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.*

Activies	Months	1st year				2nd year				3rd year			
		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						▓	▓	▓	▓	▓	▓	▓	▓

3   Selected References

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