2: 0.67, 0.83 and 0.80) (Adj-R^2: 0.6, 0.79 and 0.74) (table2). As it was expected the crust color turn to be yellower when the ratio NaCl/*Salicornia* and savory enhancer amount are below the 20% and 0.8% respectively. This could be attributed to the intensification of Maillard browning reaction with augmented salt content. As it is well known, the presence of salt inhibits yeast growth. Subsequently, more free sugar would be available for the crust coloring during baking (Moreau et al., 2009). As stated by Czuchajowska et al. (1989), bread baked without salt has a lighter colored crust. For the crumb, both colorimetric indices L* and a* showed the highest values 72 and -0.9 with low percentage of substitution (around 20%) regardless from the savory enhancer amount. Controversially, b* parameter reached its maximum 16.7 when the NaCl/*Salicornia* substitution and salt amount were nearly at the peak. By way of explanation, the crumb color tends to be darker and greener with the crescent addition of *Salicornia* powder, which is completely reasonable knowing its natural green color.

Table 2. *Regression coefficients of predicted polynomial models for bread properties*

	Constant	X_1	X_2	X_1X_2	X_1^2	X_2^2	Lack Of fit	R^2	Adj-R^2
Specific volume	3.22	-0.21^{***}	-0.0003^{***}	0.002^{***}	-	-	1.13^{ns}	0.97	0.95
Textural parameters									
Hardness	11.25	8.32	0.03^{**}	-0.04^{**}	-2.59^{*}	-	6.03^{ns}	0.8	0.7
Gumminess	9.55	6.88	-0.0008^{*}	-0.03*	-2.23*	0.0001	6.38^{ns}	0.8	0.65
Chewiness	9.37	6.7	-0.003*	-0.02*	-2.18*	0.0001	6.32^{ns}	0.78	0.63
Color parameters									
L_{crumb}	72.75	-0.71	-0.04^{**}	-	-	-	3.03^{ns}	0.67	0.6
a_{crumb}	-0.93	0.003	0.003^{***}	-	-	-	1.41^{ns}	0.83	0.79
b_{crumb}	14.94	0.01^{**}	-0.008^{**}	0.014^{*}	-	-	1.16^{ns}	0.8	0.74
b_{crust}	37.27	-2.18^{*}	0.04	0.02^{*}	-	-	0.38^{ns}	0.67	0.56

ns: not significant ($p < 0.05$); * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.
X_1: Savory enhancer amount; X_2: NaCl/*Salicornia* ratio.

3.3 Optimization

The optimization method or desirability consist of selecting the desired goal for each of the established variables in order to achieve the ideal responses. Savory enhancer amount was desired to be retained in the range and the

degree of substitution to be at the maximum. The chosen dependent variables were desired as follow: stability, G′, G″, h, specific volume, a* crumb and b* crumb were maximized whereas MTI, (Hm-H)/Hm, hardness, gumminess and chewiness were minimized. Forwardly, water absorption, DDT, b*crust and L* crumb were kept in range. The results of optimization suggested that 1.8 % of savory enhancer amount and 68% of degree of substitution might be the most suitable composition to fulfill the study objective while preserving dough and bread quality with a coefficient of desirability D= 0.73.

4. Conclusion

According to the obtained data, the use of *Salicornia* powder as a potential partial substitute of NaCl seems like a promising approach since it has been shown able to cover most of salt major functionalities. Indeed, with respect to dough rheological properties, a ranging substitution between 50 and 70% preserved dough quality and did not negatively alter it. Regarding the bread quality characteristics, the salt reduced bread fortified with *Salicornia* powder showed to have an improved textural quality. Nevertheless. the sensory characteristics of bread in relation to color analysis need to be adjusted in order to meet the consumer's expectations. Our optimum values procured through RSM methodology provided a degree of substitution equal to 68 % that is adequately in line with the WHO recommendations concerning sodium daily intake reduction and the use of a natural substitute.
The idea of producing a high-quality low-salt bread enriched with Salicornia is justifiable in alliance with our findings. The evaluation of the sensory profile by panel members of the produced bread with the optimal composition is noteworthy as a next step. That would help providing a full comprehensive study treating all significant perspectives.

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Grape pomace as an innovative flour for the formulation of bakery products: how nutritional, textural, and sensorial properties were affected?

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This PhD project has been focused on the enhancement of grape pomace, the main by-product of wine industry, in order to obtain high-added value foods by creating at the same time a second life for waste wineries. Grape pomace, rich in dietary fiber and antioxidant compounds, has been exploited as innovative flour to improve the nutritional, chemical, textural, and sensory characteristics of bakery products.

La vinaccia come farina innovativa per la formulazione di prodotti da forno: come sono influenzate le proprietà nutrizionali, strutturali e sensoriali?

Il presente progetto di dottorato si è focalizzato sulla valorizzazione della vinaccia, il principale sottoprodotto dell'industria enologica, al fine di ottenere prodotti alimentari ad alto valore aggiunto dando allo stesso tempo una seconda vita ai sottoprodotti enologici. La vinaccia, una ricca fonte di fibre alimentari e composti antiossidanti, è stata sfruttata come farina innovativa per migliorare le caratteristiche nutrizionali, chimiche, strutturali e sensoriali dei prodotti da forno.

Key words: Grape pomace; bakery products; by-products; dietary fiber; bioactive compounds; functional foods

1. Introduction

The vinification is one of the main agro-industrial activities in the world, and *Vitis vinifera* L. is the most used for industrial wine production. Winemaking generates a large quantity of waste and by-products corresponding to 30% *w/w* of the starting grape used for wine productions, represented by grape pomace, grape seeds, grape stalks, and wine lees (Beres et al., 2019). These substances are considered to be highly polluting due to the presence of organic substances, pH, salinity and heavy metal content, thus having negative repercussions on environmental and economic sustainability (Troilo et al., 2021). Grape pomace is the most abundant solid by-product, obtained following pressing and fermentation process, and consist of skin, pulp, seeds, and residual stalks (Bordiga et al., 2019). Traditionally, is used to produce distillates, feeds, and fertilizer; however, due to its interesting composition, is considered an alternative to obtain high-added value products. In fact, grape pomace is characterized by high concentration of phenolic compounds, dietary fiber, proteins, and minerals. Moreover, grape seeds are rich in antioxidant compounds, such as vitamin E, phytosterols, fibers, lipids, and proteins (Barba et al., 2016).
In the recent years, many studies have focused on the enrichment of different types of foods with by-products of food industries, with the aim to formulate functional foods with beneficial effect on human health related to the presence of dietary fiber and polyphenols. Muffins are one of the most eaten bakery products in the word, highly appreciated both for their texture and taste, and for ease of use and storage. However, muffins are high-calorie products with a low dietary fiber and protein content (Shih et al., 2020), not in line with the expectation of consumers for healthy foods. At the same way, pizza is one of the most appreciated and consuming foods in the word, for its economical price and quick preparation. Refined wheat flour is the main ingredient used in commercial pizza bases, consisting mainly of carbohydrates, and low amount of dietary fiber, vitamins, and minerals. As a result, the nutritional intake it provides is limited, contributing to increase in blood glucose.
In this perspective, the activities of these years have been focused on the enrichment of bakery products such as muffins and pizza bases, by using grape pomace as functional ingredient.
In particular, the research was divided into these following steps:

A1) characterization of grape pomace (skin and seeds) in terms of proximate composition, antioxidant activity, anthocyanins, and total phenolic content;
A2) production of muffins, assessing the influence of different particle size fractions on chemical, nutritional, textural, and sensory properties;
A3) evaluation of the influence of addition of grape skin and seeds in the formulation of pizza bases on the techno-functional, textural, and sensory characteristics.

2. Materials and Methods

2.1 Pre-treatment of grape pomace

Red grape pomace (*Vitis vinifera* L.) was dried at 120 °C for 60 min in a ventilated oven to obtain a moisture content of 3-4%. Then, the grape pomace was separated from the grape seeds by a 5 mm sieve, and ground separately by an electric mill equipped with a sieve of 0.6 mm. For muffin formulation, the grape pomace without

seeds, was sifted by different sieves to obtain four particle size fractions: 600-425, 425-300, 300-212, and 212-150 µm; while for pizza bases formulation, the grape skin and grape seeds were sieved separately by stainless steel sieves into a particle size <300 µm.

2.2 Muffins preparation

Muffins were produced replacing 15% of wheat flour 00 with grape pomace powder (GP). After cooking, four types of muffins were produces: M425 (grape pomace powder at 600-425 µm); M300 (grape pomace powder at 425-300 µm), M212 (grape pomace powder at 300-212 µm), and M150 (grape pomace powder at 212-150 µm).

2.3 Pizza bases preparation

The production of enriched pizza bases was performed replacing the wheat flour 00 with different grape by-products flour. In particular, were considered two types of flour: GS consisting of grape skin, and GM consisting of mix of skin/seeds in 70/30 *w/w* ratio. These two alternative flours were added replacing 15, 20, and 25% of wheat flour 00. Doughs were kneaded for 15 min, places overnight at 4 °C for proofing, and then places in a proofing cell at controlled temperature of 30 °C for 1 h. Finally, the doughs were cooked at 280-290 °C for about 5 min, to obtain seven types of pizza bases: CTR (control with only wheat flour 00), 15S (addition of 15% of grape skin), 20S (addition of 20% of grape skin), 25S (addition of 25% of grape skin), 15M (addition of 15% of mix skin/seeds), 20M (addition of 20% of mix skin/seeds), and 25M (addition of 25% of mix skin/seeds).

2.4 Chemical proximate composition of grape pomace powder and fortified bakery products

Grape skin and seeds powders, muffins, and pizza bases, were characterized by the determination of total anthocyanins content (TAC), and by ABTS and DPPH assays to assess antioxidant activity, while the total phenol content (TPC) was determined according to the Folin-Ciocâlteu method, as described by Difonzo et al. (2021). Moreover, the samples were subjected to the proximate composition for the determination of total dietary fiber (TDF), protein, ash, and lipid content according to the AOAC methods n. 985.29, 979.09, 979.03, and 945.38 F respectively (AOAC, 2006).

2.5 Instrumental and sensorial analysis of muffins and pizza bases

The analysis of color in terms of lightness (L^*), redness (a^*), and yellowness (b^*) was carried out on crust and crumb of muffins sample, using a colorimeter; while the textural profile analysis (TPA) was performed on muffins cut into 2 × 2 × 2 cm pieces, and on pizza bases cut into 4 × 4 cm pieces, according to Dingeo et al. (2020) and Pasqualone et al. (2019), respectively. In addition, muffins were subjected to image analysis, elaborating the picture through the ImageJ software, to discriminate the muffins in terms of pores area, mean area, circularity, and solidity. Finally, the sensory analysis of bakery products was carried out by a trained panel composed by nine judges. Samples were evaluated indicating the intensity of appearance, olfactory and taste, and textural attributes using a linear unstructured scale of 10 cm for muffins and using a 9-point scale for pizza bases.

2.6 Statistical analysis

Statistical analysis was performed by analysis of variance (one-way ANOVA) and Tukey's comparison test for grape pomace powder and muffins datasets. The data regarding pizza bases, on the other hand, were subjected to Dunnet test, one-way and two-way ANOVA variance analysis, followed by Tukey's comparison test. Differences were considered statistically significant at $p < 0.05$. Dunnet test was performed for multiple comparisons with control, and the differences were considered statistically different at $p < 0.001$.

3. Results and Discussion

3.1 Muffin formulation

Chemical characterization of the grape pomace powders

Grape pomace powders at different particle size used for muffin formulation, were characterized for total phenolic content (TPC), as well as for antioxidant activity (Table 1). The TPC did not show statistical differences varying the particle size, while the antioxidant activity, evaluated with ABTS and DPPH assays, showed lower values in coarser powders.

The same trend was confirmed for total anthocyanins content (TAC), with lower values in the grape pomace powder >425 µm. These differences could be due to a higher fragmentation of the structural components of grape pomace during the milling process leading to a greater release of these components into thinner fractions.

Table 1 *Chemical composition of grape pomace size powder.*

Particle size	TPC (mg/g)	TAC (mg/g)	ABTS (µmol TE/g)	DPPH (µmol TE/g)
600-425 µm	10.39 ±0.14^a	1.52 ±0.02^c	40.12 ±1.46^b	38.16 ±0.90^b
425-300 µm	12.39 ±0.77^a	1.71 ±0.03^b	50.79 ±2.10^a	45.43 ±0.77^a
300-212 µm	11.69 ±0.09^a	1.78 ±0.01^a	52.01 ±2.25^a	44.51 ±0.24^a
212-150 µm	11.18 ±0.74^a	1.76 ±0.01^a	52.54 ±1.69^a	46.21 ±0.52^a

Different letters in the same column mean a significant difference at $p < 0.05$.

Characterization of fortified muffins

Table 2 reports the results of proximate composition, TPC and TAC, antioxidant activity, and colorimetric indexes of the experimental muffins. The moisture content did not show significant differences among the samples; lipid and ash contents were significantly higher in obtained muffins with thinner grape pomace powders (M212 and

M150); on the contrary, proteins and carbohydrates were generally significantly higher in M425.

The total dietary fiber content was significantly higher in M300, although slight differences were found among the samples. In all cases, the incorporation of 15% of grape pomace powder allowed to label the muffins as "source of fiber", according to the Regulation (EC) 1924/2006; in fact, this claim is attributed to foods that contain at least 3 g of total dietary fiber per 100 g of product. Moreover, muffins made with thinner powders (M150) showed slightly higher values of total polyphenols and total anthocyanin and, consequently, higher antioxidant activity.

Regarding color parameters, the crust showed significantly higher lightness (*L**), redness (*a**) and yellowness (*b**) in M425. The values of color indexes generally significantly decreased with decreasing of powder particle size. Also the crumb showed a significant decrease in lightness of muffins as the particle size decreased and, contrary to crust, significantly higher values of *a** and *b**, probably due to the slightly higher anthocyanins content. The different *L** values of crust and crumb could be attributed to the Maillard reaction between reducing sugars, abundant in grape pomace, and amino acids.

Table 2 *Chemical and proximate characterization (expressed as g/100 g) and colorimetric parameters in terms of lightness (L*), redness (a*) and yellowness (b*) of crust and crumb of muffins.*

Parameters	M425	M300	M212	M150
Moisture	23.34 ±2.70^{a}	22.31 ±0.54^{a}	21.90 ±0.58^{a}	24.90 ±1.07^{a}
Lipids	22.70 ±0.57^{b}	24.90 ±0.77^{a}	25.45 ±0.73^{a}	25.88 ±0.71^{a}
Proteins	9.72 ±0.26^{a}	9.03 ±0.17^{b}	8.76 ±0.14^{b}	8.81 ±0.10^{b}
Ashes	1.80 ±0.18^{b}	1.83 ±0.05^{b}	2.12 ±0.05^{a}	2.09 ±0.01^{a}
TDF	3.40 ±0.25^{ab}	3.92 ±0.24^{a}	3.10 ±0.21^{b}	3.06 ±0.16^{b}
Carbohydrates	40.04 ±0.81^{a}	38.01 ±0.96^{a}	38.67 ±0.98^{a}	35.26 ±0.82^{b}
TPC (mg/g)	0.64 ±0.01^{b}	0.65 ±0.01^{ab}	0.68 ±0.01^{ab}	0.69 ±0.01^{a}
TAC (µg/g)	25.20 ±0.20^{b}	24.50 ±0.20^{b}	28.03 ±0.15^{a}	28.03 ±0.55^{a}
ABTS (µmol TE/g)	2.23 ±0.02^{b}	2.21 ±0.02^{b}	2.50 ±0.02^{a}	2.49 ±0.06^{a}
DPPH (µmol TE/g)	1.72 ±0.01^{b}	1.75 ±0.02^{b}	1.74 ±0.02^{a}	1.78 ±0.02^{a}
Crust				
*L**	43.85 ±2.39^{a}	40.82 ±1.70^{b}	37.76 ±3.38^{c}	36.73 ±1.33^{c}
*a**	13.34 ±1.42^{a}	8.76 ±2.23^{b}	7.96 ±1.74^{b}	7.60 ±2.09^{b}
*b**	26.81 ±2.37^{a}	20.90 ±2.63^{b}	17.10 ±1.92^{bc}	14.06 ±3.96^{c}
Crumb				
*L**	61.64 ±2.72^{a}	54.60 ±2.75^{b}	44.18 ±3.35^{c}	46.34 ±2.60^{c}
*a**	1.14 ±0.15^{d}	2.21 ±0.25^{c}	3.40 ±0.36^{b}	5.82 ±0.30^{a}
*b**	9.17 ±0.81^{b}	9.50 ±0.63^{b}	9.90 ±0.85^{b}	11.53 ±0.62^{a}

Different letters in the same row mean a significant difference at $p < 0.05$.

Figure 1 shows the results of texture analysis of the examined muffins. The results showed that varying the particle size of grape pomace powder, significantly affected hardness and cohesiveness. In particular, the values of hardness and cohesiveness were inversely correlated and M425 showed significantly lower values of hardness, and significantly higher values of cohesiveness.

The mean value related to cell area distribution, mean area, circularity, and solidity of pores of muffin crumb, are shown in Table 3.

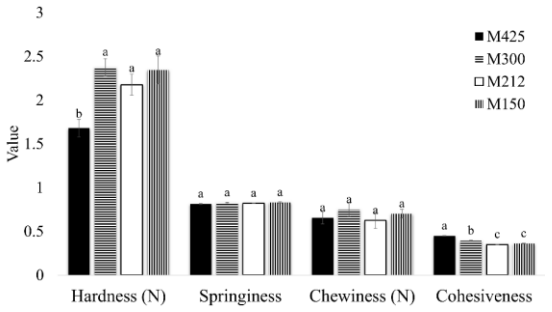

Figure 1 *Texture parameters of muffins.*

Table 3 *Mean values of cell area distribution (mm^2) and mean area (mm^2), circularity, and solidity of pores in the muffin crumb.*

Range of area (mm^2)	Cell area distribution (%)			
	M425	**M300**	**M212**	**M150**
0.05-0.07	21.56 ±0.93^{c}	21.07 ±0.76^{c}	25.91 ±0.62^{a}	22.85 ±0.03^{b}
0.07-0.1	17.65 ±0.27^{b}	19.80 ±1.79^{b}	22.92 ±0.54^{a}	19.45 ±0.66^{b}
0.1-0.2	244.91 ±0.62^{a}	25.79 ±0.28^{a}	25.01 ±1.06^{a}	26.02 ±0.84^{a}
0.2-0.3	10.06 ±0.37^{a}	10.28 ±0.06^{a}	8.38 ±0.48^{b}	8.52 ±0.38^{b}
0.3-0.6	11.76 ±0.36^{a}	10.96 ±0.65^{ab}	7.66 ±0.29^{c}	9.94 ±0.47^{b}
0.6-2	10.50 ±0.56^{a}	8.35 ±0.09^{ab}	5.86 ±0.17^{c}	7.82 ±0.66^{ab}
2-8	3.55 ±0.13^{a}	3.52 ±0.68^{ab}	2.79 ±0.26^{b}	3.45 ±0.61^{ab}
8-18	-	0.23 ±0.01^{ab}	0.40 ±0.03^{a}	0.50 ±0.09^{a}
18-30	-	-	0.44 ±0.06^{a}	0.49 ±0.04^{a}
30-50	-	-	0.41 ±0.06^{a}	0.73 ±0.09^{a}
50-80	-	-	0.22 ±0.03^{a}	0.23 ± 0.01^{a}
Mean area	0.54 ±0.01^{b}	0.56 ±0.13^{b}	1.07 ±0.15^{a}	1.13 ±0.10^{a}
Circularity	0.47 ±0.01^{a}	0.47 ±0.02^{a}	0.37 ±0.02^{b}	0.34 ±0.01^{b}
Solidity	0.67 ±0.01^{a}	0.66 ±0.01^{a}	0.64 ±0.01^{b}	0.64 ±0.01^{b}

Different letters in the same row mean a significant difference at $p < 0.05$.

Muffins formulated with the smallest particle sizes showed significantly higher values of mean area, than the muffins formulated with the coarsest particle sizes. These variations could be related to the presence of larger pores, with areas in the range between 18 and 80 mm^2. In addition, thinner powders used in the muffin formulation, led to the development of less regular pores in shape compared to the granulometry >300 µm. Otherwise, the solidity showed values inversely proportional to the particle size, indicating the presence of pores with smoother edges in M425 and M300. Therefore, it emerges that the reduction of particle size of powder has induced a crumb with a heterogeneous porosity due to the presence of large and irregular shape pores (Figure 2). Figure 3 shows the results of the sensory analysis of muffins. The crust and crumb color were perceived significantly more intense in M212 and M150 ($p < 0.05$). The pore homogeneity, instead, was directly proportional to the granulometry; M425, in fact, was characterized by the presence of small and evenly distributed pores, compared to M212 and M150, as shown in Figure 2. The perceptions of toasted, must, spicy, and astringent, related to the presence of tannins in grape pomace, significantly increased with decreasing the particle size (M150) ($p < 0.05$); while sweetness and acid showed increasing intensities as particle size decreased. Finally, the texture attributes perceived in the mouth in terms of stickiness showed a significant increase in M212 and M150 ($p <$

0.05). Softness, instead, showed no differences among samples, despite slightly higher values were observed in M212 and M150, with a score equal to 5-6 of 10.

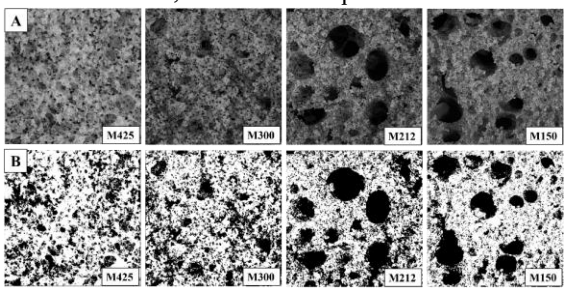

Figure 2 *Picture of longitudinal section of muffin crumb (A); binary images of muffin crumb (B).*

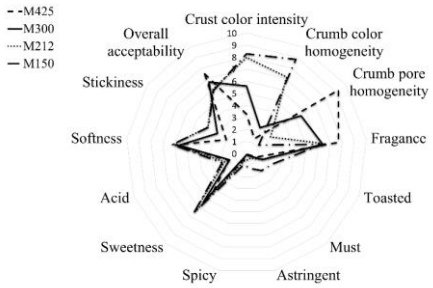

Figure 3 *Sensory evaluation of muffins.*

3.2 Pizza bases formulation

Chemical composition of flour

The powders used for the formulation of pizza bases, were characterized to evaluate the proximate composition, TPC, TAC, and antioxidant activity. As shown in Table 4, the wheat flour 00 (WF), showed the lowest content in total dietary fiber (TDF), lipids and ashes, compared to grape skin flour (GS) and mix of skin e seeds flour (GM). In contrast, both the protein and carbohydrate content were higher in refined flour. While, comparing the oenological by-products flours, it emerged a higher lipids content in GM, due to the presence of seeds which led an increase in fat fraction. In addition, the total dietary fiber content (TDF), was statistically different in the alternative flours; in fact, GS showed a quantity of 46.56% compared to 35.03% found in GM. Polyphenols were higher in GM sample (31.37 mg/g) compared to GS and wheat flour (18.53 and 4.21 mg/g, respectively). Similarly, the antioxidant activity had the same trend of TPC, with higher values in GM. TAC, as expected, was higher in GS and was not detected in wheat flour.

Table 4 *Chemical composition in terms of TPC and TAC (mg/g), ABTS and DPPH assays (µmol TE/g) and proximate composition (expressed as g/100 g) of pizza bases.*

Flour	Moisture	Lipids	Proteins	Ashes	TDF	Carbohydrates	TPC	TAC	ABTS	DPPH
WF	12.23 ±0.21^{a}	1.35 ±0.02^{c}	15.08 ±0.00^{a}	0.68 ±0.04^{c}	3.3 ±0.08^{c}	67.36 ±0.09^{a}	4.21 ±0.08^{c}	-	0.63 ±0.01^{c}	0.23 ±0.01^{c}
GS	4.75 ±0.29^{b}	5.97 ±0.03^{b}	12.21 ±0.27^{b}	13.78 ±0.04^{a}	46.56 ±0.11^{a}	16.73 ±0.24^{c}	18.53 ±0.52^{b}	7.4 ±0.05^{a}	110.07 ±0.24^{b}	68.89 ±0.23^{b}
GM	4.91 ±0.32^{b}	6.79 ±0.02^{a}	10.58 ±0.13^{c}	10.43 ±0.10^{b}	35.03 ±0.12^{b}	32.26 ±0.30^{b}	31.37 ±0.63^{a}	5.55 ±0.05^{b}	160.52 ±1.89^{a}	123.48 ±1.86^{a}

Different letters in the same column mean a significant difference at $p < 0.05$.

Characterization of pizza bases

Table 5 shows the chemical and proximate composition of the different pizza bases formulated.

Table 5 *Chemical composition in terms of TPC and TAC (expressed in mg/g), ABTS and DPPH assays (expressed in µmol TE/g), and proximate composition (expressed as g/100 g) of pizza bases.*

Parameters	CTR	15S	20S	25S	15M	20M	25M	*p*-value P*OF
Moisture	25.31 ±0.65	24.30 ±0.41^{ab*}	22.79 ±0.50^{cd*}	22.51 ±0.29^{d*}	24.92 ±0.11^{a*}	23.73 ±0.56^{bc*}	22.44 ±0.30^{d*}	$p < 0.001$
Lipids	0.81 ±0.01	1.11 ±0.02^{d*}	1.44 ±0.01^{b*}	1.50 ±0.01^{b*}	1.28 ±0.03^{c*}	1.42 ±0.02^{b*}	1.88 ±0.02^{a*}	$p < 0.001$
Proteins	11.24 ±0.19	10.60 ±0.40^{b*}	10.85 ±0.42^{b*}	11.08 ±0.01^{a}	11.34 ±0.13^{a}	11.06 ±0.23^{a}	11.15 ±0.04^{a}	$p = 0.001$
Ashes	2.28 ±0.09	3.75 ±0.00^{d*}	4.29 ±0.06^{c*}	4.81 ±0.13^{b*}	3.28 ±0.13^{e*}	3.90 ±0.06^{d*}	5.42 ±0.03^{a*}	$p < 0.001$
Carbohydrates	59.97 ±0.67	54.66 ±0.10^{ab*}	53.34 ±0.73^{bc*}	50.91 ±0.51^{d*}	53.13 ±0.19^{a*}	52.77 ±0.84^{c*}	50.14 ±0.54^{e*}	$p < 0.001$
TDF	0.39 ±0.11	5.78 ±0.01^{d*}	7.29 ±0.10^{b*}	9.19 ±0.12^{a*}	6.05 ±0.10^{c*}	7.12 ±0.10^{b*}	8.97 ±0.12^{a*}	$p < 0.001$
TPC	0.25 ±0.00	1.70 ±0.01^{e*}	2.44 ±0.10^{c*}	3.10 ±0.06^{a*}	2.02 ±0.04^{d*}	2.66 ±0.07^{b*}	3.15 ±0.01^{a*}	$p < 0.001$
TAC	-	0.45 ±0.02^{c*}	0.54 ±0.01^{b*}	0.72 ±0.03^{a*}	0.31 ±0.00^{e*}	0.39 ±0.00^{d*}	0.51 ±0.01^{b*}	$p < 0.001$
ABTS	0.71 ±0.01	1.70 ±0.01^{e*}	2.71 ±0.10^{de*}	3.10 ±0.06^{d*}	8.02 ±0.06^{c*}	10.32 ±0.31^{b*}	12.87 ±0.60^{a*}	$p < 0.001$
DPPH	0.34 ±0.02	5.98 ±0.06^{e*}	7.72 ±0.22^{c*}	8.22 ±0.15^{a*}	6.76 ±0.32^{d*}	8.35 ±0.20^{b*}	9.99 ±0.06^{a*}	$p < 0.001$

All values were compared with the CTR with * $p < 0.001$ by two-way ANOVA test. Different letters in the same row mean a significant difference at $p < 0.05$. P= percentage; OF= oenological flour.

The lipid content was influenced by the type of flour and percentages of substitution. The trend is directly proportional to the percentage added, and greater in 25M, due to the presence of seeds. In contrast, the protein content did not differ from the control, although lower values were found in 15S and 20S. Finally, the quantity of total dietary fiber, in all cases (except for CTR), allowed to attribute the claim "high fiber content" (EC Regulation 1924/2006), highlighting a greater impact linked to the percentage rather than the types of flour. In addition, the

different fiber content impacted the moisture level of the sample, which decreased as the fibers increased, due to ability of the latter to bind water. As expected, the incorporation of oenological flours modified the phenolic content; for both GM and GS, the addition of 25% led to the highest concentration of polyphenols. Similarly, the antioxidant activity was positively influenced by the addition of GM. Finally, the anthocyanin content showed a trend opposite, with values statistically higher in the products obtained with GS.
Figure 4 reports the data related to texture analysis of pizza bases. The addition of GM and GS influenced hardness and chewiness more than other parameters; in particular hardness values have increased as replacement percentages increase. As regard cohesiveness, the value decreased in products formulated with GM, probably due to higher level of dietary fiber. The results of the sensory analysis are shown in Figure 5. From the appearance point of view the fortified pizza bases obtained high scores of crust and crumb color intensity compared to the control ($p < 0.05$), especially at the highest added percentage of GS. The thickness, however, showed a trend inversely proportional to the increase in substitution. Olfactory analysis, on the other hand, showed the highest values of must and pungent scent in 25S and 25M. Sweetness, acid, bitter and salty showed no difference; however, the perception of astringency increased with the presence of grape seeds (25M) ($p<0.05$). Softness and humidity showed the highest values when GM was added.

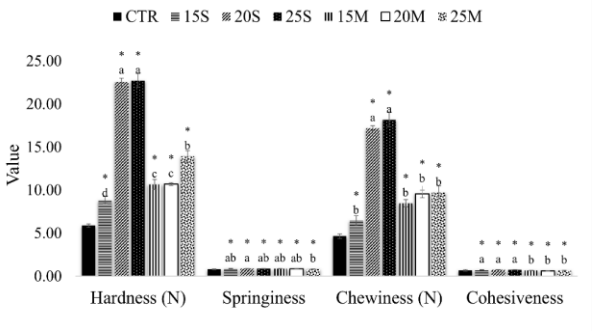

Figure 4 *Texture analysis parameters of pizza bases.*

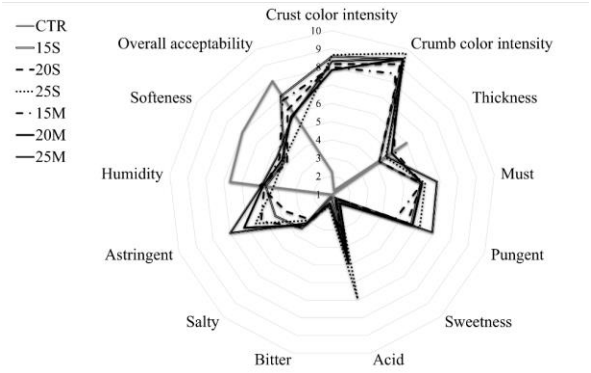

Figure 5 *Sensory evaluation of pizza bases*

4. Conclusions and Future Perspectives

These studies have proposed strategies for the enhancement of the main oenological by-product, highlighting its important nutritional composition and its use as innovative ingredient for the formulation of bakery products.
The addition of grape flour by-products in muffins and pizza bases has allowed to attribute the health claims "source of fiber" and "high fiber", respectively, as well as to increase the content of antioxidant compounds. However, from a textural and sensory point of view, fortification has altered the values of hardness, color and perceptions of acid and astringent; nevertheless, the products were not unpleasant for consumers.
Overall, the use of these by-product has made possible to obtain foods with high added value, contributing to the reduction of wineries waste. Future research could focus on the study of *in vitro* and *in vivo* nutritional effects and tests to extend the shelf-life of easily perishable foods.

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Definition and validation of a healthy and sustainable dietary pattern, enriched with plant-based foods rich in bioactives compounds, in the context of the MIND FoodS Hub project

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This PhD research project is aimed at setting up and analyse a healthy and sustainable dietary pattern, called EAT-IT, based on EAT-Lancet proposal, and including bioactive-rich plant-based foods selected within the MIND FoodS Hub project. Among the foods included in the pattern under study is present a cultivar of blueberries rich in anthocyanins whose protective effect was evaluated on markers of vascular function in a group of elderly subjects, as a target of vulnerable population. A pilot study is currently underway to evaluate the feasibility, adherence, and effect of the developed dietary pattern.

Definizione e validazione di un modello salutare e sostenibile, arricchito con alimenti vegetali ricchi di composti bioattivi, nel contesto di MIND FoodS Hub

Questo progetto di dottorato ha lo scopo di sviluppare e analizzare un modello alimentare sano e sostenibile, chiamato EAT-IT, basato sulla proposta EAT-Lancet e inclusivo di alimenti vegetali ricchi di bioattivi, selezionati nell'ambito del progetto MIND FoodS Hub. Tra gli alimenti inclusi nel pattern in studio è presente una cultivar di mirtilli ricca di antociani, il cui effetto protettivo è stato valutato su marker della funzione vascolare in un gruppo di soggetti anziani, individuati quali target di popolazione vulnerabile. È attualmente in corso uno studio pilota per valutare la fattibilità, l'aderenza e l'effetto del consumo del modello alimentare sviluppato.

Key words: Sustainable healthy diets, sustainability, nutrition, functional validation, dietary patterns.

1. Introduction

In accordance with the activities included in this PhD project and previously described, this oral communication reports the main results of the following activities:
(A1) to define a Mediterranean dietary pattern in line with the EAT-Lancet Commission reference diet (Willett *et al.*, 2019), adapted to the Italian food habits (EAT-IT)
(A2) to compare the levels of nutritional adequacy and environmental impact of this dietary pattern with another one based on the Italian Dietary Guidelines (IDG)
(A3) to implement a procedure of adaptation of the EAT-IT dietary pattern to different energy targets, also including selected bioactive-rich food while maintaining its nutritional adequacy and increasing feasibility
(A4) to develop a pilot intervention study, evaluating the actual feasibility of the EAT-IT dietary pattern, as well as its effect on nutritional status and eating behaviour.
(A5) to study the protective role of a bioactive-rich product, within the dietary pattern, on specific functions in a target group (also considering potential mechanisms of action).

2. Materials and Methods

2.1 Definition of the EAT-IT dietary pattern

A sustainable Italian-Mediterranean dietary pattern (EAT-IT) providing 2500 kcal was developed as already published (Tucci *et al.*, 2021). Briefly, the EAT-Lancet Commission reference diet provides the daily intake for eight different food categories (whole grain, tubers or starchy vegetables, vegetables, fruits, dairy foods, protein sources, added fats, and added sugars), which are expressed as both grams/day and kilocalories/day considering a diet of 2500 kcal/day. We rearranged these data, calculating feasible portion sizes and weekly frequencies of consumption for these food categories, and then allocated them into different meals according to Italian/Mediterranean food habits. Finally, we have summarized these elaborations, to obtain a dietary scheme that can be easily used for the management of meals.

2.2 Comparison of the nutritional adequacy and environmental impact of an EAT-IT-based dietary plan and an IDG one

To evaluate the nutritional adequacy of the EAT-IT dietary pattern, two different mid/long term dietary plans, comprehensive of different recipes and alternatives for every meal, were developed to obtain a realistic evaluation of the nutritional profile of the two different dietary plans were elaborated using a software (MètaDieta professional 4.1.1 METEDA Srl–Roma, Italy): one consistent with the EAT-IT dietary plan, and the other one on

the IDG, as a control. Then, the resulting nutritional composition was compared and their compliance with the Italian recommendations for macro- and micronutrients (LARN) assessed.
Data obtained from the elaborations were used to settle an ad hoc calculator sheet to allow the adaptation of the dietary pattern to lower energy requirements, optimizing the levels of nutrient intake adequacy and feasibility by revising food portions and recipes, but not changing the amount of protein other than plant-based sources. The nutritional adequacy of a new EAT-IT dietary plan, based on 2000 kcal, in comparison with a IDG one and the Italian recommendations (LARN) was newly assessed using the software.
The same dietary plans were used to calculate the associated levels of environmental impact. Briefly, a multilevel database of carbon and water footprint values for different food items, named SU-EATABLE LIFE database, has been recently released (Petersson *et al.*, 2021). Based on this database, we calculated the carbon footprint (CF) and water footprint (WF) of the foods included in the two dietary plans. The average values of CF and WF between the foods included and their possible alternatives for each day of the week was calculated and finally central tendency and measures of statistical dispersion of the data were calculated. The statistical significance of any differences found within the total levels of environmental impact and within specific food categories were assessed using a parametric statistical test (t-test) or a non-parametric one (Mann–Whitney U test), after using the Shapiro–Wilk test to verify the distribution of the data. Finally, data about the actual food consumption for the Italian population were collected from the latest available validated survey, the INRAN-SCAI 2005–06 (Leclercq *et al.*, 2009), to calculate a reference value for the actual average levels of CF and WF.

2.3 Development of a pilot intervention study, evaluating the actual feasibility of the EAT-IT dietary pattern
A pilot study, aiming to evaluate the actual feasibility of the EAT-IT dietary pattern, was performed as part of a main dietary intervention trial developed to assess the environmental and health related benefits of the EAT-IT pattern. The pilot study consists of a randomized controlled trail with a cross-over design involving a small group of volunteers. The eating habits of this group of young healthy subjects were assessed through a 7-day weighted food diary and then elaborated by the Metadieta software as reported before. Energy and nutrient intake and frequency of consumption of different food groups were compared with reference values (LARN) and with both the IDG and EAT-IT dietary patterns. The subjects enrolled have been randomly allocated to consume a 6-week EAT-IT dietary pattern or a control diet (in which IDG recommendation are provided). In both cases, the dietary patterns have been adapted to individual energy requirement and eating habits, assessed by the elaboration of food diaries. To define the dietary recommendation for the participants, the ad hoc calculator sheet previously settled was used to allow the adaptation of the EAT-IT or IDG dietary pattern to the different energy requirements, optimizing the levels of nutrient intake adequacy and feasibility by revising food portions and recipes, but not changing the amount of protein other than plant-based sources. Before and after the intervention, blood samples (25 ml) have been collected to analyse: i) biochemical markers (i.e., fasting glycemia, lipid profile, complete blood count with formula); ii) markers of inflammation and nutritional status (e.g., by ELISA kits and chromatographic techniques); iii) levels of DNA damage by the Comet assay in peripheral blood mononuclear cells (PBMCs). Subjects enrolled were also asked to provide before and after the intervention a 24 h urine collection that will be used to explore metabolomic profiles. Body composition was also assessed before and after the intervention through anthropo-plicometric measurements (height, weight, waist circumference, skinfolds according to reference methods). Finally, these subjects have been asked to complete an adapted version of a validated questionnaire (Barnard *et al.*, 2000) to evaluate the feasibility and acceptability of the EAT-IT dietary pattern.

2.4 Functional validation of a variety of a bioactive-rich blueberry in the improvement of vascular function in a specific target of population
The EAT-IT dietary pattern include different bioactive-rich foods selected based on their potential impact on metabolic and functional effects. Specifically, a polyphenol-rich cultivar of blueberry was investigated for its effectiveness in acutely improving vascular function considering its role in a at risk target group, such as the older subjects. This study followed a crossover design and involved 17 healthy older volunteers with more than 60 years and free from major diseases (e.g., diabetes, medical history of thrombosis or myocardial infarction), apart from mild hypertension, which has not considered as an exclusion criterion to allow the evaluation of blueberry effect on the average older subjects, and not just those who were able to maintain optimal vascular homeostasis despite aging. The subjects recruited have been randomly allocated to one of the two groups who will consume the blueberry product or the control product. Blueberry product consisted in a mousse of 250 g of frozen blueberry from the species *Vaccinium corymbosum* cv. Legacy, able to provide at least 300 mg of anthocyanins (ACNs). The control product consisted in a sugar drink (250-300 mL) containing the same sugars and amount present in the blueberries. The study was divided into two different phases. One of them involved the measurement of kinetics of absorption of anthocyanins and other bioactive, as well as the modulation of several plasma markers of vascular function (i.e., endothelin-1, NO, ICAM-1, VCAM-1 and VEGF) and inflammation (i.e., IL-6, IL-8, and TNF-α), evaluated by ELISA kits, and oxidative stress (i.e., levels of DNA damage), evaluated by the Comet assay, as well as glycemia and insulin. In this phase participants were asked to allow blood samples (7 mL) at baseline (t0) and after 1 h (t1), 1 h 30 '(t2), 2 h (t3) and 4 h (t4) from the consumption of blueberry or control product. Tubes containing silicon for serum and tubes containing heparin as anticoagulant for peripheral blood mononuclear cells (PBMCs) are used. An aliquot of blood (500 µL) is immediately processed to obtain PBMCs, while the rest of blood is maintained at room temperature (22 °C) for 30 min before to be processed by centrifugation at 1088 g for

15 min at 4 °C. The serum obtained is collected, divided in aliquots, and stored at -80 °C until analysis. For the second phase, participants were asked to be evaluated for their levels of endothelial-dependent vasodilation in the small finger arteries is assessed by a non-invasive plethysmographic method, using the Endo-PAT 2000 (Endo-PAT 2000, Itamar Medical Ltd, Caesarea, Israel) at baseline and after 2 h from the consumption of blueberry or control product. Briefly, the Endo-PAT equipment consists of two finger-mounted probes that sense the pulsatile volume changes of the fingertip. For the evaluation, subjects are requested to lay down in a supine position, with both hands on the same level, in a comfortable and thermoneutral environment. Then, a blood pressure cuff is placed on one upper arm (study arm), while the contralateral arm serves as a control arm. After a 10 min equilibration, the blood pressure cuff on the study arm is inflated to 200-220 mmHg for 5 min. The cuff is then deflated to induce reactive hyperemia while the signals are recorded. RHI, the primary outcome of the study, is an index of endothelial-dependent flow-mediated dilation derived automatically by the device. In fact, RHI is calculated as the ratio of the average pulse wave amplitude during hyperemia (60-120 s of the post-occlusion period) to the average pulse wave amplitude during baseline in the occluded hand, divided by the same values in the control hand and then multiplied by a baseline correction factor. Apart from the RHI, the Endo-PAT device allow to assess other markers of vascular function and arterial stiffness i.e., Augmentation Index (AIx), Framingham reactive hyperemia index (fRHI), and AI@75. The day before each appointment participants must follow dietary indications to avoid consumption of foods rich in anthocyanins or phenolic acids (e.g., berries, citrus fruits, dark colored vegetable, coffee, tea, wine, chocolate). This protocol received ethical approval by the Ethics Committee of the University of Milan at 14/12/2020 and has been registered on clinical trial registry ISRCTN (ISRCTN18262533 - https://doi.org/10.1186/ISRCTN18262533).

3. Results and discussion

3.1 EAT-IT dietary pattern definition

The EAT-IT dietary pattern was formulated as a concrete scheme providing both portion sizes and frequencies of consumption, and it has been already described and published (see Tucci *et al.*, 2021). Briefly, the EAT-IT dietary pattern appears as a plant-based diet rich in whole grains, with an important contribution of legumes and dried fruit. Animal protein sources, such as dairy (but not beef, lamb, and pork) are limited compared with the IDG.

3.2 Comparison of the nutritional adequacy and environmental impact of an EAT-IT-based dietary plan and a IDG one

Overall, the nutritional profile of the developed EAT-IT dietary plan resulted balanced, except for fibres levels (higher than the recommendation) and for calcium (below recommendation). Vitamin D levels resulted strongly below recommendation in both EAT-IT and IDG-based dietary plan (see Tucci *et al.*, 2021).

The application of the protocol to other energy targets evidenced that levels below 2000 Kcal increased the possibility of inadequacies for several micronutrients (e.g., calcium, Vitamin D, iron).

Regarding the levels of environmental impact, the EAT-IT dietary plan resulted associated with a statistically significant lower level of CF, compared with the IDG one ($p<0.05$), and a lower but not significant level of WF ($p = 0.06$), see Figure 1a and b. Both dietary plans resulted associated with a lower CF and WF than the average Italian diet. Considering specific food categories, the EAT-IT plan was more sustainable ($p<0.05$) in terms of CF for the categories of cereals, vegetables, and dairy, while, in terms of water footprint, for the categories of fruit, dairy, and cereals. CF and WF, on the other hand, were not different for protein sources as the two models have an almost overlapping quantity of meat.

Figure 1a

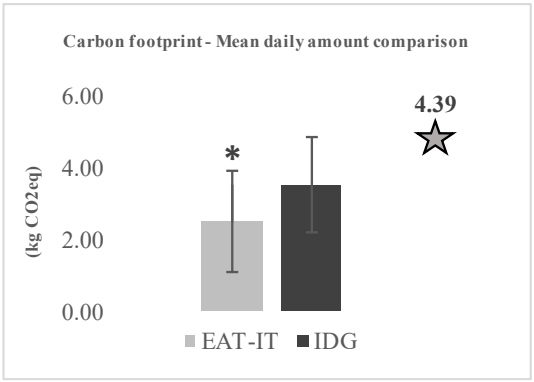

Figure 1b

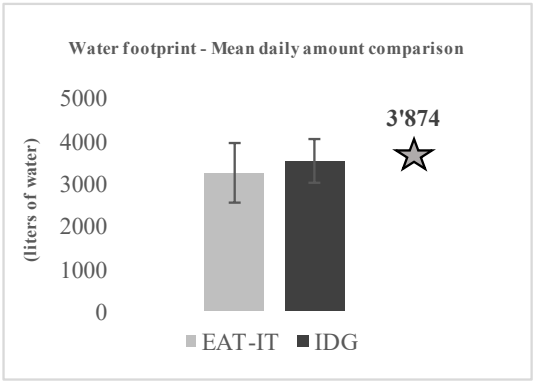

Figure 1 *Daily mean carbon (CF – Figure 1a) and water footprint (WF – Figure 1b) comparison between the two dietary plan (EAT-IT and IDG). The bars indicate standard deviation of carbon or water footprint between different days of the dietary plan. The asterisk indicate that the difference is statistically significant ($p<0.05$). The stars indicate mean daily impact of actual Italian food consumption, based on the INRAN-SCAI 2005–06 data.*

3.3 Assessment of dietary habits in a small group of healthy, young adults within the pilot intervention study evaluating the actual feasibility of the EAT-IT dietary pattern

For the pilot study, a group of 10 healthy subjects (mean age = 26 years, 24-30 years, 4 M + 6 F) that completed a 7-days weighted food diary were assessed for their dietary habits and food intake. These elaborations showed a nutritional intake far from the Mediterranean pattern being higher in fat (and saturated fat specifically) and lower in carbohydrate intake (about 47%). Also, a low level of PUFA was found. The intake of micronutrients was lower with respect to the reference intake for calcium, iron, and vitamin D, as expected. Considering the general dietary habits of these subjects, synthesized for the main food categories in Table 1, they resulted far from both EAT-IT and IDG dietary pattern specifically for the low consumption of legumes, nuts, fruits and vegetables, and higher consumption of red and processed meat. Subjects enrolled have been receiving personalised dietary recommendations to follow during the intervention study to test the feasibility of the EAT-IT and IDG-related diet that will be completed by July 2022.

Table 1 *Food habits of selected food categories resulting from the analysis of 10 healthy subjects, recruited within master's degree and PhD students.*

Food categories	Average portion and weekly frequencies of consumption	Comparison with EAT-IT diet and IDG
Dairy	8 portions/week of 150 ml for milk and yogurt and 75 g for cheese	↓ both EAT-IT dietary pattern and IDG
Cereals	3 portion/day of 90 g for pasta, 95 g for rice, 75 g for other cereals (e.g., spelt), 50 g for bread, and 30 g for breakfast cereals	↓ whole grains with respect to EAT-IT
Beef, lamb, and pork	1,5 portion/week of 140 g for beef, lamb, and pork + 2 portion/week of 90 g for cured meat	↑ both EAT-IT and IDG dietary pattern
Legumes	3 portions of 100 g for fresh legumes (equivalent for approximately 35 g of dried legumes)	↓ both EAT-IT dietary pattern and IDG
Nuts	3 portions/week of 16,5 g	↓ both EAT-IT dietary pattern and IDG
Vegetables	1 portion day of 200 g	↓ both EAT-IT dietary pattern and IDG
Fruits	1 portion day of 150 g	↓ both EAT-IT dietary pattern and IDG
Olis	1 portion day of 24 g	↓ both EAT-IT dietary pattern and IDG

3.4 *In vivo* functional validation of a variety of blueberry included in the dietary pattern

The acute study on the health-promoting effect of blueberry was performed on 17 older subjects, with a mean age of 69 years (62-74 years). The characteristics of the enrolled subjects are synthetized in Table 2. After completing the study, we found a statistically significant higher ΔRHI after blueberry intake, compared to control product intake, but not for AI, as shown in Figure 2a. This improvement was also associated with a significant reduction, highlighted by the post hoc analysis by time, within the single treatment, in the levels of Endothelin-1, an important vasoconstrictor peptide produced by the endothelium, after blueberry, but not control product consumption (Figure 2b), thus further mechanistically supporting the effect found on RHI. However, no differences were found for other markers of vascular function or inflammation, such as VCAM-1, ICAM-.1, NO, IL-6 and TNF-α.

Table 2 *Characteristics of the enrolled subjects for the acute study on the effects of blueberry on vascular function in older subjects.*

Characteristics		(n = 17)	
Age (yr)	69 ± 5	Fasting glycemia (mg/dL)	100 ± 13 (77 – 124)
Male	10 (58,8%)	Total cholesterol (mg/dL)	210 ± 29 (154 – 260)
Female	7 (41,2%)	LDL cholesterol (mg/dL)	124 ± 21 (86 – 158)
Height (cm)	168,6 ± 7,6	HDL cholesterol (mg/dL)	51 ± 12 (33 – 73)
Weight (kg)	70,5 ± 9,1	Tot. chol. / HDL chol. ratio	4,3 ± 1 (2,8 – 6,4)
BMI (kg/m2)	24,8 ± 2,4	Triglycerides (mg/dL)	124 ± 55 (71 – 242)
Systolic blood pressure (mmHg)	120 ± 7	C-reactive protein	1,5 ± 1,5 (0,2 – 4,2)
Diastolic blood pressure (mmHg)	78 ± 6	Use of drugs	9 (52,9%)

Figure 2 a

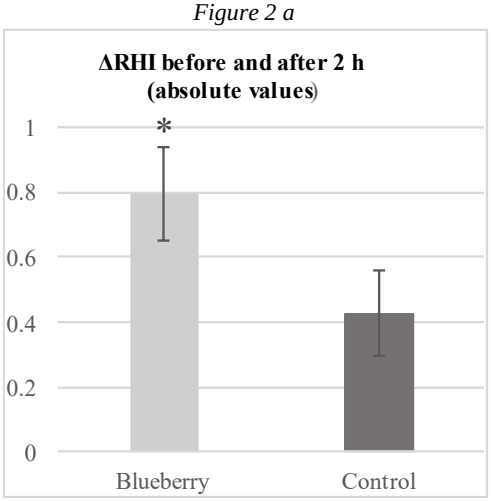

Figure 2 b

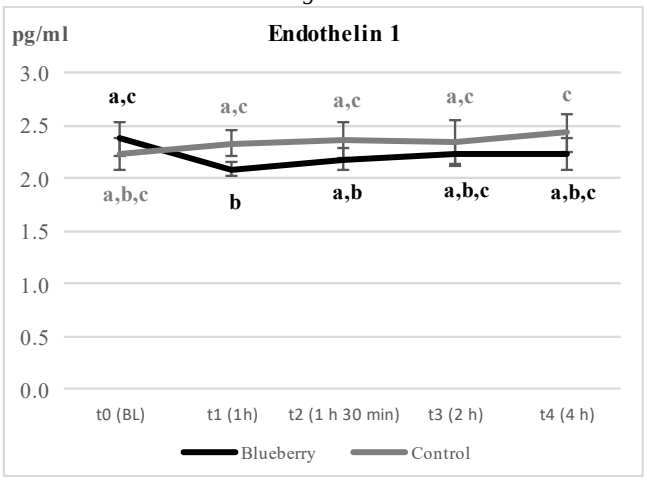

Figure 2 ***a)*** *Mean variation ΔRHI before and after blueberry intake or control product, expressed in absolute values. Data are expressed as ΔRHI ± standard error. The asterisk indicates statistical significance ($p<0.05$) based on the repeated measures analysis of variance (RANOVA)* ***b)*** *Mean levels of endothelin 1. Data are expressed as mean ± standard error for the two different treatments over time. The repeated measures ANOVA did not indicate an effect of the treatment, time, or interaction. The letters indicate the differences found through the post hoc analysis by time, within the single treatment, showing a significant reduction at 1h.*

4. Conclusion and future perspectives

The necessities to identify the elements of a more sustainable way of eating is urged by the scientific community given that world population is still growing and that the Food Systems represent one of the main factors responsible for environmental impact, contributing to global warming and climate change. At the same time, sustainable diets need to be healthy and nutritionally adequate to allow the satisfaction of nutritional needs. On these issues, the EAT-Lancet Commission on Healthy Diet from Sustainable Food Systems has published an authoritative and extensive report in which it indicates how it promotes the possibility of obtaining a food pattern that simultaneously satisfies the nutritional adequacy requirements (for the purpose of preventing chronic diseases) and of limited environmental impact, showing that these two constraints are not incompatible. However, the reports did not indicate how to adapt the proposal to different food context. In this PhD project, an Italian-Mediterranean adaptation, and the protocol to achieve this aim has been realized, as well as the evaluation of its theoretical nutritional adequacy and levels of impact, compared to national food-based dietary guidelines. The results show that the EAT-IT dietary pattern result as overall adequate and about 29% less impacting, in terms of theoretical CF emission, compared with an isocaloric IDG-based pattern, but also that include high frequencies of consumption of legumes and nuts, and reduced consumption of dairy. Since these characteristics could preclude many subjects with eating habits far from those expected from adhering to these patterns and that evidence on the actual health effects of these patterns are lacking, this PhD project also involves the evaluations of their effective feasibility, through a pilot study that is currently underway on a group of ten healthy subjects. Future perspectives involve the extension of this evaluation to a much larger and more representative sample of the general population, also to evaluate the effects on health and eating behaviors, after having clarified the possible experimental limits through the pilot study. Finally, given that such patterns are plant-based, the *in vivo* evaluation of the health promoting effects of selected foods are also considered. The results of a crossover RCT evaluating the vasoactive properties of a selected variety of blueberry in a group of older subjects support the hypothesis that acute blueberry consumption counteracts the detrimental effects of aging on vascular reactivity, resulting in higher RHI and supporting the role of bioactive in the optimization of healthy and sustainable dietary pattern.

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Identification and characterization of bioactive plant extracts and evaluation of viable use in the food industry

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This PhD project concerned the study of extracts from agri-food chain waste. In particular, the activities were focused on the identification of plant matrices of potential interest for the presence of bioactive components, the standardisation of the extraction process using solvent-free methods, the chemical characterisation of the extracts obtained by gas-chromatography and liquid-chromatography, as well as the biofunctional activities (such as antioxidant, antibacterial and antiproliferative). Several waste matrices were evaluated and the most promising were those derived from the raspberry chain (leaves and powder).

Individuazione e caratterizzazione di estratti di piante bioattive e valutazione di un possibile impiego nell'industria alimentare

Questo progetto di dottorato ha riguardato lo studio degli estratti da matrici di scarto provenienti dalla filiera agroalimentare. In particolare, le attività sono state focalizzate sull'identificazione di matrici vegetali di potenziale interesse per la presenza di componenti bioattivi, la standardizzazione del processo estrattivo mediante metodiche solvent-free, la caratterizzazione chimica degli estratti ottenuti attraverso gas-cromatografia e cromatografia liquida, così come le attività biofunzionali (come quella antiossidante, antibatterica e antiproliferativa). Sono state valutate diverse matrici di scarto e le più promettenti sono risultate essere quelle derivanti dalla filiera del lampone (foglie e polvere).

Key words: Raspberry, leaves infusion, extraction, parameters evaluation, green tea comparision

1. Introduction

During the PhD course, several waste derived from the agro-food chain were examined and the extracts obtained were investigated using chemical-free methods. In particular, steam distillation using distilled water was used as the preferred methodology. Different matrices deriving from the waste of the supply chain have been studied and selected for their feasibility in being subjected to extraction. Of the different matrices examined (*Humulus lupulus*, *Cannabis Sativa*, *Carthamus tinctorius*, *Rubus idaeus*), waste from the raspberry chain was the most interesting. This production chain mainly involves two types of waste: peels and seeds from the extraction of fruit juice and the leaves. The residues of the juice production were dried, ground and used to obtain a pasta supplemented with raspberry flour (manuscript under submission). Regarding the leaves, the volatile molecules obtained by steam distillation extraction were investigated (De Santis, Carbone, Garzoli, Masci, & Turchetti, 2022). The data obtained showed interesting activities on cell proliferation in tumour and healthy cell models, inhibiting the growth of cancer cells and promoting healthy ones, antibacterial activity on various strains and a moderate antioxidant capacity measured by DPPH, ABTS and FRAP assays. Based on this data, we investigated this matrix using a method that would allow an extraction of the non-volatile component. The leaves were used to obtain different extracts by infusion and compared with extracts from green tea leaves (*Camellia sinensis*) by the same process. Because infusion is a low-cost, quick and easy process requiring no solvents except water, it is applicable on both an industrial and domestic scale, encouraging its use by consumers. With a view to recovering waste from the agri-food chain, reusing waste instead of disposing of it would give added value to producers and processors by allowing the production of novel foods or products for the cosmetic or pharmaceutical industry (Ispiryan & Viškelis, 2019). Herbal infusions have been used by humans for centuries, in particular those derived from the leaves of the tea tree (*C. sinensis*) date back over 2000 years for their strong antioxidant properties. Scientific studies on the composition, biological properties and beneficial effects of *C. sinensis* infusions are increasing year after year, highlighting the importance of dietary supplementation with these extracts for the prevention of certain diseases (Samanta, 2020). In line with a thought of circularity and recovery, this study focused on the infusion of *R. idaeus* leaves taken from a local farmer. *R. idaeus* L., also called "Raspberry", is a plant belonging to the Rosaceae family, widely cultivated in Europe, North America, and Asia. The properties of *R. idaeus* leaves have been well known since time, recommended for the treatment of various diseases (Chwil & Kostryco, 2018). In addition, thanks to its functional properties, raspberry leaf extracts are used as an food additive and as dietary supplements (Belščak-Cvitanović et al., 2012; Ponder & Hallmann, 2019). The secondary metabolites produced by plants are subject to great variability, due to agronomic and environmental aspects, and to the possible alteration caused by aggressive solvents. We decided to investigate the non-volatile component of the same matrix using distilled water as an extraction solvent. Raspberry leaves infusion was investigated both chemically and

functionally, in relation to infusion times and temperatures, comparing it to green tea from two varieties (*C. sinensis* var. *Gunpowder* and *C. sinensis* var. *Chun Mee*).

2. Materials and methods

2.1. Raspberry (*R. idaeus*) and green tea (*C. sinensis* var. *GunPowder* and *Chun Mee*) leaves and extraction

For the study, raspberry leaves, collected at the 'Lamponi dei Monti Cimini' company, were dried at room temperature and leaves of two green tea varieties (*GunPowder* and *Chun Mee*) purchased from an herbalist shop in Viterbo (Italy) were examined. Leaves were shredded and the extraction was performed according to: 1,5g of plant material were immersed in 150 mL of distilled water at room temperature and 80°C for 2, 5 and 8 minutes. The extracts were centrifuged at 6000 rpm and filtered using Whatman laboratory paper and kept at -20°C until use. Eighteen extracts were produced according to RI (*R. idaeus*), CM (*C. sinensis* var. *Chun Mee*), GP (*C. sinensis* var. *Gun Powder*), RT (Room Temperature), 80 (80°C), 2-5-8 (infusion time). The moisture was 31±1% for raspberry, 4,90±0,35% for *C. sinensis* var. *Chun Mee and* 3,01±0,05% for *C. sinensis* var. *GunPowder*

2.2. HPLC analysis

The phenolic chemical profile investigation was carried out according to the method described by Ritchey and Waterhouse (Ritchey & Waterhouse, 1999) using Dionex apparatus with Ultimate 3000 DAD detector (Thermofisher) equipped with a c18 reverse phase column (5 µm, 120 Å, 4,6x250mm - Thermofisher).

2.3. Total Phenolic Content (TPC) and Antioxidant activity (DPPH and FRAP)

TPC and antioxidant capacity by DPPH assay were estimated spectrophotometrically according to the methods described by De Santis and colleagues (De Santis et al., 2022). The results were expressed as the µM of Trolox Equivalents (TE) g^{-1} of sample.

FRAP of samples was determined according to the method described by Xiao and colleagues (Xiao, Xu, Lu, & Liu, 2020), with minor modification in the final part of the method: a volume of 50 µL of sample/standard solution was mixed with 150 µL of working solution and the absorbance was measured spectrophotometrically at 593 nm after an incubation of 15 min at 37°C. Trolox was used for the calibration curve. The results were expressed as µM of TE g^{-1} of sample used.

2.4. Antibacterial activity

Two strains were used to evaluate the antibacterial activity of the extracts: *Escherichia coli* ATCC 25922 and *Bacillus cereus* ATCC 10876. All strains were investigated according to Turchetti and colleagues (Turchetti et al., 2020). MIC and MBC values were expressed as %, reported in Table 4. All the experiments were repeated three times.

2.5. Statistical analysis

Data were reported as mean±standard deviation of at least two independent experiments with three replicates. A one-way analysis of variance (ANOVA) was used to analyze the data, followed by Tukey Pairwise Comparisons test ($p<0.05$) and Principal Component Analysis (XL-stat Addinsoft, Paris, 2019).

3. Results and discussion

As can be seen from the results obtained through the different investigations, temperature and infusion times lead to statistically important differences.

Regarding total flavonols, a time-dependent increase can be observed for both room-temperature and 80°C infusion, but the most important increase is temperature-dependent, especially for raspberry (Table 1).

Table 1. Total flavonols detected by HPLC expressed as mg quercitin equivalents L^{-1} of extract. RI (*R. idaeus*), CM (*C. sinensis* var. *Chun Mee*), GP (*C. sinensis* var. *GunPowder*), RT (Room Temperature), 80 (80°C), 2-5-8 (infusion time).

Sample	mg QE/L	Sample	mg QE/L	Sample	mg QE/L	Sample	mg QE/L	Sample	mg QE/L	Sample	mg QE/L
RI_RT_2	13,42	**RI_80_2**	43,37	**CM_RT_2**	7,26	**CM_80_2**	8,42	**GP_RT_2**	7,15	**GP_80_2**	41,45
RI_RT_5	12,81	**RI_80_5**	74,59	**CM_RT_5**	8,95	**CM_80_5**	12,59	**GP_RT_5**	8,59	**GP_80_5**	60,65
RI_RT_8	26,03	**RI_80_8**	102,31	**CM_RT_8**	21,72	**CM_80_8**	18,62	**GP_RT_8**	21,18	**GP_80_8**	86,62

Looking at the total phenols content there is an increase directly related to both temperature and infusion timing, as shown in table 2. The increase in total phenol content is evident when the infusion temperature is 80°C compared to room temperature, with a 2-3-fold increase for raspberry samples, while for green tea var. Chun Mee the increase was 4 times higher. Green tea var. GunPowder was the most affected by infusion temperature with values increased 5-7 times compared to those obtained at room temperature. The infusion time allowed a TPC increase between 1.4-2.6 if the data is compared between 2 and 5/8 min, as can be seen in table 2.

Table 2. TPC and antioxidant activity. Means that do not share a letter in the same column are significantly different (p<0,05) with Tukey post-Anova test. RI (*R. idaeus*), CM (*C. sinensis* var. *Chun Mee*), GP (*C. sinensis* var. *Gun Powder*), RT (Room Temperature), 80 (80°C), 2-5-8 (infusion time).

ROOM TEMPERATURE				80°C			
Sample	**TPC (mg GAE/g)**	**DPPH (µM TE/g)**	**FRAP (µM TE/g)**	**Sample**	**TPC (mg GAE/g)**	**DPPH (µM TE/g)**	**FRAP (µM TE/g)**
RI_RT_2	2,85±0,15 ^{a,b}	0,86±0,002 ^{a}	10,69±0,37 ^{a}	**RI_80_2**	8,05±0,29 ^{a}	5,45±0,30 ^{a}	49,22±1,56 ^{a}
RI_RT_5	4,17±0,57 ^{b,c}	1,95±0,12 ^{b,c}	22,64±0,15 ^{b}	**RI_80_5**	14,25±0,35 ^{b}	9,99±0,21 ^{b}	97,90±0,59 ^{b}
RI_RT_8	5,09±0,47 ^{c}	2,46±0,12 ^{c}	32,90±0,82 ^{c}	**RI_80_8**	14,49±0,09 ^{b}	14,16±0,98 ^{d}	134,06±1,71 ^{c}
CM_RT_2	2,55±0,31 ^{a,b}	1,87±0,36 ^{b,c}	12,33±0,74 ^{a}	**CM_80_2**	12,36±0,76 ^{c}	11,95±0,54 ^{c}	85,43±4,69 ^{b,d}
CM_RT_5	4,00±0,75 ^{b,c}	3,09±0,12 ^{d}	24,33±1,04 ^{b}	**CM_80_5**	17,95±0,58 ^{d}	14,43±0,06 ^{d}	129,85±5,43 ^{c}
CM_RT_8	4,94±0,41 ^{c}	5,22±0,15 ^{e}	35,27±1,64 ^{c}	**CM_80_8**	20,53±1,02 ^{e}	16,21±0,18 ^{e}	135,43±2,75 ^{c}
GP_RT_2	1,16±0,28 ^{a}	0,18±0,005 ^{f}	1,38±0,03 ^{d}	**GP_80_2**	8,70±0,67 ^{a}	6,93±0,06 ^{a}	57,17±4,47 ^{a}
GP_RT_5	1,75±0,43 ^{a}	1,07±0,06 ^{a}	5,48±0,30 ^{e}	**GP_80_5**	12,16±0,38 ^{c}	10,11±0,51 ^{b}	74,75±1,19 ^{d}
GP_RT_8	2,60±0,20 ^{a,b}	1,38±0,15 ^{a,c}	9,17±0,74 ^{a}	**GP_80_8**	13,09±0,13 ^{b,c}	11,29±0,21 ^{b,c}	89,96±2,01 ^{b}

Table 3. Increase in TPC and antioxidant activity in relation between time and temperature. *RI (R. idaeus), CM (*C. sinensis *var. Chun Mee), GP (C. sinensis var. Gun Powder), RT (Room Temperature), 80 (80°C), 2-5-8 (infusion time).*

TPC

RT	**mg GAE/g**	**Ratio time (*5/2 and **8/2)**	**80°C**	**mg GAE/g**	**Ratio time (*5/2 and **8/2)**	**Ratio 80°C/RT**
RI_RT_2	2,85	-	RI_80_2	8,05	-	2,82
RI_RT_5	4,17	*1,46	RI_80_5	14,25	* 1,77	3,42
RI_RT_8	5,09	**1,79	RI_80_8	14,49	**1,80	2,85
CM_RT_2	2,55	-	CM_80_2	12,36	-	4,85
CM_RT_5	4	*1,57	CM_80_5	17,95	*1,45	4,49
CM_RT_8	4,94	**1,94	CM_80_8	20,53	**1,66	4,16
GP_RT_2	1,16	-	GP_80_2	8,7	-	7,50
GP_RT_5	1,75	*1,51	GP_80_5	12,16	*1,40	6,95
GP_RT_8	2,6	**2,24	GP_80_8	13,09	**1,50	5,03

DPPH

RT	**µM TE/g**	**Ratio time (*5/2 and **8/2)**	**80°C**	**µM TE/g**	**Ratio time (*5/2 and **8/2)**	**Ratio 80°C/RT**
RI_RT_2	0,86	-	**RI_80_2**	5,45	-	6,34
RI_RT_5	1,95	*2,27	**RI_80_5**	9,99	*1,83	5,12
RI_RT_8	2,46	**2,86	**RI_80_8**	14,16	**2,60	5,76
CM_RT_2	1,87	-	**CM_80_2**	11,95	-	6,39
CM_RT_5	3,09	*1,65	**CM_80_5**	14,43	*1,21	4,67
CM_RT_8	5,22	**2,79	**CM_80_8**	16,21	**1,36	3,11
GP_RT_2	0,18	-	**GP_80_2**	6,93	-	38,50
GP_RT_5	1,07	*5,94	**GP_80_5**	10,11	*1,46	9,45
GP_RT_8	1,38	**7,67	**GP_80_8**	11,29	**1,63	8,18

FRAP

RT	**µM TE/g**	**Ratio time (*5/2 and **8/2)**	**80°C**	**µM TE/g**	**Ratio time (*5/2 and **8/2)**	**Ratio 80°C/RT**
RI_RT_2	10,69	-	**RI_80_2**	49,22	-	4,60
RI_RT_5	22,64	*2,12	**RI_80_5**	97,9	*1,99	4,32
RI_RT_8	32,9	**3,08	**RI_80_8**	134,1	**2,72	4,07
CM_RT_2	12,33	-	**CM_80_2**	85,43	-	6,93
CM_RT_5	24,33	*1,97	**CM_80_5**	129,9	*1,52	5,34
CM_RT_8	35,27	**2,86	**CM_80_8**	135,4	**1,59	3,84
GP_RT_2	1,38	-	**GP_80_2**	57,17	-	41,43
GP_RT_5	5,48	*3,97	**GP_80_5**	74,75	*1,31	13,64
GP_RT_8	9,17	**6,64	**GP_80_8**	89,96	**1,57	9,81

For antioxidant activity, a similar trend is shown with the values increasing in relation to time and infusion temperature. As can be seen from table 3, for the DPPH assay µM Trolox equivalents increase from 1.21 to 7.67 times in relation to infusion timing, while the 80°C/RT ratio increases from 3.11 to 38.5 fold. About FRAP assay, a similar trend to the previous one can be observed, with increases from 1.31 to 6.64 times as a function of time. Temperature, as in the other cases, is the most influential factor in increasing activity, with an improvement in antioxidant capacity from 3.84 to 41.43 times. Regardind antibacterial activity, no significant inhibitory capacity was observed with extracts obtained at room temperature on the strains tested. On the other hand, with regard to extracts obtained at 80°C, the only strain found to be sensitive was *B. cereus*, with a MIC/MBC of 50% for all samples, with the exception of RI_80_2 which showed no activity and CM_80_5 and CM_80_8 which showed MIC/MBC value at 25%, as shown in table 4.

Table 4. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of infusions on *E. coli* and *B. cereus* expressed as % of extract used. RI *(R. idaeus)*, CM *(C. sinensis* var. *Chun Mee)*, GP *(C. sinensis* var. *Gun Powder*), RT (Room Temperature), 80 (80°C), 2-5-8 (infusion time), NA (Not Applicable), NE (No Effect).

ROOM TEMPERATURE					80°C				
	E. coli		*B. cereus*			*E. coli*		*B. cereus*	
Sample	**MIC**	**MBC**	**MIC**	**MBC**	**Sample**	**MIC**	**MBC**	**MIC**	**MBC**
RI_RT_2	NE	NA	NE	NA	RI_80_2	NE	NA	NE	NA
RI_RT _5	NE	NA	NE	NA	RI_80_5	NE	NA	50%	50%
RI_RT _8	NE	NA	NE	NA	RI_80_8	NE	NA	50%	50%
CM_RT_2	NE	NA	NE	NA	CM_80_2	NE	NA	50%	50%
CM_RT_5	NE	NA	NE	NA	CM_80_5	NE	NA	25%	25%
CM_RT_8	NE	NA	NE	NA	CM_80_8	NE	NA	25%	25%
GP_RT_2	NE	NA	NE	NA	GP_80_2	NE	NA	50%	50%
GP_RT_5	NE	NA	NE	NA	GP_80_5	NE	NA	50%	50%
GP_RT_8	NE	NA	NE	NA	GP_80_8	NE	NA	50%	50%
Gentamicin	31,25 µg/mL	31,25 µg/mL	15,625 µg/mL	15,625 µg/mL					

As reported in Fig. 1, Principal component analysis (PCA) showed that 99.17% of the total variance was explained by the first and second components. The first component (PC-1) explained 82.74% of the total variance, while PC-2 explained 16.43%. The antioxidant capacity (FRAP, TPC, DPPH) clustered the samples into three groups according to PC-1, while PC2 discriminated higher quercetin content samples. Observing the score plot shows how infusion time affects samples differently. Although all treated at 80 ° C samples increase the extraction of the antioxidant component from the matrices, this occurs at different times, as evident for Rubus after 8 minutes (RI_80_8). As was expected, the treatment at room temperature delays the extraction and reduces the antioxidant capacity of the extract for all the samples nevertheless of the variety or plant species.

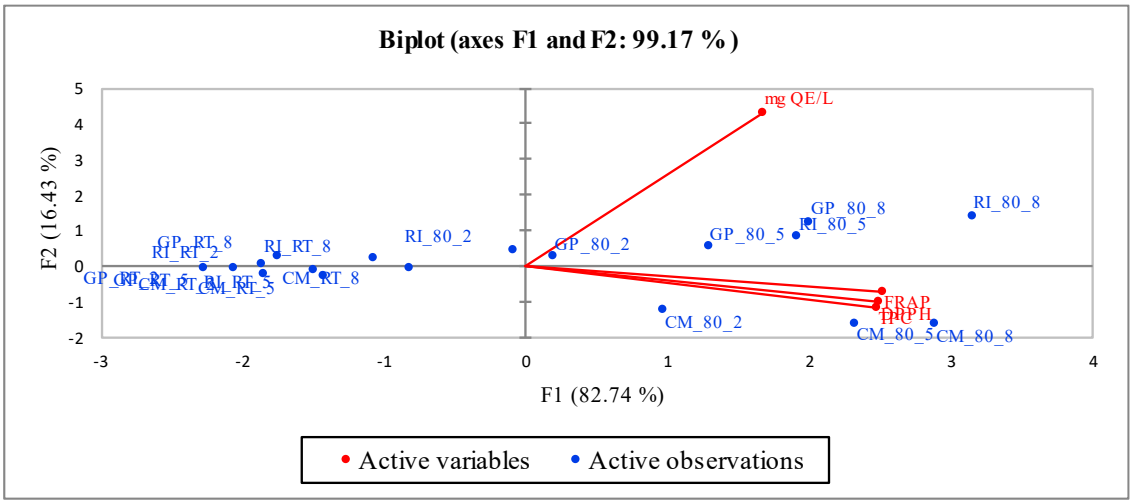

Figure 1. Principal Component Analysis of influence factors temperature and time of infusion on matrices.

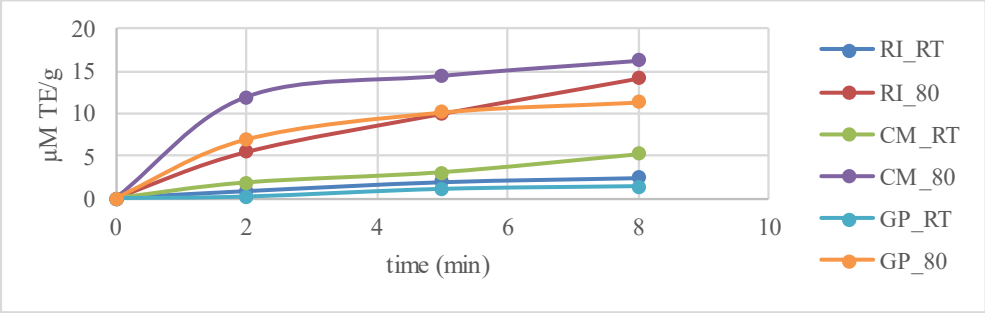

Figure 2. DPPH activity in relation to temperature and time for infusion.

4. Conclusion

Infusions obtained from waste matrices have great potential for use in various fields, especially in the agro-food industry. In this view, we focused on the study of the biofunctional properties of matrices, paying particular attention to two aspects: the extraction method and the extraction parameters. In the first case, it was necessary to select the extraction solvent. In our case, water, a non-toxic and environmentally sustainable solvent, was chosen. With regard to the extraction parameters, we wanted to understand what the optimum was in terms of temperature and extraction time for the different matrices, optimising the extraction of the bioactive molecules of interest. In this regard, three similar matrices (raspberry and two green tea varieties) were compared. The study of parameters is particularly important with plant matrices, especially in an industrial production view, optimising the use of resources and avoiding waste. As the results of this study show, time and temperature factors significantly influence both the composition of the extracts and their biological properties. Furthermore, the data show that raspberry leaves, a waste matrix from a production process that aims to cultivate this plant to obtain the fruit rather than the leaf, are a promising base for consumption as infusion, comparable to the two varieties of green tea examined. More attention needs to be paid to the extraction conditions and the matrices used, especially with regard to timing and temperature, which play a crucial role in the extraction and in the intrinsic properties of the drink itself. As shown in Fig. 2 regarding to the DPPH assay, in the case of Rubus temperature (as expected) and time lead to an increase in antioxidant activities, reaching values of a matrix well known for its properties, such as green tea. These parameters should be monitored, through both quantitative (TPC and other analyses) and qualitative (antioxidant activities and other biological properties) kinetics, in order to estimate the best extraction process, according to the structural characteristics of the matrix (dried or fresh, if the leaves are leathery, waxy, etc.).

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Biopolymer active coating to extend the shelf-life of minimally processed fruits and vegetables

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The aim of PhD thesis work is to extend the shelf-life of minimally processed fruits and vegetable applying a biopolymer active coating. Respiration and/or transpiration rate were used as response parameters to select the best coating formulation. Subsequently, the impact of the coating on the degradation kinetics of the product has been quantified. Three case study has been carried out: fennel, pear and apple. The developed coatings (sodium caseinate; sodium caeinate/guar guam/beeswax; chitosan/insect protein) enriched with propyl gallate were able to preserve the products quality from 10 to 63%.

Utilizzo di coating attivi a base di biopolimeri per il prolungamento della shelf-life di frutta e verdura minimamente processata

L'obiettivo del progetto è estendere la shelf-life di frutta e verdura minimamente processata mediante l'utilizzo di rivestimenti biopolimerici attivi. Le velocità di respirazione e/o di traspirazione sono state utilizzate come parametri per selezionare la formulazione migliore del coating. Successivamente, è stato quantificato l'effetto del coating sulle cinetiche di degradazione del prodotto. Tre casi studio sono stati affrontati: finocchio, pera e mela. I diversi coating sviluppati (caseinato di sodio; caseinato di sodio/gomma guar/cera d'api; chitosano/proteine d'insetto) arricchiti con il gallato di propile hanno consentito di preservare la qualità dei prodotti dal 10 al 63%.

1. Introduction

Minimally processed fruits and vegetables (MPF&V) have a shorter shelf-life than fresh ones (Ahvenainen, 1996). Biopolymer coatings are a promising preservation technology that allows to extend the shelf-life of food products, slowing down their quality decay through the reduction of mass transfer of gas, moisture or aromas. In addition, coatings can be used as carrier of active additives that can contribute to the shelf-life extension and/or improve food nutritional quality of MPF&V (Yousuf *et al.*, 2018, Khan *et al.*, 2021). The aim of the PhD work was to extend the shelf-life of MPF&V applying a biopolymer active coating. Respiration and transpiration rate were used as response parameters to select the best coating formulation. Subsequently, the impact of the coating on the degradation kinetics of the product has been quantified. Samples, with and without coating, were stored at specific temperatures and chemical-physical and nutritional quality indices were monitored during storage.
This oral communication reports the main results of the following three activities:
A1) To study the effect of sodium caseinate (SC) active coating on the quality of MP fennel. SC coatings were prepared at different concentration of SC and enriched with propyl gallate (PG). To select the optimal SC concentration, respiration rate (RR) and transpiration rate (TR) of samples has been quantified at 10 °C. Then, a stability study has been performed to quantify the effect of the selected SC active coating on the degradation kinetics of the fennel critical quality indices (colour, antioxidant capacity, total polyphenols, and vitamin C).
A2) To study the effect of a coating based on SC, guar gam, beeswax and PG on the quality of MP pears. RR and TR was measured at 10°C and critical quality indices (firmness, antioxidant capacity, total polyphenols content, and vitamin C content) were monitored for 30 days at 10 °C.
A3) To evaluate plasma activated water (PAW) as antioxidant medium to prepare active coating. A low molecular weigh chitosan (CH) and proteins extracted by house cricket (PE) has been used at different concentrations to optimize the solution stability of the polymers in PAW or 1% acetic solution. Then, the optimal coating formulation obtained with and without PAW was applied on fresh-cut apples to study its effect on the quality of the product. RR and TR has been determined at 5°C. Moreover, nutritional quality indices (total polyphenols content and antioxidant capacity) were studied during storage at 5°C for 13 days.

2. Materials and Methods

SC from bovine milk, guar gum, bees wax, emulsifiers (tween 80 and span 20), glycerol, PG, riboflavin, Folin-Ciucalteu, and DPPH reagent were purchased from Sigma-Aldrich (Milan, Italy). A1) Fennel heads (*Foeniculum vulgare* Mill. subsp. *vulgare* var. *azoricum* cv *Augusto* and *Tiziano*) were supplied by COMEX Company (Pagani, Italy). SC solutions with protein concentrations at 8%, 10%, 12%, and 14% (g mL^{-1}) were prepared and applied on MP fennels as reported by Valentino *et al*. (2020); PG (0.13 mg mL^{-1}) was added to SC solution at 8% to obtain active coating (SC+PG).
A2) 8% SC, 0.2% guar gum, 2% bees wax, emulsifiers ratio 4:1 respect to bees wax, and 0.3 mg mL^{-1} PG was used to prepare the blend (BLEND-1). *Conference* pears were acquired at the supermarket in Portici (Italy).

A3) Solubility and stability of CH and PE at 1% and 2% in PAW or 1% acetic solution were studied. CH was unsoluble in PAW, thus the blend was prepared by mixing at 1:1 ratio 2% CH solubilized in 1% acetic solution with 2% PE solubilized in PAW (BLEND-2). Blend obtaining without PAW was used as control (BLEND-3). BLEND 2 and 3 were applied on fresh cut apples acquired at supermarket in Potsdam (Germany).
The respiration rate was measured in a closed system, as reported by Torrieri *et al*. (2009), on:

- MP fennels uncoated and coated with SC at different concentrations (8%, 10%, 12%, 14%) at 10°C;
- MP pears uncoated and coated with BLEND-1 at 10°C;
- Fresh-cut apples uncoated and coated wih BLEND-2 and 3 at 5°C.

The transpiration rate was measured as reported by Volpe *et al*. (2018) on:

- MP fennels coated with SC at 8% and without coating at 76, 86, 92 and 100% RH at 10°C;
- MP pears coated with and without BLEND-1 at 50 and 86 % RH at 10°C;
- Fresh-cut apples coated with and without BLEND-2 and 3 at 60, 76, 86 and 96 % RH at 5°C.

Furthermore, fennels coated with SC+PG were packaged in polypropylene trays, wrapped with LDPE film, and stored at 10°C for 0, 5, 8 e 12 days. Pears coated and uncoated were storage at 70% RH at 10°C for 0, 3, 6, 9, 15, 20, 24 and 30 days; fresh cut apples with and without coatings were storage at 5°C for 0,1, 3, 6, 9 and 13 days in polyethylene bags. Physico-chemical and nutritional parameters were monitored during storage. Duncan's test and t-test were used to determine significant differences among samples ($p \leq 0.05$). The statistical analyses were performed using the SPSS software (SPSS Inc. 17.0, Chicago, IL, USA, 2002). The determination of the kinetic constants of the physical and nutritional properties were carried out by non linear regression of the data using the solver Excel tool (Office, 365). Variation of chemical and nutritional indices were described by a pseudo-first order kinetic model:

$$Q = Q_f - (Q_f - Q_i) * exp\,(-kt) \qquad (1)$$

Where Q is the quality index, the *f* and *i* subscripts refer to the final and initial quality of the samples, k is the kinetic costant and t is the storage time.

3. Results and Discussions

3.1 Case study: fennel

In table 1 were reported RR and TR of fennel at 10°C. The results showed that SC coatings at different concentrations did not significantly affect the RR of MP fennels, that assumes an average value of 12 ±1 mL kg^{-1} h^{-1} and 9±2 mL kg^{-1} h^{-1}, respectively for RR_{O2} and RR_{CO2}. SC coating at 8% did not affected the TR of MP fennels, that, on the contrary, increased with increasing the relative humidity. Thus, the lowest concentration (8%) of SC was chosen to prepare active coating with PG. Fig. 1 shows that pseudo first order model was able to describe the changes of antioxidant capacity and vitamin C during time. Pseudo first order model were also appropriate in describing the data of the other physical and nutritional properties (data not shown). The values of kinetic costants of the MP fennels quality indices are reported in Table 2.

Table 1. *Average and standard deviation of RR and TR of fennels stored at 10°C, pears and apples stored at 5°C, with and without coating.*

		Physiological parameters							
		RR ml $kg^{-1}h^{-1}$		TR g $kg^{-1}h^{-1}$					
Product	Treatment	RR_{O2}	RR_{CO2}	50% RH	60% RH	76% RH	86% RH	96% RH	100% RH
Fennel	Control	12.3±0.3[a]	10.1±1.9[b]			1.1±0.2[a]	0.5±0.1[a]	0.3±0.1[a]	0.02±0.00[a]
	8% SC	11.4±0.5[a]	9.2±0.2[a]			1.2±0.2[a]	0.5±0.1[a]	0.3±0.1[a]	0.02±0.01[a]
	10% SC	11.9±0.7[a]	10.1±0.6[b]						
	12% SC	12.7±0.3[b]	11.9±0.3[c]						
	14% SC	12.1±0.8[a]	8.1±0.9[a]						
Pear	Control	10.3±0.6[b]	7.2±0.7[b]	0.11±0.01[a]			0.03±0.01[a]		
	BLEND-1	4.7±1.1[a]	4.4±0.5[a]	0.11±0.01[a]			0.03±0.01[a]		
Apple	Control		17.1±0.4[b]		3.6±0.1[b]	2.9±0.1[a]	1.6±0.1[c]	0.8±0.1[b]	
	BLEND-2		9.9±0.7[a]		3.4±0.1[a]	2.9±0.1[a]	1.0±0.1[a]	0.6±0.1[a]	
	BLEND-3		11.1±1.3[a]		3.5±0.1[a]	2.9±0.1[a]	1.1±0.1[b]	0.6±0.1[a]	

Table 2. *Values of model parameters of quality decay of fennels stored at 10°C, pears and apples stored at 5°C, with and without coating.*

		Fennel			Pear		Apple		
		Control	8% SC	8% SC+PG	Control	BLEND-1	Control	BLEND 2	BLEND 3
Antioxidant capacity	Q_0 (mg $_{Trolox}$ gr $_{dm}^{-1}$)	1	1	1	1.9	1.9	2.3	2.3	2.3
	Q_f (mg $_{Trolox}$ gr $_{dm}^{-1}$)	0	0	0	0	0	0	0	0
	k (mg $_{Trolox}$ gr $_{dm}^{-1}$day^{-1})	-0.067	-0.064	-0.060	-0.17	-0.06	-0.76	-0.25	-0.10
Total polyphenol content	Q_0 (mg $_{GAE}$ gr $_{dm}^{-1}$)	35	35	35	61	61	141	141	141
	Q_f (mg $_{GAE}$ gr $_{dm}^{-1}$)	65	65	65	26	37		0	
	k (mg $_{GAE}$ gr $_{dm}^{-1}$day^{-1})	0.092	0.073	0.037	-0.17	-0.10	0.05	0.04	0.04
Vitamin C content	Q_0 (mg $_{vit\ C}$ gr $_{dm}^{-1}$)	0.14	0.14	0.14	39	39			
	Q_f (mg $_{vit\ C}$ gr $_{dm}^{-1}$)	0	0	0	5	5			
	k (mg $_{vit\ C}$ gr $_{dm}^{-1}$ day^{-1})	-0.055		-0.037	-0.14	-0.07			
Firmness	Q_0 (N)				0.13	0.13			
	Q_f (N)				0	0			
	k (N day^{-1})				-0.41	-0.06			
ΔE	Q_0	15	15	15					
	Q_f	0	0	0					
	k (day^{-1})	0.13	015	0.30					

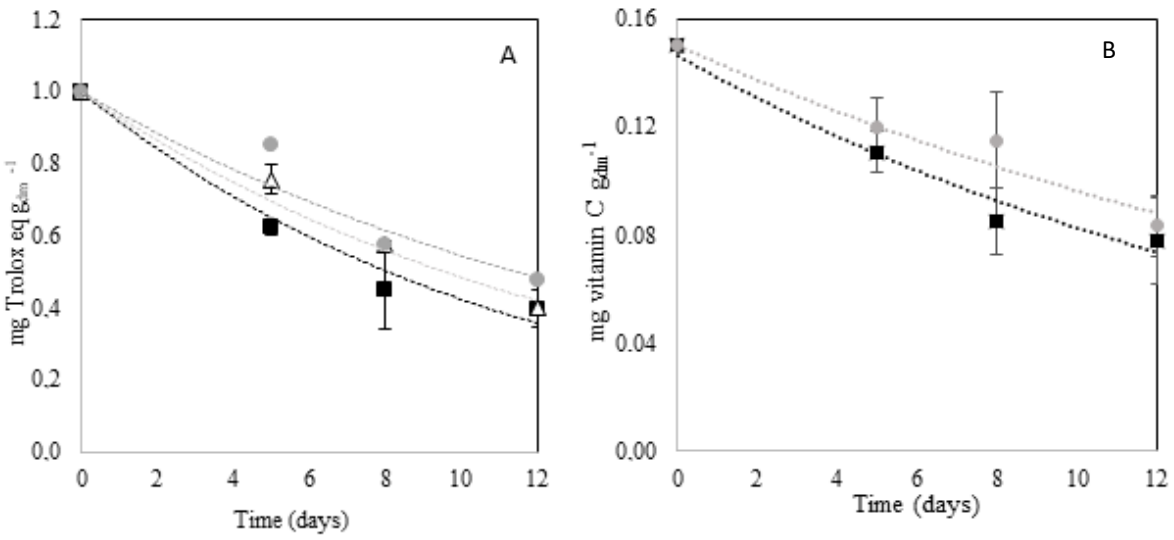

Figure 1. (A) *Antioxidant capacity of control (■) SC8% (▲) and SC+PG (●) and* **(B)** *Vitamin C content of control (■) and SC+PG (●) of fennels storage at 10 °C for 12 days.*

Antioxidant capacity decreased during storage time for all the samples (Fig. 1A) according with Capotorto *et al*. (2018), however active coating slows down the decrease rate of 10% (Table 2). The content of vitamin C decreased of 50% after 12 days for the control samples, whereas active coating preserved vitaminc C degradation about 31% (Fig. 1B). Active coating slows down the increase of polyphenol content of 47% (Table 2). On the other hand, the active coating did not preserve the color, indeed costant kinetic of control increased of 13%, whereas costant kinetic of active coating increased of 30%. As reported in literature, the color of cutted fennel increases during the storage (Artes *et al*., 2002, Escalona *et al*., 2005, Escalona *et al*.,2014 Capotorto *et al*., 2018); cutting causes a series of reactions physical and physiological both in injured tissues and in adjacent ones. The physical effects following the cut are immediate and cause mechanical shocks to the tissues, removal of the protective epidermal layer, accumulation surface of water and expose the tissues to contaminations (Ahvenainen, 1996, Capotorto *et al*., 2018).

3.2 Case study: pear

The results of RR and TR of pears at 10°C were reported in Table 1. The BLEND-1 applied on the product showed significativly effect ($p \leq 0.05$) on the O_2 consumption and CO_2 production, that assumes an average value of 10 ±1 mL kg^{-1} h^{-1} and 7±1 mL kg^{-1} h^{-1}, respectively for RR_{O2} and RR_{CO2} for control samples and an average value of 5 ±1 mL kg^{-1} h^{-1} and 4.4±0.5 mL kg^{-1} h^{-1}, respectively for RR_{O2} and RR_{CO2} for samples with coating. No effect of the coating on TR was observed. Fig. 2 shows that pseudo first order model was able to describe the changes of vitamin C during time. Pseudo first order model were also appropriate in describing the data of the other physical and nutritional properties (data not shown). Kinetic constants of firmness, antioxidant capacity, total polyphenol content and vitamin C content are reported in Table 2. The coating has a significant effect on the kinetic constants of all quality indices studied (Table 2). The antioxidant capacity decreased during storage time for all the samples, according with Manzoor *et al*., (2013). The BLEND-1 applied on the samples reduces the decrease of this parameters of 63% comparing with control sample ($p \leq 0.05$). Furthermore, the total polyphenols content decreased during time, but coating reducing the degradation about 30% compared with control sample ($p \leq 0.05$). Similar results were obtained with vitamin C content, that decreased during the time but the BLEND-1 reduced degradation of 33% at 10°C ($p \leq 0.05$). This result agrees with Lin *et al* (2008), that showed a smaller vitamin C reduction during storage in presence of coating respect to control sample. Active coating showed a good effect on firmness, reducing the kinetic costant of 47% compared to control samples.

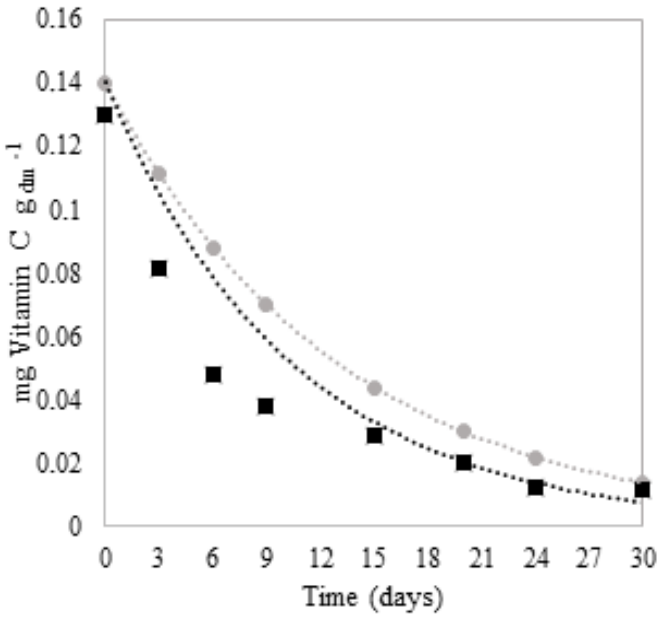

Figure 2. *Vitamin C content of control (■) and BLEND-1 (●) of pears storage at 10 °C for 30 days.*

3.3 Case study: fresh cut apples

The RR_{CO2} of uncoated samples was 17.1 ± 0.4 mg kg^{-1} h^{-1}, whereas the values of samples with BLEND-2 and BLEND-3 were 9.9 ± 0.7 and 11.1 ± 1.3 mg kg^{-1} h^{-1}, respectively (Table 1). There were no significant differences ($p \leq 0.05$) between the samples with BLEND-2 and BLEND-3, but differences were significant compared to the control sample ($p \leq 0.05$). TR results were reported in Table 1. As the relative humidity increases, there was a decrease of TR, furthermore the samples with coatings showed lower values than the sample without coating at 60, 86 and 96% RH. Statistically significant differences ($p \leq 0.05$) emerged between the samples with and without coatings, with the exception of the sample stored at 76% RH.

As for fennel and pear, a pseudo first order model well describe the antioxidant capacity change during storage (fig.3). The BLEND-2 reduced the kinetic constant of antioxidant capacity and total polyphenols content of about 17% compared to control samples.

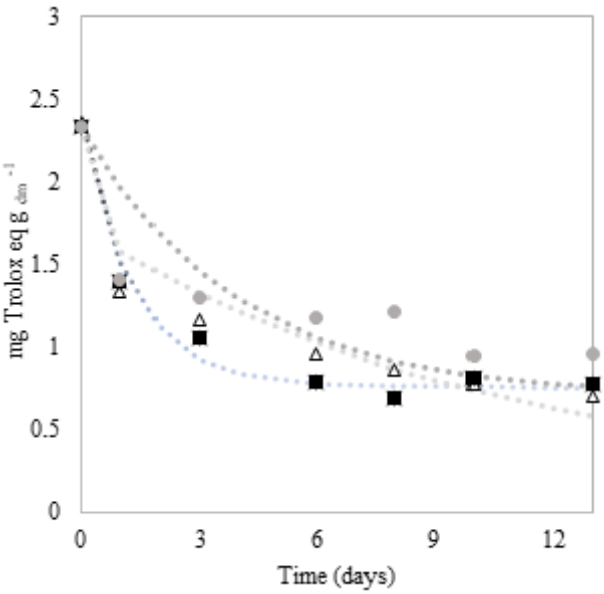

Figure 3. *Antioxidant capacity of control (■)BLEND-2 (●)BLEND-3 (▲) of fresh cut apples storage at 5 °C for 13 days.*

4. Conclusions and future perspectives

The SC coating did not affect the quality of MP fennels; however, SC can be a good substrate for dispersion of active compounds for the development of bioactive coating. SC+PG active coating was able to preserve the antioxidant capacity and the total polyphenol of MP fennels during storage at 10°C. However, active coating showed a negative impact on fennel colour. Coating based on SC, gum, bees wax and PG was able to reduce the respiration rate and preserve (+35%) the physical and nutritional quality of MP pears. PAW showed a potential application to develop edible coating. The solubility of the biopolymer in the PAW is a critical parameter for the coating development, however PAW was a good solvent for PE. Blend of CH (2%) and PE (2%) were successfully applied on fresh-cut apples. Coating obtained with PAW reduced RR_{CO2}, TR and preserved nutritional quality of fresh-cut apples. In conclusion, the coatings developed were able to retard the senescence and preserve the nutrition quality of MPF&V. Further research on the impact of coating on sensory properties of MPF&V and consumers acceptability should be quantify before to scule-up the technology at industrial level.

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Validation of Microbiome Mapping Strategies for the Food Industry

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This PhD project aimed at applying a microbiome mapping strategy in the environment of several food processing facilities, in order to prove the advantages of Whole Metagenome Sequencing techniques to decipher the complex communities inhabiting food contact and non-food contact surfaces after the routine sanitation (i.e., the residential microbiome), as well as their metabolic potential.

Validazione di strategie di mappatura microbiologica negli ambienti di lavorazione per l'industria alimentare

Questo progetto di dottorato ha l'obiettivo di sviluppare una strategia di mappatura del microbioma nell'ambiente di lavorazione delle industrie alimentari, e di validarla in situ, con lo scopo di dimostrare i vantaggi di tecniche basate sul sequenziamento dell'intero metagenoma nella mappatura delle comunità microbiche residenti che popolano superfici a contatto e non con gli alimenti anche dopo la sanificazione, nonché il loro potenziale metabolico.

Key words: metagenomics; food production environment; whole metagenome sequencing; sanitation procedures.

1. Introduction

In this oral communication, the results of the following activities will be presented and discussed:

A1) validation of the mapping strategy and assessment of the most suitable microbial DNA extraction protocol from low-biomass surface swabs;
A2) application of the developed protocol to describe the microbial composition and the functional potential of the microbiome inhabiting:
A2.1) dairy facilities;
A2.2) minimally-processed vegetables (MPV) producing facilities.

2. Microorganisms residing in the food industry

Cleaning and disinfection (i.e., sanitation) of the food production environment help producers to guarantee the safety of their product, as well as to control the development of food spoiling microbes. The main purpose of cleaning is to remove food residuals that accumulate during the processing steps, whereas disinfection aims at reducing the concentration of microorganisms on the surfaces.
Even though more and more strict sanitation protocols have been applied throughout the years in the food industry, evidence suggests that several bacterial taxa can overcome the contact with biocides, thus permanently establishing themselves on food production surfaces (Møretrø and Langsrud, 2017). These microorganisms become a residential microbiota of the food industry.
Cultural-dependent methods (i.e., isolation and phenotypic characterization of microorganisms) are the most common techniques to study microorganisms inhabiting the food industry. However, limitations of these techniques have been described (Bourdichon *et al*., 2021), namely their inefficiency in differentiating properly bacterial isolates, as well as their inability in providing information about the unculturable fraction of the community, which might be substantial. Also, more than one week could be necessary to identify a microorganism, which represent an important limitation if an outbreak occurs.
Hence, there is a strong need of novel and faster Standard Operating Procedures (SOPs) offering a wider view of both taxonomic and functional composition of the residential microbiome in the food industry.
Whole Metagenome Sequencing (WMS) consists in the untargeted fragmentation and sequencing of total DNA extracted from a microbial community (De Filippis *et al*., 2021). This technique has the potential to overcome limitations of the cultured-based approach. It may provide a strain-level description of the microorganisms inhabiting the food production environment, highlight long-term routes of contamination of the final product.

3. Experimental Procedure

In order to demonstrate the ability of WMS approach to unveil taxonomic and functional composition of microbiomes residing in the food industry, we first tested four different DNA extraction protocols, in order to select the one that guarantee the highest yield for such low-biomass samples. Thereafter, we visited 19 food companies producing cheeses (n = 16) or minimally processed vegetables (n = 3). Cheesemaking facilities produced: i) Buffalo Mozzarella cheese (n = 10), ii) Caciocavallo cheese (n = 5) and iii) goat cheese (n = 1).

Environmental samples from each facility were collected after the routine sanitation of surfaces and prior to a new processing shift. The total number of collected samples is reported in Table 1. Also, details about the sanitation procedures (e.g., disinfectant's active ingredient, solution concentrations) were recorded. Besides environmental samples, we collected ingredients, intermediate (when available) and final products of the first processing after the environmental sampling. Also, hands/aprons swabs of an employee from the facility were collected.
Microbial pellets were obtained from each product, then DNA extraction, WMS and bioinformatic analyses were performed.

Table 1 *Samples collected from the visited facilities.*

Facility type	Surfaces		Operator swabs	Raw materials	Final products
	Food contact	Non-food contact			
Cheeses	61	25	8	46	88
Vegetables	12	5	3	6	6
Total	73	30	11	52	94

4. Materials and Methods

Samples from environment and operator's hands/aprons were collected by swabbing with 5 Whirl-Pak® Hydrated PolyProbe swabs (Madison, Wisconsin, United States), covering an area on 1 m^2 or a sampling unit. Ingredients and final products were stored into sterile bags. All the samples were homogenized in 1:10 Phosphate Buffered Saline (NaCl 137 mmol L^{-1}, KH_2PO_4 1.8 mmol L^{-1}, KCl 2.7 mmol L^{-1} and Na_2HPO_4 10 mmol L^{-1}, pH = 7.4), then cellular pellets were obtained. DNA was extracted from pellets using the DNeasy PowerSoil Pro Kit (Qiagen, Hilden, Germany), with a modified version of the protocol. Thereafter, DNA was quantified using a fluorometric method, then it was sequenced on an Illumina NovaSeq platform, leading to 2x150 bp reads.
Raw reads were quality filtered, then metagenome-assembled genomes were reconstructed. In addition, several bioinformatics tools were used to profile the taxonomic composition of the metagenomes, as well as to assess the metabolic potential of the community.

5. Results and Discussion

5.1 DNA extraction protocol optimization

To guarantee the maximum DNA extraction yield from environmental samples, four different variants of the standard DNeasy PowerSoil Pro protocol were tested: i) protocol with no modifications; ii) cell pellet wash with 10 mL of 100% EtOH prior to the DNA extraction; iii) addition of 70% EtOH to the wash buffer; iv) both modifications ii) and iii). The protocols were tested on mock samples.
Addition of 70% EtOH to the wash buffer performed better than all the other protocol variants, with a minimum yield of 1 ng/µL (Figure 1). EtOH might increase the yield of extraction from low biomass samples (such as swabs made on sanitized surfaces) by removing salts and impurities from the column, as reported by Li *et al*. (2020). Therefore, we selected the protocol version iii) to extract DNA from our samples.

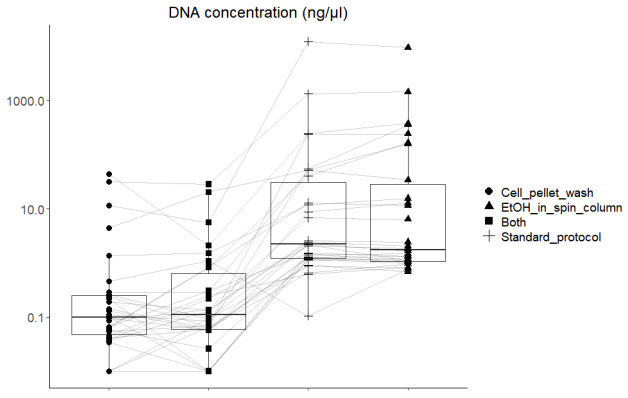

Figure 1 *Boxplot showing the DNA extraction yield achieved with each protocol. DNA concentration is reported in ng/µL.*

5.2 Microbiome characterization in the environment of dairy facilities

After MetaPhlAn (Beghini *et al*., 2019) taxonomic profiling of the metagenomes from cheesemaking facilities, a higher biodiversity (as measured with Shannon's and Simpson's indices) was observed on surfaces than in ingredients/final products (Figure 2). This suggests that a highly diverse microbiome might resist environmental stresses such as the use of biocides, probably by forming biofilms. This result was also supported by a Principal Coordinate Analysis based on the binomial distance, that shows a clear separation between food (ingredients and final products) and surfaces (both food contact and non-food contact; Figure 3A). Interestingly, *Acinetobacter* and

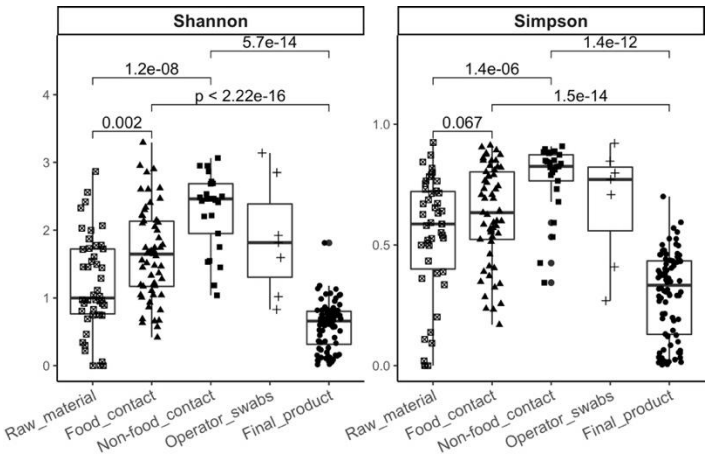

Figure 2 *Alpha diversity indices compared among sample types from cheesemaking facilities. Groups' medians were compared using pairwise Wilcoxon rank-sum test.*

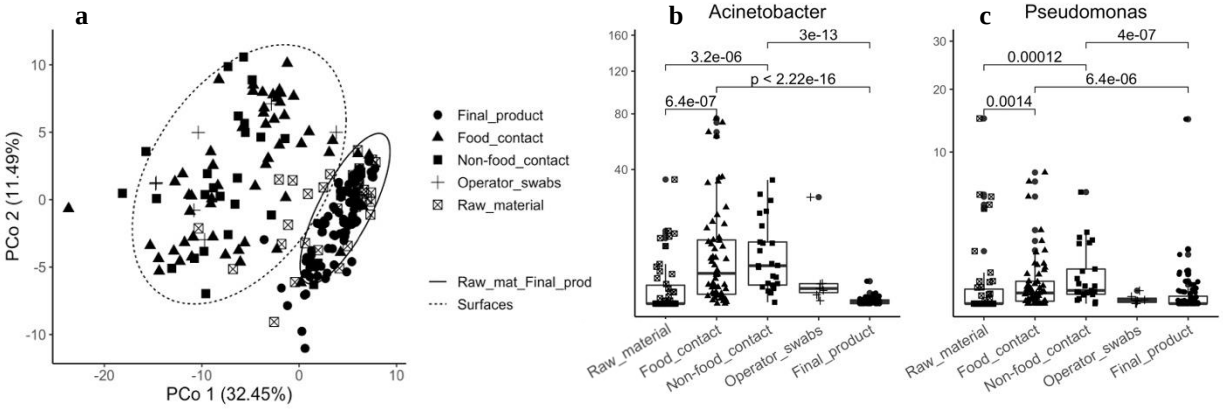

Figure 3 *A) PCoA based on taxonomic composition of samples using the Binomial distance metric; boxplots showing the relative abundance of Acinetobacter (B) and Pseudomonas (C) on the different types of samples. Groups' medians were compared using pairwise Wilcoxon rank-sum test.*

Pseudomonas were more abundant on surfaces (Figure 3B & C). These taxa have been associated with biofilm formation and with food spoilage (Cui *et al*., 2022; Wang and Xie, 2020), and their persistence in food production plants might cause food loss. The higher abundance of these taxa of surfaces might be a result of a selection exerted by the long-term usage of biocides, such as disinfectants.

As expected, we also observed a high relative abundance of Lactic Acid Bacteria (LAB). Interestingly, strain-level analysis of MAGs from *Streptococcus thermophilus* suggested the presence of product-specific, and even facility-specific, strains (Figure 4). Also, functional analysis highlighted that the genomic strain-diversity of *S. thermophilus* might lead to different metabolic activities exerted by members of this species. LAB are well known to contribute to the sensorial profile of cheeses, and our data suggest that they might represent a facility-specific fingerprint of the product.

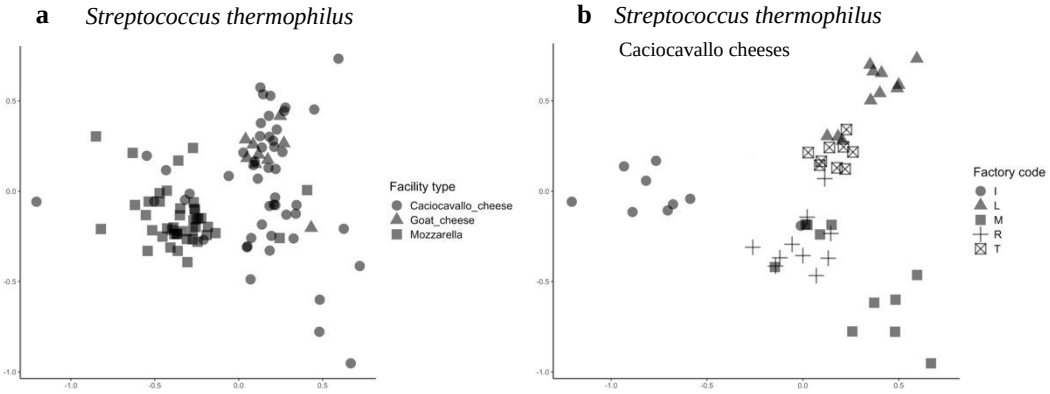

Figure 4 *PCoAs based on Average Nucleotide Identity (ANI) distance between S. thermophilus MAGs. MAGs are shape-coded according to A) facility type and B) Caciocavallo cheese factories where they originated.*

5.3 Microbiome characterization in the environment of minimally processed vegetables producing facilities

Taxonomic composition of samples collected in minimally processed vegetables producing facilities was assessed following the same approach used for dairy samples. Vegetables and surfaces harbour different microbial compositions, according to a PCoA based on the Bray-Curtis distance, although their biodiversity was comparable. In addition, no differences were observed in the abundance of several spoilage or potentially pathogenetic taxa, such as *Acinetobacter* and *Bacillus*.

Furthermore, we screened our metagenomes to assess the virulence potential of microorganisms residing in both vegetables and surfaces. We found more than 600 genes related to microbial virulence, with more than 80% of these attributable to *Pseudomonas*. In particular, virulence genes that were associated with biofilm formation had a similar abundance on both vegetables and surfaces, highlighting a potential adaption pattern (Figure 5).

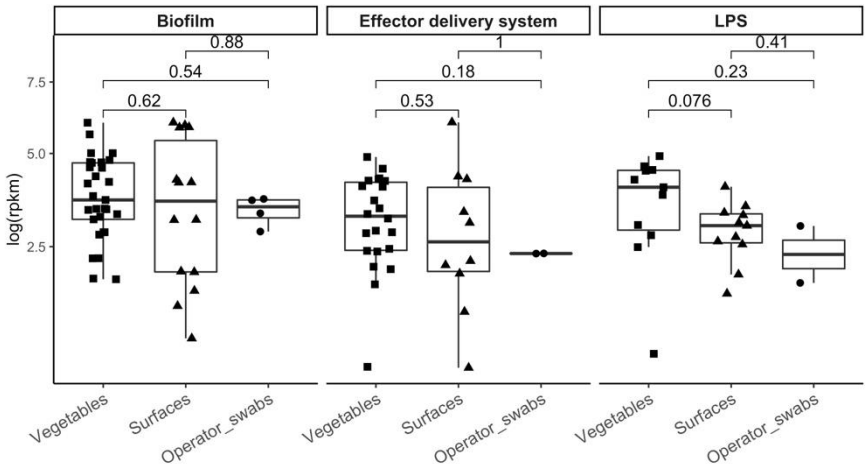

Figure 5 *Boxplots showing the log(RPKM) abundance of genes associated with biofilm formation and lipopolysaccharide secretion in different samples types from MPV facilities. Medians were compared with pairwise Wilcoxon rank-sum test.*

This result suggests that sanitation procedures adopted in minimally processed vegetables producing facilities might be ineffective in removing biofilms. Bacteria embedded into biofilms are up to 1000-fold more resistant to disinfectants compared with planktonic cells (Carrascosa *et al*., 2021), thus industrial disinfection might expose microorganisms to sub-minimal inhibitor concentration of these compounds, enhancing not only their biofilm formation ability, but also their antibiotic resistance (AR). Indeed, we found more than 250 genes associated with antibiotic resistance and attributable to several taxa (e.g., *Pseudomonas*, *Acinetobacter* and *Bacillus*). Interestingly, surfaces hosted a wide range of antibiotic resistance genes, with multidrug resistance being the most prevalent group (Table 2).

Table 2 *The table summarizes the AR genes found in MPV facilities according to sample type, resistance class and taxa from which they derive.*

Sample type	Genes	Most prevalent AR classes	Most contributing taxa
Food contact	143	Multidrug, β-lactams, glycopeptide	Bacillus, Acinetobacter, Enterobacter
Final products	49	Multidrug, quinolones, β-lactams	Pseudomonas, Pantoea
Raw materials	35	Multidrug, quinolones	Pseudomonas, Pantoea
Non-food contact	30	Glycopeptide, multidrug	Pseudomonas
Operator swabs	20	β-lactams	Bacillus

In addition, analysis of MAGs allowed us to identify 4 genomes attributable to *Bacillus cereus sensu strictu*, associated with food contact surfaces or swabs made on operator's hands/aprons. This microorganism is a well-known pathogen, which can produce emetic or diarrheal exotoxins (Baldwin, 2020). To further assess the pathogenesis of these genomes, we aligned the genes predicted from the MAGs to the nucleic sequences associated with *B. cereus* enterotoxins secretion, i.e., *hblCDA*, *nheABC*, *cytK*, and *entFM* operons. From the alignment results, we observed that all the MAGs had the operons, thus further confirming their pathogenesis (Table 3).

Table 3 *Each line of the table refers to a B. cereus MAG. For each MAG, presence/absence of pathogenic operons and % of identity and coverage are reported.*

Sample	Sample type	hblCDA	nheABC	cytK	entFM	identity	coverage
G5	Food contact	Incomplete	Yes	Yes	Yes	> 98%	> 97%
G6	Food contact	Yes	Yes	Yes	Yes	> 96%	> 97%
G7	Food contact	Yes	Yes	Yes	Yes	> 97%	> 97%
J8	Operator swabs	Yes	Incomplete	Yes	Yes	> 98%	> 98%

6. Conclusions and Future Perspectives

Unveiling the microbial communities that inhabit the food production environment is a primary task. Information gained from such investigation is useful for the food business operator to better understand metabolic activities exerted by beneficial microorganisms (e.g., LAB), as well as to track spoilage/pathogenic microbes and verify the effectiveness of sanitation procedures.

Our results show that WMS can provide more taxonomic/metabolic details about the food plant-adapted microbiome than culture-based methods, as well as in shorter analyses times. With this technique, strain-level detection and characterization of alterative and pathogenic taxa can be reached.

Although promising, the use of Whole Metagenome Sequencing strategies to study the microbiome of the food processing environment is still at a preliminary step. The bottleneck of this approach is represented not only by the high (although constantly decreasing) cost of sequencing, but also by the need for bioinformatics skills to correctly analyse and interpret data.

Also, since this approach targets the microbial DNA, it cannot provide any information about the actual metabolic processes occurring in a microbiome, but it can just illustrate the potential activities that microorganisms might exert.

Hence, further technological improvements (such as the validation of innovative procedures based on the analysis of RNA) and new professional figures specialized in metagenomic data analysis are needed to fully implement WMS as a routine mapping strategy in the food industry.

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Multifactorial traceability and characterization of green and roasted coffee

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The main steps of PhD project are exposed. Coffee sampling, as first, had been crucial to identify the suitable samples to submit to our process of characterization and traceability. Green and roasted coffee were characterized, both macro-nutrients and volatile profile were assessed to evaluate their trend and changings once coffee underwent to 3 different roasting profiles. Sugar (sucrose, glucose, and fructose) together with asparagine were also tested to evaluate their impact on acrylamide (AAM) formation and degradation during roasting processes differing for length and end temperature. Additionally, a preliminary screening with IR spectroscopy had been performed and correlated with chemical profile and qualitative classification of coffee was performed. A study on differences and similarities of cup profile of coffee beverages tastes via cupping and after espresso extraction was published, moreover a work on bitter taste to investigate different kind of bitter perception in coffee as well as their liking level is now on going. Finally, a sensory-chemical work aimed to identify differences provided on specialty coffee flavors and chemical profile by different water filtration systems will be carried out.

Tracciabilità e caratterizzazione multifattoriale di caffè verdi e tostati

Per il progetto di dottorato cruciale è il ruolo del campionamento del caffè per identificare i lotti conformi per il lavoro di tracciabilità elementare. Dei caffè verdi e tostati sono stati analizzati il contenuto in macronutrienti ed il profilo dei composti volativi per valutarne le evoluzioni dopo 3 diverse tostature con lunghezza e temperatura finale incrementali. Asparagina e zuccheri (glucosio, fruttosio e saccarosio) sono stati quantificati nel prodotto verde per verificare relazioni con la quantità di acrilamide prodotta in tostatura. Inoltre, è stato eseguito uno screening preliminare con spettroscopia IR correlato al profilo chimico e alla classificazione qualitativa del caffè. I risultati di uno studio comparativo delle caratteristiche sensoriali di caffè estratti alla brasiliana e con macchina espresso sono stati pubblicati, inoltre è in corso uno studio sul gusto amaro per indagare diversi tipi di percezione amara nel caffè e il loro livello di gradimento. Infine, sarà eseguito un lavoro con approccio sensoriale-chimico volto a identificare le differenze fornite sui sapori e sul profilo chimico di caffè Specialty da diversi sistemi di filtrazione dell'acqua.

Key words: green coffee, roasting, acrylamide, sugar, asparagine, headspace, odorants, Arabica, Robusta, IR,

1. Introduction

In accordance with the PhD thesis project described here above, this oral communication reports the main results and the progresses of the following four activities directed to:

A1) identify similarities and differences, depending on roasting level, between sensory profiles of specialty coffee extracted via cupping and espresso machine;

A2) evaluate the concentration of acrylamide and its precursors in roasted and green high-quality and specialty coffee and the influence played on them by origin, post-harvesting processes and roasting level;

A3) the identification of volatile aroma compounds of green and roasted coffee, analyzed via SPME-GC/MS, the differences caused by origin, post harvesting processes on green coffees and the influence that roasting levels provide for roasted samples;

A4) determine the elemental profile of green and roasted coffees to identify, whether present, a set of stable elements that could be used as traceability tools from the origin to the roasted coffee.

2. Specialty Coffees: traceability, volatile aroma compounds, sensory profile and safety

Since the last two decades, coffee market and the communication on coffee quality continuously increased and spread worldwide (Specialty Coffee Association, 2018b). Consumer have now the opportunity to buy cups brewed with different extraction methods by lots of single origin, single variety and even single farm coffees of known post harvesting process, altitude of the plantation and guaranteed flavor notes(Credence Research, 2018).

Parallel with the increase of SC market shares, frauds and loss of traceability are threats that, as for other high added value goods, overhang the authenticity of the products sold and bought through the whole supply chain that can be contrasted with reliable methods to track back roasted coffee to fields(Núñez et al., n.d.).

In this scenario, being coffee a processed good, the impact of post-harvesting processes on aroma and nutrient concentration and the effect of roasting on the volatile molecule profile released by roasted and ground coffee, the aftertaste, and the presence of regulated molecules as Acrylamide is in final beverage are of crucial importance.

All that considered, it is needful to improve the knowledge on specialty and high-quality coffee with the aim of giving solid scientific base at the development of this premium products market that, also in term of social and

environmental sustainability, would be the key for the future of coffee sector.

3. Experimental Procedure

In this PhD thesis a multiplicity of experimental procedure was set up to accomplish the objective of a multifactorial characterization of a big set of specialty coffees as detailed in paragraph 5. The work had a preliminary study in which 8 samples of specialty coffee were roasted at three different roasting levels then characterized for phenolic content (Folin test), melanoidins concentration, pH and acidity of brewed coffee and then sensory analyzed to identify the best roasting to match the flavor descriptors provided by the supplier.
Traceability data provided by supplier on 74 coffee samples, namely country of origin, region, farmer, specie, variety, year of the crop, post harvesting process, average altitude of coffee plantation were all recorded together with a score of reliability of these information. Physical features of green coffee (moisture, aW and defect presence/absence) were analyzed then, after freezing, a portion was ground and analyzed for volatile aroma profile, asparagine, sugar, macro, micro and rare earth element content. Whole green coffee beans were roasted at three roasting levels,. Roasting processes were evaluated by the % of weight loss. Roasted coffee samples (three for each green lot) were characterized for volatile profile, acrylamide, macro, micro and rear earth element content.

4. Materials and Methods

4.1. Samples

Green Arabica and Canephora coffee were sampled (1kg) from GrainPro bags of 60 kg. All Arabica coffees were "Specialty" or "Premium" coffee according to the Specialty Coffee Association protocol. Robusta coffee samples were Fine Robusta or selected among higher quality coffee for each country (Supremo NV, 2021). Coffee samples were from varieties representing the vast variability available in coffee market and processed with the three main Post-harvesting Processes.

4.2. Elemental composition

After grinding and storage at -20°C, 0,5g of both green and roasted coffee were weighted and mineralized in a Teflon tube with 5 mL of ultrapure HNO3 65% and 1 mL of H2O2 30% using a microwave system (Mars 5 Express, CEM) at power of 800 W for 20 min at 140°C followed by 20 min at power of 800W at 200°C. After cooling, the mixtures were filtered (porosity 0,45µm) using DIGIfilter system and completed to 50 mL final volume with ultrapure water. Elements were determined by ICP-OES 5800 Agilent Technologies (Santa Clara, CA, USA) for microelement, ICP-MS 7800 Agilent Technologies for microelement and ICP-MS/MS 8900 Agilent Technologies for rare earth element. Certified material from rice and tea were analysed to recovery test and blank samples were used for MDL calculation (3*std.dev AVG black value).

4.3. Asparagine and sugar

Asparagine was quantified by LC-MS/MS (Thermo Fisher Scientific) as reported by (Bertuzzi et al., 2020). 2 g of green coffee was extracted with 50 ml 0.01 M formic acid for 40 min and diluted (1+9 v/v) with H2O:CH3CN= 90:10 (v/v), asparagine was separated on a X-Select HSS T3 column (2.5 µm particle size,150×2.1 mm i.d., Waters Corporation) with a gradient elution of H2O-CH3CN (acidified 0.2% formic acid; pH = 2.6). The ionisation was performed in positive mode (ESI) and the ions fragment detected were 116, 87 and 74 m/z. LOD= 0.5 mg kg-1, LOQ=1.5 mg kg-1.
Sugars were extracted from 0.5 g of ground green coffee with 50 ml of 80% (v/v) ethanol for 10 min in an ultrasonic bath; after centrifugation (3500 g, 5 min), the extract was diluted (0.5 + 9.5 v/v) using H2O:CH3CN = 25 + 75 (v/v). Quantification was performed by LC-MS/MS. (Thermo Fisher Scientific, San Jose, CA, USA), Chromatographic separation was obtained using Xbridge BEH Amide column (2.5 µm particle size, 100 × 2.1 mm i.d., Waters Corporation, MA, USA) and a gradient elution 8 mM ammonium formate - CH3CN. The flow rate was 0.2 ml min-1. Fructose and glucose was ionized in negative mode (ESI), considering the [M+HCOO]− ion (225 m/z), sucrose in positive mode, considering the [M+Na]+ ion (365 m/z). Ions fragment were 90, 113 and 179 m/z for fructose, 90, 119 and 179 m/z for glucose, 185 and 203 m/z for sucrose. All sugars had LOD= 100 mg kg−1 an LOQ= 300 mg kg−1.

4.4. Roasting

Sample were roaster with IKAWA Model V2-PRO (IKAWA Ltd., UK, 2018). Each roasting batch was of 50 g (±0,5g). Three roasting profiles, namely "light" (I roast), "medium" (II roast) and "dark" (III roast), were applied reaching commercial roasting levels (Vezzulli et al., 2021). Preheating of chamber before coffee inlet was at 174-175°C, "light" roasting ended at 205°C (5.46 min), "medium" at 210°C (6.16 min) and the "dark" at 215°C (6.46 min). After roasting coffee was cooled, weighted, and stoared at -20°C. Before analyzing coffee was ground using Moulinex blender (Model AR110830).

4.5. Acrylamide

Acrylamide quantification was performed following the method by (Bertuzzi et al., 2017). 2 g coffee was weighed, added of 20 ml of Milli-Q water, 5 ml of hexane, 1ml of Carrez I and 1 ml of Carrez II and agitated for 45 minutes on rotatory and shaking stirrer. centrifugation at 4500 g (10 min) was applied, then 5 ml of aqueous phase was

added of 10 g of MgSO4, 1 g of NaCl and 10 ml of CH3CN. After 5 minutes shaking, vial was centrifuged and 3 ml of the organic phase were added of 150 mg of basic Al2O3, shaken and centrifuged for 3 minutes. 1 ml of the organic phase was purified on OASIS HLB column (Waters Corporation, Bedford, MA, USA) conditioned with 3 ml of H2O and 3 ml of CH3CN. AA was eluted into a vial. After evaporation, the residue was immediately re-dissolved in 1 ml of CH3CN: formic acid 0.2% (v/v) aqueous solution 10:90 (v/v). 100 µl of a deuterium-labelled AA (AA-d3) internal standard solution (1 mg l-1) was added to 900 µl of the extract; then, 20 µl were injected into the LC-MS/MS (Thermo-Fisher Scientific) in positive mode. Chromatographic with X-Select HSS T3 column (2.5 µm particle size, 150 × 2.1 mm i.d., Waters Corporation) and a gradient elution H2O-CH3CN (0.2% formic acid) was performed. The ions fragment detected were 55 and 44 m/z for AA and 58 m/z for AA-d3. LOD=5 µg kg-1; LOQ 15 µg kg-1. Results were corrected for the recovery (83.2%). 30% of samples for each roasting level was analyzed in duplicate.

4.6. Sensory Analysis

Twenty-four samples were split into three groups. Tasting was set to avoid draft effect given by bitter compounds. A panel of six coffee tasters (three Q-Arabica Graders, two experts from Istituto Italiano Assaggiatori Caffè and a master graduated in sensory analysis) was asked to evaluate with M34 Trialcard Plus form by "Centro Studi Assaggiatori – Italian tasters". The validation and replicability were evaluated via analytical replicate. Panel calibration was made with 100% arabica coffee and median of scores was kept as panel central value. Data were recorded with ADS System by Horizon Design and Centro Studi Assaggiatori Brescia.(Vezzulli et al., 2021)

4.7. Volatile compounds

Volatile compounds were detected using HS-SPME/GC-MS. 1.5 g of ground coffee was placed into a 15 mL vial immediately sealed with a Teflon-lined septum and screw cap (Supelco, Bellefonte, PA, USA). After 10 min of equilibration at 50 °C under magnetic stirring, the headspace of the ground coffee was sampled (30 min) by using an SPME fiber coated with DVB/CAR/DMS (75 µm) (Supelco, Bellefonte, PA, USA),. Extraction was under continuously magnetically stir at 50°C for 30 min. Fiber was thermally desorbed at 220 °C for 5 min in splitless mode. Volatile compounds analysis was made with TraceGQ Ultra coupled with an ISQ single quadrupole mass spectrometry (Thermo-Fisher Scientific, San Jose, CA, USA). Capillary column Rtx-5MS, 30 m x 0.25 mm i.d., 0.25 µm film thickness (Restek Corporation, Bellefonte, PA, USA was used). He (carrier gas) flow rate was 1.0 L/min. Temperature was set at 40 °C and increase of 12°C/min up to 180 °C (held 12 min); then at 40°C/min up 280°C (held 5 min). Transfer line T= 240 °C and the MS source T=230 °C. Mass spectra were acquired in the EI mode at 70 eV, m/z range of 40–450. Compound was identified with spectra database (NIST) when reference compounds were not available. In addition, linear retention indices (LRI) of volatiles in our experimental conditions were compared with data from the literature. Data acquisition was performed by a ThermoQuest Xcalibur 1.2 software. Each analysis was carried out in duplicate.

5. Results and Discussion

5.1. Sensory profiles

In the pilot work that was the frame of the PhD project, after espresso extraction of 8 different lots of coffee roasted at 3 levels (light, medium and dark) and the sensory analysis of samples, followed by the comparison with the sensory profile provided by supplier (Specialty Coffee Association, 2018a), medium roasting level was identified to be the most suitable to replicate the cup profile expected from the Origin.

Additionally, the 8 coffees also matched the sensory profile already available in literature for the analyzed countries and the different post harvesting processes (Hoffmann, 2018).

Therefore, coffee were also analyzed for their phenolic, melanoidins, caffein and acrylamide content that, together with pH and titratable acidity and the provided roasting profiles can play the role as a good and accessible method of standardization in the roasting process also for SME.

5.2. Acrylamide concentration and correlation with precursors

As reported in Table 1. the post harvesting process has a strong and significant ($p<0,05$) impact on the concentration of monosaccharides (Knopp et al., 2006) in green beans depending on the time of contact that seeds had with the mucilage and the mesocarp of the drupe during drying. Sucrose is not impacted by the post harvesting processes, the slight difference in our sample is due to the sample variability. Asparagine is always low in our sample set thanks to the lack of immature and defected seeds, majorly concentrated in this AA(Schouten et al., 2020) as in specialty and high-quality coffees is by definition.

Acrylamide concentration follows what already provided in literature (Lantz et al., 2006), particularly it picks in light and medium roasted coffee and the pathway of degradation is favored in dark roasting (Table 2).

Table 1: *Average sucrose (g kg^{-1}), glucose (mg kg^{-1}), fructose (mg kg^{-1}) and asparagine (mg kg^{-1}) content in dry, honey, wet Arabica and Robusta green coffee.*

	Dry Arabica	Honey Arabica	Wet Arabica	Robusta
Sucrose (g kg^{-1})	65.1 ± 4.6 A	71.6 ± 4.3 B	70.8± 7.7 B	32.6 ± 2.0
Glucose (mg kg^{-1})	1760.8 ± 636.2 B	707.9± 563.7 A	515.9 ± 270.6 A	941 ± 291.1
Fructose (mg kg^{-1})	3472.2 ± 1437.4 B	814.6 ± 755.6 A	560.9 ± 408.8 A	1093 ± 305.0
Asparagine (mg kg^{-1})	231.0 ± 35.0 a	242.0 ± 55.5 a	246.4 ± 61.9 a	365.6 ± 99.1

^{A,B,a,b} *Letters provide with significant differences from Games-Howell (uppercase) and Waller Duncan's (lowercase) test among Arabica coffees on the same line.*

Table 2: *Average acrylamide content (µg kg^{-1}) in dry, honey, wet Arabica and Robusta coffee at three different roasting levels.*

	Dry Arabica	Honey Arabica	Wet Arabica	Robusta
AA I roast (µg kg^{-1})	212.1 ± 70.6 b	148.2± 48.0 a	193.3 ± 56.6 a,b	300.4 ± 97.4
AA I roast-replicate (µg kg^{-1})	212.8 ± 87.6 b	130.3 ± 53.5 a	196.0 ± 81.5 a,b	309.5 ± 188.8
AA II roast (µg kg^{-1})	197.9 ± 45.6 b	159.0 ± 34.8 a	199.2 ± 38.2 b	229.9 ± 68.4
AA III roast (µg kg^{-1})	150.7 ± 32.8 b	104.2 ± 28.3 a	134.3 ± 40.4 a,b	213.5 ± 78.9

^{a,b} *Letters provide with significant differences from Waller Duncan's test among Arabica coffees on the same line.*

It was also found a correlation, for natural and honey coffees at light and medium roasting level, between monosaccharides concentration, the Aw of green coffee and the final content of acrylamide as in equation (1)

$$\text{Acrylamide} = m[(\text{fructose}+\text{glucose})*Aw\ ]+ q \quad (1)$$

where "m" and "q" are coefficients depending on the post-harvesting process and the roasting level.
Linearity (y=mx+q) of the equation is to be considered as deriving from the small range of precursors and acrylamide concentrations investigated and the finite amount of energy provided by the roasting process.

5.3. Volatile aroma compound

Green and roasted coffee provided with respectively 53 and 58 identifiable volatile molecules, more than a half of them known to be aroma active compounds. Even if data analysis in still on going, a preliminary statistical discrimination between origins (Risticevic et al., 2008) via green coffee volatile spectra, and among roasting levels, considering those from roasted coffee, was achieved.

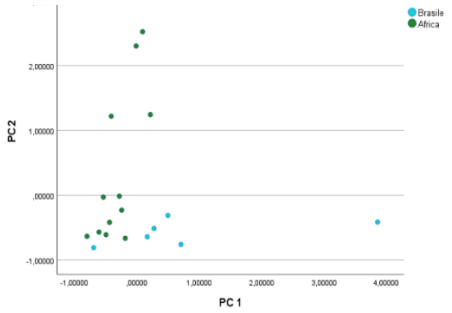

Figure 1 *discrimination between African and Brazilan coffee via PCA*

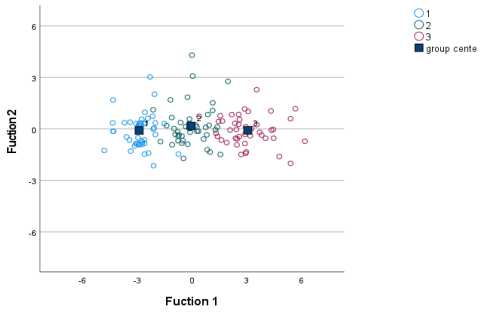

Figure 2 *discrimination among roasting levels via DA*

PCA (Figure 1) discriminated between African and Brazilian Arabica green coffees thanks, at first, to the higher abundance of limonene in the former. Additionally, head-space analysis of coffee roasted at 3 different roasting levels, confirmed that roasting strongly impact on aroma development in coffee (Abdelwareth et al., 2021; Liberto et al., 2013): figure 2 shows the discriminant analysis (DA) among roasting levels (total predictability of 88.8%).

5.4. Elemental analysis

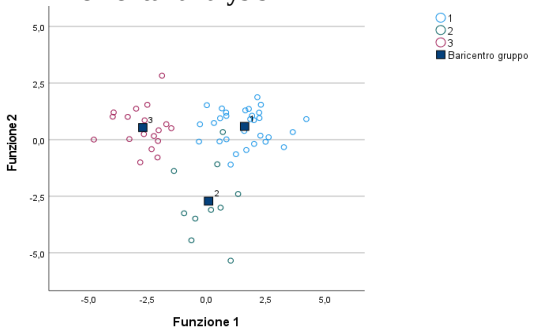

Figure 3 *discrimination among continents of origin in roasted coffee via DA (1=america; 2=asia;3=africa)*

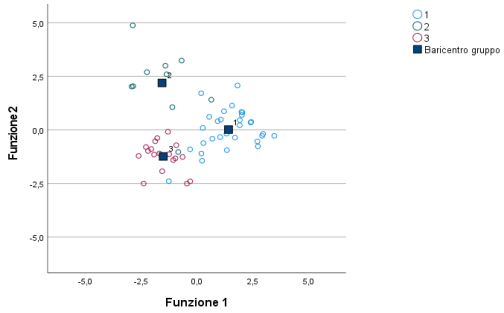

Figure 4 *discrimination among continents of origin in green coffee via DA (1=america; 2=asia;3=africa)*

Elemental characterization of coffee and the subsequent preliminary data analysis provided with a good discrimination of continent of origin of coffee lots both in green (93.1% of correct clustering) and roasted (98.3% of correct clustering) coffee, as reported in figure 3 and 4 and already described in literature (Bitter et al., 2020; Habte et al., 2016; Santato et al., 2012). In addition, our work found that some elements could be used to trace back roasted coffee to the green one and, finally to the origin, not mentioned in literature before. Several further analyses are now needed to confirm the hypothesis.

6. Conclusions and Future Perspectives

To conclude, this work, even it is just an extract of the whole PhD project aimed to give a deep and wide overview on peculiarities of specialty and high-quality coffee, it gave an insight on hazard and strength of a new and in continuous spreading sector of coffee market.
In next month, analysis of data from volatile compounds and elemental characterization will be deepen and several new investigations on the power of this two analytical methos as traceability and authentication tools will be conducted. Lastly, also considering the increasing interest on fine Robasta coffees, some of the experimental procedures above mentioned will also be applied to a set of samples of this specie.

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Yeast strain and processing technology affect the composition of yeast autolysates: characterization and potential effects on wine evolution

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This PhD project concerns the production of innovative yeast derivatives for winemaking, considering the use non-*Saccharomyces* yeasts and the optimization of manufacturing process. Autolysates from *S. cerevisiae* and *T. delbrueckii* obtained by using traditional and emerging technologies were characterized and their effect on wine ageing was evaluated after six months. Ultrasounds and enzyme addition led to higher release of soluble molecules, whereas high hydrostatic pressure and thermal inactivation determined higher insoluble solids content and poorer volatile profile, also depending on the strains. Protective effect against oxidation and no impact on filterability and volatile profile were observed in wines during ageing.

Ceppo e tecnologia di processo influenzano la composizione degli autolisati di lievito: caratterizzazione e potenziali effetti sull'evoluzione del vino

La presente tesi riguarda la produzione di derivati di lievito innovativi per vinificazione, dall'impiego di lieviti non-*Saccharomyces* all'ottimizzazione del processo di produzione. Autolisati di *S. cerevisiae* e *T. delbrueckii* ottenuti mediante tecnologie tradizionali ed emergenti sono stati caratterizzati e il loro effetto sul vino valutato dopo sei mesi di affinamento. Ultrasuoni e aggiunta di enzimi consentivano un maggior rilascio di molecole solubili, mentre alte pressioni e inattivazione termica un maggiore contenuto di solidi insolubili e profilo aromatico più povero, in funzione anche del ceppo. Nei vini, sono stati osservati buona protezione dall'ossidazione e nessun impatto su filtrabilità e profilo aromatico.

Key words: *Saccharomyces*, *Torulaspora*, yeast autolysates, wine ageing, chemical composition, volatile profile

1. Introduction

Yeast derivatives are a group of additives largely used in food industry as flavor enhancers, dietary supplements (Podpora et al., 2016), emulsifiers (da Silva Araújo et al., 2014) and, in winemaking, as fermentation and quality enhancers (Pozo-Bayón et al., 2009). Their addition in wine might simulate the ageing on lees, possibly improving the quality in less time and shortening ageing time (Barrio-Galán et al., 2019). Currently the only yeast employed for their production belongs to the genera *Saccharomyces*, even if there is an increasing interest in non-*Saccharomyces* yeast strains thanks to their capacity to release high amounts of polysaccharides (Domizio et al., 2014) and antioxidant compounds (Binati et al., 2021). Till now their contribute to wine ageing, composition and quality was not fully elucidated; however, their impact on wine might be not negligible since they represent a consistent part of natural microflora of grapes and must. Thermal inactivation and enzyme addition are the most common methods used to produce yeast derivatives, leading to obtain products with different chemical composition and volatile profile (Comuzzo et al., 2012). Emerging technologies such as ultrasounds and high pressure processing have been recently introduced in food industry as alternative to the traditional thermal treatments: ultrasound is useful for improving food quality and safety (Abid et al., 2013) and for accelerating yeast autolysis during wine ageing on lees (del Fresno et al., 2019). Interesting results were observed also concerning high pressure technologies, both for the production of yeast extracts and for polysaccharides extraction from yeasts (Dimopoulos et al., 2020; Voce et al., 2021a). The aim of this work is to evaluate how the yeast strains and processing technologies might affect the chemical composition and volatile profile of yeast autolysates; their effect on wine after six months of ageing was also investigated.

2. Materials and methods

2.1 Yeast autolysates: strains and treatments

Two commercial active dry yeasts (*S. cerevisiae* and *T. delbrueckii*, hereafter referred to as S and T) were rehydrated in 10 volumes of distilled water and processed by conventional methods, thermal inactivation and enzyme addition (hereafter referred to as THERM and ENZ, respectively) and emerging technologies, ultrasounds and high hydrostatic pressure (hereafter referred to as US and HHP, respectively). Thermal inactivation was carried out by autoclaving yeast suspensions for 2 hours at 120°C, whereas enzymatic lysis was induced by adding a commercial preparation of β-glucanase (0,5% w/w); ultrasounds treatment was performed as previously described (Voce, 2021b) in a temperature range of 20-45°C, whereas for high hydrostatic pressure yeast suspensions were treated at 600MPa for 15 minutes at 30°C. Except for thermal inactivation, the pH of the treated suspensions was adjusted to 4.5, then incubated at 45°C for 24 hours to allow yeast autolysis. The suspensions were then freeze-

dried and subjected to chemical analysis. For wine ageing, 300 mg/L of each autolysate were added to a white table wine and the analysis were carried out after six months of ageing at 20°C. A control sample (sulfur dioxide addition up to 150 mg/L) was also prepared. All treatments were performed in triplicate.

2.2 Characterization of yeast autolysates

The chemical characterization of autolysate powders was carried out in model wine (tartaric acid 33 mM, pH 3.2, ethanol 12% v/v). Free amino acids, proteins and polysaccharides were determined on the soluble fraction of autolysates as reported by Voce (2021b), whereas riboflavin was evaluated by RP-HPLC as described by Fracassetti et al. (2019). Total insoluble solids were determined by weighting. Antioxidant properties were evaluated by DPPH assay as reported by Dilna et al. (2015), both on soluble and insoluble fractions; furthermore, total content of cysteine, glutathione and reducing proteins containing cysteine (RPC) was evaluated by RP-HPLC (Tirelli et al., 2010).

2.3. Analysis on wine

Oxidizability was evaluated by the browning assay (POM-test) developed by Müller-Späth (1992). Catechins were determined as described by Zironi et al. (1992) and total polyphenol index (TPI) by measuring the OD at 280 nm (10 dilutions). Wine filterability was evaluated by measuring the filtration flow-rate trough a 0.45 µm pore size cellulose acetate membrane (47 mm diameter), whereas polysaccharides content was determined as described in Voce (2021b).

2.4. Volatile profile of yeast autolysates and wines

Two grams of freeze-dried powder and 10 mL of wine were introduced in 20 mL glass vials sealed with PTFE/silicone septa. The volatile profile was analyzed by SPME-GCMS, using a GC2030 Nexis gas chromatograph, coupled with a QP2020NX mass spectrometer (Shimadzu, Kyoto, Japan) and equipped with a GC autosampler (HTA, Brescia, Italy). The conditions for sample conditioning, microextraction and GC separation were those reported by Voce et al. (2021a).

2.5. Statistical analysis

Mean and standard deviation, one-way ANOVA and Tukey test, factorial analysis and Principal Component Analysis were carried out by the statistical software Statistica for Windows (StatSoft, Tulsa, OK, USA) version 8.0. The statistical differences between samples were considered significant at $p<0.05$.

3. Results and Discussion

3.1 Chemical composition and volatile profile of yeast autolysates

The chemical composition of autolysates is reported in table 1. All treatments caused a similar release of nitrogen compounds in model wine, except for US for which the highest amount was observed: only S-US was statistically different from all the other autolysates (248 mg/g), followed by T-US (91mg/g). US efficiency on cell disruption is due to permeabilization and damage to cell membrane, with following enhanced release of intracellular enzymes that may facilitate the hydrolysis of cell components (Voce, 2021b). This could explain the highest amount compared to traditional methods and HHP, whose effects seem to be mostly related to cell wall breakage (De Iseppi et al., 2019; Dimopoulos et al., 2020). Regarding polysaccharides, the different concentrations seemed to be related not only to the treatment but also to the strain: by considering the effect of treatment, the highest content was obtained with US for *S. cerevisiae* (217 mg/g) and with enzyme addition for *T. delbrueckii* (337 mg/g). On the other hand, by comparing the two yeasts treated with the same technology, US seemed to be more efficient in favoring the release of cell wall polysaccharides in *S. cerevisiae*, being probably dependent on a different susceptibility of the strain, composition and structure of cell wall, concentration of yeast suspension or cell size.

Table 1 *Chemical characterization of autolysates in model wine. Nitrogen compounds: sum of amino acids and proteins; radical scavenging activity: soluble and insoluble fractions; Cys+GSH+RPC: sum of cysteine, glutathione and reducing proteins. *SD: standard deviation.*

Yeast	Treatment	Nitrogen compounds (mg/g)	Polysaccarides (mg/g)	Riboflavin (µg/g)	Totale insoluble solids (mg/g)	Radical scavenging activity (µmol GSH/g)	Cys+GSH+RPC (µmol/g)
		Mean ± SD*	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
S. cerevisiae	S_ENZ	68 ± 4 ab	112 ± 17 a	11,0 ± 1,0 bcd	641 ± 25 c	29,4 ± 1,9 a	9,2 ± 0,4 bc
	S_THERM	67 ± 4 ab	191 ± 17 cd	6,7 ± 0,4 ab	678 ± 8 c	46,8 ± 6,7 bcd	10,1 ± 0,6 cd
	S_HHP	62 ± 15 ab	109 ± 7 a	19,0 ± 2,4 f	450 ± 41 b	36,0 ± 4,0 abc	13,2 ± 0,4 e
	S_US	248 ± 43 c	217 ± 26 d	17,8 ± 3,2 ef	289 ± 2 a	51,1 ± 7,4 cd	7,0 ± 1,2 a
T. delbrueckii	T_ENZ	39 ± 5 a	337 ± 9 e	13,8 ± 1,1 de	350 ± 10 a	60,6 ± 7,1 d	11,1 ± 0,7 d
	T_THERM	52 ± 4 ab	160 ± 14 bc	6,2 ± 0,4 a	659 ± 40 c	51,9 ± 5,9 cd	6,2 ± 0,7 a
	T_HHP	37 ± 8 a	141 ± 6 ab	12,9 ± 1,0 cd	823 ± 10 d	31,3 ± 3,4 ab	7,1 ± 0,1 a
	T_US	91 ± 17 b	173 ± 10 bc	8,6 ± 1,4 abc	675 ± 56 c	36,1 ± 7,5 abc	7,9 ± 0,7 ab

Riboflavin content was affected both by strain and treatment, with the lowest concentration detected in autolysates obtained by THERM for both yeasts, whereas the highest concentration was observed in autolysates obtained from

S. cerevisiae by emerging technologies (up to 19µg/g). In the case of white wine, in certain conditions that include riboflavin concentration (more than 30 µg/L) and presence of methionine, riboflavin may trigger light-struck defect (Fracassetti et al., 2019); even if statistical differences emerged, from the practical point of view the amount detected were below the limit level for triggering these reactions. By considering the insoluble solids content, ENZ, THERM and HHP showed the highest amount of such fraction; this trend was particularly evident in the case of *S. cerevisiae*, with an amount of about 641, 678 and 450 mg/g respectively, resulting significantly different from US (289 mg/g). In the case of *T. delbrueckii*, autolysates obtained by HHP showed the highest insoluble solids content (823 mg/g) resulting significantly different from all other autolysates. Thermal inactivation is generally employed for extracting cell wall components (De Iseppi et al., 2019), as well as the high pressure can allow a high cell disruption but pressure above 600 MPa might cause inactivation of intracellular enzymes, thus decreasing or preventing the induction of autolytic process (Dimopoulos et al., 2021). On the other hand, the higher permeability of cell walls and membranes induced by addition of exogenous enzymes and by US may enhance the release of intracellular content and the activity of endogenous enzymes, thus inducing autolysis and increasing the concentration of soluble compounds. Regarding antioxidant properties, treatment and strain affected the radical scavenging activity of the autolysates and the total content of cysteine, glutathione and reducing proteins. Concerning *T. delbrueckii*, autolysates obtained by both the traditional methods showed the highest radical scavenging activity, with statistical differences compared to T-HHP, whereas for *S. cerevisiae* the best results in terms of antioxidant activity were obtained by US and THERM. On the other hand, cysteine, glutathione and reducing proteins containing cysteine residues, known for their antioxidant properties, were detected in the highest concentration in S-HHP, resulting significantly different from all the other autolysates.

The high temperatures, potential secondary activity of the exogenous enzymes or the enhanced proteolytic activity caused by US might lead to a higher protein hydrolysis with following release of amino acids and small peptides that seem to exhibit antioxidant properties (Mirzaei et al., 2015); this might in part explain the highest radical scavenging activity observed in the autolysates obtained by traditional methods and US.

In figure 1 (a and b), the results of PCA carried out on the total absolute area of yeast autolysates are reported and the volatile compounds detected are grouped by chemical class. The volatile profile of yeast autolysates seemed to be dependent on the treatment; in general, autolysates obtained by ENZ and US were mainly characterized in terms of aroma compounds for both yeasts, whereas HHP and THERM allowed to obtain autolysates with lower odor impact, as observed in previous experiments (Voce et al., 2021a). The concentration seemed to be related to the treatment, whereas the aromas that mainly characterized the different autolysates seemed to be influenced by the strains: acids, pyrazines and esters mainly characterized T-US and T-ENZ, whereas S-US and S-ENZ seemed to be more characterized by higher alcohols and minor compounds that included diols and lactones. The poorer volatile profile of autolysates obtained by THERM and HHP make them more suitable for wine ageing because of their potential, lower impact on the aroma profile of the final product.

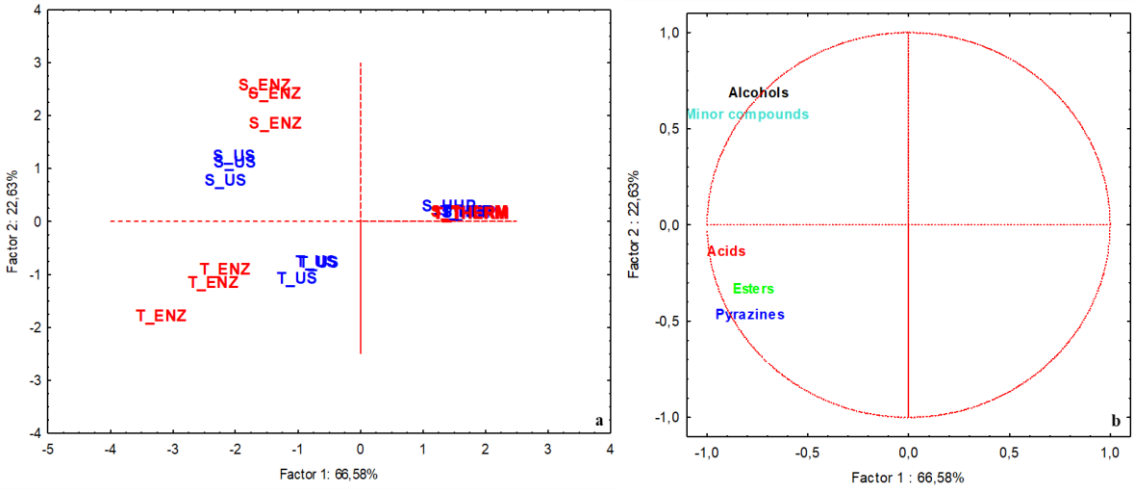

Figure 1 *Results of PCA carried out on the absolute area of volatile compounds detected in autolysate powders and grouped by chemical class. Projection of case (samples) (**a**) and variables (chemical class) (**b**) on the factor-plan are reported. In blue, yeasts treated with emerging technologies, in red yeasts treated with traditional methods.*

3.2 Effect of yeast autolysates addition on the chemical composition and volatile profile of white wine after six months of ageing

The chemical composition of white wine after six months of ageing is reported in table 2. All autolysates gave a good protection against wine oxidation, with no significant differences in terms of POM-test, TPI and catechins content compared to the control.

Table 2 *Chemical composition of white wine added with yeast autolysates after six months of ageing. TPI: total polyphenols index. *SD: standard deviation.*

Yeast	Treatment	POM-test				IPT index				Catechins (mg/L)				Poliysaccharides (mg/L)			
		Mean	±	SD*		Mean	±	SD		Mean	±	SD		Mean	±	SD	
S. cerevisiae	Con (SO_2)	71	±	2	a	5,3	±	0,3	a	8,1	±	0,5	a	1485	±	114	b
	S_ENZ	94	±	1	b	4,9	±	0,7	a	7,6	±	0,9	a	1519	±	174	b
	S_THERM	76	±	13	ab	5,3	±	0,2	a	8,3	±	0,4	a	1369	±	230	ab
	S_HHP	81	±	8	ab	5,0	±	0,6	a	8,5	±	0,4	a	1766	±	125	b
	S_US	73	±	13	ab	5,8	±	0,2	a	8,2	±	0,3	a	1812	±	72	b
T. delbrueckii	T_ENZ	80	±	6	ab	5,6	±	0,3	a	8,0	±	0,3	a	1762	±	207	b
	T_THERM	80	±	7	ab	5,9	±	0,7	a	8,4	±	0,4	a	1469	±	393	ab
	T_HHP	58	±	5	a	5,5	±	0,2	a	7,9	±	0,7	a	906	±	57	a
	T_US	79	±	9	ab	5,7	±	0,1	a	8,9	±	0,1	a	1484	±	185	b

Regarding POM-test, this index is related to the potential oxidizability of the wine; the highest value was observed for S-ENZ, thus resulting significantly different from control and wine added with T-HHP. The adsorption phenomena of phenolic compounds by the insoluble fraction of yeast derivatives as well as their interaction with soluble polysaccharides (Barrio-Galán et al., 2019) might explain the tendential lower TPI and catechins content observed in wine added with S-ENZ. The slight differences observed in terms of oxidizability were probably related to the fact that the wine used was young and enough stable; in this case, a more prolonged ageing time would have been necessary in order to observe more evident effects.
Regarding polysaccharides content, the mean higher amounts were observed in wines added with S-US and T-ENZ that, as discussed above, were the autolysates with the highest concentration of soluble polysaccharides. However, wine added with T-HHP (906 mg/L) resulted the only significantly different from the control: the relative lower amount of such soluble macromolecules detected in the autolysate (141 mg/g) coupled with the highest total insoluble solids content (823 mg/g) might explain the lowest polysaccharides amount and the lower potential of oxidizability in the resulting wine. Lastly, the addition of autolysates seemed not to influence the filtration of wine after ageing and no statistical differences were observed (data not shown).
The aroma profile of wines after six months of ageing is reported in figure 2 (a and b). Considering the results of PCA, the control wine seemed to be the most characterized in terms of volatile compounds, whereas wines added with autolysates obtained by THERM and HHP were the poorest. This may probably link to the lower concentration of aroma compounds in these autolysates, as reported above, thus reflecting in a lower odor impact on wine. However, also the autolysates obtained by means US and ENZ seemed not to particularly influence the aroma profile of the resulting wines, even if the powders were the most characterized in terms of volatile compounds. This might be explained by the ability of polysaccharides, especially mannoproteins, and of the insoluble fractions of powders to adsorb aroma compounds, thus modulating their volatility and consequentially affecting the volatile profile of wine (Barrio-Galán et al., 2019; Juega et al., 2012).

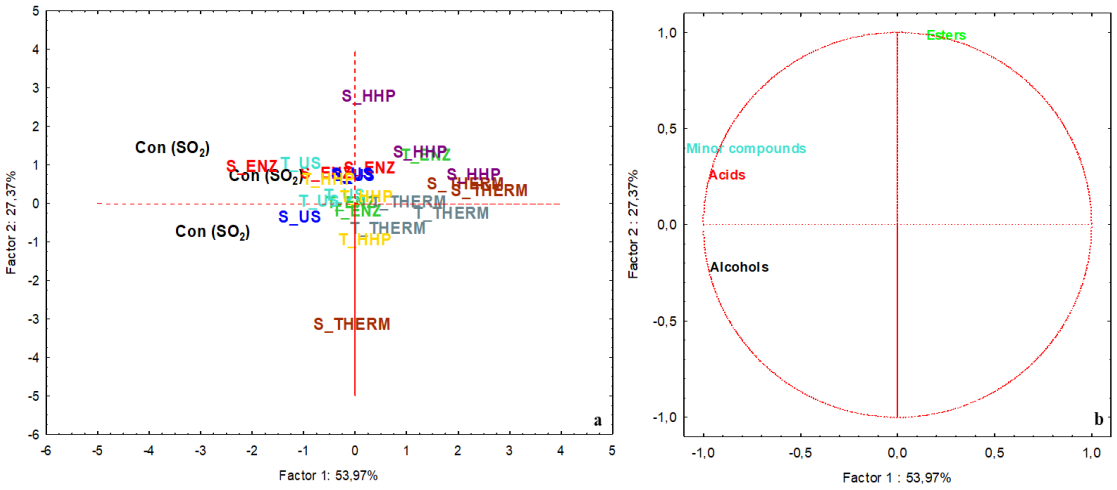

Figure 2 *Results of PCA carried out on the concentrations of volatile compounds detected in wine and grouped by chemical class. Projection of case (samples) (**a**) and variables (chemical class) (**b**) on the factor-plan are reported. Black: control; red: S-ENZ; brown: S-THERM; violet: S-HHP; blue: S-US; green: T-ENZ; grey: T-THERM; yellow: T-HHP; light blue: T-US.*

4. Conclusion and future perspectives

Strain and processing technologies may strongly affect the chemical composition and volatile profile of yeast autolysates. Considering the results obtained, non-*Saccharomyces* strains may be an interesting source of compounds, especially polysaccharides and antioxidant molecules. Concerning the process, emerging technologies may be efficient and low-cost methods for producing yeast autolysates and extracts by Ultrasounds, or yeast cell walls or hulls by High Hydrostatic Pressure, thus being able to replace enzyme addition and thermal inactivation, respectively.

This wide variety in terms of chemical composition make these products suitable for different food applications: as dietary supplement and as prebiotics as concern nitrogen compounds and polysaccharides, and as quality enhancer due to polysaccharides that positively affect stability, color, astringency and structure of wine during ageing. Lastly, the antioxidant activity showed by autolysates makes them also suitable for food and beverages protection against oxidation.

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