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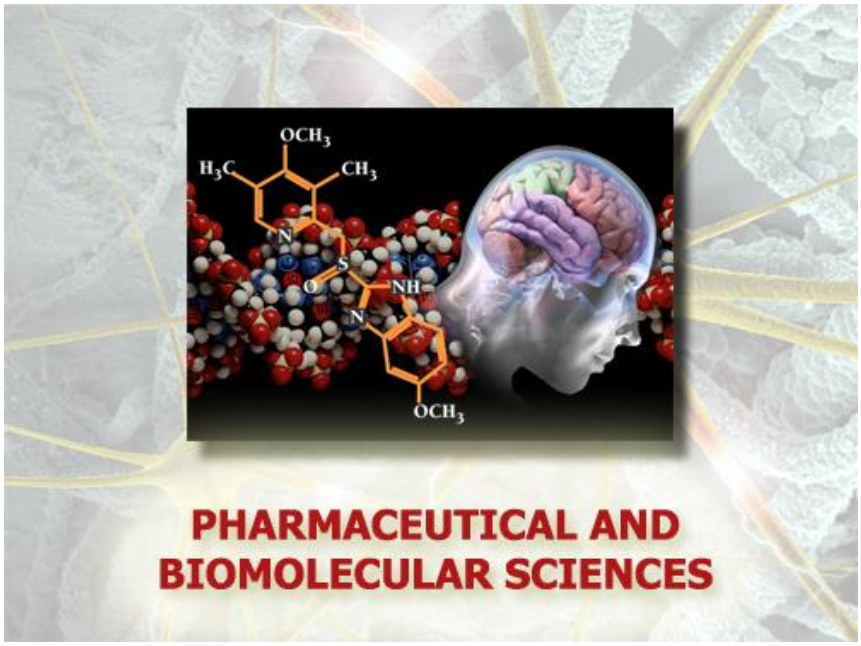

New strategies and technologies for the extraction and formulation of nutraceuticals and cosmeceuticals

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Preface

This thesis is an original intellectual product of the author, Alessandro COLLETTI, which is submitted to filfill the degree of Doctor in Pharmaceutical and Biomolecular Sciences at the University of Turin (Italy). The research herein was conducted under the supervision of Prof. Giancarlo Cravotto from the Department of Drug Science and Technology of the University of Turin (Italy), between November 2019 and November 2022. The present thesis saw the author involved in three clinical studies at the Department of Medical and Surgical Sciences (DIMEC) of the Alma Mater Studiorum University of Bologna (Bologna), SC Pediatric Urology of the SS Antonio e Biagio and Cesare Arrigo Hospital (Alessandria), and Studio Dentistico Pisano Procchio (Alessandria).

The motivation for this research stems from my passion for nutraceuticals and preventive medicine. With the continuous increase of the prevalence of chronic degenerative diseases, lifestyle changes are an increasingly important aspect requiring attention. In this regard, nutraceuticals constitute an important component of preventive medicine, as confirmed by nearly17,000 published scientific papers with the term “nutraceutical” in the title. It is not sufficient to add “years to life”; we need to focus on adding “life to years”.

Abstract

Nutraceutical is a synchratic neologism from "nutrition" and "pharmaceuticals" coined by dr. Stephen de Felice in the late 1980s. It is the discipline that studies enriched foods, functional foods, novel foods, food supplements (including botanicals), and foods for special medical purposes, which may have a preventive or, in some cases, a therapeutic role on one or more pathologies or risk factors.

The worldwide size of the nutraceutical market was estimated in about $500 billion at the end of 2021, with expectations of growth at an average annual rate at 6.9%, which would take the sector to $750 billion in 2027. Italy has a particularly prominent position with reference to the supplements market, the size of which is about 3.8 billion € in 2020. It is the leading European market, estimated to be worth 14.6 billion, with a 26% share, ahead of Germany (18.8%), France (14.7%), the United Kingdom (9.5%) and Spain (7.2%). The European market growth expectations are in the range of 6% annually, with Italy expected to reach a size of 4.8 billion in 2025.

Current guidelines for the prevention of the major chronic degenerative diseases suggest the importance of a correct lifestyle which include the optimization of physical activity, an appropriate nutrition plan and, if necessary, a nutraceutical treatment. In this context, in recent years, a great interest has been applied to nutraceutical supplements, which include a heterogeneous class of molecules with great potential to reduce inflammation, oxidative stress, and pain.

Among the most interesting nutraceuticals, bromelain is a mixture of proteolytic enzymes that is extracted primarily from pineapples (*Ananas comosus*) that is well known and used in several fields, especially in the nutraceutical and cosmeceutical sectors. The demand for bromelain is increasing quickly, and the reason for this great interest in the clinical field is related to its anti-inflammatory, antiedematous, fibrinolytic, anticancer, anticoagulative and antithrombotic properties that have been thoroughly described in the literature. In addition, this enzymatic complex is used in other sectors, including cosmetics, breweries, flesh processing and tenderisation, and textile industries. However, the isolation and purification of bromelain from pineapple (fruit, stem, core, leaves) is a challenge and constitutes 70–90% of the total

production cost of the final extract. To date, the commercial cost of bromelain extracts is high, with prices hovering around 2400 USD/kg. Despite the new feasible methods of protein purification (e.g., membrane filtration, reverse micellar systems, aqueous two-phase extraction, chromatographic techniques) and the new biotechnological processes developed to mitigate production costs, several limitations still create problems for the efficiency of product recovery from crude-plant extracts and the effectiveness of the obtained extract. In fact, the enzyme complex tends to be irreversibly inactivated at high temperatures (e.g., during the pasteurization process), while the progressive concentration of bromelain in crude pineapple juice during the purification process can induce spontaneous enzymatic deactivation. In this context, the use of a freeze-dried extract of pineapple juice obtained from by-products (core and peel of *Ananas comosus*) and thus, respecting the concept of "zero waste approach" and the "circular economy", has been shown to preserve a good quantity of total bromelain (8% of dry weight) in active form.
In this regard, three clinical studies have been conducted to evaluate the potential efficacy of a lyophilized pineapple extract (standardized and titled in bromelain) in order to reduce pain and improve quality of life in people with orchiepididymitis, or gonarthrosis, or subjected to surgical removal of lower third molars.

- The first pilot, interventional, double-blind, single-center, randomized study involved the enrollment of pediatric patients diagnosed with orchiepididymitis, who will be randomized 1:1 to receive a lyophilized pineapple extract or placebo in addition to the antibiotic treatment for the disease. The study population included 60 male patients belonging to the SC Pediatric Urology of the SS Antonio e Biagio and Cesare Arrigo Hospital, with a documented diagnosis of orchiepididymitis. After the obtainment of the informed consent, urinalysis and urine culture, urine examination of renal function on single spot, urinary ultrasound and quality of life (QoL) questionnaires were included before the start of treatment. After that, subjects were randomized in order to receive the nutraceutical (based on pineapple extract) or placebo for 15 days in addition to the antibiotic therapy. The

primary endpoint of the study was to investigate the comparison of the need for ibuprofen intake between the pineapple group and placebo. Secondary endpoints were the evaluation of the ultrasound parameters (oedema, scrotum size, epididymal commitment, vascularity) and the intensity of pain by using the Wong-becker pain rating scale (smileys: children from 6 to 10 years) and the Numerical Rating Scale (NRS) pain rating scale (for children >10 years) in addition to the Visual Analogue Scale (VAS) pain rating scale. A significant reduction of pain (VAS, NRS, Wong-becker) was observed in pineapple group from day 1 to day 15 (VAS-10: $p<0.0001$, NRS: $p=0.0006$, Wong-Becker: $p=0.0009$). Despite that the pain reduction was not statistically significant compared with placebo group, patients of active group reported a halved average intake of ibuprofen compared with placebo group. Regarding the secondary endpoints, pineapple extract demonstrated a better regression of the oedematous component and improvement of vascularity evaluated by Echo Doppler analysis. In addition, through both the prader orchidometer and palpatory analysis, there was a faster normalization of testicular volume in the pineapple group compared to the placebo group. In conclusion the results underlined the effectiveness of pineapple lyophilized extract on young people with orchiepididymitis by reducing the perceived pain and the need for ibuprofen intake. Moreover, the safety profile and the palatability of the nutraceutical treatment were excellent.

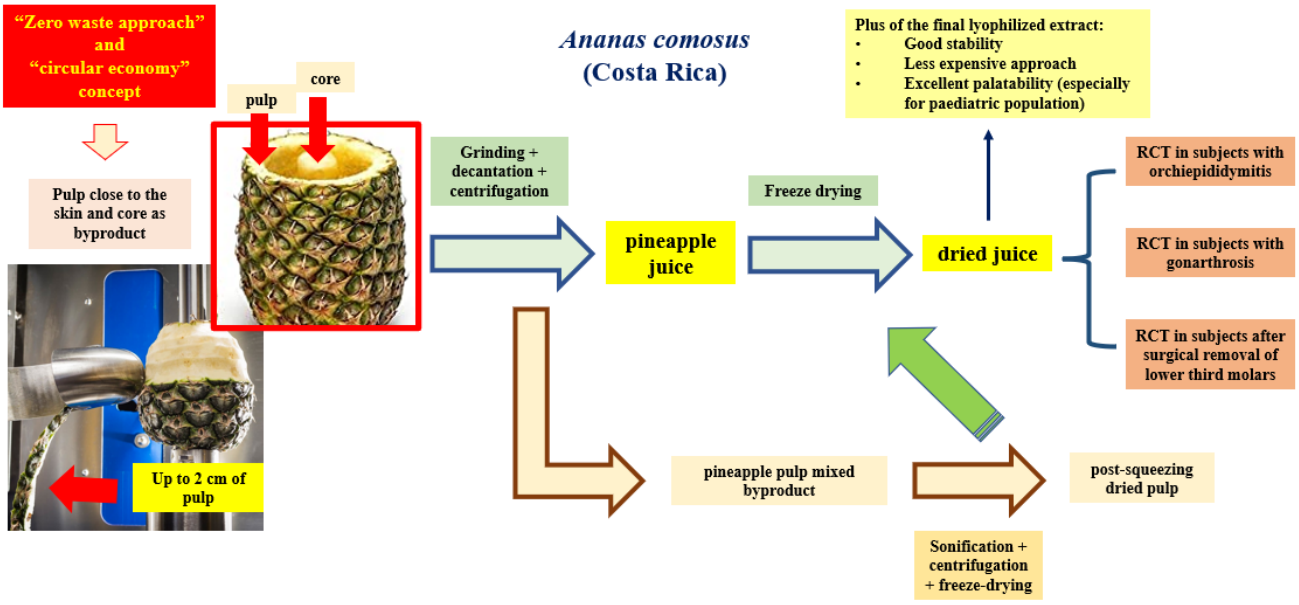
- The second double-blind, randomized clinical trial, was conducted at the Department of Medical and Surgical Sciences (DIMEC) of the Alma Mater Studiorum University of Bologna, on 40 subjects with gonarthrosis, for 8 weeks, to evaluate the effectiveness of a treatment based on lyophilized pineapple extract. The primary outcome was to assess the need of non-steroidal anti-inflammatory drugs (NSAIDs) (diclofenac 100 mg) in comparison with placebo group (after 4 and 8 weeks of treatment). The improvement of the total Western Ontario McMaster Universities Osteoarthritis (WOMAC) index, WOMAC pain and WOMAC stiffness

subscales, Lequesne Functional Index (LFI) and VAS were also evaluated. At the end of the study, the assumption of diclofenac (from day 0 to day 56) was statistically higher in the placebo group ($p<0,05$). Pineapple group showed a significant improvement in WOMAC index and WOMAC pain subscales, and VAS scale, after 8 weeks of treatment, compared with placebo. Moreover, the quality of life measured with the quality-of-life assessment (SF-36) was superior in the active group. The administration of pineapple extract titrated in bromelain in people with gonarthrosis showed a significant analgesic and anti-inflammatory effect, demonstrating to be a good alternative to NSAIDs to provide a more comfortable quality of life to these patients.

- In the third randomized, three arms, placebo controlled, clinical study (conducted in Studio Dentistico Pisano Procchio, Alessandria), it was evaluated the effect of a lyophilized pineapple extract titrated in bromelain or bromelain as mono-component, or placebo on pain, swelling, trismus, and quality of life (QOL) after surgical removal of lower third molars. Moreover, the need of NSAIDs (ibuprofen 600 mg for a maximum of t.i.d.) was also evaluated compared with the placebo group. The study included 42 people requiring extraction under local anaesthesia of a single mandibular third molar. The patients were randomized and distributed to receive the pineapple extract, bromelain or placebo and starting the treatment the day of surgery and continuing it for the next 7 days. The primary outcome was the need of NSAIDs between the two groups. The outcome variables were pain, swelling, and trismus, which were measured at 1, 3, and 7 days postoperatively. Differences in efficacy between freeze-dried pineapple extract and one-component bromelain were also evaluated. At the end of the study, the assumption of ibuprofen (from day 1 to day 7) was statistically higher in the placebo group ($p<0,05$). In addition, all groups showed a reduction in pain and swelling at all intervals, despite that the assumption of ibuprofen was statistically higher in the placebo group. Active groups also showed a

significant difference in the effect on QOL in most subscales and total scores compared with placebo ($P<0.05$). The supplementation of pineapple extract titrated in bromelain showed a significant analgesic and antioedema effect, in addition to improve QOL in the postoperative period for patients who had undergone lower third molar surgery. Moreover, both bromelain and pineapple supplementation reduced the need of ibuprofen to a comparable extent, demonstrating to be a good alternative to NSAIDs to provide a more comfortable postoperative course to these patients.

Graphical abstract



Chapter 1
Introduction of dietary supplements and nutraceuticals

1.0 Introduction of nutraceuticals

Nutraceutical is a synchratic neologism from "nutrition" and "pharmaceuticals" coined by dr. Stephen de Felice in the late 1980s. It is the discipline that studies enriched foods, functional foods, novel foods, food supplements (including botanicals), and foods for special medical purposes (Table 1), which may have a preventive or, in some cases, a therapeutic role on one or more pathologies or risk factors.

If, from a clinical point of view, nutraceuticals is a discipline widely recognized by the scientific community (as evidenced by the more than 100,000 works on PubMed with the term "nutraceutical" present in the title at the beginning of May 2023), from a legislative point of view nutraceuticals do not exist.

Clinicians define nutraceuticals by law as a dietary supplement that falls within the sectoral legislation (Directive 2002/46/EC, implemented in Italy with the Legislative Decree of 21 May 2004, n. 169) (12-13) as: "*food products intended for the supplementation of the common diet and which constitute a concentrated source of nutrients, such as vitamins and minerals or other substances, having a nutritional or physiological effect, in particular, but not exclusively, amino acids, essential fatty acids, fibers and extracts of vegetable origin, both as single and multi-compounds, commercialized in pre-dosed forms*".

According to Directive 2002/46/EC, the dietary supplement is something that "integrates the common diet"; however, some members of the scientific community prefer the term nutraceutical to subdivide the substances that have a normal integration role (e.g., the magnesium or potassium salts used during the physical activity) and therefore classifiable as dietary supplements, from the active ingredients that may have a preventive or therapeutic role (e.g., curcumin from *Curcuma longa* as adjuvant to chemotherapy in people with cancer), the so-called nutraceuticals.

Table 1. Nutraceuticals: subcategories and classification

FUNCTIONAL FOODS	ENRICHED FOODS	NOVEL FOODS	FOODS FOR SPECIAL MEDICAL PURPOSES	DIETARY SUPPLEMENTS
				
Foods **naturally rich in "substances"** which can be useful for maintaining health	Foods with **added substances** to enhance the nutritional or physiological effects	Foods or food substances which are **"new" for use**, which have recognized nutritional and/or physiological effects	Complete or not nutritionally, foods for special medical purposes may have adjustments focused on **specific needs**	Food products intended to supplement the common diet, and which constitute a **concentrated source of nutrients**
Example: the tomato naturally rich in lycopene	Example: Selenella potato with added selenium	Example: some insects such as grasshoppers	Example: formulation based on essential amino acids and micronutrients for sarcopenic patients	Example: supplements with magnesium, passionflower and valerian

As discussed above, the dietary supplement is considered a food by the regulatory authority. As such, the first characteristic that distinguishes it is the **safety**, established through the history of consumption that characterizes that particular substance. Simultaneously, a dietary supplement does not necessarily have to be effective. However, the efficacy is an extremely important parameter in the choice of a nutraceutical, especially in individuals with pathologies or risk factors in which the prescription of a supplement justifies the advice, use, and the expense by the patient.

The non-obligation of clinical testing of nutraceuticals represents the greatest limitation of this category of molecules and, as a direct consequence, it is now

possible to find on the market products of all types, with highly heterogeneous substances, in various combinations, at dosages and in extremely different pharmaceutical forms. For this reason, it therefore becomes essential to make use of scientific studies, preferably randomized and controlled clinical trials or, better still, meta-analyses of randomized and controlled clinical trials (supporting the “Evidence Based Medicine”).

1.1 Nutraceuticals and foods

It was underlined that from legislative perspective nutraceuticals are "foods". Indeed, several of the active ingredients that are currently registered as dietary supplements are naturally contained in foods. The Mediterranean diet, for example, is rich in functional foods: extra virgin olive oil, spices, some vegetables such as the *Brassicaceae* family and the dried fruit are nutraceuticals. Not surprisingly, it has long been linked to a reduced risk of developing chronic degenerative disorders, such as cardiovascular and neurological diseases as well as some types of cancer [1]. The potential benefits of the Mediterranean diet are attributable especially to the presence of molecules with antioxidant, anti-inflammatory, antibacterial, pro or anti-apoptotic, and vasodilatory activity.

The question is: if some foods are "nutraceuticals" and nutraceutical compounds are mostly present in the Mediterranean diet, why does it become necessary to "integrate"?

- **The preventive-therapeutic dosages of some molecules are difficult to assume exclusively with the diet**: for example, coenzyme Q_{10}, potentially used in the prevention of heart failure, is found in some foods such as eggs, beef, sesame, and herring, but at concentrations that require multiple servings not sustainable from a nutritional point of view. Furthermore, the pathological or pre-pathological subject is often deficient in precisely those molecules that would exert a protective action for a given problem: in such patients, the use of nutrition alone is often insufficient for the restoration of

deficiencies established over time due to multiple factors such as an altered behavioural diet, age, drug intake, and/or the presence of comorbidities.

- Optimization of "**bionutraceutical strategies**" to improve the pharmacokinetic profiles of nutraceuticals: the success of a nutraceutical treatment, in fact, does not depend exclusively on the correct choice of the active ingredient and on the dosages of administration, but also on its correct formulation from a technical and bio-nutraceutical perspective. Bionutraceuticals is the discipline that studies the relationships between the pharmaceutical form and the "*in vivo*" bioavailability of the active ingredient. Using specific pharmaceutical forms, depending on the nature of the active ingredients enables the enhancement of the effectiveness of the final treatment.

- Possibility of associations of different supplements in **nutraceutical poly-pills**: the use of specific pharmaceutical forms allows the inclusion of more active ingredients within the formulation and, consequently, the possibility of action on different molecular pathways (inflammation, oxidation, analgesia etc.)

- **Standardization and titration of active ingredients**: the use of standardized and titrated extracts is essential for the treatment to be effective and reproducible over time. Standardize means to "make uniform". The use of standardized extracts, which guarantee a constant and repeatable content of active ingredients in each production batch, permit the reproducibility of the health action of the nutraceuticals. Given the normal tendency to variability of natural products as a consequence of different factors (plant origin, cultivation conditions, climate, etc.), the standardization process must first of all concern the raw material. The selection in the field of uniform plant populations based on the content of functional substances; therefore, represents the first fundamental step in the process of standardization of

botanical drugs and all products derived from it. The subsequent transformation process, which concentrates and confers the desired characteristics to the extract must then guarantee, through the use of codified methods (G.M.P.) and conducted in parallel with analytical laboratory controls, a finished product always with the same chemical (title in active ingredients) and physical (density, appearance, consistency, and solubility) characteristics. Only with the use of standardized extracts it is possible to always ensure the same qualitative and quantitative active molecules. The capacity of a product to exert a beneficial effect (efficacy) is, in general, directly related to the quantity of active ingredients present. This quantity is indicated by a percentage called "**title**". The title, which is the index of the quantity of active ingredients per unit of weight, expresses the concentration of an extract (or a drug). However, a high title is not sufficient to guarantee the efficacy: it is important to consider a product that provides, at the indicated dosage, a quantity of active ingredients sufficient to ensure the effect.

- **Compliance with and persistence to treatment**: the intake of a nutraceutical poly-pill, compared with the daily and repeated intake of a pool of predetermined foods, contributes to compliance with and persistence to the treatment.

1.2 Development requirements of a nutraceutical

The development of a nutraceutical product should be based on 3 pillars that unite the world of integration with the world of pharmaceutical: quality, safety and efficacy.

1.2.1 Quality

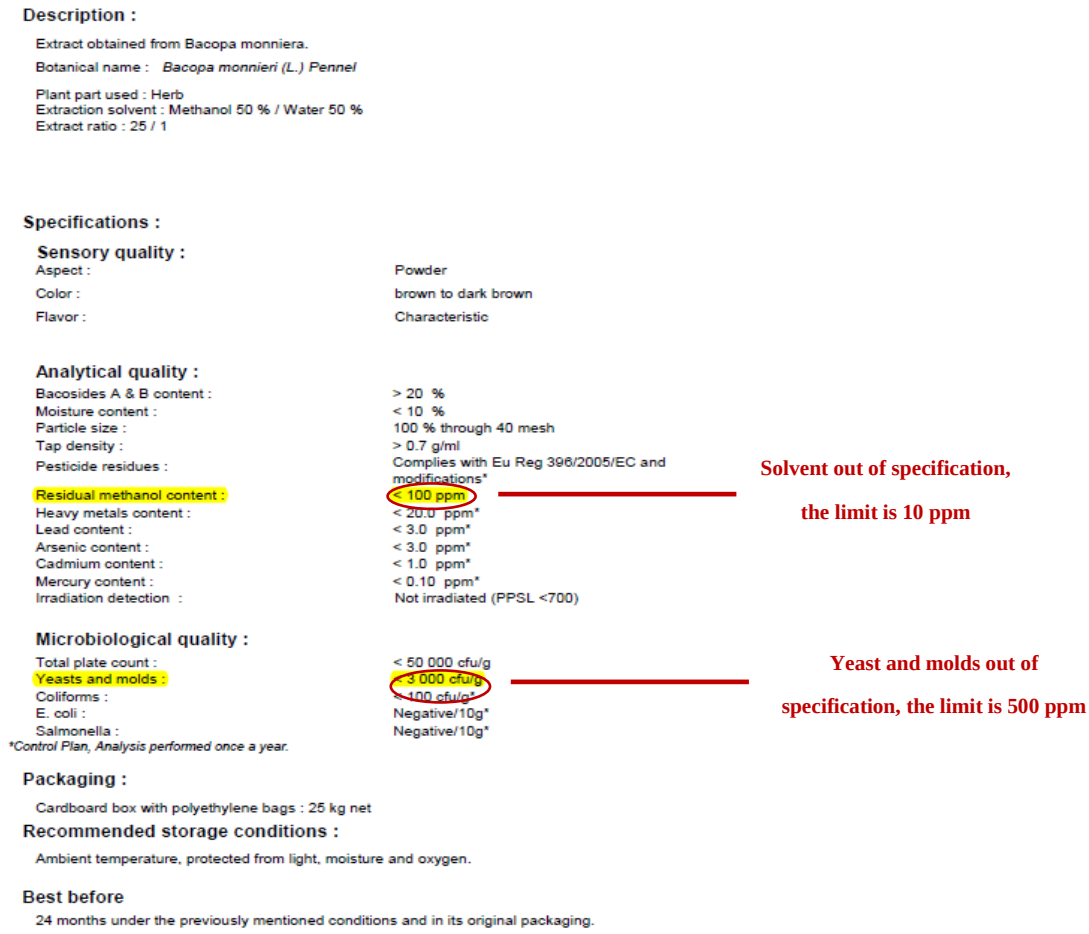
The quality of a nutraceutical is a condition "*sine qua non"* for its efficacy and safety. However, the quality must necessarily be defined by objective values that rely on validated criteria and not on subjective and somewhat imaginative considerations. Therefore, the quality of a nutraceutical cannot be defined if the raw materials, formulation strategies and production processes are not clearly known.

The quality of raw material should meet the legal requirements regarding the absence or presence within certain limits of potential contaminants such as pesticides (Reg. 839/2008/EC), heavy metals (Reg. 629/2008/EC; lead <3 ppm, cadmium <1 ppm, mercury <0.1 ppm), residual solvents (Dir. 32/2009/EC), polycyclic aromatic hydrocarbons (Reg. EU 1933/2015), mycotoxins (aflatoxin B1 ≤ 5ppb , aflatoxins B1 + B2 + G1 + G2 ≤ 10 ppb), microbial load ($\leq 10^4$ CFU/g, with the absence of *E. coli* and *salmonella*), yeasts, and molds ($\leq 10^3$ CFU/g).

Furthermore, the controls on the raw material also concern the possible presence of allergens according to the regulation of Annex II Reg. 1169/2011/EU (celery and products based on celery, sesame and products based on sesame, mustard and derivatives, lupins and products based on lupins, molluscs or crustaceans and products based on molluscs, sulfur dioxide and sulphites in concentrations >10 mg/kg or 10 mg/L in terms of SO_2, soy and products based on soy, milk and derivatives, nuts, cereals containing gluten, egg and egg products, fish and fish products, peanuts and peanut products). If one or more allergens are present, there is an obligation to highlight their presence on the label.

Figure 1 reports an example of how a *Bacopa monnieri*-based extract, according to current legislation, does not comply with the specifications on residual solvents and contaminants.

Figure 1. Technical data sheet of a *Bacopa monnieri* extract.



Description :

Extract obtained from Bacopa monniera.

Botanical name : *Bacopa monnieri (L.) Pennel*

Plant part used : Herb
Extraction solvent : Methanol 50 % / Water 50 %
Extract ratio : 25 / 1

Specifications :

Sensory quality :

Aspect :	Powder
Color :	brown to dark brown
Flavor :	Characteristic

Analytical quality :

Bacosides A & B content :	> 20 %
Moisture content :	< 10 %
Particle size :	100 % through 40 mesh
Tap density :	> 0.7 g/ml
Pesticide residues :	Complies with Eu Reg 396/2005/EC and modifications*
Residual methanol content :	< 100 ppm
Heavy metals content :	< 20.0 ppm*
Lead content :	< 3.0 ppm*
Arsenic content :	< 3.0 ppm*
Cadmium content :	< 1.0 ppm*
Mercury content :	< 0.10 ppm*
Irradiation detection :	Not irradiated (PPSL <700)

Solvent out of specification, the limit is 10 ppm

Microbiological quality :

Total plate count :	< 50 000 cfu/g
Yeasts and molds :	< 3 000 cfu/g
Coliforms :	< 100 cfu/g*
E. coli :	Negative/10g*
Salmonella :	Negative/10g*

Yeast and molds out of specification, the limit is 500 ppm

Control Plan, Analysis performed once a year.

Packaging :

Cardboard box with polyethylene bags : 25 kg net

Recommended storage conditions :

Ambient temperature, protected from light, moisture and oxygen.

Best before

24 months under the previously mentioned conditions and in its original packaging.

• **Plant powders**: characterized by the dried and shredded plant. It has the advantage that conveys the entire phytocomplex, but the disadvantage that it is not a concentrated source of active ingredients.

• **Vegetable extracts**: it can be obtained from fresh or dried plants using different extraction techniques (conventional and non-conventional), and with different solvents. It can be extracted as fluid, soft, or dry extract. It has the advantage that it constitutes a concentrated source of active ingredients and standardization of metabolites (most of the clinical studies of nutraceuticals conducted in the world use

these types of extracts). The disadvantage is related to the cost of the raw material, which is higher compared with the other types of extracts.

• **Tinctures and macerates**: obtained from dry drug (macerated) or fresh drug (tincture), they are well known for their traditional use. Mother tinctures are also a concentrated source of active ingredients. However, they are not standardized and are alcoholic.

The use of **standardized and titrated plant extracts** has made it possible to significantly reduce the variability of the composition of the extract physiologically due to the plant (moisture content, plant origin, method, and time of harvest), the extraction (extraction method, type of solvent, and solvent concentration), and production processes (batch size and extraction speed). Consequently, the choice of a quality raw material also concerns knowing how to identify the correct botanical extract by considering the cultivation area of the plant (which should have climatic characteristics similar to those of the native geographical area of the plant), the botanical part correct for the active ingredient to be used, the extraction method, and the extraction solvent (ideally extraction methods and green solvents free from toxicity but simultaneously effective in extracting the components of interest). Finally, it is important also the standardization process to ensure the repeatability of characteristics of the extract and, consequently, of the product.

As mentioned above, only with the use of titrated and standardized extracts is it possible to always make the same qualitative-quantitative formula. Standardizing means defining the specifications of the extract to make it as repeatable as possible [plant species, part of the plant used, place and period of collection, extraction solvent used, form of the extract (solid, soft, or liquid), extraction method, DER (drug/extract ratio), markers for titration].

Below (Figures 2 and 3) shows an example of how hawthorn extract can be significantly different from another obtained from the same plant (*Crataegus oxyacantha*).

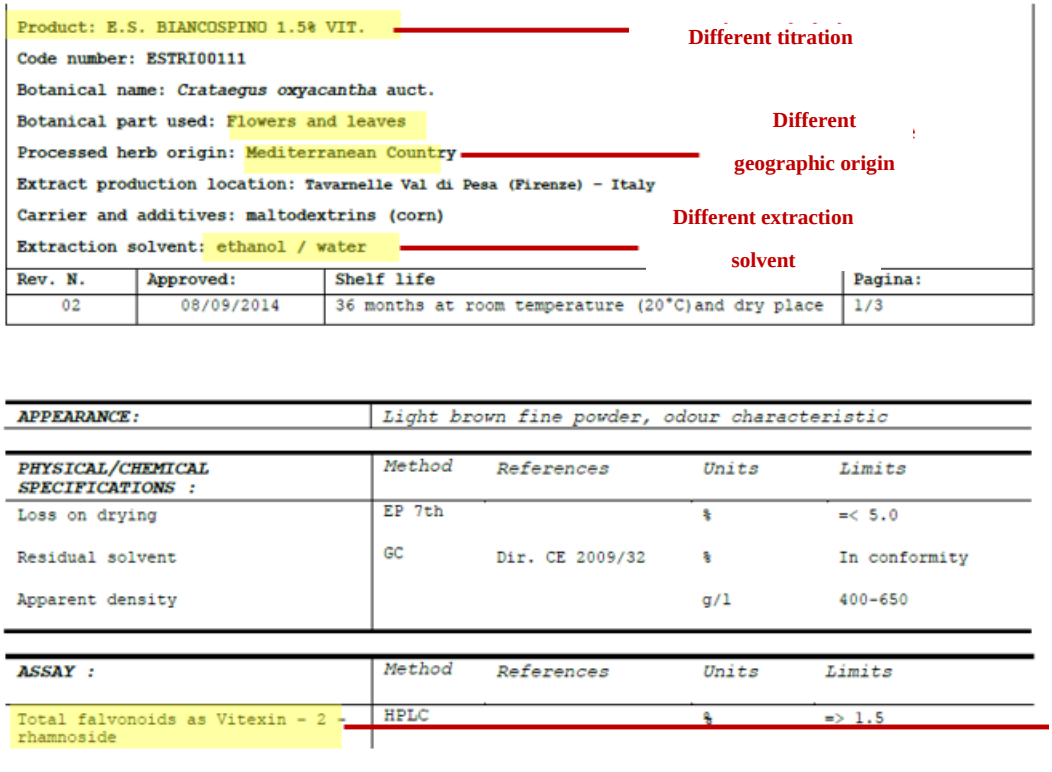
Figure 2. Data sheet of the first hawthorn extract (Extract 1)

Prodotto:	**Biancospino 2.5% estratto secco acquoso**
Codice:	3105402
Nome botanico:	*Crataegus curvisepala Lind. (C. oxyacantha L.)*
Nome INCI:	CRATAEGUS OXYACANTHA EXTRACT
Numero CAS:	94891-21-1 / 84603-61-2
Numero EINECS/ELINCS:	305-620-8 / 283-262-0
Solvente di estrazione:	acqua
Rapporto E/D	fino al titolo dichiarato (aprox 1/3-4)
Eccipienti e sostanze ausiliarie:	maltodestrina (da mais) max 30%
Famiglia botanica:	Rosaceae
Origine della pianta:	tutta Europa
Provenienza materia prima impiegata nel prodotto:	Europa orientale
Condizioni di crescita:	spontaneo
Stadio di crescita:	alla fioritura
Periodo di raccolta:	da giugno a luglio
Tipologia di essiccazione:	essiccatore sottovuoto
Parte della pianta impiegata:	fiori e foglie
Tipo di preparazione utilizzata:	estratto secco
Destinazione d uso:	Materia prima ad uso alimentare e cosmetico.

Costituenti attivi della pianta e titolo relativo:
flavonoidi (iperoside, vitexina 2-rhamnoside), procianidine oligomeriche, ammine, acidi fenolcarbossilici, acidi terpenici, steroli e amminopurine

Marker biologico:
Iperoside

Figure 3. Data sheet of the second hawthorn extract (Extract 2)



Product: E.S. BIANCOSPINO 1.5% VIT.
Code number: ESTRI00111
Botanical name: *Crataegus oxyacantha* auct.
Botanical part used: Flowers and leaves
Processed herb origin: Mediterranean Country
Extract production location: Tavarnelle Val di Pesa (Firenze) - Italy
Carrier and additives: maltodextrins (corn)
Extraction solvent: ethanol / water

Different titration

Different geographic origin

Different extraction solvent

Rev. N.	Approved:	Shelf life	Pagina:
02	08/09/2014	36 months at room temperature (20°C)and dry place	1/3

APPEARANCE:	Light brown fine powder, odour characteristic			
PHYSICAL/CHEMICAL SPECIFICATIONS :	Method	References	Units	Limits
Loss on drying	EP 7th		%	=< 5.0
Residual solvent	GC	Dir. CE 2009/32	%	In conformity
Apparent density			g/l	400-650

ASSAY :	Method	References	Units	Limits
Total falvonoids as Vitexin - 2 - rhamnoside	HPLC		%	=> 1.5

Different marker

Starting from a raw material of quality, the second step concerns the **formulation**: the success of a nutraceutical/pharmacological treatment does not depend exclusively on the correct selection of the active ingredient and its dosage of administration, but also on the correct bio-pharmaceutical formulation.

A quality raw material is therefore not sufficient to guarantee the quality of the product. Some active ingredients, in fact, have problems of **bioaccessibility** and bioavailability, which may compromise the effectiveness of the treatment. Bioaccessibility (B∗), which is the amount of active substance readily available for the enteric absorption, is defined by three important variables: release, solubilization, and interactions of the active ingredient. In other words, an active ingredient with reduced solubility, or conveyed by a tablet that does not disintegrate, or an active that negatively interacts with organic matrices (e.g., food or drinks) may have a

reduced bioaccessibility. A bioactive substance with a relatively high overall bioaccessibility [>75%] can be classified as B∗[+], whereas an active with a low overall bioaccessibility can be classified as B∗[-] L, B∗[-] S, or B∗[-] I, depending on whether its bioaccessibility is limited mainly by release (L), solubilization (S), or interactions (I).

After the release of the active ingredients from the matrix that contains it and its solubilization within the gastrointestinal fluids, it must be transported through the enterocyte and then be absorbed into the bloodstream [2]. Another important factor that defines the final bioavailability of the active ingredients, therefore, concerns its **absorption** (A∗) and the variables that may influence its distribution in the blood stream. It therefore becomes essential to consider the anatomy and physiological processes that regulate the intestinal absorption, not neglecting the layer of mucus present as support to the enterocytes, the tight junctions that regulate the paracellular permeability of some active substances, the phospholipid bilayer membrane that characterizes the enterocytes and transcellular absorption processes, and finally the active transporters and efflux pumps that can influence the absorption processes. The absorption of a nutraceutical by the epithelial cells lining the gastrointestinal tract can be limited by one or more factors, depending on its molecular and physicochemical characteristics. For example, it can be classified as A∗[-] ML, A∗[-] BP, A∗[-] TJ, A∗[-] AT, or A∗[-] ET if its absorption is limited by transport through the mucus layer (ML), reduced permeability in the phospholipid bilayer (BP), tight junctions (TJ), active transporters (AT), or efflux transporters (ET), respectively. A nutraceutical with relatively high absorption [>75%] by enterocytes can be classified as A∗ [+] [3].

Finally, the transformation processes (T*) defined by the phenomena of chemical degradation or by the microbiota metabolism that occur in the gastrointestinal tract are a further important aspect in the correct formulation choice with a view to better oral bioavailability.

A nutraceutical can therefore be classified as T∗[-] C, T∗[-] M, or T∗[-] CM depending on whether its bioaccessibility is limited by chemical degradation (C),

metabolism (M), or from both (CM). If a nutraceutical is relatively stable toward molecular transformations in the gastrointestinal tract (>75% remaining in a bioactive form), it can be designated as T∗ [+].

The **overall bioavailability** (BA) of the nutraceutical is represented by the formula [4]: BA = B* × A* × T*

Table 2 illustrates the **BAT classification** proposed by McClements and collaborators, which characterize the main limiting factors for the oral bioavailability of nutraceuticals [5].

Table 2. Bionutraceutical classification system (BAT)

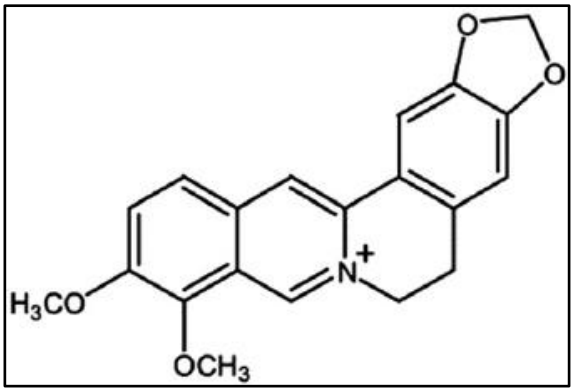
Biopharmaceutical classification system	
Class	**Subclasses**
B* (bioaccessibility)	L: liberation
	S: solubility
	I: interactions
A*(absorption)	ML: mucus layer
	TJ: tight junction
	BP: phospholipid bilayer permeability
	AT: active transporters
	ET: efflux transporters
T* (transformation)	C: chemical degradation M: metabolism

Based on what has been expressed, the biopharmaceutical classification system (BCS), strongly linked to the BAT classification, divides the active ingredients into: type I (high permeability and high solubility), type II (high permeability and low solubility), type III (low permeability and high solubility), and type IV (low permeability and low solubility). It is agreed that an active ingredient of class II, III, or IV may be treated from a bionutraceutical perspective to improve the aspects related to poor enteric bioaccessibility.

For example, berberine (Figure 4) is a quaternary benzylisoquinoline alkaloid found in the root, rhizome, stem, fruit, and bark of several plant species such as Coptis (*Coptis chinensis, Coptis japonica*), Hydrastis (*Hydrastis canadensis*), and Berberis

(*Berberis aristata*, *Berberis vulgaris*, *Berberis croatica*), well known for its antioxidant, anti-inflammatory, anti-proliferative, lipid-lowering, and insulin-sensitizing properties [6,7,8,9,10,11].

Figure 4. Molecular structure of berberine



The bioavailability of berberine is <1% due to the poor intestinal absorption caused by the tendency to induce a self-aggregation of particles which reduces its solubility in this tract, to the low permeability of the molecule (BCS class III), and to the first pass metabolism in the intestine and liver (43.5% and 0.14%, respectively). The effect of the first pass intestinal metabolism is not clear yet, despite that it is probably of enzymatic origin, linked to the cytochromes CYP2D6 and CYP3A4 involved in the hepatic metabolism. In addition, berberine is also the substrate of P-glycoprotein (P-gP), an ATP-dependent protein, better known as Multi Drug Resistance Glycoprotein (MDRG), capable of expelling the substrate from the enterocyte towards the enteric lumen (*pumping off*).

In recent years alternative approaches have been studied to increase the bioavailability of this molecule using: permeability enhancers (e.g., sodium caprate, sodium deoxycholate), P-gp inhibitors such as silymarin and particular salts of cationized chitosan with mucolytic substances such as acetylcysteine (NAC) [12], or modified release dosage forms (nanoemulsions, micelles, liposomes, and nanoparticles) with quite satisfactory results [13].

The formulation quality is therefore also extended to the excipients included in the formulations with specific technological purposes (diluents, preservatives, coating, etc.) and to the material used for the primary packaging. The use of specific excipients can permit the development of non-conventional nutraceutical forms (modified release). This means that dosage forms in which the absorption profile of the active ingredient is determined by the pharmaceutical form; the rate of release of the active ingredient is the determining factor in the rate of absorption. With non-conventional nutraceutical forms, we have a preparation capable of modifying the speed and/or time and/or place of release of the active ingredient, to achieve certain therapeutic objectives that cannot be obtained with traditional pharmaceutical forms intended for the same route of administration.

For example, for a patient with colorectal cancer, the use of site-specific release forms may improve the effectiveness of the therapy, acting selectively in the context affected by the disease. Another example includes the delayed-release forms containing botanicals such as lemon balm, valerian and hops which could be particularly useful in patients suffering from morning insomnia who, taking a tablet in the evening, will benefit from a delayed release of the active ingredients in the early hours of the morning. Alternatively, a patient with the *Helicobacter pilory* infection treated with nutraceutical gastro-retentive systems (in addition to drug therapies) could obtain better results if compared with the same conventional dosage form.

Quality of the raw material and the formulation must be accompanied by the production quality. The production processes, in fact, together with both the packaging and storage can influence the stability of the product. Stability refers to the maintenance of a product within defined limits and its storage and shelf life as well as the chemical, physical, therapeutic, and toxicological characteristics it possessed at the time of its preparation. In fact, if a product is not stored correctly or if the production processes are not adequately studied according to the type of active ingredients present in the formulation, phenomena of chemical (degradation of the active ingredient, pH variations, oxidation, hydrolysis, complexation, chelation, etc.), physical (phase separations, variation of solubility or state of aggregation, etc.), or

microbiological (contamination with molds, yeasts, pathogens, etc.) instability may occur.

1.2.2 Effectiveness

From a legislative perspective, the effectiveness of a nutraceutical is unnecessary because the food supplement is a substance or a complex of substances which integrate the common diet, with a nutritional or physiological role on the body. By law, therefore, the supplement does not have to be effective in treating or preventing a disease or a risk factor. However, from a clinical perspective, different randomized and controlled clinical trials have shown how the nutraceutical approach may be useful in the prevention and co-management of various risk factors or diseases [14,15,16].

A bridge between the legislation and the clinical world is the European Food Safety Authority (EFSA) which, on the basis of the published work, is responsible for the claims that can be spent on the label. The EFSA decides what can be declared about the health properties of a supplement. The claims are regulated and refer to the components of the product (not the product itself), and always describe a "contribution" that the component "can" make to the "good physiological functioning" of some anatomical areas. For example, "*Vitamin C contributes to the normal collagen formation and normal cartilage function*".

However, these claims present grey areas that may lead to confusion of the health worker in the final selection of the product:

- **Not all claims are dose-dependent**: For several substances there is no minimum effective dose for the declaration of the claim. For example, a product based on dry extract of devil's claw 50 mg (title 10% harpagosides) can declare the same claim on the label ("*it supports the joint function"*) as a product with devil's claw 500 mg (title 10% harpagosides).

- **Claims are attributed to the single active ingredient and not to the final product**. Consequently, clinical trials and preclinical testing that are discouraged as a claim endorsed for a substance can be used by all products containing that

particular substance and not only by the company that produced data for the claim request.

- **Claims do not refer to the quality of the raw material or product**.
- **Claims do not refer to the efficacy of the product** but to the "nutritional-physiological" functions.

No obligation of clinical and preclinical trials for the food supplement, in addition to the grey areas of the current legislation, has led to a significant increase on the market of ineffective, poor quality, and potentially unsafe products, conveyed by dubious commercial and marketing messages, as well as ethical and scientific value.

However, despite that the Directive 2002/46/EC (implemented with the Legislative Decree 21/05/2004 n° 169) defines the supplement as a food substance that supplements the common diet, the interest of the health worker is that the "nutraceutical approach" improves the client/patient state of health and, possibly, is effective on health.

How is it possible to recognize a serious nutraceutical? It is not enough to say "red yeast rice", "coenzyme Q_{10}", "curcumin" ...

For the plant, for example, it is necessary to understand if the literature is based on a specific extract, with a specific titration in active principle (if known, if associated with active molecules, even toxic), administered in a specific context. For the nutraceutical it is necessary to also understand the pharmaceutical formulation (type of release, oxidation/reduction state, excipientistics, etc.).

Guidelines for recognizing a "serious" nutraceutical are provided below:

- For **mono-components** the proposed nutraceutical must have a bibliographic support that includes at least:

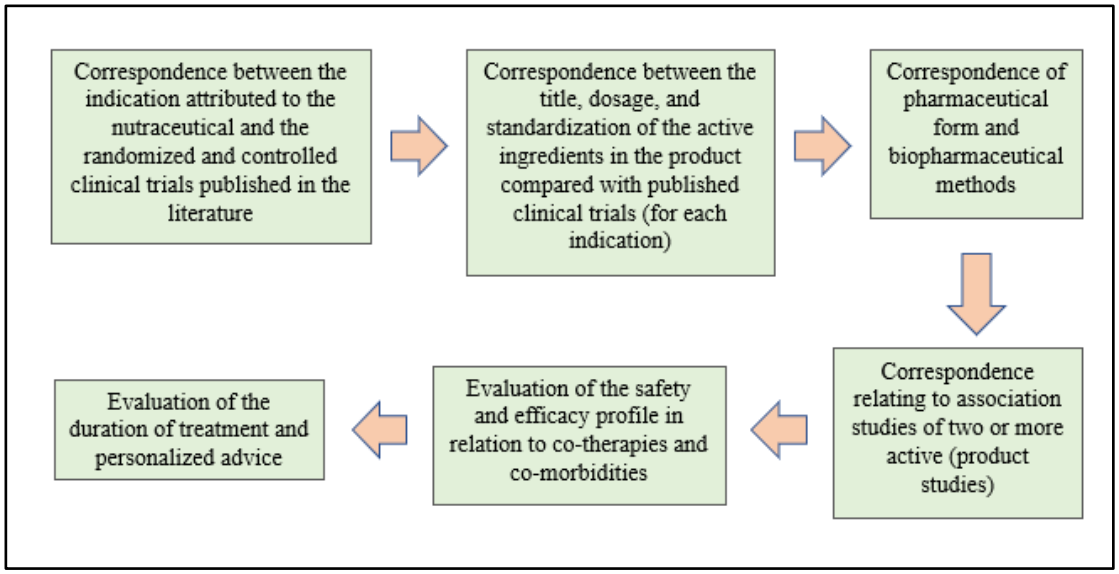
1) the description of the mechanism of action possibly associated with pharmacokinetic studies (for example, a good share of polyphenols has an oral bioavailability close to 0%; therefore, the usefulness of supplementation is doubtful);

2) the preventive/therapeutic indication has been proven by clinical studies, preferably conducted in double-blind against placebo or other active ingredient, on populations similar to ours for ethnicity and lifestyle;

3) the dosage and quality (title and standardizations) similar to those proven effective and safe in clinical studies;
4) the pharmaceutical quality control.
- For the **multi-component** the roles are similar to the previous ones if all the active ingredients are present in the product at the dosage proven by clinical studies. Furthermore, in case of a declaration of a synergistic action of the components (pharmacologically speaking, the effectiveness of two components together is greater than expected by individuals), this should be demonstrated by studies "*ad hoc*" and not simply extrapolated from pre-existing data on single molecules.
How to suspect a less "serious" product: the (unscientific) advertising claims reported in the information material proposed by the companies are not false, but lend themselves to dubious interpretations if they:
- do not refer to the geographical origin of the product, titration and/or quality control (often not existing for small producers);
- do not refer to a biologically plausible or proven mechanism of action;
- do not refer to minimum effective doses;
- do not refer to the duration of the treatment;
- do not refer to a specific pathophysiological condition to manage (therapeutic indication);
- extrapolating the results obtained with different formulations or dosages from those advertised;
- extrapolating the results obtained *in vitro* and *ex vivo* to humans;
- extrapolating the results in populations other than ours;
- omit any risks of adverse events.

The choice of a nutraceutical product should therefore go through a flowchart designed to produce advice according to science and conscience (Figure 5).

Figure 5. Flow chart for the correct choice of a nutraceutical



.2.3 Safety

The dietary supplement should be safe "for definition" as it is considered a food with a history of consumption on the national territory prior to May 15, 1997 (Directive 2002/46/EC implemented with the Legislative Decree 21/05/2004 n°169). However, although the food supplement is typically considered as a "natural product" with the meaning of the total safety of itself, the scientific literature is not exempt from reports of adverse effects caused by nutraceuticals, especially in frail or pluri-pathological patients.

A nutraceutical, in fact, is characterized by active substances that have mechanisms of action sometimes similar to pharmacological. As such, it can cause adverse effects directly or indirectly, interacting with the absorption, metabolism and excretion of other drugs or supplements, and modifying its natural pharmacokinetic profile.

The most common cause of adverse effect of a drug or a supplement, excluding the intrinsic safety profile of the active principle contained in the product (for example the alkaloids conin, conhydrin, pseudoconhydrin, conicein and methylconicin contained in hemlock are naturally toxic because induce the non-depolarizing type

neuromuscular blockade causing the neuromuscular paralysis), as it concerns the modulation of enterohepatic cytochromes (cytochrome P450 family).

Several active ingredients contained in food supplements can have an inductive or inhibitory effect on the activity of one or more cytochrome P450 isoforms, altering the therapeutic action and toxic effects resulting from the simultaneous intake of different active ingredients.

The inhibition of one or more cytochrome P450 isoforms occurs when two active substances (drugs, supplements and foods) are both substrates of the same isoform (for example CYP 3A4). In this case, the active principle will have a lower binding affinity and will be metabolized over a longer time than when taken alone. This phenomenon can lead to an increased risk of overdose effects and the occurrence of adverse effects.

Similarly, an active ingredient can induce an increase in the concentration and activity of one or more cytochrome P450 isoforms, increasing the clearance of other active substances which are substrates, and consequently its therapeutic action [17].

These aspects are particularly important in the oncology field where the therapeutic windows of chemotherapy drugs are very narrow, and an alteration of their metabolism could seriously compromise the efficacy or safety profile. Examples of potentially harmful botanical-antiblastic interactions are reported below (Table 3).

Table 3. Examples of botanicals-antiblastic interactions

Botanicals	Attention
Echinacea (inhibition CYP3A4)	Camptothecins, cyclophosphamide, TK inhibitors, epipodophyllotoxins, taxanes, and vinca alkaloids
Ginkgo (inhibition CYP3A4/CYP2C19 and scavenger action)	Camptothecins, cyclophosphamide, taxanes, and vinca alkaloids N.B. reduction in the effectiveness of alkylating agents, platinum and derivatives, and oncological antiblastics
Ginseng (stimulation of growth)	Attention: breast and endometrium positive estrogen receptors
Green tea (induction CYP1A2)	Erlotinib and bortezomib
Soy (growth inhibition antagonism)	Tamoxifen, breast, and endometrium positive estrogen receptors
Hypericum (power inductor CYP450)	All drugs
Valerian (inhibition CYP2C19)	Tamoxifen, cyclophosphamide, and teniposide
Vitis vinifera (induction CYP3A4 and scavenger action)	Camptothecins, cyclophosphamide, TK inhibitors, epipodophyllotoxins, taxanes, vinca alkaloids, platinum, and derivatives

Finally, it should be highlighted that some adverse events may be attributable to the presence of unwanted contaminants (for example citrinin, a mycotoxin contained in fermented red yeast rice, which can cause hepatotoxicity) [18]. Therefore, the choice of quality raw materials becomes fundamental (as described in the quality section), supported by the analyses on contaminants that certify its compliance.

1.2.4 A summary example regarding the importance of quality: curcumin

Curcuma longa L. is a perennial herbaceous plant with a rhizome that has been well known since ancient times for its beneficial properties and is one of the most studied

botanicals. The typical yellow-orange colour of turmeric derives mainly from the three active components of the plant: curcumin, monodemethoxycurcumin, and bisdemethoxycurcumin. Curcumin is a powerful multi-target polyphenol capable of modulating the expression of genes involved in cell survival, cell proliferation, angiogenesis as well as inhibiting various protein kinases, pro-inflammatory cytokines such as Tumour Necrosis Factor alpha (TNFα), interleukins (IL-1, -2, -6, -8, and -12), and inflammatory enzymes such as cicloxigenase-2 (COX-2), and lipoxygenases (LOX) [19].

The quality of turmeric depends first of all on the quality of the raw material and the purity of the extract. The analysis that allows the identification of the plant is DNA analysis (DNA barcoding) of the plant itself. A fundamental qualitative aspect concerns the absence of accidental or voluntary contamination with:

• species other than *Curcuma longa*, such as *Curcuma zeodaria*, *Curcuma aromatica*, and *Curcuma xanthozzhiza*

• azo dyes such as metanyl yellow and Sudan I and IV, which are organic compounds not allowed as additives and prohibited for food use but used as adulterants of turmeric

• synthetic turmeric

• contaminants such as polycyclic aromatic hydrocarbons (Reg. (EU) No. 2015/1933), pesticides (Reg. (EC) No. 396/2005), or solvents (Dir. 2009/32/EC) outside the acceptable limits, as required by current regulations

• GMOs

From a chemical perspective, the curcumin has a poor solubility in water and low intestinal bioaccessibility, with a consequent limitation of its bioavailability. To overcome this problem bionutraceutical strategies have been identified to improve the pharmacokinetic profile of this nutraceutical. One of the most popular strategies is the association of curcumin with piperine. Piperine is able to increase the bioavailability of some nutritional substances such as resveratrol or berberine, through the inhibition of P-gP, which is able to expel the substrate from the enterocyte towards the enteric lumen (*pumping off*).

Piperine in combination with curcumin therefore improves the activity of that supplement in the body, enhancing the amount of substance dissolved in the plasma and its absorption. However, it is notable that piperine increases the absorption of curcumin but with a non-selective mechanism; therefore, it can increase the absorption of other substances, of natural or synthetic origin, foreign to the body. In addition, studies conducted on mice have shown that the improvement in the pharmacokinetic profile is significant at doses of piperine >50 mg kg body weight and is not free from adverse effects on the gastrointestinal tract [20].

Another strategy concerns the use of the **phytosome** turmeric. The phytosome is a formulation of curcumin complexed with lecithins as surfactants which, together with bile salts, participate in the physiological process of absorption of lipophilic compounds, making even compounds that are not highly soluble in water more bioavailable. The efficacy of the phytosome turmeric is demonstrated by 35 scientific studies in humans, of which at least a third conducted with the randomized and controlled scheme, relating in particular to the areas of cardiovascular, intestinal and ocular health, nutrition in athletes, osteoarthritis, diabetes, and side effects of cancer therapy. In these studies involving over 2000 subjects, no adverse hepatic reactions were reported [21].

Nanocurcumin is a polymer formulation encapsulated in curcumin nanoparticles with a narrow size distribution of approximately 50 nm. Unlike curcumin, nanocurcumin is readily dispersed in aqueous media and has been shown to exert a greater inhibition of transcription factor NF-κB in different cell lines compared with normal curcumin, with significantly higher absorption kinetics with respect to turmeric powder.

Another type of nanoformulation, the **solid lipid nanoparticle** (SLN) loaded with curcumin, has also shown an improvement in the bioavailability of this molecule.

Finally, another formulation of curcumin aimed at increasing its absorption is the **liposome**. Liposomes are double-layered spherical vesicles that protect hydrophobic compounds (such as curcumin) and interact with aqueous environments by increasing aqueous solubility [22].

1.3 Conclusions

- **Nutraceutical**, expressed as the science which studies the effects of natural and functional substances on human health, is becoming increasingly important and considered in the panorama of the prevention and maintenance of health.
- Nutraceutical as a branch of medicine must make use of the principles of development: **quality, efficacy, and safety**.
- The **quality** includes the correct choice of raw materials, formulation strategies suited to the selected active ingredients, and the production and storage processes, which do not compromise the stability of the product.
- **Advanced formulation techniques** that allow the promotion of the best bioavailability of the active ingredients, the real weak point of many substances, are recommended to demonstrate the high biological potential of these molecules.
- The **efficacy** of a nutraceutical, evaluated through randomized and controlled clinical trials, may vary depending on the combination of the active ingredients and excipients present in the formulation that may or may not interact with the transport systems of the gastrointestinal tract or with the metabolism of CYP450.
- The **safety** of a nutraceutical is not absolute but can be influenced by several factors, which include the presence of contaminants in the chosen raw material, fractions of active ingredients present in the extract naturally toxic to the organism, and the modulation of the clearance systems by co-intake of drugs or other supplements.

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Chapter 2
The nutraceuticals market

2.0 Introduction of nutraceuticals market

The worldwide size of the functional foods market is estimated at approximately $500 billion at the end of 2021, with expectations of growth at an average annual rate of 6.9%, which would take the sector to $750 billion in 2027. The largest category is weight control foods, at $214 billion, with growth forecast at an average annual rate of 6%, followed by supplements, which are worth $140 billion globally (expected to increase 7.7%). Baby foods are estimated at $73 billion (+6.5%), but it is the vegan specialty foods ($25 billion, +9%) that show the brightest expectations [1].

Different long-term trends are candidates to support the growth of the functional foods market. First, the longer life expectancy has led to an increase in the share of the long-lived population with the consequent increases in health care costs. This has made it clear to public health systems the need to encourage the population to enter the older age group in relatively good health and overall well-being. A dietary regimen in which the intake of necessary nutrients occurs in a proper and balanced manner certainly contributes to this goal, reducing the likelihood of the onset of the physical and intellectual diseases typically associated with advancing age (cardiovascular disease, osteoporosis, vision disorders, deterioration of brain function, etc.).

However, there is increasing evidence of disordered and unbalanced, high-calorie, and hyperlipidic eating styles. According to the World Health Organization (WHO), 39% of individuals over the age of 18 are overweight, a substantial increase from 20% in 1975 [2]. In addition, approximately 13% of the world's population is obese, a value that has tripled since 1975. The overweightness and obesity among children and adolescents aged 5-19 years have increased worldwide from 4% in 1975 to over 18%. With approximately 900 million undernourished individuals in the world, there are 1.5 billion who are obese or overweight, confirming that the annual deaths from lack of nutrition (about 36 million) are not too far behind those from its excess (29 million). The direct and indirect costs associated with the eating disordered and related metabolic problems are enormous. Uncertain estimates put them at a total of $4.8 trillion per year, close to 3.5% of world *Gross Domestic Product* (GDP), with

peaks of 44.8% in Latin America (about $500 billion) and 4.3% in North America ($1 trillion). The economic burden for Europe is estimated at approximately 900 billion, which is more than 3% of its GDP [3].

Moreover, beyond the excessive caloric and lipid intake, there is also the problem of food quality. Significant portions of the population follow a diet characterized by deficiencies in nutritional components essential to the maintenance of adequate health. A balanced diet required, for example, a 50% incidence in fruit and vegetable consumption, while in the European adolescent population this portion is limited to 17% [4]. Also in Europe, sugar consumption is 15% above recommended levels and 47% in North America; meat consumption exceeds these levels by 36% in Europe (38% red meat, 51% sausages) and 48% in North America (46% and 50%, respectively) [5]. Dietary reassignment would reduce eating disorder-related deaths by 15%; however, a large portion of the population does not appear able to organize their daily diet to meet the recommended thresholds [6].

Finally, it is worth mentioning that a significant segment of the world population harbors a distrustful attitude toward drugs, fearing their addictive nature and side effects. This tendency is enhanced by the growing evidence of microbial resistance to drugs, which develops when microorganisms such as bacteria, viruses, fungi, and parasites mutate in such a way as to render the pharmacological treatments used to combat them ineffective. This is a natural phenomenon that is accelerated by improper behaviors, such as the abuse of antibiotics, their accidental release into the environment with re-entry into the food chain, or, again, the uncontrolled disposal of unused or expired drugs. The phenomenon of antimicrobial resistance may help push consumers toward nutraceuticals, particularly those with associated immune system response boosting effects. The pandemic emergency has acted as a further accelerator because the epidemic has particularly caused a surge in demand for foods and supplements with immune system support functions. Vitamin C supplements have been particularly sought after [7]. Although no vitamin or food, in any amount, can prevent Covid-19 infection once a person has been exposed to the virus, it is still true that individuals suffering from nutritional deficiencies are more likely to suffer from the complications induced by any infection or disease. In this context, a poor

nutrition is among the many factors that contribute to a weak immune response. Italy has a particularly prominent position with reference to the supplements market, the size of which is approximately 3.8 billion € in 2020. It is the leading European market, estimated to be worth 14.6 billion, with a 26% share, ahead of Germany (18.8%), France (14.7%), the United Kingdom (9.5%), and Spain (7.2%). The European market growth expectations are in the range of 6% annually, with Italy expected to reach a size of 4.8 billion € in 2025. Between 2008 and 2020, the Italian supplement market tripled in size, with an average annual growth rate of >9%. The strong propensity of Italian consumers for supplements is evident considering that their average per capita expenditure is about 64€ compared to Germany's 33€, France's 32€, and the UK's 21€. It is estimated that 54% of the population in Italy uses supplements, compared with shares of between 20% and 25% in Germany, France, and the United Kingdom. It should be noted that in Italy supplements are sold essentially through the pharmacy and parapharmacy channel (87% by value), with the presence of the large-scale retail trade at 8%, whereas in France (55%) and Germany (67%) the pharmaceutical and parapharmacy channel is more contained [8].

2.1 Recent trends in conventional and functional feeding

The interest in the protection of physical and mental well-being through an adequate diet has over time taken on an upward relevance that is part of an increased sensitivity to behaviors and attitudes, food and supplements, of preventive health protection. The result has been an elongation of the health chain, traditionally confined to ex-post curative, clinical, and hospital-based interventions, causing it to encroach into a sphere that extends from the dietary regimen to the entirety of lifestyles.

The original concept of diet as a deprivation to curb overweightness has been replaced with the concept of a balanced variety of food to improve overall well-being. Food, from a simple vehicle of nutritional satisfaction and sensory pleasure, has become an active tool for preserving and improving physical and cognitive health. The great popularity that nutraceuticals, functional foods, and innovative foods in general (novel foods) have had since their introduction in the late 1980s has

expanded in several demographic and consumer preference trends, some of which are indicated in the summary below:

1. The **longer life expectancy** has led to an increase in the share of the long-lived population with consequent gains in health care costs. This has made it clear to public health systems the need to encourage the population to enter the older age group in relatively good health and overall well-being. A dietary regimen in which the intake of necessary nutrients occurs in a proper and balanced manner contributes to this goal, reducing the likelihood of the onset of the physical and intellectual diseases typically associated with advancing age (cardiovascular disease, osteoporosis, vision disorders, deterioration of brain function, etc.) [9].

2. A significant segment of the world population harbours a **distrustful attitude toward drugs**, fearing its addictive nature and side effects [10]. Moreover, in some countries, especially in the East, the preference for foods with beneficial properties is intertwined with local traditions based on the use of natural remedies. All of these trends aim to increase preventive attitudes as well as to favour a substitution (moreover, not always appropriate) of the pharmacological approach with that based on food-based remedies.

3. There is growing evidence of **microbial resistance to drugs**, which develops when microorganisms such as bacteria, viruses, fungi, and parasites mutate in such a way as to render the pharmacological treatments used to combat them ineffective [11]. This is a natural phenomenon which is accelerated by improper behaviours, such as the abuse of antibiotics, its accidental release into the environment with re-entry into the food chain, and the uncontrolled disposal of unused or expired drugs. The phenomenon of antimicrobial resistance may help push consumers toward nutraceuticals, particularly those with associated immune system response-boosting effects.

4. The **pandemi**c emergency has acted as a further contingent accelerator in terms of a focus on dietary styles focused on the intake of substances useful in increasing resistance to **Covid-19 infection**. In particular, the epidemic has caused a surge in the demand for foods and supplements with immune system support functions. Although no vitamin or food, in any amount, can prevent Covid-19 infection once a

person has been exposed to the virus, good nutrition can help support the normal role of the immune system, increasing its ability to respond appropriately. In general, people with nutritional deficiencies are more likely to suffer from the complications induced by any infection or disease, and poor nutrition is among the many factors that contribute to a weak immune response. In turn, food and supplement manufacturers must implement special care to ensure that their references do not contain misleading claims in terms of immunity. The U.S. Food and Drug Administration (FDA) and the European Food Safety Authority (EFSA) have repeatedly warned manufacturers against associating its products with effects against Coronavirus.

5. Moreover, there is increasing evidence of the spread of **high-calorie and hyperlipidic eating styles**. Public information and awareness campaigns have been launched in many countries with the aim of making the prevention of food-borne disorders a priority over their treatment, as the latter entails direct costs that burden the health system and indirect costs that affect the entire community. According to the WHO, 39% of individuals over the age of 18 are overweight, a substantial increase from 20% in 1975. In addition, approximately 13% of the world's population is obese, a figure that has tripled since 1975. Overweight and obesity among children and adolescents aged 5-19 years have increased worldwide from 4% in 1975 to just over 18%. Compared with approximately 900 million undernourished individuals worldwide, there are 1.5 billion who are obese or overweight, such that the annual deaths from lack of nutrition (about 36 million) are not too far behind those from its excess (29 million) [12]. These are problems once considered exclusive to high-income countries; however, they are now increasing in low- and middle-income countries as well, particularly in urban settings. In industrialized countries, the average daily energy intake now exceeds 3,400 kcal, compared with a recommended level of approximately 2,500 kcal [13]. The direct and indirect costs associated with eating disordered and related metabolic problems are enormous. Uncertain estimates put them at a total of $4.8 trillion per year, close to 3.5% of world GDP, with peaks of 4.8% in Latin America (about $500 billion), and 4.3% in North America ($1

trillion). The economic burden for Europe is estimated at approximately 900 billion € [14].

6. Beyond the excessive caloric or lipid intake, there is also the problem of **food quality**. Significant portions of the population follow a diet characterized by deficiencies in nutritional components essential to the maintenance of adequate current and, more importantly, prospective health. A balanced diet would require, for example, a 50% incidence in fruit and vegetable consumption, whereas in the European adolescent population this portion is limited to 17% [15]. Sugar consumption in Europe is 15% above the recommended levels and 47% in North America; meat consumption exceeds these levels by 36% in Europe (38% red meat, 51% sausages) and 48% in North America (46% and 50%) [16]. In contrast, the fruit and vegetable intake is expected to increase by 56% in Europe and 21% in North America. Dietary reassignment would reduce the eating disorder-related deaths by 15% [17]. Several world authorities, and among them the EFSA for the European Union, have considered of launching extensive awareness and information campaigns, for example by establishing and disseminating recommended daily intake (RDI) levels of specific substances (so-called Nutrient Reference Values or Dietary Reference Values), differentiated by age and gender. Consumers should align these rules with appropriate planning of their daily diet. However, several clinical studies have shown that a large portion of the population is unable to organize their daily diet to meet the recommended thresholds [18]. None of the European countries exceeds 40% in terms of adherence to optimal doses. With reference to vitamin D, there is a suboptimal intake by 40% of the European population [19], plus an additional 15% who are even in a state of clinical deficiency. Between 2016 and 2020, cardiovascular disease is estimated to have resulted in 38.5 million cases of hospitalization in the EU in the population over the age of 55, at a direct and indirect cost of 1,330€ billion over the 5-year period. Regular consumption of omega-3 (1 g/daily), for example, would have led to a reduction of about 5% in the risk of heart disease, with fewer hospitalizations matched by an estimated savings of 64.5 billion € (approximately 370.000 fewer hospitalizations per year) [20,21].

Similarly in the United States, a reduction in the risk of hospitalizations of 6.9% (approximately 140.000 fewer hospitalizations per year) has been estimated [22].

2.2 The nutraceuticals global market

It is difficult to draw a precise perimeter of the economic size of the nutraceutical market, because of the multi-product areas it touches and the inevitable areas of overlap of some of its specialties. Table 1 isolates some specialties that fall under the broad spectrum of functional nutrition and nutraceuticals, offering an estimate of the worldwide size of its respective markets. Collectively, this is $500 billion in 2021, with projected growth over the long term in the range of 6.9% average per year, with an expected value of about $745 billion in 2027.

The first category refers to **diet food** (for slimming or weight management), a segment valued about $215 billion in 2021, with growth estimated to be approximately 6% annually until 2027. Thirty percent of diet food refers to products for the replacement of the entire daily food ration (so-called TDRs: Total Diet Replacements), for use by those suffering from severe overweight, and 70% of dietary supplement products for weight control (MRPs: Meal Replacement Products). According to the taxonomy adopted by the Total Diet & Meal Replacements Europe association, TDRs products include low-calorie diets (LCDs: Low Calorie Diets, with energy content 800−1,200 kcal) and very low-calorie diets (VLCDs: Very Low-Calorie Diets, which contain <800 kcal in a daily dose). In general, these are foods balanced with vitamins, minerals, protein, essential fats, fiber, and other nutrients, designed to replace the conventional foods and facilitate the weight loss. Typically, the diet foods are administered in the form of shakes or soups, rehydrated meals, bars, or desserts, or even ready-to-drink solutions.

Table 1. The world market of functional foods (Rsp, Usd bln, 2019-2027E)

	2019	*in %*	2020	2021	2022	2023	2024	2025	2026	2027	*Cagr 27/20*
Diet food	197,9	*43,6*	201,8	213,7	226,4	239,9	254,3	269,6	285,8	303,1	*6,0*
Dietary supplements	138,5	*30,5*	141,3	152,1	163,8	176,4	190,0	204,7	220,6	237,7	*7,7*
Baby food	64,5	*14,2*	68,8	73,4	78,3	83,4	89,0	94,9	101,3	106,7	*6,5*
Free from ex vegan	16,7	*3,7*	17,7	19,0	20,2	21,6	23,0	24,5	26,1	27,9	*6,7*
Vegan food	21,2	*3,3*	23,0	25,0	27,3	29,7	32,4	35,3	38,5	42,0	*9,0*
Totale free from	*37,9*	*7,0*	*40,7*	*44,0*	*47,5*	*51,3*	*55,4*	*59,8*	*64,6*	*69,9*	*8,0*
Sport food	14,8	*4,7*	15,2	16,5	17,9	19,3	21,1	23,1	25,2	27,3	*8,8*
Totale	**453,6**	***100,0***	**467,9**	**499,7**	**533,8**	**570,3**	**609,7**	**652,0**	**697,4**	**744,8**	***6,9***

MRPs, however, are single meal replacements to be consumed in addition with conventional food as part of a low-calorie diet. They can be consumed instead of breakfast, lunch, or dinner, containing between 200-400 kcal, and are available in pre-rationed form. MRPs contain protein, carbohydrates, and fat and can be fortified with vitamins and minerals. MRPs are also offered in the form of snacks, bars, drinks, powder-based shakes, and soups. Smoothies are available in a prefabricated or powdered form and contemplate a variety of flavours.

Dietary supplements have areas of overlap with the previous category to some extent; these molecules are used to correct nutritional deficiencies, maintain adequate intake of certain nutrients, and support specific physiological functions, assuming that lifestyles, eating habits, or aging make it difficult to consume those substances through the ordinary diet. From the packaging perspective, supplements are marketed 'in dosage forms': pills, tablets, capsules or chewable gum, liquids, and soluble powders and are never presented as a conventional food, except when offered in the form of bars.

The categorization of dietary supplements according to their contents is complicated by their extreme variety, the mixing with which they are combined, and the way in which both factors can vary over time. In 2021, the global supplement market is estimated to be approximately $150 billion, with growth expectations approximately 7.7% annually through 2027 [23].

Products for the feeding of infants (<12 months of age) and **young children** (1-3 years of age) form a global market estimated at $73 billion in 2021, with expected growth rates in the range 6-7% annually on average. Although it is true that the global demographic trend is characterized by a declining birth rate, the growing emancipation of women and their increased participation in the world of work are fuelling the need for alternative forms of infant feeding to maternal feeding. An estimated 70% of infants in Europe are formula-fed before the age of 6 months, and infant formulas are the only suitable alternative for children whose mothers are unable to breastfeed or choose not to. Infant formulas include three main categories:
1. infant formulas: intended for feeding infants in the first few months of life. In the event that maternal breastfeeding is not possible, they constitute the only products that, on the advice of the paediatrician, can be used as breastfeeding substitutes.
2. follow-on formulas: intended for the feeding of infants at the time when adequate complementary feeding is introduced (from 6 months) and which constitutes the main liquid element within a progressively diversified diet.
3. baby food: aimed at the progressive diversification of infant and young child feeding in early childhood. They are foods adapted both to the functional immaturity of organs and apparatuses inherent to age as well as to the consumption of foods that are at a much higher ratio per unit of body weight than in adults, and thus increase the risk of exposure to contaminants.

Sports products (sport food supplements) are worth approximately $17 billion globally, with expected growth rates in the range of 9% on average annually. Sports drinks should not be confused with energy drinks, which are characterized by stimulant properties due to the presence of compounds such as caffeine, taurine, B vitamins, along with sugars and sweeteners and, in some cases, plant extracts. In contrast, sports drinks have nutritional characteristics adapted to physical activity and the rapid restoration of salts and minerals that are lost during exercise. The products are sold in powder, bar, ready-to-drink, gel, and tablet formats.

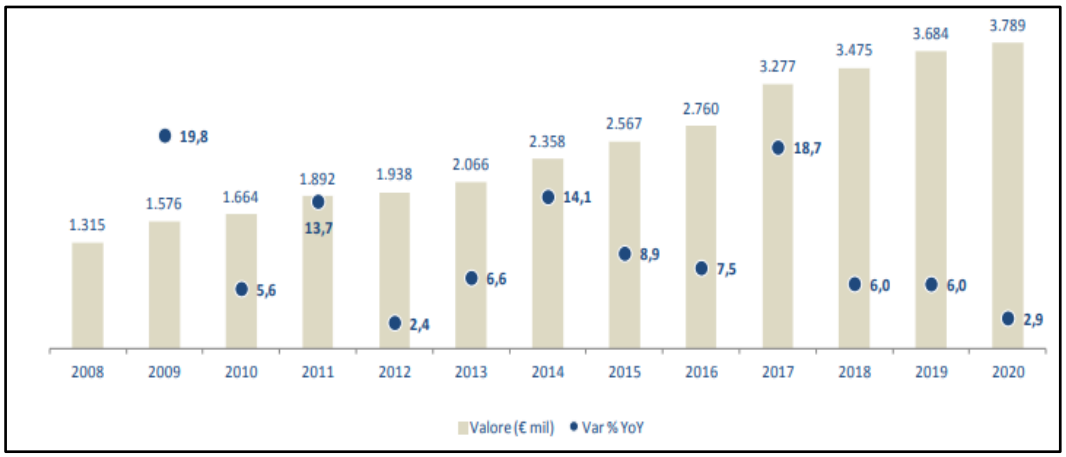
'Free from' products have a global market estimated at $19 billion in 2021, for an expected growth of 6.7% on average per year. In this case, the pool of potential users ranges from those with celiac disease to those who suffer from other forms of

intolerance, e.g., toward lactose or other allergens, or feel they do not want to consume specific substances (e.g., dyes, palm oil, etc.). It is also evident that a large part of the free from market, in the taxonomy followed here, includes diet foods.

2.3 The market in Italy and Europe

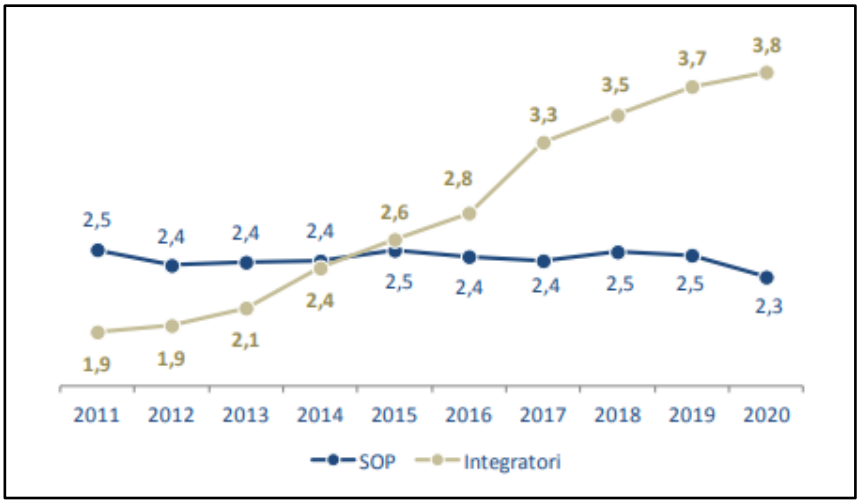
Specialized nutrition with a health purpose was worth approximately 4.8 billion € in Italy in 2020 [24]. Infant food, including infant formulas, follow-on formulas (from 6 months of age) and, in general, the entire baby food complex that is consumed by the infant up to 3 years of age, represents a 0.3 billion € market. Specialized nutrition in the narrower sense is worth an additional 0.7 billion, whereas is largely represented by dietary supplements, which stand at 3.8 billion €. Within baby food, the highest value segments are baby food (52%) and milk (26%). In specialty food, solutions for celiacs are worth 400 million €, whereas those for special medical purposes (AFMS) amount to 170 million. The rest, approximately 70 million, relates to sports nutrition or dedicated to weight management. Supplements, which make up as seen the bulk of the market, achieved a cumulative growth of 9.2% in 2008-2020, with double-digit increases in some years and a significant advance of 2.9% even in 2020. In essence, their market tripled from €1.3 billion in 2008 to €3.8 billion in 2020 (Figure 1). The positive momentum was reinforced in 2021 with 4.5% value growth during the first half of the year. Supplements are conveyed to the public essentially through the pharmaceutical channel, which in 2020 accounted for sales of 3 billion (79% of the total), parapharmacies (8%), and large-scale retail (8%) intermediate approximately 0.3 billion each, whereas the remaining 5% of the product reaches the consumer through the online channel represented by marketplaces run by pharmacies and parapharmacies (0.2 billion). In 2020, dietary supplements accounted for 12.7% of total pharmacy sales, a share that has grown steadily over time and stood at 10.6% in 2017.

Figure 1. The Italian nutraceutical market (€ mil and var%YoY) (data from Federsalus 2021)



The growth of the nutraceutical market has been impetuous as is evident when comparing its dynamics with that of non-prescription (SOP) medicines within which the categories of over the counter (OTC), also known as self-medication medicines, which can be advertised and have direct access to the shelf (self-service), and so-called SP medicines, which can also be advertised but are not accessible at the shelf in self-service mode (so-called behind the corner) converge. SOP medicines have shown a weakly declining dynamic over the past decade, whereas supplements, which were worth less than SOPs in 2011, surpassed them in 2015 and then increased their lead through 2020 to a size of 3.8 billion € against 2.3 billion € for SOPs (Figure 2).

Figure 2. The Italian market for nutraceuticals and SOPs (2011-2020 € bln)



Note that the latter are sold almost entirely through the pharmacy and parapharmacy channels (97.5%), whereas supplements also enjoy some penetration in the large-scale retail channel (8%). The success of supplements, as well as homeopathic and herbal products, can also be linked to the fewer constraints governing their marketing and advertising, which are aspects that facilitate their perception by consumers as alternative prescriptions to SOPs. The use of supplements has become a characteristic feature of the consumption habitus of Italians, as documented by the Censis according to which there are about 32 million compatriots who use them (54% of the population), represented 60.5% by females and 39.5% by males. The age group most affected is between 35 and 64 (62.8% of the total), followed by citizens between 18 and 34 (20.3%) and those over 65 (16.9%). Fifty eight percent of users connote being habitual, with frequency of intake either daily or on several days during the week [25]. In other countries, the figures are more modest: in the United Kingdom, for example, supplement intake involves 25% of the population, 41% of them habitually [26]. The percentages in France stand at 22% among adults and 14% among young people [27]. In Germany the overall percentage can be estimated at 25%. The health emergency of COVID-19 acted as an accelerator: it is estimated that one in ten Italians decided to assume supplements during the pandemic [28]. In

addition, there were 28.6 million medical prescriptions for supplements in 2019 alone, an average annual growth of 12.1% from 20.3 million in 2016. The positive dynamics of the supplement market appears even more remarkable when noting that its development has taken place despite the fact that these are specialties with a retail price per package that is significantly higher than that of SOP drugs and even notified products (Figure 3).

Figure 3. Average selling price (€) and number of packs per capita of supplements

Since 2008, the per capita consumption of supplements has risen from 1.6 to 4.1 packs per person based on the entire population, a figure that would double to approximately 8 packs per person when considering only the 32 million active users (Table 2). The active ingredients and molecules of which supplements are composed of, have seen a wide proliferation of substances led by vitamins and minerals, which, at 746 million, make up 19.7% of the total, followed by probiotics at 387 million (10.2%). Multivitamins and minerals include both nonspecific uses, i.e., in the absence of a specific deficiency, and specific uses (e.g., folic acid in pregnancy, iron and vitamin D in non-breastfed infants, vitamin B12 deficient in vegans, etc.).

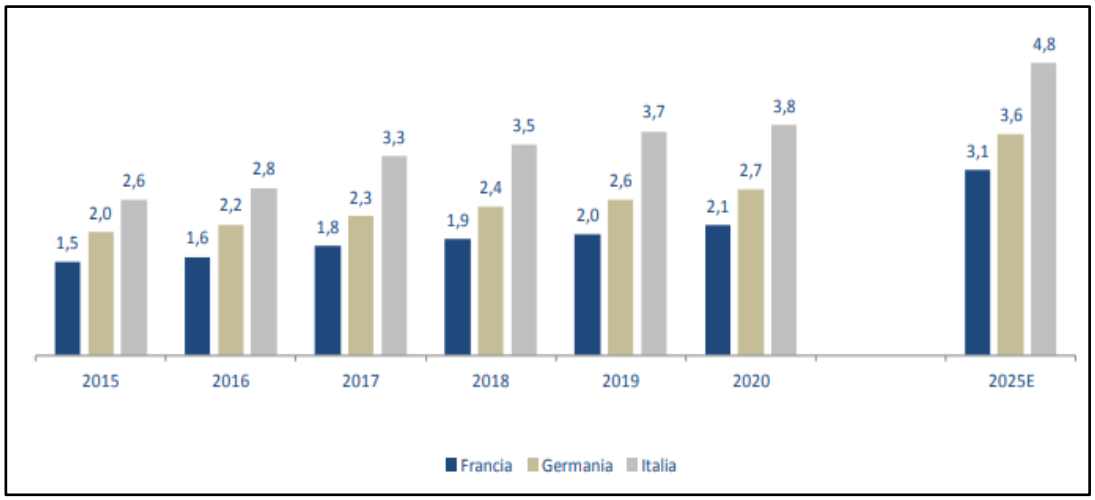
Table 2. Principles and specialties of food supplements in Italy (Rsp, € mil.)

	2019 (€ mil)	%	2020 (€ mil)	%	Var. % 2020/2019
Minerals and multivitamins	673	18,3	746	19,7	+10,8
Gastroenteric system	427	11,6	413	10,9	-3,3
Probiotics	418	11,3	387	10,2	-7,4
Circulatory system	296	8,0	292	7,7	-1,4
Urinary/Reproductive system	258	7,0	256	6,8	-0,8
Sport/Stimulants	251	6,8	236	6,2	-6,0
Throat and respiratory system	238	6,5	219	5,8	-8,0
Relax and sleep	159	4,3	189	5,0	+18,9
Weight management	93	2,5	113	3,0	+21,5
Muscles and joints	92	2,5	100	2,6	+8,7
Eyes	93	2,5	89	2,3	-4,3
Other	686	18,7	749	29,8	+9,2
Total	3684	100	3789	100	+2,9

Regarding the benefits for individual functionalities, the products for intestinal and digestive system wellness prevail, accumulating sales of 413 million € (10.9% of supplements), ahead of products for the circulatory system with 292 million € (7.7%), for the urinary and reproductive system with 256 million € (6.8%), tonic, stimulant and sports references amounting to 236 million € (6.2%), those for the respiratory system at 219 million € (5.8%), and finally sleep aids with sales of 189 million € (5%). The reversal of habits and anxieties brought by 2020 have clearly favoured certain types: vitamin and mineral products (+10.8%) and those with relaxing and rest-promoting functions (+18.9%). These are some of the main specialties that allowed the market to progress in 2020, given the declines that affected several other product lines. Among the latter, specialties for weight loss deserve a mention, which have been moving for years along a waning ridge that discounts consumer

disillusionment about the ability of these products to promote weight loss without having to undergo either diets or physical activity. As a reminder, in Italy the marketing of dietary supplements is subject to a label notification procedure with the Ministry of Health (Notified Products), which may require changes to protect consumer health as well as the withdrawal of the product from the market. If the procedure is favourably concluded, the products are included in the Register of Food Supplements periodically updated and published on the Ministry of Health website. In the field of supplements, Italy emerges as the largest European market, estimated as a whole at approximately 14.6 billion € in 2020, with a market share of 26% (3.8 billion), ahead of Germany credited with 2.7 billion € (18.8%), France at 2.1 billion € (14.7%), Russia at 1.6 billion € (11%), the United Kingdom at 1.4 billion € (9.5%), and Spain at 1.1 billion (7.2%). The trio of the leading countries alone account for 59.5% of the European market (Figure 4), a share that rises to 87.2% when Russia, the United Kingdom and Spain are also considered. A growth rate of approximately 6% is projected for the entire European market, which would take it to over 19 billion € in retail sales in 2025.

Figure 4. Size of the top three European markets (Rsp, € bn, 2015-2025E)



Italy's leadership is evident, and looking ahead, our market is poised to remain the largest in Europe: in 2025 it could reach 4.8 billion €, maintaining a large lead over the German (3.6 billion €) and French (3.1 billion €) markets. Expected average annual growth rates are 5-7% for these major markets. The product innovation is set to be one of the drivers of market growth in the future: the increase of approximately 3% in 2020 is, in fact, the balance of the decline in sales recorded on mature products (-0.8%) rather than the offset by the increase in volumes on new launches (+3.7%). The size of the Italian market is well documented, in addition to its absolute value, by the amount of per capita spending, which is roughly double (63.6€) that of Germany (32.9€) and France (31.7€) and three times greater than that of the United Kingdom (20.6€). The two largest European markets after Italy have their own specificities (Figure 5). In Germany, the physical pharmacy channel conveys 67% of the market (2.3 billion €), to which is added an estimated 17% of sales that pass through online pharmacy portals (Table 3). The lower expenditure per inhabitant compared to Italy appears to be a consequence of lower use per inhabitant (about 3.2 packs per person) and lower unit cost (approximately 9.7 €). In France, the market appears much more diversified with pharmacies and parapharmacies standing at 55%.

Figure 5. Per capita spending in major European markets (Rsp, €, 2020)

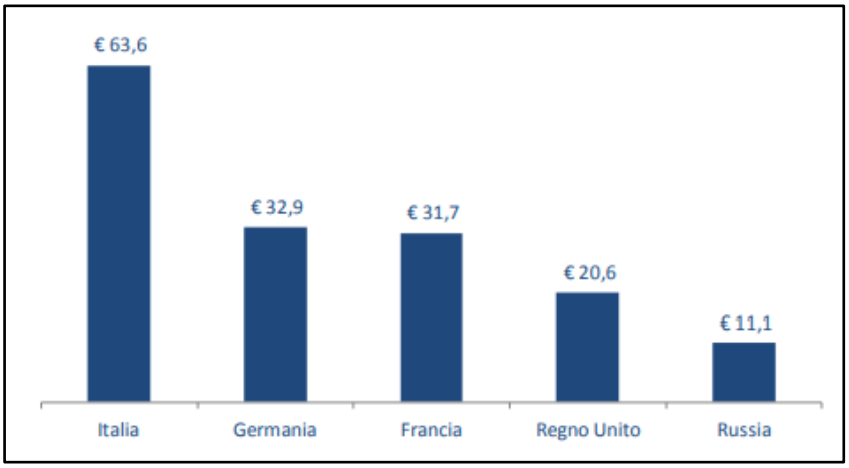


Table 3. The sales channels of food supplements

	Italy	France	Germany
Pharmacy	79%	50%	67%
Parapharmacy	8%	5%	-
Pharmacy + Parapharmacy	87%	55%	67%
Drugstore / specialized shop	-	16%	11%
GDO	8%	9%	-
Direct sales	-	11%	-
On-line	5%	9%	17%
Other	-	-	5%
Total	100	100	100

2.4 Conclusions

- Demographic trends show how the Italian population and Western countries lead to a net **increase of mature and elderly subjects in fair health conditions** who rightfully want to remain healthy and active in the years to come, without necessarily resorting to pharmacopreventive approaches. In this context, the attention of the community and health professionals regarding the potential preventive applications of a targeted dietary supplement with nutraceuticals is developing rapidly.
- This growth is evidenced by the **increase in companies** that deal with nutraceuticals (with consequent potential for use), by the increase in global turnover related to the purchase of these products by patients and healthy individuals, and by the constant increase of projects research and scientific articles that reveal the potential of these products.
- The **worldwide size of the nutraceutical market** is estimated at approximately **$500 billion** at the end of 2021, with expectations of growth at an average annual rate at 6.9%, which would take the sector to $750 billion in 2027.

- **Italy emerges as the largest European market with a 3.8 million € in 2020** and a market share of 26%, ahead of Germany credited with 2.7 billion € (18.8%), France at 2.1 billion € (14.7%), Russia at 1.6 billion € (11%), and the United Kingdom at 1.4 billion € (9.5%).
- The size of the Italian nutraceutical market is well documented by the amount of per capita spending, which is roughly double (63.6 €) that of Germany (32.9 €), and France (31.7 €).

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Chapter 3
The use of by-products in the nutraceutical field

3.0 The use of by-products in the nutraceutical sector

According with the Food and Agriculture Organization (FAO), one-third of all food produced in the world is wasted, which represents approximately 1.3 billion tons [1]. The ever-increasing production of food requires a huge amount of resources, and raw materials are not yet fully exploited. This leads to the generation of waste, which adds to the waste generated by the leftovers of consumption. Reducing food waste and making suitable use of resources can help to meet the demand for the estimated 60% higher food production that will needed by the world's population in 2050. Global food waste generated by food processes, such as vegetable oil extraction, starch, juice and sugars production, also contributes significantly to environmental issues, because of its extensive use of energy and resources, as well as the associated greenhouse gas emissions [2].

There are conspicuous food losses within the food chains of the most developed countries, including 39% during food production and a significant 42% by the consumer [3]. A large number of by-products of the various phases of food production have been studied to find ways to limit food production's environmental and economic impact and researchers have experimented with new processes for the recovery of valuable components. Moreover, the conversion of many primary metabolites can generate new resources [4].

In this context, fruits and vegetables have the highest waste rates of any food, i.e., 45% [5], which in developing regions such as Asia, Africa, and Latin America are concentrated in agriculture and processing. Because of how they are processed, a significant amount of these foods is traditionally discarded. When fruit is processed, parts such as the core, peel, pips, and kernel are discarded.

For example, in the fruit juice industry, more than 50% of raw material becomes by-products that are rich in active compounds and have high nutritional content. Improved use of these by-products could represent a key strategy for a circular economy [6]. Moreover, the fruit juice by-product industry could represent a strategy to elevate the level of "fruit" intake and its associated compounds, resulting in the development of new natural ingredients for the food industry and lower food wastes during process stages, and therefore increased efficiency. For this, an adequate process for developing the by-product and its complete characterization, in terms of composition, and also in relation to functional properties, is necessary before its

application in real and easily manufactured food.

3.1 The example of functional juices obtained from fruit waste products: from byproduct to clinical investigation.

3.1.1 Preamble

The important role of dyslipidaemia, especially hypercholesterolemia, in the development of atherosclerosis-related cardiovascular diseases has been fully documented in genetic, pathologic, observational and intervention studies [7-9]. International guidelines recommend improving lipid profiles through lifestyle modification and the use of appropriate drugs with the common goal of reducing low-density lipoprotein cholesterol (LDL-C) to the lowest possible level to prevent the development and progression of atherosclerosis [10,11]. Although statins are the most frequently used drugs for improving lipid profiles and lowering LDL-C levels, their use is limited by their side effects and interactions with other drugs [12,13]. While complete statin intolerance is estimated to occur in less than 5% of the population, the number of people that are intolerant to conventional treatment ranges from 45,000 to 290,000 individuals/year worldwide, with statin intolerance being one of the main reasons for statin discontinuation, poor adherence, and resulting failure of lipid-lowering treatment [14,15]. In addition, the guidelines indicate that the benefit-risk ratio of these drugs is favorable for secondary prevention of cardiovascular diseases or primary prevention in subjects at high or very high overall cardiovascular risk, but unfavorable in the majority of subjects who have only moderately high LDL-C levels and are not at high cardiovascular risk. There is therefore an unmet therapeutic need in two classes of subjects: patients who are unable or unwilling to take cholesterol-lowering medication despite their high cardiovascular risk; and, health-conscious subjects at low cardiovascular risk who would like to reduce their LDL-C levels, but are not eligible for pharmacological therapy [16]. In this context, the European guidelines for dyslipidemia management consider the possibility of using lipid-lowering nutraceuticals in support of the possible use of a relatively large number of natural compounds [17]. As highlighted in a report by the CTT Collaboration on more than 170,000 subjects, in cholesterol-lowering drug therapy, each further reduction of LDL-C by 1 mmol/l (~40 mg/dl) decreased the risk of revascularization, coronary artery disease and ischemic stroke by about one-fifth. It was underlined that a LDL-C reduction of 3.2 mmol/l (125 mg/dl) could lead to a decrease in risk of about 40–50%, in the absence of increased

risk of cancer or non-cardiovascular-related death [18]. One mmol/l is a reduction that is achievable through lifestyle improvements associated with lipid-lowering nutraceuticals [19]. Moreover, it has been estimated that every 1% reduction in LDL-C level corresponds to a reduction in the relative risk of cardiovascular events of greater than 1% [20,21].

It has been known, since the middle of the 20th century, that fruit juices can have a favorable effect on blood lipid profiles and are particularly capable of reducing LDL-C because of the remarkable antioxidant effects of polyphenols and phytosterols [22]. Moreover, the ever-increasing production of food requires a huge number of resources with raw materials not yet being fully exploited. This leads to the generation of waste, adding to the waste generated by consumption leftovers. Reducing food waste and making suitable use of resources can help to meet the issue of the estimated 60% higher food production that will be needed by the world's population in 2050 [23]. Global food waste also contributes significantly to environmental issues, because of its extensive use of energy and resources as well as the associated greenhouse gas emissions.

Conspicuous food loss is present within the food chains of the most developed countries, including 39% during food production and a significant 42% by the consumer [24]. A large number of the by-products of the various phases of food production have been studied to find ways to limit the environmental and economic impact of food production, and researchers have experimented with new processes for the recovery of valuable components. This may be interesting, in this work's context, as lipid-lowering metabolites can be extracted from fruit waste, using the so-called "zero-waste approach". In fact, several studies have underlined the cardiovascular–disease prevention activity of a number of fruit extracts (containing polyphenols), including kiwi, the Annurca apple, bergamot and grape, which have been demonstrated to regulate lipid profiles, while exerting anti-inflammatory, antioxidant and hypoglycemic effects [25,26,27,28,29].

The main aim of this study is to evaluate the effects of a blended drink containing kiwi, Annurca apple, bergamot and grape juice (obtained from fruit by-products), phytosterols, red yeast rice and berberine on the lipid profiles of patients with acquired hypercholesterolemia.

3.1.2 Results and Discussion

In comparison with baseline, four weeks of nutraceutical juice administration led to a significant reduction in total cholesterol (16%, from 276.9 ± 60.8 to 233.6 ± 50.6 mg/dl; p<0.001), LDL-C (18%, from 181.5 ± 44.4 to 149.7 ± 42.2 mg/dl; p<0.001) (Figure 1), triglycerides (27%, from 167.9 ± 102.5 to 122.4 ± 59 mg/dl; p=0.011), non-HDL-cholesterol (21% from 216.2 + 57.7 to 171.4 + 48.9, p<0.001), and apolipoprotein B levels (12%, from 131.0 ± 33.6 to 113.9 ± 27.3; p<0.001), and there were no differences in the HDL-cholesterol, apolipoprotein A1 and HbA1c levels (Table 1).

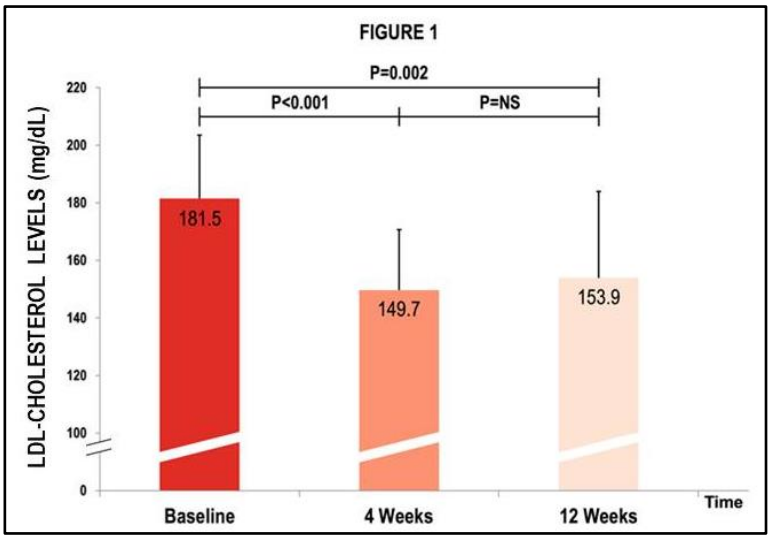
In comparison with baseline, 12 weeks of nutraceutical juice administration led to significant reductions in total cholesterol (15%, from 276.9 ± 60.8 to 245.1 ± 63.1 mg/dl; p<0.001), LDL-cholesterol (18%, from 181.5 ± 44.4 to 153.9 ± 60.5; p=0.002) (Figure 1), non-HDL-cholesterol (14% from 216.2 + 57.7 to 185.4 + 65.7, p<0.001) and apolipoprotein B levels (12%, from 131.0 + 33.6 vs 118.8 + 3 2.9; p=0.009), and there were no differences in HDL-cholesterol, apolipoprotein A1, triglyceride and HbA1c levels (Table 1).

No significant differences in any of the study parameters were observed between the fourth and twelfth week of follow-up. No adverse events were recorded and none of the patients were forced to discontinue the nutraceutical juice. Overall compliance over the study period was 96%.

Table 1. Biochemical evaluations at baseline and after four and 12 weeks of follow-up.

	Baseline	**4 weeks**	**12 weeks**	**p Baseline *vs* 4 weeks**	**p Baseline *vs* 12 weeks**	**p 4 weeks *vs* 12 weeks**
Total cholesterol (mg/dL)	276.9 ± 60.8	233.6 ± 50.6	246.1 ± 63.1	<0.001	<0.001	NS
LDL-cholesterol (mg/dL)	181.5 ± 44.4	149.7 ± 42.2	153.9 ± 60.5	<0.001	0.002	NS
HDL-cholesterol (mg/dL)	60.7 ± 15.7	62.1 ± 16.6	60.7 ± 16.0	NS	NS	NS
Triglycerides (mg/dL)	167.9 ± 102.5	122.4 ± 59	168.6 ± 163.1	0.011	NS	NS
Non-HDL cholesterol (mg/dL)	216.2 ± 57.7	171.4 ± 48.9	185.4 ± 65.7	<0.001	<0.001	NS
Apolipoprotein A1 (mg/dL)	155.8 ± 24.0	163.4 ± 27.6	163.8 ± 31.4	NS	NS	NS
Apolipoprotein B (mg/dL)	131.0 ± 33.6	113.9 ± 27.3	118.8 ± 33.0	<0.001	0.009	NS
HbA1c (mmol/mol)	37.3 ± 5.6	37.6 ± 5.2	38.4 ± 4.6	NS	NS	NS

Figure 1. LDL-cholesterol levels at baseline and after four and 12 weeks of follow-up.



Atherosclerotic cardiovascular diseases are chronic degenerative pathological conditions that can be prevented by changing modifiable risk factors. This can normally be achieved by means of drug administration and/or by effecting lifestyle changes. However, although major advances have been made in the development and use of risk-factor-modifying drug treatments, the role of lifestyle intervention has been relatively less explored and is generally under used [30]. An exception is the recent growing interest in the research and use of nutraceuticals and functional foods as agents for the prevention and treatment of atherosclerosis, which play an important role in primary prevention and may further increase the efficacy of both primary and secondary prevention. Multiple cholesterol-lowering nutraceutical products of different types have been developed [31]. In this study, a novel nutraceutical beverage was used to lower LDL-cholesterol by an average of 18% within one month and has several advantages over other nutraceutical products. The use of entirely natural ingredients makes it more attractive to people who wish to avoid artificial substances and, unlike the many nutraceuticals packaged as pills or vials, its presentation as a fruit juice allows it to be considered a food rather than a quasi-pharmacological product. Pharmaceutical-like packaging can act as a barrier, especially in subjects that are reluctant to take medication as a matter of principle.

Finally, its value in terms of vitamin and fiber intake can positively contribute to a more balanced diet.

There are two main categories of subject who may benefit from the nutraceutical beverage used in this study. The first includes those who refuse to take medicinal products. Previous studies have shown that up to 50% of subjects that are prescribed a statin stop taking it within a year [32]. Although non-compliance is a multifactorial phenomenon, it is known that some of these subjects stop taking their medication because they have a personal dislike of taking a pill daily, and these people may well find the nutraceutical drink described in this study an acceptable alternative, not least because its packaging and presentation does not resemble that of a pharmacological product.

The second category includes subjects who wish to lower their LDL-cholesterol levels further than the level obtained by the administration of their on-going drug treatment, and health-conscious subjects who wish to reduce their LDL-C levels despite their low cardiovascular risk for purposes of primary prevention. Neither of these groups would meet guideline-based criteria for starting a new or additional drug treatment, but both might benefit from additional lowering of LDL-cholesterol for which there is no currently known protocols. For these subjects the nutraceutical beverage developed in this study could be an interesting option.

We herein provide preliminary data on a nutraceutical product composed entirely of natural ingredients and is presented as a fruit juice, and how that it significantly improved cholesterol profiles (including LDL-C levels) after only four weeks. The product's non-pharmacological appearance, natural origin, and nutritional value could help increase compliance among individuals seeking to avoid pharmacological agents and could be a viable option for individuals who do not have an indication for drug treatment but want to improve their cardiovascular risk profile.

Despite the relevant findings and practical implications, this study is not without limitations. We acknowledge that the small sample size and open-label format mean that the study should be confirmed by new randomized double-blind trials. In addition, the relatively short follow-up period means that assessment of the possible occurrence of adaptation phenomena is possible. However, these have not been documented to date. Further research is needed to uncover the reasons and mechanisms for the effects observed during the study. For instance, the effect on serum lipids is likely mediated in part by a change in the gut microbiota induced by fruit polyphenol supplementation. Available experimental data suggest that supplementation with bergamot and polyphenolic fractions is able to exert a

beneficial effect on the composition of the gut microbiota [33]. However, no specific evidence for simultaneous supplementation with these nutraceutical compounds is available to date [34].

3.1.3 Materials and Methods

3.1.3.1. Subjects

Fourteen subjects were enrolled in the study: five males and nine females with a mean age of 65.5±9.4 years. Table 1 shows their demographic and anthropometric characteristics at baseline and during follow-up. There are no significant differences in any of these variables throughout the course of the study. The subjects underwent clinical, anthropometric and biochemical evaluations at baseline and after four and 12 weeks (Table 1). The study population consisted of hypercholesterolemic subjects aged >18 years who required primary prevention treatment because of hypercholesterolemia, but who refused to take, or could not tolerate, statins. Subjects were excluded from the study if they showed any clinical signs of chronic infection; hepatic, renal or gastrointestinal disease; or any acute disease requiring treatment. The exclusion criteria also included diabetes, a history of significant metabolic disease and the use of lipid-lowering or anti-coagulation drugs over the previous six months. All subjects provided their informed consent before entering the study.

3.1.3.2. Study protocol

The study protocol involved the daily administration of a nutraceutical juice for 12 weeks. All the enrolled subjects were given 100 mL bottles of the juice and were instructed to shake and drink one bottle every morning between breakfast and lunch. All the unused bottles were retrieved for inventory purposes, and compliance was assessed by counting the number of empty bottles returned at specified clinic visits. The selection of these four fruits was related to their intrinsic properties to ameliorate the lipidic profile in blood serum as supported by recent papers or meta-analysis.

3.1.3.3. The juice

Each 100 mL bottle contained:

1) Kiwi [35], Annurca apple [36], bergamot [37] and grape juice [38];
2) two grams of phytosterols;
3) red yeast rice containing 2.9 mg of monacolins from *Monascus purpureus;*
4) 100 mg of berberine complexed with β-cyclodextrin.

The phytosterol dose was chosen on the basis of the average efficacious dose identified in a meta-analysis of randomized clinical trials [29]. The red yeast rice extract was certified to contain purified monacolins, without any chromatographically detectable levels of dehydromonacolins, decalin derivatives, or contaminants. Although berberine was complexed with β-cyclodextrin, it retained its bitter taste, thus limiting its dose. At the beginning of our investigation, we used a water solution of monacolin, berberine and phytosterols. Unfortunately, the unpleasant taste had a bad impact on the compliance and the volunteers left soon the trial.

3.1.3.4. *Juice processing and storage*

The raw materials and semi-finished products were purchased from qualified suppliers, and each product was accompanied by a specific technical data sheet indicating the origin of the raw material, the microbiological, physicochemical and organoleptic characteristics of the product, the type of packaging, the storage methods, the presence or absence of GMOs and/or allergens, and the expiring date.
The raw materials in the nutraceutical juice do not require any particular storage conditions. They are aseptic and non-perishable (except for mint, which is frozen and stored at -18°C) and are stored on shelves in the designated cool and dry area of the organic raw materials warehouse.
The raw materials used to prepare the nutraceutical juice are weighed and placed in a Roboqbo vacuum cooking system. In the first cooking phase, the oily active ingredients are mixed with soy lecithin and part of the water and kiwi fruit (i.e. about 10% of the finished product). Once the mixture reaches a temperature of 70 °C, it is passed through a GEA homogenizer, which is ideal for high-pressure treatment of nanodispersions and cell lysis and guarantees high performance at a pressure of 500 bar.
At this point, the active ingredient mixture is mixed with the other raw materials before being heated to a temperature of 85 °C and then conveyed to the filling area, where it is used to fill the 100 mL vials.
Before unloading the product, the RCQ manager takes a sample in the laboratory and checks that the physicochemical properties meet the required specifications. the nutraceutical juice is pasteurized at 85°C for 10' and then cooled until the temperature in the middle of the bottle is < 40°C.

3.1.3.5. Assessments

The clinical evaluations included a determination of height, body weight, waist circumference and arterial blood pressure, and an electrocardiographic examination. Blood samples taken after a 12-hour fast were used to measure total cholesterol, LDL cholesterol, high-density lipoprotein cholesterol (HDL-C), apolipoprotein A1, apolipoprotein B, triglyceride and HbA1c levels using standard clinical procedures. Upon enrolment, the subjects were asked to maintain their dietary habits and to not change their physical-activity routines over the course of the study.

Table 2. Clinical and anthropometric characteristics of the study population at baseline, and after four and 12 weeks**.**

	Baseline	**4 weeks**	**12 weeks**
Age (years)	65.5±9.4	-	-
Males/females (n)	5/9	-	-
Family history of cardiovascular diseases (n)	12	-	-
History of hypertension (n)	6	-	-
Obesity (n)	1	-	-
Smokers (n)	0	-	-
Heart rate (beats per minute)	62.7±9.4	63.1±8.9	62.6±9.1
Systolic blood pressure (mmHg)	128.9±17.7	125.9±19.7	127.9±18.7
Diastolic blood pressure (mmHg)	80.3±11.1	79.7.1±9.9	80.6±12.1
Height (cm)	171.3±22.7	172.3±21.4	171.8±21.7
Weight (Kg)	73.3±8.7	74.2±10.1	73.0±8.9
BMI (kg/m^2)	24.7±1.7	24.6±1.9	24.7±1.8
Waist circumference (cm)	88.3±7.9	89.1±7.7	88.9±8.1

3.1.3.6. Statistical analyses

Statistical analyses were carried out using R Studio, Version 4.2.1. The baseline characteristics of the study population as a whole are expressed in absolute numbers (percentages) in the case of binary or categorical variables, mean values (standard deviation) in the case of normally distributed continuous variables, and median values (interquartile range) in the case of non-normally distributed continuous variables. Distribution normality was ascertained by visually inspecting histograms. Mean baseline blood-test values were compared separately with those obtained after

four and 12 weeks using two-tailed paired t-tests, whereas trends across the three time points were analyzed using three-way analysis of variance (ANOVA). Statistical significance was considered at a nominal alpha value of 0.05, and all tests were two-sided.

3.1.4 Conclusions of the study

In conclusion, the study shows that 100 ml of dietary supplementation with standardized kiwi, Annurca apple, bergamot and grape juice extracts with phytosterols, red yeast rice and berberine complexed with β-cyclodextrin, safely provides significant improvements in serum lipids in subjects with moderate hypercholesterolemia. Although the potential diseases prevention and therapeutic effects of polyphenols is documented in the literature [39], this is the first study with this peculiar combination.

Our study should be considered preliminary and it had some limitations such as the small sample size of the pilot study and the relatively short duration of the treatment. For these reasons, these results should be considered preliminary, not definitive, and should be confirmed with the rigor of long-term randomized clinical trials to be conclusive.

In addition, future research is needed to understand the mechanistic role that the polyphenols contained in the fruit-by-product extracts play in the reduction of cholesterolemia and in cardiovascular-disease prevention.

3.2 Conclusions

- According with the Food and Agriculture Organization (FAO), **one-third of all food produced in the world is wasted**, which represents approximately **1.3 billion tons**.
- Global food waste generated by **food processes**, such as vegetable oil extraction, starch, juice and sugars production, also contributes significantly to environmental issues, because of its extensive use of energy and resources, as well as the associated greenhouse gas emissions.
- A large number of by-products of the various phases of food production have been studied to find ways to limit food production's environmental and economic impact and researchers have experimented with new processes for the recovery of valuable components.

- In **fruit juice industry**, more than **50% of raw material becomes by-products** that are rich in active compounds and have high nutritional content.
- The preliminary **study conducted on fruit by-products** shows that 100 ml of dietary supplementation with standardized kiwi, Annurca apple, bergamot and grape juice extracts with phytosterols, red yeast rice and berberine complexed with β-cyclodextrin, safely provides significant improvements in serum lipids in subjects with moderate hypercholesterolemia.

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Chapter 4
Valorisation of potato peel

4.0 Introduction

The potato (*Solanum tuberosum*) is the fourth largest food crop in the world after rice, wheat and maize, and is a very important part of human diets. It was estimated that overall world potato production was 388 million tons in 2017, with more than 40% being produced in China and India (FAO, 2020). This staple crop contains a wide range of molecules with relevant functions in human nutrition, such as vitamins, amino acids and minerals. In particular, nutritional intakes of potassium (up to 693.8 mg/100 g), ascorbic acid (up to 42 mg/100 g), dietary fibre (up to 3.3%) are provided by several typologies of potato, together with smaller amounts of protein (0.85%–4.2%) and others bioactive compounds [1].

The worldwide use of potatoes is increasingly shifting away from fresh and towards machined products, and this leads to huge amounts of potato peel (PP) being produced as industrial waste to be managed. Moreover, recycling and disposal of this waste poses quite the challenge because of legal restrictions to avoid undesirable consequences such as decomposition with bad smell and being a source of late blight inoculum, leaf roll virus, and other diseases that can spread in neighbouring fields in case of winter field spreading or burial [2].

4.1 Chemical composition of potato peel

To fully understand the physicochemical properties of PP, it is crucial to focus on its whole composition (both physical and chemical). The knowledge of these features will support the development of an environmentally friendly approach for the utilisation of PP. Table 1 illustrates the main components [3,4]. In addition, PP contains various polyphenols and phenolic acids, which are responsible for its antioxidant activities, whereas the fatty acids and lipids show antibacterial properties [5]. The lipid fraction includes triglycerides, alcohols, long-chain fatty acids and sterol esters. Moreover, lignin units have been detected in the cell walls of potatoes [6]. Although PP is rich in starch (52% on dry material), the total amount of fermentable reducing sugar is limited (0.6% on dry material) [4].

Table 1. Chemical composition of raw PP, g per 100 g (adapted from: AIMS Agriculture and Food, 2019, 4(3): 807–823).

Compound	Values range
Water	83.3-85.1
Protein	1.2-2.3
Total lipids	0.1-0.4
Total carbohydrate	8.7-12.4
Starch	7.8
Total dietary fibre	2.5
Total phenolic content	1.02-2.92
Total flavonoids	0.51-0.96
Ash	0.9-1.6

4.1.1 Phenolic Compounds in Potato Peel

PP is a great source of phenolic compounds as approximately 50% of these molecules are situated in the peel and adjacent sections [7]. The growing demand of natural antioxidants comes from their applicability as functional ingredients in food formulations as they can ensure the protection of cells against oxidative damage and reduce the risk of oxidative-stress-linked degenerative diseases [8]. For these reasons, the use of by-products to produce food ingredients with excellent nutritional features gained much interest and, consequently, their recovery acquired economic attractivity [9]. In this respect, several studies highlighted PP as a source of natural antioxidants [10,11]. These bioactive metabolites can be added to functional foods and can be exploited to produce nutraceuticals by virtue of their possible health benefits [12].

As already mentioned, *Solanum tuberosum* shows an interesting concentration of phenolic compounds that may well integrate the diet. Potato germplasms contain an outstanding variety of polyphenols, in terms of both composition and concentration [13], confirming the presence of active metabolites in all the parts of the tuber [14]. In details, the detected classes count phenolic acids and flavonoids, including flavanols, flavonols and anthocyanins [3]. A list of phenolic compounds that are present in potatoes can be found in Table 2.

Table 2. Qualitative profile of phenolic compounds in *Solanum tuberosum* (Adapted from: Akyol *et al.*, 2016 [15])

Hydroxycinnamic acids	- Chlorogenic acid (CGA) Crypto-CGA Neo-CGA - Ferulic acid (FA) - Caffeic acid (CA) - *p*-Coumaric acid (p-CUA)
Hydroxybenzoic acids	- Gallic acid (GA) - Protocatechuic acid (PCA) - Vanillic acid - Salicylic acid
Non-anthocyanin flavonoids	- Catechin (CAT) - EpiCAT - Eriodyctiol - Naringenin - Kaempferol glycosides - Quercetin glycosides
Anthocyanins	- Petunidin glycosides - Malvidin glycosides - Pelargonidin glycosides - Peonidin glycosides
Dihydrocaffeoyl polyamines	- Kukoamine A - N^1,N^8 *Bis*(dihydrocaffeoyl)spermidine - N^1,N^4, N^{12}- *Tris*(dihydrocaffeoyl)spermine - N^1,N^4,N^8 -*Tris*(dihydrocaffeoyl)spermidine

The most common method for the recovery of polyphenols from potatoes is **solid-liquid extraction** with ethanol, methanol and aqueous alcohol mixtures. However, this approach requires long extraction times and led to moderate yields [15]. Hence, new extraction and isolation techniques have been developed to overcome these issues. Ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE), and pressurised-liquid extraction (PLE) represent only a limited example of these intensification techniques [14]. PPs contain larger amounts of several nutrients than the pulp; almost 50% of phenolic compounds are found in the skin and adjacent

tissues, suggesting that this by-product has a wide range of potential uses [10,15]. PPs have traditionally been used to produce high quality and nutritive animal feeds. The polyphenol-containing matrix is generally subjected to different types of **pre-treatments** before the actual extraction step [16]. The main pre-treatments listed in the literature include the physical modification of the biomass (grinding in planetary mills, hammer or blender mills, pre-treatment by ultrasonic or hydrodynamic cavitation and the use of homogenisers), which is useful for increasing mass transport (reduction of the average size of the matrix to be extracted) and the permeability of the material to the extraction solvents (creation of porosity, freeze-drying, cell-wall destruction). However, these processes might be overly harsh for certain compounds, such as in the case of polyphenols, which are labile to different stimuli to varying degrees (temperature, light, etc.) [17]. Desaccharification is a further type of pre-treatment and is used to remove, from the matrix, components, such as salts and sugars (compounds with little or no activity) that are easily soluble in water at low temperatures. The aim is to enrich the final extract by increasing the activity/dry-extract selectivity without depleting the matrix of polyphenols, the latter of which are normally soluble at temperatures above ambient temperature [18].

4.1.2 Glycoalkaloids in Potato Peel

Unfortunately, the recovery of phenolic compounds, in the case of potato extracts, may entail a significant issue as toxic glycoalkaloids might be concentrated throughout the process [9]. During the germination phase, glycoalkaloids are naturally generated in the tuber, and they can potentially exhibit both adverse and positive effects (acetylcholinesterase inhibition and anticarcinogenic action respectively). PP glycoalkaloid (PGAs) content varies according to a number of different factors and conditions (e.g. agrotechnical processes, seasonality, maturation state in the harvest and post-harvest manipulation) and more than eighty different alkaloids have been identified including *alpha*-solanine, *alpha*-chaconine, dehydrocommersonine, atomatine, demissine, dihydro-b-chaconine and dihydrosolanine [19]. Over 330 mg/kg sample concentration, these molecules can cause death [20]. For consumer safety, it is therefore crucial to verify the presence of these metabolites in the final product. In particular, it is recommended that potato tubers should not contain more than 100 mg kg^{-1} fresh weight of these compounds (upper limit of safety: 200 mg kg^{-1} fresh weight) [21].On the other hand, besides their toxicity and harmful effects, several *in-vitro* and pre-clinical studies have investigated the role that glycoalkaloids may play against many diseases, such as

inflammation, glycemia, allergies, microbial infections, fever and even specific types of cancers [22].

For example, a study by *Ding et al*. (1993) has demonstrated the antitumor activity of solasonine, b1-solasonine, solamargine and solanigroside P against MGC-803 cells, and highlighted the possible role of steroidal glycoalkaloids in the treatment of gastric cancer, probably via the down-regulation of p53 mutation, an increase in the Bax-to-Bcl-2 ratio and the activation of caspase-3 to induce apoptosis [23]. For this reason, as reported by *Benkeblia* (2020), the development of efficient extraction and purification techniques for PGAs, and the enhancement of glycoalkaloid content in PPs via breeding or the molecular engineering of new varieties to increase extraction yield, making PP waste (PPW) a more valuable by-product [22].

4.1.3 Starch, non-starch polysaccharide and other valuable compounds in potato peel

The most important chemical components of PPW are starch, non-starch polysaccharides (cellulose, hemicelluloses and pectin), lignin, proteins, lipids and ash [24]. Several different sugars and uronic acids have been identified after sequential extraction, and these include mannose, galacturonic acid, xylose, glucose, fucose, glucuronic acid, galactose, rhamnose and arabinose. During the storage, these compounds displayed good stability under acidic conditions, facilitating their further purification and eventual commercialisation or conversion into bioproducts [25]. In this regard, and in light of the growing worldwide energy requirement together with the environmental sustainability awareness, carbohydrate waste streams, such as PPW [26], could be a promising alternative for the production of biofuels and chemicals via the biochemical conversion of sugars [27].

In addition, a significant portion of polysaccharides have long been exploited to enhance the texture, water retention and stabilisation of emulsions, and are being ever more frequently integrated into health foods owing to their prebiotic effects and the presence of dietary fibres and mimetic fats [28]. In particular, PP fibres are well known as nutraceuticals in cardiovascular prevention. In fact, many studies have underlined the effects of fibre supplementation in lipid-lowering and as hypoglycaemic agents [29,30,31,32].

Water-soluble polysaccharides that are extracted from PPW are also a promising source of natural antioxidants and can be used as additives in food, pharmaceutical and cosmetic preparations as highlighted in a study by *Jeddou* and colleagues [5]. In fact, these bioactive molecules show interesting water-holding and fat-binding

capacities in addition to exhibiting a variety of biological activities, including immune-system regulation, inflammation reduction as well as anti-tumour and anti-oxidative properties [33].
Lactic acid is an organic acid that can be obtained from PPW. It is widely used in food, pharmaceutical, cosmetic, and industrial applications. Its production generally starts with glucose that is obtained from starch or lignocellulosic biomass that has either undergone separate hydrolysis and fermentation or coupleds saccharification and fermentation in the simultaneous presence of enzymes and a pure culture [26].

4.2 Current strategies for Potato-Peel valorisation

Simple solid/liquid extraction (SLE) is still the widespread method reported for the extraction of bioactive compounds and, in particular, polyphenols from PPs (Table 3). In fact, although its longer extraction times and higher solvent consumption are drawbacks, the equipment utilized is simple and does not require high capital investment. Traditionally, polyphenols are extracted from PPs with organic solvents, such as ethyl acetate, acetone, methanol, and ethanol [34]. Even If these solvents have amazing extraction capacity and a low price, their use has some disadvantages, including high flammability and toxicity (solvent-dependent) [7,35,36]. However, of these organic solvents, ethanol (EtOH) is considered a "GRAS" solvent (generally recognised as safe and harmless) and can therefore be used in the food field [37].
In recent years, the development of new techniques, such as UAE, MAE, pressurised-liquid extraction (PLE) and subcritical water extraction (SWE) (Table 4), for the valorisation of by-products has led to significant reductions in the use of organic solvents, which has improved extraction efficiency and reduced potential toxicity [38,39].

UAE is well known as an efficient unconventional technique for the recovery of several compounds, such as pectin, hemicellulose, polysaccharides, proteins, glycoalkaloids, unsaturated fatty acids and phenolic compounds [40,41]. It is considered to be a versatile, flexible and simple technique that requires relatively small capital investment and is scalable for commercial use [42]. It intensifies extraction by quickening diffusion phenomena and enhancing solvent penetration and mass transfer. UAE has been demonstrated to significantly improve the recovery of polyphenol extracts from PPs, compared to conventional extraction methods alone. *Kumari et al*. have investigated the UAE, at 33 and 42 kHz, of polyphenols from the PPs of the varieties cream-skinned Lady Claire and pink-skinned Lady

Rosetta. Compared to SLE processes alone, the UAE-treated extracts had higher total phenolic content, in particular at lower ultrasonic frequency (33 kHz) better than higher frequency treatment (42 kHz). The study also highlighted the fact that the Lady Rosetta extract had higher phenolic contents (7.67 mg GAE gdb^{-1} for chlorogenic acid (CGA), as the most representative) and higher antioxidant activity (DPPH value 5.86 mg TE gdb^{-1}, FRAP 22.21 mg TE gdb^{-1}) than Lady Claire peel (particularly rich in CA, CA). Finally, Peleg's model of diffusion (R2 >0.92) was found to be a valuable tool with which to understand UAE kinetics and to estimate the extract's phenolic yield at a variety of extraction time ranges [36]. Although 80% aqueous methanol is the most suitable solvent for the extraction of phenolics from PPs, as was underlined in the above-mentioned study, other examinations have shown that water/glycerol mixtures can be very efficient for polyphenol extraction. A study by *Paleologou et al*. has assessed the optimisation of potato-peel extraction and evaluated the extraction efficiency using aqueous mixtures of two bio-solvents, ethanol and glycerol [43,44]. The extractions were assisted by ultrasound (US). The study showed that, under improved conditions, the extraction yields in total polyphenols were 9.11 mg and 8.71 CA equivalents per gram dry weight, for water/ethanol and water/glycerol mixtures, respectively. The kinetic assay showed the water/ethanol system faster than water/glycerol (diffusion coefficients of 0.46×10^{-11} and 0.33×10^{-11} $m^2\ s^{-1}$ respectively) [45]. *Wang et al.* have also used UAE (US power 400 W for 4 minutes, solid-to-liquid mass ratio 1:25, ethanol concentration 80%) to extract potato-peel flavonoids with satisfactory results (maximum extraction yield of flavonoids 2.92%) [46]. These results confirmed those obtained by *Samarin et al.*, in which UAE improved the quantity of total phenolic compounds in the PP extract [34].

Moreover, it is interesting to state that the effects of US power density could deeply influence the extraction of the different polyphenols. According to Alves Filho *et al.* [47] this technique can be exploited to selectively extract specific caffeoylquinic acids (CQAs) and feruloylquinic acids. In particular, it has also been established how US could promote the hydrolysis of triCQA at 20–50 W/L power density meanwhile that of 3,4-CQA at 50 W/L.

Finally, the strong potential of using UAE in combination with SLE has also been tested on other components, such as some steroidal alkaloids, that are present in the potato-peel waste. Several methods for the extraction of alkaloids from potato have been described, and the most commonly used include polar solvents, such as methanol and ethanol, acid solvents, such as acetic acid, trichloroacetic acid and

sulphuric acid, or combined alcohol–acidic solutions [22]. Nevertheless, the use of the UAE with SLE has shown the most promising results and technical efficiency. In particular, a study by *Hossain* and colleagues, identified the optimal UAE conditions using response surface methodology (amplitude: 61 µm, extraction time: 17 minutes), which resulted in a recovery of 1,102 µg steroidal alkaloids/g dried PP compared to 710.51 µg with only SLE. In terms of individual glycoalkaloids, the yields were 273, 542.7, 231 and 55.3 µg/g dried PP for alpha-solanine, alpha-chaconine, solanidine and demissidine, respectively, using UAE [48]. In addition, this technique proved the viability of the concomitant extraction and chemical conversion of alpha-solanine and alpha-chaconine into beta-solanine and beta-chaconine using US [49].

In recent years *Dai et al.* [50,51] have evaluated **MAE** as an alternative to conventional methods for the extraction of the bioactive compounds present in PPs [2]. MAE is a novel process that utilises microwave (MW) energy to heat solvents and samples to extract target compounds from the sample into the solvent and can reduce extraction times and solvent consumption as well as promoting higher selectivity towards target molecules [3]. When MW pass across a biological medium, their energy is absorbed and switched into thermal energy. The capability of a medium to absorb and convert MW energy into heat is defined by its dielectric properties. In a study conducted by *Singh et al*., MAE was demonstrated to be effective in the extraction of ascorbic acid and selected phenolics, as it used less solvent and considerably reduced the extraction time, although methanol concentration and extraction time played an important role in the extraction of single phenolics. A maximum total phenolics content of 3.94 mg g^{-1} dry weight was obtained with 67.33% methanol and a MW power level of 14.67% for 15 minutes. However, the highest contents of ascorbic acid, CA and ferulic acid (FA) as well were obtained with 100% methanol and a MW power level of 10% for 15 minutes, while the highest antioxidant activity (evaluated by using the DPPH assay) was obtained under the same conditions, but reducing the treatment time to 5 minutes [35]. The same research group, in another study, concluded that the yield of the total phenolics extracted during the MAE process is drastically influenced by solvent concentration, extraction time and the dissipation factor of the solvent [52].

Sequential Hydrothermal Extraction (SeqHTE) is another unconventional technology and is a versatile “green alternative” for repurposing PPs as a resource.

It enables the stepwise fractionation of the biomass to extract several bioactive molecules according to the different affinities between water and the compounds at different temperatures. This decreases the residual solid content and thus contributes to mitigating environmental and handling problems. In a recent study by *Martinez-Fernandez et al.*, a SeqHTE process was shown to recover 22.48 and 32.87 mg/g dry peel of polyphenols, and 20-450 and 35-610 mg/kg dry peel of alkaloids from Russet Burbank and peel mixture samples, respectively [53].

Pulsed electric field (PEF)-assisted extraction, a well-known cell-disintegration technique, is based on external electric fields that cause the electroporation of cell membranes, boosting the diffusion of solutes. This permeabilization of cell membranes can be carried out at moderate electric fields (<10 kV/cm) and low specific energies (<10 kJ/Kg). *Frontuto et al.* have conducted a study to assess the effectiveness of the PEF-assisted extraction, in association with SLE, of total phenolic compounds from both pre-treated (with PEF) and non-pre-treated potato-peel extracts. The results showed that the combination of PEF and SLE granted higher total phenolics yields (10%) and antioxidant activity (9%), compared to the control extraction. In addition, the association of PEF with SLE led to reductions in duration, temperature and solvent consumption (optimised conditions: 52% ethanol, 230 min and 50 °C for PEF; and 54% ethanol, 233 min and 50 °C for SLE). As highlighted in the study, no significant degradation of polyphenols after PEF (such as chlorogenic, syringic, protocatechuic, caffeic, and *p*-coumaric acids) was revealed by the HPLC-DAD analyses [54]. This interesting result confirms results obtained by *Puértolas et al.*, who investigated the effects of PEF-assisted treatment on the anthocyanin extraction yield from purple-fleshed potato (*Solanum tuberosum*, variety ‘‘Vitelotte’’) at different extraction times (60–480 min) and temperatures (10–40 C°), using water and ethanol (48% and 96%) as the solvents. In particular, after treatment, it was found that PEF can be performed with water without decreasing the anthocyanin extraction yield from purple-fleshed potato, compared to ethanol (untreated sample using 96% ethanol: 63.9 mg/100 g fw; PEF-treated sample using water: 65.8 mg/100 g fw) [55].

A novel approach, called **ohmic heating**, has recently been proposed by *Pereira, et al.*. It allows water to be used as a solvent for the recovery of phenolics from PPs. By contrast to PEF, ohmic heating applies a constant electric field, and is used as a novel technique for heating foods. Its action is based on the electroporation of cells

and their simultaneous heating, which facilitate increased mass transfer into the extracting solvent. Nevertheless, ohmic heating is used less frequently than UAE and PEF because it may degrade thermally labile compounds, although most polyphenols present in PP seem to be heat stable [56].

The importance of green solvents, such as water, and the future perspectives of their use have been highlighted by *Singh and Saldana*, who examined the application of subcritical water, under high pressure and temperature, to the extraction of polyphenols from PPs. In their study, they registered good recovery rates for phenolic compounds (81.83 mg/100 in 30 min at 180 °C) related to 3 h of extraction with an organic solvent (methanol) [18].

Pressurised Liquid Extraction (PLE) is a further innovative and "green" technique for the valorisation of by-products. PLE is a technique in which pressure is applied during extraction to allow temperatures above the boiling point of solvents to be used. These higher temperatures increase mass transfer and extraction rates, meaning that PLE generally involves shorter extraction times and lower organic-solvent consumption than conventional techniques. Although PLE did not enhance extraction compared to SLE, the use of aqueous ethanol as the extraction solvent, in a recent study, led to the recovery of a higher amount of polyphenols compared to the use of 100% methanol [57]. *Hossein et al.* have shown that a higher yield of glycoalkaloids was recovered from potato-peel PLE (1.92 mg/g dried PPs) than from conventional SLEn (0.981 mg/g dried PPs). In particular, under two optimum PLE conditions (89% methanol and 80 °C), the levels of individual steroidal alkaloids obtained were 873, 597, 374 and 75 µg/g dried PP for α-chaconine, α-solanine, solanidine and demissidine, respectively. Related values for SLE were 46%, 59%, 40% and 52% lower for α-chaconine, α-solanine, solanidine and demissidine, respectively [58].

Table 3. Analytical methods used for phenolic compounds extracts from potatoes.

Potato cultivar	**Extract analysis**	**Target class of compound**	**Ref.**
Cufri chandromukhi	HPLC-DAD	CGA, CA, GA	[59]
9 Italian cultivars	HPLC-UV-Vis	CGA	[60]

Ranger Russet, Norkotah Russet	HPLC-MS	Neo-CGA, CGA, CA, quercetin-3-o-glu-rut, rutin, kaempferol-3-o-rutinoside, cryptoCGA, quinic acid	[61]
23 Native Andean cultivars	HPLC-DAD, HPLC-MS, HPLC-FLD	CGA, neo-CGA, crypto-CGA, CA, PCA, vanillic acid, FA, petanin, rutin, kaempferol-3-*O*-rutinoside	[62]
320 specialty potato genotypes	HPLC-DAD	CGA, CA, GA, CAT	[63]
Russet Burbank	Not cited	CGA, FA, vanillic acid, CA, benzoic acid	[64]
Jasim, Atlantic, Jawan, Superior, Jopung	HPLC-MS	CGA, CA, FA, p-CUA, *trans*-cinnamic acid	[65]
Nicola, Sieglinde F, Isci 4052, Isci 67	HPLC-DAD	CGA, CA, FA, CAT	[66]
Not cited (Indian cultivar)	HPLC	GA, CA, CGA, PCA	[67]
13 native Andean genotypes	HPLC-DAD	Neo-CGA, crypto-CGA, CGA, kaempferol-3-o-rutinoside, quercetin	[68]
Karlena	HPLC	GA, neo-CGA, PCA, CAT, crypto-CGA, CGA, vanillic acid, CA, FA, p-CUA	[69]
Siecle, Purple Majesty, Dakota pearl, FL 1533, Vivaldi, Yukon gold	HPLC-UV-Vis	CGA, CA	[7]
Goldrosh, Nordonna, Dakota pearl, Norkotah, Red Nordland, Sangre, Viking, Dark Red Nordland	HPLC-DAD, HPLC-MS	CGA, CA, GA, FA, CAT, p-CUA, o-CUA	[70]
8 cultivars	HPLC-DAD	CGA, CA, epiCAT, p-CUA, vanillic acid, quercetin	[71]
Sava, Bintje	HPLC-DAD	PCA, gentisic acid, GA, CGA, salicylic acid, CA, FA, p-CUA	[11]

Bintje, Piccolo, Purple Majesty	HPLC-DAD-MS	CGA, neo-CGA, crypto-CGA, kaempferol rutinose, rutin	[72]
16 cultivars	HPLC-DAD / APCI-MS	CGA, CA, 3-o-CQA, 1-o-CQA	[73]
13 Italian cultivars	HPLC-DAD-MS	5-o-CQA, 4-o-CQA, 3-o-CQA, FA, anthocyanins	[74]
Purple majesty, Yukon gold, Atlantic	UPLC-MS	CGA, CA, FA, sinapic acid	[75]
50 cultivars	HPLC-DAD-MS	CGA, rutin, kaempferol-3-rutinose	[76]
Vitelotte, Luminella, Charlotte, Bintje	UPLC-DAD	CGA, neo-CGA, crypto-CGA, CA, FA, p-CUA, syringic acid, vanillic acid, CAT, rutin, kaempferol-3-o-rutinoside	[77]
Sava	HPLC-DAD	GA, PCA, gentisic acid, CGA, vanillic acid, syringic acid, CA, salicylic acid, p-CUA, FA	[78]
Not cited	HPLC-DAD	CGA, neo-CGA, crypto-CGA, CUA, genistin, quercetin-3-β-D-galactoside, naringin, naringenin, luteolin, genistein, kaempferol, flavan-3-ol	[79]
Not cited	UPLC-MS	CGA, quinic acid, CA, methyl caffeate	[80]
15 Colombian cultivars	HPLC-DAD-MS	CGA, neo-CGA, crypto-CGA, CA	[81]
Agria	HPLC-UV	CGA, FA, GA	[10]
Valfi, Blaue Elise, Bore Valley, Blue Cango	HPLC-UV	CGA, CA, FA, CUA, crypto-CGA, neo-CGA, p-CUA	[82]

HPLC: High Performance Liquid Chromatography; UPLC: Ultra Performance Liquid Chromatography; DAD: Diode Array Detector; MS: mass spectrometer.

Table 4. Non-conventional extraction methods for phenolic compounds in potatoes.

Potato cultivar	Extraction system	Experimental conditions	Target class of compound	Ref.
Nicola, Timo, Siikli, Rosamund, Van Gogh	UAE	MeOH and 10% acetic acid (85:15), 30 min	CGA, CA, FA, sinapic acid, vanillic acid, syringic acid	[83]
20 potato cultivars		MeOH (80%), acetic acid (1%), 20 min	CGA, petunidin-3-glucoside chloride, pelargonidin-3-glucopyranoside	[84]
Purple, Innovator, Russet, Yellow		MeOH–acetone-water (7:7:6, v/v/v), 20 min, 30 °C	CGA, CA, p-CUA, FA	[85]
Penta, Marcy		MeOH and 10% acetic acid (85:15), 30 min	CGA, CA, GA, p-CUA, FA	[8]
Diamond		MeOH (70%), ultrasonic water bath with ice, 15 min	CGA, caffeic, 4-hydroxybenzoic, *p*-coumaric, and trans-o-hydroxycinnamic acids	[86]
Russet		Solvents used for extraction: solvent A (25% water, 70% MeOH, 5% acetic acid) solvent B (24% water, 67%	CGA, CA, neo-CGA	[87]

		EtOH, 9% acetic acid), solvent C (46% water, 51% EtOH, 3% acetic acid), 20min		
BP1		MeOH: acetone: ultra-pure water (7:7:1; v:v:v), 5 min	CGA, CA, FA	[88]
Netherlands #7		MeOH (80%) and formic acid (1%), 30 °C, 30 min	GA, PCA, CGA	[89]
Ramus		Continuous air stream ultrasonic bath, 15 min	Total phenolics content	[46]
Calwhite	MAE	EtOH (60%), 80 °C, 2 min, solid-to-solvent ratio 1:40 (g/ml)	CGA, CA, neo-CGA, crypto-CGA, FA, p-CUA	[90]
Russt Burbank		MeOH (67.33%), 15 min and a MP of 14.67%	Total phenolics content	[35]
Agria		150-1000 W, 1-7 min	PCA, CGA, neo-CGA, crypto-CGA	[91]
Lady Claire	Pressurized liquid extraction (PLE) + solid-	10.3 MPa, 125 °C, EtOH (70%)	CA	[57]
Red		40 bar, 190 °C, 9 min of static	GA, GCA and syringic acid	[92]

	liquid extraction	holding time using a flow rate of 3 mL min^{-1}		
Red	Subcritical water extraction	180 °C, 30 min	GA, CGA, CA, PCA, syringic acid, *p*-hydroxyl benzoic acid, FA, CUA	[18]
Vitelotte	PEF aided extraction	3.4 kV/m and 105 µs (35 pulses of 3 µs), water	Anthocyanins	[55]
Vitelotte	Ohmic heating assisted	100 °C for 1 s 200 V/cm, water	Anthocyanins, CGA, FA, ellagic acid, catechin, rutin	[56]
Russet Burbank (dark brown skins)	SeqHTE Sequential Hydrothermal Extraction	Stage 1: 150 or 170 °C; Stage 2: 200 or 220 °C for variable residence times from 10 to 20 min	Total phenolics content (CGA, CA, p-CUA, FA, GA, salicylic acid, catechin, epicatechin, naringenin, syringic acid, and ellagic acid)	[53]

HPLC: High Performance Liquid Chromatography; UPLC: Ultra Performance Liquid Chromatography; DAD: Diode Array Detector; UV: Ultraviolet detector; MS: mass spectrometer; SeqHTE: Sequential Hydrothermal Extraction, PEF: Pulsed electric field; MP: MW power (watts)

4.3 Recent advances in potato-peel valorisation

Potato is one of the most abundantly produced vegetables in the world, and large quantities of waste are created because of its extensive use in various industries. The peeling process alone can produce 6-10% of the total potato-peel waste, with 0.16 tons of waste produced per ton of processed potato [93]. In the age of circular economy this waste could represent a real feedstock.

Several studies have demonstrated that it is possible to successfully replace (at least in part) the concentrated feed mixture in sheep and fish rations with potato-peel-based products giving improvements in nutritional parameters, including protein and

fat in muscles and liver [94,95]. Potato-peel waste can also be used as biofuels, biofertiliser, biogas and biosorbents after procedures such as fermentation, extraction and others [3]. However, one of the most promising applications for PPs is the production of bioactive compounds. In this regard, phenolic acids, of all the phenolic compounds, have raised great interest as both nutraceuticals and drugs [96]. Gallic acid (GA), chlorogenic acid (CGA), FA, vanillic acid, *p*-coumaric acid (*p*-CUA), CA, protocatechulcgentlsic acid, *p*-hydroxybenzoic acid, syringic acid and salicylic acid are the principle phenolic acids that have been identified in PPs using HPLC [11]. Most of these phenolic substances have been found to present preliminary evidence for antioxidant and anti-inflammatory action in the literature and might be subjects for further study. For example, CGA offers several positive properties, such as antioxidant, antitumoral, anti-inflammatory, antimicrobial, analgesic, neuro- and cardio-protective effects, as highlighted in both *in-vitro* and animal studies [15]. Nevertheless, human randomised clinical trials of potato-peel polyphenols have not yet been performed despite phenolic molecules in PPs being well known, and the number of clinical trials (which have tested these compounds from other food sources) that have documented their potential health applications.

The main limitation to the use and commercialisation of phenolic bioactive that are extracted from PP is the fact that most of the proposed conventional extractive methods are expensive and based on laboratory studies. Thus, the concept of Green Extraction acquired relevance. This sustainable approach indicates the development of extraction procedures able to reduce energy consumption and providing at the same time a high-quality product. Usually, renewable natural products, alternative energy sources and solvents are the milestones of Green Extractions [97]. Sustainable extraction would also, theoretically, be much more advantageous in economic terms. However, for this to be true, existing processes must be improved and optimised, and new processes that should also consider using alternative solvents, must be tested [98]. In fact, although the unconventional processes for extracting value-added products are well established in the laboratory, the industrial-scale production with specific cost-effective analyses is still a challenge. In this regard, the uninterrupted availability of PPs and the selective separation of desired components are the major barriers to scale-up. The ideal extraction method for potato-peel polyphenols should be based on: little capital investment, low energy consumption, water as a solvent, high yield and easy integration into current processing lines. Unfortunately, none of the methods described in the literature satisfy all of these criteria. In particular, even though significant improvements in

extraction efficiency have been obtained using unconventional extraction techniques such as UAE, MWAE and PLE, they still involve high costs compared to chemical methods, and new proposals and solutions to reduce these constraints are, at this moment, still lacking. For this reason, potato processors should adapt the method that best suits their production to optimise extraction yields, sustainability, and high through-put.

Another problem is the high moisture content of PPs, which affects collection, storage, handling, and transportation. The drying of PPs is essential before any use and an effective dryer for this purpose is important. Even the storage conditions of PPs can influence the antioxidant properties of the polyphenols. In a study conducted in Ontario (Canada), the levels of polyphenolic compounds and their antioxidant activity in the PP were influenced by storage temperature with highest loss observed at 25 °C, compared to -20.4 °C (minimum loss), which highlights the importance of proper storage conditions in maintaining antioxidant properties [8]. Similar conclusions were made in a study by *Lachman et al*., who underlined that total antioxidant capacity was modified by both the storage conditions and the potato cultivar. For example, it was reported that cold storage (4 °C) differently influenced the total anthocyanins content of Violette and Highland Burgundy Red cultivars compared to Valfi ones: in the formers the total antioxidant capacity increased by 18.5% and 12.1%, respectively (if stored at 4°C instead of room temperature), meanwhile in the latter it decreased by 33.9%

5.0 Comparative Potato-Peel (PP) Extraction under non-conventional technologies using bio-based solvents: a case study

The design of sustainable procedures for biomass valorisation (mainly agricultural, industrial, and forest residues) [99,100], using efficient extraction technologies [101], coupled with bio-based solvents [102], is one of the hottest topics in current scientific literature. To partially address this issue, we report herein a comparative, although preliminary, study on potato-peel valorisation under green extraction procedures carried out in our laboratories at the University of Turin. Extractions of *Solanum tuberosum* peels have been carried out in the presence of sustainable solvents (mainly ethanol, water and bio-based solvents) under both conventional and non-conventional technologies (such as US and MW irradiation) in order to identify the best protocol for the recovery of residual bioactive compounds. This preliminary work aimed to demonstrate the synergism that can exist between so-called enabling

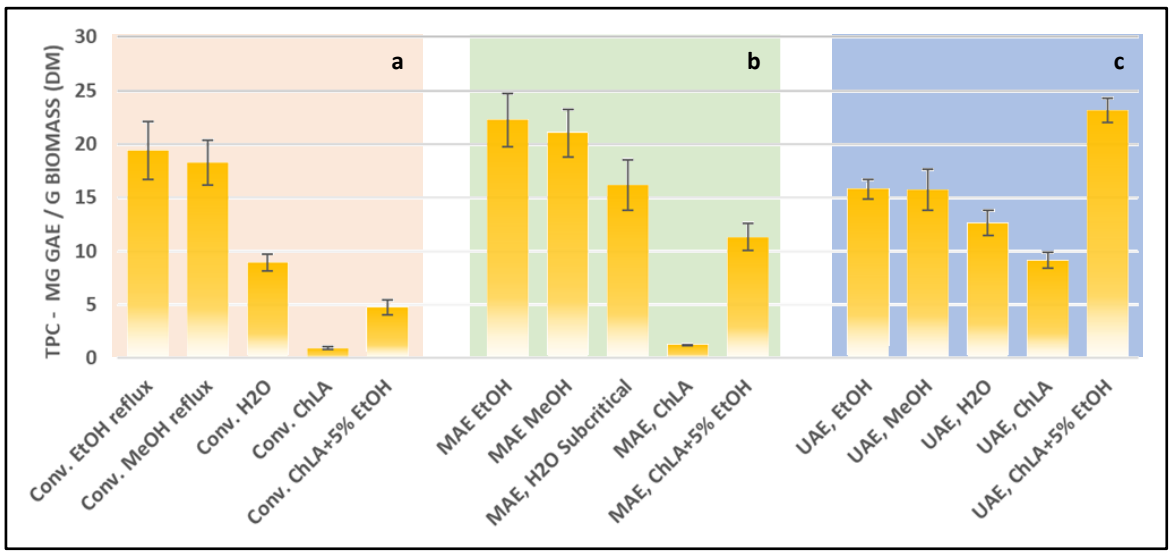
technologies (MW and US) and bio-based solvents, and that can produce an extract enriched in polyphenols from a food-processing waste benchmark, such as PPs. The general extraction procedures adopted for this comparative study have been described further in Appendix A and draw from previously reported procedures. Three different kinds of solvents have been considered and compared in terms of extraction efficiency (see Graph 1,), under both conventional and non-conventional extraction procedures, at a 1:20 S/L ratio: i) an hydroalcoholic mixture (ethanol or methanol/water 70:30); ii) distilled water (also applied under subcritical conditions in MAE); and iii) a choline chloride: lactic acid (1:1) mixture (ChLA).

In the presence of a hydroalcoholic mixture, both the MW and US processes halved the extraction time (from 30 to 15 min), compared to classical extraction conditions (reflux), and almost reached the same value of TPC (Total Phenolic Compounds) extracted. A comparison of the data obtained exclusively using non-conventional processes showed that UAE was found to be much more efficient than MAE. Despite granting a slightly lower quantity of TPC recovery under hydroalcoholic conditions (17 vs 22 mg GAE/g biomass (DM)), the operating temperatures of UAE were significantly lower than those adopted for MAE (50° *vs.* 120°C respectively for 15 min extraction time). This confirms the crucial role played by mass transfer, which was significantly enhanced by cavitation during the fast-extractive process under US conditions. Interesting results were only obtained for the application of water as a green extractive solvent under subcritical conditions and MW irradiation (180 °C); good TPC recovery was observed (18 mg GAE/g biomass (DM)).

In addition to conventional hydroalcoholic mixtures and water, a new class of environmentally friendly solvents, namely natural deep eutectic solvents (NaDES), has been explored in potato-peel-extraction experiments. The concept of green solvents is strongly associated to the principles of green chemistry, and NaDESs have recently gain much more consideration than the others available [103], including for use as extraction solvents for phenolic compounds [104]. In brief, a deep eutectic solvent (DES) is a fluid that is usually made of two or three safe and inexpensive components that are cpable of self-association, often through hydrogen-bond connections, to create a eutectic mixture with a lower melting point than that of each individual component. Moreover, their production is 100% atom-economic and, unlike ionic liquids (ILs), they are mostly nontoxic and biodegradable. The NaDES ChLA (choline chloride and 1:1 lactic acid) has been synthesised and tested in potato-peel extractions under non-conventional conditions and has been discussed in this comparative work. Unfortunately, only moderate results have been achieved

for potato-peel extraction in ChLA under US irradiation (9 mg GAE/g biomass (DM) of TPC). The better results found in UAE, compared to MAE and conventional extractions, can be explained by the boosted mass transfer effect that is induced by cavitation within the viscous extractive mixture due to the presence of NADES. Better results were achieved by adding a small amount of EtOH (5%) during the ChLA extraction of PP with the best TPC recovery (19 mg GAE/g biomass (DM)) occurring under US in only 15 min of irradiation.

Graphic 1. Comparative extractions of PP under non-conventional technologies and sustainable solvents.



In a typical experiment, 10 g of dry yellow PP (previously milled) was extracted using the proper solvent at a 1:20 S/L ratio: a) conventional extraction was performed under reflux in a hydroalcoholic mixture at 100°C using water, and at 120°C using ChLA; b) MAE was performed in 15 min at 120°C (or 180°C for subcritical water extraction) using a pressurisable MW multimode reactor; c) UAE was performed in 15 min at 50°C using an immersion sonotrode working at 21kHz and 500 W.

Starting from these preliminary results for the US-assisted extraction process, it will be necessary to perform an accurate screening of the most influential extraction parameters in NADES, such as times, temperatures, matrix/solvent ratios. Moreover, different natural deep eutectic mixtures could be tested for this purpose. In addition, it would also be desirable to conduct a rapid evaluation of synergistic NaDES/extract

effects, due to the known stabilizing effects that DES have on extraction products. This comparative study could pave the way for the development of a synergistic process that combines enabling technologies together with green solvents for the recovery of high-added-value products from residual biomass.

4.4 Conclusions

- Potato is one of the most abundantly produced vegetables in the world, and large amounts of potato waste are generated because of its widespread use in various industries.
- The several advantages of potato waste mean that it can serve as the best response for eco-friendly industrial products.
- One of the most promising applications of PPs concerns its content of polyphenols, which can be extracted using different technologies that are based on the "green chemistry" concept, leading to economic and environmental advantages. However, further investigations are needed to optimise capital investment, energy consumption, the nature of the solvent, yield and integration into current processing lines.
- To date, none of the unconventional methods described in the literature fulfils all of these criteria, and industrial-scale production with specific cost-effective analyses is still a challenge. In addition, the standardisation of cultivation and storage methods is also important to ensure process reproducibility.
- Finally, there is a strong need for *in-vitro* and *in-vivo* studies to help better understand the pharmacodynamic and pharmacokinetic properties of these bioactive compounds and for the development of new nutraceutical and/or pharmaceutical products.

4.5 Appendix A.

4.5.1 Materials and methods of Comparative Potato-Peel (PP) Extraction under non-conventional technologies using bio-based solvents

4.5.1.1 Biomass material

The *Solanum tuberosum* L. cv. Agria peel used in this work was bought at city market (Turin-Italy). Before use, PP was freeze-dried and milled using a laboratory blender (HGBTWTS360, Waring Blender). Sieving was applied to select <1000 µm granulometry (Giuliani, Italy).

4.5.1.2 *Chemicals*

All chemicals were purchased from Sigma-Aldrich and used without further purification. NaDES was obtained via heating: ChLA was prepared with equimolar ratios of choline chloride (ChCl) and lactic acid (LA) [105]. The two components were stirred and heated at 50°C in a round-bottom flask without adding water until a homogeneous liquid was formed. ChLA was finally collected for biomass extraction without further purification.

4.5.2 General procedures of Comparative Potato-Peel (PP) Extraction under non-conventional technologies using bio-based solvents

4.5.2.1 Conventional extraction

For the sake of comparison, conventional reflux extraction was performed with a EtOH hydroalcoholic solution (70:30 alcohol/water ratio). The result of this test was used as a benchmark [[22]]. In a typical extraction, 10 g of dry yellow PP (previously milled) was mixed inside a round-bottom flask with the correct amount of EtOH or MeOH hydroalcoholic solution, at the 1:20 S/L ratio. The mixture was continuously stirred while reflux conditions were reached by means of an oil bath. The extraction was carried out for 35 min. After extraction, the solutions were filtered under vacuum, and fresh extraction solvent was used to thoroughly wash the matrices. The alcoholic fraction was removed using a rotary evaporator, and the crude extracts were then freeze-dried (LyoQuest–85, Telstar, Spain), and the dry material was exploited for total polyphenol content (TPC). For sake of comparison, the same procedure was applied and the hydroalcoholic solution was replaced with ChLA. All the other parameters were kept unchanged. ChLA solutions were extracted three times with

chloroform after the addition of an aliquot of distillate water. The organic fractions were analysed after evaporation. Every test was performed in triplicate and results are reported as average value ± SD.

4.5.2.2 Microwave-assisted extraction (MAE)

MAE was performed in a SynthWAVE reactor (Milestone Srl, Italy), which is a pressurisable multimode microwave (MW) system that can work under an inert atmosphere (N_2). Tests were performed by mixing 1 g of dry yellow PP (previously milled) with the desired solvent, at the 1:20 S/L ratio, and then suitable agitation was performed. The protocol was applied to different solvent systems, namely EtOH and MeOH hydroalcoholic solutions (70:30 alcohol/water ratio), deionised water and ChLA NaDES. Before each run, the system was purged with nitrogen three times to reduce oxygen-derived degradations. The reactor was finally pressurised with 20 bar of N_2 to avoid solvent evaporation at the working temperature. All tests were performed at 1500 W of irradiation with a heating ramp of 5 minutes. An process temperature of 120°C was applied for the hydroalcoholic solutions and ChLA, whilst 180°C was used for subcritical water. The use of a subcritical working temperature has been supported by Singh *et al.* [18]. The system was stirred at 650 rpm and the temperature held for 15 minutes. The MW extraction time has been supported by Singh *et al.* [35]. After extraction, the solutions were filtered under vacuum, and fresh extraction solvent was used to thoroughly wash the matrices. Where necessary, the alcoholic fraction was removed by a rotary evaporator, and the crude extracts were then freeze-dried (LyoQuest–85, Telstar, Spain) and the dry material was analysed for total polyphenol content (TPC) determination. ChLA solutions were extracted three times with chloroform after the addition of an aliquot of distillate water. The organic fractions were analysed after evaporation. Every test was performed in triplicate and results are reported as average value ± SD.

4.5.2.3 Ultrasound-assisted extraction (UAE)

UAE extractions were performed using an immersion sonotrode (HNG-20500-SP, Hainertec Suzhou, China), working at 500 W at a frequency of 21 kHz. Extractions were performed by mixing 5 g of dry yellow PP (previously milled) with the desired solvent at the 1:20 S/L ratio. The mixture was placed in a Pyrex® thimble and cooled by means of an ice bath. The temperature was measured throughout UAE and was maintained under 50°C to maintain cavitation efficiency [106]. The solution was sonicated for 15 minutes to avoid overheating. The abovementioned protocol was

applied to different solvent systems: hydroalcoholic solutions (70:30 alcohol/water ratio) with EtOH and MeOH, deionised water, and ChLa NaDES. After extraction, the solutions were filtered under vacuum, and fresh extraction solvent was used to thoroughly wash the matrices. Where necessary, the alcoholic fraction was removed using a rotary evaporator, the crude extracts were then freeze-dried (LyoQuest–85, Telstar, Spain) and the dry material was analysed for total polyphenol content (TPC). ChLA solutions were extracted three times with chloroform after the addition of an aliquot of distillate water. The organic fractions were analysed after evaporation. Every test was performed in triplicate and results are reported as average value ± SD.

4.5.3 Antioxidant activity of Comparative Potato-Peel (PP) Extracts produced under non-conventional technologies using bio-based solvents

4.5.3.1 Total phenolic contents (TPC)

TPC was determined according to previously developed method [107]. The procedure require a standard curve of GA, used as the reference for phenolic compound quantification. Calibration curve is included between 5 and 250 µg/mL in a H_2O/DMSO 1:1 mixture. Dried extracts were dissolved in a H_2O/DMSO 1:1 mixture at a concentration of ~0.8 mg/mL. The GA and analyte (250 µL) are collected into test tubes, together with reactive mixture, as it follows: Folin–Ciocalteu (250 µL, diluted equally with distilled H_2O), 10% p/v Na_2CO_3 solution (500 µL), distilled H_2O (4 mL). Test tubes were then vigorously shaken and left at room temperature. After 25 min, absorption of the solutions was measured at 740 nm with a Cary 60 UV-Vis spectrophotometer (1 cm cuvette, Agilent Technologies, Santa Clara, CA, USA). TPC was expressed as GA equivalents (GAE, mg/g) over the dried matrix (DM). All tests were carried out three times and expressed as averages.

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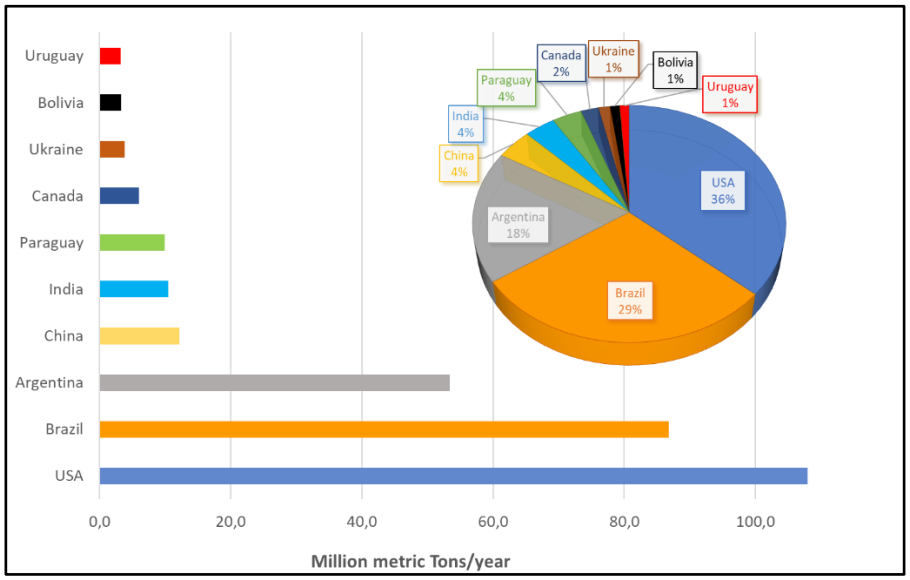
Chapter 5
Valorisation of soy products

5.0 Introduction

Soybeans (SBs) are currently one of the most important food crops worldwide. Although they originated in Asia and have been cultivated there for thousands of years, 7 of the top 10 producers are presently found on the other side of the Pacific region, in North and South America [1] (Figure 1).

Figure 1. World leaders in soy production, based on annual data released by FAOSTAT.



Despite the ancient tradition of soy foods, it is only in the last 15 years that they have also been shown to be beneficial in the prevention and treatment of various chronic diseases [2]. However, individuals can develop an allergy to this important legume [3].

It is now well established that SBs are an abundant source of proteins due to their high nutritional value and excellent physical and chemical properties (Table 1). Moreover, SBs and SB products are rich sources of minor non-nutrient components with potential health benefits, which are often referred to in the literature as phytochemicals. Within this group of substances, we find biologically active proteins and peptides, such as protease inhibitors (which include trypsin inhibitors, low molecular weight proteins that bind trypsin and thus interfere with protein hydrolysis during digestion) [4], lectins (tetrameric glycoproteins that make up 1–2% of the seed and that are able to agglutinate red blood cells and, once ingested, stimulate the

secretion of pancreatic enzymes and enterocytes, interfering with the intestinal absorption of nutrients) and lunasin (a recently discovered peptide, a major component of Bowman-Birk's protease inhibitor, particularly effective in suppressing carcinogenesis) [5]. SB products are also important sources of isoflavones, phytosterols, phytic acid and saponins. In traditional nutritional theory, many of these components have been considered anti-nutrients. However, in the last two decades, it has been discovered that they can exert beneficial health and therapeutic effects, from cholesterol-lowering effects to anticancer effects, a controlling effect of diabetes mellitus and the reduction of postmenopausal osteoporosis [6].

As far as processing is concerned, SBs are first classified, cleaned, dried at about 10% humidity and split to remove the shell. SB shells are processed to create fibre additives for bread, cereals, snacks and livestock feed. After husking, the beans are rolled into fat-rich flakes that can be used in animal feed or processed into wholemeal flour for food use. Crushing breaks up the oil cells of the seed, improving the oil-extraction process. The next step is to extract the raw oil, which is then refined to produce cooking oil, margarine and pastry fat.

Table 1. Nutritional composition of traditional unfermented soy-based foods, such as fresh, dried and boiled soybeans (SBs), soy flour, SBs meal, soy protein (SP) concentrate and isolate, soymilk and okara (100 g portion, based on USDA Nutrient Database) [7].

Compound (Amount Unit/100 g)	**SBs Raw [1]**	**SBs Dried [2]**	**SBs Boiled [3]**	**Soy Flour [4]**	**SB Meal [5]**	**SP conc. [6]**	**SP Isolate [7]**	**Soy Milk [8]**	**Okara [9]**
Water (g)	8.54	0.8	62.6	5.2	6.94	5.8	4.98	93.3	81.6
Energy (kcal/KJ)	446/1866	449/1880	172/721	434/1816	337/1409	328/1373	335/1401	33/138	76/320
Protein (g)	36.5	43.3	18.2	37.8	49.2	63.6	88.3	2.8	3.52
Total lipid (g)	19.9	21.6	8.97	206	2.39	0.46	3.39	2.0	1.73
Tot saturated FA (g)	2.88	3.13	1.30	2.99	0.27	0.05	0.42	0.21	0.19

Tot monounsaturated FA (g)	4.40	4.78	1.98	4.56	0.41	0.08	0.64	0.33	0.30
Tot polyunsaturated FA (g)	11.3	12.2	5.06	11.7	1.04	0.20	1.65	0.83	0.76
Ash (g)	4.87	5.28	1.91	4.46	5.58	4.7	3.58	0.27	0.88
Carbohydrate (by diff., g)	30.2	29.0	8.36	31.9	35.9	25.4	0	1.8	12.2
Fibre (total dietary, g)	9.3	8.1	6	9.6	NR	5.5	0	1.3	NR
Sugars (total, g)	7.33	NR	3	7.5	NR	20	0	NR	NR
Minerals									
Calcium (mg)	277	140	102	206	244	363	178	4.0	80
Iron (mg)	15.7	3.95	5.14	6.37	13.7	10.8	14.5	0.58	1.3
Magnesium (mg)	280	228	86	429	306	140	39	19.0	26
Phosphorus (mg)	704	649	245	494	701	839	776	49.0	60
Potassium (g)	1.80	1.36	0.52	2.5	2.49	0.450	0.081	0.141	0.213
Sodium (mg)	2	2	1	13	3	900	1005	12	9
Zinc (mg)	4.89	4.77	1.15	3.92	5.06	4.4	4.03	0.23	0.56
Copper (mg)	1.66	1.08	0.41	2.92	2	0.98	1.60	0.12	0.2
Manganese (mg)	2.52	2.18	0.82	2.28	3.8	4.19	1.49	0.17	0.40
Selenium (µg)	17.8	19.3	7.3	7.5	3.3	0.8	0.8	1.3	10.6
Vitamins									
Ascorbic acid (C) (mg)	6	4.6	1.7	0	0	0	0	0	0
Thiamine (B1) (mg)	0.87	0.43	0.16	0.58	0.69	0.32	0.18	0.161	0.02

Riboflavin (B2) (mg)	0.87	0.78	0.28	1.16	0.25	0.14	0.1	0.07	0.02
Niacin (B3) (mg)	1.62	1.06	0.40	4.32	2.59	0.72	1.44	0.15	0.1
Pantothenic acid (B5) (mg)	0.79	0.47	0.18	1.59	1.98	0.06	0.06	0.05	0.09
Pyridoxine (B6) (mg)	0.38	0.22	0.23	0.46	0.57	0.13	0.1	0.04	0.12
Folate (B9) (µg)	375	205	54	345	303	340	176	1.5	26
Retinol (A) (IU) *	22	0	9	120	40	0	0	10	0
α-Tocopherol (E) (mg)	0.85	NR	0.35	1.95	NR	NR	0	0.01	NR
Phylloquinone (K) (µg)	47	37	19.2	70	NR	0	NR	NR	NR

* 1 IU = 0.3 mcg retinol. NR = not reported, FA = fatty acids. [1–8] Full description, Food Data Central Identifier (FDC ID), National Nutrient Database Identifier (NDB ID) for the Standard Reference Legacy Release (SR Legacy) of all soy-based foods are cited in the corresponding reference [7].

The defatted soy flakes are used to produce animal feed and form the basis of a variety of products for human consumption, including SB meal, SB concentrates and SB isolates. These products are widely used in food to help retain moisture and improve shelf life, as well as acting as emulsifiers and meat substitutes in food products. SB meal is produced by grinding the defatted flakes. The protein content of the flour is about 50%, which makes it a source of protein, while also improving the colour of the crust and the shelf-life of baked goods.

SB isolates are produced in a chemical process that removes most proteins from the defatted flake, resulting in a product with about 90% protein content that is free from fibre and carbohydrates. Isolates are used in many dairy-like products, including cheese, milk, non-dairy frozen desserts, coffee whiteners and meat products. SB concentrates, on the other hand, are prepared by removing soluble sugars from defatted flakes and contain about 70% protein and retain most of the dietary fibres of the seed. They are used in protein drinks and as a basis for soups and sauces. SB

meal and soy-protein concentrates are used in meat products, mainly because of their fat- and water-absorbing properties [8]. Nutritional values of traditional fermented soy-based foods are reported in Table 2.

Table 2. Nutritional composition of traditional fermented soy-based foods, such as tempeh, miso, soy sauces, natto and tofu (100 g portion, based on USDA Nutrient Database) [7].

Compound (Amount Unit/100 g)	**Tempeh**	**Miso**	**Natto**	**Soy Sauce**	**Tofu**
Water (g)	59.6	43.0	55.0	66	70.0
Energy (kcal/KJ)	192/803	198/828	211/883	60/251	116/484
Protein (g)	20.3	12.8	19.4	10.5	8.92
Total lipid (g)	10.8	6.0	11	0.1	8
Tot saturated FA (g)	2.54	1.02	1.59	0.011	1.16
Tot monounsaturated FA (g)	3.2	1.12	2.43	0.017	1.77
Tot polyunsaturated FA (g)	4.3	2.88	6.21	0.044	4.52
Ash (g)	1.62	12.8	1.9	17.8	8.7
Carbohydrate (by diff., g)	7.64	25.4	12.7	5.6	4.38
Fibre (total dietary, g)	NR	5.4	5.4	0.8	NR
Sugars (total, g)	NR	6.2	4.9	1.7	NR
Minerals					
Calcium (mg)	111	57	217	20	46
Iron (mg)	2.7	2.49	8.6	2.38	1.98
Magnesium (mg)	81	48	115	40	52
Phosphorus (mg)	266	159	174	130	73
Potassium (g)	412	210	729	212	75
Sodium (mg)	9	3728	7	5586	2873
Zinc (mg)	1.14	2.56	3.03	0.43	1.56
Copper (mg)	0.56	0.42	0.67	0.14	0.38
Manganese (mg)	1.3	0.86	1.53	0.50	1.17
Selenium (µg)	0	7	8.8	0.8	17.3
Vitamins					
Ascorbic acid (C) (mg)	0	0	13	0	0.2
Thiamine (B1) (mg)	0.08	0.098	0.16	0.06	0.16
Riboflavin (B2) (mg)	0.36	0.23	0.19	0.152	0.10
Niacin (B3) (mg)	2.64	0.091	0	3.95	0.38
Pantothenic acid (B5) (mg)	0.28	0.34	0.22	0.38	0.13

Pyridoxine (B6) (mg)	0.22	0.20	0.13	0.2	0.09
Folate (B9) (µg)	24	19	8	18	29
Cobalamin (B12) (µg)	0.08	0.08	0	0	0
Retinol (A) (IU) *	0	87	0	0	0
α-Tocopherol (E) (mg)	NR	0.01	0.01	0	0
Phylloquinone (K) (µg)	NR	29.3	23.1	0	0

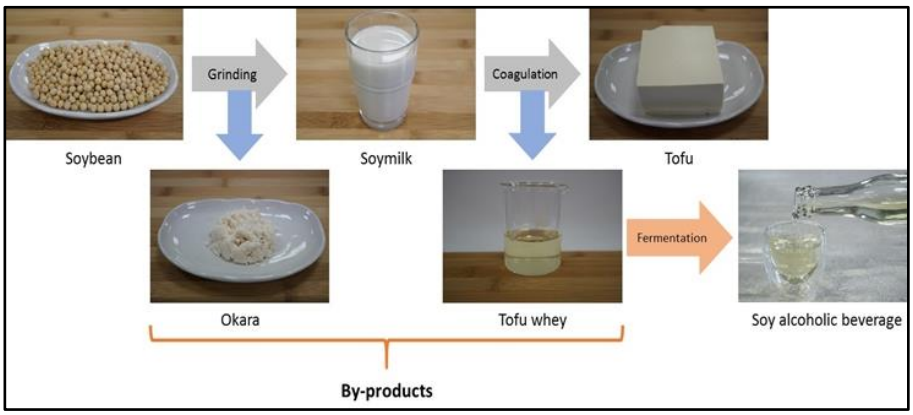
* 1 IU = 0.3 mcg retinol. NR = not reported, FA = fatty acids. Full description, FDC ID, NDB N. (SR Legacy) of all the soy-based foods are cited in the corresponding reference [7].

SB-based foods are generally divided into two categories: unfermented and fermented foods. Traditional unfermented soy-based foods include fresh SBs, whole dried SBs, soy nuts, SB sprouts, whole SB meal, soymilk and soymilk products, such as tofu, okara and yuba. Fermented products include tempeh, miso, soy sauces, natto and finally tofu and fermented soymilk products [9].

5.1 Okara: between Production and Consumption

Increased awareness of the health benefits associated with SB food consumption, knowledge of milk-related allergies and a move towards more sustainable food production have led to an increase in the amount of soy products available. Soymilk is one of those products, the production and consumption of which has increased in most countries and is continuously accompanied by the accumulation of the okara by-product. Current statistics on soymilk production are not widely available. In 1983, soymilk production was estimated at about 1 million tonnes [10], but it has dramatically increased in recent decades. In 2006, SB beverage production reached over 1 million tonnes in Western Europe, North America and Japan alone [11]. Traditional soymilk is produced at a SB to water ratio of 1:5, although sweetened and flavoured soy drinks are made at a seeds-water ratio of up to 1:20. However, it can be estimated that about 170,000 tonnes of okara are produced from 1 million tonnes of soymilk for dairy-type soymilk (protein content 3.5%) at a seed-to-water ratio of 1:7, and assuming that 1.2 tonnes of SB are produced per tonne of SB during soymilk production [12]. Tofu and other soy products are not included in this estimate, but they are similarly based on primary soymilk production (Figure 2). It is therefore reasonable to conclude that several million tonnes of okara are produced each year [13-15].

Figure 2. Soybean by-product production.

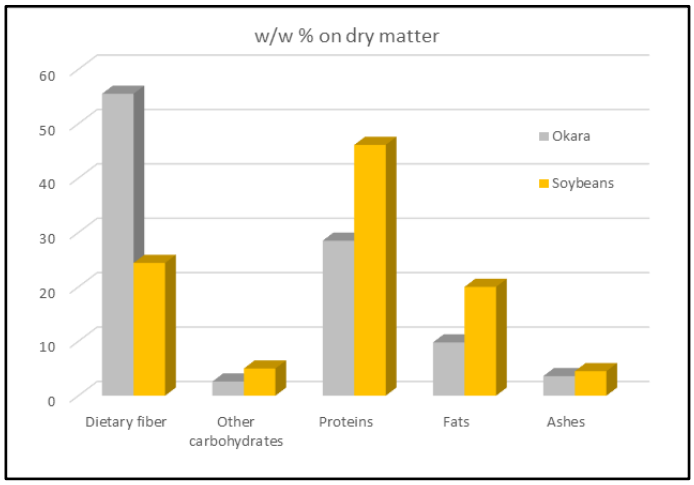


5.2 Composition of Okara, a By-Product of Soymilk Production

Soybean curd residue (SCR), also known as okara (Japan), biji (Korean) or douzha (Chinese), is a by-product of SB processing. For each kilogram of SB used to produce soymilk or tofu, about 1.1-1.2 kg of okara are formed [16]. Its main components are broken cotyledon cells and the coating of SBs. Currently, SCR is treated as waste and is either used as feed or fertiliser or is landfilled. In Japan in particular, most of this is burned, creating carbon dioxide [17].

As indicated in Table 1, okara consists of about 80% water, 3.5 to 4.0% protein and most of the insoluble SB components. Its high moisture content means that okara also contains water-soluble components. Its exact composition mainly depends on the variety of SB used, the incidence of sunshine and other environmental factors, as well as the processing conditions used during soymilk production. Therefore, the characteristics of the water-soluble fraction may vary depending on the raw material used [18]. When moisture free, okara contains near 10% fats, 55% crude fibre and 30% protein (Figure 3) [19].

Figure 3. Dry composition of okara, compared to SBs.



5.2.1 Dietary Fibre

Although okara has a high moisture content (nearly 70–80%), most of the water is linked to the dietary fibre, resulting in a pasty texture that is similar to waterlogged sawdust. Fibre, mainly insoluble fibre (in the form of cellulose and hemicellulose), accounts for most of the dry matter content (40–60%) [19] which can be fermented by microbes in the large intestine, although it cannot be digested in the small intestine. By comparison, the amount of free carbohydrates (such as arabinose, glucose, galactose, fructose, sucrose, raffinose and stachyose) is low (4–5%) and the lack of fermentable carbohydrates is the main factor limiting efficient fermentable microbial growth. In particular, it contains 1.4% stachyose and raffinose, which can cause flatulence and swelling in some individuals. The monomers that make up the polysaccharide fraction of the cell wall are mainly galacturonic acid, galactose, arabinose, glucose, xylose, fucose and a small amount of rhamnose and mannose [20] (Table 3). Their influence on the water retention and swelling capacity of okara places it is a potential source of texturizing additives [21]. Mateos-Aparicio et al. have subjected okara to high hydrostatic pressure (HHP) leading to the amount of soluble dietary fibre increasing more than 8-fold, which is useful in ensuring that okara has anti-inflammatory and anti-carcinogenic effects on the digestive tract [22].

Table 3. Composition of total fibre in okara (low molecular weight carbohydrates g/100 g dry matter).

Carbohydrates	References			
	[25]	[26]	[27]	
Rhamnose	0.85	0.3 ± 0.1	1.0 ± 0.1	
Fuchose	0.45	0.5 ± 0.1	0.1	
Arabinose	6.35	5.7 ± 0.1	-	
Xylose	5.14	2.7 ± 0.1	-	
Mannose	1.26	1.5 ± 0.3	-	
Galactose	10.83	10.4 ± 0.2	0.2	
Glucose	15.01	11.9 ± 0.4	0.2	
Sucrose	-	-	0.6 ± 0.1	

A combination of okara and soft wheat flour has been found to result in an increase in the contents of protein, dietary fibre and isoflavones, compared to the use of soft wheat alone [23]. The importance of dietary fibre is linked to the regulation of intestinal functions [24]. Their presence in okara has been associated with potential hypolipemic and hypocholesterolaemic effects [13,25], as well as hypocaloric effects, thus making it effective and useful for the improvement of metabolic syndrome [26]. The prebiotic action that it provides is also interesting [27]. Finally, research on Syrian hamsters fed with okara has suggested how its main components, dietary fibres and proteins, may be related to a reduction in total lipids and cholesterol and an increase in faecal production in animals fed a fat-rich diet [28].

5.2.2 Protein Component

Proteins make up between 15.2 and 33.4% of okara (dry matter). The main proteins are globulin 7S and globulin 11S [29]. The okara protein isolates contain all of the essential amino acids, and have a protein efficiency index that is even higher than that of soymilk (2.71 vs 2.11) and tofu, but with low water solubility [30,31] (Table 4). It has also been found that the protein fraction of SCR is able to withstand complete digestion by the gastrointestinal enzymes, pepsin and pancreatin (the latter of which mainly consists of trypsin, amylopsin and steapsin). The low molecular weight fraction (less than 1 kDa) of these digestible peptides has a more potent ability to inhibit the angiotensin-converting enzyme and shows great antioxidant activity, probably because of its high percentage of hydrophobic amino acids [32]. Trypsin

inhibitors can range from 5.2 to 14.4% of the protein content, although they can be inactivated with appropriate heat treatment [33].

The bioconversion of high molecular weight okara proteins into smaller proteins may increase the solubility of protein isolates and generate bioactive peptides or amino acids. Chan and Ma [34] have shown that the solubility of the protein fraction, when treated with acid, can increase considerably (recovery of 53% when extracted at pH 9 and 80 °C for 30 min), and thus lead to improvements in other functional characteristics, such as emulsifying and foaming properties. In addition, it has been found that maximum protein recovery (93.4%) is achieved with an okara powder fineness of less than 75 µm [35].

Table 4. Amino acid composition (mg/g of protein) of unmodified okara protein isolate.

Amino Acids	Content
Aspartic acid	117
Threonine	41
Serine	50
Glutamic acid	195
Glycine	46
Alanine	46
Cysteine + methionine	26
Valine	51
Isoleucine	51
Leucine	81
Tyrosine + phenylalanine	95
Lysine	65
Histidine	28
Arginine	75
Proline	36
Tryptophan	N.D.*

* Not determined.

Trypsin inhibitors can also be degraded by microorganisms in order to improve the nutritional quality of the residue. However, microorganisms can catabolise proteins and amino acids, reducing the amount of essential amino acids present. The various effects of fermentation on the molecular weights of peptides, the amino acid profile

and the inhibitory activity of trypsin should therefore be considered, as they can influence the overall functional characteristics (such as solubility and foaming properties) and bioactivity of fermented okara.

5.2.3 Lipid Fraction

SCR also contains a considerable amount of lipids, between 8.3 and 10.9% (dry matter). Most fatty acids are mono- or polyunsaturated and consist of linoleic acid (54.1% of total fatty acids), oleic acid (20.4%), palmitic acid (12.3%), linolenic acid (8.8%) and stearic acid (4.7%) [21].

Lipoxygenase and hydroperoxide lyase react with unsaturated fatty acids, mainly linoleic acid, during SB grinding, leading to the formation of aromatic compounds, such as hexyl and nonyl aldehydes and alcohols. These low-detection-threshold odours represent the aromas of raw soymilk. Since these enzymes are normally denatured at temperatures above 80 °C, the Chinese method of making soymilk (in which the SBs are ground before the filtrate is boiled) produces an okara in a greener manner [36]. The variant obtained using the Japanese procedure for soymilk production is therefore relatively more palatable and probably has lower trypsin-inhibitor content, meaning that it can be more easily used for cooking and processing [33]. This may explain the phenomenon by which okara is often sold as a packaged product in Japanese supermarkets, but rarely found in Chinese shops.

Fermentative microorganisms can metabolise fatty acids and their derivatives to produce more desirable aromatic compounds. Quitain et al. have investigated the recovery of okara oil components via extraction with supercritical carbon dioxide, modified with ethanol [37]. The results indicated that 63.5% oil-component recovery was obtained at a relatively low temperature of 40 °C and a pressure of 20 MPa in the presence of 10% mol EtOH. This oil component mainly consisted of fatty acids, phytosterols and traces of decadienals. EtOH proved itself to be useful in increasing the yield and amounts of phenolic compounds and the two primary soy isoflavones, genistein and daidzein, in the extracts. These compounds are known antioxidants that can increase both the stability and value of the oil, making the process attractive for the cosmetic, pharmaceutical and food industries.

5.2.4 Isoflavones

Isoflavones are present in many legumes, and SB can be considered an important source [12,38]. Wang and Murphy have shown that there are more aglycons in okara than in soymilk. Generally, the isoflavone content in SCR varies between 12% and

40% of the original isoflavone content in the beans. This leads to isoflavone concentrations in the pulp of between 0.02% to 0.12% (dry weight), depending on SB type [23]. The role of isoflavones as phytoestrogens has made them a topic that has been widely discussed in the literature, as they are attributed with important activity against hormone-derived cancers, osteoporosis and menopausal syndrome disorders [39–41].

SBs contain up to twelve different categories of isoflavones, classified into three main groups (daidzein genistein and glycitein), all of which can take four different forms: aglycones (15.4%), β-glucosides, malonyl-glucosides (28.9%) and acetyl-glucosides. Okara may contain the same twelve isoflavones, although the processing conditions during the production of soymilk and its residue may change the original isoflavonic profile [42]. Isoflavones are polyphenols with a structure that is similar to flavones [43,44]. Both flavones and isoflavones are subclasses of flavonoids, one of the largest groups of polyphenols [45,46].

The total concentrations of isoflavone and the different forms of isoflavone in SBs and their products depend on SB variety, its cultivation, the processing and storage conditions [38,47–49] (Table 5). Malonyl-glucosides and β-glucosides are the primary forms in SBs. However, these components can be transformed into aglycones and acetyl glucosides during processing, following either enzymatic conversion or thermal stress [50]. The most common chemical changes in isoflavones include the decarboxylation of malonyl to acetyl-glucosides, and the ester hydrolysis of either acetyl or malonyl-glucosides into β-glucosides. In addition, the splitting of their glucosidic bond leads to an increase in the amount of aglycones [51], which show greater bioavailability in humans [52].

Table 5. Contents of the twelve isoflavones in okara (mg per g dry weight).

Groups	Forms	Content (mg)
Aglycones	Daidzein	22
	Glycitein	1.1
	Genistein	31
β-glucosides	Daidzein	48
	Glycitein	2.2
	Genistein	53
Malonyl glucosides	Daidzein	64
	Glycitein	2.8
	Genistein	130
Acetyl glucosides	Daidzein	-

	Glycitein	3.2
	Genistein	-
Total		355

Fermentative microorganisms are also able to secrete β-glucosidase, thus bioconverting isoflavonic glucosides into aglycones, and are thus an opportunity for value addition [53].

Producers use different methods for the small-scale and large-scale production of soymilk and okara. This change in processing conditions may affect the isoflavonic profile in the resulting products. The most significant differences are in the temperatures used during SB immersion, the, either hot or cold, grinding of the SB suspension, cooking temperature and sterilisation before or after separating soymilk from its residue [54–56]. Another factor that complicates isoflavone behaviour in okara is their interactions with other matrix components, such as non-covalent interactions between polyphenols and macronutrients, mainly proteins [57,58].

The health effects of polyphenols, including a reduction in the risk of cardiovascular and cancer diseases [41], antioxidant and anti-inflammatory properties [59,60] and useful effects against type 1 and type 2 diabetes mellitus [61], have been widely proven.

The exploitation of by-products for the recovery of polyphenols has aroused particular interest with a view to contributing to more sustainable agriculture and food production [62]. In fact, by-products are often very rich in phenolic compounds, due to their presence in peels and seeds, which are often retained in the residues. Their relatively low water solubility and tendency to associate with other components may contribute to these by-products being rich in polyphenolic content. The potential applications of polyphenols are plentiful: food flavourings and colours, bioactive ingredients for health and antioxidant stabilisers.

Several conventional and unconventional technologies have been proposed for the separation of these high-value components. Conventional solid-liquid extraction commonly uses hydroalcoholic mixtures [63]. However, many other solvents, such as acetone, acetonitrile, methanol and ethyl acetate, are still widely studied in the extraction of polyphenols, due to the relatively easy solubilisation offered by these solvents and mixtures [64]. Acid, alkaline and sub- or supercritical fluid extraction are common alternatives. Modern technologies, such as pulsed electric fields, ultrasound-assisted extraction and microwave-assisted extraction, have been suggested as means to increase yields and overcome some difficulties in polyphenol

extraction. Examples of possible obstructions include kinetic limitations in cell matrix extractions, component instability and solvent residues in the final product.

Soy molasses, a by-product of soy-protein concentrate preparation, is a popular starting material for the production of isoflavone. Being an alcoholic extract of soy flakes, it contains isoflavones in a slightly more concentrated form. However, many patented processes use SBs or SB meal as a starting material when the recovery of isoflavones from side products, such as okara, would require less valuable resources.

5.2.4 Nutritional and Anti-Nutritional Elements

Soyasaponins are a group of non-volatile amphiphilic molecules that are present in a wide variety of legume seeds, such as SBs, peas, lentils and lupins [65]. Soy-based products are the main food sources of soyasaponins [66]. These are mainly contained in the cells of SB cotyledons and released into the okara after processing. It was reported by Gurfinkel and Rao, in 2003, that they possess immunostimulating, antiviral, hepatoprotective and chemopreventive properties [67].

Other components include minerals, lignans, coumestans (the latter two belonging to the category of phytoestrogens, which are inhibitors of enzymes involved in the metabolism and biosynthesis of estrogens, such as aromatase, 17-β-hydroxysteroid reductase, sulfatase and sulfotransferase), phytosterols and phytates (responsible for the chelation of calcium and iron in the intestine).

Anti-nutritional factors present in okara, such as phytates, saponins and trypsin inhibitors, limit its use in animal feed [68]. Fermentative microorganisms can metabolise these and other factors, such as allergenic proteins (containing glycine and β-conglycine), by providing a final fermented product of improved nutrition and digestibility [69].

Okara also contains a variety of minerals, such as potassium, calcium and iron [21,70], whose consumption can improve human health (see Table 1). For example, potassium reduces systolic blood pressure [71], copper promotes antioxidant defence and immune function [72], and magnesium mitigates hypertension problems and helps prevent diabetes complications. The absence of iron has been associated with fatigue and an increased risk of cardiovascular/thromboembolic events [73], while calcium prevents osteoporosis and fractures in adulthood and old age [74].

4.3 Production and Use of Okara

Soymilk can be made from whole SB or fat soy flour. Its production usually consists of five main stages:

1) SB washing to remove impurities
2) SB soaking/hydration for 12 h at 25 °C, then draining and rinsing with water
3) cooking at 98 °C for 5 min, with the aim of both sterilising and improving aroma and nutritional value via the inactivation of trypsin inhibitors
4) grinding in a blender with distilled water (1:10 ratio *w*/*v* SBs/water) for the preparation of a slurry
5) separation of the slurry into soymilk (water-soluble SB extract) and okara by mechanical means (usually filtration) [10,15,75].

The moisture content primarily depends on the soymilk-okara separation method. Wet okara, due to its high moisture content, deteriorates very quickly, making its use difficult. Every element in soymilk processing is fundamental. In the Japanese manufacturing method, whole soaked SBs are first cooked and then ground and filtered. In the Chinese method, raw SBs are first ground and then extracted with water, filtered and finally heated [76].

Two main approaches to the use of okara in foodstuffs have been described in the literature. In the first, whole SCR is used as a component in finished products. In the second approach, several constituents are isolated from it and then used as ingredients.

The inclusion of okara as a complete ingredient in foodstuffs often requires the drying of the fresh residue. Okara is a difficult material to dry because of its high tendency to agglomerate, leading to spattering phenomena, and the low energy efficiency of the drying process [77–79]. In the dry state, it is used to improve the consistency of a product, since it has a good retention capacity for water and oil, and to enrich foods with nutritional components, such as fibre and proteins. Examples of products that contain whole SCR are: a soy-based snack [80], a cheese bread [81] and a French bread [82].

The addition of wet okara to food products is being considered even less than previously by producers. Its direct incorporation into feed or food is possible, but it is limited by the presence of enzyme inhibitors, regardless of whether SBs have been heat treated before grinding [22,69,83]. Turhan et al. and Su et al. have described its use in the production of low-fat beef burgers [84,85] and to reduce the fat content in a coconut-based baked snack [86]. Furthermore, in 2000 Rinaldi et al. have introduced freshwater products that are enriched with okara [23].

As regards the use of SCR ingredients, more attention has been paid to the polysaccharide fraction. Its polysaccharides have been characterised in detail by several authors, as described in Section 2.1. Okara fibres, which are mainly insoluble,

have a high water- and oil-holding capacity, and also swell in water. The indigestible fraction shows good suitability for fermentation by *bifidobacteria*, indicating that it may potentially be used as a prebiotic ingredient [87]. To increase fibre solubility (from 38.1% to 64.8%), okara is either treated with enzymes and a high-pressure process (HPP) [22] or by extrusion [13,88].

Regarding the protein fraction of okara, past research has focused on methods of extraction and modification that can make it suitable as an ingredient [30,31,34], while recent literature has reported detailed characterisation and composition [33,89]. In addition, Vishwanathan et al. have investigated the extractability of proteins in SCR and the production of a protein concentrate by membrane separation [90]. A study on peptide preparation via protease hydrolysis has reported that the obtained products showed antioxidant activity [91].

Finally, okara has been studied for its potential ability to provide natural antioxidants. Methanol, acetone and water have been used to extract polyphenols and oligosaccharides, which are precious components with antioxidant and prebiotic properties [39,92–94].

5.4 Application of Okara in Functional Foods

The chemical and nutritional qualities of SB, which are, in particular, high protein content, a fatty composition that is rich in unsaturated fatty acids (for example, linoleic acid), and high isoflavones contents, have, over the years, aroused great interest from the food industry. Specifically, the incorporation of soy-derived ingredients into a variety of products with the aim of providing beneficial properties to the body has been the focus. These are called ‘functional foods’ [95]. In Western countries, for example, water-soluble SB extract (soy drink or soymilk), one of the main products, has been used as an important alternative for people who are intolerant to lactose or allergic to cow’s milk proteins [96]. Furthermore, other traditional soy-based foods, such as tofu, miso, tempeh and soy sauces, should be mentioned in the same context.

5.4.1 Production of Food for Human Consumption

Even okara has been used for many years for food purposes in China and Japan, both in its raw and processed forms, to more easily provide a fair intake of fibre and protein. SCR has solvent-binding properties, making it an ideal low-cost ingredient with which to increase yields in meat products [18]. It has also been shown to have a positive effect on the shelf life of chocolate cookies at an optimal concentration of

5%, as well as preventing syneresis in cheese ravioli filling during freezing and defrosting. At the same time, its insipid taste allows it to be used at relatively high levels without adversely affecting the taste or texture profiles of meat and bakery products.

Okara can partially replace wheat flour, SB flour and other ingredients in food production to increase fibre and protein contents. Several studies have indicated its usefulness in the production of bread, pancakes, puff pastries, pasta, candies, drinks, sausages and nutritional flour.

In a study by Wickramarathna et al., bread that was made by replacing wheat flour with 10% okara powder had almost the same sensory qualities and physical-chemical characteristics as normal bread [97]. A significant difference in crust colour was found in the control (white) and the 10% SCR-enriched bread (red), which had a caloric value that was higher (15.9 kJ/g) than the control value (14.4 kJ/g), due to the higher protein and fat content in the okara.

Suda et al. have produced pancakes and bread with the addition of okara powder (50.4% dietary fibre, 21.3% vegetable protein and 0.45% calcium) to the food ingredients, with the aim of developing fortified foods for medical use [98]. Three different types of bread were processed, including the 10% SCR bread, and other additives and preservatives were needed to promote yeast fermentation and allow storage at room temperature. After freezing without preservatives, the flavours of the three breads were modified. A soft pancake with 20% okara was prepared using a mixture of pancake powders: the preservative-free pancake was suitable for storage in the refrigerator before being eaten, while both the fresh pancakes and those containing the preservatives made with 20% okara were acceptable as supplements for the hospitalised elderly. The study showed that the soft pancake that was based on SB pulp and contained 40% water was more useful as a convenient supplement for dietary fibre, vegetable protein and calcium than the okara breads.

The quality of bread produced with the addition of okara is significantly increased by adding enzymes (pentosanase, lipase and glucose oxidase). The increase in powder substitution from 4% to 8% has also been found to lead to a quality increase [99]. Bread with 5% fresh SB pulp fibre, treated with 1% NaOH for 1 h and 1% HCl at 60 °C for 2 h, had a quality and appearance that was very similar to those of normal bread [100]. Okara can also replace part of the wheat flour needed to make cookies and cakes [101–104].

Blown food is produced by subjecting cereals, potatoes or SBs to preheating at 300–400 °C in an autoclave at high pressure with steam (15 bar). Using okara and starch

as raw materials, blown soy-food can be processed via extrusion and frying. The fresh residue was first immersed in water at pH 3–5 and 80–100 °C for 2 h. Subsequently, after neutralisation, filtration, drying and crushing, the okara powder was mixed with starch, water, 2% salt and 1% spices. The optimal conditions for the extrusion were: a temperature of 160 °C, and the presence of 30% water and 40% starch. The extruded food was fried at 180 °C for 40 s. The processed product had a crisp texture and an attractive taste, and was devoid of the typical okara taste, which is reminiscent of broad beans [105]. 54% starch, 8% SCR mixed with sweet potato residue (6:4 ratio), 31% water and 7% additional materials were used to give a high-quality product via frying at 160–165°C for 3.5 min after extrusion [106]. Finally Yu (2001) has proposed the use of 100 g okara, 500 g potatoes, 150 g wheat flour and 20 g powdered milk as raw materials, which, after fermentation with yeast at 28 °C for 1 h, drying at 60 °C for 1–2 h and frying at 180 °C for 1 min, afforded an okara-fibre-based food [107].

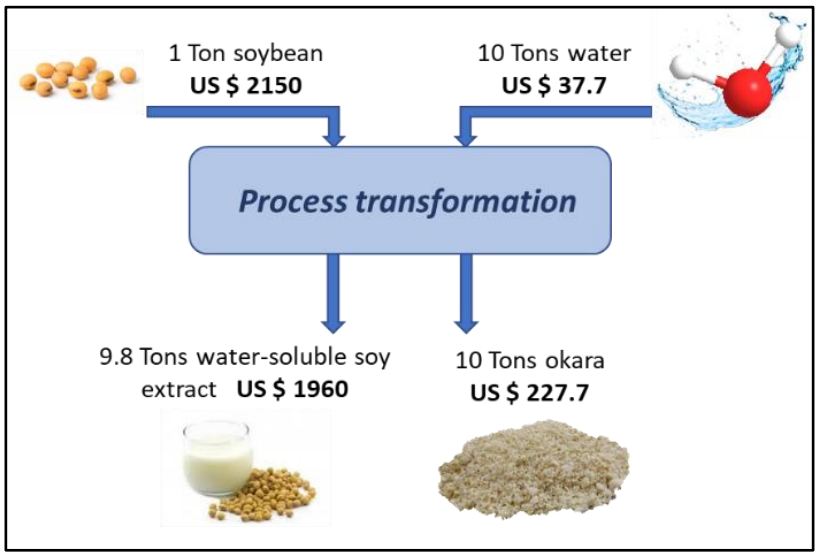
Dried noodles that contain dietary fibre and have a lower glycaemic index can also be prepared using SB pulp [108]. The noodles were of good cooking quality when okara was added at 9% (with a particle size of 100 mesh), 0.25% sodium alginate and 4% salt [109]. Bedani et al. have increased the nutritional and functional properties of soy yoghurt with the addition of okara [110], while Waliszewski et al. have added it to corn tortillas to improve their amino acid profile [111]. In Argentina, SCR-containing candy (nougat) has been tested as well as peanuts, glucose, hydrogenated oil, sugar and natural essences with the aim of increasing the supply of available vegetal proteins [112]. In addition, it may also be possible to produce steamed bread [113], a healthy drink [114], sausage [115] and sliced vegetables [116].

On the other hand, the development of a composite okara powder is an ideal way to use SCR. Xie and Li have invented an enriched nutritional flour that is suitable for diabetics; fresh okara was dried and ground into a powder (80–200 mesh), then mixed (5–25%) with either wheat flour, wheat gluten or buckwheat flour to improve food-processing properties [117]. This composite flour can partially replace wheat flour in the daily preparation of foods, such as bread, steamed bread and pasta for diabetics. Rotem and Almog have developed a premixed powder that contained protein-rich SCR for health-food applications; ground and dried okara (70 mesh) was mixed with gluten, in proportions of between 3:1 and 12:1 [118]. The product contained soy proteins, at between 10% and 30%, and total proteins were between 15% and 50%.

5.4.2 A Study on Paste Production

Even SCR, once dried, is an excellent alternative for incorporation into food products such as pâté, process adjuvant or food fortification [119]. An economic analysis of the potential exploitation of okara for food enrichment has been carried out. 1 kg of SB and 10 L of water are used in the production process of soymilk, resulting in 1.2 kg of okara (Figure 4). Therefore, the okara generated by the process corresponds to 10.9% of the raw material used and is usually discarded without generating financial resources. An estimation of the total cost of processing SBs to produce soymilk means that 227.7 US $ per tonne of raw material is discarded during the generation of okara. The feasibility of incorporating okara into food is therefore a solution for waste, whose disposal generates costs and environmental impact.

Figure 4. Costs (US $/Ton) of the production processes of water-soluble SB extract (soymilk) and okara (data from Guimarães et al.) [120].



Vegetable paste is a homogeneous mixture of vegetables to which various aromas are added to improve sensory properties. Once packaged and subjected to a suitable thermal process (4 °C/48 h), it is eaten cold. Guimarães et al. have prepared and analysed 3 formulations with different SCR contents (F1 at 33.87, F2 at 43.45, F3 at 50.60 *w*/*w* percentage, respectively), in which carrots and mayonnaise were liquefied to produce a homogeneous mass and to which, subsequently, SB pulp, spices and lemon juice were added in appropriate quantities to give a product with good acceptability and nutritional quality. The increase in the okara fraction in the

formulations led to an increase in the total dietary fibre, protein and lipid contents, as well as the energy of the samples (Table 6). The calcium, potassium and total isoflavone contents also increased with the incorporation of greater amount of okara in the formulations (from F1 to F3).

Table 6. Comparison of the chemical composition of okara and okara-based paste (100 g portion).

Nutrients	Okara	Formulations		
		F1	F2	F3
Humidity	80.25 ± 0.04	81.26 ± 0.04	81.42 ± 0.05	80.77 ± 0.06
Protein (g)	7.91 ± 0.25	3.07 ± 0.70	4.00 ± 0.26	4.72 ± 0.19
Lipids (g)	6.22 ± 0.45	5.62 ± 0.86	6.20 ± 0.09	7.62 ± 0.46
Ash (g)	0.86 ± 0.00	1.98 ± 0.00	1.76 ± 0.01	1.72 ± 0.00
Total fibre (g)	13.83 ± 0.49	5.79 ± 0.17	7.17 ± 0.22	8.00 ± 0.25
Soluble fibre (g)	3.25 ± 0.09	1.67 ± 0.03	1.99 ± 0.04	2.19 ± 0.05
Insoluble fibre (g)	10.58 ± 0.40	4.13c ± 0.14	5.18 ± 0.18	5.82 ± 0.20
Carbohydrates (g)	2.44 ± 1.29	5.8 ± 0.49	4.61 ± 0.32	3.50 ± 1.37
Energy (kcal)	100.17	89.65	94.20	105.81

The formulation of the vegetable paste enriched with 33.9% okara (F1) has a low energy value (89.65 kcal/100 g) and lipid percentage (5.6%), but high protein (3.1%), β-carotene (0.411 mg/100 mL), dietary fibre (5.8%) and isoflavone (0.15 μmol/g fresh matter) contents, and demonstrated high sensory acceptability. In conclusion, the use of okara in the development of a vegetable paste is an interesting and feasible alternative means to increase the nutritional value of a product and to effectively exploit the residue generated by the production of soymilk.

5.4.3 Fibre Recovery and Enhancement

The dietary fibre market is highly competitive because of its beneficial effects in reducing the risk of cancer and coronary heart disease (Larrauri, 1999) [121]. Due to its high fibre content, low costs and the abundant volumes produced, SCR is an excellent raw material from which to recover dietary fibre.

A great deal of research has been published on the supply of okara fibre. In one of these studies, dietary fibre was prepared from wet okara via the following steps: drying, porphyrisation, soaking with alkaline solution, enzymatic hydrolysis, bleaching, precipitation with ethanol and drying. The conditions for alkaline soaking

were 4% NaOH at 80 °C for 80 min. The enzymatic hydrolysis of trypsin was performed for 30 min to remove the protein content. Whitening was performed with 3% H_2O_2 at 50 °C for 60 min. After processing, the okara fibre was composed of 41% insoluble fibre, 15% soluble fibre, 6% lipids, 1% protein, 2% ash and 8% moisture. The water-retention capacity and hydration capacity were 10.08 g H_2O/g of product and 18.66 mL H_2O/g of product, respectively [66]. Li et al. have reported that optimal bleaching was performed with 3 g of H_2O_2/100 g of dry okara at pH 10 and 80 °C for 3.5 h [122]. After bleaching, the whiteness of the fibre residue reached 88%, the water-retention capacity increased 1.7-fold and the expansive capacity improved 1.9-fold. The following treatments have been tested to improve soluble fibre yields from okara: extrusion [123,124], high pressure [22], chemical and enzymatic [125–127] and fermentation by microorganisms [128].

5.4.4 Applications in Animal Nutrition

SCR contains a high content of proteins and non-fibrous carbohydrates, making it attractive for use with dairy cattle and goats [129]. It is also much cheaper than SB meal and can therefore be used as feed for cattle, pigs, sheep, fish [130] and poultry [131] with the aim of replacing part of their normal feed.

When half of SB meal was replaced with okara to feed dairy cattle and yellow cattle for 30 days, there were no significant differences in milk production, milk-fat content, feed consumption and daily profit (for yellow cattle) between the groups fed on milk and those fed on SB meal. The feeding cost of the okara-fed group was significantly lower than that of the SB-fed group [133,134].

Meeting the high protein requirements of young pigs is a challenge for organic pig producers: the price of organic feed is up to 4 times higher, and has limited availability. Okara is a potential source of alternative organic proteins and its intake in up to 25% of young pigs' diets had no effect on average daily profit, average daily food intake and gain/feed ratio compared to the control [135].

Many food by-products have a high moisture content and, in order to avoid the high energy costs of drying, are therefore often stored via silage, a forage storage technique that consists of acidifying the plant mass using anaerobic microorganisms, with the aim of preventing other microorganisms from colonising it resulting in the loss of its nutritional value. Therefore, a typical practice in Japan is to mix wet by-products with dry feed to prepare low moisture total mixed ration (TMR) silage. The combination of okara and peanut shells can therefore have a synergistic effect on the silage mix to achieve the right dry matter and fermentable carbohydrates for optimal

silage fermentation. Silage with an okara/peanut waste ratio of 78:22 has been found to reduce fibre content and lignification, and improve the efficiency of both silage fermentation and *in-vitro* ruminal fermentation models after 8 weeks [129].

SCR can also be used to produce microbial proteins, which are synthesised by solid state fermentation. During fermentation, the mould degrades the okara fibre into low molecular weight carbohydrates, which are further used by yeasts to synthesise proteins. In addition, some anti-nutritional factors in okara (such as trypsin inhibitors, saponin and lectin) can be decomposed or reduced via fermentation [135]. After solid fermentation for 3 days by a mixed bacterial culture using *Aspergillus oryzae*, *Aspergillus niger* and *Saccharomyces cerevisiae* yeasts, the crude protein content of okara increased by 43.1% compared to the original [136]. It has also been found that crude protein content doubled, compared to the original material, when using okara and wheat bran (ratio 8:2) as a substrate, and *Aspergillus niger*, *Trichoderma viride*, *Saccharomyces cerevisiae* and *Candida utilis* (ratio 1:1:1:3) as a mixed crop, after fermentation at 32 °C for 3 days [137].

5.4.5 Application in Ecological Materials

It has recently been discovered that sedimentation materials, such as okara, wheat bran, rapeseed and flaxseed, can effectively absorb organochlorine compounds. When these were applied to wastewater (pH 10) that contained 0.1 g/L of dichloromethane, this organic solvent was removed from the wastewater with an efficiency of 70–90% after 90 min. High correlation was found between the removal efficiency and the number of spherosomes, which are intracellular particles used to absorb organochlorine compounds [138]. It has also been observed that okara (10 g/L) can absorb 96% Cd^{2+} and 89% Zn^{2+} from water that contained either 50 mg/L Cd^{2+} (pH 6) or Zn^{2+} (pH 7). The maximum absorption quantity was 19.61 mg of Cd^{2+} and 11.11 mg of Zn^{2+} per g of okara, respectively [139]. The use of okara as an adsorbent is therefore an efficient and economical method for the removal of organochlorine compounds and heavy metal ions from wastewater.

Okara is a good material for the production of edible packaging and biodegradable materials. Zhang et al. have separated the fibre from okara using protease and lipase treatments [140]. Edible wrapping paper was then produced by mixing the fibrous material with Chinese sweet potato, dextrin, sucrose and carrageenan, according to the conventional paper-processing method. The stress resistance, flammability and dissolution rates of edible paper were 11.6 × 105 Pa, 138 g/cm^2 and 7.97 mg/s, respectively. Edible paper had a similar softness to conventional paper, but higher

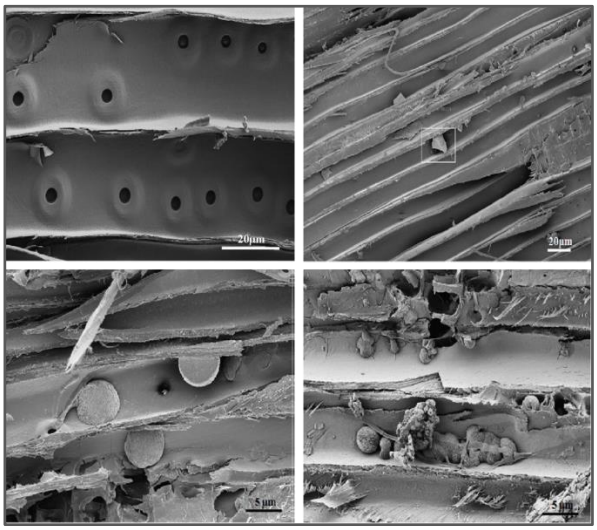
strength and brittleness. Moreover, its water absorption and solubility were higher than that of normal wrapping paper.

In another study, Wen and Liu have prepared an edible film by mixing okara fibre with 2% dextrin and 1.5% glycerol, throwing the mixture on a glass plate and drying it to remove water [141]. Li et al., however, etherified the residue with epichlorohydrin and prepared degradable composites using modified okara and polyvinyl alcohol, with the addition of $CaCO_3$ [142]. The water-absorption capacity of composite materials decreased as the degree of etherification increased. Composite materials degraded in soil, with a weight-loss rate of more than 70% after 60 days. Chen has prepared a biodegradable plastic using corn gluten flour and okara. The corn gluten flour was first mixed with okara and the mixtures were extruded into pellets using a twin-screw extruder. The pellets were then extruded again as a final preparation [143]. When 20% okara was mixed in, the sample showed the maximum tensile strength and maintained thermal stability of less than 240 °C. The plastic can be used in agriculture thanks to its low cost and biodegradability.

Finally, okara has a potential application in the ecological preservation of wood, and can be useful as a replacement for copper azole (CuAz) and quaternary ammonium copper salt (ACQ), which are widely used as replacements for copper arsenate chromate (CCA). Enzymatic-hydrolysed okara wood preservatives showed good stability against hot-water leaching.

The leached wood blocks treated with formulations of okara/$CuCl_2$ (OK/CC, Figure 5 bottom-left) and okara/$CuCl_2$/$Na_2B_4O_7$ (in the presence of NH_4OH, used as a dissociating agent) (OK/CC/B, Figure 5 bottom-right) were highly resistant to fungal decay, against both *Postia placenta* and *Gloeophyllum trabeum*, compared to the control and $CuCl_2$-treated wood blocks (CC, Figure 5 top-right) especially when okara was hydrolysed by cellulase (Celluclast® with an enzyme loading of 0.1 mL/g) [14].

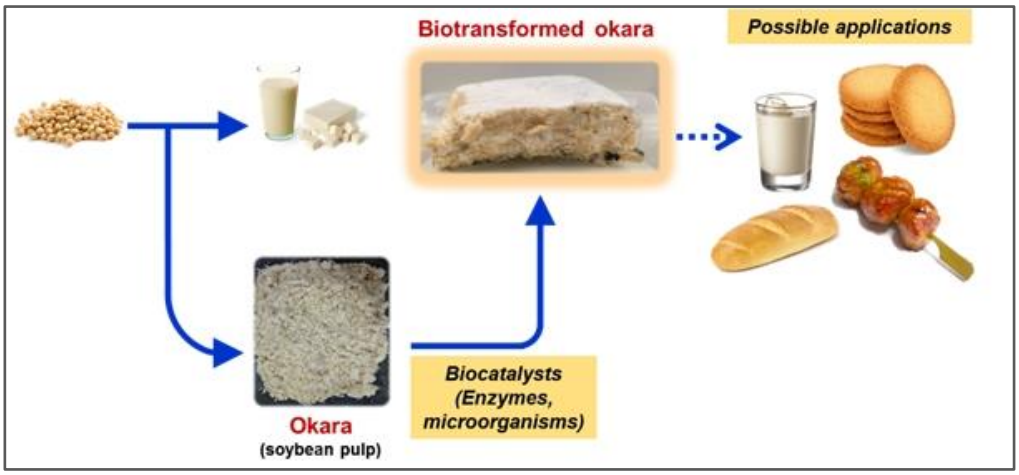
Figure 5. Scanning electron microscopic (SEM) images of control wood block (top-left), wood blocks treated with CC formulations (top-right), leached wood blocks treated with OK/CC (bottom-left) and OK/CC/B (bottom-right) formulations with permission from [15].



5.5 Biovalorisation through Fermentation

Okara is rich in carbohydrates, proteins and other nutrients, making it a potentially useful substrate for microbial fermentation. Fermentation can reduce the content of raw fibre, increase the content of soluble fibre, proteins, amino acids and isoflavones, as well as decomposing phytic acid (which is a deposit of phosphorus in the seeds that is indigestible to humans and able to chelate other nutrients, while in ruminants it is lysed by bacterial flora), leading to improvements in the nutritional value and processing properties of okara. Fungi, bacteria and yeasts are therefore very important for the production of functional ingredients and food products (Figure 6) [144].

Figure 6. Flowchart illustrating process flow from SB to biotransformed okara, with potential applications in food products (Image credit: Vong Weng Chan) [145].



5.5.1 Fungal Fermentation

SB pulp is suitable for fungal fermentation as it provides a physical surface for the adhesion and growth of fungi. Filamentous fungi excrete cellulolytic enzymes, including endoglucanase, esoglucanase and β-glucosidase, breaking down lignocellulosic biomass and thus promoting better digestibility.

5.5.*2 Production of Bioactive Compounds*

The fungal fermentation of okara has been studied for the extraction of bioactive substances. Fujita, Funako and Hayashi have fermented okara with *Aspergillus* sp. HK-388, a strain isolated from soil samples in Osaka (Japan) [146]. The bioactive compound that was isolated in the methanolic extracts was 8-hydroxydaidzein. 8-Hydroxydaidzein can inhibit aldose reductase and tyrosinase, indicating that it has potential pharmaceutical and cosmetic applications. Since it was not detected in unfermented okara, the authors assumed that it was a product of the biotransformation of daidzin and daidzein, both of which are present in the starting material. In another study [146], okara was fermented with *Monascus purpureus* IFRPD 4046, a red purple mould, with the aim of producing monacolin K, a hypolipidemic agent that has been approved in Europe [148,149]. The yield of monacolin K was 192 mg/kg okara, which is about 2.5 times lower than that obtained using rice as a substrate. A carbohydrate supplement may therefore be needed to improve the production of monacolin K from SCR.

Okara was also used as a substrate for the cultivation of edible mushrooms [150–152]. In these studies, a selected fungus was grown on okara and the fungal polysaccharide was then extracted from the fermented biomass. The results were then compared with those of the unfermented SCR extracts. Fermented okara extracts showed increased antioxidant capacity in vitro and, where *in-vivo* tests were performed, improved immunomodulatory activity [153,154]. It has also been shown that ultrasonic-assisted extraction improves the yield of fungal polysaccharides compared to hot water extraction. Li et al. (2016) suggested that fungal growth may degrade okara fibres to produce low molecular weight oligosaccharides and, at the same time, release antioxidant peptides from proteins [150]. This may lead to a synergistic effect between fungal polysaccharides and bioactive okara components in the extract.

5.5.3 *Production of Food Fermented by Fungi*

SCR may be fermented by fungi to produce food for direct consumption. Meitauza, which is produced mainly in the province of Hubei, China, is an indigenous food that is based on fermented okara. Conventionally, steamed okara is shaped into blocks, cooled and covered with rice straws at a temperature below 20 °C for about 8–14 days to obtain natural fermentation [83]. *Actinomucor elegans* and *Zymomonas mobilis* are two microorganisms that are isolated from meitauza. In a study by Xu, Liu et al., pure crops of these microbes were inoculated to produce meitauza [155]. The final product showed a reduction in moisture content and crude protein, the degradation of okara dietary fibre and a significant increase in free amino acids. These compositional changes have led to a pleasant and delicate taste, as well as a smooth and rubbery texture.

Okara can also be used to prepare tempeh, a traditional Indonesian food, which is normally produced from whole SBs fermented by *Rhizopus oligosporus*. Okara-tempeh has been used as a dog snack in a study by Yogo et al. [156]. Significant increases in the concentrations of short-chain fatty acids and the levels of *Bifidobacterium* and *Bacillus* were observed after 1 week. The authors attributed this effect to SB oligosaccharides, such as raffinose and stachyose, which have prebiotic potential and can stimulate the growth of these bacterial species.

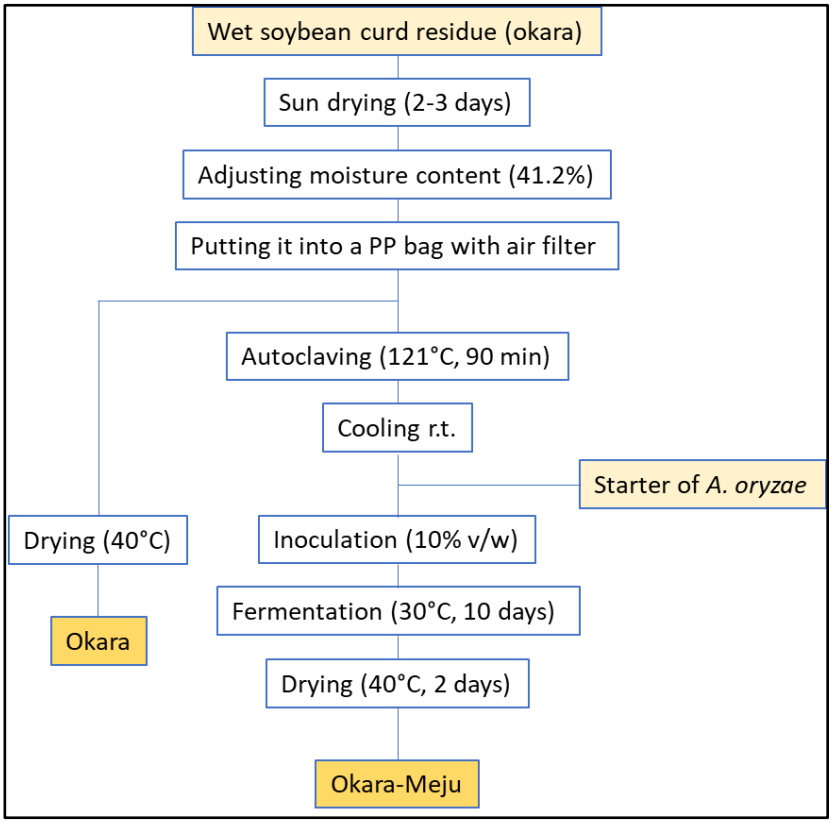
In Asia, SBs are typically fermented with *Aspergillus oryzae* as the first step in the preparation of several SB-based seasonings. SB biomass that is fermented with mould is known as koji. By replacing SBs with okara, okara-koji has been prepared, which, once dried, was added to biscuits and cupcakes [157]. The consistency and

palatability of the resulting baked products, with up to 10% substitution in biscuits and 5% in cakes, were not affected. This pastry also showed reduced lipid oxidation and starch retrogradation during storage. Dried okara-koji was then used as a flour substitute to extend the shelf life of high-fat bakery products.

The similarity between the texture of okara and fermented SB paste (i.e., miso) led to a series of studies, by Matsuo and Takeuchi, on the health benefits and palatability of an okara-miso-based condiment [158,159]. The okara-miso was prepared in several steps: okara-oncom and soy-oncom were prepared for inoculation with intermediate *Neurospora*. A mixture of 10% okara-oncom and 90% soy-oncom was then fermented with a commercial starter, *A. oryzae* [160]. Compared to miso produced entirely from steamed SBs, okara-miso showed greater antioxidant activity and greater anti-mutagenicity. In-vivo studies have also shown that rats fed with okara-miso had significantly higher hepatic catalase activity, significantly lower serum cholesterol and lower liver thiobarbituric acid reactive substance values than rats fed with normal miso [161]. It has been suggested that the antioxidant effect is due to the higher amounts of isoflavonic aglycones present in the okara-miso. The digestible aglycones and proteins are therefore able to facilitate the decomposition and removal of cholesterol, with the consequent effect of lowering it. The organoleptic properties of foods prepared with okara-miso have been defined as "acceptable". This is probably due to the addition of higher quantities of aspartate and glutamate to give a characteristic umami flavour and possibly mask the bitterness of the aglycones.

Meju is a traditional Korean SB cake that has been fermented and dried naturally, using *A. oryzae* [162]. Lee et al., following the principles of meju production, replaced SBs with okara (Ok) to obtain okara-meju (Ok-Me) [163]. Its dietary effects on body weight, blood lipids and antioxidant activity in mice that were fed on a high-fat diet (HC) were then examined. After an 8-week treatment, the body-weight gains of the Ok and Ok-Me groups were significantly lower than that of HC one, with the Ok-Me group value being lower than that of the Ok group. Furthermore, the serum TG, total-cholesterol and LDL-cholesterol contents of Ok and Ok-Me groups were lower than those of HC (Figure 7).

Figure 7. Procedure for the preparation of fermented SCR (okara-meju).



These studies highlight the additional health benefits that fermentation can confer to okara, helping to prevent obesity and improve lipid profiles.

5.5.4 Bacterial Fermentation

Most studies on the bacterial fermentation of okara involve the *Bacillus* species, probably because of its ability to produce extracellular alkaline proteases and the fact that it is commonly found in many fermented soy products [164].

5.5.5 Production of Bioactive Compounds

Okara that is fermented with *Bacillus subtilis* has been shown to possess increased antioxidant activity *in vitro*. Proteinases produced by *B. subtilis* are able to hydrolyse soy proteins. It has also been proposed that the bioactivity of *Bacillus*-fermented okara may involve contributions from γ-polyglutamic acid [165], bioactive peptides [166] and the fibrinolytic enzyme nattokinase [167,168]. The optimisation of the

fermentation and extraction of these bioactive components from fermented okara can provide greater insight into new potential therapeutic applications.

The inhibition of α-glucosidase from fermented okara is another interesting area of research. α-Glucosidase catalyses the breakdown of starch and disaccharides into glucose, meaning that its inhibition can reduce the absorption of food carbohydrates. α-Glucosidase inhibitors are one of the crucial therapeutic agents for hyperglycaemia-related diseases, such as diabetes and obesity. Zhu et al. have examined the inhibition of α-glucosidase by some microorganisms that are used to produce fermented okara [169]. *B. subtilis* B2 showed more than 90% inhibitory activity at a concentration of 0.625 mg/mL (methanol extract) and 0.313 mg/mL (aqueous extract), as opposed to the very low inhibitory activity observed in unfermented okara extracts. Subsequently, Zhu et al. purified and identified 1-deoxyrimycin (DNJ) as the α-glucosidase inhibitor in a fermented okara suspension in 2010 [170]. 1-DNJ, a naturally occurring nitro-derivative of sugar, and its derivatives are potential therapeutic agents in the management of diabetes, HIV infection and Gaucher's disease [171]. Based on the maximum inhibitory activity against α-glucosidase, the optimal yield of 1-DNJ in an aqueous mixture containing fresh okara at 4.5% (*w*/*v*) was 0.74 mg/g fermentation broth (dry basis). The industrial synthesis of 1-DNJ is generally based on a combined chemical-biotechnological approach [172], and the possible use of okara as an economical raw material for the production of 1-DNJ for food use is extremely interesting.

9-*Cis*-11-*trans* conjugated linoleic acid is another noteworthy bioactive compound that is obtained from the fermentation of SCR. In 2010, Vahvaselka and Laakso were the first to exploit the linoleic acid fraction of okara by hydrolysing it with lipolytic oatmeal for 3 weeks, then fermenting it with *Propionibacterium freudenreichii ssp. Shermanii* in an aqueous okara suspension at 5% *w*/*w* [173].

5.5.6 Using Dried Okara as A Prebiotic

When consumed, prebiotics selectively stimulate the growth and/or activity of certain intestinal microbes that can confer health benefits to the host [174]. The use of okara as a prebiotic has been investigated in in-vitro studies using *Bifidobacterium bifidum* and *Lactobacillus acidophilus* [87,175]. Okara provided a surface for bacteria-cell adhesion, thus facilitating substrate absorption and cell growth. Treatment with β-glucanase (Ultraflo L®) increased the content of okara-soluble dietary fibres and subsequently increased fermentation by *B. bifidum* [176]. The conversion of the insoluble dietary fibres of okara into soluble fibres was also

observed when *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subspecies *bulgaricus* were used [177].

Several researchers have studied the dietary effects of SB yoghurt, produced via the lactic fermentation of soymilk and okara, on lipid and cholesterol blood levels in rats [178]. This yoghurt was prepared by mixing soymilk and dried okara powder, in a 2:1 ratio, and then fermenting the mixture with *L. delbrueckii* subspecies *delbrueckii*. The final product was then freeze-dried and incorporated into rat diets. Regardless of their diet, rats fed with this SB yoghurt had a significantly lower level of total plasmatic cholesterol than the control group and groups fed only with a standard soymilk yoghurt or with a mixture of unfermented soymilk and okara. This suggests that the use of okara provided additional benefits to soymilk, as the fibre-rich okara facilitated the excretion of bile acids, through the adsorption on faecal matter, thereby improving the hypocholesterolaemic effect [178,179]. Moreover, fermentation played a key role in the cholesterol-lowering effect as it caused the production of bioactive peptides via the enzymatic hydrolysis of soy proteins. Results of DNA micro-array analyses also showed that the consumption of this SB yoghurt reduced lipid and cholesterol synthesis and stimulated the β-oxidation of fatty acids and cholesterol disintegration.

The organoleptic qualities and structural profiles of SB yoghurt were then evaluated. Soymilk with either dried okara alone or inulin (a carbohydrate polymer indigestible to humans, classified as soluble fibre) and dried okara were fermented with a starter culture of yogurt that contained *L. acidophilus*, *Bifidobacterium animalis* subsp. *lactis* and *S. thermophilus* [110]. These yoghurts showed significantly higher physical stability, but a low pleasantness index, probably due to the relatively large size of the dried okara particles. Nevertheless, the addition of inulin seems to increase palatability.

5.5.7 Fermentation Using Yeasts

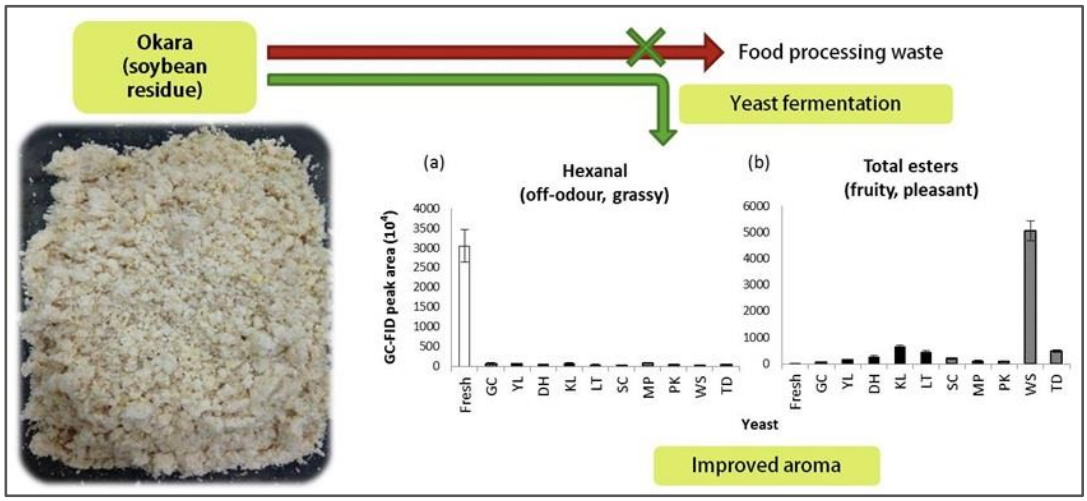
The strong metabolic activity and diversity of yeasts mean that they offer great potential for the biotransformation of SCR. In fact, the use of okara fermentation by yeasts has so far been concentrated mainly on final consumer products, with the aim of providing better nutritional or aromatic effects.

In 2011, Rashad et al. studied the feasibility of producing a yeast-fermented okara food and paid particular attention to improving nutritional quality [180]. A mixture of yeasts, *Candida albicans*, *Candida guilliermondii*, *Kluyveromyces marxianus* NRRL Y-7571 and NRRL Y-8281, *Pichia pinus* and *Saccharomyces cerevisiae*, was

inoculated in the okara to evaluate solid state fermentation (SSF). Yeast fermentation generally increased the amount of protein and ash and reduced the content of fibre, carbohydrates and crude lipids. The changes were caused by the metabolic activities and action of extracellular yeast enzymes. The in-vitro antioxidant activities of the fermented product were also about 1.5–2 times higher than those of the control.

Okara was also individually fermented with ten different yeasts in an SSF, and changes in the aroma profile were then evaluated [181]. The yeasts used were typically associated with dairy products (*Geotrichum candidum*, *Yarrowia lipolytica*, *Debaryomyces hansenii*, *Kluyveromyces lactis*) and wines (*S. cerevisiae*, *Lanchancea thermotolerans*, *Metschnikowia pulcherrima*, *Pichia kluyveri*, *Torulaspora delbreuckii*, *Williopsis saturnus*), collectively referred to as "dairy yeast" and "wine yeast", respectively. This study exploited the proteolytic and lipolytic qualities of dairy yeast and the ability of wine yeast to produce esters. After fermentation, the main odorous molecules present in fresh okara, saturated and unsaturated C6 aldehydes, were transformed into methyl ketones and/or esters, and the unwanted, grassy okara odour was greatly reduced. In particular, the okara fermented by *W. saturnus* contained the largest quantity and widest variety of esters, particularly C6 esters, and a significantly fruity odour (Figure 8). This study illustrates the possibility of obtaining esters and modifying the aroma of okara, via biotransformation, to improve its organoleptic quality.

Figure 8. Changes in (**a**) hexanal and (**b**) total esters in fresh and yeast-fermented okara (GC = *G. candidum*, YL = *Y. lipolytica*, DH = *D. hansenii*, KL = *K. lactis*, LT = *L. thermotolerans*, SC = *S. cerevisiae*, MP = *M. pulcherrima*, PK = *P. kluyveri*, WS = *W. saturnus*, TD = *T. delbrueckii*) (Image credit: VONG Weng Chan) [182].



The direct fermentation of okara, sometimes with the addition of nutrients, however, is not the only way forward. There have also been numerous studies on the compositional modification of raw okara, including physical processing methods (e.g., high hydrostatic pressure, extrusion), as well as chemical and enzymatic treatments [7,183–184] The general increase in the amount of soluble dietary okara fibres following these treatments highlights their feasibility as a pre-fermentation step, increasing the amount of fermentable carbohydrates and oligosaccharides.

Studies have also been conducted on the fractionation of specific components of okara, such as dietary fibres, proteins and isoflavones [90,185–190]. This contributes both to the biovalorisation of these isolated components and to higher efficiency and yield than the direct fermentation of okara.

Finally, microbial transformation of the protein fraction, or its hydrolysates, can produce active peptides and single-cell proteins, while fermentation of the lipid fraction, using lipolytic and oily microorganisms, can be exploited for the production of microbial oils and other substances that are derived from lipids.

5.6 Nutrition and Health

As mentioned above, okara contains high levels of dietary fibre and proteins, as well as significant amounts of isoflavones and mineral elements, which gives it a high nutritional value and a potential prebiotic effect. It is therefore potentially useful as a functional ingredient with health-promoting properties [189].

5.6.1 Diabetes

In recent decades, the incidence of diabetes, particularly type 2, which accounts for more than 90% of cases, has increased rapidly (from 151 million in 2000 to 285 million in 2010). By 2030, the overall prevalence of diabetes will be 7.8%, with about 438 million adults suffering from it [191]. Researchers believe that consuming large amounts of soluble fibre can help control blood sugar levels after meals as it slows down the rate of carbohydrate uptake in the intestine. Soluble fibre also reduces blood cholesterol and triglyceride (TG) levels by binding to cholesterol and helping to eliminate it from the body. In addition, a protein-rich diet may be useful for people with hyperglycaemia [192]. Okara, which contains about 50% fibre and 25% protein, is an ideal food for diabetics. In fact, it has been used for many years by the Chinese population to prevent this chronic disease.

In 2000, Xu et al. studied the effect of SB fibres on the blood sugar, lipid levels and hepatic-nephritic histomorphology of mice with streptozotocin-induced diabetes [193]. The glycaemia, total serum cholesterol and TGs of diabetic mice fed with okara-containing food for 5 weeks decreased significantly, while high-density lipoprotein (HDL) cholesterol increased significantly. Their study showed that okara can significantly reduce the plasma levels of sugar and lipids, improve blood sugar and lipid metabolism, and protect the liver and kidneys of diabetic mice.

5.6.2 Hyperlipidaemia

Okara may play an interesting role in preventing hyperlipidaemia. In a study by Villanueva et al. [29], male Syrian golden hamsters were fed a high-fat diet supplemented with okara for 3 weeks. The different diets contained either 13% or 20% okara fibres (Ok-13 and Ok-20), low protein okara with 13% fibre (Ok1-13) and isoflavone-free okara with 13% fibre (Ok2-13). The plasma levels of TGs, VLDL plus LDL cholesterol, and total cholesterol in hamsters fed with OK-20 decreased significantly. Total lipid, TG, total and esterified cholesterol

concentrations in the liver were reduced by the OK-20 diet. All tested okara diets increased the faecal excretion of total lipids, TGs, free cholesterol and total nitrogen. The results suggest that the main components of okara, dietary fibre and protein, may be related to total-lipid and cholesterol reduction in plasma and the liver, as well as increased faecal production in hamsters fed a high-fat diet.

Wang and Li studied the effect of okara fibre on lipid metabolism and the hemorheology of rats fed with a cholesterol-rich diet [194]. In groups fed with either okara or pectin, total cholesterol and LDL cholesterol levels decreased significantly after 8 weeks, while the pure cellulose-fed group had no hypolipidemic effect. In all groups, TGs and HDL cholesterol underwent no noticeable changes. Therefore, a diet containing either 6% okara or pectin may reduce blood viscosity and platelet aggregation in hyperlipidaemic rats. More specifically, SCR showed a more pronounced effect on these aspects than pectin.

5.6.3 Obesity

Okara may be effective as a weight-loss dietary supplement with a potential prebiotic effect. Préstamo et al. have reported that growth rate and nutritional efficiency were lower in an okara-fed group than in a control group, even though SCR, in the form of a dietary supplement, had no influence on the food intake in rats [195]. An increase in faecal weight and moisture was observed in the SCR-fed group. Moreover, the in-vivo colonic fermentation of okara resulted in a lower pH and the higher total production of short-chain fatty acids, demonstrating that it might be effective as a weight-loss dietary supplement with a potential prebiotic effect.

In another study, mice were fed either a high-fat diet (14% crude fat) or a high-fat diet supplemented with dry okara (10%, 20% or 40%) for 10 weeks [196]. The intake dose of okara suppressed the development of body weight and white epididymal adipose tissue in a dependent manner, and prevented an increase in plasma lipids, including total cholesterol, LDL cholesterol and unesterified fatty acids. Consuming okara also prevented liver steatosis. The real-time reverse-polymerase transcriptase chain reaction (RT-PCR) revealed that the intake of okara led to an under-regulation of the fatty-acid synthesis gene in the liver and an overregulation of the 7α-hydroxylase cholesterol gene.

5.6.4 Antioxidant Activity

In-vitro experiments have shown that okara is a potential source of antioxidant components, [92], in particular protease hydrolysate [91]. Zhu et al. have studied the antioxidant activity of meitauza, a traditional Chinese food derived from SB residue that is fermented by *Bacillus subtilis* B2 [169]. Compared to the aqueous soy koji extract, the aqueous okara-koji extract showed higher scavenging activity against the 1,1-diphenyl-2-picrylhydrazyl radical (DPPH).

The alkali-soluble polysaccharide fractions extracted from okara showed in-vitro antioxidant power (11–26 μmol Trolox equivalent (TE)/g dry weight) and free-radical scavenging activity (63–78 μmol TE/g dry weight). The potential antioxidant activity of the polysaccharides in the okara cell wall can be attributed to pectins, although the contribution of residual proteins cannot be excluded [20].

An ethanolic okara extract has shown greater scavenging activity against DPPH radicals than petroleum ether, ethyl acetate and acetone extracts. Synergism with vitamin C and citric acid was also reported [197].

5.7 Limiting Factors for Okara Valorisation

Although the worldwide production of okara is high, its utilisation index is low, which means poor yields from rich resources. More worryingly, management mistakes are causing significant environmental pollution. Some of the factors that limit its efficient use are summarised below.

5.7.1 Humidity and Drying

Despite the wealth of nutritional components, the high moisture content of okara, of between 70% and 80%, and the 25% protein (dry base) hamper proper preservation. As a result, it decomposes rapidly once produced [198]. To overcome these limitations, the fresh residue should be dried as soon as possible, under appropriate drying conditions, to facilitate its movement and transport. The natural drying process is not suitable because of the time required and the influence of the weather, meaning that the product begins to rot before drying is complete. General preservation methods for okara include freezing (low temperatures from 0 °C to −18 °C or lower inhibit the microorganisms' growth), oven drying and vacuum freeze-drying.

Indirectly heated conductive drying under agitation is a good choice for drying the residue, which is initially in the form of sticky lumps but disintegrates into small

pieces or particles during drying [199]. Cui and Luo used a flash dryer with a rotating mill to dry okara and obtained satisfactory results [200].

Taruna and Jindal have studied the use of a continuous fluidised bed of inert particles, similar to a vortex, to dry SB pulp [198]. Although this drying technique provided acceptable results, both in terms of drying kinetics and energy consumption, the process suffered from okara agglomeration when a high okara-feeding speed was used; the residue, in fact, joined the inert particles together to form lumps, resulting in reduced drying rates.

According to a study by Wachiraphansakul et al. SCR that is dried in a fluidised bed has acceptable qualities in terms of colour, protein content and, above all, urease activity, when a water jet dryer is used [201]. However, a limitation in the blower used means that only a small amount of okara can be introduced into the drying chamber, leading to unsatisfactory specific energy consumption values. The addition of silica gels, used as absorbent particles, has been found to facilitate the drying process, both in terms of drying kinetics and the quality of the dried okara [79].

Treatment with a high voltage electric field can significantly improve drying speed, compared to kiln drying [202]. In fact, the drying time under high electric field conditions has been reduced by 15–40%, compared to the control [203]. Microwave vacuum drying is a potential and competitive option for drying okara because of its fast drying and low volume. This combined drying method has a drying time that is 90% lower than that of hot-air drying and freeze drying, while the quality of the dried product is similar to that obtained by freeze drying [204]. Drying methods have a significant effect on the sensory qualities and functional properties of okara. In terms of water-retention capacity, swelling capacity and the ability to bind lipids, freeze-dried SCR has proven to be the best, followed by vacuum drying and hot air drying. On the other hand, in terms of cation exchange capacity, hot-air-dried okara is the best, followed by vacuum drying and freeze drying [205]. However, all the common drying methods are energy intensive and cause major changes to the taste, colour and aroma of the product.

5.7.2 Anti-Nutrients

Raw SBs have high anti-nutritional factors, in particular, trypsin inhibitors that are partially inactivated during the solvent extraction and the roasting process [206,207]. Trypsin inhibitors are one of the most important factors that limit the application of okara in feed. Cattle fed with okara have digestion problems due to the antitrypsin, a further factor that limits its use in animal development. Experimental results show that fresh okara, used as a direct feed, has an impact on growth, behaviour and physiological activity [208]. Inactivation methods for trypsin inhibitors include physical, chemical, bioreduction and fermentation processes, as well as complex methods that make use of natural compounds [209].

5.8 Conclusions

- The disposal of okara is still an unsolved problem caused by the increased global production of soymilk, tofu and their derivatives.
- Although numerous studies have been carried out on this topic, only a few propose a means of cost-effectively exploiting SCR. Furthermore, most tofu and soymilk factories are small and widely dispersed, making it difficult to collect an abundant quantity of okara for rapid, centralised processing before it degrades. The development of suitable dryers is therefore the fundamental prerequisite for this substance's full exploitation.
- Although SCR can be used in many contexts, it is also important to adopt appropriate methods for its use. Drying and grinding into a powder, then mixing with wheat flour or other ingredients to prepare composite flours, for the nutrition of people with specific needs (such as obesity and diabetes), or functional foods is an interesting choice for its use. Moreover, chemical and enzymatic treatment, fermentation, extrusion, high pressure and micronisation can improve the nutritional quality, taste and processing properties of okara and consequently the acceptance of the food as a whole.
- Future long-term randomized clinical trials are needed to confirm the healthy benefits of nutraceutical components and functional foods from SCR. In addition, pharmacodynamic studies will be useful to understand the mechanisms of action of polyphenols, isoflavones or bioactivated peptides isolated from SCR.

- In this regard, more efficient extraction processes and standardized protocols will guarantee high-quality products for food and nutraceutical industrial preparations.
- Although considerable progress has been made in recent years, the development of devices for the proper conservation of SCR and techniques for its proper use are challenges for the future. According to “circular economy” principles, transforming by-products from waste materials into in new added-value products is an ever more urgent task.

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Chapter 6
Valorization of highly active cranberry's polyphenolic fraction: new advances in processing and clinical applications

6.0 Introduction

Vaccinum macrocarpon (Ait. Ericaceae) called “large cranberry”, “North American cranberry” “bearberry”, or simply “cranberry” is a fruit originally from New England and currently grows throughout the east and northeast parts of the USA and Canada. The USA produces 436.691 tons/year of cranberries equal to 58% of the world’s total cranberry production, with $3.5 billion estimated in economic activity [1,2,3]. However, only 3-5% of cranberries are used for fresh fruit consumption, while 95-97% are destinated for processing, which include both the food and nutraceutical industries [2]. In fact, cranberries are known as a rich source of phenolic compounds and used for a long time in traditional folk medicine, especially for the treatment of bladder and kidney ailments [4]. Starting from the second half of the 20th century the use of *V. macrocarpon* juice to treat urinary tract infections (UTIs) and wounds has been reported in different manuals of phytotherapy. According to subsequently observational and interventional studies in humans, consumption of cranberry demonstrated to be associated with beneficial effects firstly in UTIs prevention, but also in other conditions [5]. In this regard, cranberries possess antioxidant and anti-inflammatory activity, and its supplementation have shown to promote overall gut and oral health and reduce or prevent some cardiovascular risk factor or chronic conditions (e.g., hyperglycaemia, dyslipidaemia, diabetes type II, cardiovascular diseases) and even cancer [6,7,8]. During the last years, the new trend of studies has focused the attention also on the effects of cranberry on gut microbiota composition and gastrointestinal health [9]. In this sense, the consumption of 2 bottles of cranberry juice (each of 250 mL) for 90 days was found to suppress *H. pylori* infection of susceptible people [10].

After a description of the main bioactive components of cranberry, this chapter will describe the state of the art of the main extraction and purification methods of cranberry, discussing the advantages and limitations of these strategies. Clinical applications that have arisen from RCTs are also discussed. Finally, future perspectives for cranberry extracts will be presented.

6.1 Phytochemical composition of cranberry

V. macrocarpon phytochemical composition is complex and includes many polyphenols like A-type procyanidins (PACs), anthocyanins, benzoic acid, but also terpenes like ursolic acid. The amounts of different classes of phytochemicals in cranberry fresh fruit, dried fruit, juice and sauce are synthesized in Table 1. PACs represent about 85% of the total weight of the flavan-3-ols [11] and comprise a group of different chemical structures based on the common constitutive unit represented by the (−)-Epicatechin. The several types of PACs derived by different types of linkage and degree of polymerization, in particular the presence of A-type or B-type, influences the efficacy of the extract against urinary tract infections (UTI). Indeed, the A-type is significantly more effective than the B-type in inhibiting *Escherichia coli* adhesion to uroepithelial cells [12]. Moreover, while PACs are contained in high amounts in several plant foods such as apples or grapes, A-type PACs are contained in high amounts only in cranberries and lingonberry (*Vaccinum vitis-idaea* L.) [11]. The anthocyanins mainly present in cranberries are cyanidin and peonidin glycosides. Among them four anthocyanins, i.e. cyanidin-3-galactoside, cyanidin-3-arabinoside, peonidin-3-galactoside and peonidin-3-arabinoside have been identified as the most representative while cyanidin-3-glucoside, peonidin-3-glucoside and other various anthocyanins are present only in small amounts [13]. Anthocyanins are mainly responsible for the colour of the berries and the naturally occurring glycosylation in position 3, increasing the stability of its aglycone portion, the anthocyanidins, which is highly unstable [13].

Phenolic acids are not specific components; indeed, it is present in many other plant food and berries. In cranberries, phenolic acids are mainly represented by hydroxybenzoic and hydroxycinnamic acids while ellagic acid and ellagitannins have not been detected. The hydroxybenzoic acids are the most abundant and benzoic acid is more prevalent than *p*-hydroxybenzoic, *o*-hydroxybenzoic and 2,4-dihydroxybenzoic acids. As concerns the hydroxycinnamic acids, cranberries contain *p*-coumaric, caffeic, ferulic and sinapic acids [14].

Cranberry fruits also contain phytochemicals not included in the polyphenols class such as triterpenoids compounds like ursolic, oleanolic and betulinic acids. Among them the most abundant is the ursolic acid (UA) which is present also in the peels of several fruits and which has anti-inflammatory recognized properties [15] but cranberry fruits also contain two rare derivatives of UA such as *cis*- and *trans*-3-*O*-

p-hydroxycinnamoyl ursolic acid. On the other hand, in a study focused on cranberry juice fractions tested through a bacterial adherence assay, two coumaroyl iridoid glycosides and a depside have been identified [16].

Among berries and plant food, *V. macrocarpon* is probably the richest species for flavonols and the most abundant flavonols in cranberries are represented by the glycosylated form of quercetin, myricetin and a small amount of kaempferol. In particular, quercetin 3-galactoside represents the predominant flavanol, even if other glycosides are present in traces [17].

The exact amount of each bioactive compound in cranberry depends on the variety of American cranberry which is analysed and from the exact phase of the plant life. In general, PACs and flavonols have been found high in the earliest stage and decrease during the growing of the fruits. The decrease of PACs is more rapid than the decline of flavanols. Anthocyanins production starts when cranberry fruit has finish its growth and continues during ripening [18].

All the phytochemicals are present in the cranberry fresh fruits but often cranberries are consumed in processed forms to obtain juice, sauce or dried fruits and these processes could affect the content of bioactive compounds (Table1). Anthocyanins are particularly affected, and its loss could reach 50% of the total amount due to removal of skin and seeds, high temperatures and oxidation of polyphenols. PACs and flavonols are more resistant against the high temperatures and during pasteurization but not against the very high heat used during the process necessary to obtain cranberry powders [19].

Table 1. Polyphenols content of cranberry foods (adapted from *Blumberg et al.* 2013 [18])

Food source		Flavan-3-ol monomers and dimers [11,20]	Proanthocyanidins [11,21]	Anthocyanins [21,17]	Hydroxybenzoic acids [20,22]	Hydroxycinnamic acids [20,22]	Terpenes [23]	Flavonols [22]
Cranberry fruit	*mg/100 g*	7–33	133–367	13–171	503–602	73–82	65–125	20–40
	mg/serving (80 g whole fruit)	5.6–26.4	106–293	10.4–136.8	402–482	57.6–65.6	52–100	16–32
Cranberry juice	*mg/100 g*	6–35	89–230	27–132	64	12–19	Trace	11–58
	mg/serving (200 ml juice)	7	17.8–46	5.4–26.4	12.8	2.4–3.8	Trace	2.2-11.6
Canned cranberry sauce	*mg/100 g*	112.8	16–54.4	0.6–11.8	476	47.5	1.1–22.8	—
	mg/serving (70 g sauce)	78.9	11.2–38	0.4–8.3	333.2	33.2	0.8–16	—
Sweetened, dried cranberries	*mg/100 g*	—	64.2	10.3	—	—	98.5	—
	mg/serving 40 g dried fruit)	—	25.6	4.1	—	—	39.4	—

6.2 Extraction methods

Several types of cranberry products have been tested in pre-clinical and clinical trials even if data regarding the comparison of the relative composition of the extracts and the bioactivity for different products is still few or lacking [7].

American cranberry is rarely consumed as fresh fruit, due to the sour, astringent flavour of the berry [18]. For this reason, a wide range of cranberry products are available on the market for both the consumers and the researchers with different chemical profiles, according to the extraction methods used (Figure 1).

The most common use of cranberry is the production of juice, which causes an increase of food industry waste, well known as pomace (berry press residues) that contain berry skin and seeds [24]. In a concept of circular economy, the use of cranberry by-products represents an interesting strategy for the valorisation of food industry wastes, recovering from the berry press residues different nutraceutical molecules, such as the phenolic compounds (Table 2) and the oligosaccharides fractions (usually discarded), which are known to have health properties [24]. However, the optimisation of extraction technique is necessary in order to selectively extract the molecules of interest, with an acceptable yield. In fact, the extraction of phenolic compounds from cranberry seeds is often difficult, especially using "green solvents" and unconventional extraction methods [25]. Pomace may be used also as an animal feed additive [26].

Depending on the nature of the chemical and physical extraction processes and the sequence of processing steps adopted, it is possible to obtain several cranberry extracts with different bioactive components (Table 3). Whole berries are generally stored frozen after harvest and a rapid wash, increasing the storage period and allowing subsequent treatments for several months after the fruit has been harvested [27].

Cranberry juice is extracted by pressing or decanting from frozen berries which have been cut and macerated and include the separation of solid and liquid fruit components, followed by centrifugation and filtering, in order to remove the suspended solid particulates [28]. The freezing, cutting and maceration processes are important to increase the extraction of phenolic components, improving the surface area of fruits by breaking down cell wall components [29].

After the separation of solid and liquid fruit components, the juice is particularly rich in water soluble molecules including several flavonoids and organic acids (tartaric, fumaric, citric, and shikimic acid) as well as sugars (fructose, glucose, sucrose), while the pomace is composed mainly of cranberry skins and seeds is rich in proteins, insoluble polymers and polyphenols adhered within the physical structures of such residues [7]. The extraction of bioactive components can be improved also using a mixture of pectinase enzymes in addition to water and heat to biochemically degrade cell wall structures [30,31].

In general, the liquid achieved from the juice production undergoes the process of pasteurization with the use of heat to facilitate the long-term storage. The concentration of juice exploits the mechanism of reverse osmosis or counter current chromatography, obtaining the cranberry juice concentrate also known as cranberry syrup with a remarkably high concentration of solute (50 Brix) [32]. Concentrated juice syrup is then diluted with water and sweetened or blended with other fruit juices to obtain respectively sweet cranberry juice and mixed fruit juices [28].

Cranberry juice can be treated with proprietary methods to remove sugars, especially for nutraceuticals preparations, in order to reduce the caloric content of juice and avoid any problems with gluco-intolerant patients [33,34].
Fresh berries can also be processed to obtain dried cranberries, canned cranberry sauce and other foods with added cranberry materials. Sweetened dried cranberries prepared from the frozen, cut berries are instead used in food industries [27].
However, during the drying process, there is a partial loss of nutritional value as a consequence of exposure of cranberry fruits to high temperature and air for long time. In this regard, during the extrusion process, characterized by the exposition of cranberries at high-temperature for a short-time, in order to reduce the moisture and then prolong the shelf-life, a significant loss of total anthocyanins have been observed [24].
For this reason, and in addition to reduce energy expenditures, new unconventional technologies and pre-treatment have been developed [22]. The study by *Nowacka et al.* analysed the quality of microwave-vacuum dried, osmodehydrated cranberries processed by the means of blanching and ultrasound or blanching followed by pulsed electric field and sonication in comparison with the traditionally treated material. The microwave-vacuum drying process was demonstrated to be very short (25-38 min) if compared with the convective drying, that lasted several hours (13.2 h). Most

of the samples subjected to ultrasound and pulsed electric field and sonication treatments before osmodehydrated and drying were characterized by similar or higher amounts of bioactive compounds such as polyphenols, anthocyanins and flavonoids, and better colour and taste, as compared to cut osmodehydrated cranberry fruits subjected to convective drying (reference samples) [22].
An interesting technique to reduce deteriorating reactions and increase the shelf life is represented by the food coating, which is characterized in the application of an edible layers on the surface of cranberries. In this regard, *Lozano-Navarro et al.* [35] reported the antimicrobial impact when 0.5% of cranberry extract was added to chitosan and starch-based film.

An interesting product from cranberry pomace or enriched juice (from the whole berries) is the hull extract powder, which can be used both for food and nutraceutical applications. Starting from the pomace or the whole berries cut, after pressing and filtering, the enriched juice is treated with additional enzymes and heat beyond those typically used for juice production. After that, the resulting enriched juice extract is typically spray-dried to obtain the hull extract powder [7].

The composition of cranberry extracts is extremely variable, depending on several factors, such as the treatments with different enzymes, temperature and time conditions and the concentration and drying steps which affect the qualitative-quantitative composition of the final extracts [36]. Considerable evidence suggest that flavonoids could be easily degraded during processing due to different factors which affect its stability (temperature, light, pH, and the presence of endogenous enzymes such as polyphenol oxidase or glycosidases). Flavonoids are generally stable at a lower pH, reducing the conversion from the stable flavilium cation to less stable carbinol pseudobases or quinodal bases which are more easily oxidized. Even the presence of polyphenol oxidase and/or glycosidase can reduce the stability of anthocyanins reacting with simple phenolics to form quinones or cleaving the sugar from many flavonoids respectively. Several studies showed that flavonoid stability appears to be dependent primarily on the sugar attached rather than the aglycone, and glucosides are more stable than galactosides, which are in turn more stable than arabinosides [37,38].

In this regard, the great variability of bioactive molecules present in the extracts could influence the results of cranberry effects administered in clinical practice. In addition, even if a major part of the clinical studies has investigated the effects of

cranberry as nutraceutical, marketed in the form of dry extracts (some of these titrated and standardized in phenolic compounds) in different dosage forms (syrups, sachets, capsules, tablets, etc.), the lack of information which describe the methods used to obtain the extracts make impossible to predict the difference of bioactive compounds which may be present in the final product [39,40,41,42].

Figure 1. General processing steps and relative relationship for different cranberries materials (Adapted from *Coleman et al.* 2020 [7])

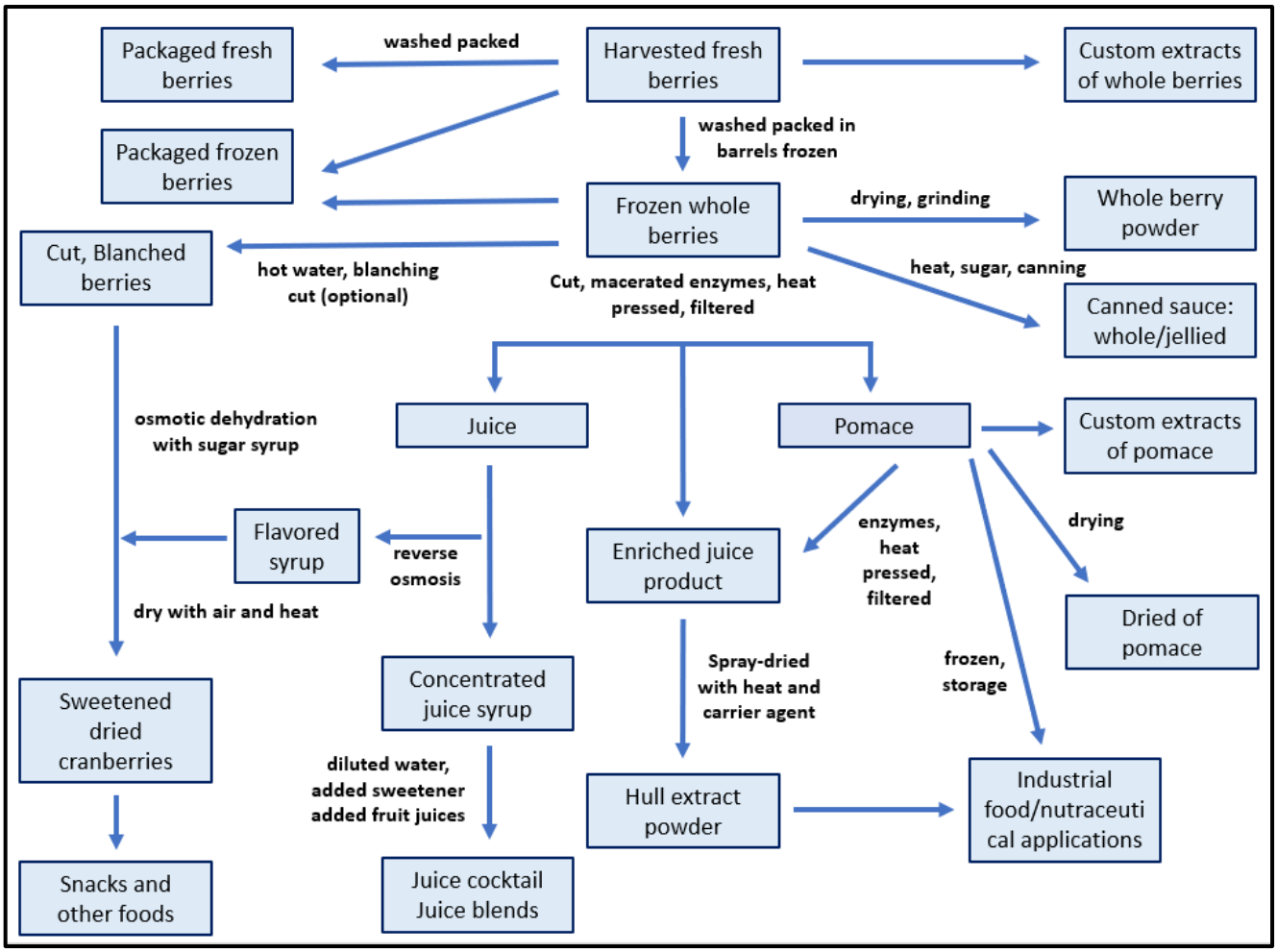


Table 2. Content of polyphenolic compounds in cranberry pomace (adapted from *White et al.* 2011 [43])

Polyphenols	**Concentration (mg/100 g dw)**
Anthocyanins	
Cyanidin 3-arabinoside	49.6 ± 6.8
Peonidin 3-arabinoside	26.6 ± 0.5
Peonidin 3-galactoside	20.1 ± 0.5
Cyanidin 3-galactoside	13.2 ± 0.2
Peonidin 3-glucoside	7.4 ± 0.3
Cyanidin 3-glucoside	4.5 ± 0.2
Total anthocyanins	121.4 ± 5.9
Flavonols	
Quercetin	146.2 ± 22.7
Myricetin	55.6 ± 2.6
Quercetin 3-benzoyl galactoside	27.5 ± 3.4
Quercetin 3-rhamnoside	18.5 ± 3.4
Quercetin 3-arabinofuranoside	16.7 ± 3.5
Quercetin 3-arabinopyranoside	15.2 ± 3.6
Quercetin 3-galactoside	12.8 ± 3.6
Unidentified	12.1 ± 3.5
Methoxyquercetin 3-xyloside	11.4 ± 3.7
Quercetin 3-xyloside	5.5 ± 0.3
Quercetin 3-coumaroyl galactoside	2.3 ± 0.3
Myricetin 3-arabinoside	1.8 ± 0.1
Myricetin 3-xyloside	1.5 ± 0.3
Total flavonols	358.4 ± 16.3
Procyanidins	
Dimer (DP2)	52.7 ± 1.7
Trimer (DP3)	30.7 ± 1.4
Hexamer (DP6)	25.6 ± 1.2
Pentamer (DP5)	22.7 ± 1.2
Heptamer (DP7)	16.6 ± 1.2
Octamer (DP8)	16.1 ± 2.9
Tetramer (DP4)	16.1 ± 1.3

Nonomer (DP9)	13.2 ± 1.1
Monomer (DP1)	5.12 ± 0.0
Total procyanidins	186.5 ± 8.8

Table 3. Content of polyphenolic compounds depending on cranberry processing step and type of pre-treatment (adapted from *White et al.* 2011 [43])

Processing step	**Pre-treatment**	**Polymer concentration (mg/100 g Fresh Berries)**	**% Polymer**
Fresh	-	206.2 ± 9.3	81.7
Blanched	Grinding + blanching	251.8 ± 8.4	80.7
	No grinding + blanching	245.3 ± 18.5	82.6
Enzyme treated mash	Grinding + blanching	261.4 ± 20.9	81.2
	Grinding + no blanching	319.6 ± 11.5	85.3
	No grinding + blanching	172.7 ± 8.6	76.1
Unclarified juice	Grinding + blanching	104.2 ± 17.7	82.3
	Grinding + no blanching	103.5 ± 8.2	76.0
	No grinding + blanching	100.7 ± 4.3	78.5
Clarified juice	Grinding + blanching	107.7 ± 3.6	80.4
	Grinding + no blanching	74.0 ± 10.9	67.5
	No grinding + blanching	86.2 ± 4.5	78.0
Pasteurized juice	Grinding + blanching	76.1 ± 4.0	76.5
	Grinding + no blanching	69.4 ± 2.7	68.0
	No grinding +	75.3 ± 7.2	73.6

	blanching		
Pomace	Grinding + blanching	109.7 ± 1.7	88.9
	Grinding + no blanching	127.7 ± 3.9	92.5
	No grinding + blanching	130.4 ± 0.5	90.0

6.2.1 Unconventional Extraction techniques

A consolidated method to extract polyphenols from cranberry pomace provides the use of petrochemical-based solvents, including methanol, acetone, ethyl acetate and chloroform. In particular, methanol/hydrochloric acid and acetonitrile/trifluoroacetic acid/water are the most employed mixtures; nevertheless, this technique requires extensive time and the solvent residue is toxic and then represents a limit for the extracts application, since these are non-GRAS solvents [44]. Therefore, development of effective methods by using green techniques, addressed extraction of polyphenols from cranberries, but even more to conversion of cranberry pomace into higher added value products, is of a great importance. In this regard, an alternative method to extract anthocyanins and phenolic compounds from cranberry, and in general thermally sensitive phytochemicals, is the use of subcritical water and pressurized fluids. Subcritical water extraction consists of exposing water to high temperatures (above 100°C), and under enough pressure to remain in the liquid state. Beyond the water, other solvents such as ethanol and mixtures, can be used and the technique is called pressurized fluids. This is an environmentally friendly technique. At such conditions, the main physicochemical changes include an increase in both ionization and self-diffusivity and a decrease in surface tension [45]. Changes in the temperature and solvent type are critical on the total phenols and anthocyanins, while no significant difference was reported changing pressure. However, the best condition to extract anthocyanins from cranberry is pressurized ethanol at 60-100°C and 50 bar [43]. In addition to cranberry, this technique has been successfully applied to extract several bioactive compounds, including grape pomace and bilberry [46,47]. Moreover, the choice of acid can influence the stability of anthocyanins and in this contest organic acids are preferred, and the optimal seems to be hydrochloric acid (2.4 g/100 g cranberries) [45].

There is evidence that many procyanidins are not able to be extracted by conventional methods and they remain in the cranberry pomace, thus some authors suggest using an alkaline hydrolysis to improve the extraction of procyanidins from the treated residues through the breaking of their bonds [43].

A new method proposed by *Roopchand and colleagues* consists of the extraction of polyphenols from cranberry pomace with 50% ethanol (at 80° C, pH 2, for 2 hours) and stabilizing the extracted compounds *via* complexation with soy protein isolate. By co-drying cranberry pomace extract with a protein-rich food matrix, such as soy protein isolate, unlike dried cranberry pomace extract alone, proanthocyanidins, anthocyanins and total polyphenols were found to be highly stable at 37 °C [25].

The ultrasound-assisted technique is often used in polyphenols extraction [48,49,50] and consists in the shaking of the sample in the extraction solvent for extended periods of time [51]. During the treatment with ultrasounds, the cell wall matrix is disrupted and various compounds, including polyphenols, are released into the medium. The results obtained from ultrasound-assisted extraction showed that this has the greatest potential [44]. Interestingly, *Klavins et al.* compared the ultrasound, microwave assisted and Soxhlet techniques to extract polyphenols from press residues of cranberry. The results confirmed the highest potential of ultrasound assisted extraction compared with the other methods, being fast, low-cost and convenient to use. The best extraction solvents were aqueous ethanol and methanol, even if in the presence of acid (trifluoroacetic acid or HCl for anthocyanins or polyphenols respectively), which may limit the use of the final extracts as functional foods or nutraceutical substances [52]. The microwave-assisted technique has also been successfully tested in the extraction of carbohydrates from pomace of cranberries. Indeed, the total sugars yield reached the value of 21.3% and contained mostly oligosaccharides in the degree of polymerization range of 7 to 10 [53].

Very recently, a supercritical carbon dioxide extraction followed by pressurized fluid extraction has been proposed and tested to obtain a “zero waste” processing of cranberry pomace. Supercritical extraction with carbon dioxide is considered a green technique, since it avoids the use of organic solvents. Moreover, carbon dioxide is a non-toxic, safe and manageable solvent. It is optimal to extract lipophilic compounds, therefore combined with pressurized fluid extraction, the efficiency of the process can be maximized [54].

6.3 Clinical applications

6.3.1 Urinary tract infections

UTIs are the second most common type of bacterial infections worldwide, following otitis media [55]. It affects more than 150 million people/year worldwide and causing an economic burden >$2.6 billion in annual health care expenditures [56].

UTIs are categorized as pyelonephritis and kidney infections when it affects the upper urinary tract (ureters and kidney parenchyma), and as cystitis and urethritis when it affects the lower urinary tract (bladder or urethra). It is also generally divided into sporadic uncomplicated and complicated infections [57]. Complicated UTIs are less common and associated with functional or structural abnormalities such as immunosuppression, urinary obstruction, catheterization, pregnancy or renal dysfunction [58]. Uncomplicated UTIs are more prevalent in women who have a 50% risk of at least an episode of cystitis (vs 12% risk in men) during their life and 20-30% risk of recurrent UTIs [59]. The antibiotic therapy represents the most common approach to UTIs, even if it presents some limits including the risk of antibiotic resistance, well documented by the scientific community, and the damage of the intestinal microbiota [60]. In fact, recurrent UTIs require multiple antibiotics for several periods of the year, used also as prophylactic agents. In this regard, the rate of fluoroquinolone resistance is >20% in different nations and the FDA, in 2016, pointed out how the serious side effects associated with fluoroquinolones generally outweigh the benefits for people with uncomplicated UTIs [61]. Relapses in UTIs could be caused by the same microorganism or by a different microorganism. *E. Coli* is responsible for 85% of cystitis even if other Gram-negative bacteria such as *Klebsiella pneumoniae* and some Gram-positive bacteria such as *Staphylococcus saprophyticus* and enterococcal species might be implicated in the pathogenesis of uncomplicated UTIs, because it is able to join directly to the bladder epithelium [57,62].

The study by *Bodel et al.* in 1959 described for the first time the use of cranberry in prevention of UTIs, attributing its efficacy to the presence of hippuric acid [63]. However, as early as the 1600s, Native Americans used cranberry consumed as a food for the treatment of UTIs and for wound and blood poisoning [64,65]. From 1990s to 2000s, cranberry research was focused on UTIs prevention, as highlighted by the first robustly designed RCT including 153 females with frequent bacteriuria and randomized to receive 300 ml of cranberry juice or placebo. The results indicated

the potential use of cranberry in UTIs, obtaining a reduction by more than 50% of bacteriuria only in cranberry group [66]. Although the consumption of cranberries has been extensively recommended for UTIs prophylaxis and relief of adverse symptoms, the recommendation coming from the systematic-reviews and meta-analyses of the literature seem to be in part contrasting [67,68,69,70,71,72]. The motive for different conclusions and recommendations of cranberry use in UTIs prevention could be explained by a lack of knowledge regarding the role of cranberry constituents and its impact on urinary tract and gut microbiota, but also the limited characterization of the cranberry materials used in clinical trials, as well as a lack of systematic protocol for the selection of subjects and clinical assays [7]. In fact, cranberry was later found containing different types of bioactive compounds, including anthocyanins, flavonols and phenolic acids. However, among the polyphenols of this fruit, type A proanthocyanidins have shown the greatest bioactivity because it is able to inhibit binding of uropathogenic *E. coli* to the uroepithelial cell receptors, attenuate the uropathogen reservoir in the gastrointestinal tract and suppress the inflammatory cascade. In this regard, A-type proanthocyanidins contain an additional ether interflavan bond between C2 →O →C7 if compared with B-type proanthocyanidins, which probably do not exert any effect on UTIs prevention (Figure 2) [73].

A RCT demonstrated the ability of type A proanthocyanidins of cranberry to inhibit the ex vivo adherence of both P type and type 1 uropathogenic *E. coli* (Figure 3) [74]. Similar result was obtained against *Candida albicans* strain, with a significant reduction in the adherence and biofilm formation after cranberry supplementation [75]. The daily recommended amount of PACs to decrease the episodes of UTIs is at least 36 mg [18]. However, cranberry is well known to contain also organic acids (e.g., citric, malic, shikimic, quinic), terpenes and carbohydrates which might exert pleiotropic activities in UTIs prevention. In particular, D-mannose has demonstrated to reduce the adherence of *E. Coli* to uroepithelial cells *in vitro* [76], while vitamin C and other organic acids promote the acidification of the urine with a bacteriostatic effect [77]. Even cranberry xyloglucan oligosaccharides were found to play a role in UTIs prevention, reducing *E. coli* adhesion to the bladder [78].

Figure 2. Proanthocyanidin A- (left) and B-type (right).

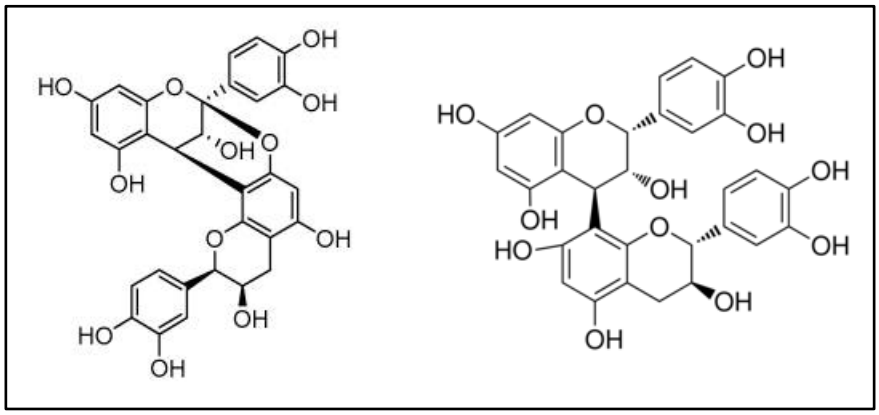
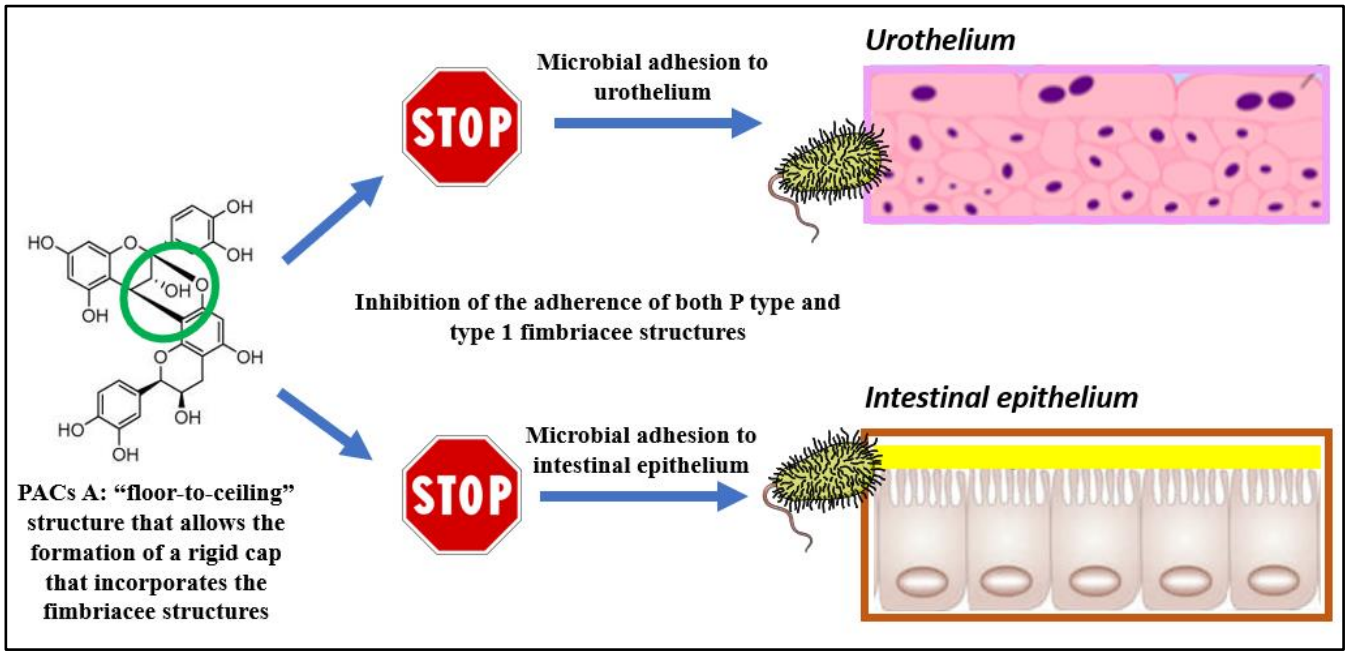


Figure 3. A-type proanthocyanidins (PACs A) and anti-uropathogenic mechanism of action.



The meta-analysis by *Fu et al*., including 7 RCTs conducted on healthy women (n = 1498) at risk of UTIs showed that cranberry reduced the risk of UTIs by 26% (pooled risk ratio: 0.74; 95% CI: 0.55, 0.98), suggesting the possible effect of this nutraceutical in preventing uncomplicated UTI recurrence in healthy people [72]. Similar results were obtained by another meta-analysis of 28 RCTs (RR: 0.67, 95%CI 0.55-0.79, p <0.0001). [79]. However, data on complicated UTIs prevention

with cranberry remains unclear and in part contrasting as reported by four previous meta-analyses [69,80,81,82], among which only three reported a trend of relative risk reduction after cranberry supplementation [69,81,82].

Cranberry proanthocyanidins and their metabolites also act by reducing the intestine reservoir of potential uropathogenic bacteria. In particular, cranberry flavonoids might decrease the intestinal colonization of opportunistic extra-intestinal *E. coli* and thus, the risk of UTIs incidence [83]. At the same time, the gut microbiota has been shown to interact positively with cranberry flavonoids in a "two-way interaction", improving the conversion of active metabolites which might enhance the anti-UTI effects and promote the intestinal eubiosis [84]. For example, phenyl-γ-valerolactones are one of the most important metabolites of cranberry found in the urine, with exhibited antiadhesive activity *in vitro* [85]. Therefore, the heterogeneity of results of cranberry supplementation against UTIs might be also attributed to the inter-variability of gut microbiota composition, which might influence the conversion of cranberry flavonoids into bioactive molecules.

Finally, the combination of cranberry with some probiotic strains (*Lactobacillus spp*.) has been proposed to be effective for the management of recurrent UTIs [86]. *Lactobacillus* spp. seem to improve the production of biosurfactants, bacteriocins, lactic acid and hydrogen peroxide and to inhibit the intestinal adherence of uropathogenic bacteria [87]. However, RCTs are still inconclusive and need further investigation as a consequence of the small samples size, the different dosages of both cranberry extracts and probiotics, the probiotic strains used and the typology of enrolled populations [88,89,90].

In conclusion, cranberry supplementation could be used and recommend in order to reduce the incidence of UTIs in particular in people with recurrent infections, reducing the use of antibiotics and potentially the economic burden which affects UTIs. However, both *in vitro* and long-term clinical trials are necessary to investigate the impact of cranberry supplementation in UTIs relief symptoms, as well as to clarify the mechanisms that contribute to the efficacy of cranberry's PACs in the reduction of UTIs, the duration of treatments, the dosages of administration, the impact of gut microbiota on the conversion of active metabolites and the differences of the different standardized extracts.

6.3.2 Oral, Gastric and Intestinal health

Over the last years, several researchers have focused their attention on the effects of cranberry regarding the intestinal health for its well-known antiadhesion activity against various microbes in the stomach, small intestine and colon [91]. However, contrary to the studies about UTIs prevention, the effects of cranberry on the gastrointestinal tract have been mainly investigated with *in vitro* or animal studies [91].

Cranberry proanthocyanidins act first as anti-inflammatory molecules, reducing the levels of interleukin (IL)-1β, IL -6 [93], bacterial lipopolysaccharide induced expression of iNOS and cyclo-oxygenase-2 (COX-2) in macrophages [94] as well as to improve the anti-inflammatory IL-10 [93]. These results were confirmed in a study including mice (eight-week-old) divided into three groups in order to receive a chew diet, a high-fat high-sucrose diet, or high-fat high-sucrose diet and cranberry extract (0,2 g/Kg) for 8 weeks. At the end of the treatments, cranberry supplementation showed to protect gut inflammation, measured by increasing *Akkermansia* spp. population, reducing the oxidative stress and intestinal triglyceride content [95].

A-type proanthocyanidins and its metabolites also interacted positively with the intestinal microbiota composition, preventing microbial dysbiosis. As demonstrated by *Bekiares* and colleagues, who investigated the impact of dried cranberries (42 g/day) consumption on human gut microbiota (n =10) using the faecal microbiome test, an improvement of *Firmicutes/Bacteroidetes* ratio as well as of the count of *Akkermansia* has been observed after the nutraceutical assumption [96]. In addition, the cranberry powder supplementation (30 g/day) showed to enhance the production of short chain fatty acids (SCFAs), particularly studied for the post-biotic effect [97].

In the last 15 years, cranberry supplementation was investigated to improve the success of eradication of *H. pylori* infection, which represent the major cause of peptic ulcer disease and gastric cancer [98]. In fact, *in vitro* studies demonstrated the ability of cranberry constituents to exert anti-adhesion activity on *H. pylori* [99]. In a prospective RCT, 189 Chinese people with *H. pylori* infection were randomly divided to receive cranberry juice (250 ml) or placebo for 90 days. At the end of the study, 14 of the 97 subjects (14.43%) in the cranberry juice treatment group had negative results for the 13C-urea breath test (vs. 5 of the 92 in the placebo group) ($p<0.05$) [10]. Consumption of high-proanthocyanidins cranberry juice twice daily (44 mg proanthocyanidin/240-mL serving) resulted in decreased *H. pylori* infection rate by 20% as compared with low dosages of proanthocyanidins ($p<0.05$) [100].

In the study by *Seyyedmajidi and colleagues*, which included 200 patients with *H. pylori* infection and peptic ulcer disease, in treatment with the triple therapy (lansoprazole, clarithromycin, and amoxicillin), the addition of cranberry to triple therapy for *H. pylori* had a higher rate of eradication if compared with the conventional therapy alone (up to 89% and significant) [101]. In another RCT, 177 patients with *H. pylori* infection and treated for the first week with the triple therapy (omeprazole, amoxicillin and clarithromycin) were randomized to receive 250 mL of either cranberry juice (cranberry + triple therapy, n=89) or placebo (placebo + triple therapy, n=88) twice daily and only cranberry juice or placebo for the next 2 weeks. At the end of the treatments, analysis by gender revealed that the eradication rate was higher in the cranberry arm for female subjects. However, no significant differences were observed in male subjects [102].

In a multicentric RCT including 295 asymptomatic children (6-16 years of age) who tested positive for *H. pylori*, they were allocated in four groups: cranberry juice/Lactobacillus johnsonii La1 (CB/La1), placebo juice/La1 (La1), cranberry juice/heat-killed La1 (CB), and placebo juice/heat-killed La1 (control). Cranberry juice (200 mL) and La1 product (80 mL) were given daily for 3 weeks. At the end of the study, *H. pylori* eradication rates significantly differed in the four groups: 1.5% in the control group compared with 14.9%, 16.9%, and 22.9% in the La1, CB, and CB/La1 groups, respectively ($p<0.01$) [103]. Finally, an ongoing 4-week study will investigate the effects of cranberry juice fortified with omega-3 intervention on *H. pylori* eradication [104].

Cranberry PACs demonstrated to prevent the formation of *P. gingivalis* biofilm, and thus to be useful also for oral health. PACs act through three main mechanisms of action: the inhibition of bacterial and host-derived proteolytic enzymes, the regulation of host inflammatory response and osteoclast differentiation and activity [105]. The studies of *Bodet and colleagues* suggested that cranberry extract has the potential to reduce the proliferation of *P. gingivalis*, *T. forsythia* and *T. denticola* in periodontal pockets or its protienase mediated destructive processes occuring in periodontitis, reducing the inflammatory cytokine response of macrophages induced by the LPS fraction of Gram- [106,107]. In addition, cranberry has also shown to reduce the expression of COX-2 and matrix metalloproteinases (MMPs)-1 and -9, produced by resident and inflammatory cells in response by periodontopathogens such as *Aggregatibacter actinomycetocomitans* [108]. The supplementation of

mouthwash cranberry juice in volunteers demonstrated to reduce the salivary counts of oral streptococci (*S. mutans*), acting as an oral anti-adhesive nutraceutical and inhibiting the extracellular polysaccaride synthesis that promote the sucrose dependent adhesion of oral bacteria [109,110]. Similar results were shown by *Yamanaka et.al* [111], *Durate et al.* [112], and *Weiss et al.* [113] who examined the effects of cranberry polyphenols (supplemented through mouthwashes, tooth paste or chewing gum) on biofilm formation and bacterial growth of *S. mutans*. The research groups concluded by highlighting the possible preventive action of cranberry polyphenols in the development of dental plaque.

The impact of cranberry supplementation on oral health, gut microbiota and *H. pylori* eradication represents one of the most interesting research points of the coming years, in order to prevent oral health and both gastro-intestinal and extra-intestinal diseases. However, longer RCTs are still lacking and urgent to better understand the role of bioactive molecules of cranberry in oral and gut microbiome modulation and the future perspectives for this nutraceutical beyond the UTIs prevention.

6.3.3 Cardiometabolic effects

Cardiovascular diseases (CVDs) are the leading cause of mortality and disability in developed countries [114]. The impact of CVDs is estimated to have been US $906 billion in 2015 and is expected to rise by 22% by 2030 [115]. In this sense, cranberries which are a rich source of proanthocyanidins, anthocyanins, flavanols, flavonols, and phenolic acids, may contribute to reducing or preventing some cardiovascular modifiable risk factors [116]. However, to date, despite several studies have explored the effect of cranberry supplementation on CV risk factors in different categories of people (healthy subjects [117], patients with type 2 diabetes [118], with coronary artery disease [119], overweight or metabolic syndrome [120]), the produced results have been contrasting and in part remain unclear.

24 Chinese people with type 2 diabetes and treated with 160 mg/day of anthocyanins from bilberries and blackcurrants showed a reduction in fasting plasma glucose and HOMA-IR [121]. Similar results were obtained with cranberry juice (240 mL/day) consumed by overweight, older adults [117]. In another randomized and placebo-controlled pilot study, 8 overweight or obese men and women with abdominal adiposity consumed 450 mL/day high polyphenol cranberry extract beverage or placebo for 8 weeks. The results showed a reduction of endothelin-1, fasting C-reactive protein and reduced oxidized glutathione ratio, and an improvement of nitric

oxide ($p<0.05$ for all) compared with the placebo. Cranberry also reduced serum insulin and increased HDL cholesterol compared with the placebo ($p<0.05$) [116]. However, data on insulin improvement after cranberry treatments are still contrasting, as highlighted in a study which investigated the effects of cranberry juice in subjects with coronary artery disease (no improvement of fasting glucose, insulin, or HOMA-IR) [119]. In a RCT, 35 obese individuals with elevated fasting glucose or impaired glucose tolerance were treated with 450 mL/day of low-calorie cranberry beverage or placebo for 8 weeks. At the end of the study, a significant reduction of lipid peroxidation (measured as levels of 8-isoprostane group) was shown in the cranberry group (-2.18 pg/mL), while it was increased in the placebo group (+20.81 pg/mL) ($p=0.02$). In addition, an improvement in levels of triglycerides (-13.75% vs +10.32%; $p=0.04$) and nitrates (+3.26 µM/L vs -6.28 µM/L; $p=0.02$) was also observed. However, cranberry beverage consumption showed no impact on insulin sensitivity [122]. Another RCT, partially in contrast with this finding, included 41 overweight patients with non-alcoholic fatty liver disease who were randomly allocated to receive either a cranberry supplement (288 mg/day of extract, equivalent to 26 g of dried cranberry) or a placebo for 12 weeks. At the end of the study, alanine aminotransferase and insulin decreased significantly especially in the active group (alanine aminotransferase: $p<0.05$ compared with placebo; insulin: $p<0.001$ compared with baseline for active group and $p=0.020$ compared with baseline with placebo) [22].

Cranberry supplementation has no lipid-lowering properties as demonstrated by several RCTs, even if anthocyanins have been reported to improve LDL cholesterol in dyslipidemic people through the inhibition of cholesterol ester transfer protein [116]. Data on HDL cholesterol improvement remains still contrasting. *Ruel et al.* reported an improvement by 8% of HDL cholesterol after cranberry juice consumption in people with abdominal adiposity [123]. Other studies reported no significant effects on lipid profile [119,120, 124,125,126]. Nevertheless, differences in the duration of the studies, populations, baseline subject characteristics, medication use, polyphenol composition of the juice may underline the great heterogeneity of the results obtained. An interesting ability of cranberry, due to its antioxidant capacity, is the reduction of biomarkers of oxidative stress. Cranberry juice supplemented in people with metabolic syndrome [120] as well as in healthy subjects [127] demonstrated to reduce oxidized LDL (which contributes to the progression of atherosclerosis), inflammatory markers (hs-CRP, endhotelin-1) [117],

and increase the nitric oxide (NO) release suggesting a temporal benefit to vasodilation [128,129].

In conclusion, to date, data regarding the supplementation of cranberry on glycemia and insulinemia is still contrasting and need further investigations also to understand the glucoregolation mechanism of actions. Cranberry seems not able to exert significant lipid-lowering activities even if it may contribute to reduce lipid peroxidation, oxidative stress and inflammation.

6.4 Discussion and future perspectives

Today, the first indication for cranberry prescription regards the prevention of UTIs, which are responsible for 7 million doctor visits, 1 million emergency room visits and 100000 hospitalizations each year (only in the USA), with an estimated economic burden of $ 1.6 billion [130]. In this context, based on the results of evidence present in the literature, cranberry supplementation could be used and recommend in order to reduce the incidence of UTIs in particular in people with recurrent infections and reducing the use of antibiotics in people with UTIs. However, the impact of cranberry supplementation in UTIs relief symptoms is still unclear and in part contrasting. Even the mechanisms which contribute to the efficacy of cranberries' PACs in the UTIs prevention and the impact of gut microbiota on the conversion of active metabolites need to be clarified. Possible cranberry indications beyond UTIs include the prevention of oral health, intestinal eubiosis and the *H. pylori* eradication in addition with the conventional treatments. In this regard, despite that it represents one of the most interesting research points of the coming years, longer RCTs are urgent to better understand the role of bioactive molecules of cranberry in oral and gut microbiome modulation before recommending it in clinical practice [100]. Similar discourse concerns the use of cranberry in cardiovascular prevention where it seems to contribute to reduce lipid peroxidation, oxidative stress and inflammation [117].

One of the most important limits in cranberry supplementation regards the high costs to obtain titrated (in PACs) and standardized extracts. The abovementioned conventional techniques do not allow the production of inexpensive quality extracts and thus low-cost strategies to prevent UTIs and other diseases [131]. In the cost-effectiveness analysis by *van den Hout* and colleagues, including high-UTI-risk residents, the supplementation of cranberry capsules demonstrated to be effective in preventing UTIs but was not likely to be cost-effective in the investigated dosage,

frequency and setting [22]. In this regard, not all cranberry products have been shown to comply with the laws of European and non-European countries. The study conducted by *Wang et al.* and *Mannino et al.* emphasized the urgency of standardized product quality control and labelling for cranberry dietary supplements manufacture and marketing, reporting some cases of adulteration by other botanical extracts, non-uniformity between the test of dosage forms and the contents of PACs, and the low quality of extracts [132,133].

The problem of cheap but poor-quality cranberry extracts is a serious, underestimated and potentially dangerous problem for people's health, not only for the absence of PACs or for the low quality un-titrated extracts, but above all for the presence of contaminants. A Chinese study which used the gas chromatography-triple quadrupole tandem mass spectrometry demonstrated a possible contamination with pesticide residues of cranberry plant extracts [134]. However, a report by the EFSA Panel considering the composition, manufacturing process, anticipated intake, history of consumption of the source and human studies concluded that the cranberry extract powder is safe as a food ingredient at the proposed uses and use levels. Safety data including long-term studies is still lacking and need further investigations [135].

An important challenge for the next years is also to develop a food-compatible method for the extraction of cranberry phytochemicals and thus produce qualitatively effective and safe extracts. The study by *Klavins et al.* demonstrated the great potential of unconventional techniques such as ultrasound or microwave-assisted extraction associated with hydroalcoholic solvents, being potentially fast to use and ensuring good yields [52]. Nevertheless, although the unconventional processes for extracting value-added products are well established in the laboratory, the industrial-scale production with specific cost-effective analyses is still a challenge. In this context, the uninterrupted availability of cranberries and the selective separation of desired components are the major barriers to scale-up. The ideal extraction method for cranberry PACs should be based on: little capital investment, low energy consumption, water as a solvent, high yield and easy integration into current processing lines. Unfortunately, none of the methods described in the literature satisfy all of these criteria. In particular, even though significant improvements in extraction efficiency have been made using unconventional extraction techniques such as ultrasound or microwave-assisted extraction, they still involve high costs

compared to chemical methods, and new proposals and solutions to reduce these constraints are, at this moment, still lacking.

6.5 Conclusion

- Cranberry extracts supplementation as a functional food and nutraceutical, may help the prevention of urinary tract infections, and probably even the prevention of cardiovascular and gastroenteric diseases.
- Aiming to validate efficacy and safety of clinical applications as long-term RCTs, further investigations of the mechanisms of action are required.
- The quality of cranberry's polyphenolic fractions starts from the raw material, then extraction processing and formulation: a "*conditio sine qua non*" for a good food supplement.
- New non-conventional extraction methods are welcome to improve the quality of the extracts and reduce the overall costs.

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Chapter 7

Valorisation of bromelain from pineapple by-products

7.0 Introduction

Bromelain is a mixture of proteolytic enzymes that is extracted primarily from pineapples (*Ananas comosus*) that is well known and used in several fields, especially in the nutraceutical and cosmeceutical sectors [1]. It was first identified by *Marcano* in 1891 in pineapple juice, and *Heinecke* discovered higher amounts of bromelain in the stem than the fruit in 1957 [2]. Although bromelain was initially defined as the mixture of enzymes produced in the fruit, today, this term includes any protease extracted from any member of the *Bromeliaceae* family [3]. Pineapple is the most common plant from which bromelain is extracted. The proteases that constitute bromelain are cysteine endopeptidases, which catalyze the hydrolysis of the peptide bonds of non-terminal amino acids [4]. Bromelain usually includes glycosidases, phosphatases, ribonuclease, cellulases, peroxidases and glycoproteases, which primarily cleave alanyl, glycyl and leucyl peptide bonds. These enzymes are present in different ratios that depend on a number of factors, including the geographic location of the plant, the land and mode of cultivation, the extraction method, and the part of the plant used [5]. In fact, although bromelain is present in almost all of the aerial parts of the plant—the peels, leaves, stems and fruit—only the fruit and stems are rich in bromelain. In particular, the bromelain in pineapple stems (EC number 3.4.22.32) is the most abundant protease, with an isoelectric point (pI) of 9.5 and optimum pH range of 6–7, while the bromelain found in pineapple fruit (EC number 3.4.22.33) is present in lower amounts, with a pI of 4.6 and an optimum pH range of 3–8 [6,7]. These significant differences in bromelain composition, probably due to the presence of different thiol-endopeptidases, may, in part, explain the great heterogeneity of clinical and preclinical results for potential effects on a wide category of conditions, including inflammatory diseases, cancer, immune dysfunction and others [8].

The demand for bromelain is increasing quickly, and the reason for this great interest in the clinical field is related to its anti-inflammatory, antiedematous, fibrinolytic, anticancer, anticoagulative and antithrombotic properties that have been thoroughly described in the literature [3]. In addition, this enzymatic complex is used in other sectors, including cosmetics, breweries, flesh processing and tenderisation, and

textile industries [7,9,10]. However, the isolation and purification of bromelain from pineapple (fruit, stem, core, leaves) is a challenge and constitutes 70–90% of the total production cost of the final extract [11]. To date, the commercial cost of bromelain extracts is high, with prices hovering around 2400 USD/kg [12]. The development of methods to achieve highly purified bromelain in fewer steps is therefore necessary to decrease overall production costs [3]. Despite the new feasible methods of protein purification (e.g., membrane filtration, reverse micellar systems, aqueous two-phase extraction, chromatographic techniques) and the new biotechnological processes developed to mitigate production costs, several limitations still create problems for the efficiency of product recovery from crude-plant extracts and the effectiveness of the obtained extract [12]. In fact, the enzyme complex tends to be irreversibly inactivated at high temperatures (e.g., during the pasteurization process), while the progressive concentration of bromelain in crude pineapple juice during the purification process can induce spontaneous enzymatic deactivation [5].

7.1 Physicochemical Properties of Bromelain

Pineapple-stem bromelain belongs to the class of α + β proteins that are characterized by a single polypeptide chain, either 211 or 212 amino acids in length, and have an estimated molar mass of 22.8 kDa. The amino acid sequences of bromelain are homologous to those of papain, chymopapain and actinidin [13]. A single oligosaccharide chain has been reported to be conjugated to this polypeptide, making the molar mass of stem bromelain around 23.8 kDa [14].

Many studies have shown that the proteolytic activity of bromelain is only partly connected to its pharmacological effects, suggesting that evaluating the whole phytocomplex, including the non-protein factors [7], is of great importance. The most prominent cysteine endopeptidases in pineapple tissues, other than stem and fruit bromelain, are ananain and comosain, whose physicochemical characteristics are summarized in **Table 1** [15,16].

Bromelain remains intact for a relatively long time when stored at −20 °C, while its proteolytic activity is lost, via bromelain degradation, when it is heated to 100 °C for 10 min [15,17]. Khan and colleagues have demonstrated that the maximum activity

of glycosylated bromelain occurs at pH 7.0 and 30 °C [18]. The activity of the protease complex decreases by 17% at 40–60 °C and, upon lowering the pH to 3.0–4.0, the optimum temperature was found to decrease with a higher sensibility for the deglycosylated rather than the glycosylated form [18].

Bhattacharya et al. have investigated the activity of bromelain after storage at 30 °C for 30 days, and 4 °C for 60 days. Their results show a retention of activity after storage of 22 ± 2% and 44 ± 2% under these respective conditions, highlighting that there is an inverse correlation between storage temperature and the retention of enzymatic activity [19]. As for pH, bromelain activity was shown to increase up to pH 7.0, showing a decreasing trend thereafter [20].

Table 1. Physicochemical characteristics of pineapple endopeptidases and amino acid composition of both green and ripe fruits (adapted from Rowan and Buttle [15] and Murachi [21]).

Endopeptidase	Amino Acid Composition %			Molar Mass (kDa)	pI	$A^{1\%}_{280\ nm}$	Presence of Glycoproteins
		Green Fruit	Ripe Fruit				
Fruit bromelain	Lysine	7.8	8.3	25–31	4.6	19.2	Yes
	Histidine	1.4	1.3				
	Arginine	8.6	9.1				
	Aspartic Acid	29.8	29.8				
	Threonine	13.8	13.8				
	Serine	32.2	32				
	Glutamic Acid	23.2	23.4				
	Proline	11.6	12				
	Glycine	32.6	32.2				

	Alanine	23.8	24.4				
	Cysteine	10.0	10.0				
	Valine	19.8	20.1				
	Methionine	6.0	5.8				
	Isoleucine	16.4	16.2				
	Leucine	10.0	10.0				
	Tyrosine	22.4	22.2				
	Phenylalanine	7.6	8.0				
	Tryptophan	5.6	-				
Stem bromelain	-			23.8–27	9.5	20.1	Yes
Ananain	-			23.4–25	>10	16.5	No
Comosain	-			24.5	>10	-	Yes

pI: isoelectric point, $A^{1\%}_{280\ nm}$: absorbance.

6.2 Extraction and Purification Methods

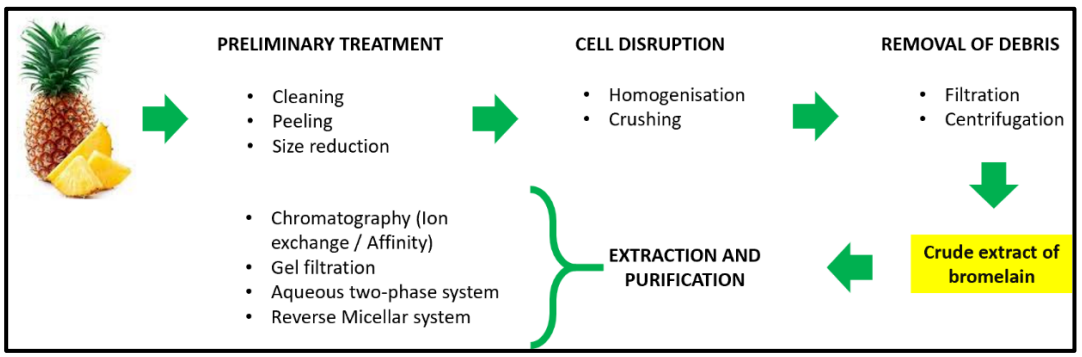
In general, plant proteases are produced in the early stages of fruit development, as has been demonstrated for papayas [22]. However, bromelain is extracted from pineapple when the fruit is mature, with the highest concentration of proteases only being reached at that point [23].

As highlighted in the introduction, the production of bromelain is expensive (price for extract: 2400 USD/Kg) [12], as it entails several manufacturing processes, including extraction, purification, drying and packing. Purification is the most significant of all these processes, with it accounting for at least two-thirds of the total cost of bromelain production [5]. Furthermore, no guidelines for bromelain purification are currently available.

The starting product, which can include the fruit, stem and other parts of the pineapple, is usually ground, without adding water, to obtain a raw crude extract. Subsequently, the extract (cooled pineapple juice) is processed further using

conventional methods, such as centrifugation, ultrafiltration and lyophilisation, giving a yellowish powder (Figure 1) [24]. However, several unconventional methods have been developed in recent years to improve both enzymatic yield and purity (**Table 2**) [25].

Figure 1. Overview of bromelain extraction and purification methods. Conventional approach: centrifugation, ultrafiltration and lyophilisation.



The conventional approach to bromelain extraction is currently the most widely used. It consists of a first phase in which the raw extract is homogenized to disrupt pineapple cells and it is then centrifuged to remove the particulate material without denaturing the proteases [26]. The enzymatic concentration of stem bromelain has been reported to be higher than that of fruit bromelain [24]. Although centrifugation produces a homogenous extract, subsequent processing is required to eliminate or decrease the quantities of impurities [27]. Ultrafiltration (~100 min) is followed by centrifugation, which is the gold standard for protein concentration, using membranes with molecular weight cut-offs from 3 to 100 kDa, leading to an ~8.9-fold increase in bromelain concentration. Convection (driven by difference in pressure applied to the membrane) and diffusion (driven by concentration gradient across the membrane) are the two main mechanisms that influence mass transfer during membrane filtration [28]. Other effects that can influence mass transfer include dielectric exclusion, steric effects and the Donnan equilibrium [29].

Membrane filtration, used in combination with other processes, has been proven to improve bromelain purification. A study by Doko et al. has reported a yield of 98%

for combined microfiltration, ultrafiltration, ammonium sulphate precipitation and ultracentrifugation, although proteolytic activity was found to be lower by the end of the processes [30]. Chao et al. have obtained 64.7% activity recovery and a 5.3-fold concentration using nano-TiO_2 and ultrafilters [31]. The simultaneous use of both microfiltration (plain membrane) and ultrafiltration (10 kDa Millipore kit) resulted in activity recovery of 90% and bromelain that was concentrated 10-fold [32]. Hebbar et al. have used the combined processes of a reverse micellar system and ultrafiltration to purify bromelain from pineapple stems. They demonstrated that bromelain is retained using membranes of 5 kDa with an activity recovery of 95.8% and a 5.9-fold concentration [33].

The last purification step entails lyophilisation, also known as freeze drying, which removes water from the ultrafiltered bromelain [34]. During the transition of water from a solid to a gas, some proteins and in particular enzymes may be denatured as they shift from a "molecular state" (proteins soluble in water) to a "particle state" (proteins without water). A decrease in bromelain enzymatic activity of more than 50% has been observed when a lyophilisation step was carried out at the end of a purification protocol [9]. For this reason, stabilizers, such as cryoprotectants, are required to maintain protein stability during the lyophilisation process.

Bresolin and colleagues have used ammonium sulphate precipitation in combination with the desalting and freeze-drying of pineapple peel and obtained an activity recovery of 75% (after lyophilisation, alone, the specific activity of bromelain decreased to 5.2% of its original value) [8]. Similar results have been obtained by Devatake et al. with a bromelain fruit extract [35].

7.2.1 Unconventional Approaches

Although conventional methods are frequently and widely used both in laboratories and on an industrial scale, they often present limitations, principally in terms of yield, purification and processing costs. Modern techniques permit the above-mentioned conventional approaches to be used in association with unconventional processes, including gel filtration, aqueous two-phase extraction, extraction via reversed micelles, ion exchange and affinity chromatography. The use of both types of

approaches allows an initial pre-purification step, which concentrates the enzymatic proteases to be performed with a subsequent step to remove the remaining impurities [5]. The most appropriate technique for the isolation of bromelain also depends on the intended application of the purified enzyme. Nevertheless, a combination of low-resolution (filtration and liquid–liquid extraction) and high-resolution techniques (chromatography) is probably the best approach to limit costs, provide high yields and low-impurity extracts and to retain enzymatic activity.

7.2.1.1 Aqueous two-phase systems

Aqueous two-phase systems are a low-cost, environmentally friendly, rapid and scalable technique for the extraction and purification of many compounds, and involves a reusable polymer and a salt, or two reusable polymers [36]. In general, this approach separates proteins and prevents denaturation while removing undesirable contaminants, such as pigments and polysaccharides [37]. However, the structure of bromelain means that few organic solvents are well tolerated by the enzymes in the extract [38]. Using this method Sankaran et al. have obtained pure bromelain with significant protease activity (stem: 4.0 units/mg; fruit: 3.6 units/mg) when a mix of polyethylene glycol (PEG) (8% *w*/*w*) and ammonium sulphate (15% *w*/*w*) was used [39]. Similar results have been obtained using a PEG/potassium phosphate system [27].

Ketnawa et al. have used several biphasic systems, containing PEG and salts at different concentrations, to test the purification of bromelain from pineapple peel. The highest enzymatic activity and yield was obtained with PEG-3000 (15% *w*/*w*) and magnesium sulphate (20%) in water (enzyme activity: 5.23 units/mg) [40].

Finally, an aqueous two-phase system used to extract and purify bromelain with polyphenol oxidase provided a four-fold concentration from the starting material [27].

7.2.1.2 Chromatographic techniques

Ion exchange chromatography is one of the most commonly employed of the chromatographic methods used for bromelain extraction, and has proven to be a low-

cost, highly specific, scalable and reliable technique [41]. Cation exchange chromatography has been used to separate ananain in combination with affinity chromatography [42]. However, better purification (85% of the initial active proteases) has been obtained using acetone precipitation in combination with the two above-mentioned chromatographic techniques [17].

Bresolin et al. have demonstrated the efficacious separation of the enzymatic complex from polysaccharides using ion exchange chromatography (removal of 97.7% of the polysaccharides initially present in the crude extract) [8].

Devakate et al. have separately employed salt precipitation and ionic exchange chromatography to extract bromelain from clarified pineapple juice. The latter technique led to a 10-fold enrichment in bromelain (from 58 to 590 units/mg) and gave a three-fold higher yield than salt precipitation [35]. A bromelain preparation with comparable enzymatic activity (390 units/mg) has been obtained by Costa et al. using a combination of cation exchange and size exclusion chromatography [11]. Hernández et al. have also tested the combination of cation exchange and size exclusion chromatography, and this approach resulted in the recovery of 41.15% of enzymatic activity, in relation to the initial stem extract [43].

Affinity chromatography is another successful technique for bromelain extraction. Bobb et al. have managed to isolate stem bromelain from other impurities by eluting pineapple-stem extracts through agarose-ε-aminocaproyl-D-tryptophan methyl ester (ACTME) [44]. Similar results have been obtained by Rowan et al. using a Sepharose-Ahx-Phe-GlySc column [17]. The effective separation of recombinant bromelain has also been performed using a different type of affinity chromatography, based on a nickel ligand (Ni-NTA His Bind resin) [25]. Indeed, Amid et al. obtained an increased purity grade (41-fold) for the recombinant bromelain expressed in the BL21-AI E. coli strain using this method [45].

High-speed counter-current chromatography has been tested by Yin et al. in combination with a reverse-micelle solvent system to purify bromelain; 3.0 g of bromelain was generated from 5 kg of pineapple fruit [46].

7.2.1.3 Reversed micellar extraction

Reversed micellar extraction (RME) is a feasible, relatively low-cost and energy-efficient process that is simple to scale-up and is characterized by protein solubilisation towards micellar inner polar cores. This technique can selectively separate enzymes and proteins such as those contained in the raw bromelain extract. This method involves two steps named forward and backward extraction; the first is a selective solubilization of proteins into reverse micelles and the second is the release of proteins from the micelles to an aqueous phase. Chaurasiva et al. have reported a recovery of 85% (four-fold purification) of bromelain using this approach [47]. Hebbar et al. have obtained significant bromelain recovery using small-scale (10 mL) RME purification methodologies. Similar results have been obtained at a larger scale, although a small loss in bromelain recovery was observed [48].

7.2.1.4 Precipitation methods

Precipitation is a technique that is based on the addition of a range of precipitating agents: (1) salts, (2) non-ionic polymers, (3) organic solvents (ethanol, propanol, methanol, ketones, etc.). An alternative method is based on pH change, which alters the nature of the solution.

Organic solvents have been used successfully for bromelain purification. In fact, ethanol (30–70%) has been used for extraction, leading to a recovery of 98% of the initial enzymatic activity [9]. Ethanolic extraction gave higher yields than sulphate and isoelectric precipitation [49]. Soares et al. have compared different precipitation agents; ethanol, (NH4)2SO4, and PEG. The most effective agent was ethanol, which gave higher enzyme recovery (30–70% vs. 20–40%) than ammonium sulphate, whereas PEG failed to precipitate bromelain [9].

These methods have also been extensively combined with other purification techniques (e.g., chromatography) to remove impurities and contaminants, as well as to improve enzyme purity and specific activity [5,8,20,35].

7.2.1.5 Novel strategies with recombinant technologies

Recombinant DNA technology is a well-known methodology used to produce about 90% of the enzymes on the market as a whole [50]. The production of recombinant cysteine proteases, such as papain from Pichia pastoris [50] and glycyl endopeptidase from E. coli [51], has been reported in the literature. The first attempt to obtain recombinant bromelain was carried out by Jung et al. who cloned the BAA1 gene that encodes fruit bromelain in Brassica rapa [51]. The stem-bromelain gene, from Ananas comosus, has been cloned by Muntari et al. and gave higher enzymatic activity than commercial bromelain, with the optimum temperature and pH being 45 °C and 4.6, respectively [50]. Recombinant bromelain from Ananas comosus showed higher thermal stability, as highlighted in a study by Nurul et al. [52]. Recombinant stem bromelain also showed higher potential as an anti-microbial agent (in particular vs. *E. coli* O111), compared to the commercial product [53].

Table 2. Unconventional techniques for the extraction and purification of bromelain (adapted from Manzoor et al. 2016 [54]).

Purification Technique		Activity Recovery (%)	Purification Fold/Factor	Reference
Aqueous two-phase extraction	PEG/K_2SO_4	228	4.0	[27]
	PEG/polyacrylic acid	335.27	25.78	[55]
	PEG/$(NH_4)_2SO_4$	-	11.80	[38]
	PEG/$MgSO_4$	113.54–206	2.23–62	[10,39,56,57]
	Block copolymers	79.5	1.25	[36]
Filtration	Microfiltration and ultrafiltration	85–100	10	[32]
	Microfiltration, ammonium sulphate precipitation, ultracentrifugation	50	-	[30]

	Nano-TiO_2, ultrafiltration	64.75	5.3	[31]
Reverse micellar systems	Reverse micelle systems	85–97.56	4–5.2	[45,46,48,58]
	Affinity-based reverse micelle system	185.6	12.32	[59]
Chromatography	High speed counter-current chromatography, reverse-micelle system		-	[45]
	Immobilized metal affinity membrane	94.6	15.4	[60]
	Precipitation, ion exchange chromatography	-	3.3	[35]
	Cation exchange chromatography	-	10	[61]

7.3 Comparison of Bromelain Extraction Techniques

The direct comparison of the published data on bromelain extraction techniques is a difficult task because several types of bromelain extracts and test conditions were used. Purification level and activity are the two main parameters used to assess the differences between extraction techniques (Table 3). The aqueous two-phase extraction technique showed the highest purification factor even if it involved a strong reduction in enzymatic activity [62]. Moreover, despite this strategy being considered a rapid and scalable technique, one of the main disadvantages of this process is the high salt concentration and the critical recovery that hamper recycling. Precipitation has a high activity recovery, second only to ultrafiltration, in addition to being a low-cost technique and, for this reason, the most used for commercial bromelain extracts. Nevertheless, the purification fold with this method is generally low and it presents the same problem as the aqueous two-phase extraction technique of high precipitant content (waste environmental impact) [20]. Ion exchange chromatography is the most expensive technique for several reasons: the low recovery and separation efficiency, and the small sample loading capacity [35]. However, it can produce satisfactory results especially when combined with other

techniques such as gel filtration. In this regard, one of the challenges for the coming years is the study of new hybrid extraction techniques to optimize costs, reduce environmental impact and increase extract yield and purity. Limited information is available for the use of affinity membranes, despite the fact that the potentially high selectivity could represent an important advantage for bromelain extraction [63]. Lastly, the ultrafiltration technique seems to be the most promising for bromelain large-scale extraction operation because it is easily scalable to high product throughput and environmentally friendly [32]. To date, the most promising and economically viable results have been obtained by combining different processes such as the reverse micellar systems with ultrafiltration [33] or microfiltration prior to ultrafiltration [64].

Table 3. Comparison of the different bromelain separation techniques.

Separation Method	Purification Fold (-)	Activity Recovery (%)	Advantages	Limitations	Reference
Aqueous two-phase extraction	16.3	55.6	Aqueous medium Low cost	Poor knowledge on mechanisms; High salt content	[62]
Ion exchange chromatography	10	84.5	Mild operation condition	High cost; Difficult optimization due to the complexity	[35]
Precipitation	4.9	85.97	Low energy needed Many alternative as precipitants	High precipitant (salt) content, hardly reciclable	[20]
Ultrafiltration	10	90	High product throughput Environmentally	Membrane fouling	[32]

			friendly Easy scaling up		
Affinity membranes	2.5	-	High selectivity	High costs and difficult monitoring	[63]

7.4 Bromelain Enzymatic Activity Measurement

The detection of the enzymatic activity of the various bromelain extracts and preparations is fundamental to assessing their bromelain content, which reflects the yield efficiency of the extraction and purification techniques, as well as the stability of the proteolytic activity under operational and storage conditions (pH, temperature). A number of different substrates can be used to measure bromelain activity, ranging from casein, gelatin and hemoglobin to the more sensitive casein and albumin azo-derivatives and various artificial model peptides.

Casein, which can also be used in the form of sodium caseinate, is the most frequently used substrate for proteolytic activity measurements [65], due to its high availability and low cost. Proteolytic-activity measurements are usually performed on 0.5–1% casein solutions in buffers at neutral pH over various time periods, ranging from 5 to 30 min, after which the reaction is stopped via the addition of trichloroacetic acid (TCA) [9,35,40,48]. The amount of soluble peptides released by bromelain is detected at 275–280 nm and usually compared with a standard curve obtained using tyrosine solutions at increasing concentrations to calculate the enzymatic activity. It is worth noting that thiol-based reducing agents and EDTA are required for the optimal measurement of enzymatic activity [66]. The reducing agents prevent the oxidation of catalytic cysteine, whereas the EDTA chelates metals that may interfere with the enzymatic activity [67,68].

The proteolysis of azocasein (1% solution in buffers at pH 6.5–7.5) results in the formation of peptides with histidine and tyrosine diazo sulphonic acid analogues that remain in solution after the reaction mixture is treated with 5–10% TCA. Azo-derived soluble peptides can be detected spectrophotometrically at 440 nm after the addition of an alkali solution with no significant interference from other colored

compounds [8,19,55]. Activity measurements are usually carried out at 37 °C, both in the case of casein and azo-casein, although a number of studies have reported that the optimum temperature for stem and fruit bromelain lies in the 50–60 °C range [55].

Bromelain proteolytic activity can be conveniently measured using a number of synthetic substrates that simulate model peptides, which release molecules that are easily detectable by fluorescence and/or spectrophotometry. Although more expensive than the previously described substrates, Z-Arg-Arg-p-nitroaniline (pNA), Bz-Phe-Val-Arg-pNA, pGlu-Phe-Leu-p-nitroanilide and N-Cbz-L-Gln-p-nitrophenyl ester are widely used as they allow continuous measurements of enzymatic activity to be performed, with free pNA being detected at 400–410 nm [15,69–71]. The peculiar chemical structure of these synthetic compounds also facilitates the easy detection of bromelain sources, since the enzymes derived from the various plant parts have shown preferential hydrolytic activity towards these substrate types [15]. In particular, the Z-Arg-Arg substrate is specific for stem bromelain, whereas fruit bromelain and ananain have shown more preferential activity towards the Bz-Phe-Val-Arg substrate [15,72,73].

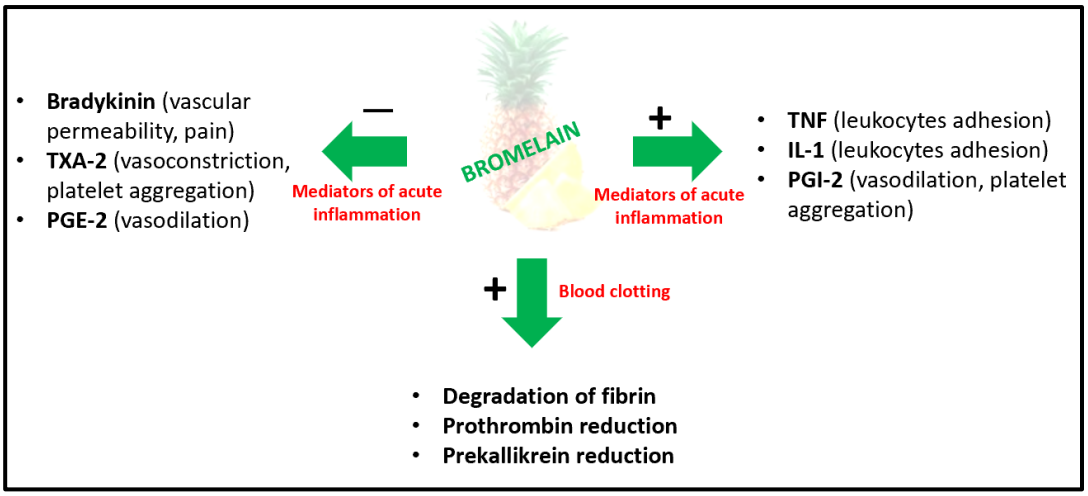
7.5 Pharmacodynamics and Pharmacokinetics Profiles of Bromelain

The mechanisms of action of bromelain have not yet been fully established. However, the anti-inflammatory activity of this supplement on three different pathways has been highlighted in several in vivo and in vitro studies. The first is the kallikrein-kinin pathway; bromelain regulates the plasma fibrinogen levels and blood levels of bradykinin and improves serum fibrinolytic activity by activating factor XI, which subsequently activates plasma prekallikrein [54,74]. Secondly, bromelain acts on the arachidonic acid pathway (Figure 2); it modulates proinflammatory prostaglandins (via the inhibition of prostaglandin E2 and thromboxane A2) and enhances the anti-inflammatory mediators, increasing platelet cyclic adenosine monophosphate (cAMP), and, thus, the levels of prostaglandin (PG) I2 and PGE1. Finally, bromelain modulates cell-migration immunity: acting on the migration of neutrophils to inflammation sites [75]. In detail, it removes several cell-surface molecules

(including CD128a/CXCR1, CD128b/CXCR2, CD14, CD44, CD16 and CD21) that are vital to leukocyte trafficking, cellular adhesion, the induction of pro-inflammatory mediators and immunomodulatory effect on T cells. It also reduces P-selectin-mediated neutrophil recruitment (Figure 1) [76]. These actions permit bromelain to be potentially effective in several conditions associated with inflammation, with or without oedema, and alterations in blood coagulation. In fact, RCTs that have been carried out highlight the activity of bromelain in diseases such as rheumatoid arthritis, osteoarthritis, perioperative sport injuries, cancer, cardiovascular diseases, chronic rhinosinusitis and skin wounds and burns, which are all conditions with an inflammatory component [3].

The pharmacokinetics profile of bromelain is still unclear because of difficulties determining this component in serum and the poor knowledge of its bioaccessibility and bioavailability. It would appear that bromelain is absorbed into the small intestine, rapidly forming a complex with anti-proteases (α2-macroglobulin and α1-trypsin) [7]. On the other hand, the possibility that bromelain penetrates the lamina propria intact has been underlined, with α2-macroglobulin-bromelain complexes being formed, leaving bromelain proteolytic activity untouched [15]. Bromelain is considered to be a safe nutraceutical. As reported by Moss et al., it is impossible to determine the LD50 (mg/kg body weight) of this supplement at doses above 10 g/kg per os in mice, rats and rabbits. However, the LD50 for intraperitoneal administration is 37 mg/kg and 85 mg/kg for mice and rabbits respectively, while for intravenous administration it is 30 mg/kg and 20 mg/kg [77]. No relevant side effects have been observed at doses of up to 2000 mg/Kg per os, even with prolonged periods of administration [7]. However, although bromelain is well tolerated and considered a safe nutraceutical, clinical trials have reported a few side effects, including transitory diarrhea, nausea and vomiting, allergic reactions and a risk of bleeding, especially in people treated with anticoagulant drugs, such as warfarin and clopidogrel [78].

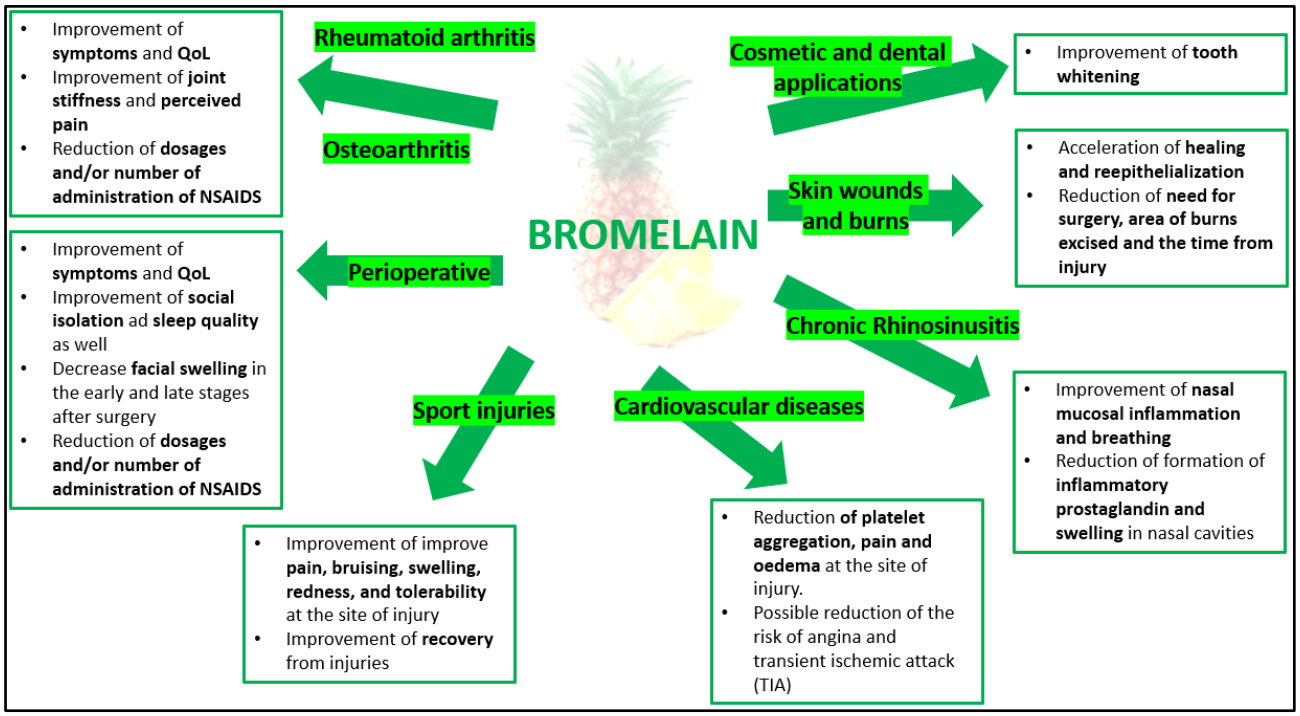
Figure 2. Bromelain anti-inflammatory and anticoagulant mechanisms of action.



7.6 Bromelain Studies

The clinical applications of bromelain include a wide range of conditions that are generally characterized by an inflammatory component that may be associated (or not) with oedema and alterations in blood coagulation. These therapeutic applications are discussed below and summarized in Figure 3.

Figure 3. Bromelain and its potential clinical applications.



7.6.1 Perioperative

A recent meta-analysis of six RCTs demonstrated that bromelain alleviates postoperative pain seven days after mandibular third molar surgery (p = 0.002) and decreases facial swelling in the early and late stages after surgery (respectively p = 0.02 and p = 0.0004) [79]. Similar results were obtained in previous meta-analyses carried out by Mendes et al. [80], de Almeida et al. [81] and de Souza et al. [82], that also showed improvements in social isolation and sleep quality.

In another RCT, sixty patients with long bone fractures were treated with 810 mg/day bromelain for the first three days after surgery and 480 mg/day in the remaining follow-up period, with the antioedematous drug aescin being used as the control. A significant decrease in post-traumatic and post-operative swelling and pain was observed in patients who were administered bromelain. Healing acceleration was also observed compared to the control group (to whom aescin was administered) [83].

In another RCT, eighty-two primiparous women were treated with bromelain and benefited from reductions in pain (measured on the VAS scale), ecchymosis and oedema caused by episiotomy compared with a placebo group ($p < 0.05$ for all). In addition, wound healing was faster in the bromelain group than in the placebo group ($p < 0.05$) [84]. Howat et al. also demonstrated the existence of a trend of reduction in pain and ecchymosis, although the differences did not reach statistical significance [85].

Seltzer and colleagues reported the ability of bromelain to decrease perioperative oedema and ecchymosis in 53 rhinoplasty cases [86].

7.6.2 Osteoarthritis and Rheumatoid Arthritis

The anti-inflammatory and analgesic properties of bromelain were first reported in 1964 for the treatment of both osteoarthritis (OA) and rheumatoid arthritis [87]. Several RCTs underlined the potential ability of this nutraceutical to decrease pain and inflammation in people with knee and hip OA [88]. In one of the most recent RCTs, 16-week supplementation with bromelain (500 mg/day) and diclofenac (100 mg/day) was tested in mild-to-moderate knee OA patients. At the end of treatment, the effects of bromelain and diclofenac in mitigating the symptoms of mild-to-moderate knee OA were comparable [Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC)] [89]. Similar results have been obtained in people with hip OA [81]. Another study included forty men and women with lumbar spine osteoarthritis who were divided into two groups to receive, for six weeks, either aceclofenac 100 mg b.i.d or aceclofenac 100 mg b.i.d with enzyme supplements (bromelain) 250 mg b.i.d. The patients treated with bromelain reported significantly diminished pain scores (visual analogue scale and Oswestry low back pain questionnaire, respectively $p = 0.001$ and $p = 0.000$) and improved life quality, compared with patients treated with aceclofenac alone [90]. Bromelain's potent analgesic and anti-inflammatory properties were highlighted when administrated to patients suffering from chronic osteoarthritis (both pain-associated and not), even when used in association with other nutraceuticals including *Curcuma longa*, *Harpagophytum procumbens* and/or Boswellia serrata [91]. Bromelain potentially

acts as a non-steroidal anti-inflammatory drug (NSAIDS) painkiller, as it reduces the dosage and/or the number of administrations of conventional treatments [92].

7.6.3 Sport Injuries

In recent years, bromelain's properties have been tested in the treatment of sport-related muscle injuries and in relieving occasional post-exercising muscle pain [93]. Muscle pain and apparent stiffness that is experienced after training (so-called "delayed onset muscle soreness" (DOMS)) is one of the most important limitations for athletes who perform repeated strenuous exercises [94]. In this regard, bromelain has been shown to attenuate the occurrence of contraction-induced skeletal muscle injury, which is considered to be one of the causes of DOMS [95]. However, in a recent study, forty subjects that were treated with either bromelain (300 mg t.i.d.), ibuprofen (400 mg t.i.d.), placebo (t.i.d.) and a control group demonstrated no significant improvement in their active range of motion and perceived pain after an eccentric exercise protocol (bromelain and ibuprofen seem to be ineffective and comparable to the placebo) [96]. In addition, the supplementation of bromelain with rutoside before and after a marathon race failed to attenuate post-race inflammation, neither did it decrease the incidence of upper-respiratory illness in runners [97].

In a multicentre, double blind RCT involving 27 European hospitals, 721 patients with acute unilateral sprains of the lateral ankle joint were treated with a triple combination (bromelain, rutoside and trypsin), double combinations, single substances or a placebo. The results showed that bromelain had no remarkable impact on decreasing symptoms compared to either the double combinations and/or the other single substances. However, significant improvements in pain, swelling and range of motion were observed compared with the placebo [98]. In contrast to these results, patients with sport-related ankle injuries that were treated with bromelain in a double-blind placebo-controlled trial had faster recovery times than the control group [99]. Even the double-blind studies of Zuschlag [100], Deitrick [101] and Rathgeber [102] demonstrated significant improvements in healing from sports injuries and faster haematoma resorption.

Finally, bromelain treatment has been shown to improve pain, bruising, swelling, redness and tolerability at the site of injury when administrated to patients suffering from blunt trauma injuries to the musculoskeletal system [103]. Moreover, in subjects with haematoma, bromelain induced more rapid resorption with a significant decrease in haematoma volume compared to the placebo group [104].

7.6.4 Acute Sinusitis, Rhinitis, Rhinosinusitis

Bromelain also acts as a mucolytic agent, decreasing mucus production while promoting natural drainage, the formation of inflammatory prostaglandin and decreasing swelling in nasal cavities. Several trials have investigated the role of this supplement in sinusitis, rhinitis, rhinosinusitis and chronic rhinosinusitis, although there is a lack of long-term data [105,106]. Seltzer et al. have treated 49 patients affected by sinusitis with either bromelain or a placebo. In association with the conventional treatment, the bromelain group showed general improvements in overall rating, breathing difficulties and nasal discomfort [107]. Similar results have been obtained by Ryan et al. who observed that bromelain administration improved nasal mucosal inflammation and breathing in people with acute sinusitis [108]. Finally, the administration of bromelain (average intake: six tablets = 3000 Fédération Internationale Pharmaceutique (FIP)) to 12 patients with chronic rhinosinusitis, both with and without nasal polyps, in a prospective, open-label, observational pilot study provided a significant improvement in total symptom scores (TSS), total rhinoscopy score (TRS) and quality of life (Sino-nasal Outcome Test 20) [98].

7.6.5 Cancer

In vitro and in vivo studies have demonstrated the anti-cancer effects of bromelain; it quite likely switches-off the NF-kB gene signal, thus inhibiting Cox-2 expression, activating several caspases and promoting apoptosis [109,110]. In addition, bromelain exhibits anti-metastatic properties, which it exerts via the upregulation of p53 and the activation of the autophagy mechanism [67]. The potential effects of this supplement, in terms of tumor-size reduction and/or regression and improvements in

chemotherapeutic-agent efficiency, have been highlighted in many studies carried out on several cell lines, including A-375 melanoma, P-388 leukemia, ADC-755 breast cancer, sarcoma (S-37), A431 epidermoid carcinoma and Lewis lung cancer [111–113]. Although there is a lack of RCTs to investigate the effects of bromelain on tumor progression, bromelain-related decreases in the side effects caused by conventional treatments have been evaluated by a number of research groups.

In a clinical investigation, 494 patients with breast cancer that were undergoing adjuvant hormone therapy and suffering from severe arthralgia as well as severe mucosal dryness were treated with a complementary adjuvant, which included bromelain in addition to papain, sodium selenite and Lens culinaris lectin. Two-thirds of patients showed an improvement in both arthralgia and mucosal dryness after four weeks, with the conventional treatments causing limited side effects ($p < 0.001$) [114]. Similar results have been obtained by the group of Uhlenbruck and colleagues [115]. Bromelain also stimulates the deficient monocytic cytotoxicity of mammary tumor patients [116] and promotes the apoptosis process [117].

Finally, bromelain supplementation, along with α-lipoic acid, Boswellia serrata and methylsulfonylmethane, has been shown to decrease chemotherapy-induced peripheral neuropathy in patients treated with neurotoxic chemotherapy [118].

7.6.6 Blood Coagulation and Cardiovascular Disease

Bromelain is effective, as an adjuvant, in the treatment of cardiovascular diseases, especially as it is known to possess antithrombotic and anticoagulant activities [67], although clinical trials are, in part, conflicting and require further confirmation. Bromelain has been demonstrated to reduce platelet aggregation, as induced by adenosine phosphate, blood viscosity and the risk of thrombus formation, while also improving the phosphorylation of Akt, and thus inhibiting cell apoptosis [119]. In vivo studies have shown that bromelain decreases the risk of angina pectoris and transient ischemic attack (TIA), although long-term RCTs are required to confirm this preliminary evidence [2]. Furthermore, bromelain possesses antihypertensive properties [7]. It has been reported that the oral administration of bromelain significantly reduces the risk of acute thrombophlebitis [2,120]. On the other hand,

bromelain supplementation did not significantly affect blood clotting in another clinical trial that included healthy volunteers [121]. Similar conclusions have been obtained in another trial in which supplementation with 160 mg/day of bromelain (for one week in patients with oedema and inflammation) did not affect bleeding, coagulation and prothrombin [122]. These contradictory results might, in part, be explained by both the limited sample size and dosage of supplemented bromelain. In fact, Errasti and colleagues have highlighted the dual action of bromelain on blood coagulation; at low dosage, this supplement seems to have a pro-coagulant effect, while having an anticoagulant effect at a high dosage [123].

7.6.7 Other Applications

Bromelain is also an interesting nutraceutical for cosmetic dental applications, in particular for tooth whitening. In a recent study, tooth bleaching gels containing bromelain, used in association with papain, have been demonstrated to be potentially effective in the development of peroxide-free tooth whitening gels [124].

Bromelain, used in association with other active ingredients, has also been observed to increase the function of macular pre-ganglionic elements, as demonstrated in a recent RCT that included thirty patients with intermediate age-related macular degeneration [125].

In a multi-center, open-label, non-comparative clinical trial, 398 women with endometriosis-associated pelvic pain were treated for six months with bromelain, N-acetyl cysteine and alpha lipoic acid. The treatment provided a significant improvement in endometriosis-associated pelvic pain (visual analogue scale >4 diminished from 92.7% to 82.7% ($p < 0.05$)) [126].

Bromelain supplementation in people with inflammatory bowel disease is another potential indicator of its efficacy. Although new RCTs are urgently needed to better understand the effects of bromelain on the prevalence, severity and sternness of colitis, quality of life and markers of inflammation, this supplement decreased the harshness of colonic inflammation. This effect is probably due to its proteolytic action, which most likely eradicates the cell-surface receptors involved in leukocyte defection and activation [127].

Bromelain has also shown relevant activity in allergic airway diseases [128], decreasing the serum levels of interleukins (IL)-4, -12, -13, -17 and interferon-alpha, bronchoalveolar lavage leukocytes (eosinophils and lymphocytes), cellular infiltrates and bronchoalveolar lavage CD4+, CD8+ T CD4+ and CD25+T cells [129]. However, there is also a lack of RCTs in this case.

Topical applications of bromelain have been tested for skin wounds, burns and the debridement of necrotic tissues [130]. Bromelain includes a specific fraction of escharase, which is considered to be the main debriding agent and can selectively act upon damaged tissues without harming healthy ones [2,131]. The escharase fraction has been demonstrated to accelerate healing and reepithelialization by simplifying the debridement process [132]. In a multi-center, open-label RCT, patients (4–55 years old) with deep partial and full thickness burns, covering 5–30% of their total body surface area, were either treated with a topical application of bromelain (for 4 h) or with the standard of care, which included either surgical excisional or non-surgical debridement. Treatment with bromelain significantly decreased the need for surgery, area of burns excised and the time from injury to complete debridement compared with standard care ($p < 0,0001$ for all) [133]. The updated guidelines of a multi-professional expert panel of plastic surgeons and burn-care specialists recommended the use of bromelain as an enzymatic debriding agent for burns [134]. In a preliminary study, eight patients with pityriasis lichenoides chronica were treated with bromelain (40 mg three times a day for one month, 40 mg twice a day for one month and 40 mg a day for one month) and showed an improvement in the condition that can probably be traced to its anti-inflammatory, immunomodulatory and/or anti-viral properties [135].

Although bromelain supplementation seems to be effective in many diseases, some limitations should be emphasized. Firstly, the sample populations considered in the studies were small and heterogeneous, the treatment period was limited and the bromelain dosages and types of extracts used were extremely variable, even when administered for the same disease. In addition, only a single clinical trial is available for most of the conditions mentioned in the literature. Finally, few data on bromelain safety are available, especially for long-term treatment. For these reasons, new long-

term RCTs are needed to allow the prescription of this supplement in clinical practice and shed light on the pharmacokinetic and pharmacodynamic profiles of this complex nutraceutical.

7.7 Discussion and Future Perspectives

Bromelain is one of the most thoroughly investigated proteolytic complexes that is characterized by several thiol endopeptidases [136]. It is extracted predominantly from pineapple stems and fruit [53]. However, a number of studies have underlined the possibility that bromelain can be extracted from by-products, such as the peels, core or crown, which would fulfil the requirements of a zero-waste approach [3].

Several studies have been carried out to evaluate the stability, purification and potential applications of bromelain, especially in the pharmaceutical and nutraceutical sectors [5,137]. While bromelain has been demonstrated to improve several conditions, thanks to its anti-inflammatory and anti-oedema properties, its mechanisms of action are yet to be completely understood [113].

Extraction techniques for bromelain include both conventional and unconventional methods (Table 4), which can also be combined in some cases [1]. Alternative methods for bromelain purification can be applied to achieve improved cost-effectiveness, purity and yields. In fact, one of the most important limitations to bromelain commercialization is the high cost of the extract (about 2400 USD/Kg), which is mainly due to the required purification processes [12]. Although unconventional technologies, such as chromatography, reverse micellar systems, gel filtration, aqueous two-phase systems and others, have been thoroughly investigated and demonstrated to improve purity and yield, compared with conventional techniques, none have presented an economic assessment of the processes involved. Moreover, there is still a lack of standardized international guidelines for bromelain extraction.

One of the most promising current strategies involves the production and purification of recombinant bromelain, which may decrease the overall process cost and improve bromelain yield and purity [50].

Considering the great medical and industrial interest in bromelain, future studies on convenient and effective extraction and purification techniques, the drafting of new international guidelines, an extensive costs analysis and the evaluation of process scaling-up are necessary and are a challenge that may decrease production steps and costs. In the clinical setting, new pharmacodynamics and pharmacokinetics studies are needed to better understand the potential applications of this nutraceutical in terms of efficacy and safety.

Table 4. Advantages and limits of bromelain purification using classic or unconventional methods.

Purification Approach	Technique	Advantages	Limits
Classic methods	Centrifugation Ultrafiltration Lyophilisation	↑ yield during pre-purification steps	↓ enzymatic yields
Unconventional methods	Gel filtration Ion exchange chromatography Affinity chromatography Aqueous two-phase extraction Reversed micelle extraction	↑ selectivity and purity of the final extract ↑ enzymatic yields ↓ number of processes for final bromelain extract ↑ efficacy of bromelain purification	↑ costs of product recovery stability (?)

7.8 Conclusions

- Bromelain extract is an interesting nutraceutical to be potentially used as an adjuvant in several conditions, including cardiovascular diseases, skin disorders, sport injuries, sinusitis and inflammatory diseases.
- However, the poor quality of clinical studies and the high cost of bromelain extraction and purification represent the main limitations to the use of these extracts.
- New comprehensive studies are needed to validate the efficacy and safety of clinical applications and long-term RCTs must be designed before therapeutical recommendations.
- Further investigations of the mechanisms of action are also required.
- In addition, despite the broad array of studies on the applications of unconventional extraction methods, there is still a lack of guidelines for the processing steps required to obtain the highest yields and enzymatic activity.
- Finally, new studies on the same matrices, comparing classic and non-conventional techniques or their combination, are also needed to optimize extract yield and activity, which may lead to an economically viable product.

6.9 References

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Chapter 8
Evaluation of the effect of a treatment based on bromelain extract from pineapple in paediatric subjects with orchiepididymitis: a randomized two-arm clinical study

8.0 Protocol synopsis

Title of the study	"**Evaluation of the effect of a treatment based on bromelain extract from pineapple in pediatric subjects with orchiepididymitis: a randomized two-arm clinical study"**
Trial code	ALTO2022
Rational	A) The orchiepididymitis represents an inflammatory condition of the epididymis and testicles particularly prevalent in pediatric children. B) Conventional treatments include the antibiotic therapy (amoxicillin + clavulanic acid) in combination with symptomatic therapies (anti-inflammatory/analgesic drugs). C) Among the most prescribed analgesic/anti-inflammatory drugs in children with orchiepididymitis, the ibuprofen represents the gold standard of conventional symptomatic therapies. However, the ibuprofen therapy is not free from side effects (e.g. dyspepsia, epigastralgia, diarrhea, nausea and vomiting, skin rashes). D) Bromelain is a cysteine protease complex known to the scientific community for its anti-inflammatory, anti-oedema and analgesic power, with an excellent safety profile. E) In this sense, although to date there are no clinical studies relating to the use of bromelain in orchiepididymites, this supplement has been particularly studied in various inflammatory conditions associated or not with the oedema component, demonstrating that it may reduce the doses or the number of administrations of conventional anti-inflammatory/analgesic drugs. F) Bromelain in the form of freeze-dried pineapple is also a functional food with an excellent palatability especially for the pediatric population.
Research center	Cesare Arrigo Children' Hospital, Alessandria
Researchers involved	Dr. Luciano Sangiorgio, Dr. Alessandro Colletti, Prof. Giancarlo Cravotto, Dr.ssa Marzia Pellizzato, Prof. AFG Cicero, Dr. Federico Leoni
Study design	This is a pilot, interventional, non-pharmacological, double-blind, single-center, randomized study

Tested products	Pineapple extract or placebo in addition to the antibiotic treatment for the disease
Study arms	Arm 1: 5,1 g pineapple extract + antibiotic therapy (amoxicillin + clavulanic acid 50 mg Kg/day) Arm 2: placebo + antibiotic therapy (amoxicillin + clavulanic acid 50 mg Kg/day)
Sample size	60 males between (ages 6-18)
Goals	• Comparison of anti-inflammatory/analgesic assumption between the pineapple group and the placebo group • Ultrasound parameters (oedema, scrotum size, epididymal commitment, vascularity) • Wong-becker pain rating scale (smileys) from 6 to 10 years • NRS pain rating scale (children >10 years) • VAS pain rating scale • Reporting adverse events • Adherence to therapy (card-diary)
Inclusion criteria	• Male subjects • Age between 6 - 18 years • Diagnosis of orchiepididymitis • Normal kidney function • Obtaining informed consent
Exclusion criteria	• Complex malformations of the urinary tract • Acute pyelonephritis • Any medical or surgical condition that makes the patient's adherence to the study protocol complex or inconstant • Allergies / intolerances to the active ingredient / excipients
Scheduled visits	• T0 (day 0): 1. Medical examination + Urinalysis and urine culture + Urine examination of renal function on single spot + Urinary ultrasound + QoL questionnaires 2. Randomization

	3. Delivery of diary sheet (bromelain / placebo YES NO, ibuprofen YES NO and if yes, daily quantity) 4. Start of treatment • T1 (day 7): 1. Medical examination + QoL questionnaires + Compliance assessment • T2 (day 15): 1. Medical examination + Urinalysis and urine culture + Urine examination of renal function on spot signs + Ultrasound of the urinary tract + QoL questionnaires 2. Evaluation of compliance 3. End of treatment
Variables collect	• Anamnesis and personal data (T 0): • Medical examination (T 0): • Drug history (T0, T1, T2) • Adherence to the proposed treatment (T1, T2) • Consumption of anti-inflammatory / analgesics • Tolerability and acceptability of the tested product (T1, T2) • QoL questionnaires (T0, T1, T2) • Urine analysis, urine culture, urine analysis of renal function on single spot (T0, T1, T2) • Echography of the urinary tract (T0, T1, T2)
Statistical analysis	The following statistical analyses will be conducted: - Complete descriptive analysis of all parameters studied: mean, mode, median, standard deviation, standard error of the mean, 95% confidence intervals, normality test - Comparative tests: comparison between treatment periods and merging data on the basis of randomization - Analysis by subgroups and advanced statistics will be conducted on the basis of the results obtained from the analysis of data in its entirety A significance level of $p < 0.05$ will be considered acceptable for all tests conducted
Timing	o Start of the study: July 2022

	o Enrollment deadline (indicative date): August 2022 o End of the study: March 2023

8.1 Abstract

Orchiepididymitis (OE) are characterized by the inflammation of the testis and epididymis and represent one of the most common causes of acute scrotal pain in adults and in paediatric populations. Among the most prescribed analgesic/anti-inflammatory drugs in children with orchiepididymitis, the ibuprofen represents the gold standard of conventional symptomatic therapies. However, despite those children with OE the NSAIDs shown a spontaneous resolution of the inflammation generally within a week, the ibuprofen therapy is not free from side effects. In the last years, clinical studies suggest that oral supplementation of nutraceuticals may help to reduce inflammation, pain and/or oedema in subjects with chronic inflammatory diseases, reducing the need for NSAIDs. Among the nutraceutical compounds, Brome-Inf is a freeze-dried extract of pineapple, highly concentrated in bioactive peptides and bromelain, marketable as a food supplement or functional food. In this sense, we performed a pilot, interventional, double-blind, single-center, randomized study, with the enrollment of 60 pediatric patients diagnosed with orchiepididymitis, who will be randomized to receive Brome-Inf or placebo, for 15 days, in addition to the antibiotic treatment for the disease. The primary endpoint of the study was to investigate the comparison of the need for ibuprofen intake between the pineapple group and placebo, and the intensity of pain by using the Wong-Becker pain rating scale and the Numerical Rating Scale NRS pain rating scale in addition to the Visual Analogue Scale (VAS) pain rating scale. At the end of study, a significant reduction in VAS, NRS and Wong-Becker pain rating scale was observed in the active group from day 1 and day 15. In addition, people in the pineapple group reported a slower need for ibuprofen compared to the placebo, without side effects and 100% of compliance of treatment. In conclusion, oral supplementation with pineapple extract could represent a potential option in the co-management of orchiepididymitis, improving quality of life. Palatability, safety and compliance were excellent.

8.2 Introduction

As discussed in the chapter 6, bromelain is a mixture of proteolytic enzymes which is extracted primarily from pineapples (*Ananas comosus*) [1]. The demand for bromelain is increasing year by year, and the reason for this great interest in the clinical field is related to its anti-inflammatory, antioedematous, fibrinolytic, analgesic, anticoagulative and antithrombotic properties which have been thoroughly described in the literature [2]. The proteases that constitute bromelain are cysteine endopeptidases, which catalyse the hydrolysis of the peptide bonds of non-terminal amino acids [3].

Despite that the mechanisms of action of this enzymatic complex are not fully understood, several *in vitro* and *in vivo* studies underline three different pathways of action: the improvement of the fibrinolytic activity by activating factor XI and regulating the kallikrein-kinin pathway, the modulation of arachidonic cascade and the production of inflammatory cytokines, and the limitation of the neutrophil's migration to inflammation sites [4]. These actions permit bromelain to be potentially effective in several conditions associated with inflammation, with or without oedema. In fact, RCTs that have been carried out highlight the activity of bromelain in diseases such as rheumatoid arthritis, osteoarthritis, perioperative sport injuries, cardiovascular diseases, chronic rhinosinusitis and skin wounds and burns, which are all conditions with an inflammatory component [5]. In this context, bromelain may contribute to also reduce the inflammation and oedema caused by orchiepididymitis.

Orchiepididymitis (OE) are characterized by the inflammation of the testis and epididymis which can include epididymitis, orchitis and true orchiepididymitis. They represent the most common cause of acute scrotal pain in adults [6]. However, in paediatric populations the ethology of OE differs from the adult form and remains poorly understood. In fact, despite that adult OE are most often of infectious origin, current literature offers several possible pathophysiological explanations for paediatric OE including a pre-existing urinary tract malformation or vesical dysfunction, bacterial infection from the urinary tract, post-infectious inflammatory condition, viral infection, auto-immune disease or vasculitis, trauma, or idiopathic

origin [7]. Other studies suggest the possible correlation between specific urinary tract malformation such as vesicoureteral reflux, ectopic ureters, prostatic utricle, posterior urethral valves, meatal stenosis, urethral stenosis and urinary reflux in the genital tract; nevertheless, clinical data is still lacking and in part conflicting [8,9,10]. For this reason, it is difficult to recommend specific investigation guidelines (urinary analysis, testicular and/or urinary tract and kidney ultrasonography, or micturating cysto-urethrogram) as well as the impact of antibiotic therapy that is systematically recommended is still debating, depending on several factors such as the age of the patient and the presence or not of the urinary tract infection [11,12].

In the pediatric department of the SS Antonio e Biagio and Cesare Arrigo Hospital, the local practice guidelines currently mandate to investigate every boy presenting a suspected OE with i) a blood test, ii) an urinalysis and iii) a testicular, urinary tract and kidney ultrasonography. All children diagnosed with OE receive a prescription of ibuprofen. Regarding the antibiotic treatment, it is limited to urinary tract infections (positive urinalysis).

Among the most prescribed analgesic/anti-inflammatory drugs in children with orchiepididymitis, the ibuprofen represents the gold standard of conventional symptomatic therapies [13]. However, despite those children with OE the NSAIDs shown a spontaneous resolution of the inflammation generally within a week, the ibuprofen therapy is not free from side effects (e.g. dyspepsia, epigastralgia, diarrhea, nausea and vomiting, skin rashes) [14]. In the last years, clinical studies suggest that oral supplementation of nutraceuticals may help to reduce inflammation, pain and/or oedema in subjects with chronic inflammatory diseases, reducing the need for NSAIDs [15]. Among the nutraceutical compounds, Brome-Inf is a freeze-dried extract of pineapple, highly concentrated in bioactive peptides and bromelain, marketable as a food supplement or functional food. In this sense, although to our knowledge there are no clinical studies relating to the use of bromelain in orchiepididymitis, this supplement has been particularly studied in various inflammatory conditions associated or not with oedema component, demonstrating that it could reduce the doses or the number of administrations of conventional anti-

inflammatory/analgesic drugs [16,17,18]. Moreover, bromelain in the form of freeze-dried pineapple is also a functional food with a good palatability especially for the paediatric population, and an excellent safety profile.

The aim of the study is to investigate the potential role of bromelain supplementation associated with the antibiotic therapy in paediatric population, in order (when possible) to reduce the need of non-steroidal anti-inflammatory drugs (NSAIDs) as well as to improve the quality of life in children with OE.

8.3 Materials and Methods

8.3.1 Study design and partecipants

This was a pilot, interventional, double-blind, single-center, randomized study. It involves the enrollment of pediatric patients diagnosed with orchiepididymitis, who will be randomized 1:1 to receive bromelain extract or placebo in addition to the antibiotic treatment for the disease. The study population will include 60 male patients (Figure 1) belonging to the SC Pediatric Urology of the SS Antonio e Biagio and Cesare Arrigo Hospital, with a documented diagnosis of orchiepididymitis. Participants were required to be aged between 6 and 18 years, with diagnosis of orchiepididymitis, but with preserved renal function. The presence of complex malformations of the urinary tract, acute pyelonephritis, any medical or surgical condition that makes the patient's adherence to the study protocol complex or inconstant, assumption of other supplements, allergies or intolerances to the active ingredient or excipients were the exclusion criteria.

Enrolled subjects presented a diagnosis of orchiepididymitis before the randomization of the study. After the obtained of informed consent (T=0), urinalysis and urine culture, urine examination of renal function on single spot and urinary ultrasound were included before the start of treatment. After that, subjects were

randomized to receive the nutraceutical (based on pineapple extract) or placebo for 15 days in addition to antibiotic therapy. The intervention period lasted 15 days. At T=1 (day 7) and at the end of treatment (T=2, day 15), patients were evaluated for clinical status, and by the execution of same the analysis in T=0, in addition to the evaluation of the compliance and the tolerability of the products. The study timeline is described in detail in Figure 2.

The study fully complied with the ethical guidelines of the Declaration of Helsinki and with The International Council for Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) Harmonized Tripartite Guideline for Good Clinical Practice (GCP). The study protocol was approved by the Ethical Committee of the SC Pediatric Urology of the SS Antonio e Biagio and Cesare Arrigo Hospital. All patients provided written informed consent to participate.

Figure 1. Flowchart of participants in the study

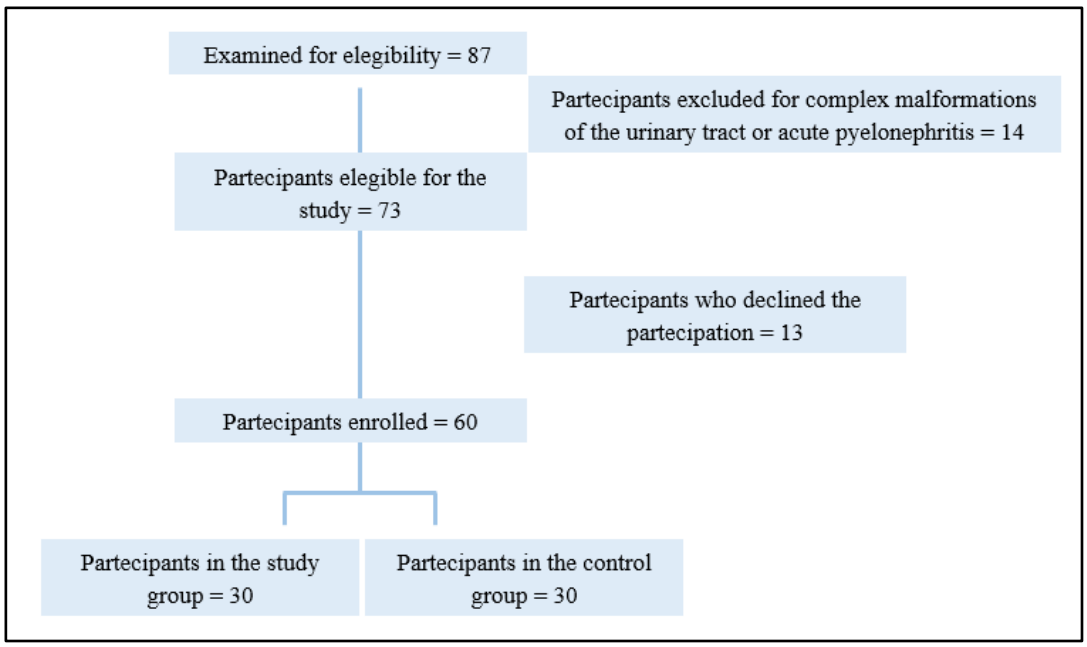
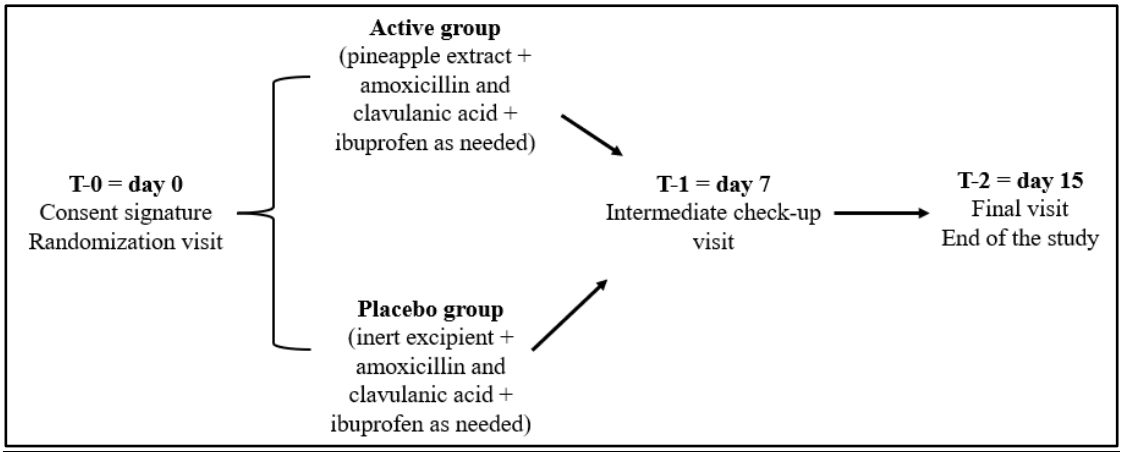


Figure 2. Study timeline



8.3.2 Treatment

At T=0, the enrolled subjects were randomized to receive daily either an indistinguishable powder of placebo (5,1 g/day) with amoxicillin + clavulanic acid (50 mg Kg/day) or Brome-Inf (5,1 g/day) containing a lyophilized pineapple extract titrated in bromelain (400 mg/day) with amoxicillin + clavulanic acid (50 mg Kg/day). The study products were manufactured and packaged by Studio 3 Farma srl (Torre di Mosto, Italy), in accordance with Quality Management System ISO 9001:2015. At the time of randomization, each patient was provided with boxes containing 76,5 g of powder (either active ingredients or placebo) and an appropriate measuring cup to take the correct amount of daily product. In addition, subjects were instructed to take ibuprofen 200 mg as needed, if pain became significant (for a maximum of 3 t.i.d.).

Randomization was performed centrally, by computer-generated codes. Participants and investigators were blinded to the group assignment. The alphanumeric codes (X and Y) of randomization were kept closed inside an envelope kept in the locked drawer of the main investigator's desk. They were opened at the end of the study by the principal investigator.

For the entire duration of the study, patients were instructed to take the assigned treatment once daily, at about the same time each day, preferably during the morning, in fasted state.

Participants' compliance was assessed by using a delivery of diary sheet (bromelain / placebo YES NO).

8.3.3 Assessment of the efficacy

The primary endpoint of the study was to investigate the comparison of the need for ibuprofen intake between the pineapple group and placebo and the intensity of pain by using the Wong-Becker pain rating scale (smileys: children from 6 to 10 years) and the Numerical Rating Scale NRS pain rating scale (for children >10 years) in addition to the Visual Analogue Scale (VAS) pain rating scale. Secondary endpoints were the evaluation of the ultrasound parameters (oedema, scrotum size, epididymal commitment, vascularity).

8.3.4 Assessment of the safety and tolerability

Safety and tolerability were evaluated through continuous monitoring during the study to detect any adverse event and clinical safety of treatments. A blinded, independent expert clinical event committee was appointed by the principal investigator to categorize the adverse events that could possibly be experienced during the trial as not related, unlikely related, possibly related, probably related, or definitely related to the tested treatment. The compliance of treatments and the occurrence of adverse effects were also monitored by using a delivery of diary sheet (bromelain / placebo YES NO; Ibuprofen YES NO, and if YES number of daily administrations; antibiotic YES / NO).

8.3.5 Statistical analysis

Personal data and physiological/pathological anamnesis were detected only at the enrollment visit (T-0), treatment compliance only in T2, the rest of the parameters at each visit from T0 to T2. As this is a pilot study, it is believed that a sample size of

60 subjects in the context of a randomized study design may be sufficient to evaluate the feasibility of the study, pending the planning of a possible larger controlled clinical trial. The following statistical analyses, conducted with the help of the SPSS 26.0 software, version for Windows, were provided:

- Complete descriptive analysis of all parameters studied with continuous distribution: mean, mode, median, standard deviation, standard error of the mean, 95% confidence intervals, Kolmogorov-Smirnov test of normality**;** the prevalence of factors in categorical variables have been described as absolute numbers and percentages.
- Comparative tests: the direct comparison has been carried out by treatment period and by combining data by type of treatment; for the normally distributed parameters a t-test for paired samples have been carried out in order to compare the parameters evaluated before and after each treatment phase, while a T-test for independent samples will be used to evaluate any differences between treatment groups; for variables not normally distributed the Mann-Whitney-U and Wilcoxon tests were performed, respectively for paired and independent samples. Data with categorical distribution was compared with the application of the chi-square test followed by Fisher's exact test.
- Analysis by subgroups and advanced statistics were conducted on the basis of the results obtained with the basic analysis.
- A significance level below 0.05 was considered statistically significant for all tests conducted.
- Statistical analysis was conducted for both ITT and PPT.

8.4 Results

Seventy-three people with acute orchiepididymitis were considered for the study. However, 13 patients were later excluded for the presence of complex malformations of urine system or because they declined the informed consent. Thus, 60 patients,

who had attended the follow-up visits and completed the study, were included in the final analysis.
The mean patient age was 12.8 (range 7 to 17). No statistically significant differences were found in the demographic characteristics of the people (Table 1).
Regarding the perceived pain a significant reduction of pain (VAS-10, NRS, Wong-becker) was observed in Brome-inf group from day 1 to day 15 (VAS-10: $p<0.0001$, NRS: $p=0.0006$, Wong-Becker: $p=0.0009$) (Table 2). Despite that the pain reduction was not statistically significant compared with placebo group, patients of active group reported a halved average intake of ibuprofen compared with placebo group (Table 3).

Regarding the secondary endpoints, Brome-Inf demonstrated a better regression of the oedematous component and improvement of vascularity evaluated by Echo Doppler analysis. In addition, through both the prader orchidometer and palpatory analysis, there was a faster normalization of testicular volume in the Brome-Inf group compared to the placebo group.

No side effects were reported during the treatments. Moreover, Brome-inf supplementation showed a good palatability and an excellent compliance and acceptability (100%).

Table 1. Average age and BMI of the subjects enrolled in the two groups.

	Placebo (n=30)	**Brome-Inf (n=30)**	**P value**	**Significativity**
Age (years)	12.7±3.4	12.9±4.0	0.9697	n.s.
BMI	23.7±0.4	23.5±0.3	0.6906	n.s.

Table 2. Number of people who have taken ibuprofen and average intake of grams of ibuprofen taken per person in the two groups.

	Placebo (n=30) From T=0 to T=1	**Brome-Inf (n=30) From T=0 to T=1**	**Placebo (n=30) From T=1 to T=2**	**Brome-Inf (n=30) From T=1 to T=2**	**P value**
Number of people who have taken ibuprofen	27	11	19	3	n.c.
Average intake of grams of ibuprofen taken per person	2,2 g±1.4	1.1±0.9	1,0 g±0.8	0,5 g±0.4	Placebo T0-T1 vs Brome-Inf T0-T1 P= 0.6327 n.s. Placebo T1-T2 vs Brome-Inf T1-T2 P=0.8107 n.s.

Table 3. Variation of VAS, NRS and Wong Becker scores of the subjects enrolled in the two groups.

Variable	**Placebo (n=30)**	**Brome-Inf (n=30)**	**P value***
VAS-10			
Day-1	8,6±1,4	8,7±0,7	0.9493 n.s.
Day-7	5,9±1,2	3,5±1,0	0.1299 n.s.
Day-15	3,5±1,0	1,4±0,7	0.0907 n.s.
P value (D1 vs D15)	0.0044**	<0.0001***	
NRS			
Day-1	8,2±1,4	8,3±1,2	0.9569 n.s.
Day-7	6,2±1,7	3,9±1,5	0.3146 n.s.
Day-15	2,6±1,7	1,3±1,5	0.5686 n.s.
P value (D1 vs D15)	0.0137*	0.0006***	
Wong-Becker			
Day-1	8,2±1,6	8,1±1,8	0.9670 n.s.
Day-7	5,6±1,8	3,5±1,3	0.3482 n.s.
Day-15	2,8±1,1	1,2±0,8	0.2443 n.s.
P value (D1 vs D15)	0.0073**	0.0009***	

n.s. not significant

n.c. not calculable

*significant p<0.05

**significant p<0.01

***significant p<0.001

8.5 Discussion

To our knowledge, this is the first clinical trial which investigated the analgesic effects of oral pineapple extract in people with orchiepididymitis. In this context, the reduction in VAS, Wong-Becker and NRS was more pronounced in the active group compared with the placebo group, suggesting that the nutraceutical supplementation reduced the need of ibuprofen. The analgesic effect of bromelain reported in this clinical trial is in agreement with other studies which investigated the utility of this supplement in different conditions associated with pain. Zatuchni et al. showed an improvement of rate of oedema, inflammation and pain in people affected by perineal pain [19]. These results were confirmed also in people subjected to a laparotomy [20], and in individuals with acute knee pain [21] or acute lateral ankle sprain [22]. In addition, Bromelain has favorable effect on well repaired episiotomy wound healing [23]. A meta-analysis of six RCTs demonstrated that bromelain significantly alleviates postoperative pain seven days after mandibular third molar surgery (p = 0.002) and decreases facial swelling in the early and late stages after surgery (respectively p = 0.02 and p = 0.0004) [24]. A significant decrease in post-traumatic and post-operative ecchymosis, swelling and pain was also observed in patients with long bone fractures [25] or under rhinoplasty [26] who were treated with bromelain. In addition, several RCTs underlined the potential ability of this nutraceutical to decrease pain and inflammation in people with knee and hip OA [27] with comparable effects to NSAIDs in mitigating the symptoms of mild-to-moderate OA [28]. In recent years, bromelain's properties have been tested in the treatment of sport-related muscle injuries and in relieving occasional post-exercising muscle pain [29]. Moreover, it showed to improve pain, bruising, swelling, redness and tolerability at the site of injury when administrated to patients suffering from blunt trauma injuries to the musculoskeletal system [29].

The mechanisms of action of bromelain have not yet been fully established. However, this enzymatic complex seems to act by removing different cell-surface molecules (including CD128a/CXCR1, CD128b/CXCR2, CD14, CD44, CD16 and CD21) which are important for leukocyte trafficking, cellular adhesion, induction of pro-inflammatory mediators and immunomodulatory effect on T cells. Bromelain also reduces P-selectin-mediated neutrophil recruitment, and it modulates proinflammatory prostaglandins though the inhibition of prostaglandin E2 and

thromboxane A2. In addition, it regulates the plasma fibrinogen levels and blood levels of bradykinin and improves serum fibrinolytic activity by activating factor XI, which subsequently activates plasma prekallikrein [30].

Compliance with Brome-Inf treatment was 100%, facilitated by the fact that the product was a lyophilized extract of pineapple, dispersible in water, with excellent palatability. Moreover, no side effects were reported during the study.

This study presents some limitations: the first one is the relatively small sample of enrolled patients. This was, however, just the first preliminary study aiming to evaluate efficacy and tolerability of pineapple extract in children with orchiepididymitis. Moreover, we did not measure the possible changes of plasmatic inflammatory markers which may be reduced following the treatment with nutraceutical, and thus elucidating the action of bromelain on inflammation. Definitely, the current study is preliminary, and further researches are needed to more deeply investigate the long-term effect of pineapple extract on a broader number of inflammatory parameters and before to definitively consider bromelain supplementation in clinical practice.

8.6 Conclusion

In conclusion the results underlined the effectiveness of Brome-Inf on young people with orchiepididymitis by reducing the perceived pain and the need for ibuprofen intake. Moreover, the safety profile and the palatability of the nutraceutical treatment were excellent. Further RCTs with larger sample size is necessary to consider the bromelain treatment in children with orchiepididymitis using different dosing strategies and possible combination with other nutraceuticals.

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Chapter 9
Evaluation of the effects of a dietary supplement based on pineapple bromelain extract on joint inflammation and on the intake of pain-relieving/analgesic drugs in subjects suffering from symptomatic knee osteoarthritis

9.0 Protocol synopsis

Title	**"Evaluation of the effects of a dietary supplement based on pineapple bromelain extract on joint inflammation and on the intake of pain-relieving/analgesic drugs in subjects suffering from symptomatic knee osteoarthritis"**
Code of the study	Brom_2022
Rational	G) Knee osteoarthritis is a highly prevalent and disabling chronic-degenerative disease. H) Since there is no pharmacological cure, the objective of the available treatments is to reduce the symptoms. I) Some nutraceuticals seem to improve the symptoms associated with gonarthrosis. J) Recent data suggest that oral Bromelain supplementation can help reduce the symptoms associated with gonarthrosis. However, the effect of bromelain in the form of freeze-dried extract directly from pineapple juice (functional food) has not yet been studied. K) There are no studies aimed at finding a better formulation to make the freeze-dried extract of bromelain more economically sustainable, exploiting the concept of the circular economy, starting from pineapple waste from the food chain.
Research center	Department of Medical and Surgical Sciences (DIMEC) of the Alma Mater Studiorum University of Bologna
Researchers involved	Prof. Arrigo F.G. Cicero, Prof. Claudio Borghi, Dr. Alessandro Colletti, Prof. Giancarlo Cravotto, Dr.ssa Marzia Pellizzato
Study design	Single center, randomized, double-blind, interventional study
Tested products	Dietary supplement based on freeze-dried pineapple extract (5 g, title 8% bromelain) vs placebo
Study arms	1 scoop/morning (empty stomach)
Sample size	N 40 subjects affected by symptomatic osteoarthritis of the knee
Goals	• Short (30 days) and medium (60 days) term evaluation of the effect of the nutraceutical tested on the Western Ontario McMaster Universities Osteoarthritis Index (WOMAC) scale and related subscales compared to the baseline.

	• Short (30 days) and medium (60 days) term evaluation of the effect of the nutraceutical tested on the Lequesne Functional Index (LFI). • Short (60 days) and medium (120 days) term evaluation of the effect of the nutraceutical tested on the Visual Analogue Scale (VAS) on the pain of the affected knee. • Short (30 days) and medium (60 days) term evaluation of the effect of the tested nutraceutical on the joint range of motion (ROM). • Short (30 days) and medium (60 days) term evaluation of the quality-of-life through the Short Form Health Survey (SF-36) questionnaire. • Short (30 days) and medium (60 days) term evaluation of the effect of the nutraceutical tested on the number of anti-inflammatories/analgesics used (diclofenac 100 mg) to reduce pain associated with knee osteoarthritis.
Inclusion criteria	• Aged between 40 and 85 years old • BMI<35kg/m2 • Symptoms ascribable to arthritic phenomena • Obtaining informed consent
Exclusion criteria	• Indication for infiltrative or surgical treatment of the affected joint. • Previous resistance to oral or systemic anti-inflammatory/painkiller treatments. • Oral infiltrative or chondroprotective treatment with hyaluronic acid in the 6 months prior to enrolment. • Any medical or surgical condition that makes patient adherence to the study protocol complex or erratic. • Previous intolerance to pineapple.
Scheduled visits	• T-1 (day -7/14): Screening visit • T0 (day 0): Randomisation visit • T1 (day 30): Intermediate check-up • T2 (day 60): Study closing visit
Variables collect	• Personal data* • Pharmacological history • Weight/Height/BMI • WOMAC and relative subscales compared to the baseline

	• Lequesne Functional Index • ROM of the knee under study • Use of anti-inflammatories/analgesics used to reduce pain associated with the study knee osteoarthritis • Adherence to the proposed treatment • Tolerability and acceptability of the tested product (Scale from 1 to 10)
Statistical analysis	The following statistical analyzes will be performed: - Complete descriptive analysis of all parameters studied: mean, mode, median, standard deviation, standard error of the mean, 95% confidence intervals, normality tests - Comparative tests: comparison between treatment periods and merging data based on randomization - Analysis by subgroups and advanced statistics will be conducted on the basis of the results obtained from the analysis of the data as a whole A significance level of $p<0.05$ will be considered acceptable for all tests conducted
Timing	• Start of the Study: December 2022 • Enrollment closure (indicative date): March 2023 • End of Study (indicative date): May 2023

9.1 Abstract

Osteoarthritis (OA) is the most common of joint pathologies, affecting approximately 58 million adults in the world. It is a pathological process that originates from the loss of the physiological balance between degenerative and reparative phenomena, primarily at the level of the articular cartilage, causing a global joint decompensation with the appearance of pain, stiffness and worsening of the quality of life. These symptoms can cause in the long term a greater risk of overweight/obesity, diabetes mellitus and falls and fractures. Despite the current guidelines for the treatment of OA suggest as the gold standard for this condition the pharmacological treatment characterized by non-steroidal anti-inflammatory drugs (NSAIDs), opioids and COX-2 specific drugs, a great interest has been applied to nutraceutical supplements which include a heterogeneous class of molecules of great potential to reduce inflammation, oxidative stress, pain, joint stiffness and improve the cartilage formation. In this context, we performed a double-blind, randomized clinical trial, conducted on 40 subjects with knee osteoarthrosis, for 8 weeks, in order to evaluate the effectiveness of a treatment based on lyophilized pineapple extract Brome-Inf (titred in bromelain). The primary outcome was to assess the need of NSAIDs (diclofenac 100 mg) in comparison with placebo group (after 4 and 8 weeks of treatment). Moreover, the improvement of the total Western Ontario McMaster Universities Osteoarthritis (WOMAC) index, WOMAC pain and WOMAC stiffness subscales, Lequesne Functional Index (LFI) and VAS were also evaluated. At the end of the study, the assumption of diclofenac (from day 0 to day 60) was statistically higher in the placebo group ($p<0,05$). All groups showed an improvement in WOMAC index, WOMAC pain and WOMAC stiffness subscales, LFI and VAS scale, both after 4 and 8 weeks of treatment, despite that the assumption of diclofenac was statistically higher in the placebo group. The administration of pineapple extract titrated in bromelain in people with knee osteoarthrosis showed a significant analgesic and anti-inflammatory effect, demonstrating to be a good alternative to NSAIDs to provide a more comfortable quality of life to these patients. Additional research is needed, with larger samples in order to evaluate the analgesic and anti-inflammatory effects of the entire phyto-complex of pineapple in inflammatory conditions other than knee osteoarthrosis.

9.2 Introduction

Osteoarthritis (OA) represents the most common of joint pathologies and the main reason of joint pain and functional impairment in the world [1]. It is a degenerative inflammatory pathological condition that originates from the loss of the physiological balance between degenerative and reparative phenomena, primarily at the level of the articular cartilage, causing global joint decompensation. It currently affects approximately 58 million adults, with an estimated increase to 78.4 million by 2040 [2], and it is classified into primary (or idiopathic) and secondary (attributed to causative factors such as recurrent trauma or overweight). OA in Italy affects about 4 million people. As many as 80% of elderly and about 18% of people of working age (18-60 years) would be affected [3].

The pathophysiological process of OA is progressive, involving in succession and in its entirety the entire joint including the capsule-ligamentous component and subchondral bone with metaplasia of the synovial cells and the formation of osteophytes. Macroscopically, there is a fissuring of the articular cartilage up to the formation of gross loss of substance initially focal and subsequently diffuse; later there is a synovial proliferation with a generally mild synovitis which could be the consequence of the exposure of cartilage components to the immune system with consequent inappropriate activation of the cytokine network and triggering of the inflammatory cascade and subsequent joint damage [4]. This inflammation is characterized by pain, stiffness, and a reduced range of motion regarding the arthritic joints. The onset of OA-related symptoms can compromise the quality of life of people by reducing the ability to perform physical activity and thus increasing the risk of fractures and developing overweight/obesity and diabetes mellitus [5]. Factors predisposing to OA could be classified as local biochemical factors, including joint injury, joint space and physical activities, and general factors such as sex, age, comorbidities like obesity and nutrition disorders [6].

The objectives of the treatment of knee arthrosis include the reduction of the inflammatory state and, as far as possible, a slowdown in the progression and joint damage while preserving joint mobility, improving the quality of life and cenesthesia. Physical activity and a diet program promote the reduction of body weight (first

recommendation), functional overload and joint stress [7]. Generally, the lifestyle change aims to reduce pain, improve joint stiffness and slow down the progression of the disease [8]. In addition to the lifestyle change, which represents the first non-pharmacological step of OA treatment, it is possible to associate the conventional treatment that is characterized by non-steroidal anti-inflammatory drugs (NSAIDs), opioids and cyclooxygenase (COX)-2 specific drugs. Nevertheless, the pharmacological treatment has only a "palliative" role by reducing symptoms but not considering the essential problem of the cartilage disorder. In addition, it can cause (especially for long period of consumption) possible side effects which could reduce the compliance for the appearance of gastrointestinal problems, cardiovascular effects and others [9]. The last approach of OA treatment is surgery when the lifestyle changes and medications are not enough.

In recent years, a great interest has been applied to nutraceutical supplements which are well known to reduce inflammation, oxidative stress, pain, joint stiffness and improve the cartilage formation [10]. Several nutraceutical compounds have demonstrated to reduce pain, which is typically chronic in OA and represents the main cause of disability for this condition [11]. Among these, bromelain finds a useful application for the reduction of pain, inflammatory reactions and the oedema component associated with the disease. Bromelain is a crude, aqueous extract obtained from the stem of the pineapple (*Ananas comosus*) that contains numerous proteolytic enzymes. The anti-inflammatory mechanism is mediated by an increase in serum fibrinolytic activity, a reduction in plasma fibrinogen and bradykinin levels, with lowering of vascular permeability, oedema and pain. Moreover, bromelain reduces the levels of prostaglandin (PG)-E2 and thromboxane (TX)-A2 and it modulates the surface adhesion molecules of some types of immune cells [12]. It is frequently used for acute inflammation, in the treatment of osteoarthritis, rheumatoid arthritis and in sports traumatology [13]. Specifically, the anti-inflammatory and analgesic properties of bromelain were first reported in 1964 for the treatment of osteoarthritis (OA) and rheumatoid arthritis [14]. The supplementation with bromelain as single component showed a comparable effect to NSAIDs in mitigating the symptoms of mild-to-moderate lumbar spine, knee or hip OA [15,16,17].

Bromelain potentially acts as a NSAIDS painkiller, as it reduces the dosage and/or the number of administrations of conventional treatments [18].
Recent research is aimed at the best formulation to make this extract more economically sustainable, exploiting the concept of the circular economy, starting with pineapple waste from the food chain. In this context, Brome-Inf is a freeze-dried extract of pineapple by-products, highly concentrated in bioactive peptides and bromelain, marketable as a food supplement or functional food.
The aim of the study is to investigate the potential role of bromelain supplementation (in form of functional food) in people with knee osteoarthrosis, in order (when possible) to reduce the need NSAIDs as well as to improve their quality of life.

9.3 Materials and Methods

9.3.1 Study design and partecipants

This was an interventional, double-blind, single-center, randomized study. It involves the enrollment of patients with gonarthrosis, who will be randomized 1:1 to receive pineapple extract Brome-Inf (titled in bromelain) or placebo for 8 weeks. The study population will include 40 subjects affected by symptomatic osteoarthritis of the knee (Figure 1) belonging to the Department of Medical and Surgical Sciences (DIMEC) of the Alma Mater Studiorum University of Bologna. Participants were required to be aged between 40 and 85 years, BMI<35kg/m^2, and symptoms ascribable to arthritic phenomena.

The exclusion criteria were previous intolerance to pineapple, indication for infiltrative or surgical treatment of the affected joint, previous resistance to oral or systemic anti-inflammatory/painkiller treatments, oral infiltrative or chondroprotective treatment with hyaluronic acid in the 6 months prior to enrolment and any medical or surgical condition that makes patient adherence to the study protocol complex or erratic. The patients were excluded as study subjects if they had

any missing data or recall visits, or they reported the use of nontrial drugs during the observation period.

After the obtained of informed consent and the screening visit (T=-1, day -7), people were randomized (T=0) to receive the nutraceutical (based on pineapple extract) or placebo for 60 days. At T=1 (day 30) and T=12 (day 60) patients were evaluated for clinical status, in addition to the evaluation of the compliance and the tolerability of the products. The study timeline is described in detail in Figure 2.

Figure 1. Flowchart of participants in the study

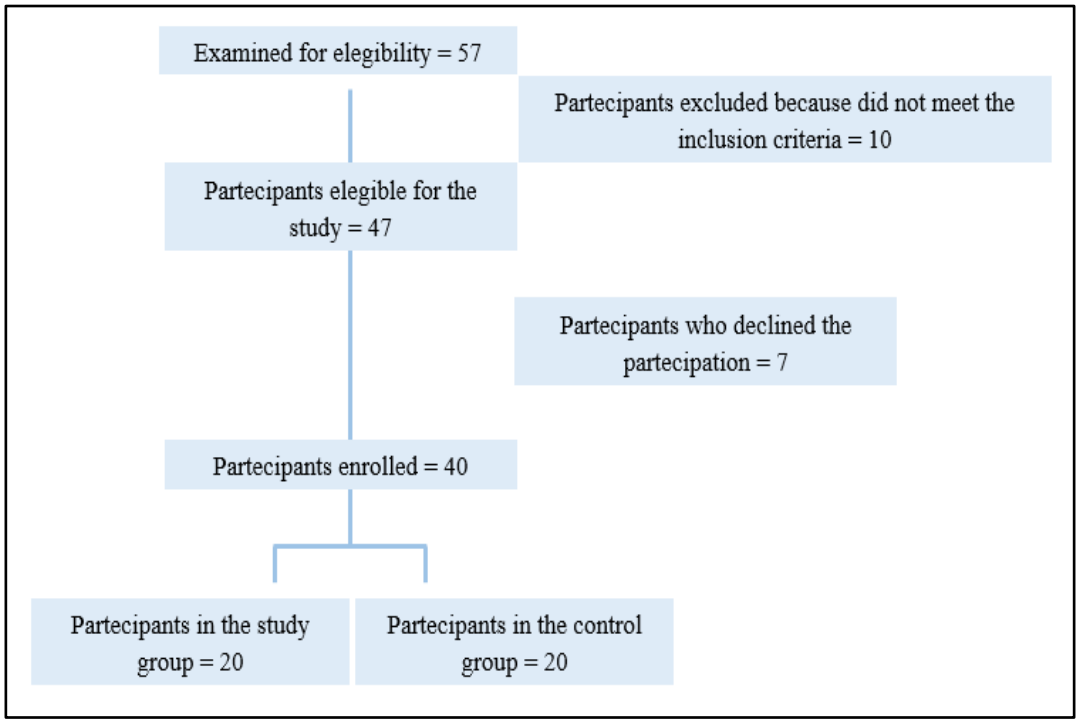
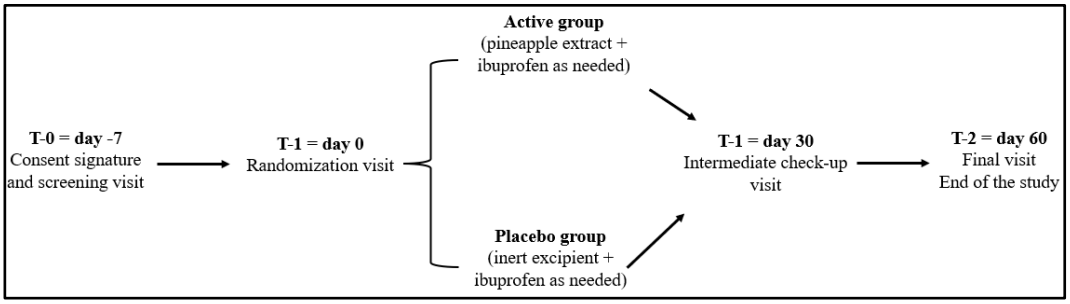


Figure 2. Study timeline



9.3.2 Treatment

After the consent signature (T-1), at the time of randomization (T0), every patient was given Brome-Inf (freeze-dried pineapple powder, with a spoon) or placebo (similar for taste and shape) to be taken orally, 5 g/day for 60 days. The lyophilized pineapple contained 400 mg of bromelain for 5 g of extract. In addition, subjects were instructed to take diclofenac 100 mg as needed if pain became significant (for a maximum of t.i.d.). The study products were manufactured and packaged by Studio 3 Farma srl (Torre di Mosto, Italy), in accordance with Quality Management System ISO 9001:2015.

Randomization was performed centrally, by computer-generated codes. Participants and investigators were blinded to the group assignment. The alphanumeric codes (X and Y) of randomization were kept closed inside an envelope kept in the locked drawer of the main investigator's desk. They were opened at the end of the study by the principal investigator.

For the entire duration of the study, patients were instructed to take the assigned treatment every day, at approximately the same time each day, preferably in fasted state.

Participants compliance was assessed by using a delivery of diary sheet (bromelain / placebo YES NO).

9.3.3 Assessment of the efficacy

The clinical efficacy outcomes were monitored at baseline, week 4 and week 8 of treatment. The primary outcome was change in Western Ontario McMaster Universities Osteoarthritis Index (WOMAC), Visual Analog Scale (VAS), joint range of motion (ROM), and Lequesne Functional Index (LFI) scores from baseline. WOMAC includes 24 items of pain (0–20), stiffness (0–8), and physical function (0–68) with the total scores of 96 [19]. The lower WOMAC score represents the better clinical outcome.

VAS is a validated, subjective measure for acute and chronic pain. Scores are recorded by making a handwritten mark on a 10-cm line that represents a continuum between “no pain” and “worst pain” [20]. The Lequesne OA index is a 10-question interview format questionnaire. This index has an interview format including 10 questions about pain, stiffness and function. The score ranges from 0 (no pain, no disability) to 24 (maximum pain, stiffness and disability) [21]. ROM is a measure widely used because it is an easily understood, direct measure of the joint’s condition, for its intuitive connection between joint flexibility and operative success [22].

Mean difference at weeks 4 and 8, was calculated as %change from baseline values in bromelain group - %change from baseline values in placebo group.

Short (30 days) and medium (60 days) term evaluation of the effect of the nutraceutical tested on the need of diclofenac used to reduce pain associated with knee osteoarthritis was also assessed.

The secondary clinical outcome was the quality-of-life assessment (SF-36) [23]. SF-36 was assessed using two summary scores (physical and mental component summary). SF-36 was scored from 0 to 100; 0 score indicated extreme problems and 100 score indicated no problems.

9.3.4 Assessment of the safety and tolerability

Safety and tolerability were evaluated through continuous monitoring during the study to detect any adverse event and clinical safety of treatments. The compliance of treatments and the occurrence of adverse effects were monitored by using a delivery of diary sheet (bromelain / placebo YES NO; diclofenac YES NO, and if YES number of daily administrations). Tolerability and acceptability of the tested product was also assessed by using a scale from 1 to 10 (1=poor tolerability/acceptability, 10=excellent tolerability/acceptability). Adverse effects reported by patients were recorded at week 4 and 8 of trial.

9.3.5 Statistical analysis

Personal data and physiological/pathological anamnesis were detected only at the enrollment visit (T-1), treatment compliance in T1 and T2.

The sample size was determined to achieve a power of 80%, with a level of significance equal to 0.05 for a specified difference in pain at a mean of 1 cm recorded on the VAS. A desired sample size of 20 patients per group was found necessary to fit a statistical model for analysing the differences among the study groups. Data were incrementally entered during the study period into an electronic sheet (Excel, Microsoft, Windows 2003, Redmond, WA), double checked for errors, and then processed using the SPSS 26.0 software, version for Windows. A descriptive analysis of each of the variables was made. The demographic and clinical characteristics of the patients were analysed using analysis of variance or Pearson's χ^2 test, as appropriate. The outcome variables were compared among the groups using

analysis of variance and within the groups by multiple measures analysis of variance. A significance level below 0.05 was considered statistically significant for all tests conducted.

9.4 Results and Discussion

The treatment with Brome-Inf reduced symptoms of mild knee pain and improved well-being in healthy adults (Table 1). There was a significant difference in symptomatic relief (WOMAC pain and VAS scores) of knee OA compared between bromelain and placebo treatments at week 8 ($p<0,05$ for all compared with placebo). SF-36 were significantly improved only in bromelain group ($p<0,05$ at week 8 compared with the placebo group). Eight weeks treatment with Brome-Inf was considered safe, without reported side effects. Brome-Inf palatability and compliance was considered excellent. The limitation of our study is the small sample size which may give rise to overestimation of treatment effect. In addition, self-reported outcome and continuous endpoints in our study would lead to potential bias.

Table 1. Changes in VAS, WOMAC (and related subscales) indexes, Lequesne functional index and knee extension range of motion (ROM) in the enrolled subjects during the trial

	Bromelain			Placebo		
	T0	T28	T56	T0	T28	T56
VAS	6.9±1.1	6.5*±0.9	5.9*°±0.8	6.7±1.0	6.5±1.1	6.4±1.3
Pain WOMAC	9.7±1.2	9.2*±1.1	8.9*°±0.9	9.5±1.3	9.4±1.2	9.2±1.2
Function WOMAC	21.8±2.4	20.1±2.5	20.3±1.4	23.1±2.5	22.7±2.4	22.5±2.0
Total WOMAC	40.1±3.6	36.4*±3.3	33.2*°±4.2	41.5±3.7	39.4±3.9	38.5±4.1
Lequesne Functional Index	6.6±0.9	6.4±1.1	6.1*±0.9	6.5±1.0	6.3±1.1	6.4±1.0
Extension ROM	86±11	88±12	87±14	84±12	85±10	85±12
SF-36-PCS (0-100)	49.4±9.8	65.2*°±8.4	59.4*°±7.3	50.1±9.8	54.2±9.1	51.6±10.8

*p<0.05 vs. baseline, °p<0.05 vs. placebo

Previous study showed similar results with the single mono-component (bromelain alone) [24], despite that bromelain even at high dose 800-mg/day for 12 weeks was ineffective for severe knee OA [25]. Thus, bromelain can ameliorate symptoms in patients with mild-to moderate OA. This is particularly consistent with the prescriptive target of nutraceuticals, within a concept of preventive medicine. Moreover, bromelain supplementation is considered safe and well tolerated while the use of diclofenac reported different side effects such as dyspnea and heartburn [26].

The exact physiological mechanism of cartilage prevention operated by bromelain is still unclear. However, it is well known that reactive oxygen species (ROS), nitric oxide (NO), and inflammatory mediators such as prostaglandin (PG)E2 are potential mediators responsible for cartilage destruction in OA patients [27]. ROS contribute to cartilage degradation stimulating the lipid peroxidation [28], and oxidizes the arachidonic acid in F2-isoprostanes, 4-hydroxynonenal, and malonyldialdehyde (MDA). Previous studies showed that MDA levels were higher, and α-tocopherol level was lower in patients with knee OA compared to controls, highlighting that OA patients have higher oxidative stress. In this regard, bromelain extract demonstrated to reduce plasma MDA levels after 16-week treatment [29], in addition to increase serum antioxidant enzyme activities (superoxide dismutase and glutathione peroxidase) [30]. Thus, the effect of bromelain on OA symptoms may be due to its role in reducing MDA level and restoring the antioxidant capacity.

9.5 Conclusion

Treatments with pineapple lyophilized extract for 8 weeks could be considered a good strategy in reducing knee OA symptoms. Moreover, this functional food improves stiffness, and physical function compared with control and acts as painkillers, reducing the dosage and number of administrations of diclofenac. The treatment with pineapple lyophilized extract is considered safe and without relevant side effects. Larger and longer RCTs and pharmacodynamic studies are needed to understand the mechanism of action of bromelain and the entire phytocomplex of

pineapple in OA and before to definitively recommend this supplement in clinical practice.

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Chapter 10
Evaluation of the effects of treatments based on pineapple extract or bromelain after a mandibular third molar surgery: a randomized three-arm clinical study

10.0 Protocol synopsis

Title	**"Evaluation of the effects of treatments based on pineapple extract or bromelain after a mandibular third molar surgery: a randomized three-arm clinical study"**
Code of the study	ALBRO2022

Rational	A) Bromelain may contribute to reduce the inflammation and oedema, facial swelling and post-operative pain caused by oral surgery, improving their social isolation and sleep quality. B) Despite that the mechanisms of action of bromelain enzymatic complex are not fully understood, several in vitro and in vivo studies underline three different pathways of action: the improvement of the fibrinolytic activity by activating factor XI and regulating the kallikrein-kinin pathway, the modulation of arachidonic cascade and the production of inflammatory cytokines, and the limitation of the neutrophil's migration to inflammation sites. C) Among the most prescribed analgesic/anti-inflammatory drugs in dental surgery, ibuprofen is the most frequently prescribed product. However, despite that people which use NSAIDs shown a spontaneous resolution of the inflammation generally within a week, the conventional therapy is not free from side effects. D) In this context, the oral supplementation of nutraceuticals may help to reduce inflammation, pain and/or oedema in subjects with chronic inflammatory diseases, potentially reducing the need for NSAIDs. E) Among the nutraceutical compounds, Brome-Inf is a freeze-dried extract of pineapple, highly concentrated in bioactive peptides and bromelain, marketable as a food supplement or functional food. In this sense, the use of bromelain in people undergoing a dental surgery, has been particularly studied demonstrating that it could reduce the doses or the number of administrations of conventional anti-inflammatory/analgesic drugs.

	F) Bromelain in the form of freeze-dried pineapple is also a functional food with a good palatability and an excellent safety profile. G) Despite that the action of bromelain as single component is well documented, to our knowledge this is the first study which compare the activity of the pineapple phytocomplex (titrated in bromelain 8%) with purified bromelain.
Research center	Studio Pisano Procchio, Alessandria
Researchers involved	Dr. Alessandro Colletti, Dr.ssa Chiara Procchio, Prof. Giancarlo Cravotto
Study design	Single center, randomized, three-arms, double-blind, interventional study
Tested products	Dietary supplement based on freeze-dried pineapple extract (Brome-Inf) vs bromelain vs placebo Protocol: 2,5 g every 6 hours starting the morning of surgery and continued for 3 days after (T2), and 2,5 g every 12 hours for the following 4 days (T3). Subjects were instructed to take ibuprofen 600 mg as needed, if pain became significant (for a maximum of t.i.d.). Moreover, postoperatively, all patients in the study received amoxicillin + clavulanic acid (1 g/day) for 5 days after surgery
Study arms	• Brome-Inf (freeze-dried pineapple powder containing 200 mg of bromelain every 2,5 g of powder, with a spoon) • Bromelain (200 mg of bromelain 2500 GDU/g every 2,5 g of powder, with a spoon) • Placebo (similar for taste and shape)
Sample size	N 42 subjects
Goals	• Post-operative pain evaluated using a visual analogue scale (VAS) • Facial swelling in the operation evaluated using 2 facial measurements: tragus-pogonion and gonion-lateral canthus • Trismus measured as the difference in the interincisal distance at the maximal mouth opening before and after surgery • Effect on QOL measured using a questionnaire with different items addressing social isolation, working

	isolation, eating ability and diet variations, speaking ability, sleep impairment, and physical appearance • Evaluation of the effect of the nutraceuticals tested on the number and doses of anti-inflammatories/analgesics used (ibuprofen 600 mg) to reduce post-operative pain
Inclusion criteria	• Presence of a partial bony impacted mandibular third molar • Aged between 18 and 35 years old • Obtaining informed consent • Absence of pericoronitis and infection at surgery • No medication during the previous 2 weeks
Exclusion criteria	• Presence of oral comorbidity • Any medical or surgical condition that makes the patient's adherence to the study protocol complex or inconstant • Co-assumption of other supplements • Previous intolerance to pineapple
Scheduled visits	• T-1 (day -7/14): Screening visit • T0 (day 0): Randomisation visit • T1 (day 1): Day after surgery visit • T2 (day 3): Intermediate check-up • T3 (day 7): Study closing visit
Variables collect	• Personal data* • Pharmacological history • Weight/Height/BMI • Pain (VAS), sweeling and trismus • QoL questionnaire • Use of anti-inflammatories/analgesics used to reduce pain associated with the surgery • Adherence to the proposed treatments • Tolerability and acceptability of the tested product (Scale from 1 to 10)
Statistical analysis	The following statistical analyzes will be performed: - Complete descriptive analysis of all parameters studied: mean, mode, median, standard deviation, standard error of the mean, 95% confidence intervals, normality tests - Comparative tests: comparison between treatment periods and merging data based on randomization

	- Analysis by subgroups and advanced statistics will be conducted on the basis of the results obtained from the analysis of the data as a whole A significance level of $p<0.05$ will be considered acceptable for all tests conducted
Timing	• Start of the Study: October 2022 • Enrollment closure (indicative date): February 2023 • End of Study (indicative date): March 2023

10.0 Abstract

This randomized, placebo controlled clinical study was performed to evaluate the effect of a lyophilized pineapple extract titrated in bromelain on pain, swelling, trismus, and quality of life (QOL) after surgical removal of the lower third molars. Moreover, the need of non-steroidal anti-inflammatory drugs (NSAIDs; ibuprofen 600 mg for a maximum of t.i.d.) was also evaluated and compared with the placebo group. The study included 42 people requiring extraction under local anaesthesia of a single mandibular third molar. The patients were randomized and distributed to receive the pineapple extract, bromelain or placebo and started the treatment the day of surgery and continued it for the next 7 days. The primary outcome was the need of NSAIDs between the two groups. The outcome variables were pain, swelling, and trismus, which were measured at 1, 3, and 7 days postoperatively. Differences in efficacy between freeze-dried pineapple extract and single-component bromelain were also evaluated. At the end of the study, the assumption of ibuprofen (from days 1-7) was statistically higher in the placebo group ($p<0,05$). In addition, the reduction in pain and swelling was significantly superior in both bromelain and pineapple groups ($p<0,05$ for all, compared with control, at all intervals), compared with the placebo. Active groups also showed a significant difference in the effect on QoL compared with the placebo ($p<0.05$). A nonsignificant reduction in trismus occurred in treatment groups compared with the placebo group. Therefore, the administration of pineapple extract titrated in bromelain showed a significant analgesic and

antioedema effect, in addition to improving QoL in the postoperative period for patients who had undergone lower third molar surgery. Moreover, both bromelain and pineapple supplementation reduced the need of ibuprofen to a comparable extent, demonstrating to be a good alternative to NSAIDs to provide a more comfortable postoperative course for these patients. Additional research is required with larger samples to evaluate the analgesic and anti-inflammatory effects of the entire phyto-complex of pineapple in surgical procedures other than third molar surgery.

10.1 Introduction

The proteolytic complex extracted from pineapples (*Ananas comosus*), which is called "bromelain" it is well known to possess anti-inflammatory, anti-oedema and analgesic properties, suggesting its prescription for several conditions characterized by the presence of acute inflammation with or without oedema [1]. For example, in a randomized controlled trial (RCT), 60 patients with long bone fractures were treated with bromelain or placebo for the first 3 days after surgery. A significant decrease in post-traumatic and post-operative swelling and pain was observed in patients who were administered bromelain. In addition, healing acceleration was also observed compared to the control group [2]. In another RCT, 82 primiparous women were treated with bromelain and benefited from reductions in pain measured on the Visual Analogue Scale (VAS), ecchymosis and oedema caused by episiotomy compared with a placebo group ($p<0.05$ for all). Moreover, the wound healing was faster in the bromelain group than in the placebo group ($p<0.05$) [3]. Howat et al. also demonstrated the existence of a trend of reduction in pain and ecchymosis, although the differences did not reach statistical significance [4]. Seltzer et al. reported the ability of bromelain to decrease perioperative oedema and ecchymosis in 53 rhinoplasty cases [5].

Previous RCTs have highlighted the activity of bromelain in other diseases such as rheumatoid arthritis, osteoarthritis, perioperative sport injuries, cardiovascular diseases, chronic rhinosinusitis, and skin wounds and burns, which are all conditions with an inflammatory component, with or without oedema [6]. In this regard,

bromelain may contribute to also reduce the inflammation and oedema caused by oral surgery. A recent meta-analysis of six RCTs demonstrated that bromelain alleviates postoperative pain 7 days after mandibular third molar surgery (p=0.002) and decreases facial swelling in the early and late stages after surgery (p=0.02 and p=0.0004, respectively) [7]. Similar results were obtained in previous meta-analyses performed by Mendes et al. [8], de Almeida et al. [9], and de Souza et al. [10], which also showed improvements in social isolation and sleep quality.

Despite that the mechanisms of action of bromelain enzymatic complex are not fully understood, several *in vitro* and *in vivo* studies underline three different pathways of action: the improvement of the fibrinolytic activity by activating factor XI and regulating the kallikrein-kinin pathway, the modulation of arachidonic cascade and the production of inflammatory cytokines, and the limitation of the migration of neutrophils to inflammation sites [11].

Among the most prescribed analgesic/anti-inflammatory drugs in dental surgery, ibuprofen is the most frequently prescribed product, followed by naproxen and acetaminophen [12]. However, despite that people who use NSAIDs have shown a spontaneous resolution of the inflammation generally within a week, the conventional therapy is not free from side effects [13]. In this context, a percentage of patients reported excessive dosing of NSAIDs. Although doses of ibuprofen under 1200 mg/day minimally increase the risk of gastrointestinal bleeding, the prescription dose increases the risk of bleeding dramatically (relative risk of 4 vs no medication) [14]. The risk is higher with prolonged use, but one study has reported that patients starting naproxen are at higher risk than those starting ibuprofen and that difference is detectable within 14 days [15]. This suggests that even a few days of use results in increased potential for injury. Some studies estimated that up to 15,000 people die annually in the United States as a complication of the NSAIDs treatment [16], and its overuse is a potential major health issue.

As mentioned above, the oral supplementation of nutraceuticals may help to reduce inflammation, pain, and/or oedema in subjects with chronic inflammatory diseases, potentially reducing the need for NSAIDs [17]. Among the nutraceutical compounds, Brome-Inf is a freeze-dried extract of pineapple, highly concentrated in bioactive

peptides and bromelain, marketable as a food supplement or functional food. In this sense, the use of bromelain in people undergoing dental surgery, has been particularly studied demonstrating that it could reduce the doses or the number of administrations of conventional anti-inflammatory/analgesic drugs [18]. Moreover, bromelain in the form of freeze-dried pineapple is also a functional food with a good palatability and an excellent safety profile. However, despite that the action of bromelain as single component is well documented, to the best of our knowledge, this is the first study that compares the activity of the pineapple phytocomplex (titrated in bromelain 8%) with purified bromelain.

The aim of this study was to investigate the potential role of nutraceutical supplementation in people subjected to mandibular third molar surgery, to reduce (when possible) the need of non-steroidal anti-inflammatory drugs (NSAIDs) as well as to improve their quality of life. The second endpoint was to evaluate differences in efficacy between freeze-dried pineapple extract and single component bromelain.

10.2 Materials and Methods

10.2.1 Study design and participants

This was a pilot, interventional, double-blind, single-center, randomized study. It involved patients enrolled for a third molar surgery, who were randomized 1:1:1 (Figure 1) to receive pineapple extract, bromelain, or placebo for 7 days after the intervention. The study population included 42 healthy individuals belonging to the "Studio Dentistico Pisano Procchio" of Alessandria, who required third molar surgery under local anaesthesia. Participants were required to be aged 18-35 years, be healthy, have a partial bony impacted mandibular third molar, be free of pericoronitis and infection at surgery, to have received no medication during the previous 2 weeks, and to have no history of allergy to the drugs used in the present trial. The presence of comorbidity, any medical or surgical condition that makes the patient's adherence to the study protocol complex or inconstant, co-assumption of other supplements, allergies or intolerances to the active ingredient or excipients

were the exclusion criteria. The patients were excluded as study subjects if they had any missing data or recall visits, or they reported the use of nontrial drugs during the observation period.

After informed consent was obtained (T=-1), the day before the surgery, participants were randomized to receive the pineapple extract, bromelain, or placebo for 7 days. On T=0 (day of surgery), T=1 (day 1), T=2 (day 3), and T=3 (day 7), patients were evaluated for clinical status, in addition to the evaluation of the compliance, and the tolerability of the products. The study timeline is described in detail in Figure 2.

Figure 1. Flowchart of participants in the study

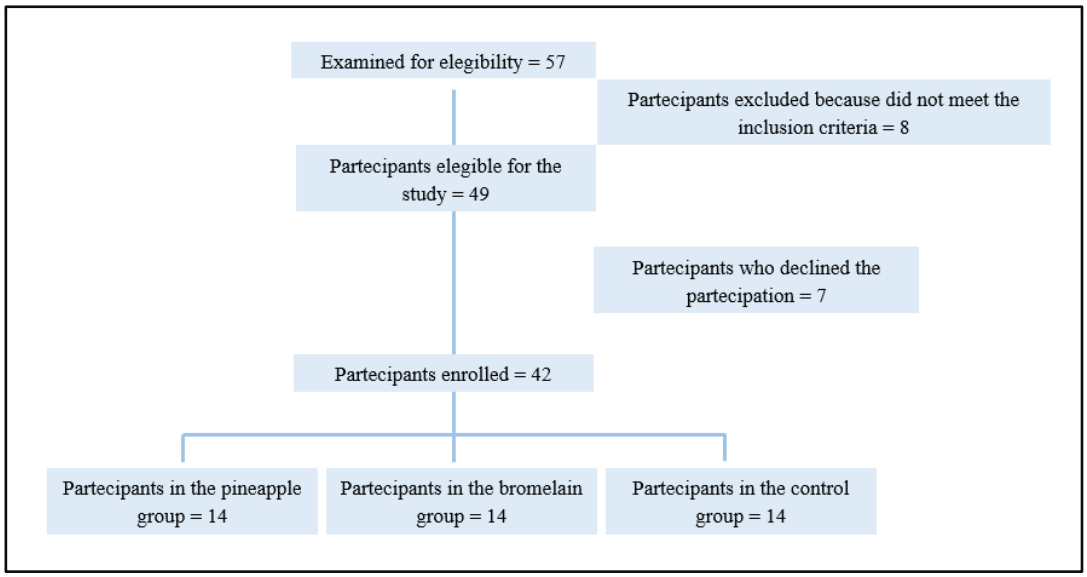
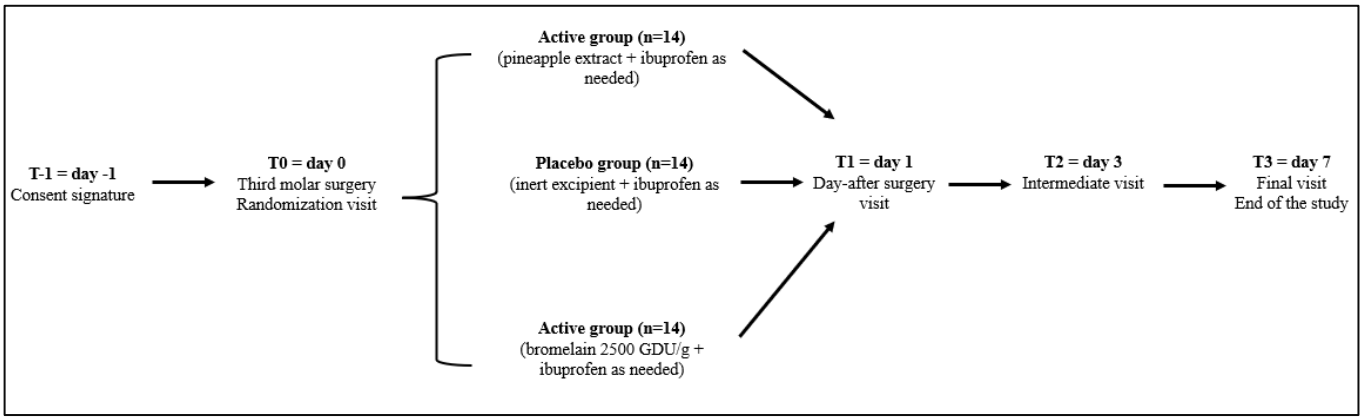


Figure 2. Study timeline



10.2.2 Treatment

After the consent signature (T-1), at the time of randomization (T0), every patient was given Brome-Inf (freeze-dried pineapple powder containing 200 mg of bromelain every 2,5 g of powder, with a spoon), bromelain (200 mg of bromelain 2500 GDU/g every 2,5 g of powder, with a spoon), or placebo (similar for taste and shape) to be taken orally, 2,5 g every 6 hours starting the morning of surgery and continued for 3 days after (T2), and 2,5 g every 12 hours for the following 4 days (T3). Subjects were instructed to take ibuprofen 600 mg as needed if pain became significant (for a maximum of t.i.d.). Moreover, postoperatively, all patients in the study received amoxicillin + clavulanic acid (1 g t.i.d.) for 5 days after surgery.

For the entire duration of the study, patients were instructed to take the assigned treatment at approximately the same time each day, preferably in fasted state. The patients were examined after 1, 3, and 7 days after surgery. At each follow-up visit, pain, swelling, and trismus were measured. The patients received the QoL questionnaire to complete on day 4 after surgery and returned at suture removal by day 7. The total number of rescue analgesic tablets taken during this period was also recorded.

The compliance of participants was assessed by using a delivery of diary sheet (pineapple / bromelain / placebo YES NO).

The study products were manufactured and packaged by Studio 3 Farma srl (Torre di Mosto, Italy), in accordance with Quality Management System ISO 9001:2015.

Randomization was performed centrally using computer-generated codes. Participants and investigators were blinded to the group assignment. The alphanumeric codes (X, Y, Z) of randomization were kept closed inside an envelope kept in the locked drawer of the main investigator's desk. It was opened at the end of the study by the principal investigator.

10.2.3 Assessment of the efficacy

The primary endpoint of the study was to investigate the comparison of the need for ibuprofen intake between the pineapple and placebo groups. The primary outcome variables were pain, swelling, trismus, and QoL scores recorded after surgery. Post-operative pain was evaluated using a VAS, 10 cm in length, ranging from 0 for ‘‘no pain’’ to 10 for ‘‘the worse possible pain’’. Facial swelling in the operation side was evaluated using two facial measurements: tragus-pogonion and gonion-lateral canthus. The preoperative sum of the two values (in millimetres) was taken as the baseline for that side. Trismus was measured as the difference in the interincisal distance at the maximal mouth opening before and after surgery.

The effect on QoL was measured using a questionnaire that has been fully described and validated in a previously published report [19]. The questionnaire includes different items addressing social isolation, working isolation, eating ability and diet variations, speaking ability, sleep impairment, and physical appearance. The recovery for each QoL item was defined as the number corresponding to a 4-point scale. The scale included the following responses: not at all (coded 0), little (coded 1), quite a lot (coded 2), and very much (coded 3). The total score range was 0-42, with higher scores indicating poorer QoL. The other outcome variables were

demographic, including age, gender, and body mass index (BMI). The intraoperative variables included the duration of surgery (in minutes from the incision to the last suture). The postoperative variables included the number of rescue analgesic tablets taken by the patients until day 7.

10.2.4 Assessment of the safety and tolerability

Safety and tolerability were evaluated through continuous monitoring during the study to detect any adverse event and clinical safety of treatments. The compliance of treatments and the occurrence of adverse effects were monitored using a delivery of diary sheet (pineapple / bromelain / placebo YES NO; Ibuprofen YES NO, and if YES, number of daily administrations).

10.2.5 Statistical analysis

Personal data and physiological/pathological anamnesis were detected only at the enrolment visit (T-1), with treatment compliance only in T3.
The sample size was determined to achieve a power of 80%, with a level of significance equal to 0.05 for a specified difference in pain at a mean of 1 cm recorded on the VAS. A desired sample size of 14 patients per group was found necessary to fit a statistical model for analysing the differences among the study groups.

Data were incrementally entered during the study period into an electronic sheet (Excel, Microsoft, Windows 2003, Redmond, WA), double checked for errors, and then processed using the SPSS 26.0 software version for Windows. A descriptive analysis of each of the variables was made. The demographic and clinical characteristics of the patients were analysed using analysis of variance (ANOVA) or Pearson's χ^2 test, as appropriate. The outcome variables were compared among the groups using ANOVA and within the groups by multiple measures ANOVA. A

significance level <0.05 was considered statistically significant for all tests conducted.

10.3 Results

Forty-nine people requiring extraction under local anaesthesia of a single mandibular third molar and had fulfilled all the inclusion criteria, were enrolled.
However, seven patients were later excluded because they did not attend the follow-up visits or had used nonstudy drugs. Thus, 42 patients, who had attended the follow-up visits and completed the questionnaire, were included in the final analysis. The mean patient age (19 men and 23 women) was 22.8 (range 19 to 27). No statistically significant differences were found in the demographic characteristics of the subjects or the parameters related to the surgical procedure among the study groups (Table 1).
Regarding perceived pain a significant reduction of pain was observed in Brome-inf and bromelain group if compared with placebo ($p<0,05$ for both) (Table 2). In addition, patients from the Brome-inf and Bromelain groups reported a halved average intake of ibuprofen compared to the placebo group.
The mean baseline measure of swelling was 257, 260, and 259 mm in the placebo, bromelain, and Brome-inf groups, respectively. The maximum swelling measures were reported 1 day postoperatively in all study groups (Table 2). The difference in the magnitude of swelling in the placebo group was significant ($p<0,05$) compared with the preoperative values. Nevertheless, the difference was not significant in the bromelain and placebo groups. The comparison among the groups revealed a reduction in swelling at 3 and 7 days in the bromelain and Brome-Inf groups. However, despite that the reduction of swelling was higher if compared with placebo, data did not reach statistical significance even though Brome-inf and Bromelain groups reported a halved average intake of ibuprofen compared to the placebo group.

The mean baseline measure of the interincisal distance was 45, 44, and 43 mm in the placebo, bromelain, and Brome-Inf groups, respectively. In all groups, trismus was

maximum 1 day after surgery and had subsided at the subsequent follow-up intervals. However, a comparison among the groups failed to reveal a significant difference (Table 2).

Regarding the QoL measures, both active groups showed a reduction of scores in all subscales compared with the placebo ($p<0,05$ for both active groups). A significant improvement was seen also in the total QoL score for both active groups compared with those for the placebo group (Table 3).

No side effects were reported during the treatments. Moreover, both Brome-Inf and bromelain supplementation showed good palatability and excellent compliance (100%). No cases of alveolar osteitis or wound infection were reported during the period of study.

Table 1. Patient demographics and intraoperative parameters

Variable	**Bromelain (n=14)**	**Brome-Inf (n=14)**	**Placebo (n=14)**	**Total**
Age (year)	22,4 ± 4,9	22,9 ± 4,5	23,1 ± 4,1	22,8± 4,5
Gender				
Male	5	6	8	19
Female	8	7	8	23
BMI (Kg/m^2)	24,4 ± 0,2	24,6 ± 0,2	24,8 ± 0,3	24,6 ± 0,2
Operation time (min)	31,2 ± 14,1	32,7 ± 18,2	31,5 ± 17,4	31,8 ± 16,5

Data presented as mean ± standard deviation

BMI: Body mass index.

Table 2. Comparison of outcome variables among and within study groups

Variable	Placebo (n=14)	Bromelain (n=14)	Brome-Inf (n=14)
VAS-10			
Day-1	3,3±1,4	2,3±0,7	2,1±0,9
Day-3	2,5±1,2	1,5±1,0*	1,4±0,8*
Day-7	0,6±1,0	0,4±0,7*	0,4±0,9*
Swelling			
Day-1	8,1±4,9	8,7±5,2	8,6±4,5
Day-3	4,2±2,8	3,0±2,7*	3,3±2,2*
Day-7	1,8±1,7	1,3±1,5*	1,2±1,2*
Trismus			
Day-1	13,1±9,2	13,4±8,9	13,1±9,7
Day-3	8,9±8,8	7,5±7,3	7,8±7,7
Day-7	5,4±6,9	4,3±6,5	4,8±6,7
Rescue tablets of ibuprofen	6,4±2,2	3,6±2,9*	3,2±2,7*

**p<0,05 compared with placebo*

Data presented as mean ± standard deviation.

VAS: Visual Analogue Scale

Table 3. Comparison of Quality-of-life subscales among study groups

Variable	Placebo (n=14)	Bromelain(n=14)	Brome-Inf (n=14)
Social	0,9±0,3	0,4±0,3*	0,3±0,4*
Work	1,0±0,5	0,6±0,4*	0,4±0,5*
Eating	8,1±2,4	5,7±1,8*	5,8±1,9*
Speech	1,8±1,5	1,2±1,3	1,4±1,6
Sleep	2,4±1,1	0,7±1,1*	0,9±0,9*
Appearance	2,9±1,2	1,2±1,4*	1,5±1,1*
Total	17,1±7,0	9,8±6,3*	10,3±6,4*

**p<0,05 compared with placebo*

Data presented as mean ± standard deviation.

10.4 Discussion

This study investigated the effect of lyophilized pineapple extract (titrated and standardized in bromelain) and bromelain as single component on postoperative sequelae and QoL measures after surgical removal of the impacted lower third molars compared with placebo. Our hypothesis, based on the study of Majid et al. [20], showed that the oral intake of bromelain in multiple daily-doses starting on the day of surgery and continued for 7 days resulted in a significant effect on the clinical and QoL status of these patients. In particular, the regular assumption of bromelain, both as a functional food or single component (200 mg every 6 hours starting the morning of surgery and continued for 3 days after, and 200 mg every 12 hours for the following 4 days), was shown to significantly reduce ibuprofen intake compared with the placebo group, acting as a killer of pain and inflammation. In this regard,

previous studies demonstrated the comparable effects of bromelain to those of pre-emptive diclofenac sodium or ibuprofen in the third molar surgery setting [21,22,23]. Moreover, both groups (pineapple and bromelain) showed a positive effect on the QoL measures after third molar removal, likely due to its anti-oedema, anti-inflammatory, and analgesic effects, demonstrating an excellent safety profile (no adverse reaction reported) and good palatability. In this context, pineapple extract and bromelain groups showed a marked antiphlogistic effect in our patients, which was higher when compared to the placebo group (characterized by a statistically higher consumption of ibuprofen). The authors chose ibuprofen in the present study as a reference drug to represent the NSAID family, and, as expected, it showed a significant analgesic and antiphlogistic effect during the early postoperative period in the placebo group.

Bromelain has shown therapeutic benefits in doses as small as 160 mg/day; however, it has been considered, for most conditions, the best results will occur at doses of 750-1000 mg/day in four divided doses [24], which was the regimen used in the present study. The mechanisms of action of bromelain have not yet been fully established. However, this enzymatic complex seems to act by removing different cell-surface molecules (including CD128a/CXCR1, CD128b/CXCR2, CD14, CD44, CD16, and CD21), which are important for leukocyte trafficking, cellular adhesion, induction of pro-inflammatory mediators, and immunomodulatory effect on T cells. Bromelain also reduces P-selectin-mediated neutrophil recruitment, and modulates proinflammatory prostaglandins though the inhibition of prostaglandin E2 and thromboxane A2 [25]. In addition, it regulates the plasma fibrinogen levels and blood levels of bradykinin and improves serum fibrinolytic activity by activating factor XI, which subsequently activates plasma prekallikrein [26]. These actions permit bromelain to be potentially effective against several conditions associated with inflammation, with or without oedema, justifying its use as a potential alternative to NSAIDs.

Several risk factors for edema, pain, and trismus after third molar surgery have been reported by different investigators and have included age, gender, operative time, and surgical experience [27]. The bias of such factors or their dominance in one group or another, which would affect the reading of our results, was minimized by randomization of the treatment allocation and the strict inclusion criteria. In addition, the surgical phase was performed by the same surgeon in all cases to avoid possible operator variability. Double-blinding also enabled us to overcome any possible personal bias from both the patients and the surgeon. It has abeen reported that surgeon-rated scores or objective testing can be significantly different from the perspective of patients [28]. This last finding emphasizes the need to consider the perception of patients in studies of third molar surgery and the effect of different drugs on its postoperative sequelae.

This study demonstrated a significant improvement in most QoL measures, highlighting that pineapple extract can be an adjuvant to improve the QoL in individuals subjected to third molar surgery. Moreover, to the best of our knowledge, this is the first study which has investigate the effect of the entire phytocomplex of lyophilized pineapple by-product on the QoL status of patients after oral surgery, and compare the effects with bromelain as single component. This may be particularly important especially in a context of circular economy; starting from the waste products from the pineapple food chain, it was possible to obtain a particularly effective titrated and standardized extract, adopting the so-called "zero waste approach". In this regard, one of the most relevant aspects of the study concerns the overlap of the results obtained with pineapple extract and single component bromelain. Although the dosages of bromelain were comparable in the two active groups, the purified bromelain exhibited a superior enzymatic activity (2500 GDU/g vs 400 GDU/g of pineapple extract). Consequently, it is important to ask whether the evaluation of the enzymatic activity through the measurement of GDU is a predictive method of the effects *in vivo*, and above all whether the impact of the entire phytocomplex is to be preferred over the single protease mixture. In fact, several studies have shown that the proteolytic activity of bromelain is only partly connected

to its pharmacological effects, suggesting that evaluating the whole phytocomplex, including the non-protein factors, is of great importance [29]. These aspects require extensive future research.

10.5 Conclusion

In conclusion, the administration of pineapple extract containing a daily oral dose of 800 mg of bromelain for the first 3 days and 400 mg for the following 4 days, or the same dosages of bromelain as single component, showed a significant analgesic and antioedema effect, in addition to improving QoL in the postoperative period for patients who had undergone lower third molar surgery. Moreover, both pineapple and bromelain supplementation reduced the need for NSAIDs, demonstrating to be a good alternative to ibuprofen to provide a more comfortable postoperative course to these patients. Additional research is required, with larger samples to evaluate the analgesic and anti-inflammatory effects of the entire phyto-complex of pineapple in surgical procedures other than third molar surgery.

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Chapter 11
Final remarks

11.0 Final remarks and perspectives

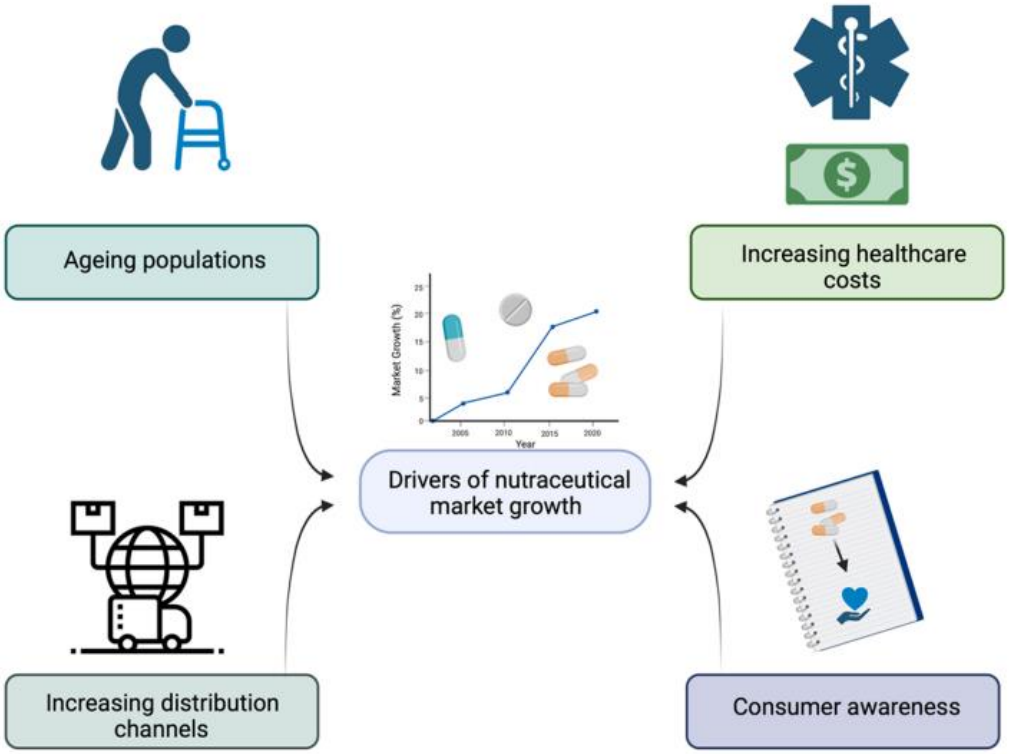
- In the last few decades, a **new paradigm** has emerged that focus the emphasis on diet and nutrition. A more health-conscious consumer pool with increased expendable income in the Western world has shifted consumer trends towards the purchase of dietary supplements, functional foods, and **nutraceuticals** with the goal of maintaining optimal health and preventing chronic pathologies which affect the quality of life and reduce the lifespan [1]. In this regard, epidemiological studies suggest an association between the assumption of nutraceuticals and the prevention of different diseases [2].
- The **nutraceutical market** represents currently a **multi-billion-euro industry** and has received an unforeseen worldwide response (Figure 1). It was valued at approximately $383 billion in 2016 and was expected to reach approximately $561 billion by 2022 prior to the coronavirus diseases 2019 (COVID-19) pandemic [2]. In addition, the value of the nutraceuticals industry is already more than 25% of the value of the pharmaceutical industry [3].
- Despite that there is an increasing interest in nutraceuticals, the **lack of universally accepted definitions and diverse regulatory frameworks** remains a challenge. Regulation of nutraceuticals varies across the globe and is unregulated in some countries. There is a need to understand the current market trends for nutraceuticals, along with variations in regulatory frameworks across different countries.
- **COVID-19 pandemic** caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) emerged in late 2019 with devastating consequences worldwide. In this context, individuals researched additional protection from infection and severe disease by purchasing nutraceuticals with potential health benefits against respiratory infections and related symptomology, causing a **growth of the U.S**. **nutraceuticals market** by 51.2% at the start of the pandemic in March 2020 compared to 2019. In Europe, China, and India, similar trends were observed [4].
- Nutraceuticals may be useful for several indications due to its multi-targeted actions. For example, bromelain from pineapple extract has antinflammatory, antioedema, analgesic, and anti-coagulant effects. In this context, nutraceutical as a branch of medicine must make use of the principles of development: **quality, efficacy,** and **safety**. The **quality** includes the correct choice of raw materials, formulation strategies suited to the selected active ingredients, and the production

and storage processes, which do not compromise the stability of the product. **Advanced formulation techniques** that allow the promotion of the best bioavailability of the active ingredients, the true real weak point of several substances, are recommended to demonstrate the high biological potential of these molecules. The **efficacy** of a nutraceutical, evaluated through randomized and controlled clinical trials, may vary depending on the combination of the active ingredients and excipients present in the formulation that may or may not interact with the transport systems of the gastrointestinal tract or with the metabolism of CYP450. The **safety** of a nutraceutical is not absolute but can be influenced by several factors, which include the presence of contaminants in the chosen raw material, fractions of active ingredients present in the extract naturally toxic to the organism, and the modulation of the clearance systems by the co-intake of drugs or other supplements.

- Among the most interesting nutraceuticals, **bromelain** is widely used for the prevention or co-gestion of different disease characterized by the presence of inflammation, oedema, and algesia. Despite that several clinical trials demonstrated the efficacy of bromelain supplementation in the reduction of pain, inflammation and the oedematous component, the commercial cost of that extract is high, with prices of approximately 2400 $/kg. In addition, the new feasible methods of protein purification (e.g., membrane filtration, reverse micellar systems, aqueous two-phase extraction, and chromatographic techniques) and the new biotechnological processes developed to mitigate production costs, several limitations still create problems for the efficiency of product recovery from crude-plant extracts and the effectiveness of the obtained extract.
- The use of a freeze-dried extract of pineapple juice obtained from by-products (core and peel of *Ananas comosus*), adhering to the concept of "**zero waste approach"** and the **"circular economy"**, has been shown to preserve a good quantity of total bromelain (8% of dry weight) in active form.
- The **clinical research** activity that I followed regarding three clinical studies conducted to evaluate the potential efficacy of a lyophilized pineapple extract to reduce pain and improve quality of life in people with orchiepididymitis, or gonarthrosis, or subjected to surgical removal of lower third molars, showed that the **nutraceutical approach may reduce the need of conventional therapies** and **improve quality of life** of both paediatric and adult populations, with **excellent safety profiles** and **palatability**.

- **Larger and more extensive studies are still needed** to:
  - Verify the **scalability** in the production of pineapple extracts from food industry by-products.
  - Analyse the **final cost of the raw material** on industrial production and conduct a detailed analysis on the cost/benefit ratio of this nutraceutical.
  - Evaluate through ***in vitro*** **studies** the active ingredients present in the freeze-dried pineapple phytocomplex that can have an additive effect to bromelain.
  - Study the **pharmacokinetics of bromelain**, almost completely unknown to date.
  - Evaluate the **long-term efficacy and safety** profile **of bromelain** (even at high doses), to consider the insertion of this nutraceutical into clinical practice.

Figure 1. Main reasons of the growth of the global nutraceutical market: ageing population, increasing costs of healthcare, increasing distribution channels, and consumer awareness.



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Appendix

1. Congresses and seminaries: main oral communications

	Type	Title of the speech	Location
"Evoluzione nella salute oculare" 5-10-2019	Congress	Il ruolo della micronutrizione e il suo beneficio in oftalmologia	Reggio Emilia
"AIDI national congress" 15-11-2019	Congress	Nutraceutica per la salute orale: applicazioni cliniche	Bologna
"National congress of SIFNut" 10-2020	FAD-Congress	Fisiologia dell'assorbimento enterico di principi attivi: cosa dimentichiamo quando formuliamo i prodotti?	-
"Pallium Marche 2020" 10-10-2020	Congress	Nutraceutici nel dolore muscolo-scheletrico	San Benedetto del Tronto
"SINut seminary" 06-2020	Seminary	Vitamin D and immune-prevention	-
"Scuola microbioma" 21-11-2020	Conference	Nutraceutici, microbioma e rischio cardiovascolare	Torino
"National congress of SIFM" 27-11-2020	Congress	Fitoterapici nelle farmacie e iter degli studi clinici	Palermo
"Pediatria online green" 30-11-2020	Conference	Micronutrienti e nutraceutica	-
"National congress of SINut" 17/18-12-2020	Congress	Nutraceutica ed immunoprotezione nello sportivo	-
"University of Pisa" 12-05-2021	Seminary	Guida all'uso dei nutraceutici	Pisa

“National congress of SIFIT” 21/23-05-2021	Congress	Botanicals a supporto del declino cognitivo: esistono dei razionali scientifici?	Siena
“NUTRIMI” 30-04-2021	Seminary	Nutraceutica e condroprotezione	Milano
“University of Turin” 18-06-2021	Seminary	Alimenti e non solo cibo: guardare oltre al microbiota	Torino
“NUCE Conference” 11-09-2021	Congress	Nutraceutici e condroprotezione	Bologna
“Ketogenic diet academy” 8/02/2020	Conference	Nutraceutici e dieta chetogenica	Bologna
“Sinseb master” 16-10-2021	Master	Nutraceutica e microbiota nello sportivo	-
“Pallium Marche 2021” 16-10-2021	Congress	Nutraceutici nel dolore LOMBO-SACRALE	San Benedetto del Tronto
“Sinseb master” 3/4-12-2021	Conference	Nutraceutica e diete low-carb nello sport	Milano
“University of Pisa” 30-11-2021	Seminary	Il counselling in nutraceutica: condroprotezione, infiammazione e dolore	Pisa
“University of Pisa” 25-10-2021	Seminary	Il counselling in nutraceutica: il sistema immunitario	Pisa

“National congress of SINut” 4/6-12-2021	Congress	Nutraceutici ergogenici a supporto dell’atleta	Bologna
“Regional congress of SINut” 22-01-2022	Congress	Le dislipidemie ed il trattamento con nutraceutici	Pavia
“NUTRIMI” 29-04-2021	Seminary	Nutraceutica e condroprotezione	Milano
“SIME national congress” 13-05-2022	Congress	Trattamento “in-out” con coenzima Q10 e benefici per la pelle	Roma
“Cosmofarma NUCE” 14-05-2022	Conference	Nutraceutica e microbiota	Bologna
“AFEN national congress” 22-05-2022	Congress	Nutraceutici e sistema immunitario nell’atleta	Roma
“SINSEB national congress” 17/19-06-2022	Congress	Nutraceuticals and Sleep in Athletes	Bologna
“Federfarma Sondrio” 22-07-2022	Seminary	Nutraceutica e medicina preventiva	Sondrio
“National congress of SINut” 15/17-09-2022	Congress	Condroprotezione nutraceutica	Bologna
“National congress of SINut” 28-09-2022	Congress	Dalla ricerca sulla materia prima alla sperimentazione clinica: focus su un estratto di Ananas comosussupporto dell’atleta	Milano
“SINut regional congress” 21-10-2022	Congress	Nutraceutici e occhio	Roma

“National congress of SIFM” 11/12-11-2022	Congress	Funghi medicinali e microbiota	Pisa
“Regional congress of SINut” 21-01-2023	Congress	Nutraceutici e NAFLD	Pavia
“ADI congress” 17/18-02-2023	Congress	Nutraceutica e tumore	Torino
“Sinseb master” 04-03-2023	Master	Nutraceutica ed infortuni a tendini, muscoli ed articolazioni	Torino
“Istituto zooprofilattico Brescia” 31-03-2023	Seminary	Integratori, nutraceutici e alimenti funzionali: ruolo per la salute	Brescia
“Cosmofarma NUCE” 06-06-2023	Conference	Nutraceutica e condroprotezione	Bologna
“Le patologie osteo articolari e lo sport” 06-06-2023	Conference	Nutraceutica e fibromialgia	Coverciano (FI)
“Sinseb master” 13-05-2023	Master	Nutraceutica ed infortuni a tendini, muscoli ed articolazioni	Roma
“University of Pisa” 15-05-2023	Seminary	La sperimentazione clinica di un nutraceutico: aree grigie e prospettive future	Pisa
“SIME national congress” 21-05-2023	Congress	Microbiota intestinale, infiammazione e pelle: ruolo dei nutraceutici ed impatto formulativo	Roma

2. Didactis

	Type	Location	Host
"Nutraceuticals teaching" 9-11-2020 13-12-2020	Didactis	Varese	UnInsubria
''Oncology pharmacy teaching'' 8/10/15/17/30-11-2020 2/11/23-12-2020	Didactis	Milano	Federfarma – Imagine
''Oncology pharmacy teaching'' 2021	Didactis	Milano	Federfarma – Imagine
"Nutraceuticals teaching" 2021	Didactis	Assago, Canosa, Roma	Farmalabor
"Nutraceuticals teaching" 2021	Didactis	Roma	Imagine – Fenagifar
"Nutraceuticals board Indena" 2019/2020	Didactis	Milano	Indena
"Nutraceuticals teaching" II level master 16-04-2021	Didactis	Palermo	Università di Palermo
"Nutraceuticals teaching" II level master 29-05-2021	Didactis	Siena	Università di Siena
"Nutraceuticals teaching" II level master 15-05-2021	Didactis	Pavia	Università di Pavia
"Preparazione esame di stato biologi" 2021	Didactis	-	ONB

“Nutraceuticals teaching” II level master 22-01-2022 + 29-04-2022	Didactis	Pavia	Università di Pavia
“Nutraceuticals teaching” II level master 22-02-2022	Didactis	Siena	Università di Siena
“Scola di Nutraceutica SINut”	Didactis	-	MediAbout-SInut
“Scuola Farmacosmesi-Lab” 08-05-2022	Didactis	-	ICQ-lab
“Phytotherapy teaching” II level master 10-2022	Didactis	Bologna	Università di Bologna
“Nutraceuticals teaching” 13-12-2022	Didactis	Varese	UnInsubria
“Nutraceuticals teaching” II level master 12-04-2023	Didactis	Pavia	Università di Pavia

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Last but not least…

I want to dedicate this thesis to myself, and to my passion for study, research, and hard work.

I have never given up in the face of life's challenges, and I will not give up in the future.

Think beyond, Go beyond, Never stop.

Università degli Studi di Torino

Dottorato in

Scienze Farmaceutiche e Biomolecolari

CICLO: XXXV

TITOLO DELLA TESI: New strategies and technologies for the extraction and formulation of nutraceuticals and cosmeceuticals

TESI PRESENTATA DA: Alessandro Colletti

TUTOR: Prof. Giancarlo Cravotto

COORDINATORE DEL DOTTORATO: Prof.ssa Roberta Cavalli

ANNI ACCADEMICI: Three years

SETTORE SCIENTIFICO-DISCIPLINARE DI AFFERENZA*: Chimica Organica (03/C1)