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PAOLA FOTI

Olive Oil By-products as A New Functional Food and Source of Nutritional Food Ingredients

Ph.D. Thesis

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"A scientist in his laboratory is not a mere technician: he is also a child confronting natural phenomena that impress him as though they were fairy tales".

Marie Curie

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SINTESI

La valorizzazione dei rifiuti e dei sottoprodotti dell'industria alimentare rappresenta una sfida e un'opportunità per uno sviluppo sostenibile e competitivo. L'implementazione di strategie produttive innovative basate sulla valorizzazione dei sottoprodotti rappresenta un modello virtuoso e un fattore discriminante per la competitività delle filiere agroalimentari al fine di ridurre l'impatto ambientale, recuperare reddito ed ottenere nuovi prodotti a medio ed elevato valore aggiunto. Inoltre, nel progettare una nuova attività d'impresa diretta a produrre e commercializzare prodotti innovativi occorre considerare vari ordini di fattori determinati dalle attività economiche ed industriali presenti nel territorio in cui l'azienda opera.

La produzione di olio d'oliva è considerata una delle maggiori attività agroindustriali nei Paesi del bacino del Mediterraneo ed è associata alla generazione di grandi volumi di sottoprodotti, solo recentemente riconosciuti come una ricca fonte di molecole bioattive. Infatti, nonostante l'elevata concentrazione di composti fenolici presenti nell'oliva, solo il 2% della concentrazione iniziale si recupera nell'olio extravergine di oliva, mentre la frazione rimanente si concentra principalmente nei sottoprodotti, sia liquidi sia solidi. I fenoli sono noti per la loro azione antiossidante, antiinfiammatoria, antimicrobica ed anticancerogenae le loro proprietà benefiche sono state riconosciute dall'EFSA.

Il presente progetto di dottorato mira alla valorizzazione sia delle acque reflue di frantoio sia del pâté di olive per la formulazione di nuovi alimenti, bevande e additivi naturali ricchi in composti bioattivi ad elevato valore nutraceutico. L'approccio strategico seguito ha previsto l'applicazione di specifiche tecnologie, quali diversi sistemi di filtrazione e l'impiego di pool microbici selezionati, allo scopo di realizzare un pronto *scale-up* aziendale per la produzione nuovi prodotti a basso costo e a basso impatto ambientale. I risultati hanno dimostrato che entrambi i sottoprodotti possono rappresentare una matrice utile nel settore alimentare come tali o come ingredienti funzionali al fine di migliorare la shelf-life e il valore nutraceutico degli alimenti e di esercitare, al tempo stesso, un effetto benefico sulla salute umana.

ABSTRACT

The valorization of food wastes is a challenging opportunity for the green, sustainable, and competitive development of a country. The interest of agri-food companies in applying a circular economy strategy is driven by the need to minimize external inputs and by the interest in valorizing both co- and by-products. In this frame, the valorization of by-products represents a virtuous model for the application of a circular economy system and contribute to the competitiveness of agri-food chain, for reducing environmental impact, recovering income by new products with medium and high added value. In addition, to design a business plan aimed at producing and marketing innovative products, it is necessary to consider various orders of factors associated to the economical and industrial profiles of the territory in which the company operates.

Olive oil production is considered one of the major agro-industrial activities in the Mediterranean countries and it is associated with the generation of a large volume of by-products, already demonstrated as a rich source of bioactive molecules. In fact, despite the high concentration of phenolic compounds in olive fruits, only the 2 % of the initial concentration is found in extra virgin olive oil, while the remaining fraction is mainly concentrated in liquid and solid by-products. The bioactive compounds, namely phenols, are known for their antioxidant, anti-inflammatory, antimicrobial and anticarcinogenic activities, and their beneficial properties are already recognized by EFSA.

The present PhD project aims at the valorization of olive mill wastewater and pâté olive cake for the formulation of new foods, beverages and natural additives rich in bioactive compounds with high nutraceutical value. The choice of the followed approaches, from the filtration techniques to the application of selected microbial pools, was motivated by the requirement of a prompt industrial scale-up and by the awareness that microbial sources represents a driver of innovation in the food system, to reduce the use of chemicals and to enhance new properties in an eco-friendly way. The results showed that both by-products are relevant matrices to be used in food industry as such or as functional ingredients in order to improve the shelf-life and nutraceutical value of foods while exerting a beneficial effect in human health against chronic-degenerative diseases.

OBJECTIVES

The main objective of the study was to valorise olive oil by-products through different approaches in order to create a virtuous recycling system for olive oil industry. In detail, the pivotal objective of the PhD thesis was the valorisation of two by-products, olive mill wastewater and pâté olive cake, characterised by a high concentration of bioactive compounds, such as hydroxytyrosol, tyrosol, oleuropein, flavonoids and other molecules of great interest in the nutraceutical and pharmaceutical fields. Today, the application of by-products used as such or as extracted, concentrated and, in some cases, fermented or microencapsulated in the food sector is of growing interest, as it represents an innovative and competitive strategy for the formulation of high value-added products.

Specifically, the objectives were:

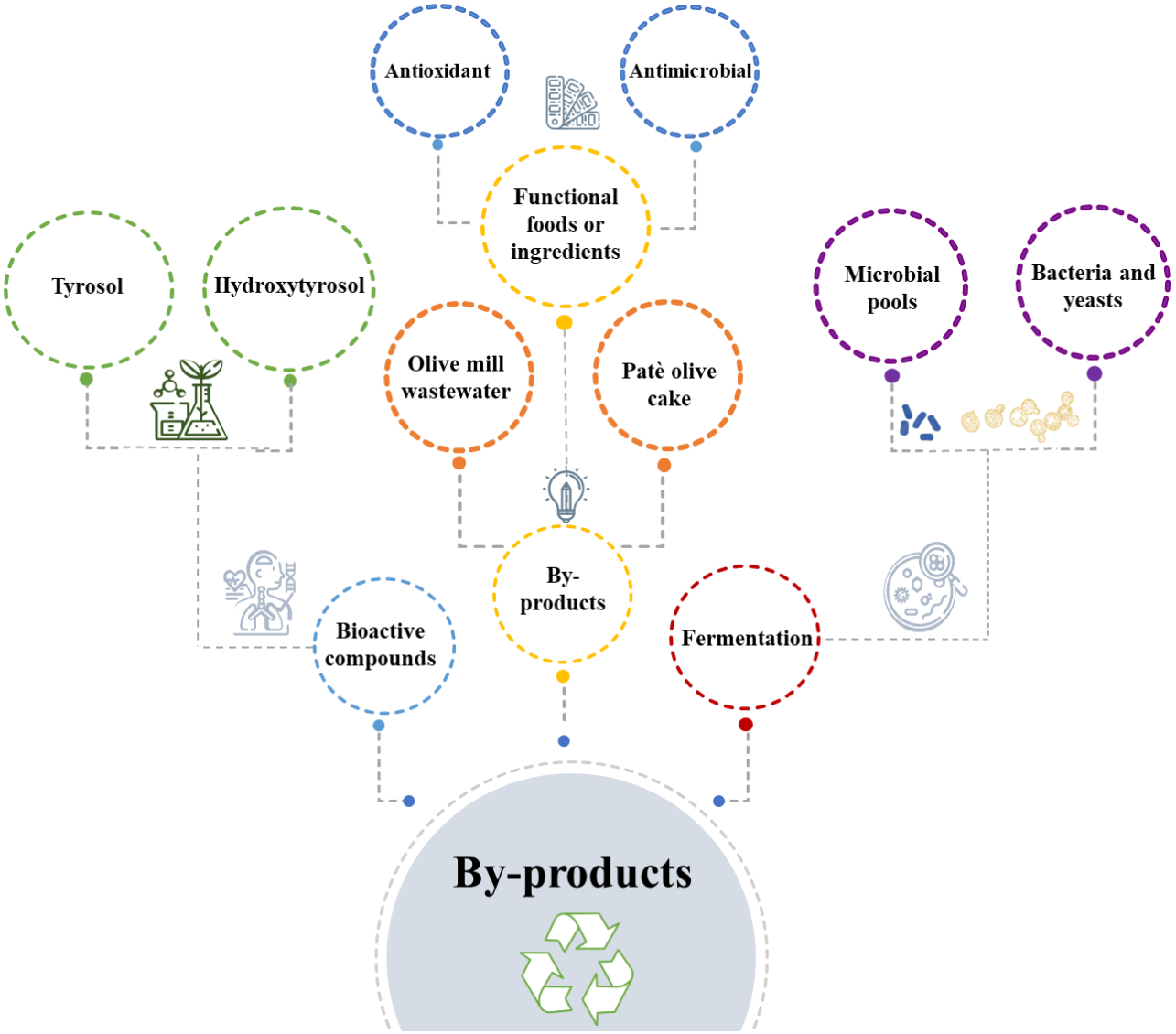
1) Valorise olive mill wastewater using an industrial system and proposing the obtained phenolic concentrates as new natural additive with a health-promoting activity for food industry;

2) To implement a valorisation strategy for the reuse of olive mill wastewater through driven fermentation using microbial pools to formulate a new fermented beverage or a new additive concentrated in bioactive compounds able to perform antioxidant and health-promoting activities;

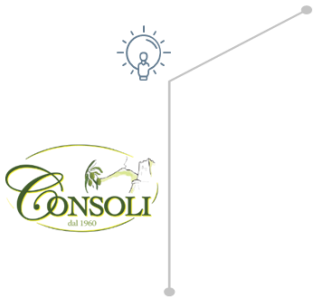
3) Enhancing the nutraceutical value of pâté olive cake through driven fermentation and assess the microbial community dynamics and the phenolic profile;

4) Replicate the fermentation, in a larger volume, of the pâté olive cake in a bioreactor and assess the process parameters, the antioxidant and the health promoting activity.

KEYWORDS


Antioxidant
Antimicrobial
Functional foods or ingredients
Tyrosol
Hydroxytyrosol
Olive mill wastewater
Patè olive cake
Microbial pools
Bacteria and yeasts
By-products
Bioactive compounds
Fermentation
By-products

AFFILIATIONS



Azienda Olearia Consoli Pasquale & F.lli s.n.c Adrano, Catania (CT), Italy.

Methodology and investigation. Set up several industrial scale trials to obtain new high value-added products from olive oil by-products in order to reduce the environmental impact and obtain cost reduction by the processing company with a view to creating a virtuous recycling system.
Period : 6 months.

Council for Agricultural Research and Economics (CREA), Research Centre for Olive, Fruit and Citrus Crops, Acireale (CT), Italy.

Conceptualization, methodology, investigation and research. Second location of the PhD research activity where physico-chemical analyses were conducted for the characterisation of various samples and metabolites produced during the fermentation process.

Department of Agriculture, Food and Environment (Di3a), University of Catania, Catania (CT), Italy.

Conceptualization, methodology, investigation and research. Principal research site of the PhD activity where microbial pools were selected for use in all fermentation trials. The different fermentations were monitored in order to assess the biotechnological aptitude of the microorganisms used in single or co-culture. Microbiological and molecular analyses were mainly carried out.

Polytechnic Institute of Bragança-IPB CIMO Bragança, Portugal.

Methodology, investigation and research. International site where various microbial DNA extraction tests and metagenetic analysis through NSG analysis were carried out to study the change in the microbial community present in fermented by-product.
Period: 8 months.

Department of Pharmacy - Pharmaceutical Sciences, University of Bari Aldo Moro (BA) Italy.

Methodology, investigation and research. Evaluation of the biological activity of by-products undergoing guided fermentation
Period: 3 months.

INTRODUCTION

CHAPTER I.
LITERATURE REVIEW

Olive mill wastewater as renewable raw materials to generate high added-value ingredients for agro-food industries

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Abstract

Olive oil production represents an agro-industrial activity of vital economic importance for many Mediterranean countries. However, it is associated with the generation of a huge amount of by-products, both in solid and liquid forms, mainly constituted by olive mill wastewater, olive pomace, wood, leaves, and stones. Although for many years olive by-products have only been considered as a relevant environmental issue, in the last decades, numerous studies have deeply described their antioxidant, anti-inflammatory, immunomodulatory, analgesic, antimicrobial, antihypertensive, anticancer, antihyperglycemic activities. Therefore, the increasing interest in natural bioactive compounds represents a new challenge for olive mills. Studies have focused on optimizing methods to extract phenols from olive oil by-products for pharmaceutical or cosmetic applications and attempts have been made to describe microorganisms and metabolic activity involved in the treatment of such complex and variable by-products. However, few studies have investigated olive oil by-products in order to produce added-value ingredients and/or preservatives for food industries. This review provides an overview of the prospective of liquid olive oil by-products as a source of high nutritional value compounds to produce new functional additives or ingredients and to explore potential and future research opportunities.

1.Introduction

Olive growing is spread over 10 million and 800 thousand hectares across the world, 97% of which are concentrated in the Mediterranean area, where the olive tree (*Olea europaea L.*) has always occupied a central role in among its population. Olive oil is one of the oldest foods and, among European countries, Spain produces about 826 thousand tons of oil, corresponding to more than 52% of world production, and Italy holds 33% of the EU production [1]. However, olive oil extraction represents a serious environmental issue due to the generation of a high quantity of waste in a very short time. The olive mill waste, both in liquid and solid forms, includes olive mill wastewaters (OMWW), wood and leaves, olive pomace (OP), and stones [2]. The worldwide production of OMWW is estimated around 6 $\times 10^6$ m^3 and 98% is produced in the Mediterranean basin. The ratio of olive oil production to OMWW is 1.0:2.5 L, reaching, in Italy, a total of 1.4 million m^3 of OMWW and 30 million m^3 in the Mediterranean basin [3,4]. In recent years, technological innovations in olive oil extraction have affected the whole supply chain, impacting the composition of OMWW, which is primarily composed of vegetation water, and water added both during malaxation and during pressing. Specifically, three different extraction processes are commonly applied: (1) the traditional press process; (2) the two- and (3) the three-phase decanter process. In the traditional process, olives are washed, crushed, mixed, and malaxed with the addition of a small quantity of water which can easily separate the oil from the other fractions. The resulting paste is then pressed to drain the residual oil and the liquid waste from the presses. It consists of a mixture of olive juice and added water and residual oil. Finally, the olive oil is separated from the water by vertical centrifugation or decanting. The traditional process is applied almost exclusively in small olive mills, with larger mills having been replaced by continuous systems. Through the use of an industrial decanter to separate all the phases, the discontinuous pressing process has been replaced by the continuous centrifugation, using a three-phase system, and later on a two-phase system [5,6]. The two-phase system, adopted in Spain and widespread in most countries, does not require the addition of water, other than during horizontal centrifugation, and results in olive oil and semi-solid olive cake [2]. The three-phase decanter process requires the

addition of hot water, in 0.6–1.3 m^3/1000 kg of processed olives [2] and results in olive oil, OMWW, and olive cake (residual solids). As a result of these differences, the three-phase extraction process presents a slightly higher yield, leading to a lower amount of olive cake but a significantly higher production of OMWW. The management of liquid wastes in olive mills has always been challenging, and extensive efforts have been carried out to find an effective strategy. Nevertheless, the disposal of OMWW in soil or waterways continues to represent a serious issue for Mediterranean countries due to its severe phytotoxicity and antimicrobial properties that can compromise the balance of ecological systems, with detrimental long-term environmental effects. In many cases, direct disposal of OMWW into lakes, rivers, and water streams has resulted in disastrous environmental consequences due to their high content of phenolic compounds, organic and long-chain fatty acids, and tannins. In addition to traditional decantation, varioussystems of purification and disposal have been proposed, such as chemical,agronomic, and biotechnological interventions. However, such approaches underestimate "waste" as a possible primary resource of high nutritional value compounds. According to EU Directive 2018/851 [7], "waste management in the Union should be improved and transformed into sustainable materials management in order to safeguard, protect and improve the quality of the environment, protect human health, ensure the prudent, efficient and rational use of natural resources, promote the principles of the circular economy, intensify the use of renewable energies, increase energy efficiency, reduce the Union's dependence on imported resources, provide new economic opportunities and contribute to long-term competitiveness". The Italian legislation, in addition to the definition of waste, identifies the conditions under which a substance or object is not to be consideredwaste, introducing the concept of by-product, which is described in Article 183- bis of the Legislative Decree n. 152/06 [8] as "the substance or object originates from a production process, of which it is an integral part, and whose primary purpose is not the production of such substance or object; it is certain that the substance or object will be used, during the same or a subsequent production or use process, by the producer or third parties; the substance or object can be used directly without any further treatment other than normal industrial practice; the further use is legal, i.e. the substance or object fulfils, for the specific use, all

relevant product and health and environmental protection requirements and will not lead to overall negative impacts on the environment or human health". As matter of fact, olive oil by-products contain a high amount of bioactive compounds, namely phenols (as reported in Table 1.). The most of the phenolic fraction present in olives is found in OMWW (up to 53%) and OP (approximately 45%), with only 2% of the initial content remaining in virgin olive oil [9]. The phenolic compounds present in OMWW are hydroxytyrosol, tyrosol, verbascoside, acids (such as caffeic, gallic, vanillic, and syringic) and polymeric substances [10,11]. Recently, the use of OMWW has been successfully proposed for different applications, and many studies have focused on obtaining compounds with high added value, i.e., phenolic extracts, through different approaches, including enzymatic and chromatographic techniques, solvent extraction methods, and membrane processes, such as microfiltration, ultrafiltration, nanofiltration, and reverse osmosis [6]. Therefore, OMWW could be considered as a potential low-cost starting matrix for extraction of antioxidants to be applied in several fields, including the food industry, where they could be used for both fortifying and prolonging the shelf life of final products [12–14]. In the present work, a literature survey was carried out taking into account a fixed timeline, between 1996 and 2020, and the keywords "olive mill wastewater". Searching on ScienceDirect, 794 records were found. Most of them fell within the scope of environmental science, such as chemical engineering, energy fuels, and agriculture, with quite constant increasing numbers in recent years, from two papers published in 1992 to 54 papers in the last five years (Figure 1a). To confirm the increasing interest in biotechnological approaches to OMWW treatment, 298 records were identified in the field of biotechnology and microbiology (Figure 1b) [15]. The aim of this review is to provide a summary of updated information on research that has been conducted using OMWW as a renewable raw material to generate high added value ingredients/products for agro-food industries, including the functional food sector.

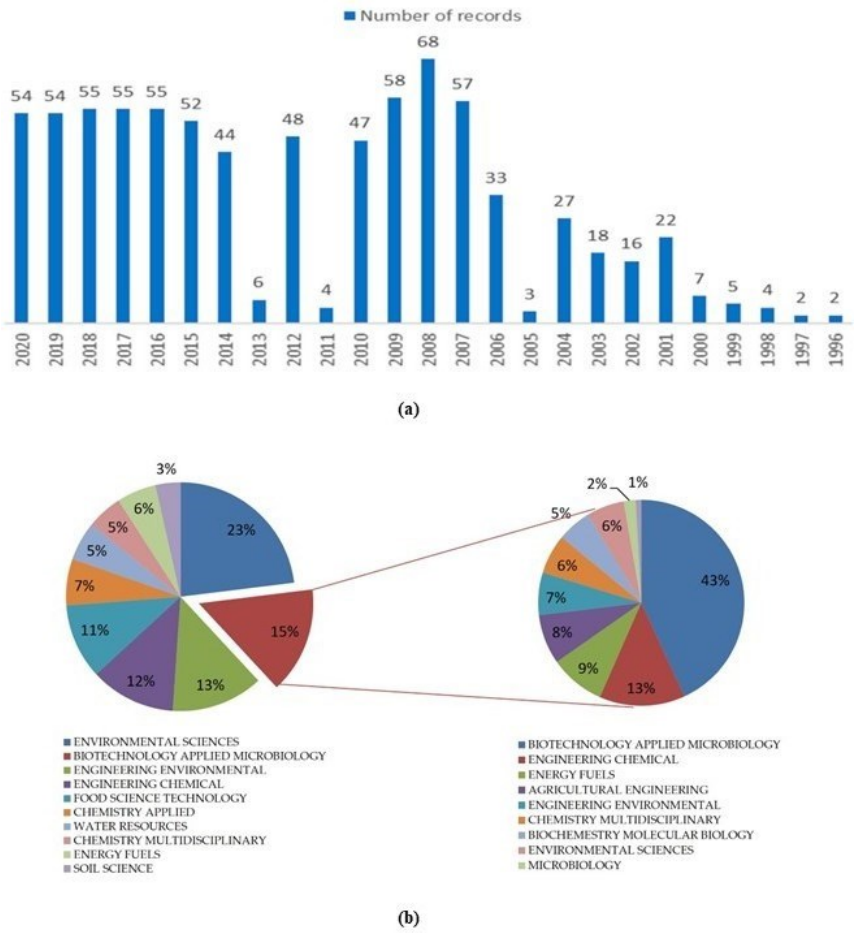

Figure 1. a) Records on olive mill wastewater found on PubMed; **(b)** distributionin different application areas of general records on OMWW, and specific records focusing on applied biotechnology and microbiology.

2. Characteristics of OMWW

2.1 Physicochemical Traits of OMWW

The OMWW is a mixture of vegetation water and soft tissues (mucilage, pectin) of olive fruits and water used in various stages of the extraction process, i.e., water added during centrifugation, and water from equipment washing [16]. The physicochemical traits of OMWW are strongly influenced by soil and climate conditions of the growing area, olive cultivar, ripeness state and, above all, by the oil extraction system. The OMWW is dark, almost black, and characterized by a typical, rather intense, odor. Due to the content of organic acids, namely malic and citric acids, OMWW presents pH values between 2.0 and 6.0 (Table 1.). Reducing sugars, essentially glucose (90%) and fructose (10%), tannins, phenolic compounds,

polyalcohols, minerals, pectins, and lipids are also present. Compared with other organic wastes, OMWW presents a higher concentration of potassium and considerable levels of nitrogen, phosphorus, calcium, magnesium, and iron [17], derived from contact with oil during the extraction phase, and due to the high hydrophilic nature of phenols [18].

Table 1. Physicochemical characteristics of OMWW, adapted by Demerche et al. [6].

Parameters	Value	Reference
pH	2.2 -5.9	[19,20]
Water (%)	80-96	[21]
Chemical oxygen demand (g/L)	30-320	[22,25]
Biological oxygen demand (g/L)	35-132	[23,25]
Dry matter (%)	6.3-7.2	[26,27]
Ash (%)	1.0	[26,28,29]
Electrical conductivity (ds/m)	5.5-10	[16,20]
Organic matter (%)	57-62	[16,20]
Total carbon (%)	2.0-3.3	[26,30,31]
Total nitrogen (g/L)	2.0-2.4	[21]
Total sugar (g/L)	5.0-12.0	[16,21,26,32,34]
Total fat (%)	1.0-23	[35]
Total suspended solids (g/L)	25-30	[36,37]
Polyalcohol (%)	9.0-15	[21,35,38]
Total phenols (g/L)	0.5-6.1	[6,26,29,33,39,41]

2.2 Microbiological Traits of OMWW

The microbial community present in OMWW is strongly influenced by several parameters, among which the ripeness state and the olive variety are the most influential [42,43]. The microbial density in OMWW varies between 10^5 and 10^6 CFU/mL (CFU: colony forming unit) and is mainly composed of yeasts, bacteria, and molds [42–45]. The yeast population includes species belonging to *Pichia, Candida*, and *Saccharomyces* genera [44,45]. A survey carried out on OMWW revealed the presence of over 100 identified fungi, mainly belonging to the genera *Acremonium, Alternaria, Aspergillus, Bionectria, Byssochlamys, Chalara, Cerrena, Fusarium, Lasiodiplodia, Lecythophora, Paecilomyces, Penicillium, Phycomyces, Phoma, Rhinocladiella,* and *Scopulariopsis* [46]. Although many studies reports that the culturable microbial population is represented by only a few bacterial communities, such as: *Firmicutes, Actinobacteria, Alphaproteobacteria, Betaproteobacteria,* and *Gammaproteobacteria*, recently, microarray analyses have revealed a high density of a larger microbial population, including

Proteobacteria, Bacteroidetes, Chloroflexi, Cyanobacteria, and *Actinobacteria*. However, the most commonly reported microbial communities, representing 50% of the 16S rRNA gene sequences deposited in GenBank, include *Gammaproteobacteria (Enterobacteriaceae, Moraxellaceae, Xanthomonadaceae, and Pseudomonadaceae*) with a percentage of almost 30%, and *Betaproteobacteria (Oxalobacteraceae and Comamonadaceae*) with a percentage of 21.5% [46]. *Alphaproteobacteria* and *Actinobacteria (Micrococcaceae, Microbacteriaceae,* and *Propionibacteriaceae*) together comprised 20%, whereas *Firmicutes (Bacillaceae, Clostridiaceae, Lactobacillaceae,* and *Paenibacillaceae)* and *Bacterioides (Prevotellaceae, Porphyromonadaceae,* and *Sphingobatteriaceae)* phyla accounted for approximately 6.8%, respectively. Furthermore, differences in microbial population have been detected, highlighting that only 15% of operational taxonomic units (OTUs) are commonly detected [47]. In addition, high densities of enteric bacteria belonging to *Porphyromonadaceae, Prevotellaceae, Lachnospiraceae, Eubacteriaceae, Peptococcaceae, Peptostreptococcaceae,* and *Ruminococcaceae spp*. or to genera *Acinetobacter, Enterobacter spp., Pseudomonas, Citrobacter, Escherichia, Klebsiella, and Serratia spp.* have been reported [48].

3. Reuse of OMWW

3.1 OMWW Management and Bioremediation

The implementation of any treatment based on the circular economy approach and "waste" reuse concept represents a competitive and innovative choice for agro-food companies for achieving a reduction in cost management and environmental impact. According to Tsagaraki and co-workers [39], 1 m^3 of OMWW corresponds to 100–200 m^3 of domestic wastewater. The COD and BOD_5 values of OMWW are very consistent and even higher when obtained by conventional systems (150 g O_2/L COD and 90 g O_2/L BOD_5 vs. 90 g O_2/L COD and 30 g O_2/L BOD_5 for conventional and two-phase extraction systems, respectively). According to the European Directive 2000/60/CE [49], the OMWW requires specific treatment prior to direct discharge to ensure environmental protection and for regenerated wastewater. Indeed, the disposal of untreated OMWW on agricultural soil causes

severe environmental damages, such as altering the color of natural water sources and exercising toxic effects on aquatic life and soil quality. OMWW is characterized by a high content of components with low biodegradability (e.g., long-chain fatty acids, lipids, and simple and complex sugars). Therefore, the most common applied systems for OMWW reuse are concerned with lowering the pollutant load and/or extracting bioactive compounds for different applications [25]. A plethora of physicochemical treatments has been developed in order to remove the phenolic compounds. However, in the majority of the studies no ecotoxicological evaluation has been reported and the success of treatment is mainly based on the reduction of color, COD, phenol content, etc. The most relevant parameter used to evaluate compost phytotoxicity is the germination index (GI). Low GI values could be attributed to the fact that at the starting stage, substrates have high concentrations of water-soluble organic substances, toxic constituents such as alcohols, organic fatty acids and phenolic compounds, high C/N ratios due to the presence of ammonia and other toxic nitrogen-based products, as well as high heavy metal and mineral salt contents [50,51]. In addition to traditional settling (conducted in tanks called “hell”), various treatments have been proposed: physicochemical, biological, or a combination. Physicochemical systems include different methods based on the use of flocculants, coagulants, membrane filtration and reverse osmosis [52], or applying oxidation cryogenesis, electrocoagulation [53,54], or a photochemical system [55]. Generally, after these treatments, the resulting products can be spread on agricultural soil as an organic fertilizer or simply subjected to evaporation in open tanks [56]. However, these practices are expensive as they produce matrices, such as sludge, that must either undergo further treatments or be disposed of. Several reports confirm that microorganisms can be proposed as a promising alternative for bioremediation of OMWW [51]. Biological methods, involving anaerobic or aerobic digestion and composting, have been applied to break complex organic compounds into simpler molecules and may lead to the production of proteins, exopolysaccharides, or energy [57,58]. The main interest in anaerobic digestion is the production of energy and reuse of the effluent for irrigation purposes [59]. However, the leading limitation is the inhibition of methanogenic bacteria by both phenolic and organic acids compounds [60]. According to Azbar and co-workers [36], anaerobic filters or up flow anaerobic

sludge bed reactors are suitable systems to remove unwanted compounds from OMWW. Filidei et al. [61] proposed sedimentation–filtration treatment of OMWW prior to anaerobic digestion as a useful method for its disposal. On the other hand, aerobic treatment is used to reduce the polluting load, responsible for certain biostatic and phytotoxic effects. Aerobic treatment has also been applied to reduce the polluting effect of municipal wastewater, focusing on the degradation of phenolic compounds. Several microorganisms, such as *Pleurotus ostreatus, Bacillus pumilus, Yarrowia lipolytica*, etc., have been tested [62–64]. Furthermore, a pool of *Candida boidinii* and *Pichia holstii* strains has been selected for its ability to reduce (up to 40%) the phenolic content of OMWW combined with 6.0 g/L of $(NH_4)_2SO_4$ at 10 °C [65]. OMWW has been proposed [66,67] as a growth substrate for *Azotobacter vinelandii* and the resultant effluents applied to cropland as fertilizer. Therefore, recent studies have shown that the biotechnological potential of indigenous microbiota should be further exploited with respect to bioremediation of OMWW and inactivation of plant and human pathogens.

3.2 OMWW Phenolic Compounds for Agricultural Use

Phenolic compounds from OMWW might be used for integrated pest management programs. Several studies have reported the use of microorganisms (as single or consortia) to degrade organic compounds in effluents [68,69]. Although OMWWs do not contain toxic substances, they are characterized by high COD values and a high concentration of compounds with biostatic activity. Recently, increasing attention has been focused on the degrading properties of microorganisms and biological aerobic treatments using yeasts and filamentous fungi, which have emerged as suitable biofertilization methods for conducting residues with lower toxicity, COD, and phenolic contents. Aissam et al. [70] treated OMWW with microorganisms isolated from the same source, such as *Candida boidinii*, *Geotrichum candidum*, *Penicillium* sp. and *Aspergillus niger*, obtaining a 40–73% reduction in phenols and a 45–78% reduction in COD value. Bleve et al. [45] identified several strains belonging to the genera *Geotrichum*, *Saccharomyces*, *Pichia*, *Rhodotorula*, and *Candida* that showed strain dependent phenol removal efficiency, decreasing phenolic and COD values, regardless of initial phenolic concentrations. In particular, *G. candidum*, both as free and Ca-alginate

immobilized cells, showed the best degradation performance, and when immobilized showed a double reduction rate ability. Indeed, Ca-alginate improved the proteolytic stability of the enzymes responsible for the degradation process. Maza-Márquez et al. [69] demonstrated that the use of a microalgal–bacterial consortium, in a photo-bioreactor, induces a decrease in pollutant load by affecting COD, BOD_5, phenolic compounds, color, and turbidity values of OMWW. The dominant green microalgae *Scenedesmus obliquus*, *Chlorella vulgaris* along with cyanobacteria *Anabaena* sp., showed a synergistic effect on resistance to toxic pollutants, leading to their decomposition. In addition, the effect of *Lactiplantibacillus plantarum* strains on decolorization and biodegradation of phenolic compounds was evaluated [69], highlighting strains able to decrease the OMWW pH within 6 days. Growth of *L. plantarum* induced the depolymerization of high molecular weight phenols, resulting in discoloration of fresh OMWW and a significant reduction in total phenols [71]. Approximately 58% of the color, 55% of the COD, and 46% of the phenolic compounds were removed when OMWW was diluted tenfold before *L. plantarum* addition. Furthermore, OMWW has also been proposed for biopesticide and compost production. The OMWW application on soil and crops resulted in a growth suppression of most phytopathogenic bacteriaand fungi and weed species without any effect on crop growth. However, certain measures should be adhered to when OMWW is used as a biopesticide, especially regarding dose and timing of use [47].

4. OMWW as a Source of Biopolymers and Bio-Energy Production

4.1 Enzyme and Exopolysaccharide Production

OMWW represents a suitable substrate for the production of enzymes by fungi. Fungi are microorganisms known for their ability to synthesize different biological catalysts that can be used in different areas. In particular, Ntougias and co-workers [46] demonstrated that ligninolytic fungi are a useful source of phenoloxidase, polyphenoloxidase, and peroxidase useful for removing recalcitrant compounds in OMWW. Several yeast strains have been characterized as highly pectolytic, xylanolytic, provided with cellulase, β-glucanase, β-glucosidase, peroxidase, and polygalacturonase activities, which could effectively degrade the complex

compounds responsible for OMWW toxicity [65,72]. Several yeasts have been described as able to reduce phenolics and sugars present in OMWW, although white-rot fungi appear to contribute more to discoloration [73]. Moreover, Giannoutsou and co-workers [74] isolated six phenotypically distinct groups of yeasts and three selected isolates were identified through biochemical tests and partial 18S rDNA gene sequence analysis as most closely related to *Saccharomyces* spp., *Candida boidinii*, and *G. candidum*. These fungal genera have been reported as able to degrade the phenolic content present in OMWW [75,76]. Several reports also propose strains belonging to different species, such as *Panus trigrinus*, *Hericium erinaceus*, and *Pleurotus citrinopileatus* for laccase (Lac) and manganese peroxidase (Mnp) production [77–79]. Filamentous fungi, such as *Aspergillus oryzae*, *Aspergillus niger*, *Aspergillus ibericus*, *Aspergillus uvarum*, *G. candidum*, *Rizhopus oryzae*, *Rhizopus arrhizus*, and *Penicillium citrinum*, have been described as lipolytic reservoirs due to their ability to produce lipase [80]. These enzymes have been used in different industries, such as dairy and pharmaceutical [81]. Moreover, OMWW has been confirmed as a suitable substrate for production of pectinase, with *Cryptococcus albidus* var. *albidus* IMAT 473 showing the best biotechnological aptitude. This enzyme, compared with other products on the market, showed a broad spectrum endopolygalacturonase activity [82–84]. Besides enzyme production, OMWWs have also been evaluated as a source of polysaccharides, especially exopolysaccharides (ESP) [85] with glucose as the main monosaccharide, followed by galactose, arabinose, rhamnose, and galacturonic acid. Xanthan, a glucose-mannose and glucuronic acid repeating unit compound, is the main ESP used in different products, such as in cosmetic formulations or as a supplement and thickening compound [86]. However, the EPSproduction through a fermentation process depends on the type of microorganism. The first production of EPS in OMWW (used at 30% v/v) was obtained through a strain of *Xanthomonas campestris* that showed a productive capacity of 4 g/L [87].Similarly, *Paenibacillus jamilae* sp. highlighted, on OMWW, the production of anEPS consisting of fucose, xylose, rhamnose, arabinose, mannose, galactose, and glucose. Morillo et al. [88] reported that *P. jamilae* CECT 5266 strain (in an 80% v/v of OMWW) produced an EPS consisting of glucose, galactose, mannose,

arabinose, rhamnose, hexosamine, and uronic acid, in agreement with results previously reported by Ruiz-Bravo et al. [89] using the strain *P. jamilae* CP-7.

4.2 Production of Bioenergy and Biofuels

The need to reduce dependence on conventional fossil fuels in favor of new alternative energy resources is a top global priority. Green energies could contribute to the reduction of greenhouse gas emissions and their consequent unfavorable impacts on global warming and climate change [90]. The high content of organic matter and the low content of nitrogen, volatile acid sugars, polyalcohols, and fats, make OMWW an attractive resource for the production of bioenergy and alternative biofuels, such as methane or ethanol [6,51]. Several microorganisms are used for biohydrogen production, through single or combined catabolic pathways (e.g., *Rhodobacter sphaeroides, Rhodopseudomonas palustris,* and *Chlamydomonas reinhardtii*). The production of these substances takes place through a process of anaerobic digestion, which consists of two phases. During the first phase, macromolecules, such as carbohydrates, proteins, and lipids, are transformed by hydrolytic and acidogenic fermentative bacteria into simple or intermediate organic compounds, volatile organic acids (acetic, propionic, and butyric acids), alcohols (ethanol), ketones, CO_2, and hydrogen. In the second step, through interactions between methanogenic and acetogenic microorganisms, these metabolites are transformed into CH_4 and CO_2 [91]. However due to the presence of oily residues or phenols responsible for antimicrobial activity, OMWW must be first treated or diluted [92]. As already known, before implementing an anaerobic digestion process, the treatment of OMWW with some fungi, such as *A. niger*, *Aspergillus terreus*, and *Pleurotus sajor-caju* play a key role in order to increase the final production of the reference bioenergy compound. Hamdi et al. [57] and Borja and co-workers [93], through a comparative kinetic study, demonstrated that the pretreatment of OMWW with *A. niger* and *A. terreus* increased the methane yield. Massadeh and Modallal [94] evaluated the ability of a *P. sajor-caju* strain to degrade the phenols of OMWW producing ethanol. For this purpose, the authors examined the effects of dilution with water (in a 1:1 ratio), heat treatment (at 100 °C), and treatment with H_2O_2. The results showed that the degradation of phenols by *P. sajor-caju* reached a level of 50% in heat-treated OMWW, 53% in heat-

treated OMWW pretreated with H_2O_2, and 58% in undiluted heat-treated OMWW. The highest ethanol yield was obtained in samples pretreated with *P. sajor-caju* and after 48 h of fermentation with 50% diluted and heat-treated OMWW. Further biological treatment was carried out with *Saccharomyces cerevisiae*. Sarris et al. [95] and Nikolaou et al. [96] confirmed the aptitude of *S. cerevisiae* to produce ethanol and optimal fermentation parameters were detected using the 1:1 OMWW/water mixture ratio. The fermentation kinetics of molasses mixed with OMWW where *S. cerevisiae* was immobilized affected the ethanol yield, reaching values up to 67.8 g/L per day. Moreover, Zanichelli et al. [97] proposed a multiphase treatment using S. cerevisiae added to OMWW with glucose, to a final sugar concentration of 200 g/L, with *A. niger* extract to hydrolyze the present polysaccharides. Although *S. cerevisiae* showed low fermentative performance, indigenous strains belonging to *Pichia fermentans* and *Candida* spp. reduced phenolic content up to 15% and 18%, respectively, without any addition or pretreatment [98]. Furthermore, Sarris et al. [99] demonstrated the ability of *Y. lipolytica* strain ACA-DC 5029 to grow on media containing a low concentration of crude glycerol and OMWW, producing a significant amount of citric acid and erythritol. In the presence of high glycerol concentration, a shift towards erythritol production was observed, simultaneously with high amounts of citric acid production. The strain showed promising characteristics to be used in the biotransformation of biodiesel derived from the combination of crude glycerol and OMWW and the subsequent production of added-value chemical compounds.

4.3 Use of OMWW in Feed Formulation

The use of agro-industrial by-products in animal feed can represent an economically and environmentally advantageous solution for the livestock sector, increasing its profitability and sustainability [100]. Olive oil by-products have been tested for the formulation of feed for lambs, pigs, and chickens by evaluating the antioxidant activity on animals and on final products. Makri et al. [101] evaluated the effect of OMWW addition in a silage formulation for lambs, containing 52.5% of solids, 7.5% of OMWW, and 40% of water. The administration of OMWW containing silage was found effective in improving animal welfare and productivity. Furthermore, several authors tested the effectiveness of a reduction in oxidative

stress and in the stimulation of the immune response of the same extract for pigs. Gerasopoulos et al. [102] studied the antioxidant effect of the addition of 4% of OMWW (representing the retentate obtained by microfiltration) in silage. Piglets fed with the fortified formulation showed an increase in tested biomarkers (as total antioxidant capacity: TAC; glutathione: GSH; catalase activity: CAT; protein carbonyls: CARB; and reactive thiobarbituric acids: TBARS) in blood and tissues and a decrease in oxidative stress, with an overall increase in productivity. In addition, Varricchio et al. [103] evaluated the antioxidant activity in piglets fed with phenol extracts, and results highlighted an increase in leukocytes and cyclooxygenase-2 (COX-2), known as markers of inflammation. Gerasopoulos et al. [104] repeated the test in chickens, highlighting markers of antioxidant activity with the same silage formulation proposed for piglet feeding. The results confirmed that such supplementation lowers the levels of lipid peroxidation and protein oxidation by increasing the total antioxidant capacity in plasma confirming that both OMWW and oil by-products (leaves and olive pomace) can be a viable alternative to fortify animal feeds.

5. Bioactive Properties of OMWW

Olive oil by-products are rich in bioactive compounds with potential health benefits [41]. Ciriminna et al. [105] investigated the relationship between phenolics and health benefits on food, pharmaceutical, and cosmetic applications. Regarding the food sector, the addition of phenols from OMWW seems very interesting not only to strengthen the beneficial effects of foods themselves, but also to extend their shelf life. In the U.S., olive pulp extracts have been approved by the Food and Drug Administration (FDA) with GRAS (Generally Recognized as Safe) (GRN No. 459) status as antioxidants in baked goods, beverages, cereals, sauces and dressings, condiments, and snacks, at a final concentration of up to 3 g/kg [106,107]. Commercial OMWW implementation in food and recovery of phenols is of great interest [108,109] and at least five companies worldwide recover phenols from OMWWs [110] to sell them as natural preservatives or bioactive additives in food products [111].

5.1 Antioxidant Properties

Many reports, both in humans and animals, confirmed that most degenerative diseases, such as cancer and cardiovascular diseases [112] are related to oxidative stress, which has also been identified as a causative agent for declining immune function and atherosclerosis [113]. Several nutraceuticals aimed to reduce the oxidative stress are currently available on the market [114]. Phenols are recognized as the main compounds responsible for the health effects of the Mediterranean dietin prevention of chronic diseases and diet-associated diseases (DRDs), such as obesity, metabolic syndrome, type 2 diabetes (T2D), cardiovascular disease (CVD), hypertension, and some cancers. Their role has been clearly recognized by the European Food Safety Authority [115] with the health claim: "Olive oil polyphenols contribute to the protection of blood lipids from oxidative stress." In recent years, an increased interest in the extraction of phenols from OMWW has been registered and different extraction techniques have been proposed [116]. Phenols are active ingredients of many medicinal plants and the mechanisms of their pharmacological activity are not yet fully understood. Beyond the mechanismof protection, based on antioxidant activities, phenols have highlighted: scavengerproperty against free radicals and reactive oxygen forms (ROS); ability to act as chelators of heavy metals (especially iron) and capability to inhibit lipoxygenase, involved in inflammatory processes. The main radical species, involved in diseases, responsible of cytotoxic effect and in damaging membranes' lipids, are superoxide anion ($O2^{-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH^{-}) [117].

5.2 Antimicrobial Properties

The main phenolic compounds present in OMWW are those derived by oleuropein hydrolysis, as hydroxytyrosol, tyrosol and elenolic acid, but also other phenols: caffeic acid, p-coumaric acid, vanillic acid, syringic acid, gallic acid, luteolin, quercetin, cyanidin, verbascoside, and other polymeric compounds [10,11]. Markovi´c et al. [118] demonstrated that hydroxytyrosol, tyrosol, oleuropein, and oleocanthal present a wide spectrum of biological effects on physiological processes, being antiatherogenic, cardioprotective, anticancer, neuroprotective,

antidiabetic, anti-obesity compounds. Furneri and co-workers [119] revealed that oleuropein was also effective against *Mycoplasma fermentans* and *Mycoplasma hominis*, which are naturally resistant to erythromycin and often also to tetracycline. Biocompounds of olive products, such as aliphatic aldehydes [120], have also been shown to inhibit or retard the growth of a range of bacteria and yeasts and could be considered as an alternative for the prevention or treatment ofinfections. Moreover, they have been evaluated for drug formulations to reduce thespread of antimicrobial resistance bacteria [121]. Bisignano et al. [122] demonstrated that hydroxytyrosol possesses an in vitro antimicrobial property against respiratory and gastrointestinal infectious agents, such as *Vibrioparahaemolyticus, Vibrio cholerae, Salmonella Typhi, Haemophilus influenzae, Staphylococcus aureus*, and *Moraxella catarrhalis*, at low concentrations.

6. OMWW as Replacer of Synthetic Additives

The strong demand for adequate nutrition is accompanied by the concern for environmental pollution with a considerable emphasis on the recovery and recycling of food by-products and wastes [9]. Several studies have focused on replacing synthetic additives with natural substances, mainly derived from plants and agro-industry by-products [123,124] with promising results. The addition of such substances not only inhibits the growth of pathogens but also prolongs the shelf life of food products. OMWWs are added as such or as extracts, concentrated and stabilized and, in some cases, microencapsulated. Specifically, encapsulation protects them from degradation due to different factors reducing the amount of compounds required to be efficient and controlling their release into the food matrix [125]. Besides therapeutic benefits, biophenols present in OMWW have been explored for their antimicrobial, antifungal, and antiviral properties. Obied etal. [14] reported that the phenolic fraction of OMWW shows antibacterial activityagainst several species, particularly *S. aureus*, *Bacillus subtilis, Escherichia coli*, and *Pseudomonas aeruginosa*. However, the antimicrobial activity was found to be higher when the whole phenolic content is used, compared with the activity of the single phenolic compound [126]. In particular, Serra et al. [127] showed that natural OMWW extracts exhibited a higher antimicrobial activity compared with the three individual biophenols (quercetin, hydroxytyrosol, and oleuropein),

suggesting a synergic effect among molecules. In most cases, to inhibit the growth of target strains, the effective tested dose was 1000 μg/mL. In addition, it has been shown that individual phenolic compounds, used at low concentrations, were not able to inhibit the growth of *E. coli, Klebsiella pneumoniae, S. aureus, and Staphylococcus pyogenes*, while whole OMWW was effective in inhibition of both Gram-positive and Gram-negative bacteria [121]. Other authors, however, reported that the bactericidal and fungicidal activities of OMWW are mainly related to the content of phenolic monomers, such as hydroxytyrosol and tyrosol [128]. Hydroxytyrosol was found to also be active against foodborne pathogens such as *Listeria monocytogenes*, *S. aureus,* Salmonella Enterica, and *Yersinia* spp. [129] and against beneficial microorganisms, such as *L. acidophilus* and *Bifidobacterium bifidum*. In addition, Fasolato et al. [130] confirmed the bactericidal effect of phenol extract purified from OMWWs. In particular, *S. aureus* and *L. monocytogenes* showed the lowest level of resistance (minimum bactericidalconcentration MBC = 1.5–3.0 mg/mL) while Gram-negative bacteria (e.g., Salmonella Typhimurium and *Pseudomonas* spp.) showed higher resistance, withMBC values ranging from 6 to 12 mg/mL. In the same study, among the tested starter species, the growth of *Staphylococcus xylosus* and *L. curvatus* was drastically reduced (at concentrations of 0.75 and 1.5 mg/mL MBC, respectively).

6.1 Application of OMWW as Food Supplement

In several studies, olive oil by-products have been added as concentrates or ingredients in the formulation of novel foods in different agro-food supply chains (Table 2). In a review, Galanakis [131] collected data related to the addition of OMWW extracts (but also of other oil industrial by-products) to fortify meat and meat products. The results showed that the obtained antioxidants induce an improvement of hygienic conditions and rheological characteristics of the final products. Olive phenols have shown better performance in raw meat treatment [132] as they were able to hinder lipid oxidation. To evaluate such an effect, an oxidation test with a thiobarbituric acid reaction (TBAR) was applied for a storage period of 72 h at 4 °C. Results in limiting lipid oxidation appear to be dependent on the concentration of phenols (500 mg ascorbic acid or catechin/L and 100 mg olive phenols/L). Lopez et al. [133] and Veneziani et al. [111] recently applied OMWW

extracted polyphenols in fermented sausages and white meat burgers, improving quality parameters and extending their shelf life. In particular, the addition of the extracts inhibited the fungal growth and spore germination in fermented sausages by performing a dose- and species -dependent activity both in vitro and in situ tests. In particular, the treatment with 2.5% of OMWW extract strongly inhibited in situ growth of *Cladosporium cladosporioides, Penicillium aurantiogriseum, Penicillium commune,* and *Eurotium amstelodami*. Veneziani et al. [111] evaluated the effect of OMWW extracted polyphenols in white meat burgers, wrapped in PVC, on improving sensorial and hygienic characteristics. The addition of the phenolic extract at different concentrations (0.75 and 1.50 g/kg) delayed the growth of mesophilic aerobic bacteria, highlighting a dose-dependent behavior, with a 24 h extension of shelf life, compared with both the control and sample treated with the lowest concentration. In addition, Fasolato et al. [130], according to Servili et al. [134], found that a 38.6 g/L concentration of phenolic extract was effective in increasing fresh chicken breast shelf life. Samples were immersed in a solution containing the extract for a few seconds, before packing and storage at 4 °C. The results showed a delay of growth of both Enterobacteriaceae and *Pseudomonas* spp. with at least a 2 day increase in shelf life, compared with the control. In addition, the treatments were shown to positively affect the odor of meat, decreasing the TBARS value. De Leonardis et al. [135] proposed the addition of lard with olive phenols as a "novel food", showing that the natural antioxidants of OMWW were highly effective in oxidative stabilization of lard. The phenol extract significantly increased the oxidative stability of lard, and the applied doses (100–200 ppm) were not cytotoxic when tested on mouse cell lines (embryonic fibroblasts). In addition, several studies have tested phenol extracts in dairy products to enhance antioxidant activity and better stabilize the products. Troise et al. [136] tested the antioxidant activity of OMWW phenolic extract in UHT milk samples, on inhibition of the Maillard reaction (MR), by adding phenolic extract at 0.1 and 0.05% w/v, revealing the reduction of reactive carbonyl species formation in samples before heat treatment, inducing a greater stability without any detrimental sensorial effects. Phenol extracts (100 and 200 mg/L) from OMWW have also been added in a functional milk drink (similar to yogurt) and fermented with a GABA producing strain (*L. plantarum* C48) and a LAB strain of human origin (*L. paracasei* 15N).

The results showed that the addition of phenolic compounds did not interfere with either the fermentation process or the activities of functional LAB [134]. The addition of extracts of both OMWW and olive pomace, at different concentrations (2, 4, 6, and 8 mg/100 g of butter) was tested in a butter formulation [137], revealing that the highest concentration confers resistance to oxidative stress during storage at 25 °C for 3 months, inhibiting the growth of *S. aureus*, total coliforms, yeast, and molds. Roila et al. [138] added biophenol extract (at 250 µg/mL and 500 µg/mL) to mozzarella cheese retarding the growth of *Pseudomonas fluorescens* and Enterobacteriaceae. The shelf life was directly proportional to the concentration, increasing by 2 and 4 days, respectively. Galanakis et al. [139] tested the antioxidant effect of OMWW phenolic extracts in combination with other antioxidants, demonstrating a reduction in oxidative deterioration during baking of bread and rusks and showing an antimicrobial effect against *S. aureus*, *B. subtilis*, *E. coli*, and *P. aeruginosa* (at 200 mg/Kg of flour). Recently, Cedola et al. [140] enriched bakery products by adding OMWW and OP previously subjected to ultrafiltration and evaluated the quality traits of final products from both a chemical and sensorypoint of view. Ultrafiltered OMWW, was used both in bread dough (1500 g of wheat flour, 900 g of OMWW, 45 g of fresh compressed yeast) and for the formulation of spaghetti at a final concentration of 30% w/w. The results showed that the addition of OMWW into bread and pasta slightly increased the chemical quality of bread and pasta without compromising their sensory traits. Zbakh et al. [141] proposed the exploitation of OMWW for setting up a functional beverage. Commercial products can include different additives, such as ascorbic acid as antioxidants, chelators including ethylenediaminetetraacetic acid (EDTA) and acidifiers, such as citric acid or carbon dioxide. The use of additional antioxidants was not required in beverages when OMWW extract were applied. Recently, a certain interest has developed in new beverages using aqueous extracts obtained with olive leaves, characterized by a high concentration of biophenols. Some of these products are already in the market and sold as integrators for human consumption. Further studies are required to investigate the effects of different formulations on the bioavailability of OMWW phenols and on their beneficial effects. These biological properties can have a significant impact on human healththrough reducing the incidence of many diseases, especially cardiovascular and chronic degenerative

diseases. As previously reported by Zbakh et al. [141], which confirmed that OMWW phenolic compounds are highly bioavailable and safe, the potential application of OMWW for setting up functional beverages as a natural concentrate of substances with antioxidant action could be a promising opportunity.To date, on the market there are beverages containing water extracts with different pharmacological indications: antioxidant, blood pressure regulator, and incidence on the metabolism of lipids and carbohydrates, although no reference legislation for the use of olive water for human consumption is currently available.

Table 2. Application of OMWW in agro-food chains.

Agro-Food Chain	Food	Quantity	Activity	Results	Reference
	Fermented sausages	2.5%	Antifungal	Inhibition of *C. cladosporioides, P. aurantiogriseum, P. commune,* and *E. amstelodami* growth	[133]
	White meat burgers	0.75–1.50 g/kg	Antimicrobial	Retarding the growth of aerobic mesophilic bacteria	[111]
	Lard	100–200 ppm	Natural antioxidant	Stabilization in oxidative of lard	[135]
	Meat	75 to 100 mg/L	Natural antioxidant	Extension of shelf life: color retaining, inhibition of microbial growth and fat deterioration	[132]
	Fresh chicken	38.6 g/L	Antimicrobial	Delay in Enterobacteriaceae and *Pseudomonas* spp. growth	[130,134]
	Milk	0.1–0.05% *w/v*	Functional ingredient	Increasing product stability	[136]
	Functional milk, fortified beverage	100–200 mg/L	Beverage fortification	Formulation of functional milk	[134]
	Butter	80 mg/kg	Natural antioxidants	Confering resistance to oxidative stress	[137]
	'Fior di latte' cheese	250–500 µg/mL	Antimicrobial	Increasing shelf life	[138]
	Bread and rusks	200 mg/Kg of flour	Antimicrobial and natural antioxidants	Inhibition of *S. aureus, B. subtilis, E. coli,* and *P. aeruginosa* growth and reducing oxidative deterioration during cooking	[131]
	Bread and pasta	900 g of OMWW (for bread) and 30% *w/w* (for pasta)	Antioxidant and food fortification	Food fortification: enhancing chemical composition without compromising the sensory characteristics	[140]

7. Conclusions and Future Perspectives

According to the future of Food and Agriculture: trends and challenges [142], about one third of all produced food is lost or wasted along the food chain, from production to consumption, highlighting an inefficiency of current food systems. The valorization and reuse of food by-products can create a virtuous recycling system in accordance with the Global Food 2030 objectives. Although the two-phase extraction system is slowly replacing the traditional olive oil extraction techniques, the disposal of OMWW remains a problem for many small olive oil mills in Italy and in other Mediterranean countries and the valorization of such a by-product appears more important than ever for the agro-food industry. The chance for agro-food companies to implement a circular economy strategy has offered new choices in by-product valorization. Despite several chemical characterizations of olive by-products, further searches are needed to fully understand the resources of such an interesting valuable raw material. Future olive oil waste management strategies should include a combination of physical and biotechnological processes, followed by further treatments, for producing valuable by-products with high functional activities. In this way, costs of treatments could be compensated by the income from useful by-products.

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Olive Pomace and Pâté Olive Cake as Suitable Ingredients for Food and Feed

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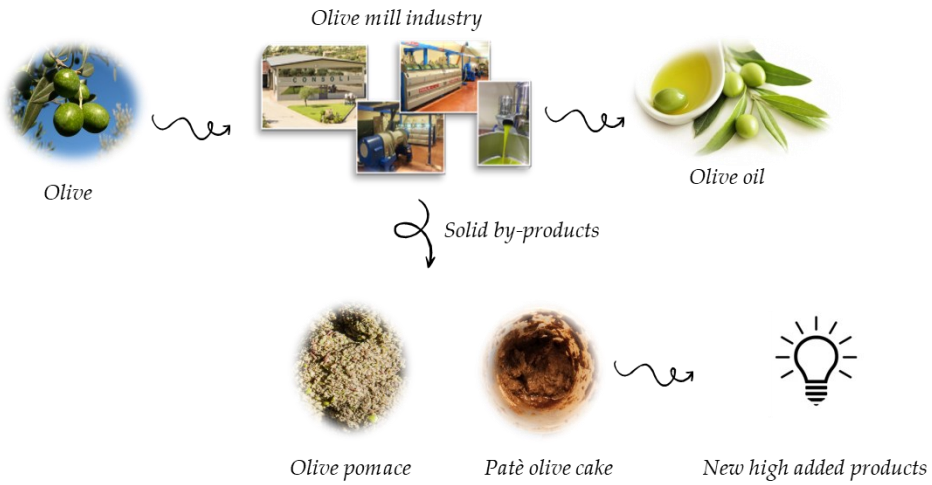
Abstract

Olive oil extraction generates several by-products that represent an environmental issue,mainly for Mediterranean countries where olive oil is mostly produced. These by- products represent an ecological issue for their phenolic components, such as oleuropein,hydroxytyrosol, and tyrosol. However, olive oil by-products can be treated and properly exploited in different fields for their health-promoting properties, and they represent great potential for the food and beverage, cosmetic, and pharmaceutical industries.Furthermore, recovery and treatment processes can contribute to efficient waste management, which can enhance the sustainability of the olive oil industry, and in turn, lead to relevant economic benefits. The solid waste, i.e., olive pomace, could be considered to be a suitable matrix or primary resource of molecules with high added valuedue to their high phenolic content. Olive pomace, at different moisture contents, is the main by-product obtained from two or three-phase extraction systems. A commonly usedcentrifugal extraction system, i.e., a multiphase decanter (DMF), does not require the addition of water and can generate a new by-product called pâté or olive pomace cake, consisting of moist pulp that is rich in phenols, in particular, secoiridoids, without any trace of kernel. Although several reviews have been published on olive wastes, only a few reviews have specifically focused on the solid by-products. Therefore, the aim of thepresent review is to provide a comprehensive overview on the current valorization of themain solid olive oil by-products, in particular, olive pomace or pâté olive cake, highlighting their use in different fields, including human nutrition.



1. Introduction

The olive oil market is constantly growing and strongly subjected to technological innovation aimed to improve the yield and quality of the final product. The European countries are the main producers, consumers, and exporters of olive oil, providing about 67% of the world's production. The cultivation of olive trees, combining traditional, intensive, and super intensive groves, in the EU Mediterranean countries, takes up about 4 million hectares. Among the EU, the largest consumers of olive oil are Italy and Spain, with an annual consumption of approximately 500,000 tons each, while Greece shows the highest per capita consumption, with about 12 kg per person per year [1]. As a result, there is an inevitable production of by-products coming from olive oil processing, i.e., mainly olive mill wastewater and olive pomace (OP). The valorization of these by-products responds to the strong demand for innovation in food system. Agrofood wastes and by-products represent a suitable matrix to be exploited in the food chain. This process involves the stabilization of wastes and by-products, and the extraction of added-value compounds that could be entirely treated or directly added as functional ingredients for designing new foods or valuable ingredients with medium and high-added value [2]. Since the highest content (in the order of 70%) of phenolic compounds is found in the unwrapped part and in the outer parts of the olive fruit [3], several studies have focused on the detection of phenolic content in olive oil by-products [4,5]. Recently, nutrigenomic approaches have largely confirmed the beneficial effect of phenols and highlighted their role in modulating the expression of different transcripts and mRNAs involved in glucose/lipid metabolism, proliferation, inflammation, and cancer [6]. The olive oil extraction process involves mainly pressure and centrifugation and can be classified into two-phase and three-phase systems. Depending on the applied extraction process, different effluents are produced (Table 1). While the three-phase system requires high amounts of water and produces considerable amounts of effluent together with a pomace consisting of woody endocarp and cuticle with a moisture content of 48–54%, the two-phase system produces wet pomace with a moisture content of 58–62% [7]. Olive pomace (OP) has been mainly subjected to hexane extraction of residual oil and the obtained product then subjected to distillation and refining. However, the extraction of oil from OP has been discontinued because it is unprofitable and because it poses issues from a

management point of view. For these reasons, several attempts have been made to use OP for the extraction of high added-value compounds or for direct use. To date, OP is used in agriculture as a soil conditioner and fertilizer; in bioenergy production; and for the extraction of hydroxytyrosol, tyrosol, oleuropein, caffeic acid, and squalene, intended for pharmaceutical, food, or cosmetic industries. However, up to now, no evidence of using such a product in human nutrition has been reported. Furthermore, an additional new by-product, called pâté olive cake (POC), is generated when olive oil extraction is carried out by using a multiphase decanter (DMF, Leopard, Pieralisi), which is an evolution of the two-phase system that combines the advantages of processing without water addition with the simplicity of three-phase extraction. The POC consists of olive pulp and vegetation water (with a moisture content of 75–90%) without any trace of kernel and it is the third product obtained by a DMF decanter together with virgin olive oil and a dried pomace (with a moisture content of 45–55%). POC is rich in bioactive compounds, and therefore, represents a promising matrix [8], potentially suitable for the formulation of new functional food. However, to reduce its bitter taste and its high perishability, due to high water content, further treatments are required.

Table 1. Olive oil and by-products from different extraction systems [7,9].

Extraction system	Olive oil (kg/100 kg olive)	Added water (%)	Olive mill Wastewater	Olive Pomace (kg/100kg olive)	Pomace Moisture (%)
Traditional		-	40	40	20
Three-phases	20	50	80-110	55-57	48-54
Two-phases	20	0-10	8-10	75-80	58-62
Two-phases DMF	20	-	-	45-55	75-90
Three-phases to savings	20	10-20	33-35	56-60	50-52

2. Physico-Chemical and Microbiological Characteristics of Olive Solid Waste

2.1 Physico-Chemical Traits of OP

OP is a solid waste that results from olive oil extraction and consists of pulp and kernel [10]. For one ton of processed olives, 0.5–0.6 tons of OP, characterized by high moisture con- tent, are produced. The OP chemical composition is influenced by the

olive cultivar, growing conditions, and by the extraction process used. Generally, the process of olive oil extraction produces, essentially, two types of OP, wet pomace (coming from a two-phase system) and dry pomace (coming from a three-phase system). The OP exhibits a dark color and pH values ranging between 4.8 and 5.2. The main components of fresh OP are water, carbohydrates, fiber, cellulose, hemicellulose, and lignin, although fats and proteins are also present, even if at smaller quantities [11]. Recently, Nunes et al. [12] reported the chemical composition of fresh OP, expressed as g/100 g. In particular, the moisture content was found to be around 60/100 g, carbohydrate content as 34/100 g, protein and ash as 2.6 and 0.7/100 g, respectively, and total fat content as 2.0/100 g. Regarding carbohydrates, they were mainly represented by lignin (43.95/100 g), insoluble and soluble fraction (2.6–17/100 g), hemicellulose (11.29/100 g), cellulose (9.55/100 g), and to a lesser extent, by arabinose, galactose, and mannose [13]. Furthermore, OP is characterized by a high content of both organic matter and carbon, high level of potassium, low content of phosphorus, and intermediate levels of nitrogen [14–16].

2.2 Microbiological Traits of OP

The microbiome of OP has been described as very similar to that of other oil by-products, such as OMWW, and composes both bacteria and yeasts. Vivas et al. [17] identified *Proteobacteria* as a dominant member, followed by *Actinobacteria* (*Streptomyces*), *Firmicutes* (*Staphylococcus*), and *Acidobacteria.* In addition, members of *Hydrocarboniphaga*, *Pseudoxanthomonas*, and *Stenotrophomonas* (*Gammaproteobacteria*) were detected, with *Comamonas* (*Betaproteobacteria*) as the main microbial group. In addition, the bacterial population has been explored by amplification of the internal transcribed spacers between the 16S and 23S rRNA genes (ITS-PCR) and by 16S rRNA sequencing. The results showed that Firmicutes were the most prevalent and diverse members [18]. Regarding the fungal population, it seemed to be strongly influenced by the olive cultivar. The dominant yeasts were *Pichia caribbica* (syn. *Meyerozyma caribbica*), *Pichia holstii* (syn. *Nakazawaea holstii*), and *Zygosaccharomyces fermented* (syn. Lachancea fermenta), followed, to a lesser extent, by *Zygosaccharomyces florentinus* (syn. *Zygotorulaspora florentina*), *Lachancea thermotolerans* (syn. *Kluyveromyces thermotolerans*), *Saccharomyces cerevisiae*, and *Saccharomyces rosinii* (syn. *Kazachstania rosinii*).

2.3 Physico-Chemical Traits of POC

DMF (multiphase decanter, Leopard) extraction technology is the only two-phase centrifugal system that produces a dehydrated pomace and recovers the POC inside the drum. This by-product shows a semi-solid consistency, and its composition is strongly influenced by the olive cultivar and ripening period. It is essentially composed of olive pulp, olive skin, wastewater, and devoid of woody parts [19]. In detail, the POC presents a high content of organic matter, fiber, and crude protein, and a low lignin content, similar to OP obtained from pitted olives. Recently, Lanza et al. [9] reported the composition of POC obtained from different cultivars such as Leccino, Carboncella, and Tortiglione. The titratable acidity, expressed as g/100 g of citric acid, ranged from 0.29 to 0.56, and the pH value ranged from 4.95 to 5.20. The moisture percentages were found to be between 81 and 84%, and the ash content was from 1.57 to 2.75%. The residual oil content, determined by Soxlet, ranged from 3.7 to 4.2%. A higher residual oil content of 10% and a lower moisture content (as 77%) was found in Frantoio and Leccino cultivars. Furthermore, the POC was characterized by high levels of essential fatty acids, such as linoleic and linolenic acids (8.5 and 1%, respectively) [8].

2.4 Microbiological Traits of POC

Currently, few studies have been carried out on microbiological characterization of POC. As recently reported by [9], the results of microbiological analyses, carried out on Mac-Conkey agar for Enterobacteriaceae counting, on malt extract agar for yeasts and molds, and on Man, De Rogosa, and Sharpe agar for lactic acid bacteria (LAB) counting, showed cell density values, expressed as colony forming units (CFUs), of around 10^5, 10^4, and between 10^3 and 10^4 CFU/g, respectively.

3. Bioactive Compounds of Solid Oil Waste Products

The use of vegetable by-products for the recovery of bioactive substances through chemical and biotechnological processes is a promising strategy [20]. The bioactive molecules found in food waste are known for their antioxidant and radical scavenging activity; for inhibiting the oxidation of DNA, proteins and lipids; and for significantly reducing the development of several diseases, such as cancer, Alzheimer's, and

Parkinson's [21]. Furthermore, the OP is widely used in different fields for antioxidant and antimicrobial properties [22]. Several phenolic compounds have been detected by liquid chromatography techniques coupled with quadrupole time-of-flight mass spectrometry (QqTOF/MS) in OP such as: oleuropein, hydroxytyrosol and tyrosol derivatives, iridoid precursors, secoiridoids and derivates, flavonoids, lignans, and phenolic acids. In particular, the most abundant phenolic compound present in olive fruit, namely the oleuropein, has been found at high concentrations in OP, reaching concentrations up to 0.9% [23,24]. The main antioxidants identified in the OP were vitamin E (α-, β-, γ-tocopherol, and α-tocotrienol); among them, α-tocopherol was the major vitamin E form (2.6 mg/100 g of raw matter). Concerning the POC obtained from the DMF system, different compositions have been reported in the literature, probably due to the different olive cultivars used for oil extraction. According to some authors, unlike other olive oil by-products, POC is mainly composed of oxidized hydroxytyrosol and luteolin, while other authors have reported mainly oleacein, hydroxytyrosol, and verbascoside. However, significant differences have been found among the different cultivars: while the POC obtained from Carboncella and Tortiglione cultivars showed the highest values of hydroxytyrosol, i.e., 977 and 720 mg/kg, respectively, and tyrosol, i.e., 57 and 67 mg/kg, respectively, the Leccino cultivar showed lower values, i.e., 243 and 21 mg/kg [9]. Further- more, Cecchi et al., 2018, confirmed that the main components of POC are hydroxytyrosol, tyrosol, 3,4- DHPEA-EDA, and verbascoside.

4. Exploitation of OP

OP has been traditionally used as a matrix to recover residual oil by solvent extraction (usually hexane) and the obtained spent pomace has been intended as a fuel or for use in the cabinetmaking sector [22]. Oil extraction from pomace is an expensive method and the use of this by-product has been proposed in different fields, in particular, in agriculture, in bioenergy production, and as a matrix for extraction of high added-value components to be used in the food, feed, and packaging sector. Regarding their use in agriculture, Innangi et al. [25] showed that despite the content of phenolics and salts, direct dispersion of OP in soil did not entail any negative impact. As a matter of fact, the long-term use of OP seemed to improve organic matter, enzymatic activities, and the soil quality index. Furthermore, several authors demonstrated that direct

application to soil could entail an increase in minerals, such as nitrogen, useful for crop production and could remove pollutants, such as heavy metals, herbicides, and triazine herbicides [26]. According to the Italian law [27], the spreading of OP must follow a suitable distribution and absorption and the recommended dosage is of 10 m^3/ha. As far as the production of bioenergy is concerned, the combustion of OP results in lowering the costs of energy conversion from non-renewable sources, for generation of thermal and electrical energy [28]. Several studies have demonstrated that aerobic digestion of OP showed good energy recovery. Furthermore, anaerobic digestion of biomass could produce biogas and recover energy. In addition, the indigenous microorganisms of OP have been evaluated for ethanol production and a 3% yield has been reported [29].

5. Use of Solid Olive By-Products in the Food and Feed Sectors

5.1 Use of OP in Food

Several applications of OP in the food and feed sectors have been proposed. In the food sector, OP has been added as an extract to preserve and increase final product stability. OP powders represent a matrix rich in various nutrients and bioactive compounds with human health and technological effects, as rheological improvement of final products [30]. However, despite their antioxidant and antimicrobial activity, the addition of vegetable by-products could alter the sensorial and technological properties of food, and for this reason, the specific amount to be added in formulations must be carefully selected [31]. An effective strategy to avoid the sensorial alteration of final products is the encapsulation of bioactive molecules. Such a strategy, in addition to improving their functional properties, provides adequate protection from thermal insults (temperature increasing), controls their release into the food matrix, and reduces the amount needed to be effective in food applications [32]. Phenols extracted from OP have been added to olive oil to further improve healthy trait and to inhibit lipid oxidation. Although results have shown increased oxidative stability of the final product, an alteration of sensory traits was observed, with bitterness and off-flavor significantly higher in phenol-enriched virgin olive oil. Regarding bakery products, OP is added (at 10% w/w) in bread and pasta. Cedola et al. [33] demonstrated that although the addition of both olive wastewater and OP improved

the nutraceutical value of final product, the OP resulted the most suitable ingredient despite higher was the adverse effect on sensory traits, related to bitter and spicy taste. Simonato et al. [34] fortified pasta by replacing durum wheat semolina with different concentration of OP (0.5 and 10 g/100 g). Fortification with OP significantly increased the content of total phenolics, and consequently, the antioxidant activity both before and after cooking. Furthermore, the OP fiber content resulted in an in vitro change in the digestibility of starch, by decreasing the rapidly digestible fractionand by increasing the slowly digestible starch fraction. Moreover, OP flour has been added to biscuits at 15% to develop functional foods with higher fiber content, high nutritional value, fewer calories, and lowered glycaemic index [35]. In a further study, volunteers eating OP supplemented biscuits exhibited a shift in their intestinal microbiota. A metagenomic analysis of 16S rRNA profile showed an increase in the abundance of *Akkermansia* and *Bifidobacterium*, both positively correlated to host physiology and protection from metabolism and cardiovascular disease [36,37]. In addition, *Bacteroides* and *Prevotella*, linked to the onset of obesity and traditional dietary paradigms, were also positively affected, whereas Enterobacteriaceae were negatively correlated [36–38]. In addition, an LC-MS analysis detected significant increases in phenolic acid concentrations in urine, 24 h after ingestion of enriched OP biscuits. Homovanillic acid and hydroxytyrosol derivates, involved in oxidative LDL-cholesterol reduction, were detected in blood [36]. These findings were in agreement with those reported by Ribeiro et al. [39], who confirmed the prebiotic activity of OP-added formulations subjected to simulated gastrointestinal digestion followed by in vitro fecal fermentation. In particular, the formulation obtained from the liquid fraction induced a positive effect on the *Prevotella* spp./*Bacteroides* spp. ratio, resulting in production of short-chain fatty acids and showing good mucin-adhesion inhibition capacity against *Bacillus cereus* (22.03 ± 2.45%) and *Listeria monocytogenes* (20.01 ± 1.93%). Other studies have reported that the addition of polyphenol rich extracts to dairy products increased their stability and prevented rancidity [40]. The phenolic extract was added to fermented milk at 100–200 mg/L ratio. The results showed that this addition did not interfere with the fermentation by *Streptococcus thermophilus* TA040 and *Lactobacillus acidophilus* LAC4 strains. Different concentrations (2, 4, 6, and 8 mg/100 g) were also added to butter and two storage temperatures (25 and 60 °C) were evaluated. The results showed that the

highest tested concentration conferred resistance against oxidative stress [41]. Furthermore, Ribeiro et al. [42] demonstrated that the addition of OP extracts (1–2%) in yoghurt provided 5 mg of hydroxytyrosol per day, increased fiber content, and improved the bioaccessibility of total phenols by 25.58%.

5.2 Use of OP in Feed

The use of OP for feed purposes is a widespread practice for the re-use of this by-product. In order to have a good market value, a feed must provide both adequate nutritional value and low cost, in order to compete with conventional feeds [43]. The addition of olive by-products in feed, used in both aquaculture and livestock, did not show any adverse effect on animal growth and improved the fatty acid profile by reducing saturated acids [44]. In aquaculture feed, the partial substitution of any of the most common vegetable oils (such as soybean, linseed, sunflower, rapeseed palm oil, and olive oil) can be proposed only if fatty acids in diet are adequately present to meet the essential fat requirements of fish and ultimately humans. Sioriki et al. [45] demonstrated that OP added at 8% in sea bream feed improved the cardioprotective properties of the final product by enriching the lipid profile of the fish with specific cardioprotective lipid compounds of vegetable origin. In addition, other authors have integrated OP in the diets of sea bass and sea bream and showed a presumed cardioprotective effect evidenced by an increased production of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in the lipid fraction of fish [46,47]. OP has been added to rabbit and lamb feed. Specifically, the introduction of OP into lamb feed increased the oxidative stability of the meat [48]. Similar results were observed for lambs [49]. The addition of 35% of OP and linseed to animal feed has shown a synergistic effect by increasing the level of polyunsaturated fatty acids and vitamin E, and decreasing the level of peroxides, thiobarbituric acid reactive substances (TBARS), and the number of conjugated dienes. In addition, oxidative stability has been monitored in pork. Phenols were detected in the meat, where they increased the sensory characteristics of the product and a 5% decrease in saturated fatty acids was observed [50,51]. Furthermore, several studies have shown that the addition of OP to animal feed is beneficial to animals and also improves the nutritional quality and quantity of the by-products, i.e., dog, milk, cheese, and eggs. Chiofalo et al. [52] demonstrated that the addition of OP, between 7.5 and 15%, positively influenced the

performance of animals, increasing meat tenderness and influencing meat quality indices, such as intramuscular fat and unsaturated fatty acids. In addition, milk produced from cows fed with fortified feed showed an increase in unsaturated fatty acids content (oleic acid, vaccenic acid, and CLA) and a decrease in SFA (short- and medium-chain fatty acids). These findings suggest that OP can improve the nutritional properties of milk and cheese without compromising their sensory profile [52]. Results related to fatty acid composition of cow's milk and cheese were confirmed by Castellani et al. [53], who indicated that monounsaturated and polyunsaturated fatty acids were increased by supplementing the feed with OP. In order to assess any shift in the flavor profile of milk or dairy products obtained from cows fed with OP, analyses were carried out using GC/MS and electronic nose. The results showed that OP did not alter the sensory profile of the product. Several studies have shown that milk produced by sheep and buffalo induced nutritional improvements in the product itself. In fact, Mannelli et al. [54] and Vargas-Bello-Pérez et al. [55] demonstrated that the addition of OP in feed improved the nutritional characteristics of sheep milk. In particular, the revealed microbiota composition showed a reduction in Anaerovibrio, a lipase-producing bacterium, which induced an inhibition of lipolysis at the expense of polyunsaturated fatty acids. Chiofalo et al. [56] also evaluated the yield and composition of ewe's milk and highlighted that the OP addition positively influenced the milk yield and improved the nutritional values by increasing the unsaturated to saturated fatty acids ratio. Finally, the use of OP at 10% also seems to have a positive effect on laying hens. Indeed, the results of a transcriptomics analysis showed that the cholesterol content of eggs was decreased as compared with controls, probably due to the modulatory effects of phenols on genes involved in the cholesterol biosynthesis pathways [57].

5.3 Use of DMF POC for New Functional Food

POC obtained from a two-phase extraction system consists of a moist pulp containing lipophilic and hydrophilic fractions and is considered to be a matrix with functional and technological properties (Table 2). Currently, the main applications of POC are in the feed sector, although several studies have evaluated strategies to process this by-product into a product intended for human consumption, as a food or nutraceutical supplement, which could represent a profitable advantage for olive oil companies. As

matter of fact, POC has been proposed as a valuable functional ingredient to fortify feed. Indeed, the inclusion of fresh POC (35%) in lamb feed demonstrated an improvement in meat quality without compromising oxidative stability [49]. Similar results were observed in chickens raised with PCO fortified feed. The results showed that the growth of chickens was better with the highest dose of POC (165 g/kg). Tyrosol and sulphate metabolites of hydroxytyrosol were found in the poultry meat and, consequently, an increase in the oxidative stability of the meat was observed [50]. POC has also been proposed as a functional additive for the formulation of bakery products. Padalino et al. [19] fortified spaghetti with POC flour at a rate of 10to 15% w/w. The spaghetti fortified with 10% w/w showed a high added-value content of phenolic com- pounds and were judged acceptable from a sensory point of view. A similar study [58], carried out on taralli, a typical Apulian product, was performed using POC, previously subjected to sequential fermentation, with yeast and LAB (namely *Saccharomyces cerevisiae* and *Leuconostoc mesenteroides*). The taralli was enriched by adding 20% fermented POC from black olives. The profiles of both the bioactive compounds and the fatty acids were monitored during storage for 180 days. The results showed significantly higher levels of bioactive compounds (hydroxytyrosol, tyrosol, verbascoside, oleacin, oleocanthal, maslinic acid, α-tocopherol, and lutein) than the control. In addition, the enriched taralli maintained a low content of saturated fatty acids and a high level of polyphenols, for up to 90 days of storage. However, the use of POC for human consumption seems to be an increasingly interesting objective for the scientific community. Indeed, several authors have already proposed POC as a new food or nutraceutical supplement, demonstrating its beneficial effects on human health. In detail, as suggested by Cecchi et al. [59], based on the total phenolic content, 1 g of dried POC provided a daily intake comparable to 200 g of virgin olive oil [59]. Tuffariello et al. [60] proposed a new product coming from a sequential fermentation, on a pilot scale, through inoculation of *S. cerevisiae* and *Leuc. mesenteroides*. The sequential inoculum improved fermentation performance significantly and demonstrated that, as in the case of table olives, the first part of fermentation was dominated by yeasts and the second part by LAB. In fermented POC, the total phenol levels were slightly reduced as compared with an unfermented sample; however, the hydroxytyrosol content was higher, while triterpene acids, carotenoids, and tocochromanols remained unchanged.

A desirable shift in volatile compounds, due to production of alcohols, esters, and acids during fermentation, was observed. The olive cultivar seems to be the most relevant factor that affects phenolic content and biological characteristics. Peršurić et al. [61] evaluated the biological activity of two POCs obtained from different cultivars, namely Frantoio and Ascolana tenera. The results obtained through chromatographic analysis (LC coupled to triple quadrupole mass spectrometry) and MALDI- TOF/MS showed that the POC presented a high content of hydroxytyrosol, verbascoside, and oleuropein aglycone derivatives and a content of triacylglycerols, rich in oleic fatty acid. The Frantoio variety, as compared with Ascolana, showed higher antioxidant activity, with phenol content of 26.66 and 17.48 g GAE/kg, respectively. The biological activity was evaluated on different enzymes, namely amylase and glucosidase, known as targets in diabetes mellitus and cholinesterase, and are the enzymes mainly involved in Alzheimer's disease [62]. Overall, POC from Ascolana showed a higher tyrosinase and amylase inhibition as compared with Frantoio. Most recently, Lanza et al. [9] confirmed a significant correlation between phenolic content and the cultivar. In agreement with previous reports, the olive cultivar from which the POC was obtained, strongly influenced the bioactive compounds detected. In detail, POC obtained from Carboncella and Tortiglione cultivars showed a higher content of total biophenols (5899 and 5543 mg/Kg, respectively) as compared with that obtained from the Leccino cultivar which exhibited a concentration of 948 mg/Kg. In the same study [9], POC extract left to spontaneous fermentation was tested on CaCo2 and HCT116 colon cancer cells, through an MTS assay, which is a colorimetric method applied for the sensitive quantification of viable cells. The rate of inhibition of cell proliferation was affected by the different tested extracts and by the used dilutions, and these results were correlated to the different contents of each phenolic compound, such as oleuropein, verbascoside, hydroxytyrosol, and the secoiridoid derivative oleacein. Indeed, in Caco2 cells, POC obtained from the Leccino cultivar induced significantly higher cell viability as compared with control cells, at any tested concentration. A similar effect was observed on HCT116 cells with POC obtained from Carboncella and Tortiglione cultivars at specific concentrations. Furthermore, other studies have focused on assessing the antiaging effect and the cardioprotective activity of different POCs. In detail, Cecchi et al. [59] evaluated the phenolic content present in fresh and freeze-

dried POCs. In fresh POC stored in tanks for 4 months, an increase in hydroxytyrosol content was observed over time, with concentrations reaching 5635 mg/Kg, whilst a decrease in oleuropein and verbascoside content was observed. In contrast, the freeze-dried POC was found to maintain the same phenolic content for several months. Tests with a hydroalcoholic extract from POC showed antiaging activity in human cells similar to the effect obtained by using pure hydroxytyrosol. Furthermore, the cardiovascular and metabolic protective effects of tablets obtained from POC (corresponding to 30 mg/day of hydroxytyrosol) was in vivo tested [63]. The results showed, in plasma, a reduction in total cholesterol (−10.8 mg/dL), LDL-cholesterol (−10.8 mg/dL), and urea, and a significant increase in calcium (+0.3 mg/dL). In addition, leukocytes, subjected to exogenous oxidative stress induced with H_2O_2, reacted by increasing the levels of the antioxidant transcription factor Nrf-2 by 88.9% and by reducing plasma levels of the proinflammatory cytokine MCP-1, a proinflammatory protein involved in the atherosclerotic process [63]. To explore the interaction between POC and human intestinal microbiota, a study using the SHIME®, an advanced gastrointestinal simulator, was proposed [64]. The study aimed to understand how the phenolic fraction present in POC influenced bacterial growth and how it could exert an antimicrobial effect in a dose-dependent manner. The main compounds found were: oleuropein-derived molecules, free hydroxytyrosol, and in a smaller amount, verbascoside and luteolin; while the fiber content was composed by both insoluble and soluble fraction (20.4% and 3.7%, respectively); whereas monosaccharide and protein contents were present at 16.8% and 9%, respectively. The same study [64] confirmed that the POC did not exhibit any antimicrobial effect on the intestinal microbial community, as the SCFA production was not reduced, while it induced a reduction in Fusobacteriaceae (usually related to inflammatory status) and an evident increase in Lactobacillaceae and Bifidobacteriaceae [64]. With regard to phenolic content, a decrease in hydroxytyrosol combined with a contextual increase in tyrosol, registered after 9 days, confirmed the presence of esterases, which are commonly active in gut microbiota and known to be involved in hydrolysis of various phenolic compounds.

Table 2. Different applications of POC.

Sector	Aim	POC-form	Amount	Results	Reference
Livestock	Feed fortification	Fresh	35%	Reduction of oxidation of cholesterol and fatty acids in lamb	[49]
	Feed fortification	Fresh	82.5 /165.0 g/Kg	Enhancement of meat oxidative stability at higher POC concentrations	[50]
Natural additives for food application	Increase the nutritional value of spaghetti	POC endured air-dried at low temperature	10 and 15% (w/w)	Increase in the content of flavonoids and total phenols	[19]
	Increase quality and nutritional value of taralli	Fermentde POC with *Saccharomyces cerevisiae* and *Leuconostoc mesenteroides*	20%	Increase in the bioactive compounds and saturated fatty acids maintained at a low level	[58]
Food	New fermented product	Fermented POC with *Saccharomyces cerevisiae* and *Leuconostoc mesenteroides*	-	Increase in hydroxytyrosol content and improved sensory notes by production of alcohols and esters	[59]
	Formulation of new functional food	Fresh, fermented or extracted POC		Carboncella, Tortiglione and Leccino cv. show different total phenol contents. The metabolic activity, tested on CaCo2 and HCT116, suggest beneficial effects related to modulation of gene expression	[9]
Nutraceutical	Formulation of new functional ingreditents	Fresh	-	The Frantoio cv show high antioxidant activity with a phenol content of 26.66 gGAE/kgThe POC obtained from As- colana cv show higher tyro- sinase and amylase inhibi- tion than those obtained from Frantoio cv.	[61]
Pharmaceutical	Evaluation of antiaging effect of phenolic extract	Fresh and dried	-	Dried POC contains high levels of hydroxytyrosol, oleuropein derivatives and exhibited a long stability, unlike fresh POC, where a breakdown of hydroxytyrosol occurred. The diluted hydroalcoholic extract shows an in vitro antiaging effect	[59]

	Evaluation of effect on cardiovascular and metabolic diseases	Tablet	4 tablets/day of POC (corresponding to 30 mg/day of hydroxytyrosol) for 2 months	Reduction in total cholesterol, LDL-cholesterol, and urea, and significant increase in plasma calcium level. Decrease in the proinflammatory protein MCP-1 in plasma	[63]
	Evaluation of interaction of POC with the intestinal microbiota through SHIME®	Powder	4 g/L of POC	No antimicrobial effect on the colonic microbial community, as SCFA production is not reduced Reduction in *Fusobacteriaceae* and increase in *Lactobacillaceae* and *Bifidobacteriaceae*.	[64]

6. Conclusions and Future Perspectives

The valorization of agro-food waste is a challenging opportunity for the sustainable and competitive development of an innovative food system and the use of by-products in the food sector remains to be a relevant challenge, with a view to the creation of virtuose recycling. Although a large number of studies have been published on olive oil by-product treatment and/or valorization, only a few studies have specifically focused on valorization of OP or POC in the food and feed sectors, highlighting that these solid by-products represent a valuable source of bioactive compounds which deserve to be reused. As a matter of fact, OP and POC applied as such or in low percentages in food formulations have proven to extend shelf-life, therefore, enhancing the functional traits of final products. Nowadays, the recovery of bioactive compounds and their addition to food formulations is a promising strategy to obtain functional food. The next challenge is the valorization of POC through biological debittering and stabilization to promote its rational use and to enrich the lipophilic and hydrophilic bioactive compounds in food, which can exert beneficial effects on human health.

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RESULTS

"Only if you are ready to consider the impossible are you ready to discover something new."

Johann Wolfgang von Goethe

The results obtained during the PhD are represented by three chapters. Each chapter is represented by a manuscript, some of them have already been published.

- **CHAPTER II. OLIVE MILL WASTEWATER**

Phenols recovered from olive mill wastewater as natural booster to fortify blood orange juice

Olive mill wastewater fermented with microbial pools as a potential new functional beverage

Protective Effect of Treated Olive Mill Wastewater on Target Bacteria and Mitochondrial Voltage-Dependent Anion-Selective Channel 1

- **CHAPTER III. PÂTÉ OLIVE CAKE**

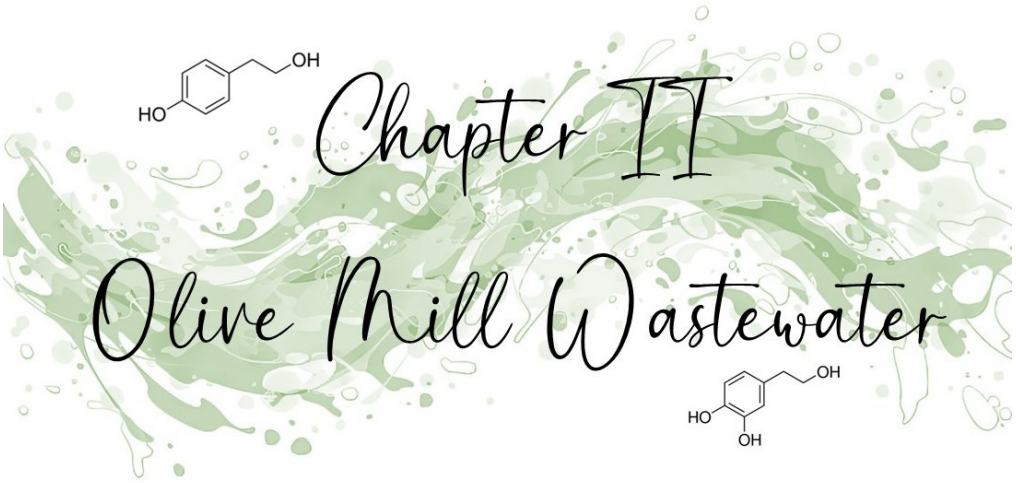
Profiling of phenol content and microbial community dynamics during pâté olive cake fermentation

Effect of microbial fermentation on functional traits and volatiloma profile of pâté olive cake

- **CHAPTER IV. STUDY RELATED TO THE PHD SUBJECT**

Microbial Application to Improve Olive Mill Wastewater Phenolic Extracts

CHAPTER II.
OLIVE MILL WASTEWATER



Phenols recovered from olive mill wastewater as natural booster to fortify blood orange juice

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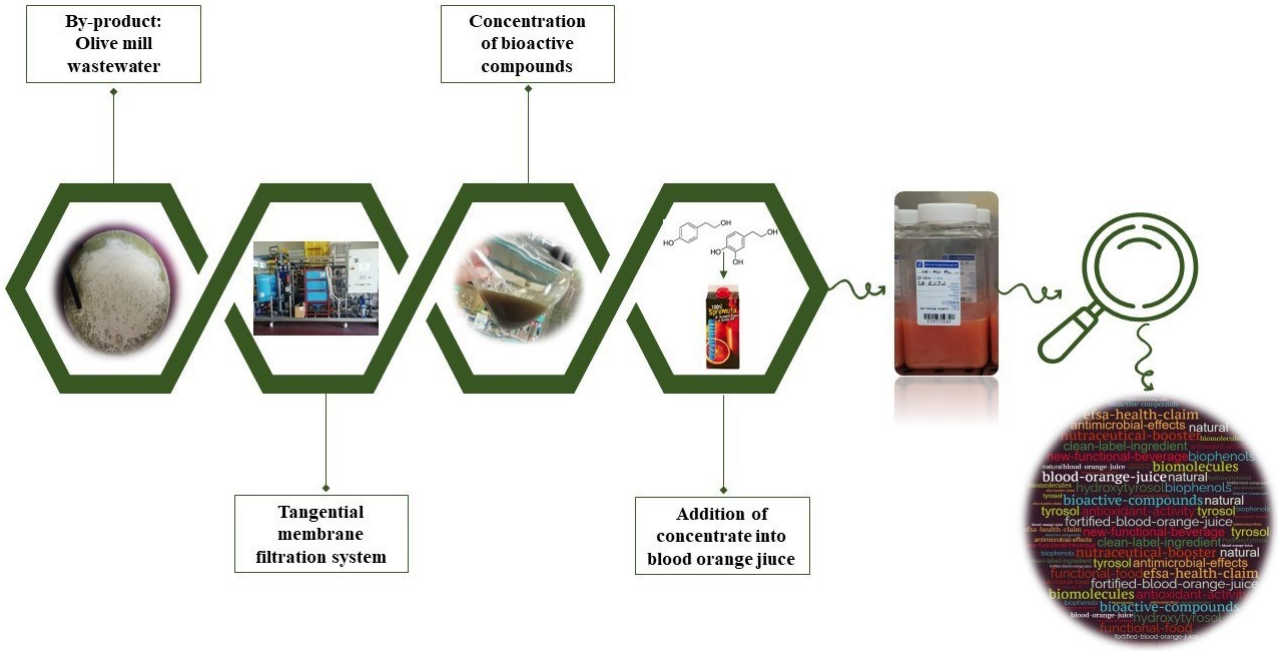
Abstract

In the present study, a tangential membrane filtration system was applied to recover phenols from olive mill wastewater. The obtained concentrates were characterised for physico-chemical traits, antioxidant activity and antimicrobial effects. Results indicated that the highest concentration of hydroxytyrosol (7203.7 mg/L) was detected in the concentrate obtained by reverse osmosis, which also showed the highest antioxidant and antimicrobial activity. Moreover, the same concentrate was added, at different ratio, up to 4:250 v/v, into a commercial blood orange juice. The fortified juice with the addition of the concentrate, up to 2:250 v/v ratio, did not show off-flavour and off-odour compared to the control. Furthermore, after 60 days of refrigerated storage, the fortified juice exhibited a hydroxytyrosol content still complying with the daily intake recommended by EFSA health claim. The obtained results can be industrially useful in producing orange juice added with a natural antioxidant concentrate as a 'clean label' ingredient.



1. Introduction

In the last years, consumers have becoming more and more aware about the ingredients in food and started actively scrutinizing the product labels. At the same time, increasing attention has been paid to the valorisation of agro-industrial waste and to the utilization of by-product, promoting their reuse to develop new functional food. In particular, vegetable by-products are considered valuable sources for the formulation of new natural food additives. Their exploitation represents a low-cost and environmentally friendly strategy that can provide alternatives to synthetic chemical compounds in food industries (Faustino et al., 2019).

Olive oil production represents the main agro-industrial activity in Mediterranean countries, and it is associated with the generation of a large amount of both liquid and solid by-products (Berbel et al., 2018). The olive mill wastewater (OMW), a liquid waste mainly obtained by the 3-phase extraction system, still represents a relevant management problem, above all for small olive oil companies but, at the same time, a high added value resource, being rich in bioactive compounds, such as hydroxytyrosol, tyrosol, oleuropein, flavonoids and others (Romeo et al., 2021). The strong antioxidant activity of these compounds turns olive oil by-products into an inexpensive source of natural antioxidants with recognised healthy effects. Hydroxytyrosol has been proven to show anti-inflammatory and antimicrobial activities, to play a role in preventing and combating cardiovascular diseases and metabolic syndromes, with neuroprotective, anticancer and chemomodulatory effects (Robles-Almazan et al., 2018).

The European Food Safety Authority (EFSA), indeed, confirmed the health claim related to olive polyphenols at dose of 5 mg of hydroxytyrosol or its derivatives, corresponding to a daily consumption of 20 g of extra virgin olive oil. Furthermore, a recent study highlighted that the addition of olive by-products to foods exhibited an effect in extending the shelf life and in inhibiting the growth of pathogens (Di Nunzio et al., 2020).

The recovery of phenols from OMW can be performed through conventional techniques, such as filtration trough membranes, solvent extractions and through emerging non-thermal technologies that reduce sensory alteration and nutritional deprecation of final product (Galanakis et al., 2018a; Barba et al., 2015). Among them, the membrane extraction technique is one of the most evaluable methods mainly at industrial scale (Caporaso et al., 2019). Furthermore, the membrane filtration technique is characterised

by a low energy consumption, good operating conditions and high efficiency in component separation. This technology, based on the capacity of substances to cross the polymeric or inorganic semipermeable membrane at different rates, allows a cost-effective purification of phenolic pool present in OMWs, thanks to the low operating temperature (Cassano et al., 2013). The filtration technique involves microfiltration (MF), ultrafiltration (UF), nanofiltration (NF) and reverse osmosis (RO) processes (Mallamaci et al., 2021). Furthermore, the fractions obtained from OMW can be added into food formulations as concentrated stabilised extracts and, in some cases, microencapsulated (Foti et al., 2021a). Therefore, this treatment makes of a by-product an alternative resource of biologically active phenols that can be used to fortify foods and/or beverages (Caporaso et al., 2019; Galanakis et al., 2017). Orange juice is a natural functional beverage thanks to the high content of vitamin C and flavonoids, the most abundant phenolic compounds present in *Citrus* fruits (Ballistreri etal., 2019). Red orange fruits represent the most important citrus product of Southern Italy. From these fruits, the obtained blood orange juice is characterized by high anthocyanin levels. Thanks to its acidity, orange juice is a suitable matrix to deliver nutraceutical molecules or probiotics and represents a promising candidate for the formulation of new functional beverages (Bonaccorso et al., 2021).

The aim of this study was to select the best concentrate, among the OMW fractions, obtained through ultrafiltration and reverse osmosis at industrial level, for fortification of blood orange juice. For this purpose, physico-chemical, microbiological, antioxidant and antimicrobial properties of different concentrates were evaluated. In addition, the most promising concentrate was added to a commercial blood orange juice, at different amounts, and its effect on physico-chemical, chemical, microbiological and sensory traits was evaluated up to 60 days of refrigerated conditions.

2. Materials and methods

2.1 Olive mill wastewater sampling

The OMW was kindly supplied by olive oil company “Azienda Olearia Consoli Pasquale & F.lli s.n.c” (Adrano, Sicily). The OMW samples, acidified with 0.6 % of food grade hydrochloric acid, were treated at farm level using the 'Permeaprocess' plant (Permeare

s.r.l., Italy). The system consists of a tangential filtration based on selective membranes suitable for purification, fractionation, and concentration of compounds. This physical method allows the elimination of water at room temperature by means of a semi-permeable membrane, capable of overcoming osmotic pressure. This technique separates the water contained in the samples, concentrating all the components present, including phenols and organic acids. Three concentrates were obtained: the ultrafiltration concentrate (C1 sample), the first osmosis concentrate (C2 sample) and the second osmosis concentrate (C3 sample). Moreover, as showed in Figure 1, the P1 sample was obtained from the C1, the P2 from the C2 and the P3 from the C3. All obtained fractions were stored at + 4 °C before analyses.

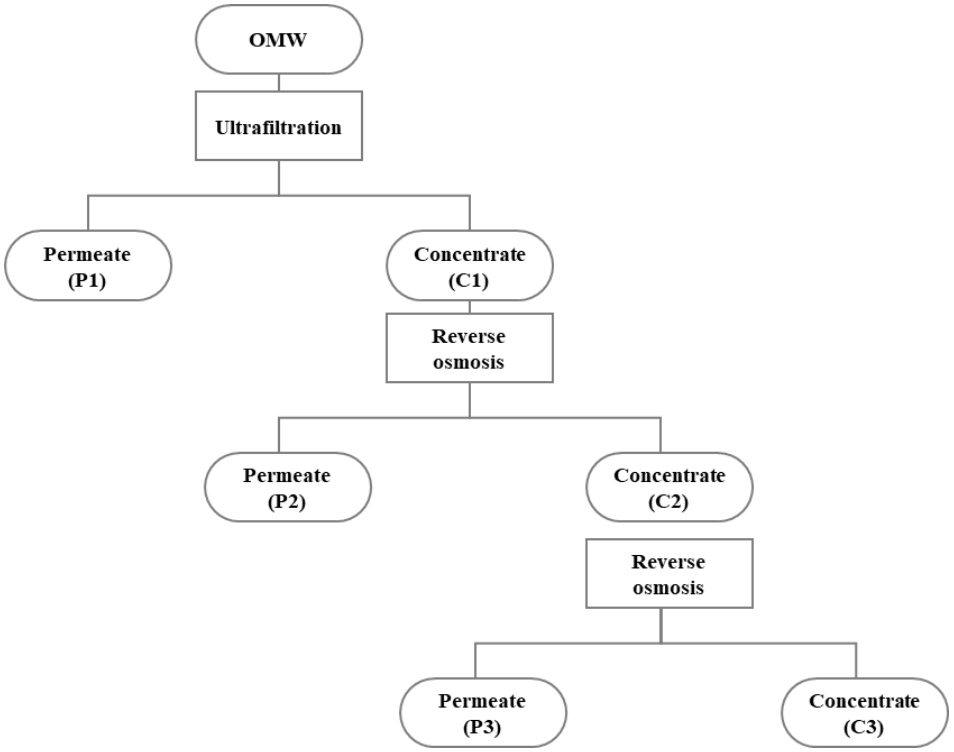


Fig. 1. Flowsheet of OMW filtration process.

2.2 Chemical analyses of OMW and obtained fractions

The pH of OMW and obtained fraction samples was measured using a Mettler DL25 pH meter (Mettler-Toledo International Inc., Columbus, OH, USA). In addition, total soluble solid (TSS) value was determined using a refractometer (Atago, RX-5000) and expressed as °Brix. The total phenolic content was detected according to the Folin-Ciocalteu's (FC)

colorimetric method. Samples were mixed with 5 mL of FC commercial reagent (Labochimica, Italy) diluted with water 1:10 v/v, added of 4 mL of a 7.5% sodium carbonate solution and left at room temperature away from light. The absorbance of samples was spectrophotometrically measured at 765 nm (Cary 100 Scan UV-Visibile, Agilent, CA, USA). The total phenolic content was expressed as mg of gallic acid equivalents (GAE)/L of sample.

2.3 HPLC analyses

2.3.1 Phenol detection

The HPLC analysis of OMW, concentrates, and the P1 permeate was performed by directly injecting the filtered samples (0.45 μm PTFE filters, Merck, Germany) into the chromatographic HPLC system. The system consisted of a liquid chromatography Waters Alliance 2695 HPLC equipped with a Waters 996 photodiode array detector (PDA) set at 280 nm and with Waters Empower software (Waters Corporation, MA, USA). The column was a Luna C18 (250 mm X 4.6 mm i.d., 5 m, 100 Å; Phenomenex, Torrence, CA, USA) maintained in an oven at 40°C. A flow of 1 mL/min was used. The chromatographic separation was performed according to Romeo et al. (2021). Theinternal standard (I.S.), a 50 mM pure gallic acid (Fluka, Switzerland), was used to quantify the phenolic compounds. The identification of phenolic compounds was obtained by comparing retention time with pure tyrosol (TYR), oleuropein (OLE) and hydroxytyrosol (HT) (Extrasynthese, Genay, France). All the analyses were carried out in triplicate for each sample.

2.3.2 Organic acid detection

For the determination of organic acids, samples were diluted with ultrapure water, at different ratios: the OMW, the C1 and the P1 samples at 1:1 v/v; the C2 and the C3 samples at 1:10 v/v; while the P2 and the P3 samples were used as they were. Each sample was then filtered, through a 0.45 μm PTFE syringe filter (Merck, Germany), before being injected into HPLC (the HPLC instruments were described in the previous section) with a DAD detector set at 210 nm (and with spectrum acquisition from 200 to 400 nm). Isocratic elution with 5 mM sulphuric acid was performed on a Rezex ROA Organic Acid H+ column (Phenomenex, Torrence, CA, USA). The run time was set at 50 minutes at

0.6 mL/min. For calibration, pure standards of lactic, citric, acetic, propionic, isobutyric and butyric acids (all purchased from Sigma-Aldrich, Italy) were injected at different concentrations. All the analyses were carried out in triplicate for each sample.

2.4 Microbiological analyses

2.4.1 Microbiological analyses of OMW and concentrate samples

The concentrates were serially diluted and poured into agar plates contained specific media and incubated at specific conditions: de Man, Rogosa, and Sharpe Agar (MRSA, Oxoid, UK) for lactobacilli count, incubated at 32 °C for 48 h under anaerobic conditions; Plate Count Agar (PCA, Oxoid, UK) for mesophilic aerobic bacteria count, incubated at 25 °C for 48 h; Sulphite-Polymyxin-Sulphadiazine Agar (SPS, Oxoid, UK) for the detection of *Clostridium perfringens,* incubated at 35–37 °C for 18–48 h under anaerobic conditions; Sabouraud Dextrose Agar (SAB, Bio-Rad, CA) for yeasts counting, incubated at 25 °C for 48 h. Microbiological analyses were performed in triplicate and results expressed as Log CFU/mL ± standard deviation (SD).

2.4.2 Microbiological analyses of permeate samples

The two permeate samples, P2 and P3, were microbiologically analysed following the membrane filtration method (Standard Methods for the Examination of Water and Wastewater: APHA, 2012). In detail, for the detection and counting of *Escherichia coli*, 100 mL of sample were filtered on membrane filters (0.45 µm pores, Cellulose, Merck, Germany) and poured in RAPID' *E. coli* 2 Agar plates (Bio-Rad, Italy), incubated at 37 °C for 24 h. For detection of *Clostridium* spores, 1 mL of sample was poured into sterile 50 mL tubes, added with 24 mL of sterile distilled water and tubes heated at 75 ± 5 °C for 10 minutes. Then, 25 mL of liquid Sulphite Polymyxin Sulphadiazine (SPS) at 45°C,at double (2X) concentration, were added and tubes incubated at 37 °C for 24 h. For detection of sulphite-reducing bacteria, 1 mL of sample was poured, by inclusion, on SPS plates and plates anaerobically incubated at 37 °C for 24 h. The counting of somatic coliphages was carried out following the "*ISO 10705-2:2000(E) - Water quality-Detection and enumeration of bacteriophages - Part 2: Enumeration of somatic coliphages*" protocol. The detection of intestinal nematodes (helminth eggs) was carried out following the "*Official method suppl. ord. g.u. n. 87"* of 13 April 2000, which foresees

a sedimentation phase and a series of centrifugations followed by flotation and observation under microscope. The detection and enumeration of *Legionella* spp. was carried out following the ISO 11731:2017 Water quality - Enumeration of *Legionella* procedure.

2.5 Antioxidant activity of OMW and fractions

Different dilutions of samples were added to the mixture of methanolic solution and 2,2-Diphenyl-1-picrylhydrazyl radical 10^{-4} M (DPPH, Merck, Germany). The absorbance was evaluated at 517 nm and the results expressed as a percentage decrease, compared to the control. Antioxidant activity was expressed with respect to sample volume and the concentration at which 50% radical scavenging occurred (IC_{50}). Stronger radical quenching results at a lower IC_{50} value. Inhibition percentage for each sample was calculated as follows:

$$\%\ inhibition = \frac{A_0 - A_x}{A_0}\ 100$$

where A_0 is the absorbance of a DPPH blank and A_x is the sample absorbance.

2.6 Antimicrobial activity of OMW and obtained fractions

The inhibitory activity of OMW and obtained fractions (C1, P1, C2 and C3) was tested, according to Foti et al. (2021b), against pathogenic strains: *Listeria monocytogenes* ATCC 19114*, Escherichia coli* ATCC 25922*, Candida albicans* ATCC 10231*, Staphylococcus aureus* ATCC 25213*, Pseudomonas aeuroginosa* ATCC 9027, *Salmonella typhimurium* ATCC 14028, *Bacillus subtilis* ATCC 19659, *Clostridium sporogenes* ATCC 11437*,* and *Enterococcus faecalis* ATCC 29212 (American Type Culture Collection). In addition, the same fractions were tested on probiotic strains*: Lacticaseibacillus rhamnosus* CRL1505*, Lacticaseibacillus paracasei* 101/37*, Bifidobacterium animalis* subsp*. lactis* BLC1 (purchased from Sacco S.r.l., Italy) and *Propionibacterium freudenreichii* DSM 4902 (Leibniz-Institute DSMZ, German collection). The test was performed on: Potato Dextrose Agar (PDA, Likson, Italy) for *Candida albicans*; MRSA, for lactobacilli; Muller Hinton Agar Base (MHA, Liofichem, Italy) for other bacteria. For the probiotic strains, each individual culture was standardised using Mc Farland 0.5 solution, which corresponded to a cell density of approximately

$1.5x10^8$ CFU/mL, while the standardised pathogenic strains were diluted to a cell density of $1x10^6$ CFU/mL. In each plate, containing the selective medium, 1 mL of cell suspension was spatulated, allowed to dry, and then sterile cellulose discs (Ø 6 mm) imbibed with each tested sample, at different dilution rates, were placed. The samples were tested as they were and at different dilution ratio (from 1:2 to 1:16). Distilled water was used as negative control. Plates were incubated at specific temperatures for 48 h and results expressed as diameter of the inhibition halo (mm).

2.7 Blood orange juice fortification

In the present study, a 100% blood orange juice, without any addition of sugar or preservatives and with an estimated shelf life of 60-65 days, was kindly provided by the Oranfrizer Company (located in Scordia, Sicily). The concentrate C2, filtered at 0.22 μm (PTFE filters, Merck, Germany), was added to the blood orange juice at different ratio [1:250 v/v (FBOJ1); 2:250 v/v (FBOJ2); 3:250 v/v (FBOJ3); 4:250 v/v (FBOJ4)] and the obtained fortified blood orange juice (FBOJ) samples were stored at +4 °C for 60 days. The FBOJ samples were analysed at different times (0, 15 and 60 days) to evaluate chemical, microbiological, and sensory traits compared to the commercial juice ascontrol. All analyses were carried out in triplicate.

2.7.1 Microbiological analysis of FBOJ

The FBOJ samples, obtained by addition of the C2 concentrate, were microbiologically analysed at 0, 15 and 60 days of storage on the following media: MRS, for the determination of lactobacilli; PCA, for mesophilic aerobic bacteria count; SAB, for yeasts and moulds. The culture conditions were the same as described in 2.4.1 section. Microbiological analyses were performed in triplicate and results were expressed as Log CFU/mL ± SD.

2.7.2 Total phenolic content and HPLC assay of FBOJ

The FBOJ samples obtained by addition of the C2 concentrate, were tested for total phenolic content as described in 2.2 section and for the quali-quantitative determination of single phenol, by direct injection into HPLC, as described in 2.3.1 section.

2.7.3 Colorimetric assay of FBOJ

The colour of the FBOJ samples, obtained by adding at different ratio the C2 concentrate, was determined at different storage times, as previously reported by Timpanaro et al. (2021). The coordinates L* (brightness), a* (green-red component), b* (blue-yellow component), were determined as the average of two transmittance measurements using a spectrophotometer CM-5 (Minolta, Milan, Italy). The parameters L*, a*, b* were determined using the illuminant D65, according to the CIELAB scale.

2.7.4 Sensory analysis of FBOJ

The standard ISO 13299:2016 provides guidelines for developing a sensory profile, which can be obtained for any products that can be evaluated by sight, smell, taste, tact, or hearing. The profile method was performed by a trained panel (EN ISO 8586:2014), and ten panellists (4 males and 6 females, aged between 28 and 45 years) were selected among the staff of CREA - Research Centre for Olive, Fruit and Citrus Crops, located in Acireale, Italy. During the training period, the judges selected the attributes to describe the colour (light orange to deep red), odour of orange, off-odour, acidity, sweetness, flavour, off-flavour, and bitterness using orange fresh juice as control. Judges evaluated the intensity of each attribute by assigning a score between 1 (absence of the sensation) and 9 (extremely intense) on a numerical unipolar scale (ISO 4121:2003). Sensory analyses were performed at the same day of C2 addition. All sensory tests were performed at the sensory analysis laboratory devised in accordance with UNI EN ISO 8589: 2014.

2.8 Statistical analyses

All analyses were performed in triplicate. SPSS software (version 21.0, IBM Statistics, Armonk, NY, USA) was used for data processing. Statistical analysis of the results was performed using one-way analysis of variance (ANOVA), and Tukey's HSD post hoc test for means separation at a significance level of $P \leq 0.05$.

3. Results

3.1 Physico-chemical traits of OMW and obtained fractions

The OMW and the obtained fractions were analysed for pH, TSS and total phenolic content. The pH ranged from 3.41 to 3.96. The TSS and total phenols values increased proceeding from ultrafiltration to reverse osmosis, reaching values of 15.17 °Brix and 8523.23 mg/L in the C3 sample (Table 1).

The P2 and P3 permeates showed the lowest values of both TSS (0.19 and 0.36 °Brix, respectively) and total phenol contents (19.42 mg/L and 55.05 mg/L, respectively).

Table 1
Physico-chemical traits of OMW and obtained fractions.

Samples	pH	TSS (°Brix)	Total phenols (mg/L)
OMW	3.92±0.07^{a}	5.40±0.02^{d}	2983.39±0.31^{d}
C1	3.91±0.08^{a}	6.29±0.07^{c}	3244.11±0.21^{c}
P1	3.94±0.06^{a}	5.05±0.07^{d}	2888.02±0.02^{e}
C2	3.96±0.05^{a}	10.35±0.24^{b}	6207.41±0.12^{b}
C3	3.90±0.14^{a}	15.17±0.04^{a}	8523.23±0.03^{a}
P2	3.45±0.01^{b}	0.19±0.04^{e}	19.42±0.01^{g}
P3	3.41±0.01^{b}	0.36±0.28^{e}	55.05±0.01^{f}
	**	**	**

Data are expressed as means ± SD. Mean values with different letters within the same column are statistically different. **Significance at $P \leq 0.01$.

3.2 Phenols, antioxidant activity and organic acid detection

Zooming on phenolic compounds, except for other phenols present in traces, HT and TYR were the only phenols detected by HPLC analysis, reaching the highest concentration in the C2 and C3 samples, with 7203.7 and 6936.2 mg/L (HT) and 1046.6 mg/L and 1613.9 (TYR), respectively (Table 2). It is interesting to point out that the C3 sample, despite the highest total phenolic content, showed a slight lower content of HT.

Table 2
Phenols and antioxidant activity detected in OMW and in the obtained fractions.

Sample	HT (mg/L)	TYR (mg/L)	IC_{50}
OMW	3321.07±61.73^{c}	508.02±20.40^{c}	87.67± 0.17^{a}
C1	3415.15±65.94^{c}	494.37±0.14^{c}	84.00±0.10^{ab}
P1	3327.68±42.58^{c}	499.11±4.07^{c}	80.18±0.18^{b}
C2	7203.67±54.85^{a}	1046.62±2.50^{b}	41.17±0.02^{d}
C3	6936.27±43.82^{b}	1613.97±6.87^{a}	50.95±0.16^{c}
	**	**	**

Data are expressed as means ± SD. Mean values with different letters within the same column are statistically different. **Significance at $P \leq 0.01$.

Results on antioxidant activity, evaluated by the DPPH method, showed that the proton removal activity of fractions was positively related to the concentration of free phenolic compounds. Lower IC_{50} values are related to a stronger radical quenching activity. As expected, the lowest IC_{50} values were detected for the C2 (41.17 IC_{50}) and the C3 samples (50.95 IC_{50}), as reported in Table 2. These results confirmed that the C2 concentrate sample, with the highest HT content, exhibited the highest antioxidant activity. Looking at organic acids, for lactic, acetic and propionic acids a general increase in their concentrations during the filtration process was observed (Table 3), whereas isobutyric acid was detected only in the OMW and C1.

Table 3
Detected organic acids.

Sample	Lactic acid (mg/L)	Acetic acid (mg/L)	Propionic acid (mg/L)	Isobutyric acid (mg/L)
OMW	3583.7±135.80^{c}	6680.9±94.59^{c}	0.00±0.00^{c}	13187.3±507.60^{a}
C1	3554.3±58.78^{c}	6540.6±40.10^{c}	0.00±0.00^{c}	12621.7±374.88^{b}
P1	3733.7±10.35^{c}	6714.7±33.29^{c}	0.00±0.00^{c}	0.00±0.00^{c}
C2	7953.7±7.93^{b}	12137.2±7.38^{b}	2984.4±89.77^{b}	0.00±0.00^{c}
C3	11860.8±107.20^{a}	17612.0±343.96^{a}	5393.9±500.56^{a}	0.00±0.00^{c}
P2	98.6±4.43^{d}	1177.0±135.83^{e}	0.00±0.00^{c}	0.00±0.00^{c}
P3	150.8±4.53^{d}	2460.4±49.56^{d}	0.00±0.00^{c}	0.00±0.00^{c}
	**	**	**	**

Data are expressed as means ± SD. Mean values with different letters within the same column are statistically different. **Significance at $P \leq 0.01$.

The highest concentration of lactic acid was detected in the C3 and C2 samples, at 11860.8 and 7953.7 mg/L, respectively, while in the other samples the mean concentration value was 3600 mg/L (in OMW, C1 and P1 samples) and 99 and 151 mg/L in P2 and P3, respectively. The C3 and C2 samples showed the highest concentrations of

acetic acid, reaching values of 17612.0 and 12137.2 mg/L, respectively, and were the only samples in which propionic acid was found (5393.9 and 2984.4 mg/L, respectively).Citric and butyric acids were never detected in any samples.

3.3 Microbiological analyses of OMW and obtained fractions

Overall, lactobacilli and *Clostridium perfringens* were not detected in OMW, C1, P1, C2 and C3 samples, whereas a mesophilic aerobic bacteria count, ranging between 4.00 and 4.35 Log CFU/mL, was detected in all samples. Furthermore, yeasts and moulds were not found in the P1 and in the C2 samples, whereas a mean value of 3.83 Log CFU/mL was counted in the other samples (Table S1, supplementary material).

Regarding the P2 and P3 permeates, *Escherichia coli* (in 100 mL), *Clostridium* spores, sulphite-reducing bacteria, somatic coliphages, intestinal nematodes (helminth eggs) and *Legionella* spp. were not detected and the results were found to comply with the limits imposed by Regulation (EU) 2020/741 on minimum requirements for water reuse (EU, 2020).

3.4 Antimicrobial activity

The antimicrobial activity of the OMW and the obtained fractions on pathogenic and probiotic strains was assessed by evaluation of inhibition zones. Overall, all the tested samples did not show any effect on probiotic tested strains, namely as *L. rhamnosus* CRL1505, *L. paracasei* 101/37, *Bif. animalis* subsp. *lactis* BLC1 or *P. freudenreichii* DSM 4902. Among the tested samples, the C2 and the C3 concentrates showed inhibitory activity against *E. coli* and *P. aeuruginosa,* with an inhibition zone of 12 and 14 mm, respectively (Table S2, supplementary material). Both the C2 and the C3 concentrates exhibited a dose-dependent antimicrobial activity against pathogens (Table S2). No inhibitory activity was observed against the other tested pathogens (*L. monocytogenes, C. albicans, St. aureus* or *Salmonella* thyphimurium).

3.5 Microbiological, chemical and sensory traits of FBOJ

The C2 concentrate, the richest fraction in the bioactive compound HY, was included in blood orange juice to fortify the nutraceutical component of the product. The C2 concentrate was added to 250 mL commercial orange juice, at a ratio of 1:250 v/v

(FBOJ1), 2:250v/v (FBOJ2), 3:250 v/v (FBOJ3), 4:250 v/v (FBOJ4), and the obtained FBOJ analysed at 0, 15 and 60 days of storage, at refrigerated conditions.

Regarding microbiological results, in all samples (both FBOJ and controls) the searched microbial groups (as lactobacilli, mesophilic aerobic bacteria and yeasts and moulds) were not detected at any sampling times, except in controls and FBJO1 which at 60 days of storage showed yeasts and moulds densities of 3.3 and 3.0 Log CFU/mL, respectively (data not shown).

Looking at physico-chemical results, no significant differences in pH and TSS values was found between control and FBOJ samples (Table 4), at any sampling points. Regarding the total phenolic content, data showed that increasing the volume of the C2 addition, a higher phenol content was found in the fortified juices.

Table 4

Chemical parameters of FBOJ samples fortified with different additions of C2 concentrate.

Samples	Time (days)	pH	TSS (°Brix)	Total phenols (mg/L)	HT (mg/L)	TYR (mg/L)
Commercial juice	0	3.38 ± 0.01	11.60 ± 0.08	3142.2 ± 0.54^{e}	0.00 ± 0.00^{e}	0.00 ± 0.00^{c}
FBOJ1	0	3.37 ± 0.01	11.55 ± 0.01	3553.2 ± 2.66^{d}	26.92 ± 0.67^{d}	43.21 ± 4.14^{b}
FBOJ2	0	3.38 ± 0.05	11.67 ± 0.01	3643.6 ± 0.54^{c}	67.43 ± 3.77^{c}	52.65 ± 3.81^{ab}
FBOJ3	0	3.39 ± 0.05	11.65 ± 0.01	3715.2 ± 1.63^{b}	82.23 ± 0.75^{b}	58.08 ± 1.62^{a}
FBOJ4	0	3.39 ± 0.00	11.56 ± 0.01	3893.2 ± 1.09^{a}	100.87 ± 1.42^{a}	56.50 ± 0.86^{b}
		n.s.	n.s.	**	**	**
Commercial juice	15	3.3 ± 0.01^{d}	11.36 ± 0.01^{c}	2900.0 ± 0.54^{e}	0.00 ± 0.00^{e}	0.00±0.00^{b}
FBOJ1	15	3.34 ± 0.01^{c}	11.75 ± 0.01^{a}	3147.9 ± 1.63^{d}	24.28 ± 0.65^{d}	60.20 ± 0.22^{a}
FBOJ2	15	3.35 ± 0.01^{bc}	11.66 ± 0.01^{b}	3174.1 ± 3.26^{c}	48.22 ± 0.38^{c}	58.89 ± 0.97^{a}
FBOJ3	15	3.38 ± 0.01^{a}	11.65 ± 0.00^{b}	3396.8 ± 0.55^{b}	81.01 ± 0.59^{b}	57.75 ± 2.44^{a}
FBOJ4	15	3.36 ± 0.01^{b}	11.67 ± 0.02^{b}	3410.6 ± 0.54^{a}	105.52 ± 1.87^{a}	59.49 ± 0.30^{a}
		**	**	**	**	**
Commercial juice	60	3.31 ± 0.00^{c}	11.70 ± 0.01^{a}	2545.0 ± 0.01^{d}	0.00 ± 0.00^{e}	0.00 ± 0.00^{c}
FBOJ1	60	3.32 ± 0.00^{bc}	11.56 ± 0.06^{ab}	2623.1 ± 0.54^{d}	21.67 ± 0.05^{d}	50.11 ± 0.16^{ab}
FBOJ2	60	3.36 ± 0.02^{a}	11.66 ± 0.04^{a}	2851.1 ± 1.09^{c}	46.29 ± 0.56^{c}	53.60 ± 0.85^{a}
FBOJ3	60	3.35 ± 0.00^{ab}	11.29 ±0.13b	2894.6 ± 0.56^{b}	75.35 ± 2.27^{b}	52.24 ± 0.20^{ab}
FBOJ4	60	3.35 ± 0.01^{abc}	11.26 ± 0.06^{b}	3100.0 ± 1.10^{a}	94.58 ± 2.91^{a}	49.20 ± 2.05^{b}
		*	**	**	**	**

Data are expressed as means ± SD. Mean values with different letters within the same column at the same time interval are statistically different. N.s. not significant; *Significance at P ≤ 0.05; **Significance at P ≤ 0.01.

In FBOJ4 sample, where the ratio C2/juice was 4:250 v/v, at the same day of fortification (T0), the total phenolic content was 750 mg/L higher than that detected in the control juice. Furthermore, it is interesting to underline that after 60 days of storage, the FBOJ4

sample showed almost the same total phenolic content (3100 mg/L) detected in the control juice at initial time (Table 4). Zooming at content of bioactive molecules, monitored at different sampling times, the FBOJ samples showed a proportional increase in HT and TYR. It is interesting to highlight that the FBOJ1 sample, obtained by adding the lowest volume of C2 extract (1 mL of C2 to 250 mL of juice), exhibited an initial concentration of HT and TYR of 26.92 and 43.21 mg/L, and a concentration of 21.67 and 60.10 mg/L of HT and TYR, respectively, after 60 days of storage. This data allows us to state that the FBOJ1 provides, up to the end of estimated shelf life, the recommended health beneficial intake of HT, as recognised by European Foods Safety Authority (EFSA).

Regarding the colour coordinates, the brightness (L*) showed an increasing trend during the shelf life in all tested sample (Table S3 supplementary material). In addition, a significant decrease in the red coordinate (a*), right after 15 days of refrigerated storage, was observed, while the yellow coordinate (b*) remains unchanged over time, reaching the highest values in FBOJ3 and FBOJ4 samples (Table S3, supplementary material).

Looking at the sensory parameters, overall, only flavour, off-flavour, bitterness and off-odour showed significant differences (Table 5). The flavour reached the highest score in FBOJ1 sample, but the statistical differences among samples were not related to the concentrate addition. Flavour descriptor decreased in FBOJ3 and FBOJ4 samples, while the perceived bitterness was higher in FBOJ4 sample, compared to other samples. Off-flavour and off-odour descriptors statistically increased in FBOJ3 and FBOJ4 samples (Figure S4, supplementary material).

Table 5

Sensory traits of FBOJ samples fortified with different additions of C2 concentrate.

	Commercial juice	**FBOJ1**	**FBOJ2**	**FBOJ3**	**FBOJ4**	
Colour	5.62 ± 0.44	5.65 ± 0.44	5.75 ± 0.38	5.68 ± 0.37	5.75 ± 0.38	n.s.
Odour of orange	5.62 ± 0.44	5.42 ± 0.57	5.18 ± 0.59	5.00 ± 0.46	5.06 ± 0.68	n.s.
Acidity	5.00 ± 0.27	5.14 ± 0.20	5.12 ± 0.23	5.06 ± 0.32	5.31 ± 0.37	n.s.
Sweetness	4.50 ± 0.38	4.40 ± 0.25	4.69 ± 0.53	4.19 ± 0.26	4.31 ± 0.37	n.s.
Flavour	5.69 ± 0.37^{ab}	5.85 ± 0.62^{a}	5.50 ± 0.38^{ab}	5.12 ± 0.64^{b}	5.25 ± 0.38^{ab}	*
Off-flavour	1.00 ± 0.00^{b}	1.00 ± 0.00^{b}	1.00 ± 0.00^{b}	2.62 ± 0.52^{a}	2.69 ± 0.46^{a}	**
Bitterness	1.31 ± 0.26^{b}	1.37 ± 0.23^{ab}	1.38 ± 0.23^{ab}	1.44 ± 0.18^{ab}	1.69 ± 0.26^{a}	*
Off-odour	1.00 ± 0.00^{b}	1.00 ± 0.00^{b}	1.00 ± 0.00^{b}	1.81 ± 0.59^{a}	2.06 ± 0.42^{a}	**

Data are expressed as means ± SD. Mean values with different letters within the same row are statistically different: ** significance at $p \leq 0.01$; * significance at $p \leq 0.05$; n.s., not significant.

4. Discussion

Polyphenols from olive fruit, olive mill wastewater or olive oil, *Olea europaea* L. extract and leaf, standardised for their content of HT, possess the health claim approved by EFSA under Article 13 (Health Claims Regulation 1924/2006), in relation to the protection of blood lipids from oxidative damage, which is known to adversely affect cardiovascular health (EFSA, 2011; EC, 2012). Furthermore, in a recent study, the safety and the effects of HT purified (99.5%) from OMW were assessed by administering HT at a daily dose of 45 mg for 8 weeks to volunteers with mild hyperlipidaemia (Lopez-Huertas & Fonolla, 2017). In particular, the authors demonstrated that the administration of HT did not affect markers of cardiovascular disease, blood lipids, inflammatory status, liver or kidney function and that electrolyte balance with vitamin C increased two fold at 4 and 8 weeks, compared to baseline levels (Lopez-Huertas & Fonolla, 2017). In the present study, the tangential membrane filtration technique produced fractions differently concentrated in bioactive compounds. The concentrate C2 showed the highest concentration of HT, known for its antioxidant activity and for playing a role as an intracellular and extracellular scavenger of reactive oxygen species (ROS) (Robles-Almanaz et., 2018). Indeed, in the present study, the higher concentration of HT was positively related to a greater antioxidant activity. Several studies have reported that the addition of OMW phenolic fraction induces a fortification of the nutraceutical component and increases the shelf life of foods (Mikdame et al., 2020; Servili et al., 2011). As a matter of facts, phenolic compounds show wide antimicrobial activity, such as antibacterial, antiviral and antifungal effects (Marković et al., 2019). Although HT has been reported to *in vitro* inhibit the growth of several pathogens, included *L. monocytogenes, St. aureus,* Salmonella enterica*, Yersinia* or beneficial microorganisms, as *L. acidophilus* and *Bifidobacterium bifidum* (Marković et al., 2019), in the present study no inhibitory activity was observed against the probiotic tested strains or against *L. monocytogenes, St. aureus,* and Salmonella. In the present study, the C2 and C3 concentrates showed inhibitory activity against *P. aeuruginosa* and *E. coli,* otherwise Medina et al. (2016) reported that a MIC value of 400 μg/mL of HT was able to affect the growth of *E. coli,* while MIC values higher than 1000 μg/mL were required to affect the growth of *P. aeuruginosa.* Among the tested samples, only the C2 showed antimicrobial activity against *B. subtilis, Cl. sporogenes* and *E. faecalis*. For *B. subtilis*, the results agreed with

those reported by Tafesh et al. (2011) and by Galanakis et al. (2018b) who showed the antimicrobial effect of OMW phenolic extracts, in combination with other antioxidants, against *B. subtilis, E. coli,* and *P. aeruginosa.* In addition, the two concentrates C2 and C3 did not exhibit any antagonist effect against *C. albicans.* This finding could be due to the hydrophilic nature of OMW concentrates, being the more lipophilic constituents partitioned into the olive oil during processing. Diallinas and co-workers (2018) reported that a lower hydrophilic/lipophilic balance could increase the cellular uptake enhancing the antioxidant or antimicrobial activities. However, the exact mechanism by which HT exerts its antimicrobial activity remains not completely understood (Wei et al., 2018) and Reverón and co-workers (2020) suggested an involvement of ROS overproduction as a mechanism of antimicrobial activity. The widest antimicrobial activity of the C2 concentrate could be related to the higher antioxidant activity even although the complex chemical composition of concentrates includes wide range of phytochemicals with synergistic effects. Nevertheless, it is relevant to underline that the controversial results reported in literature could be due to the lack of a standard method or evaluation criteria for screening antimicrobial activity in plant extracts (Nostro et al., 2000). Differences in antimicrobial assay, growth media, bacterial strains, inoculum size and cell density of the target microorganism make comparisons of antimicrobial data of plant extracts from different sources very difficult. The addition of the C2 concentrate in blood orange juice has been here proposed to obtain a functional beverage with a high content of both flavonoids and HT. Indeed, orange juice is a natural source of vitamin C, and a 200 mL dose provides up to 80% of recommended daily intake (Klimczak et al., 2007). Zooming on the effect of the C2 addition on chemical composition of juice, the results here reported confirmed that the nutraceutical value of the juice was increased and the HT was still revealed up to 60 days of storage at refrigerated conditions. Furthermore, no microbiological differences were observed in samples at any C2 additions, although the yeast and mould growth were detected after 60 days in control and in fortified juice with the lowest addition of C2 (FBOJ1 sample). The addition of any compound to food could have a detrimental effect on sensory and technological properties (Marinelli et al., 2015). The colour of orange juice influences consumers' choice, above all for blood orange juices that are rich in anthocyanins, responsible of the dark red colour. In this study, the colour of FBOJ was monitored at different times, revealing significant statistical differences on L*, a* and b* parameters. Sensory analyses were carried out at the same

day of fortification in order to assess the threshold of perception of the C2 addition to the juice. This parameter is an important preliminary step to investigate the proper concentration of a fortifying agent in designing a new functional food or beverage. Moreover, results of present study confirmed that membrane filtration techniques produce permeate fractions (the P2 and P3) suitable for irrigation, being compliant with limits imposed by Regulation (EU) 2020/741 on minimum requirements for water reuse (EU, 2020) and with the legal limits for releasing into the aquatic system (Cassano et al., 2013; Paraskeva et al., 2007; Russo 2007).

5. Conclusions

Many consumers currently require supplement with vitamins, minerals and other nutrients, and as never before research-based evidence are required to correctly inform consumer, mainly on natural compounds. At the same time, an increasing interest has been posed on the ingredients used in food products, with a major challenge for 'clean label' ingredient. The present study confirmed that the tangential membrane filtration, an eco-friendly technique, represents a suitable valorisation strategy of OMW. This extraction technology on an industrial scale can effectively represent an income-generating solution for the olive oil industry by creating a collaboration with local food and beverage companies for the formulation of new products with high nutraceutical value. In order to overcome the seasonality of olive oil by-products, a crucial point could be the creation of a continuous production, realized from different industrial by-products, to obtain a supply cycle of phenolic concentrates for food industry. The concentrates rich in HT and TYR, obtained by reverse osmosis, exhibited antimicrobial and antioxidant activity, whereas the last two permeates, thanks to the low chemical load and for complying with the limits fixed by Regulation (EU) 2020/741 on minimum requirements for water reuse (EU, 2020), are suitable for the context of a circular economy. The addition of these concentrates in orange juice formulation implies an increase of phenolic content and provides the suitable amount of molecules with healthy effect on consumer. In detail, the FBOJ samples obtained by adding 2 mL of concentrate into 250 mL of juice showed a higher nutraceutical content without any sensory change. The OMW phenol concentrate and blood orange juice combined in a new functional beverage highlight the beneficial effect of the Mediterranean diet.

Supplementary material

Table S1 Microbiological traits of OMW and obtained fractions.

Sample	Mesophilic aerobic bacteria	Yeasts and moulds
OMW	4.35±0.06	3.93±0.03[a]
C1	4.34±0.05	4.34±0.05[a]
P1	4.00±0.00	<1[c]
C2	4.04±0.05	<1[c]
C3	4.15±0.21	3.24±0.33[b]
	n.s.	**

Data are expressed as Log_{10} CFU/mL ± SD; n.s. not significant; **Significance at $P \leq 0.01$.

Table S2 Antimicrobial activity of C2 and C3 against pathogens.

Target strains	C2			C3		
	raw	**1:2**	**1:4**	**raw**	**1:2**	**1:4**
						-
Escherichia coli ATCC 25922	12	7	-	10	8	-
Pseudomonas aeruginosa ATCC 9027	14	8	-	14	8	-
Bacillus subtilis ATCC 19659	12	8	-	-	-	-
Clostridium sporogenes ATCC 11437	12	9	-	-	-	-
Enterococcus faecalis ATCC 29212	8	-	-	-	-	-

Data are expressed as halo diameter of inhibition (Ø mm).

Table S3 Colour parameters of FBOJ samples fortified with different additions of C2 concentrate.

Samples	Time (days)	L*(D65)	a*(D65)	b*(D65)
Commercial juice	T0	48.78 ± 0.12^{b}	42.31 ± 0.13^{a}	52.15 ± 0.21^{c}
FBOJ1	T0	52.45 ± 0.07^{a}	41.48 ± 0.28^{b}	49.89 ± 0.03^{d}
FBOJ2	T0	48.61 ± 0.08^{b}	40.73 ± 0.08^{cd}	55.72 ± 0.08^{a}
FBOJ3	T0	47.59 ± 0.28^{c}	40.51 ± 0.01^{d}	53.57 ± 0.04^{b}
FBOJ4	T0	52.31 ± 0.14^{a}	41.21 ± 0.16^{bc}	51.83 ± 0.21^{c}
		**	**	**
Commercial juice	T15	53.25 ± 0.07^{b}	38.96 ± 0.03^{a}	48.07 ± 0.04^{c}
FBOJ1	T15	57.45 ± 0.63^{a}	37.97 ± 0.02^{b}	45.58 ± 0.13^{d}
FBOJ2	T15	51.20 ± 0.14^{c}	37.20 ± 0.20^{c}	50.71 ± 0.11^{b}
FBOJ3	T15	51.79 ± 0.15^{c}	36.49 ± 0.01^{d}	52.81 ± 0.12^{a}
FBOJ4	T15	51.60 ± 0.28^{c}	35.68 ± 0.03^{e}	52.50 ± 0.14^{a}
		**	**	**
Commercial juice	T60	63.49 ± 0.26^{e}	16.58 ± 0.14^{a}	51.04 ± 0.08^{b}
FBOJ1	T60	70.78 ± 0.31^{a}	14.74 ± 0.21^{b}	47.93 ± 0.09^{c}
FBOJ2	T60	69.49 ± 0.26^{b}	14.48 ± 0.12^{b}	48.09 ± 0.01^{c}
FBOJ3	T60	68.46 ± 0.13^{c}	14.77 ± 0.14^{b}	51.48 ± 0.06^{a}
FBOJ4	T60	66.85 ± 0.06^{d}	14.94 ± 0.06^{b}	51.55 ± 0.02^{a}
		**	**	**

Data are expressed as means ± SD. Mean values with different letters within the same column are statistically different. **Significance at $P \leq 0.01$.

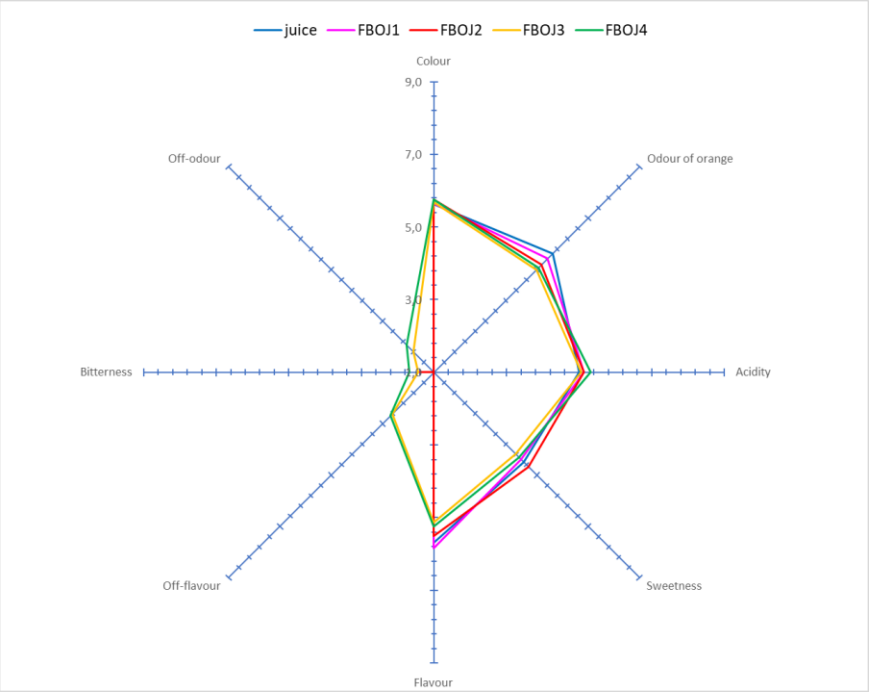


Figure S4 Spider plot of the FBOJ samples fortified with different additions of C2 concentrate.

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Olive mill wastewater fermented with microbial pools as a new potential functional beverage

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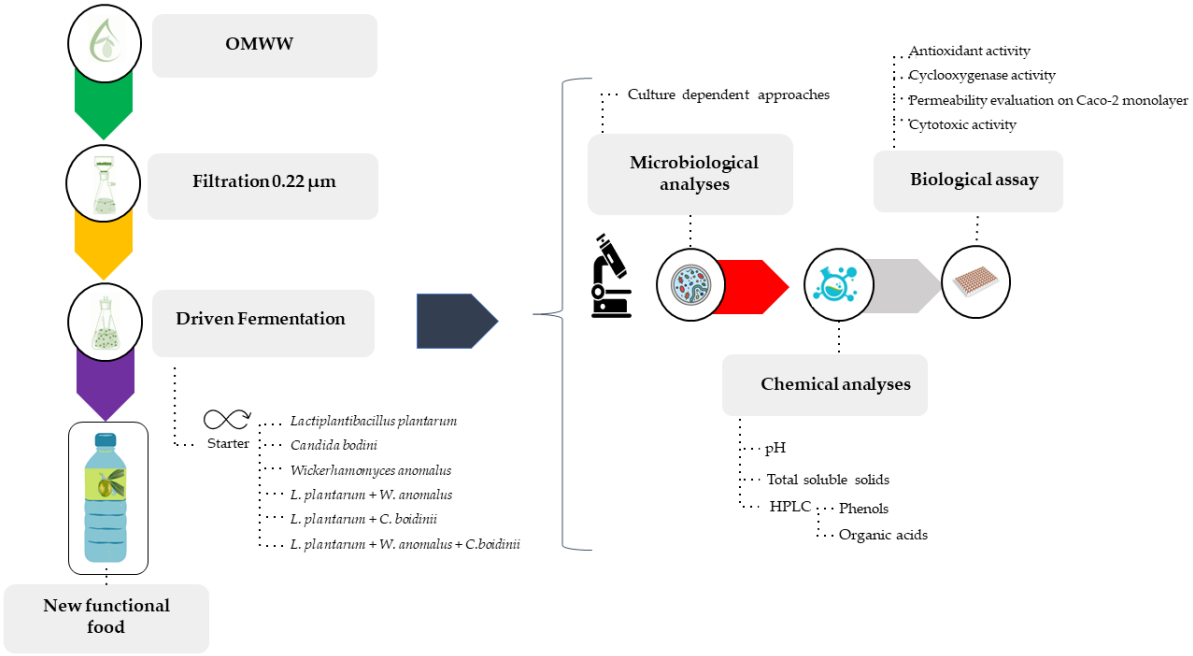
Abstract

Olive mill wastewater (OMWW) represents a by–product but also a source of biologically active compounds, and their recycling is a relevant strategy to recover income and to reduce environmental impact. The objective of the present study was to obtain a new functional beverage with a health–promoting effect starting from OMWW. Fresh OMWW were pre–treated through filtration and/or microfiltration and subjected to fermentation using strains belonging to *Lactiplantibacillus plantarum*, *Candida boidinii* and *Wickerhamomyces anomalus*. During fermentation, phenolic content and hydroxytyrosol were monitored. Moreover, the biological assay of microfiltered fermented OMWW was detected versus tumor cell lines and as anti-inflammatory activity. The results showed that in microfiltered OMWW, fermentation was successfully conducted, with the lowest pH values reached after 21 days. In addition, in all fermented samples, an increase in phenol and organic acid contents was detected. Particularly, in samples fermented with *L. plantarum* and *C. boidinii* in single and combined cultures, the concentration of hydroxytyrosol reached values of 925.6, 902.5 and 903.5 mg/L, respectively. Moreover, biological assays highlighted that fermentation determines an increase in the antioxidant and anti–inflammatory activity of OMWW. Lastly, an increment in the active permeability on Caco-2 cell line was also revealed. In conclusion, results of the present study confirmed that the process applied here represents an effective strategy to achieve a new functional beverage.



1. Introduction

Olive oil by-products, while representing a management problem for olive oil companies, may actually represent a source of high value–added compounds that can be used in pharmaceuticals, food, feed and cosmetics for their health properties [1–3]. In fact, olive mill wastewater (OMWW) is a resource rich in phenols including hydroxytyrosol (HT) and tyrosol (TYR), characterized by high antioxidant, anti–inflammatory, antimicrobial and anticarcinogenic activities [4]. The scientific community has proposed several strategies for the valorization of this by–product including solvent extraction techniques, selective resins, membrane filtration or enzymatic applications [5]. These techniques allow the extraction and/or concentration of bioactive compounds in order to increase the nutraceutical component and produce new products or functional ingredients, thus, responding to the demand of consumers who are now aware of the beneficial role that these natural products play in human and animal diets. In the food industry, OMWW have been proposed as an added functional ingredient in meat, dairy, fish, bakery products and juices [6–8]. As a matter of fact, the addition of such phenolic components in food matrices has been shown not only to fulfil a technological function (i.e., to extend the shelf life) but also to improve the health and safety properties of the food. Although the interest of the scientific community in the use of microorganisms in the bioprocessing of agro-industrial waste has grown in recent years [9], only a few microbial applications have been proposed for the valorization of this matrix. Authors have reported that the use of live microorganisms increases the content and bioavailability of the phenolic compounds, and especially of HT and TYR [10,11]. In addition, the driven microbial fermentation provides several advantages by preserving and improving food safety and shelf life due to the formation of organic acids, such as lactic, acetic, formic, propionic acids, etc. [12]. The diversities of acids are dynamic among different alcoholic beverages and fermented food, as are the synergistic effects of abiotic and biotic factors [13]. Functional microorganisms, such as lactic acid bacteria (LAB) and yeasts, are responsible for the metabolism of organic acids. Therefore, the use of selected microorganisms, especially yeasts and LAB isolated from spontaneous similar fermented matrices such as table olives, could represent a low–cost strategy to stabilize and improve the nutraceutical and sensory traits of OMWW. In detail, *Lactiplantibacillus plantarum* strains from fermented olives have been largely associated with the metabolism of phenolic compounds as they can produce degradation enzymes, such as β-glucosidase, esterase,

tannase, decarboxylase [14]. Moreover, some of them have been proposed due to their potential probiotic activity [15]. With regard to yeasts, several species show β-glucosidasic, lipasic and esterasic activity and have been used for their ability to improve sensorial profile through production of esters from fatty acids and free fatty acids. Among yeasts, *Candida boidinii* and *Wickerhamomyces anomalus* are the most commonly used as starters [16]. In addition, yeasts isolated from oil matrices, especially strains of *W. anomalus*, have demonstrated several probiotic characteristics, among which the most known is the in vitro cholesterol removal capacity [17]. Today, the functional beverage sector is steadily increasing worldwide thanks to its high nutritional value and the possibility to add flavors. Furthermore, nutraceutical beverages with added probiotics and prebiotics are of considerable interest to the consumer [18], as this matrix was shown to inhibit proliferation and induce apoptosis in several tumor cells, prevent DNA damage and exert anti–inflammatory activity [19]. The aim of this study was to set up a process to obtain a new functional beverage with a health–promoting effect starting from OMWW. For this purpose, OMWW were pre–treated through filtration and microfiltration and then subjected to fermentation with selected microbial pools, isolated from spontaneously fermented table olives. During fermentation, the biotechnological aptitude of the different strain combinations, their effect on the fermentation parameters, the increase of the phenolic content, especially as HT increase, were evaluated. Furthermore, a biological characterization to evaluate the safety profile and the antioxidant activity was performed on treated OMWW samples. Finally, the ability to cross Caco-2 cell monolayers, as a model of gastrointestinal tract absorption, was performed.

2. Results

2.1 Chemico-physical characterization of sample of different trials

The OMWW belonging to Trial I were monitored at different times (0, 8, and 30 days), through the detection of pH, total soluble solids (TSS), total phenol content and single phenols by HPLC (Table S1). Regarding pH, any significant difference was observed at the beginning of fermentation, and the lowest pH value (4.45) was reached at T8 in sample inoculated with *C. boidinii* in single culture. The TTS at the beginning of fermentation showed values between 7.08 and 8.32, reaching values between 5.60 and

6.34 at T30. During the fermentation, the total phenol content showed, to some extent, a constant trend, reaching the highest concentration at T30 in samples fermented with *W. anomalus* in single culture, with a value of 3241.9 mg/L. The results obtained by HPLC confirmed this increase, as samples treated with *W. anomalus* showed the highest concentration of HT, equal to 2630.4 mg/L. Regarding TYR, an increase during fermentation was observed, reaching, after 30 days, values between 508.6 and 679.4 mg/L in all treated samples. The chemical analyses performed on Trial I were repeated on Trial II (Table S1). The pH decreased during fermentation, showed the lowest values at T8. In detail, all inoculated samples showed a lower pH than the control sample. In particular, the lowest value was found in the samples with *L. plantarum* and *W. anomalus* in single culture, but also in the combination *L. plantarum* and *C. boidinii* and, finally, with the mix of the three strains with values ranging from 3.97 to 3.99. With regard to TSS, the greatest decrease occurred with the combination of L. plantarum in association with *W. anomalus*, going from a value of 7.84 at T0 down to 5.50 at T8. At T30, almost all samples maintained the value showed at T8 of fermentation. In addition, total phenols at T8 and T30, in all inoculated samples, showed a higher content over time compared with the control sample. In detail, the samples with significantly higher phenolic content were the three–strain association (3379.5 mg/L) and *W. anomalus* in single combination (3261.9 mg/L) at T8, while at T30 was the sample inoculated with *L. plantarum* with a value of 3577.6 mg/L. The results obtained by HPLC showed a decrease at T8 of HT in all samples except in the samples with *L. plantarum* where there was an increase of 115 mg/L of HT, and the sample inoculated with *L. plantarum* with *W. anomalus* which showed an increase equal to 262 mg/L of HT. All samples inoculated up to T30 had higher HT content. Opposingly, the TYR decreased during fermentation from an average range of values from 319.7 mg/L to 136 mg/L. In Trial III, microfiltration resulted in a clear and sterile matrix. Before starting the final fermentation, a preliminary test was carried out in a reduced volume (100 mL) to ascertain if any difference could be revealed between trials with the addition of glucose, peptone and yeast extract (added at the same concentrations) and the trials without any additions. The results showed the same pH values and cell density during fermentation. Moreover, the addition of these compounds made the OMWW turbid (data not shown). For these reasons, to improve the acceptability of the product to consumers, the thesis without additions was chosen for the final test. During

fermentation, pH, TSS and total phenol content were monitored (Table 1). Regarding pH, no significant difference was found at the beginning of fermentation. The pH at T0 was in a range of 5.12 and 5.19. Fermentation stopped at T21 for all samples examined. The end of fermentation was revealed by the stabilization of the pH value that was evaluated every three days of fermentation (data not shown). In particular, the samples inoculated with *W. anomalus* in single culture and in association with *L. plantarum* reached a pH value of 4.54 and 4.49 at T21, respectively. In addition, although slower than the previous theses, the theses containing *L. plantarum* and *C. boidinii* in single culture also reached at T21 a pH of 4.65 and 4.60, respectively. Total soluble solids showed no significant difference at any of the fermentation times. Initial values ranged from 8.30 to 10.85 °Brix, while values between 5.32 and 8.17 °Brix were reached at the end of fermentation. The sample used as a control during fermentation maintained its pH and TSS values. Regarding the content of total phenols, the highest values at the beginning of fermentation were found in the sample containing the *L. plantarum* and *W. anomalus* combination, a value that decreased during the fermentation process. In contrast, the sample with the three–strains combination showed an increase in total phenol content up to T14 with a value of 4015 mg/L, and then decreased at T21 reaching a value of 1543 mg/L.

Table 1. Chemical parameters detected in samples of trial III.

Sample	Time	pH	TSS (°Brix)	Total phenol (mg/L)
Control	0	5.18 ± 0.01	8.30 ± 0.77	3627.4 ± 0.54^{c}
L. plantarum	0	5.16 ± 0.01	10.60 ± 0.78	3711.2 ± 4.89^{b}
C. boidinii	0	5.18 ± 0.01	10.28 ± 0.70	3539.8 ± 0.54^{d}
W. anomalus	0	5.12 ± 0.08	9.76 ± 1.53	3172.9 ± 1.63^{f}
L.p+ W.a	0	5.13 ± 0.06	8.56 ± 0.80	4135.0 ± 4.89^{a}
L.p+C.b	0	5.19 ± 0.02	10.04 ± 1.13	3474.7 ± 1.09^{e}
L.p+W.a+C.b	0	5.18 ± 0.01	8.88 ± 1.39	2967.1 ± 2.18^{g}
		n.s	n.s	**
Control	8	5.17 ± 0.01^{a}	8.30 ± 0.78	1985.8 ± 3.26^{g}
L. plantarum	8	5.04 ± 0.03^{b}	10.30 ± 0.98	3032.5 ± 2.18^{b}
C. boidinii	8	4.97 ± 0. 01^{bcd}	8.88 ± 2.18	3020.6 ± 0.54^{c}
W. anomalus	8	4.87 ± 0.02^{e}	9.14 ± 1.77	2395.3 ± 1.63^{f}
L.p+ W.a	8	4.88 ± 0.04^{de}	7.52 ± 1.17	2897.2 ± 0.01^{e}
L.p+C.b	8	5.00 ± 0.01^{bc}	9.50 ± 0.32	2991.0 ± 5.44^{d}
L.p+W.a+C.b	8	4.94 ± 0.01^{cde}	8.24 ± 1.29	3268.2 ± 1.63^{a}
		**	n.s	**
Control	14	5.18 ± 0.02^{a}	8.30 ± 0.78	1809.5 ± 0.54^{f}
L. plantarum	14	4.68 ± 0.01^{cd}	9.90 ± 0.99	3282.9 ± 0.54^{c}
C. boidinii	14	4.77 ± 0.01^{b}	7.99 ± 2.82	2443.8 ± 1.63^{d}
W. anomalus	14	4.67 ± 0.04^{cd}	8.28 ± 1.44	3539.7 ± 0.54^{b}
L.p+ W.a	14	4.62 ± 0.09^{d}	6.64 ± 1.52	2199.2 ± 3.81^{e}
L.p+C.b	14	4.89 ± 0.01^{b}	9.03 ± 1.12	3545.1 ± 53.84^{b}
L.p+W.a+C.b	14	4.82 ± 0.02^{bc}	7.62 ± 1.36	4015.0 ± 2.72^{a}
		**	n.s	**
Control	21	5.19 ± 0.01^{a}	8.20 ± 0.61	1009.4 ± 0.54^{f}
L. plantarum	21	4.65 ± 0.03^{c}	8.15 ± 1.20	3392.1 ± 0.54^{a}
C. boidinii	21	4.60 ± 0.01^{d}	5.60 ± 0.57	3005.2 ± 0.54^{b}
W. anomalus	21	4.54 ± 0.04^{e}	6.36 ± 0.37	2394.2 ± 1.09^{c}
L.p+W.a	21	4.49 ± 0.01^{de}	5.32 ± 0.04	1914.6 ± 1.63^{d}
L.p+C.b	21	4.84 ±0.02^{b}	8.00 ± 1.41	3403.2 ± 10.88^{a}
L.p+W.a+C.b	21	4.82 ± 0.01^{b}	5.99 ± 0.01	1543.6 ± 1.09^{e}
		**	n.s	**

Data are expressed as mean ± standard deviations. Mean values with different letters within the same column at the same time interval are statistically different. N.s. not significant; **Significance at P≤ 0.01.

2.2 Microbiological analyses

Results on microbiological analyses (Table S2) are referred at the same sampling times reported for chemical analyses. Overall, for samples of Trial I, high microbial densities were detected for aerobic mesophilic bacteria, enterobacteria, and yeast in all sampling times. Regarding LAB, an increase of 1 Log unit at T8 was detected, and the values were quite constant until T30, with some exceptions. In detail, at the beginning of fermentation, the sample treated with *L. plantarum* showed a significantly higher cell density, with a value of 5.85 log CFU/mL,

whilst at T30 the highest LAB densities were detected in samples treated with the combination of the three strains, namely of *L. plantarum* and *C. boidinii*, *W. anomalus* in samples inoculated with *W. anomalus* in single culture, and in samples treated with *L. plantarum* and *C. boidinii* in mixed cultures. Yeasts and molds also showed a similar trend in all samples. In fact, cell density increased at T8 of fermentation and then decreased at T30, when an average value of 6.45 Log CFU/mL were detected. Aerobic mesophilic bacteria counts showed only a slight variation during fermentation, reaching a final mean value of 6.25 Log CFU/mL, whereas Enterobacteriaceae and staphylococci showed a significant decrease during fermentation. At the beginning of fermentation, the latest microbial groups showed an initial average value of 6.63 and 3.05 Log CFU/mL, respectively. These values decreased significantly during fermentation in the inoculated samples, reaching values under the detection limit. In Trial II, the LAB and yeast counts increased during fermentation (Table S2). In detail, the LAB mean value starting from 4.27 Log CFU/mL reached, after 30 days, a mean value of 7.34 Log CFU/mL, whilst in samples inoculated with *W. anomalus* it reached the lowest cell density. A similar trend was observed for yeasts that at the 30th day exhibited a mean cell density of 9 Log CFU/mL in the sample inoculated with *L. plantarum* and *C. boidinii* in mixed culture. Aerobic mesophilic bacteria were found at high density, until the end of fermentation when a final average value of 7.52 Log CFU/mL was counted. Different trends were observed for Enterobacteriaceae and staphylococci, for which after a slight increase a significant decrease was detected after 30 days in all samples. Regarding Trial III, before starting fermentation, the microfiltered OMWWs were subjected to microbiological analyses to confirm the achieved sterility. The following microbial groups were searched: LAB, yeasts, staphylococci, total mesophilic aerobic bacteria, Enterobacteriaceae and *Clostridium perfringens*. All used media and conditions are reported in Section 4. Once the OMWWs were analyzed, the selected strains were inoculated at a cell density of 108 and 107 CFU/mL for *L. plantarum* and yeasts, respectively. As shown in Figure 1, a different growth pattern between the two yeasts and the LAB strains was observed during fermentation. In fact, while in all inoculated samples LAB showed an initial decrease, during the first 14 days they increased until the 21st day; the yeasts increased their cell density during the first 18 days, when they

reached values between 7.03 and 7.78 Log CFU/mL.

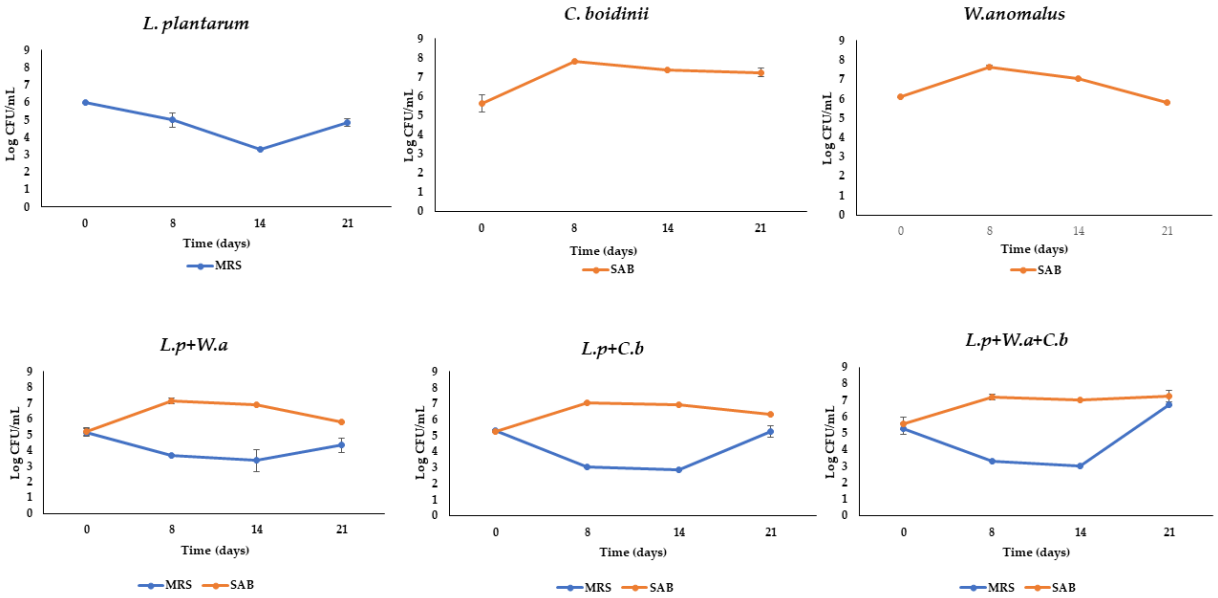


Figure 1. Microbial counts detected in MRS and SAB during fermentation in microfiltered OMWW differently inoculated. Data are expressed as means of Log CFU/mL ± standard deviation.

2.3 Phenol and organic acid detection

Regarding phenolic content, HT and TYR were the main detected compounds, found at high concentration by HPLC during fermentation (Figure 2). As for HT, at the beginning of fermentation a concentration between 341.7 and 469.1 mg/L was found. At the end of fermentation, an exponential increase of HT in all inoculated samples was observed. Particularly in the samples treated with *L. plantarum* and *C. boidinii* in single and in combined cultures, the HT concentration was found as 925.6, 902.5 and 903.5 mg/L, respectively. A slowly increase in concentration of TYR was observed during fermentation, reaching values between 315.6 and 544.7 mg/L in all inoculated samples. In contrast, the control samples showed a significant decrease in HT along fermentation, reaching values of 170.6 mg/L and a slight increase in TYR, reaching final value of 303.7 mg/L.

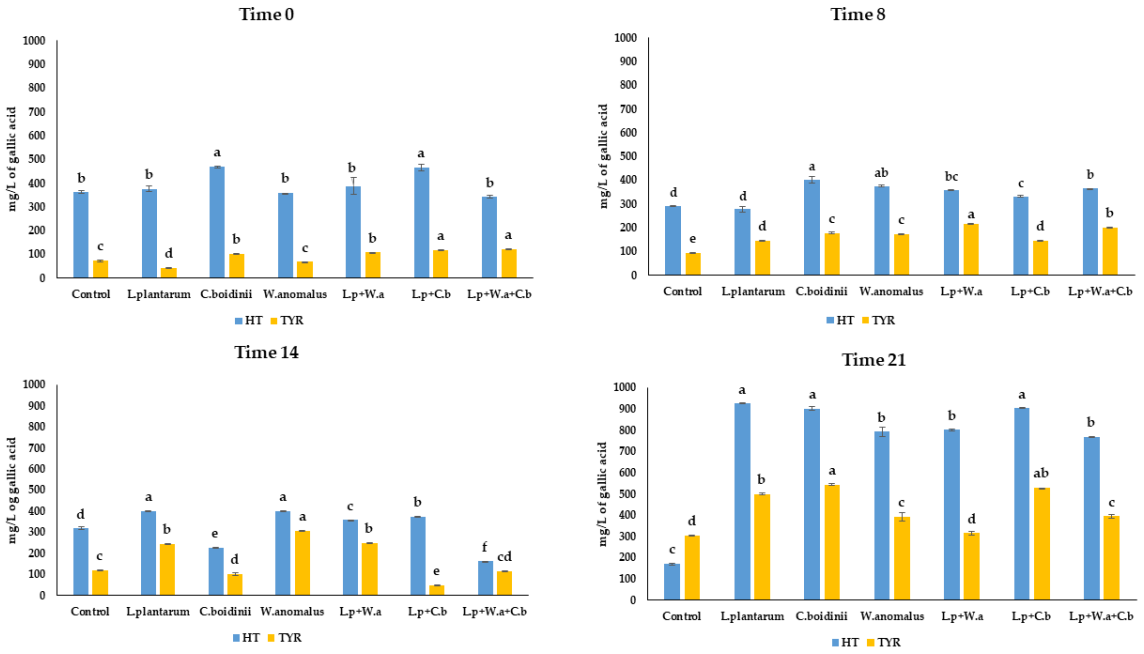


Figure 2. Concentration of hydroxytyrosol (HT) and tyrosol (TYR) during fermentation in microfiltered OMWW differently inoculated. Different letters indicate statistical differences within the columns for the same compound (significance at P≤0.01).

In addition, organic acids were evaluated at the end of fermentation in all samples. The control sample, at the beginning of fermentation, was used as an initial control (Table 2). The control, analyzed at both the beginning and at the end of fermentation, showed a constant value of acids except for butyric, for which a concentration of 566.4 mg/L was detected only at T21. For all samples inoculated with the different microbial combinations, on the other hand, an acid increase during fermentation was observed, except for isobutyric acid that decreased in sample inoculated with *W. anomalus* and in all the inoculated combinations. In detail, the sample inoculated with *L. plantarum* showed the highest increase for all the detected acids.

Table 4. Organic acids (mg/L) detected by HPLC.

Sample	Time days	Citric acid	Lactic acid	Acetic acid	Propionic acid	Isobutyric acid	Butyric acid
Control	0	4172.9 ± 96.54	1606.6 ± 99.00	416.8 ± 97.31	3865.9 ± 268.47	3136.9 ± 188.31	0.00 ± 0.00
Control	21	4529.3 ± 100.00^{de}	1219.2 ± 18.03^{f}	326.3 ± 78.89^{e}	3743.1 ± 34.21^{g}	1654.9 ± 15.21^{d}	566.4 ±48.79^{d}
L. plantarum	21	7033.4 ± 15.76^{a}	4512.6 ± 18.07^{a}	7212.8 ± 82.59^{a}	9802.4 ± 12.82^{a}	3235.3 ± 5.51^{a}	4666.4 ± 103.03^{a}
C. boidinii	21	6624.4 ± 87.69^{b}	4123.3 ± 20.03^{b}	4568.4 ± 58.78^{c}	9153.8 ± 19.41^{b}	3202.7 ± 27.72^{a}	4393.3 ± 44.23^{a}
W. anomalus	21	5214.4 ± 121.00^{c}	3774.5 ± 99.00^{c}	6214.7 ± 168.83^{b}	8219.2 ± 41.95^{c}	2072.0 ± 77.52^{b}	4239.4 ± 176.96^{a}
L.p+ W.a	21	4126.9 ± 106.79^{f}	2846.0 ± 35.53^{e}	6188.4 ± 85.52^{b}	6831.2 ± 10.08^{f}	1626.7 ± 1.41^{e}	3682.8 ± 26.00^{b}
L.p+C.b	21	4744.1 ± 16.31^{d}	3167.5 ± 33.49^{d}	4366.7 ± 132.82^{c}	7913.0 ± 24.25^{d}	2096.8 ± 16.35^{b}	2995.9 ± 54.51^{c}
L.p+W.a+C.b	21	4381.7 ± 20.88^{ef}	3075.3 ± 31.95^{d}	3334.0 ± 8.10^{d}	7342.0 ± 116.15^{e}	1810.2 ± 5.28^{c}	3550.1 ± 25.58^{b}

Data are expressed as mg/L of means ± standard deviations. Different letters indicate statistical differences within the same column (Significance at $p \leq 0.01$).

2.4 Biological assay

2.4.1 Cell culture and transepithelial transport of samples through Caco-2 cell monolayers

The intestinal permeability values, estimated with the Caco-2 cell experimental model, correlate well with human in vivo absorption data for many drugs and chemicals. Caco-2 cells are a human colon epithelial cancer cell line that, when cultured as a monolayer, differentiate to form tight junctions between cells to serve as a model of paracellular movement of compounds across the monolayer. The monolayer represents the human intestinal epithelial cell barrier and by this assay, the measured endpoint is intestinal permeability (expressed as apparent permeability—Papp value) (Table 3).

Table 3. Apparent permeability of different sample of OMWW.

Samples	Concentration of HT (mg/L)	Papp BA (nm/s) Passive transport	Papp AB (nm/s) Active transport	BA/AB	λ (nm)	ε
Control	1.70	2581	457	4.22	275	0.80
L.planturum	9.50	4015	1014	3.95	285	0.20
C. boidinii	9.00	2540	575	4.41	275	0.09
W. anomalus	7.90	1958	367	5.34	285	0.22
L.p + W.a	8.00	1905	335	5.67	284	0.19
L.p + C.b	9.00	2912	1125	2.58	275	0.19
L.p + W.a + C.b	7.70	2587	522	4.95	283	0.20

All samples were tested at a dilution of 1:100. In the table BA indicates basolateral to apical transport; AB indicates apical to basolateral transport; BA/AB values are from Papp AP−BL/Papp BL−AP.

The flux from the apical part of the monolayer to the basolateral side (BA) is referred to the passive transport, while the measurement of active transport is obtained by measuring the reversed flow (AB), since Caco-2 cells express efflux pumps in the apical side. The smaller the BA/AB ratio value, the greater the contribution of the active transport to the membrane crossing. In all tested samples, the contribution of active transport to the membrane crossing was always lower than that due to passive diffusion, as demonstrated by high values of Papp AB. This occurs mainly for samples inoculated with *L. plantarum* and *C. boidinii*, which showed the highest value (as 1125 nm/s). This value could be related to a synergic effect between the LAB and the *C. boidinii* strains, that also in single cultures showed Papp values of 1014 and 575 nm/s, respectively. This result is confirmed by the lowest BA/AB value (2.58), detected in samples fermented with *L. plantarum* and *C. boidinii*. OMWW samples and HT pure (used as a control) at the opportune dilution (1:25) have been evaluated on different cell lines, normal (HepG2) and tumoral (Caco-2), in order to evaluate their toxicological profile [20]. Since no cytotoxic effect was detected (data not shown), they resulted to be safe at a dilution of 1:25, while with higher concentrations (as such and 1:10) a cytotoxic effect was registered. These results are in agreement with data reported by Di Mauro et al. [21], confirming that the use of higher concentrations (as such and 1:10) induced a reduction in cell viability in a dose–dependent manner, while lower concentrations did not affect cell viability.

2.4.2 Activity on COX-1 and COX-2

COX, also called Prostaglandin H synthase (PGHS), is a key enzyme in the inflammatory cascade. It catalyzes the conversion of arachidonic acid (AA) in prostanoids, bioactive lipids mediating numerous physiological and pathological processes in the body. Prostanoids include thromboxane A2 (TXA2), prostaglandins (PGD_2, PGE_2, PGF2α) and prostacyclin (PGI2). Two COX isoforms are known, COX-1 and COX-2, encoded by different genes. The two isoforms show 60% homology in their amino acid sequence. COX-1 is the isoform constitutively expressed in most tissues and responsible for maintaining normal physiological functions such as gastric protection, modulation of platelet function, and renal homeostasis. COX-2, differently from COX-1, is the inducible isoform upon pro–inflammatory stimuli. The possibility of finding anti–inflammatory properties in nutraceutical compounds would make the

products under study extremely interesting, thus, the OMWW fermented sample inhibition of *ovine*COX-1 (*o*COX-1) and *human*COX-2 (*h*COX-2) enzyme activity was investigated and HT was used as positive control. Pure HT showed, at a concentration of 40 mg/L, inhibition activity on *o*COX-1 and *h*COX- 2 with a percentage of 6.41 and 26.11, respectively (Figure S1). The control OMWW sample did not show any anti–inflammatory activity, while low anti–inflammatory activity was found for the different OMWW samples. In detail, samples fermented with *L. plantarum*, *C. boidinii* and *W. anomalus* in single culture showed a moderate inhibitory activity towards both isoforms (Table S3). In particular, the sample inoculated with *L. plantarum* in single culture showed a selective inhibition of *o*COX-1, whereas samples treated with *C. boidinii* showed an inhibition towards both *o*COX-1 and *h*COX-2 with the percentage of inhibition reaching 15.96% and 12.95%, respectively. In addition, the sample inoculated with a combo of *L. plantarum* and *C. boidinii* preserves a selective inhibition towards *o*COX-1, with an inhibition of 8.20%. It could be hypothesized that *C. boidinii* produces some metabolites with a greater affinity and selectivity towards *o*COX-1 isoform.

2.4.3. Antioxidant Activity

Diabetes, cardiovascular diseases, arthritis and joint diseases, allergies and chronic obstructive pulmonary diseases are classified, according to the World Health Organization, as specific inflammation–mediated chronic diseases. The processes underlying these diseases are many, but oxidative stress is undoubtedly involved in their pathogenesis and in the development and establishment of a sustained inflammatory state. All selected samples were evaluated for their antioxidant activity by measuring their reactivity with 1,1-diphenyl-2-picrylidrazyl (DPPH), a purple–colored stable radical that strongly absorbs at λ = 517 nm, in order to determine their efficacy as scavengers of stable free radicals. Testing was carried out to compare the effect of fermented OMWW samples with the known antioxidant activity of HT (Figure 3). The data showed that the OMWW control exhibited lower antioxidant capacity, at all dilutions tested, compared with both that exerted by HT and fermented samples. In particular, at the lower tested volume (12.5 µL), the best antioxidant activity was obtained in the sample inoculated with *C. boidinii*, reaching a % RSA value higher than pure HT. The same behavior was observed for samples inoculated with L. *plantarum*

and *W. anomalus*, in single culture.

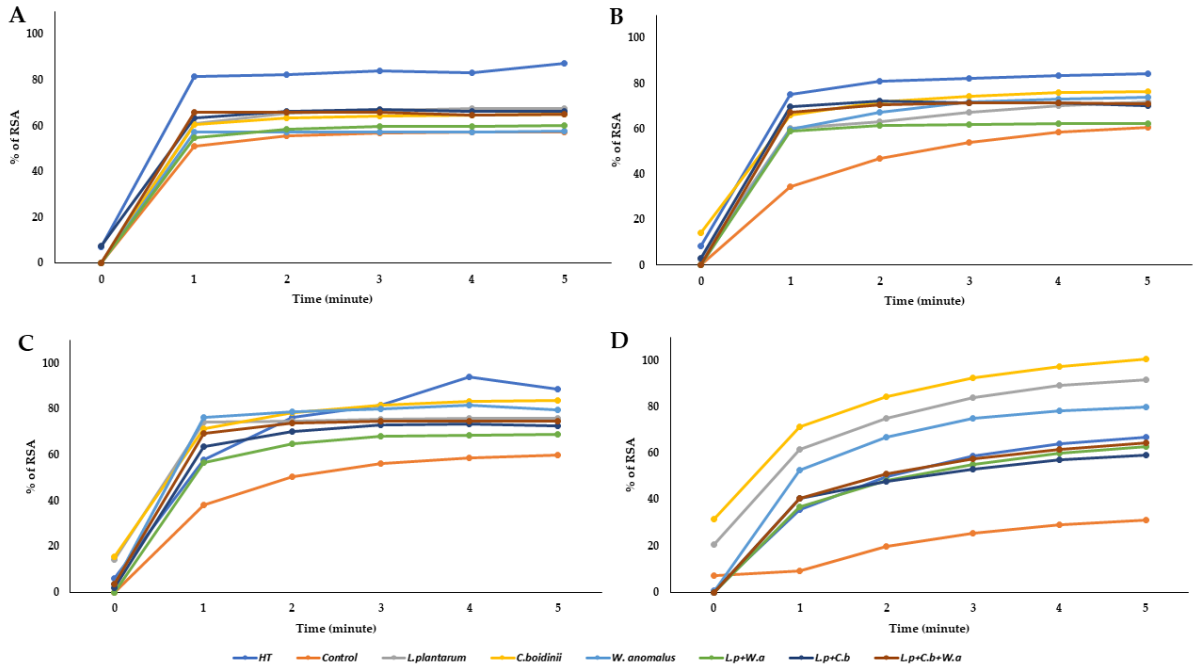


Figure 4. Evaluation of antioxidant action expressed as % RSA. Each graph corresponds to a volume (µL) used for each sample: A) 50 µL of samples; B) 37.50 µL of samples; C) 25 µL of samples; D) 12.5 µL of sample.

3. Discussion

Fermentation is widely considered a low–cost strategy to recovery and valorize agro-industrial by–products [22]. In this study, in order to obtain a suitable matrix to be fermented with selected microbial pools, different Trials were set up. For this purpose, fresh OMWWs were collected at two successive seasonal years. Samples obtained from Trial I, untreated fresh OMWW, appeared very turbid and rich in unwanted solids at both the beginning and end of fermentation. Therefore, in Trial II, the OMWWs were subjected to on farm filtration using carton filters with different porosity. To date, such a technique is used to remove unwanted solid components from the matrix, maintaining the nutritional compounds as phenolic fraction (Figure S2) [23]. Results of Trial II showed that although the OMWWs (filtered through cardboard filters) visually appeared as clear from a physical point of view, at both the beginning and end of fermentation, they were not microbiologically suitable, in relation to the high total aerobic mesophilic bacteria densities. According to the European Regulation (EC) No. 1441/2007, the absence of pathogens, such as *Salmonella* spp. and *L. monocytogenes*, is considered an

essential criterion for the microbiological safety of vegetable products, while no mandatory microbiological criterion is fixed for total aerobic mesophilic bacterial count. However, some guidelines include *Escherichia coli* and total aerobic mesophilic count as quality parameters, fixing the following thresholds (as CFU/g): *E. coli* < 10 for satisfactory; between 10 and $\leq 10^2$ for acceptable; and $>10^2$ as not acceptable [24]. The same authors, for total aerobic mesophilic count, proposed the following thresholds: $\leq 10^4$ for satisfactory, between 104 and lower or the same of 10^6 for acceptable, and $>10^6$ not acceptable, respectively [24]. Therefore, OMWW obtained through the last cardboard filter, with a porosity between 0.20 and 0.40 μm, were afterwards subjected to microfiltration (0.22 μm) in the laboratory. This procedure resulted in a microbiologically sterile, clear matrix mainly composed of phenols (Figure S3). To date, the microfiltration technique is successfully applied in food industries, such as the dairy industry, as it induces an improvement in the microbial quality of the final product [25]. In the present study, the application of such a strategy allowed the evaluation of the biotechnological aptitude of the strains, used as single or mixed cultures, and enabled an understanding of how they interact with the matrix. The results showed that the use of microbial starters drove fermentation by lowering the pH to values as low as 4.49 and inducing an increase in the phenolic compounds. In detail, the combinations of *L. plantarum* and *C. boidinii*, both in single and in mixed cultures, resulted, at the end of fermentation, in the highest HT content, with values of 925.6, 902.5 and 903.5 mg/L, respectively. No oleuropein was detected at any sampling time as found by other authors [5,26]. Although *L. plantarum* is mainly known for its β-glucosidase activity or its probiotic potential [27], in all the tests carried out, there was a slight decrease of LAB count in sample with *L. plantarum* that showed an increase only after t14 of fermentation (Figure 1). This suggests that these strains are able to utilize certain metabolic pathways to survive in difficult matrices, which is why there is an increased activity in the last sampling time. An interesting study that may explain the adaptation of *L. plantarum* is proposed by Reveròn et al. [28], who propose a study of transcriptomics and the mechanism of action of *L. plantarum* in response to treatment with pure HT. *C. boidinii* strain used as a potentially resistant strain to several hurdles present in the matrix. Recently, De Melo Pereira et al. [29] reported that the genus *Candida* is commonly found in many fermented foods and beverages obtained by the main types of fermentation (alkaline, alcoholic, acetic, lactic, and mixed processes). In addition to its

ubiquitous trait, the *Candida* genus also possesses a complex metabolic mechanism that allows it to survive, compete, and sometimes dominate fermentation processes [30]. Furthermore, it is known that a selected culture, besides the ability to control the fermentation process, should show the ability to survive in the fermentation environment and to exert acidifying activity through the production of organic acids. In the present study, results highlighted that *L. plantarum* inoculated samples exhibited the highest values of all detected acids. In a functional beverage, organic acids can play an additional role in protecting phenols, such as HT and TYR, from oxidation. In addition, different studies revealed that a lactic acid concentration of 0.5% (v/v) produced by LAB prevents pathogens' growth, such as *Salmonella* species, *Escherichia coli*, *and Listeria monocytogenes* [31,32]. This result confirmed results previously reported, namely, that the fermentation driven by LAB leads to the production of mono–, di–, and tri– carboxylic acids, i.e., acetic, lactic, and propionic acids as intermediaries of biosynthetic metabolic pathways and amino acid metabolism. In detail, Okoye et al. [33] demonstrated through genome study that LAB contain unique and shared secondary metabolite biosynthetic gene clusters with bio preservative potential and a transcription factor, namely CRP (cyclic AMP receptor protein) endowed with novel binding sites involved in organic acid metabolism. Zooming in on biological activity, results obtained from tested microfiltered fermented OMWW and from pure HT, when tested at a 1:25 dilution, were found to be safe on chosen cell lines. In the present study, the choice of cell lines was based on taking into consideration that the HepG2 is one of the most reliable experimental models for prediction human liver toxicity. Indeed, the liver is responsible for most of the orally administered xenobiotic metabolism, for its anatomical proximity to the gastrointestinal tract and for its histological structure [34], whereas the Caco-2 cell line has been chosen as the most suitable in vitro model to rapidly assess the intestinal permeability and for xenobiotic transport studies [35,36]. Caco-2 cells exhibit a well–differentiated brush border on the apical surface and tight junctions, and express typical small–intestinal microvillus hydrolases and nutrient transporters. The crossing of biological membranes must be taken into account because it correlates with the ability of a pharmacologically active compound to reach the target site where performing the biological function. The intestinal transport of polyphenols seems to be strongly influenced by several factors such as food matrix, biotransformation and conjugation that occur during absorption [37,38]. Many studies

have focused on the uptake of individual phenols, such as HT and TYR, which have shown good absorption across the cell membrane, while the uptake of a phytocomplex and how its different composition may affect the transport mechanism has been less explored [39]. In a recent study, Bartolomei et al. [40], demonstrated that a phenolic pool, extracted from extra virgin olive oil (EVOO), induced a protective effect against H_2O_2 induced oxidative stress on Caco-2 and HepG2 cell lines. This observation demonstrated a selective transepithelial transport of certain oleuropein derivatives by Caco-2 cells, confirming that the phytocomplex could be transported with different mechanisms than those involved for single phenolic compounds, separately tested. According to results previously reported both phytocomplex composition and used starter cultures can significantly influence cell membrane crossing. In the present study, microbial cultures differently modulated the response of anti–inflammatory and antioxidant activity. It has been widely reported that phenols contained in EVOO reduce the reactive oxygen species (ROS) and malondialdehyde production, the nitric oxide release and the expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX-2) [41]. Results obtained in the present study confirmed that OMWW samples affected the inhibitory activity towards COX-1 and COX-2, by a modulation of COX-2, according to previous in vitro reports on human monocytes [42]. The same authors demonstrated that HT attenuated ROS–mediated COX-2 transcription induced by bacterial lipopolysaccharide (LPS). COX catalyzes the first step in the biosynthesis of prostaglandins (PG), prostacyclin and thromboxane starting from free arachidonic acid (AA) [43]. Among prostaglandins, PGE2 is involved in inflammation, angiogenesis and in promoting the growth of several solid tumors, such as breast, ovarian, head and neck cancer, renal cell carcinoma and hematological cancers [44–46]. COX-1 and COX-2 are of great interest because they are targets of non–steroidal anti–inflammatory drugs (NSAIDs), which, when binding to the active site of COX, prevent the AA from reaching the catalytic pocket and, thus, the biosynthesis of prostaglandins. COX inhibition is therefore important in reducing the inflammatory response, tumorigenesis and cancer progression. Many of the recognized anti–cancer properties of HT are related to other activities, such as ability to modulate the antioxidant system and ROS scavenge [47,48]. Ramirez–Tortosa et al. [49] demonstrated that a supplementation with HT (15 mg/day) is effective into downregulate several transcriptional factors, as described for other antioxidant agents, able to induce, at

plasma level, a decrease of metalloproteinase in women with breast cancer. HT, as reported by the European Food Safety Authority but in general the phytocomplex present both in olive oil and in by–products, has a beneficial effect on human health. The interaction between phenols and microorganisms used as starters plays a key role in understanding the mechanism of action and how they can modulate the anti–inflammatory and antioxidant response in the development of degenerative diseases [50].

4. Materials and Methods

4.1 OMWW sampling

The OMWW samples used in the present study were obtained by a three–phase olive oil extraction system at the Consoli oil company (Adrano, Italy) and collected during a two–year period. In detail, for Trial I OMWW was collected in the 2019–2020 season and for Trials II and III OMWW samples were collected in the 2020–2021 season. All the Trials are described in Figure 3. For Trial I the fresh produced OMWW was immediately stored at −20 °C at the Di3 A, University of Catania.

For Trial II, the OMWWs were stored at room temperature in the company facilities, until further treatments. To obtain a clear matrix, OMWW samples were subjected to filtration using Oenopad® XF1, XF7 and XFSS filters (OENO S.R.L., Erbusco, Italy) suitable for food matrices and consisting of cellulose, diatomaceous earth and perlite. Different fractions were obtained: the as is sample (prefiltered or PF sample); and the three fractions (F1, F2, F3) obtained by sequential filtration with filters at different porosity, as: the "XF1" filter (8.0–20 µm) to eliminate solid particulates; the"XF7" filter (2.0–4.0 µm) for clarifying step; the "XFSS" filter (0.20–0.40 µm) for final sterilization. All fractions were collected and stored at −20 ◦C.

In addition, the Trial III was obtained from the F3 sample, in turn obtained from Trial II, by microfiltration using the Sartoclear Dynamics® kit (Sartorius, Varedo, Italy), connected to a vacuum pump. The latest process allows both the clarification/filtration and cold sterilization in a single step, as the used bottle presented a 0.22-µm polyethersulfone (PES) filter membrane. After processing samples needed for subsequent tests were frozen at −20 °C.

4.2 Set-up of fermentation process

In order to set up the fermentation process some components, such as yeast extract at 1% (w/v), peptone and glucose at 2% (w/v), were added into the fresh OMWW samples and to the F3 samples right before fermentation. All components were purchased from Liofilchem (Roseto degli Abruzzi, Italy). The fermentation process was started through the inoculum of microbial pools, consisting of yeast and lactic acid bacteria strains, belonging to the microbial culture collection of the Department of Agricultural, Food and Environmental Sciences (Di3 A) and to ProBioEtna srl, Spin off of University of Catania. In details, the *Candida boidinii* F3 30.1, *Wickerhamomyces anomalus* F5 60.5 and *Lactiplantibacillus plantarum* F 3.5 (DSM 34190) strains were used. All the strains were previously isolated from naturally fermented table olives [51]. One hundred microliters of each yeast inoculum and *L. plantarum* were spotted in Yeast Peptone Dextrose broth (YPD, Sigma-Aldrich, Milano, Italy) and de Man, Rogosa, and Sharpe broth (MRS, Oxoid, UK) and allowed to incubate overnight at a selective temperature of 25 °C and 32 °C, respectively. Then, the strains were inoculated at 0.5%, which corresponded to an initial cell density of 10^7 CFU/mL for yeasts and 10^8 CFU/mL for *L. plantarum* (Figure 4). Seven experimental samples were set up for each Trial: the un–inoculated samples (controls); three single culture inoculated samples; two samples inoculated with each yeast strain in mixed culture with the *L. plantarum* strain; one three–strain mixed culture sample. All tests were conducted in triplicate in an OMWW total volume of 400 mL. For Trial I and II, the fermentation process was monitored at regular intervals: at T0 (after about 7 h of microbial inoculation); T8 (after 8 days of fermentation); T30 (after 30 days of fermentation). For Trial III, fermentation parameters were monitored at T0, T8, T14 (after 14 days of fermentation) and T21 (end of fermentation). All fermentations were carried out at room temperature (20 ± 4 °C).

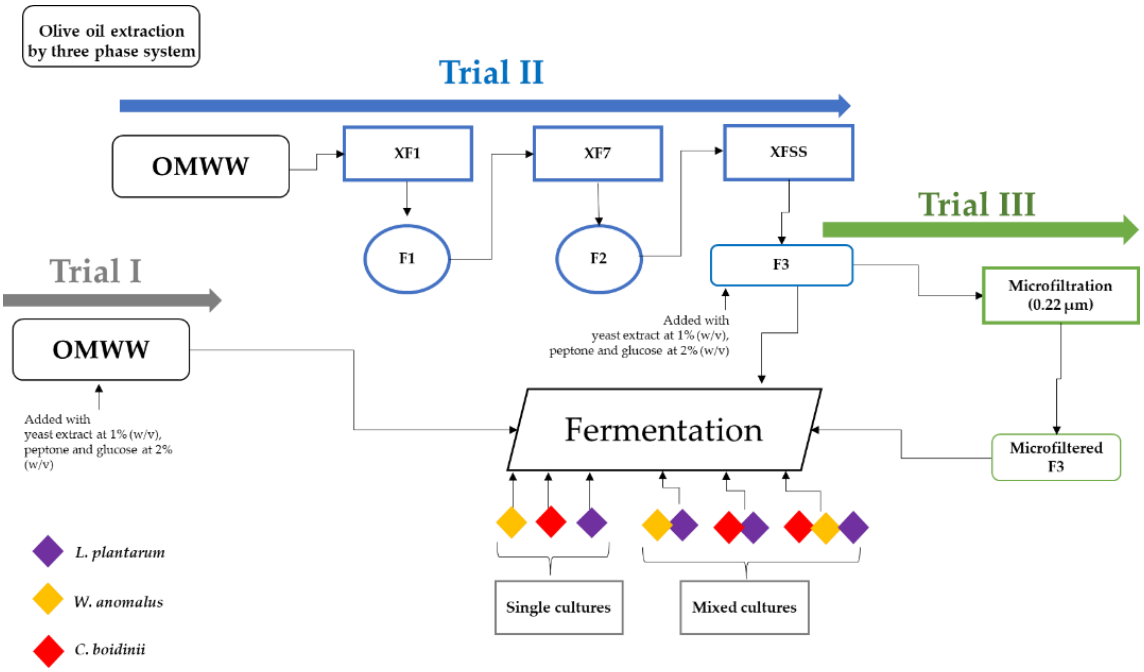


Figure 4. Fermentation process and OMWW obtained trials.

4.3 Chemical analysis

The pH, the TSS and the total phenol content were monitored for all samples during fermentation. The pH was measured with a Mettler DL25 pH meter (Mettler–Toledo International Inc., Columbus, OH, USA) and the total soluble solids (TSS), expressed as °Brix, were measured using a refractometer (Atago, RX-5000, Milano, Italy). In addition, the total phenolic content was determined according to the Folin–Ciocalteu's colorimetric method (FC). The tested samples were mixed with 5 mL of commercial FC reagent (Labochimica, Campodarsego, Italy) diluted with water (1:10 v/v) and added with 4 mL of a 7.5% sodium carbonate solution. Subsequently, samples were left in the dark at room temperature. After 2 h, the absorbance was measured spectrophotometrically at 765 nm (Cary 100 Scan UV-Visible, Agilent, CA, USA). The total phenolic content was expressed as mg gallic acid equivalent (GAE)/L of sample).

4.4 Microbiological analyses

Samples of Trial I, II and III were serially diluted and poured into agar plates containing specific media and incubated under specific conditions: de Man, Rogosa, and Sharpe

Agar (MRSA, Oxoid, Milano, Italy) for lactic acid bacteria counts, incubated at 32 °C for 48 h under anaerobic conditions; Plate Count Agar (PCA, Oxoid, Milano, Italy) for mesophilic aerobic bacteria counts, incubated at 25 °C for 48 h; Violet Red Bile Glucose Agar (VRBGA, Liofilchem, Roseto degli Abruzzi, Italy IT) for the determination of Enterobacteriaceae, incubated aerobically at 30–35 °C for 18–24 h; Sabouraud Dextrose Agar (SAB, Bio-Rad, Hercules, CA, USA) for yeast counts, incubated at 25 °C for 48 h. At the end of fermentation, the presence/absence of *Clostridium perfringens* was also determined in Sulphite-Polymyxin-Sulphadiazine Agar (SPS, Oxoid, UK), incubated at 35–37 °C for 18–48 h, under anaerobic conditions. Moreover, for starter cultures monitoring, samples of Trial III were subjected to additional counting, in MRS agar and in SAB agar media, for *L. plantarum* and yeast determination, respectively. All microbiological analyses were performed in triplicate and the results were expressed as Log CFU/mL.

4.5 HPLC analysis

4.5.1 Detection of phenols

The HPLC analyses of fermented OMWW samples were performed by directly injecting the filtered samples (0.45 μm PTFE filters, Merck, Darmstadt, Germany) into the HPLC chromatographic system, i.e., Waters Alliance 2695 HPLC liquid chromatography equipped with a Waters 996 photodiode array (PDA) detector set at 280 nm and managed through the Waters Empower software (Waters Corporation, Milford, MA, USA). The column used was a Luna C18 (250 mm × 4.6 mm i.d., 5 m, 100 Å; Phenomenex, Torrance, CA, USA) maintained in an oven at 40 ◦C. A flow rate of 1 mL/min was used. Chromatographic separation was performed according to Romeo et al. [11]. The internal standard (I.S.), 50 mM pure gallic acid (Fluka, Buchs Switzerland), was used to quantify the phenolic compounds. The identification of phenolic compounds was obtained by comparing the peak retention time with those of pure standards of tyrosol (TYR), oleuropein (OLE), hydroxytyrosol (HT) chlorogenic acid, vanillic acid, caffeic acid, syringic acid, p-coumaric acid, ferulic acid, verbascoside, luteolin-7-o-glucoside, o-coumaric acid, rutin, oleuropein, apigenin-7-o-glucoside, luteolin-4-glucoside, quercetin, luteolin, apigenin (Extrasynthese, Genay, France). All analyses were performed in triplicate for each sample.

4.5.2 Detection of organic acids

The determination of organic acids was carried out at the end of fermentation in trial III. Each sample was filtered through a 0.45 μm PTFE syringe filter (Merck, Germany) before being injected into HPLC (HPLC instruments were described in the previous section) with a DAD detector set at 210 nm (and with spectrum acquisition from 200 to 400 nm). Isocratic elution with 5 mM sulfur acid was performed on a Rezex ROA Organic AcidH+ column (Phenomenex, CA, USA). The run time was set to 50 minutes at 0.6 mL/min.For calibration, pure standards of lactic, citric, acetic, propionic, isobutyric and butyric acids (all purchased from Sigma-Aldrich, Italy) were injected at different concentrations. All analyses were performed in triplicate for each sample.

4.6 Biological assays

4.6.1 Cell culture and cytotoxic activity

Caco-2 cells were grown in Dulbecco's Modified Eagle Medium high glucose (DMEM high glucose, Euroclone S.p.A., Pero, Italy) supplemented with 10% Fetal Bovine Serum (FBS, Euroclone S.p.A., Pero, Italy), 2 mM glutamine (Euroclone S.p.A., Pero, Italy), 100 U/mL of penicillin and 0.1 mg/mL of streptomycin (Euroclone). Caco-2 cells were kindly supplied from Dr. Aldo Cavallini and Dr. Caterina Messa from the Laboratory of Biochemistry National Institute for Digestive Diseases. "S. de Bellis", Bari (Italy). Human hepatocellular liver carcinoma (HepG2) cell line was purchased from American Type Culture Collection (ATCC). HepG2 cells were cultured in Eagle's Minimum Essential Medium (MEM, Euroclone), supplemented with 10% FBS, 2 mM glutamine (Euroclone), 100 U/mL penicillin and 0.1 mg/mL streptomycin (Euroclone S), 1% Non–Essential Amino Acids (NEAA, Euroclone). Cultured cells were maintained at 37 ◦C in atmosphere containing 95% of air and 5% of CO_2. Cells were sub-cultivated every 48 h by trypsine–EDTA solution.

Determination of cell growth was performed using the 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide (MTT) assay (Sigma-Aldrich, Milan, Italy), 10.000 cells/well were seeded into 96-well plates at a volume of 100 μL. After 24 h, 100 μL of microfiltered fermented OMWW samples were added at the appropriate dilution: as such, 1:10, 1:25, 1:50 and 1:100 in triplicate. After 72 h incubation time with extracts, the plates

containing the cells were incubated with MTT for 3–4 h at 37 ◦C and 5% of CO_2. At the end of incubation time, MTT was aspirated, and the formazan crystals were solubilized by using 100 µL of dimethyl sulfoxide/ethanol (1:1) (Sigma–Aldrich). The absorbance values at λ = 570 nm were determined on the Victor Microplate Reader (PerkinElmer, Roma, Italy). Pure HT (Phytolab, Vastenbergsgreuth, Germany) was used as a positive control.

4.6.2 Transport Caco-2 Monolayer

Caco-2 cells were seeded onto a Millicell-96 assay system (Millipore, Burlington, MA, USA) in which a cell monolayer was set in between a filter cell and a receiver plate at a density of 20.000 cells/well. The culture medium was replaced every 48 h and the cells were kept for 21 days in culture. The trans epithelial electrical resistance (TEER) of the monolayers was measured daily before and after the experiment by using an epithelial voltohmmeter (Millicell–ERS). Generally, TEER values greater than 1000 Ω for a 21-day culture are considered optimal. After 21 days of Caco-2 cell growth, the medium was removed from the filter wells and the receiver plate, and they were filled with fresh Hank's balanced salt solution (HBSS) buffer (Invitrogen, Waltham, MA, USA). This procedure was repeated twice, and the plates were incubated at 37 °C for 30 min. After the incubation time, the HBSS buffer was removed and OMWW samples (dilution 1:100) were added to the filter well whereas fresh HBSS was added to the receiver plate. The plates were incubated at 37 °C for 120 min. Afterward, samples were removed from the apical (filter well) and basolateral (receiver plate) side of the monolayer to measure the permeability. The apparent permeability (Papp) referred to HT in units of nm/second was calculated using the following Equation (1):

$$P_{app} = \left(\frac{V_A}{Area \times time}\right) \times \left(\frac{[sample]acceptor}{[sample]initials}\right) \qquad (1)$$

V_A = the volume (in mL) in the acceptor well; Area = the surface area of the membrane (0.11 cm^2 of the well); time = the total transport time in seconds (7200 s); [sample]acceptor = the concentration of the sample measured by U.V. spectroscopy; [sample]initial = the initial sample concentration (1×10^{-4} M) in the apical or basolateral wells.

4.6.3 Cyclooxygenase Activity Inhibition

Preliminarily, the fermented OMWW samples obtained from Trial III were evaluated for their ability to inhibit *ovine*COX-1 or *human*COX-2 enzymes, measuring the extent (%) of enzyme activity inhibition at 50 μM, at dilution 1:25. The inhibition of the enzyme was evaluated by using a colorimetric COX inhibitor screening assay kit (Catalog No. 7601050, Cayman Chemicals, Ann Arbor, MI, USA) following the manufacturer's instructions. COX is a bifunctional enzyme exhibiting both cyclooxygenase and peroxidase activities. The cyclooxygenase component catalyzes the conversion of arachidonic acid into the hydroperoxide PGG_2 and then peroxidase component catalyzes PGG_2 reduction into the corresponding alcohol PGH_2, the precursor of PGs, thromboxane, and prostacyclin. The COX inhibitor screening assay colorimetrically measures the peroxidase activity of the cyclooxygenases monitoring the appearance of oxidized N,N,N0,N0 -tetramethyl-p-phenylenediamine (TMPD) at λ = 590 nm on the Victor Microplate Reader (PerkinElmer, Italy). Stock solutions of tested samples were dissolved in deionized distillated water.

4.6.4 Antioxidant activity

The radical scavenging activity was determined as percentage of RSA (radical scavenging activity), according to Palmeri et al., 2022. The values were expressed by using the following equation (2):

$$RSA\ \% = \left(\frac{\text{Blank Absorbance} - \text{Sample Absorbance}}{\text{Blank Absorbance}}\right) \times 100 \qquad (2)$$

Different dilutions of samples were added to the mixture of methanolic solution and 2,2-diphenyl-1-picrylhydrazyl radical 10−4 M. The DPPH absorbance values were evaluated at λ = 517 nm by monitoring the kinetics for 5 min with spectrophotometer (Shimadzu UV-1800, Denmark). Pure HT (Phytolab, Germany) was used as a positive control.

4.7 Statistical analysis

Statistical analysis of the obtained results was performed by means of one-way analysis of variance (ANOVA) and Tukey's HSD post hoc test for separation of means at a significance level of $P \leq 0.05$. For data processing, SPSS software (version 21.0, IBM Statistics, NY, USA) was used for data processing.

5. Conclusions

The microfiltration process resulted in a suitable strategy to obtain a OMWW matrix able to be fermented. The use of selected microbial pools in single and co–cultures showed an increase in HT and TYR contents at the end of fermentation, compared with the control sample. Biological analyses showed that fermentation increases the antioxidant and inflammatory activity of OMWW that resulted to be safe in HepG2 and Caco-2 cell lines. In detail, the phenolic pattern associated to starter microorganisms exhibited an increase of active permeability on Caco-2 monolayer, and a moderate inhibition towards *o*COX-1 and *h*COX-2 was observed. The results confirm that fermented OMWW can be proposed as a new beverage and/or functional ingredient that could include the addition of compounds as flavorings and probiotic microorganisms. Despite the interesting results obtained at lab scale, perspective studies should aim to replay the process at the industrial scale to standardize phenol concentration at each obtained new formulation.

Supplementary material

Table S1. Chemical parameters detected in samples of Trial I and Trial II.

Sample	Time (days)	pH	TSS (°Brix)	Total phenol (mg/L)	HT (mg/L)	TYR (mg/L)
Trial I						
Control	0	5.05 ± 0.01	7.77 ±0.02^{b}	2449.8 ± 0.25^{d}	410.1 ± 4.43^{a}	76.4 ± 0.46^{a}
L. plantarum	0	5.02 ± 0.01	7.72 ±0.01^{b}	2596.0 ± 1.42^{b}	285.4 ± 42.61^{bc}	68.2 ± 0.82^{bc}
C. boidinii	0	5.01 ± 0.01	7.73 ±0.08^{b}	2515.9 ± 0.15^{c}	415.2 ± 14.38^{a}	54.7 ± 1.49^{d}
W. anomalus	0	5.01 ± 0.01	8.21 ±0.02^{a}	2638.4 ± 0.59^{b}	373.9 ± 14.19^{a}	52.5 ± 3.28^{d}
L.p+ W.a	0	5.02 ± 0.02	7.08 ±0.01^{c}	2459.4 ± 0.79^{c}	345.2 ± 14.90^{ab}	61.5 ± 0.52^{c}
L.p+C.b	0	5.03 ± 0.01	8.32 ± 0.08^{a}	2654.7 ± 1.80^{b}	105.2 ± 2.66^{d}	54.3 ± 2.29^{d}
L.p+W.a+C.b	0	5.10 ± 0.14	8.19 ± 0.11^{a}	2744.9 ± 0.02^{a}	229.5 ± 6.62^{c}	74.6 ± 1.01^{ab}
		n.s	**	**	**	**
Control	8	4.85 ± 0.01^{a}	6.27 ± 0.08^{a}	2037.5 ± 2.08^{d}	252.5 ± 1.05^{b}	89.8 ± 6.42
L. plantarum	8	4.76 ± 0.03^{ab}	5.65 ± 0.06^{b}	1584.7 ± 0.41^{e}	174.6 ± 2.06^{d}	88.8 ± 3.14
C. boidinii	8	4.45 ± 0.07^{c}	5.79 ± 0.02^{b}	2893.4 ± 7.62^{a}	305.2 ± 14.17^{a}	98.2 ± 7.38
W. anomalus	8	4.82 ± 0.03^{a}	5.73 ± 0.09^{b}	2336.3 ± 0.41^{b}	224.4 ± 0.93^{c}	113.1 ± 16.44
L.p+ W.a	8	4.69 ± 0.01^{b}	5.19 ±0.01^{c}	2363.6 ± 3.39^{b}	267.9 ± 2.20^{b}	112.7 ± 4.63
L.p+C.b	8	4.76 ± 0.01^{ab}	6.19 ±0.05^{a}	2047.2 ± 6.69^{d}	266.8 ± 3.65^{b}	103.2 ± 1.76
L.p+W.a+C.b	8	4.64 ± 0.01^{b}	6.16 ± 0.08^{a}	2191.8 ± 2.49^{c}	300.0 ± 3.08^{a}	108.9 ± 2.12
		**	**	***	*	n.s
Control	30	4. 99 ± 0.02^{b}	6.31 ± 0.14^{a}	2642.5 ± 1.30^{b}	1283.6 ± 23.21^{c}	439.8 ± 67.33^{b}
L. plantarum	30	5.51 ± 0.14^{a}	5.99 ± 0.01^{ab}	2485.1 ± 12.90^{c}	1516.6 ± 153.76^{bc}	511.5 ± 40.51^{ab}
C. boidinii	30	4.81 ± 0.01^{bc}	5.60 ± 0.28^{b}	3135.5 ± 5.23^{ab}	2190.2 ± 155.64^{ab}	679.4 ± 9.71^{a}
W. anomalus	30	4.59 ± 0.01^{cd}	5.95 ± 0.08^{ab}	3241.9 ± 0.13^{a}	2630.4 ± 44.05^{a}	537.8 ± 9.71^{ab}
L.p+ W.a	30	4.55 ± 0.07^{d}	6.07 ± 0.09^{ab}	2555.9 ± 4.30^{b}	1622.1 ± 80.47^{bc}	529.0 ± 12.03^{ab}
L.p+C.b	30	4.71 ± 0.01^{cd}	6.05 ± 0.07^{ab}	2335.6 ± 5.34^{d}	1560.0 ± 80.26^{bc}	508.6 ± 5.84^{ab}
L.p+W.a+C.b	30	4.63 ± 0.05c^{d}	6.34 ± 0.01^{a}	3129.7 ± 4.63^{ab}	1543.3 ± 244.36^{bc}	539.4 ± 3.24^{ab}
		**	*	**	*	*
Trial II						
Control	0	5.36 ± 0.02^{ab}	7.88 ± 0.16^{bc}	3773.7 ± 7.26^{a}	727.6 ± 39.38^{bc}	382.5 ± 3.54^{b}
L. plantarum	0	5.30 ± 0.14^{ab}	7.67 ± 0.06^{c}	3655.2 ± 0.55^{b}	479.3 ± 31.42^{d}	262.0 ± 7.25^{cd}
C. boidinii	0	5.30 ± 0.01^{ab}	8.05 ± 0.07^{b}	3271.4 ± 14.62^{c}	616.6 ± 60.40^{c}	245.4 ± 12.34^{d}
W. anomalus	0	5.47 ± 0.03^{a}	8.01 ± 0.01^{b}	3778.9 ± 12.59^{a}	402.8 ± 0.08^{d}	528.4 ± 0.39^{a}
L.p+ W.a	0	5.20 ± 0.01^{b}	7.84 ± 0.08^{bc}	3653.1 ± 5.48^{b}	802.3 ± 12.26^{ab}	332.8 ± 47.82^{bc}
L.p+C.b	0	5.37 ± 0.01^{ab}	8.56 ± 0.01^{a}	2957.8 ± 10.03^{e}	874.3 ± 3.34^{a}	245.1 ± 10.16^{d}
L.p+W.a+C.b	0	5.32 ± 0.02^{ab}	8.06 ± 0.08^{b}	3077.2 ± 1.13^{d}	765.9 ± 33.78^{ab}	299.8 ± 5.06^{cd}
		*	**	**	**	**
Control	8	4.35 ± 0.07^{a}	6.47 ± 0.04^{a}	2992.6 ± 1.85^{d}	526.2 ± 4.64^{bcd}	166.2 ± 3.79^{c}
L. plantarum	8	3.97 ± 0.01^{c}	5.64 ± 0.06^{e}	3005.1 ± 7.29^{c}	594.7 ± 0.49^{bc}	211.2 ± 22.84^{b}
C. boidinii	8	4.05 ± 0.01^{c}	6.05 ± 0.07^{cd}	3201.8 ± 1.12^{d}	306.1 ± 0.16^{d}	120.0 ± 0.05^{d}
W. anomalus	8	3.97 ± 0.01^{c}	5.89 ± 0.01^{d}	3261.9 ± 4.15^{b}	393.2 ± 5.78^{cd}	121.7 ± 1.11^{d}
L.p+ W.a	8	4.18 ± 0.01^{b}	5.50 ± 0.01^{e}	3200.7 ± 3.78^{d}	1064.9 ± 15.22^{a}	280.8 ± 4.01^{a}
L.p+C.b	8	3.98 ± 0.01^{c}	6.16 ± 0.08^{bc}	3024.1 ± 28.40^{c}	314.2 ±146.37^{d}	171.8 ± 4.39^{c}
L.p+W.a+C.b	8	3.99 ± 0.01^{c}	6.36 ± 0.05^{ab}	3379.5 ± 26.15^{a}	666.9 ± 12.83^{b}	142.8 ± 3.70^{cd}
		**	**	**	**	**
Control	30	6.29 ± 0.01^{a}	6.50 ± 0.01^{a}	2796.7 ± 1.85^{e}	330.7 ± 2.17^{d}	129.5 ± 0.34^{b}
L. plantarum	30	4.15 ± 0.07^{e}	5.64 ± 0.02^{c}	3577.6 ± 12.40^{a}	840.3 ± 6.68^{b}	126.0 ± 1.38^{b}
C. boidinii	30	5.57 ± 0.01^{b}	5.84 ± 0.06^{bc}	3267.2 ± 2.28^{c}	979.2 ± 8.26^{b}	87.0 ± 0.27^{cd}
W. anomalus	30	5.64 ± 0.06^{b}	5.94 ± 0.08^{b}	3160.4 ± 41.87^{d}	596.3 ± 89.00^{c}	141.4 ± 1.63^{b}
L.p+ W.a	30	5.04 ± 0.06^{c}	5.68 ± 0.11^{c}	3364.1 ± 25.20^{bc}	1235.6 ± 38.93^{a}	306.2 ± 12.98^{a}
L.p+C.b	30	4.54 ± 0.06^{d}	6.05 ± 0.07^{b}	3426.8 ± 16.75^{b}	810.7 ± 87.83^{b}	67.5 ± 0.85^{d}
L.p+W.a+C.b	30	5.59 ± 0.01^{b}	6.36 ± 0.01^{a}	3348.4 ± 39.05^{bc}	827.2 ± 12.99^{b}	100.3 ± 0.11^{c}
		**	**	**	**	**

Data are expressed as mean ± standard deviations. Mean values with different letters within the same column at the same time interval are statistically different. n.s. not significant; *Significance at P ≤ 0.05; **Significance at P ≤ 0.01.

Table S2. Main microbial groups counted in Trial I and Trial II samples during fermentation.

Sample	Time (days)	LAB	Yeasts	Aerobic mesophilic bacteria	Enterobacteriaceae	Staphylococci
Trial I						
Control	0	5.23 ± 0.01^{d}	6.50 ± 0.04^{b}	6.29 ± 0.16^{d}	6.90 ± 0.01^{a}	1.06 ± 0.03^{e}
L. plantarum	0	5.85 ± 0.02^{a}	5.69 ± 0.01^{e}	7.36 ± 0.03^{a}	6.94 ± 0.01^{a}	1.06± 0.03^{e}
C. boidinii	0	5.31 ± 0.01^{c}	5.49 ± 0.01^{f}	5.96 ± 0.01^{e}	6.79 ± 0.01^{b}	3.98 ± 0.03^{b}
W. anomalus	0	4.78 ± 0.04^{f}	5.48 ± 0.01^{f}	7.06 ± 0.08^{b}	6.57 ± 0.03^{c}	2.63 ± 0.21^{d}
L.p+W.a	0	5.48 ± 0.02^{b}	6.19 ± 0.02^{d}	6.85 ± 0.01^{bc}	6.33 ± 0.01^{d}	4.79 ± 0.01^{a}
L.p+C.b	0	5.30 ± 0.04^{c}	6.96 ± 0.01^{a}	6.72 ± 0.03^{c}	6.36 ± 0.02^{d}	3.30 ± 0.01*c*
L.p+W.a+C.b	0	5.15 ± 0.12^{e}	6.28 ± 0.03^{c}	7.50 ± 0.04^{a}	6.57 ± 0.01^{c}	4.59± 0.16^{a}
		**	**	**	**	**
Control	8	6.74 ± 0.03^{a}	8.15 ± 0.21^{a}	8.15 ± 0.21^{a}	5.00± 0.01^{bc}	1.63± 0.40
L. plantarum	8	6.60 ± 0.01^{c}	6.88 ± 0.04^{b}	6.83 ± 0.^{01b}	4.61± 0.01^{c}	3.30 ± 0.43
C. boidinii	8	6.59 ± 0.01^{c}	7.06 ± 0.05^{b}	6.43 ± 0.07^{b}	0.00± 0.01^{d}	4.29± 0.01
W. anomalus	8	6.37 ± 0.02^{e}	6.85 ± 0.01^{b}	6.77 ± 0.01^{c}	6.02± 0.03^{ab}	3.00 ± 0.41
L.p+W.a	8	6.74 ± 0.05^{a}	7.28 ± 0.16^{b}	5.08 ± 0.01^{d}	4.85± 0.01^{c}	3.88 ± 0.03
L.p+C.b	8	6.62 ± 0.08^{b}	7.28 ± 0.31^{b}	6.57 ± 0.01^{bc}	5.34± 0.06^{abc}	4.37 ± 0.27
L.p+W.a+C.b	8	6.48 ± 0.01^{d}	6.98 ± 0.01^{b}	5.35 ± 0.01^{d}	6.17± 0.74*a*	4.12± 0.07
		**	*	**	**	n.s
Control	30	6.43± 0.01^{a}	6.56 ± 0.17^{b}	6.07 ± 0.01^{c}	2.23 ± 0.34^{a}	3.05 ± 0.06^{a}
L. plantarum	30	5.32± 0.05^{bc}	6.35 ± 0.15b^{c}	7.00 ± 0.01^{a}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}
C. boidinii	30	4.78 ± 0.06^{c}	6.04 ± 0.15b^{c}	5.72 ± 0.03^{d}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}
W. anomalus	30	6.89 ± 0.09^{a}	6.33 ± 0.01b^{c}	6.23 ± 0.02^{c}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}
L.p+W.a	30	6.05 ±0.02^{ab}	5.77 ± 0.10^{d}	6.05 ± 0.08^{c}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}
L.p+C.b	30	6.44 ± 0.04^{a}	6.41 ± 0.08^{bc}	6.07 ± 0.10^{c}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}
L.p+W.a+C.b	30	7.03 ± 0.01^{a}	7.71 ± 0.01^{a}	6.64 ± 0.01^{b}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}
		*	**	**	**	**
Trial II						
Control	0	1.30 ± 0.01^{e}	2.48 ± 0.01^{c}	5.38 ± 0.55^{bc}	0.00 ± 0.00^{d}	0.00 ± 0.00^{f}
L. plantarum	0	5.80 ± 0.17^{a}	5.41 ± 0.12^{ab}	4.15± 0.21^{e}	2.14 ± 0.09^{ab}	1.06 ± 0.03^{e}
C. boidinii	0	5.75 ± 0.03^{a}	5.51 ± 0.77^{ab}	4.23 ± 0.34^{de}	2.88 ± 0.02 ^{a}	3.97 ± 0.03^{b}
W. anomalus	0	2.00 ± 0.01^{d}	4.82 ± 0.01^{ab}	5.26 ± 0.38^{cde}	1.09 ± 0.12c	2.65 ± 0.21^{d}
L.p+ W.a	0	4.77 ± 0.01^{c}	4.64 ± 0.29^{ab}	5.65 ± 0.11^{ab}	2.77 ± 0.49 ^{a}	4.79 ± 0.01^{a}
L.p.+C.b	0	5.00 ± 0.00^{bc}	4.20 ± 0.04^{c}	7.62 ± 0.04^{a}	1.60 ± 0.09^{bc}	3.30 ± 0.01^{c}
L.p+W.a+C.b	0	5.31 ± 0.20^{b}	5.58 ± 0.19^{a}	6.71 ± 0.02^{ab}	1.02 ± 0.03^{c}	4.84 ± 0.28^{a}
		*	**	**	**	**
Control	8	5.43 ± 0.45^{b}	5.92 ± 0.04^{b}	7.38 ± 0.55^{a}	5.26 ± 0.58	4.60 ± 0.16^{b}
L. plantarum	8	7.91 ± 0.01^{a}	7.84 ± 0.09^{a}	5.82 ± 0.18^{b}	4.17 ± 0.21	0.00 ± 0.00^{d}
C. boidinii	8	7.50 ± 0.04 ^{a}	7.61 ± 0.01^{a}	7.36 ± 0.51^{a}	4.20 ± 0.24	3.00 ± 0.00^{c}
W. anomalus	8	7.91 ± 0.01 ^{a}	7.50 ± 0.65^{a}	7.84 ± 0.09^{a}	4.39 ± 0.43	3.39 ± 0.43^{c}
L.p+W.a	8	7.58 ± 0.17 ^{a}	7.47 ± 0.49^{a}	7.49 ± 0.02^{a}	4.38 ± 0.41	4.38 ± 0.41^{b}
L.p.+C.b	8	7.62 ± 0.01 ^{a}	7.00 ± 0.21^{ab}	7.00 ± 0.01^{a}	4.60 ± 0.01	3.90 ± 0.03^{bc}
L.p+W.a+C.b	8	7.61 ± 0.02 ^{a}	7.85 ± 0.19^{a}	5.30 ± 0.01^{b}	4.04 ± 0.06	5.72 ± 0.03^{a}
		**	*	**	n.s	**
Control	30	7.77 ± 0.01 ^{a}	7.84 ± 0.01^{d}	7.75 ± 0.01^{b}	0.00 ± 0.00	0.00 ± 0.00
L. plantarum	30	7.36 ± 0.08 ^{a}	7.29 ± 0.02^{f}	7.30 ± 0.01^{c}	0.00 ± 0.00	0.00 ± 0.00
C. boidinii	30	7.72 ± 0.03 ^{a}	8.00 ± 0.01^{c}	7.31 ± 0.01^{c}	0.00 ± 0.00	0.00 ± 0.00
W. anomalus	30	5.00 ±0.01^{b}	6.00 ± 0.01^{g}	7.86 ± 0.03^{a}	0.00 ± 0.00	0.00 ± 0.00
L.p+W.a	30	8.44 ± 0.66 ^{a}	8.77 ± 0.02^{b}	7.30 ± 0.01^{c}	0.00 ± 0.00	0.00 ± 0.00
L.p+C.b	30	7.80 ± 0.04 ^{a}	9.00 ± 0.01^{a}	7.86 ± 0.01 ^{a}	0.00 ± 0.00	0.00 ± 0.00
L.p+W.a+C.b	30	7.30 ± 0.01 ^{a}	7.43 ± 0.03^{e}	7.31 ± 0.03^{c}	0.00 ± 0.00	0.00 ± 0.00
		**	**	**	n.s	n.s

Data are expressed as Log CFU/mL mean ± standard deviations. Mean values with different letters within the same column at the same time interval are statistically different. n.s. not significant; *Significance at $P \leq 0.05$; **Significance at $P \leq 0.01$.

Table S3. Evaluation of tested samples inhibition (as %) on COXs enzymes.

Samples	Concentration of HT (mg/L)	Inhibition *o*COX-1 (%)	Inhibition *h*COX-2 (%)
Control	6.80	0.00	0.00
L.planturum	37.02	5 .09	0.00
C. boidinii	36.09	15.96	12.95
W. anomalus	31.72	1.32	3.27
L.p + W.a	32.06	0.19	0.86
L.p + C.b	36.13	8.20	0.00
L.p + W.a + C.b	30.71	0.00	5.78

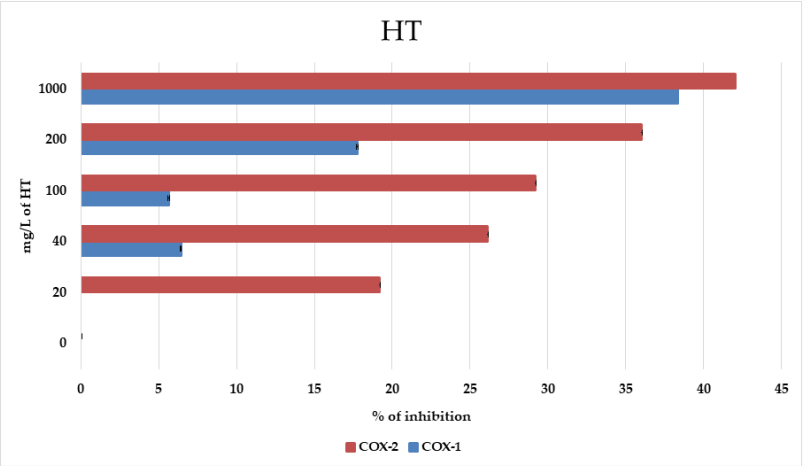


Figure S1. Evaluation of inhibition (as %) of different HT concentrations on *o*COX-1 and *h*COX-2.

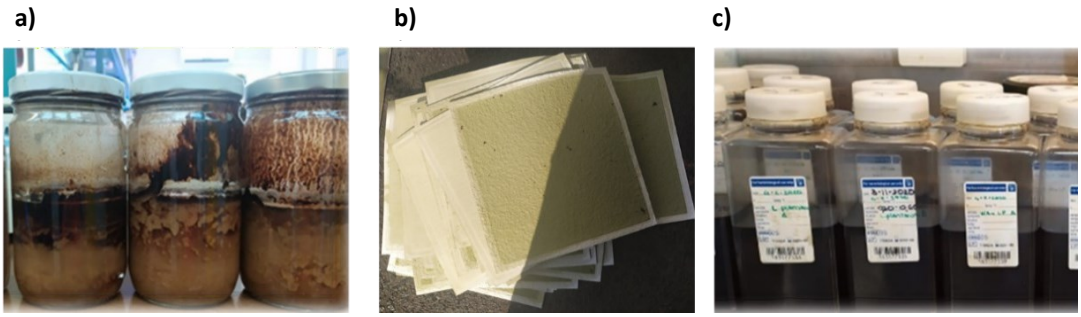


Figure S2 a) Trial I at the end of fermentation; b) Cartons filters after the spinning processand samples from trial II; c) Sample of Trial II after the fermentation process

Figure S3. OMWW samples microfiltered at 0.22 μm.

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Protective Effect of Treated Olive Mill Wastewater on Target Bacteria and Mitochondrial Voltage-Dependent Anion-Selective Channel 1

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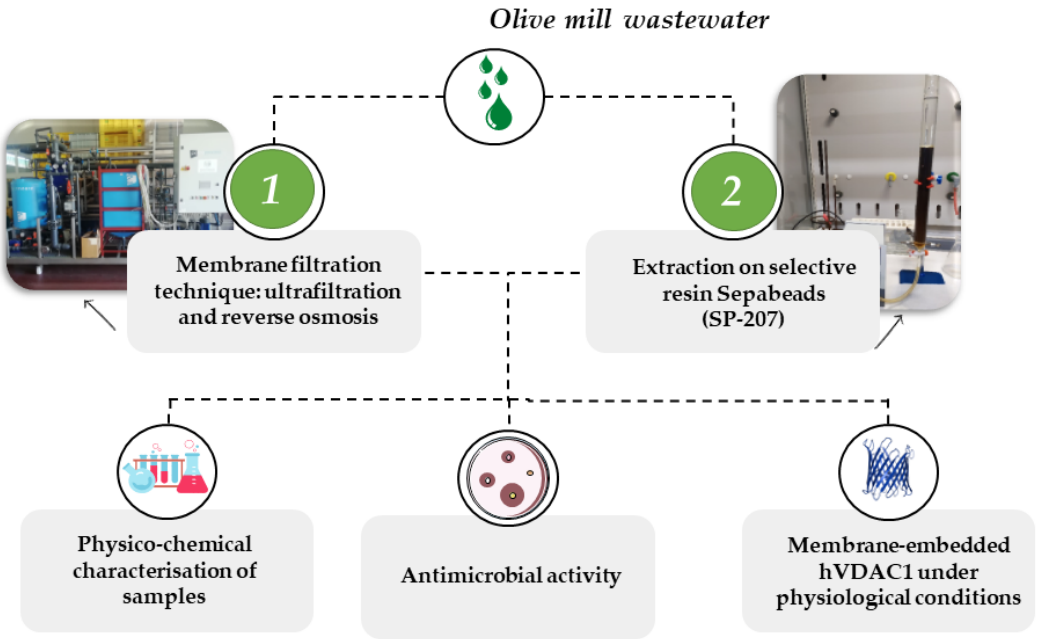
Abstract

Olive mill wastewater, a by-product of the olive oil industry, represents an important resource, rich in bioactive compounds with antioxidant activity. In this study, two strategies to concentrate the bioactive components were used: the tangential membrane filtration (ultrafiltration and reverse osmosis) and the selective resin extraction. The concentrates were evaluated for physico-chemical characteristics and antioxidant activity. Furthermore, the antimicrobial activity and the effect on the mitochondrial voltage-dependent anion selective channel 1 were evaluated. The chemical results highlighted that the highest concentration of hydroxytyrosol (as 7204 mg/L) was revealed in the sample obtained by inverse osmosis while the highest concentration of oleuropein (10005 mg/L) was detected in the sample obtained by resin extraction. The latter sample exhibited the highest antimicrobial effects against *Listeria monocytogenes*, *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Both samples exhibited a high impact on the electro-physiological parameters of VDAC1 activity. These results showed that both valorization techniques, which can be reproduced at industrial scale, provided phenolic concentrates with antioxidant and antimicrobial activity useful for different future perspectives.



1. Introduction

Several studies have highlighted the nutraceutical value associated with the Mediterranean diet, with a role in reducing the incidence of chronic degenerative diseases [1]. Among others, one of the key foods in the Mediterranean diet is extra virgin olive oil, a functional food of high nutritional value with a high content of antioxidant molecules [2,3]. However, during the olive oil extraction process only a small portion of the antioxidant content is released in the final product, as most of it is concentrated, during oil extraction, in the resulting by-products. Indeed, only 2% of the initial phenolic compounds present in olives is found in virgin olive oil, while the remaining fraction is found in olive oil by-products, such as about 53% in olive mill wastewater (OMWW) and about 45% in olive pomace (OP) [4]. The discharge of OMWW into the soil or watercourses continues to be a management and economic concern for the Mediterranean countries, due to its phytotoxicity.

In addition to the traditional decantation technique, several purification systems have been proposed as agronomic chemical and biotechnological interventions. Nevertheless, these approaches undervalue 'waste' as a potential primary resource of high-value compounds [5]. The phenolic concentrates obtained from olive oil by-products, such as OP, leaves or OMWW, can be used in different application fields, such as food, pharmaceutical and cosmetic industries or in animal feed [3]. In detail, with regard to the food industry, phenols have been proposed as a functional preservative to increase the shelf-life of foodstuffs and improve the health value of final products [6–8]. In previous studies, the use of such by-products to isolate standards of bioactive molecules, such as hydroxytyrosol (HT) and tyrosol (TYR), and to reduce toxic substances, such as acrylamide formed during Californian-style processing, has been proposed [9,10]. In particular, OMWW obtained from the three-phase system represents a relevant source of biologically active substances, including HT and TYR. Different strategies have been applied to recover phenolic compounds from OMWW, such as membrane separation [7,11], solvent extraction [12], resins treatment [2,13], centrifugation, chromatographic procedures and enzymatic reactions [14,15]. Regarding the membrane filtration technique, it is one of the most valuable methods, especially at industrial scale, and it is characterised by low energy consumption, good operating conditions and a high efficiency in component separation [16,17]. The extraction in the solid phase represents

an interesting technique as it is simple, re-producible and low cost, even if still applied mostly at lab-scale. Both the non-thermal techniques allow the recovery of bioactive compounds preserving their sensory characteristics and nutritional value [18].

Among phenolic compounds, HT is particularly relevant for its antioxidant, anti-inflammatory and antimicrobial activity [19]. In detail, different studies have widely demonstrated that HT acts as a free radical scavenger and metal chelator and presents a key role in protecting against oxidative damage, inhibiting the NADPH oxidase, the inducible form of nitric oxide synthase and the proinflammatory enzymes (5-lipoxygenase and cyclooxygenase), decreasing, in turns, the production of nitric oxide, leuko-trienes and prostaglandins [20]. Therefore, OMWW represents a relevant source of phenols to obtain new nutraceutical formulations with antioxidant and antimicrobial activity. The Voltage-Dependent Anion-selective Channel (VDAC) is the main pore-forming protein of the outer mitochondrial membrane (OMM) that, in mammals, exists in three isoforms, numbered as VDAC1, VDAC2 and VDAC3, in the order of their discovery. VDACs play a crucial role in bioenergetics and due to the interaction with Bcl-2-family proteins [21–23] and cytosolic proteins, such as hexokinases, glycolytic enzymes, neuronal and cytoskeletal proteins [24–28], they represent the major regulators of mitochondria-mediated cell death and mitochondrial metabolism. Moreover, VDACs differently regulate the homeostasis of Reactive Oxygen Species (ROS) [29–32]. VDAC1 has been suggested to be essential into ROS-induced apoptosis, as it is responsible for superoxide release from mitochondria. On the other hand, VDAC3 has recently been identified as a mitochondrial ROS sensor that protects the organelle from oxidative stress. Mitochondria are simultaneously the primary source and the main target of ROS; in particular, hydrogen peroxide (H_2O_2), a key molecule in the sensing, modulation and signaling of the redox metabolism, depolarises mitochondria and modulates ion channels. Within the well-controlled environment of an artificial membrane, H2O2 increases the single-channel conductance of VDAC1 [33] although a strong reduction in channel gating within the range of ± 50 mV applied has been registered.

The objective of the present study was to characterise the beneficial effects, as antimicrobial and antioxidant activities, of OMWW samples obtained with different techniques (by tangential membrane filtration and extraction on selective resin) on the main relevant food-related pathogens and on VDAC1.

2. Materials and Methods

2.1. Production of Phenolic Fractions from Olive Mill Wastewater

The OMWW was obtained through a three-phase extraction system, from a mix of Nocellara Etnea and Nocellara Messinese cultivars at the Consoli olive oil company (Adrano, Catania, Sicily). The three OMWW fractions, tested in the present study, were obtained by two different methods: tangential membrane filtration and extraction on selective resin (Figure 1).

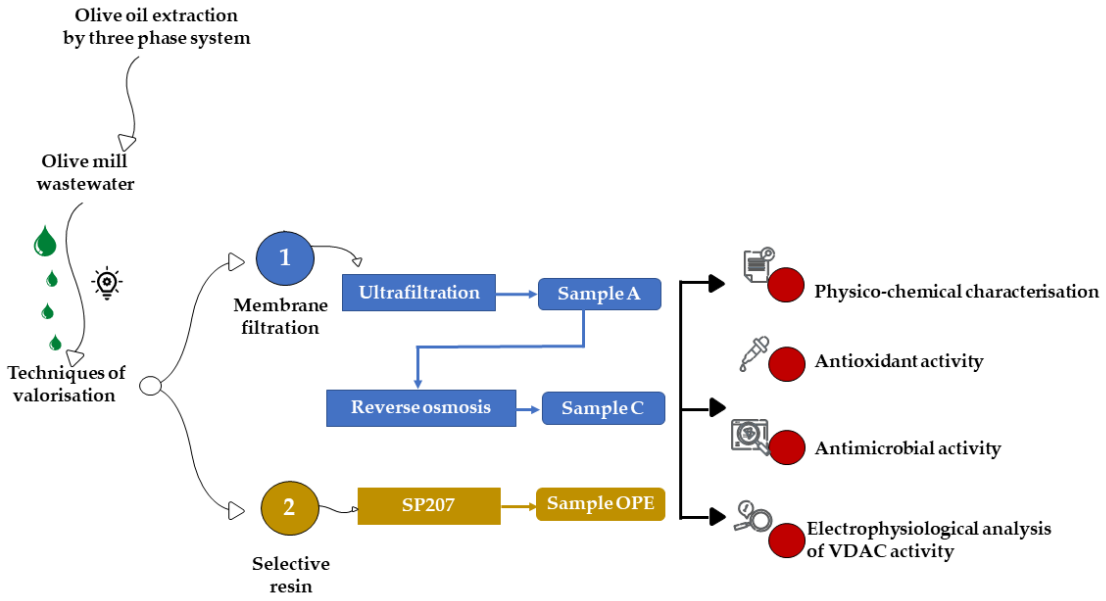


Figure 1. Experimental design.

2.1.1. Tangential Membrane Filtration System

The samples A and C were obtained by an industrial tangential membrane filtration system. In detail, sample A was obtained by ultrafiltration and sample C was obtained by reverse osmosis. The system used was the 'Permeaprocess' (Permeare s.r.l., Milan, Italy) that consists of a tangential filtration based on membranes suitable for both the purification and concentration of compounds. This technique allows the elimination of the water present in the samples (permeate), and the concentration of all the components present.

2.1.2. Extraction on Selective Resin

The OPE sample was obtained, at laboratory scale, by extraction on the selective resin Sepabeads (SP-207) (Mitsubishi Chem. Co., Tokyo, Japan). The resin was treated and loaded into a glass preparative column, according to the method described by Romeo et al. [2]. In detail, the resin was first pre-treated with 95% ethanol (food grade, Carlo Erba, Milan, Italy), then washed with water (HPLC grade, Carlo Erba, Italy) and finally dried at 70 °C until a constant weight was obtained. Then, 20 mL of the dry resin was loaded into a glass preparative column (length, 30 cm; i.d, 0.5 cm) connected to a peristaltic pump (Pump Drive, Heidolph, Schawabach, Germany). The resin was washed down with water, then the OMWW sample was charged until saturation. Be-fore the desorption step, each saturated resin was washed with 80 mL of water to re-move water-soluble compounds. The adsorbed phenol was recovered with 40 mL of a 95% ethanol/water solution (60:40, v/v). Finally, using a rotary evaporator (Rotavapor RE111, Büchi, Cornaredo, Italy), the fraction was concentrated after the vacuum distillation of ethanol at 40 °C.

2.2. Chemical Characterization of Samples

The samples, A, C and OPE were subjected to chemical characterization. In particular, pH, total soluble solids (TSS) and total phenols were determined. The pH value was measured using a Mettler DL25 pH meter (Mettler Toledo International Inc., Columbus, OH, USA), while the TSS value, expressed as °Brix, was determined using a refractometer (Atago, RX-5000 Thermo Fischer Scientific, Milan, Italy). The Folin–Ciocalteu's (FC) colorimetric method was applied to determine the phenolic content. Briefly, samples were mixed with 5 mL of FC reagent (Labochimica, Padova, Italy) and diluted in water at a 1:10 v/v ratio, with 4 mL of a sodium carbonate solution at 7.5%. After 2 h at room temperature, protected from light, the absorbance of the blue solution was spectrophotometrically measured at 765 nm (Cary 100 Scan UV-Visible, Agilent, Santa Clara, CA, USA). The total phenolic content was expressed as mg gallic acid equivalents (GAE)/L of sample. All analyses were performed in triplicate for each sample.

2.3. HPLC Analysis

2.3.1. Phenols

For HPLC analysis, the samples A, C and OPE, were filtered (0.45 µm Millipore filters, Merk Darmstadt, Germany) and injected directly into the HPLC system. The used apparatus was a Waters Alliance 2695 HPLC liquid chromatography with Waters 996 photodiode array (PDA) detector, set at 280 nm and with Waters Empower software (Waters Corporation, Milford, MA, USA). The column, a Luna C18 (250 mm × 4.6 mm i.d., 5 µm, 100 Å; Phenomenex, Torrence, CA, USA) was kept at 40 °C. Chromatographic separation was achieved by gradient elution, using an initial composition of 95% solution A (water acidified with 2% acetic acid) and 5% solution B (methanol) (Merck KGaA, Germany), at a flow rate of 1 mL/min. A solution of 5 mM of gallic acid (Fluka, Losanna, Switzerland) was used as the internal standard (I.S.). Identification of phenolic compounds was obtained by comparing the retention time with those of pure hydroxytyrosol (HT), tyrosol (TYR) and oleuropein (OLE) standards (Extrasynthese, Genay, France). All analyses were performed in triplicate for each sample.

2.3.2. Organic Acids

Each sample was properly diluted and filtered through a 0.45 µm PTFE syringe filter (Merk, Germany). A total of 5 mM sulphuric acid was eluted in the isocratic mode on a Rezex ROA Organic Acid H+ column (Phenomenex, Torrence, CA, USA). The HPLC instrument (described in the previous section) coupled with a DAD detector was set at 210 nm (with spectrum acquisition in a range of 200 to 400 nm). The run time was set at 50 min at 0.6 mL/min. Lactic, citric, acetic, propionic, isobutyric and butyric acid (Sigma-Aldrich, Milan, Italy) pure standards were injected at different concentrations. All analyses were carried out in triplicate for each sample.

2.4. Antioxidant Activity

The samples (A, C and OPE) were diluted and added to a methanolic solution added with 2,2-Diphenyl-1-picrylhydrazyl radical 10−4 M (DPPH, Merk, Germany). After 3 min, the absorbance was measured at 517 nm. The results were expressed as a

percentage decrease, compared to the control. Inhibition percentage for each sample was calculated as follows:

$$\%\ inhibition = \frac{A_0 - A_x}{A_0}\ 100\%$$

where A_0 is the absorbance of a DPPH blank, and A_x is the sample absorbance. Antioxidant activity was expressed in relation to the sample volume and the concentration at which 50% of radical scavenging occurred (IC50). All analyses were performed in duplicate for each sample.

2.5 Antimicrobial Activity

The samples A, C and OPE were tested against the following food-related target strains from ATCC (American Type Culture Collection): *Listeria monocytogenes* ATCC 19114, *Escherichia coli* ATCC 25922, *Candida albicans* ATCC 10231, *Staphylococcus aureus* ATCC 25213, *Pseudomonas aeuroginosa* ATCC 9027, according to Foti and co-workers [7]. Briefly, the tests were performed on Potato Dextrose Agar (PDA, Likson, Palermo, Italy) for *C. albicans* and Muller Hinton Agar Base (MHA, Liofichem, Roseto degli Abruzzi, Italy) for bacteria. The strains, of each individual culture, were standardised using a Mc Farland 0.5 solution for both bacteria and *C. albicans*. Each plate was spatulated with 1 mL of cell suspension and allowed to dry and sterile cellulose discs (Ø 6 mm), imbibed with each tested sample, were placed at different dilution rates. The samples were tested as they were and at different dilution ratios (from 1:2 to 1:8). Distilled water was used as a negative control. Each plate was incubated at specific temperatures for 48 h and then the results were expressed as diameter (mm) of the inhibition halo. The test was performed in duplicate for each strain.

2.6. Electrophysiological Analysis of VDAC Activity

The coding sequence of human VDAC1 (hVDAC1) cloned in the pET21 vector was heterologously expressed in E. coli to produce a recombinant His-tagged protein that was subsequently purified using Ni-NTA affinity chromatography (Qiagen, Hilden, DE) and refolded according to previous studies [27,34,35]. Electrophysiological ex-

periments were carried out at RT in the Planar Lipid Bilayer (PLB) Workstation from Warner Instruments as previously described [36–38]. In brief, a solution of 1% of 1,2-diphytanoyl-sn-glycero-3-phosphocholine (DiPhPC Avanti Polar Lipids, Alabaster, AL) dissolved in n-decane was used for bilayer membrane formation on a 200 µm-sized hole of a Delrin chamber containing two compartments (i.e., cis and trans) filled with 3 mL of 1 M KCl, 10 mM HEPES pH 7.4. Bilayer membranes, with an approximately 110–150 pF capacity, were considered satisfactory for subsequent analysis. Reconstitution of hVDAC1 was detected after the addition of ~40 ng of the purified protein to the cis side of the chamber. Channel recordings were performed with the BC-535 Bilayer Clamp amplifier (Warner Instruments) and data were digitalised using the Axon Digidata 1550 Acquisition System (Warner Instruments), with a sampling rate of 10 kHz after low-pass-filter at 300 Hz. Pore conductance (G) was calculated as the ratio of the ionic current through the channel (I) to applied voltage (V) of +10 mV. Voltage-gating parameters of hVDAC1 were investigated by measuring the average conductance of PLB containing a single channel in symmetrical 1 M KCl, 10 mM Hepes, pH 7.4 using a 10 mHz triangular voltage ramp (±50 mV). The conductance (G) at a given voltage (from −50 to +50 mV) was normalised to the conductance at the lowest applied potential (−10 mV, G0). Data are representative of at least three independent experiments and are graphited using prism 8.0 software (GraphPad Software) as the mean ± SEM. Finally, to compare the antioxidant activity the percentage of recovery of VDAC1 voltage-dependence upon the A, C and OPE samples was calculated by acquiring the Area Under Curve (AUC) from G/Gmax plots. Briefly, the AUC values of VDAC1 pre-treated with hydrogen peroxide and subsequently with A, C or OPE were subtracted from the AUC value of VDAC1 pre-treated with H_2O_2 and compared to the untreated protein.

2.7. VDAC Treatments

The effect of both N-Acetyl-L-cysteine (NAC) (Merck KGaA, Darmstadt, Germany) and tested samples (A, C and OPE) on the membrane alone, was first assessed by adding 8 µM and a final concentration of 1/100 (v/v), respectively, to both sides of a planar PLB made of 1% Di-Phytanoyl-Phosphatidyl-Choline (DiPhPC). The impact of H2O2 treatment on single-channel conductance and voltage-dependence was analysed by

incubating hVDAC1 with 8 μM of fresh chemical for 30 min at 4 °C. Each tested sample was poured at a final concentration of 1/100 (v/v) to both sides of a planar lipid bilayer containing peroxide-treated hVDAC1. As a control, NAC was added at a final concentration of 8 μM to both sides of a PLB chamber containing membrane-embedded hVDAC1 pre-treated with H_2O_2. Current vs. time traces were recorded in response to constant voltage (+10 mV) and triangular ramp application as described above.

2.8. Statistical Analyses

SPSS software (version 21.0, IBM Statistics, USA) was used for data processing. One-way analysis of variance (ANOVA) was performed to analyse data and Tukey's HSD post-hoc test for means separation at a significance level of $p \leq 0.05$.

3. Results

3.1. Physico-Chemical Characterisation of Samples

The samples (A, C and OPE) were characterised for their physico-chemical profile: pH, TSS and total phenols (Table 1). The lowest pH values were observed in samples A and C, obtained by the tangential membrane filtration technique, with values of 3.91 and 3.96, respectively. This result is related to the used technology, which concentrates what is present in the matrix, including organic acids. For the same samples (A and C), the TSS value showed the same trend of pH, while the sample OPE exhibited a Brix value lower than that found in sample C. The TSS trend was as expected considering the applied concentration system. The A and C samples, obtained through ultrafiltration and reverse osmosis, respectively, highlighted a total phenol concentration of 3244.11 and 6207.41 mg/L, respectively, whereas the OPE sample showed a total phenol concentration of 16460.42 mg/L.

Table 1. Chemical characterisation of the three tested OMWW fractions.

Sample	pH	TSS (°Brix)	Total Phenols (mg/L)
A	3.91 ± 0.03 [c]	6.29 ± 0,05 [c]	3244.11 ± 0.21 [c]
C	3.96 ± 0.06 [b]	10.63 ±0.04 [a]	6207.41 ± 0.11 [b]
OPE	4.80 ± 0.06 [a]	7.94 ± 0.03 [b]	16460.42 ± 11.3 [a]
	**	**	**

Data are expressed as means ± SD. Mean values with different letters within the same column are statistically different. ** Significance at $p \leq 0.01$. Legenda: Sample A = obtained by ultrafiltration; sample C = obtained by reverse osmosis; sample OPE = obtained by resin extraction.

3.2. Phenols and Organic Acid Detection

Regarding the phenol content, the highest concentration of HT (7203.67 mg/L) was observed in sample C (Figure S1), whereas the highest concentration of OLE was detected (10004.70 mg/L) in OPE sample (Table 2).

Table 2. Phenol detected in the three tested OMWW fractions.

Sample	HT (mg/L)	TYR (mg/L)	OLE (mg/L)
A	3414.96 ± 0.21 [c]	494.37 ± 0.12 [c]	0.00 ± 0.00 [b]
C	7203.67 ± 0.31 [a]	1046.62 ± 0.24 [b]	0.00 ± 0.00 [b]
OPE	3240.50 ± 0.25 [b]	2015.54 ± 0.31 [a]	10004.70 ±0.02 [a]
	**	**	**

Data are expressed as means ± SD. Mean values with different letters within the same column are statistically different. ** Significance at $p \leq 0.01$.

Focusing on organic acids, the highest concentration was found in the C sample (Figure S2). Citric and butyric acids were not detected in any samples. Moreover, in the OPE sample, no organic acid should be present, as resin extraction is selective for phenolic compounds. The only acid found was the propionic acid, with a concentration of 1356.50 mg/L (Table 3).

Table 3. Results of organic acids of the three tested OMWW fractions analysed by HPLC.

Samples	Lactic Acid (mg/L)	Acetic Acid (mg/L)	Propionic Acid (mg/L)	Isobutyric Acid (mg/L)
A	3554.3 ± 58.78 [b]	3554.3 ± 58.78 [b]	0.00 ± 0.00 [c]	12621.7 ± 374.88 [a]
C	7953.7± 7.93 [a]	12137.2 ± 7.38 [a]	2984.4 ± 89.77 [a]	0.00 ± 0.00 [b]
OPE	0.00 ± 0.00 [c]	0.00 ± 0.00 [c]	1356.50 ± 87.0 [b]	0.00± 0.00 [b]
	**	**	*	**

Data are expressed as means ± SD. Mean values with different letters within the same column are statistically different. * Significance at $p \leq 0.05$; ** Significance at $p \leq 0.01$.

3.3 Antimicrobial Activity

The antimicrobial activity of the A, C and OPE samples against target strains was assessed by an evaluation of the inhibition zones (Table 4). Overall, no inhibitory activity was observed against *C. albicans* for any tested sample. Regarding the sample A and C, obtained through ultrafiltration and reverse osmosis, the results exhibited a different antimicrobial activity against pathogens. In detail, while sample A showed an inhibition zone of 7 mm only against *E. coli,* sample C exhibited antimicrobial activity against *E. coli*, *P. aeruginosa* and *L. monocytogenes.* Different behaviors were observed for the OPE sample which exhibited antimicrobial activity against all pathogenic tested strains.

Table 4. Antimicrobial activity (expressed as halo diameter of inhibition in mm).

Target Strains	Sample	Sample Dilution			
		Raw	**1:2**	**1:4**	**1:8**
Escherichia coli	A	7	-	-	-
ATCC 25922	C	8	7	-	-
	OPE	11	9	7	-
Pseudomonas aeruginosa	A	-	-	-	-
ATCC 9027	C	9	7	-	-
	OPE	10	7	7	-
Staphylococcus aureus	A	-	-	-	-
ATCC 25213	C	-	-	-	-
	OPE	8	7	7	-
Listeria monocytogenes	A	-	-	-	-
ATCC 19114	C	8	7	7	-
	OPE	10	8	8	-

The concentration of OMWW fractions tested in the presence of halos corresponded to a MIC of 241 mg/mL for the OPE sample against all the tested microorganisms, 507 mg/mL for the C sample against *P. aeruginosa* and *E. coli*, and to 253 mg/mL against *L. monocytogenes*. Sample A was actively undiluted only against *E. coli*.

3.4. Tested Samples Do Not Interfere with Membrane-Reconstituted hVDAC1 under Physiological Conditions

Before evaluating the impact of the tested samples on the electrophysiological properties of hVDAC1, they were added at a final concentration of 1: 100 (v/v) to both sides of a

DiPhPC membrane, generated as previously described. This procedure en-sured the exclusion of any undesired disturbance to the phospholipid bilayer, which maintained its stability throughout the analysis time. As shown in Figure S3, the cur-rent base line remained near 0 pA upon application of a triangular voltage wave with ±50 mV amplitude. The addition of the A, C or OPE samples to hVDAC1 inserted into the membrane did not affect the single channel conductance (Figure S4B–D) while it slightly modified the voltage response profile which became noisier (Figure 2C–H), compared to the control (Figure 2A, B). Closure events starting from ±15–20 mV were less clear-cut following administration of the C sample (Figure 2C, D). The A and OPE samples, instead, in-creased the values of positive voltages required to close the channel (Figures 2E–H). In each case, however, the pore retained its ability to respond to changes in membrane potential.

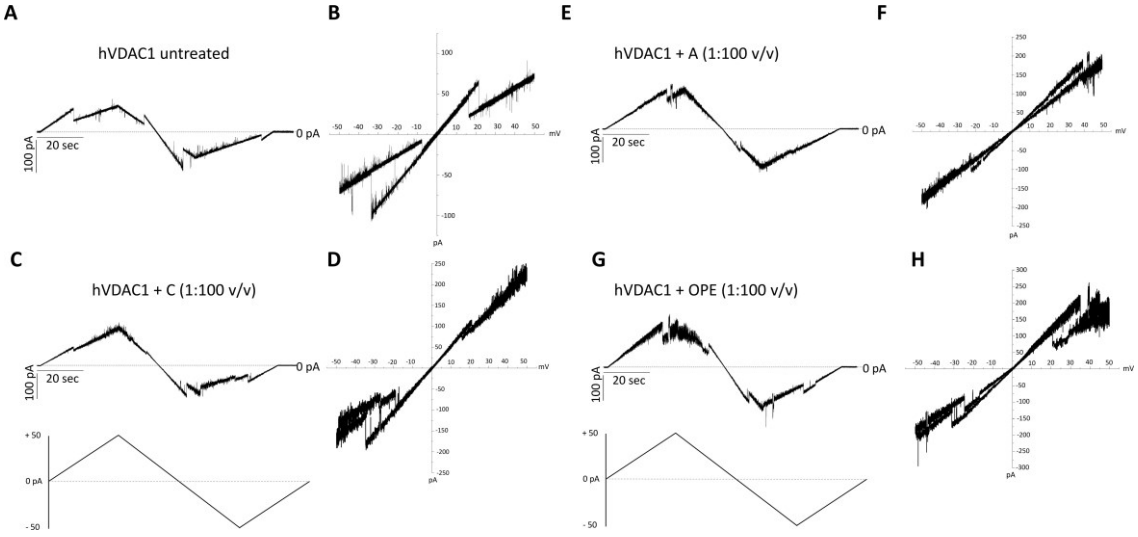


Figure 2. Analysis of hVDAC1 voltage dependence following the addition of the tested compounds by triangular voltage ramps. Sample A (panels (E, F)), sample C (panels (C, D)) and sample OPE (panels (G) and (H)) were added to both sides of a DiPhPC bilayer containing hVDAC1 at a final concentration of 1:100 (v/v) and compare with the control (panels (A) and (B)). Current measurements were performed in symmetrical 1 M KCl upon application of a triangular voltage ramp of ±50 mV. The corresponding I–V plots were obtained by plotting the current as a function of clamp voltage.

3.5. The Harmful Effect of Hydrogen Peroxide on hVDAC1 Voltage Dependence Is Reversed by A, C and OPE Samples

In order to examine the antioxidant effect of the tested samples, they were added to H_2O_2 pre-treated hVDAC1 reconstituted into planar phospholipid membranes at a final concentration of 1:100 (v/v). The treatment with hydrogen peroxide had no consequences on the single channel conductance over a long period, although it suppressed the voltage dependence as the current continuously switched between high and low conducting states, without showing any distinct gating event (Figure 3A–F). A, C and OPE samples restored the poregating of hVDAC1 affected by H_2O_2: voltages above ± 20 mV sharply closed the channel (Figure 3G–M) reestablishing the characteristic 'bell shaped' curve of untreated VDAC with lower conductance values at higher membrane potentials (Figure 4A–C).

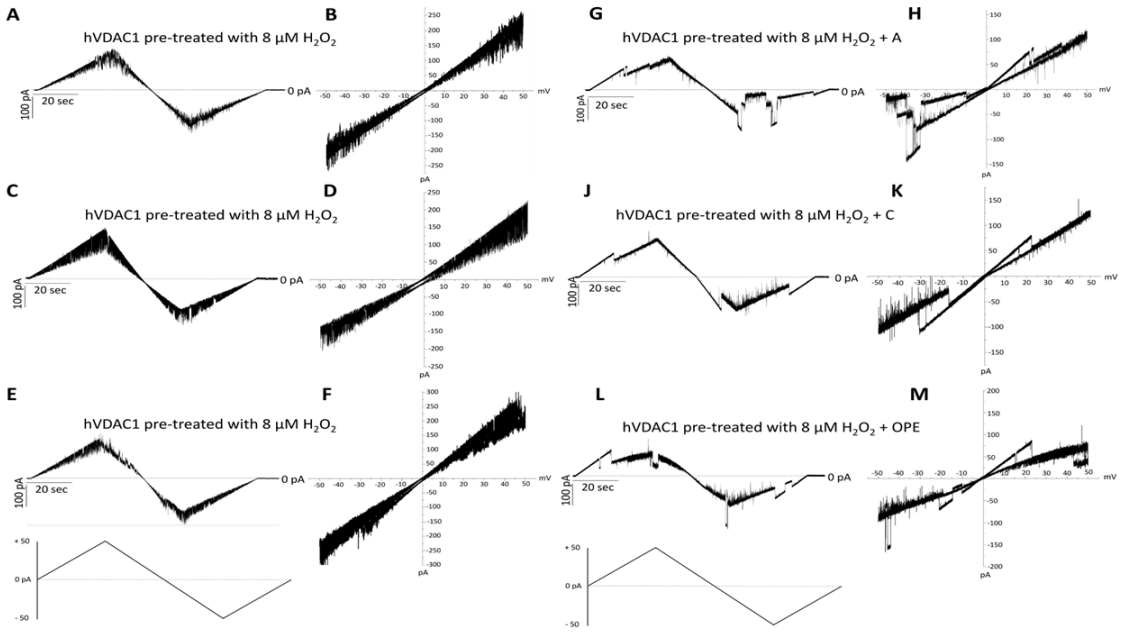


Figure 3. Analysis of the effect of tested samples on hVDAC1 voltage dependence following hydrogen peroxide treatment by triangular voltage ramps. Sample A (panels G and H), sample C (panels J and K) and sample OPE (panels L and M) were added to both sides of a DiphPC bilayer containing hVDAC1 pre-treated with 8 µM H_2O_2 (panels A–F) at a final concentration of 1:100 (v/v). Current measurements were performed in symmetrical 1 M KCl upon application of a tri-angular voltage ramp of ± 50 mV. The corresponding I–V plots were obtained by plotting the current as a function of clamp voltage.

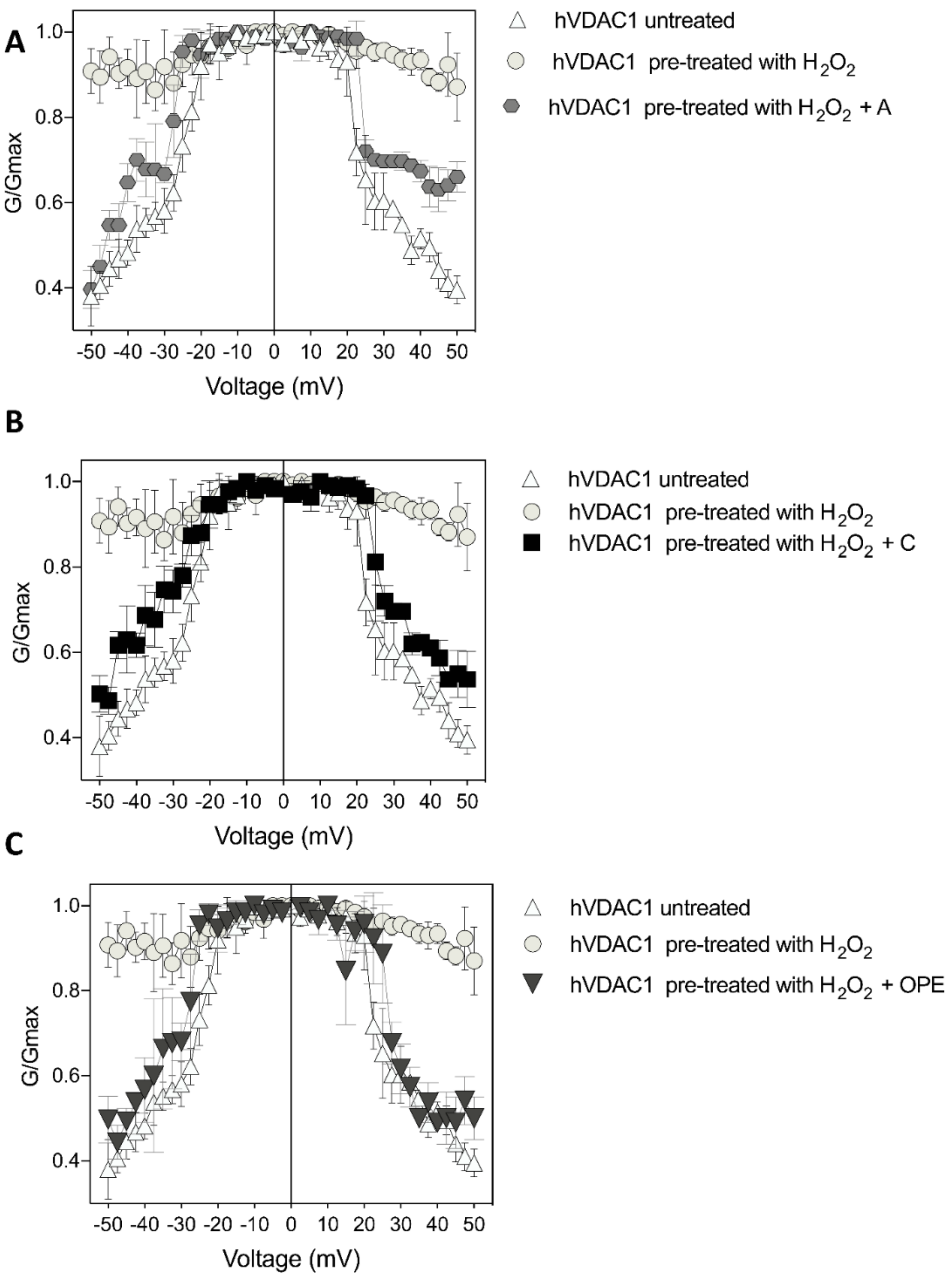


Figure 4. Conductance G/Gmax of hVDAC1 untreated (A, B and C), pre-treated with 8 μM H2O2 (A, B and C) and challenged with sample A (panel A), sample C (panel B) and sample OPE (panel C) as function of the applied voltage. Data are expressed as mean of at least 4 independent experiments.

In particular, the recovery of voltage-dependence induced by C and OPE samples was more pronounced compared to A: the normalised average conductance, G/Gmax, plotted vs. applied voltage demonstrated a complete overlap of the curves from pre-treated hVDAC1 supplemented with C and OPE and the curve from untreated protein (Figure 4B, C). N-Acetyl-Cysteine (NAC), widely known as a powerful antioxidant,

was used as a control. After verifying that it did not influence the single channel conductance nor the voltage response of membrane-reconstituted hVDAC1 (Figure 5A), we here report the effect of NAC on hydrogen peroxide-induced loss of voltage dependence which resembled that of tested samples (Figure 5E). Overall, these results suggest that the tested samples possessed antioxidant properties capable of reversing the deleterious effects of H_2O_2 on the main functional feature of hVDAC1.

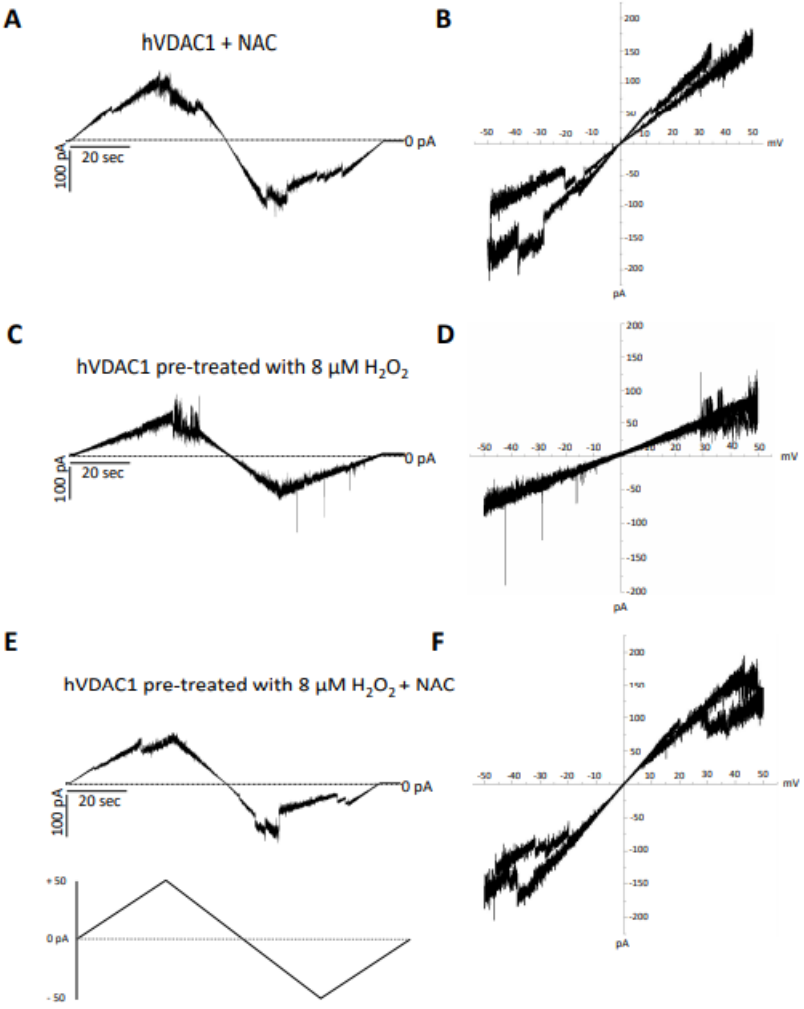


Figure 5. Analysis of the effect of N-acetyl cysteine on hVDAC1 voltage dependence following hydrogen peroxide treatment by triangular voltage ramps. NAC was added at a final concentration of 8 µM to membrane-embedded hVDAC1pA pretreated (panels C–F) or not (panels A and B) with 8 µM H2O2 (panels C and D). Current measurements were performed on symmetrical hVDAC1 pre-treated with 8 µM H_2O2 1 M KCl upon application of a triangular voltage ramp of ± 50 mV. The corresponding I–V plots were obtained by plotting the current as a function of clamp voltage.

Finally, the antioxidant activity, voltage-dependence recovery and anti-microbial activity of the A, C and OPE extracts were compared. As reported in Table 5 the highest antioxidant capacity of samples C and OPE, detected by DPPH assay (i.e., 41.71 and 50.02 IC50 values, respectively), corresponded to an enhanced recovery of the VDAC1 response to the voltage applied (76.27% for C and 78.11% for OPE) and to a broader spectrum of activity against microbial strains.

Table 5. Comparison of the antioxidant activity, voltage-dependence recovery and anti-microbial activity of each tested sample.

Sample	Antioxidant activity (IC_{50})	Recovery of VDAC1 Vdep	Anti-Microbial Activity
A	84.00 ± 0.12	62.20 ± 0.45%	*Escherichia coli* ATCC 25922
C	41.71 ± 0.03	76.27 ± 0.47%	*Escherichia coli* ATCC 25922 *Pseudomonas aeruginosa* ATCC 9027 *Listeria monocytogenes* ATCC 19114
OPE	50.02 ± 0.04	78.11 ± 0.72%	*Escherichia coli* ATCC 25922 *Pseudomonas aeruginosa* ATCC 9027 *Listeria monocytogenes* ATCC 19114 *Staphylococcus aureus* ATCC 25213

4. Discussion

In this study, two different techniques to recover and concentrate the bioactive compounds from OMWW were applied: the membrane filtration technique (ultrafiltration and reverse osmosis) at industrial scale, according to Foti et al. [7] and the ex-traction in solid phase, in lab-scale, using adsorbent resins, according to Romeo and co-workers [2]. Based on the results obtained in the present study, the lower pH values were shown by the samples A and C, a result which was highly correlated to the different chemical composition of samples, in particular to the different profile of organic acid content. In fact, while the selective resin extraction technique retained with high affinity the OMWW phenols, the tangential membrane filtration system concentrated phenols together with sugars and organic acids. In particular, a high increase in lactic and acetic acid content was observed in the samples obtained by reverse osmosis. Zooming in on phenolic content, the OMWW sample used to obtain the three tested fractions exhibited a total phenol content of 2163 mg/L (data not shown). Compared to the initial OMWW

phenol content, the highest phenol recovery was highlighted by OPE, followed by the C and A samples, with a 4.6, 2.8 and 1.5-fold concentration, respectively. Russo et al. [39], by reverse osmosis, obtained a phenol extract with 8292 mg/L total phenols. Bellumori et al. [40], using reverse osmosis, obtained an extract showing about 12000 mg/L at the same applied temperature. Regarding the HPLC analysis, the highest concentrations were detected in samples C and OPE. In detail, while sample C exhibited the highest content of HT and the absence of OLE, the OPE sample revealed a higher OLE content, reaching concentrations of 10004.7 mg/L, confirming that the concentration of each single phenol is influenced by the applied extraction method. Moreover, the concentration with reverse osmosis, at farm level, occurs over a time longer than that required at lab scale with resins. The longer duration of the extraction process could probably prolong enzymatic (esterase) activity resulting in an increase in HT in sample C. In the present study, the content of HT and TYR detected in sample C (8940 and 1238 mg/L, respectively) was quite similar to that re-ported by Bellumori et al. [40]. The phenolic compounds have extensive antimicrobial activity, such as antibacterial, anti-viral and anti-fungal effects [41], exerting a beneficial effect on human microbiota [42] and prolonging the shelf-life of food [8]. Several authors have shown that OMWW extracts exhibited an inhibitory activity against both Gram-negative and Gram-positive bacteria, but also on fungi and yeasts [39,43]. In detail, in the present study, a different antimicrobial effect for each sample was revealed, confirming that the antimicrobial mechanism is strongly related to the phytocomplex present in the different matrices. In fact, while samples A and C were characterised by all molecules present in the starting matrix (such as phenols and organic acids), the sample OPE, obtained by adsorption technology, was mostly composed of phenolic fraction. In the present study, the samples C and OPE exhibited greater inhibitory activity than sample A, which displayed modest inhibitory activity exclusively against *E. coli*. As matter of fact, both samples C and OPE showed a wider spectrum of action, with sample C able to inhibit *E. coli*, *P. aeuroginosa* and *L. monocytogenes*, and the sample OPE able to inhibit *S. aureus*. These results, related to the different composition of the two samples, appear strongly related to the different OLE content. Indeed, several studies have shown the antimicrobial effect of OLE as higher against Gram-positive than Gram-negative bacteria because of the difference in cell structures [44,45]. Topuz and co-workers [46] showed that *S. aureus* was sensitive to different OLE extracts at the different tested concentrations (3.125 mg/mL and 0.781 mg/mL).

Furthermore, in the present study, all samples showed no antagonistic effect against *C. albicans*. This result could be due to the hydrophilic nature of the tested samples according to previous reports that have shown that the alteration of the hydrophilic/lipophilic balance could affect the cellular uptake mechanisms, enhancing antioxidant or antimicrobial activities [47]. Despite the high OLE content in the sample OPE, the highest antioxidant activity was observed in sample C. This result is probably related to the higher content of HT. Several reports have praised the efficiency of HT and OLE in reducing the production of free radicals [48–51]. In particular, OLE is also known to cause mitochondria-mediated apoptosis in cancer cells [52–54]. Although many reports have emphasised the ability of HT and OLE to reduce oxidative stress and to improve mitochondrial biogenesis and function, no data regarding the effects of such antioxidants on mitochondrial proteins are still available. Therefore, samples A, C and OPE were tested to assess their antioxidant effect on hydrogen peroxide treated human VDAC1, reconstituted into a planar phospholipid membrane. As previously described, VDAC is one of the most relevant proteins of the outer mitochondrial membrane that has been demonstrated to play a key role in apoptosis and ROS homeostasis. H_2O_2, in turn, is mainly produced within mitochondria [54], which also represent the main target of its harmful effects [55]. According to Malik and Ghosh [33], the results of the present study revealed a complete loss of VDAC1 voltage-dependence upon H_2O_2-induced overoxidation. The tested samples proved to be able to antagonise and reduce the negative outcomes of hydrogen peroxide treatment on the electrophysiological features of VDAC1. In this regard, samples C and OPE, most likely due to the higher phenol content, yielded the best results in terms of recovery of the channel's ability to respond to changes in voltage applied. They completely re-shaped the normalised average conductance plotted vs. applied voltage of H2O2-treated VDAC1, making it indistinguishable from the curve of the untreated protein. Those results perfectly resembled data obtained with N-acetyl-cysteine whose ability to minimise oxidative stress is largely known. Consequently, the recovery of the oxidation of VDAC, the gateway to the mitochondrion for all substrates and metabolites, predicts a positive effect on the organelle's respiratory and bioenergetic function.

5. Conclusions

The effect of two methods, such as tangential membrane filtration and extraction with absorbent resin, in obtaining a matrix rich in bioactive compounds was evaluated. The results showed that both techniques (reverse osmosis and resin extraction) had a positive effect on the concentration of bioactive components, and although a different antimicrobial activity, related to the different phenolic profiles, was observed, no difference in antioxidant activity was detected. These results are relevant to drive the choice of the most suitable technique to treat OMWW, taking into account the criteria of cost-effectiveness and productivity. Both extraction techniques, indeed, involve several production costs related to the purchase of the necessary plants and, in the case of resin extraction, also to the use of organic solvents (food-grade ethanol). Moreover, the results of the present study suggest that further effort must be made to better explore the antioxidant mechanism of bioactive compounds present in olive oil by-products in order to propose natural food preservatives or food supplements with nutraceutical properties.

Supplementary material

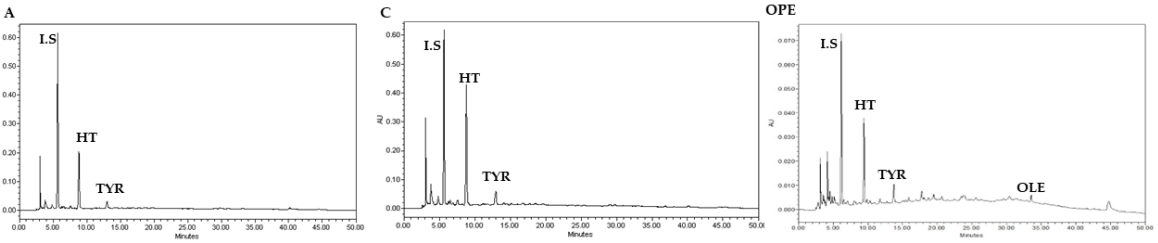


Figure S1. HPLC Chromatogram of phenols of A, C and OPE sample detected at 280 nm.

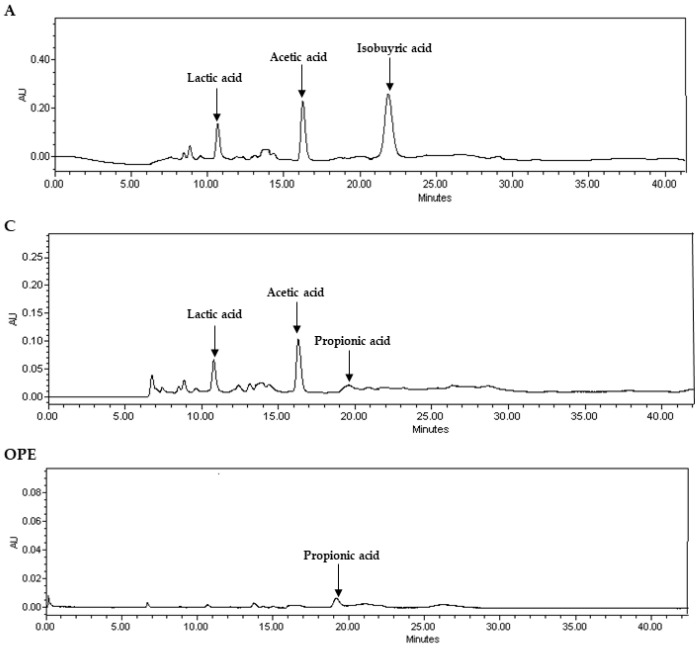


Figure S2. HPLC Chromatogram of organic acids of A, C and OPE sample detected at 210 nm.

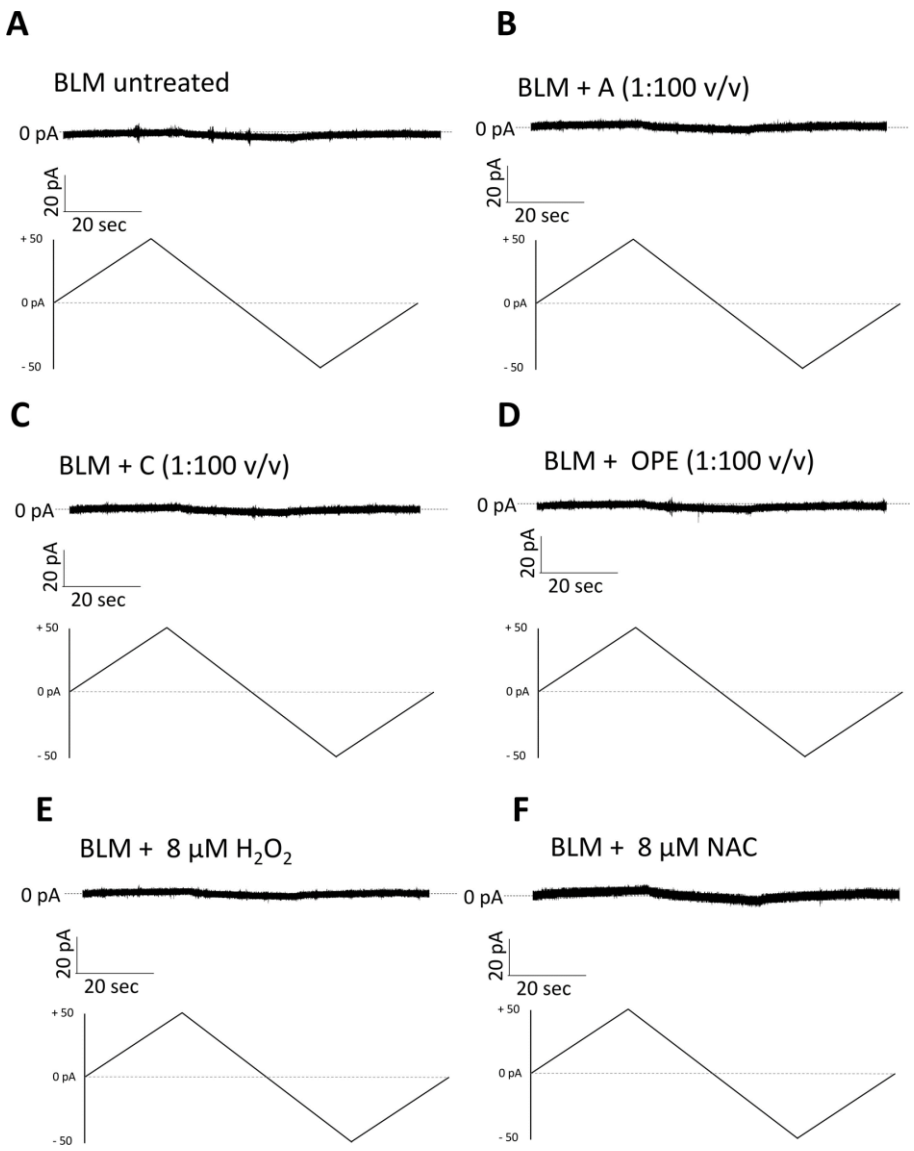


Figure S3. Evaluation of the effect of tested samples, hydrogen peroxide and N-acetyl cysteine (NAC) on membrane stability by triangular voltage ramps. Samples A (B), C (C) and OPE (D), H_2O_2 (E) and NAC (F) were added to both sides of a DiphPC bilayer at a final concentration of 1:100 (v/v) and 8 µM, respectively, to exclude any interferent effect with the lipid membrane. In each experiment, the stability of the current signal was monitored upon application of a voltage ramp of ± 50 mV in 1 M KCl.

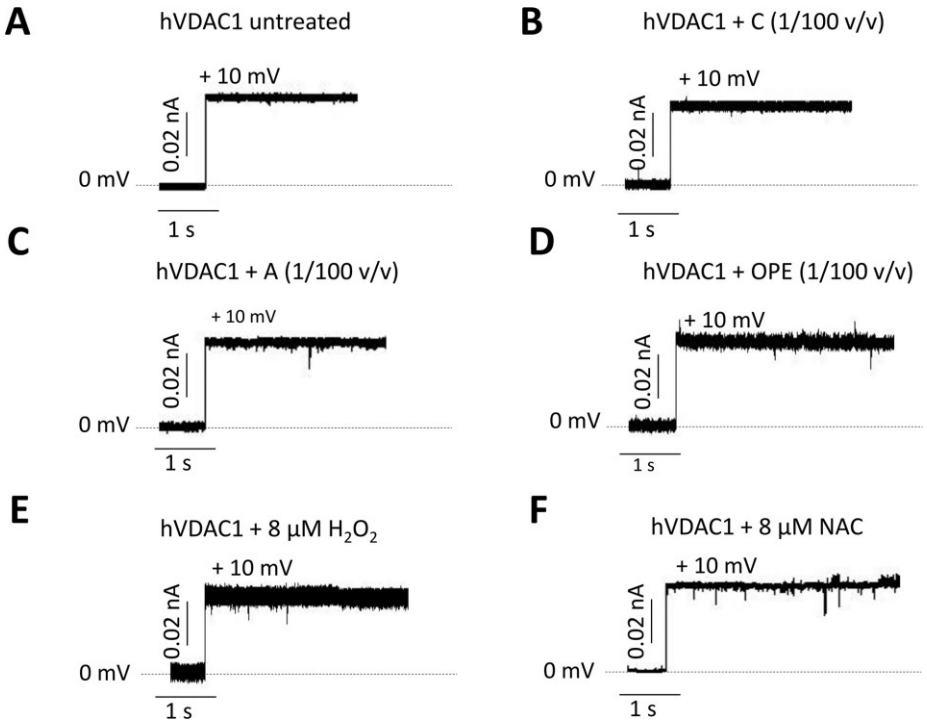


Figure S4. Single channel recordings of hVDAC1 in presence of tested samples, hydrogen peroxide and N-acetyl cysteine (NAC). Representative current traces registered upon addition of samples A (B), C (C), and OPE (D), H_2O_2 (E) and NAC (F) to both sides of a DiphPC bilayer containing hVDAC1 at a final concentration of 1:100 (v/v) and 8 µM respectively. Current measurements were performed in symmetrical 1 M KCl at + 10 mV applied.

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CHAPTER III.
PÂTÉ OLIVE CAKE

Profiling of phenol content and microbial community dynamics during pâté olive cake fermentation

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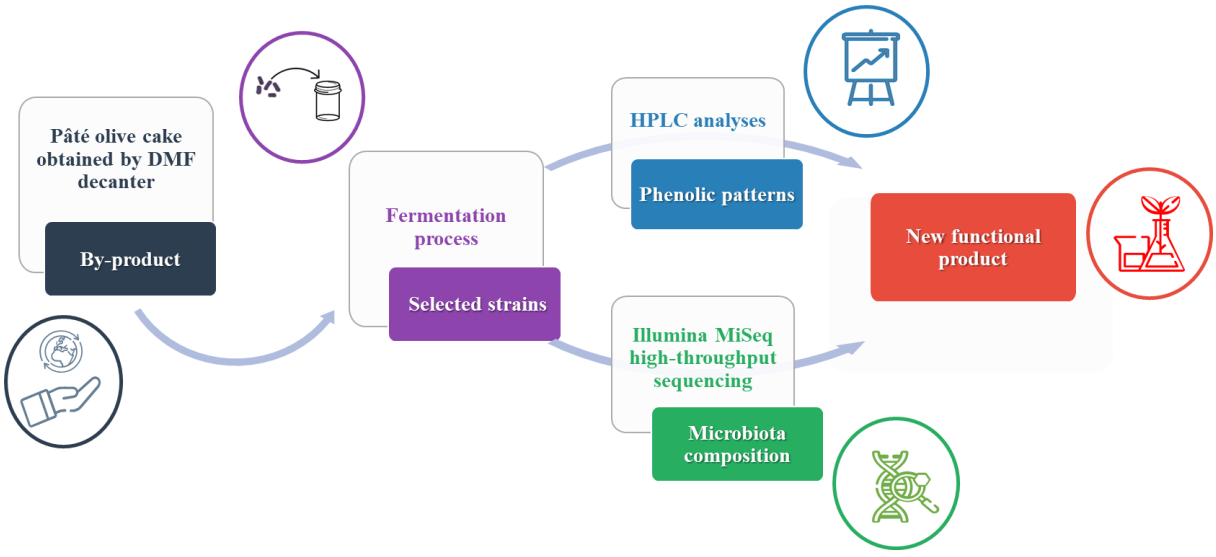
Abstract

In this study, different microbial strains, as single and mixed-cultures, were used to ferment the pâté olive cake (POC), a by-product of olive oil processing. In particular, strains belonging to *Candida boidinii*, *Wickerhamomyces anomalus* and *Lactiplantibacillus plantarum* were used. The fermentation was carried out on diluted (3:2) POC without and with glucose (2% w/v) addition. Furthermore, phenolic compounds were monitored during fermentation in POC added with glucose differently inoculated and the microbial community, at the end of fermentation, was evaluated by Next Generation Sequencing (NGS) techniques. Data highlighted that inoculated samples showed an hydroxytyrosol content higher than the un-inoculated controls. In particular, during fermentation the sample inoculated with *C. boidinii*, both in single and in mixed culture together with L. plantarum, increased the hydroxytyrosol content by 275 and 261 mg/L, respectively, after 8 days, to reach the highest content at the end of fermentation. Metagenomic analysis revealed a low abundance of 16S ribosomal RNA genes and fungal ITS in all samples at any sampling times. Furthermore, at the end of fermentation, all samples exhibited a different bacterial community with a decrease in acetic acid bacteria and an increase in Lactobacillaceae biodiversity. Finally, no effect was detected in any samples on fungal metagenomic profile, where *Dipodascus geotrichum* was found dominant both at initial and final fermentation. In conclusion, the present study confirmed that selected cultures can drive the fermentation and have an impact on the phenolic profile.



1. Introduction

Vegetable by-products represent a valuable source for obtaining new products for human consumption, as they are rich in bioactive compounds with nutraceutical traits. In particular, olive oil by-products are a source rich in bioactive compounds, i.e. phenols such as hydroxytyrosol, tyrosol and oleuropein, with confirmed health beneficial effects, contributing to the protection of blood lipids from oxidative stress (EFSA, 2011). The olive oil extraction technologies include two main systems: the three-phase system, commonly used in Italy and in Greece, which leads mainly to the production of liquid waste, i.e. olive mill wastewater; and the two-phase system, popular in Spain, which leads to the generation of a wet pomace, also called "alperujo" (Uceda et al., 2006; Veneziani et al., 2017). During the olive oil extraction process, only a small part of phenols remains in the olive oil (from 0.5% to 2.0%), while the main portion is concentrated in the liquid and in the solid by-products. Currently, both by-products are reused in various sectors, including agriculture, bioenergy, food and pharmaceutical industry (Ahmed et al., 2019). Recently the two-phase oil extraction system, based on the decanter multiphase (DMF) system (Leopard, Pieralisi, Italy) has been applied to reduce water consumption. Therefore, a dried pomace, with a moisture content of 45%–55%, and a pâté characterized by a high moisture content, between 75% and 90%, consisting of olive pulp and vegetation water are obtained. While the dried pomace is used as fuel, the pâté, without traces of stones, containing lipophilic and hydrophilic fractions is characterized by interesting technological and functional properties (Foti, Pino, et al., 2022; Lanza et al., 2020; Lozano-Sánchez et al., 2017). For food application, usually the recovery of phenols is required and their addition in food formulations is applied to provide antioxidant and antimicrobial effects or to extend the shelf life of food products. Otherwise, an alternative approach is to fortify foods by addition of pure extracts, such as hydroxytyrosol and oleuropein, however, this approach is expensive and does not completely solve the problem of by-product management (Caporaso, Formisano, & Genovese, 2018; Foti, Pino, et al., 2022). The pâté is mainly composed of triterpene acids, hydroxytyrosol, tyrosol, secoiridoid derivatives, verbascoside and fatty acids, such as palmitic acid, oleic acid and polyunsaturated fatty acids. Due to the presence of these compounds, several studies have been focused on the beneficial effects of phenols present in pâté, attributing cardio protective, antiaging and antioxidant activity (Cecchi et al., 2018; Dinu et al., 2020;

Peršurić et al., 2020), with an effect on human gastro-intestinal tract (Giuliani et al., 2019). Indeed, spontaneous or driven fermentation is a chance to stabilise and debitter the pâté, improving its nutritional and sensory properties (Durante et al., 2019). In addition, the fermentation driven by selected strains can inhibit the growth of undesirable microorganisms or pathogens reducing spoilage and shortening the debittering process (Perpetuini et al., 2020).

The metagenomic approach is useful for investigating the biodiversity of a microbial community present in a matrix, including food. The NGS techniques of microbial communities has led to the creation of a new scientific field – metagenomics – the analysis of the combined genomes of organisms co-existing in a community. The technique can reveal the main and the secondary microbial groups in fermented foods, providing information on the biodiversity of microbial population on fermentation process and on microbiota dynamics. Furthermore, the advantage of the technique is that it can obviate dependent culture techniques, which are sometimes limiting mainly in complex matrices where microbial cells can be viable but non-culturable (Medina et al., 2016). Recently, to in depth investigate the microbial composition and the metabolic processes that drive the table olives fermentation, omics approaches have been applied (Medina et al., 2016; Vaccalluzzo, Pino, Russo, et al., 2020). The aim of the present study was to combine the phenol profile detection and the metagenetic approach to insight understand the dynamic of debittering process and the shift of microbial community during fermentation in pâté olive cake differently treated and inoculated with selected lactic acid bacteria and yeast, in single and in mixed cultures.

2. Materials and Methods

2.1 Pâté olive cake and microbial strains

Pâté olive cake (POC), obtained by a multiphase decanter (Leopard, Pieralisi, Jesi, Italy) olive oil extraction, was kindly provided by *Frantoio Oleario Consoli di Consoli Pasquale e Fratelli s.n.c*, an olive oil production company located in Catania, Italy. The samples, obtained in November 2020, from a mix of Nocellara Etnea and Nocellara Messinese cultivars, were stored at −20 °C until use. In the present study, *Candida boidinii* F3 30.1, *Wickerhamomyces anomalus* F5 60.5 and *Lactiplantibacillus plantarum* F 3.5 (DSM 34190, Leibniz-Institute DSMZ, Braunschweig, Germany) strains, isolated

from naturally fermented table olives (Pino et al., 2019), belonging to the microbial culture collection of Di3A and ProBioEtna srl, Spin off of University of Catania, were used.

2.2 POC antimicrobial activity against yeasts and lactic acid bacteria

The inhibitory activity of POC against yeast and lactic acid bacteria (LAB) strains was evaluated through the disk diffusion method. In detail, the tested microbial cultures were overnight grown on specific media, as Man, Rogosa and Sharpe (MRS, Oxoid, Milan, Italy) for *L. plantarum*, and onto Potato Dextrose Broth (PDB, Merck, Milan, Italy) for yeast strains. Then, using a Mc Farland 0.5, corresponding to a cell density of 10^8 colony forming unit/mL (CFU/mL) for bacteria and 10^6 CFU/mL for yeasts, cultures were standardised and 1 mL of each suspension was streaked onto MRSA for *L. plantarum*, and onto Potato Dextrose Agar (PDA, Oxoid) for yeast. Once plates were dried, sterile cellulose discs (Ø 6 mm), imbibed with POC, at different rate dilutions (1:1 to 1:8), were placed on the plates. Distilled water was used as negative control. Plates were incubated at specified temperatures for 48 h and the results were expressed as the diameter of the inhibition halo (mm).

2.3 Fermentation trials

In order to evaluate the ability of POC to support the survival/growth of selected strains, fermentation trials were set up. Based on preliminary results (data not shown), untreated POC diluted (3:2) with sterile distilled water, was used. The fermentations were carried out into 250 mL Erlenmeyer flasks, on diluted POC and diluted POC with the addition of 2% (w/v) of glucose (Biolife, Milan, Italy) filtered at 0.22 μm (PTFE filters, Merk, Darmstadt, Germany). Diluted POC was inoculated with a 0.5 Mc Farland standardised microbial cultures (final inoculum volume of 1%) as following: three flasks were inoculated with single cultures (*L. plantarum*, *W. anomalus*, *C. boidinii*, at 1% each); two flasks were inoculated with a combined cultures of two strains (*C. boidinii* and *L. plantarum*; *W. anomalus* and *L. plantarum*, at 0.5% each) and one flak was inoculated with a mixed culture of the three strains (*W. anomalus*, *L. plantarum* and *C. boidinii*, at 0.33% each). Un-inoculated POC was used as control and indicated as spontaneously fermented sample (SF) (Fig. 1). All tests were performed in duplicate. Fermentation was

carried out at room temperature (20 ± 1 ◦C), at a 200 rpm orbital shaking on a rotary plate (Unimax 2010; Heidolph Instruments, Schwabach, Germany). POC samples differently treated were sampled at different times for further analyses. In details, the T0 time was intended as the sampling carried out few hours after POC inoculum with strains.

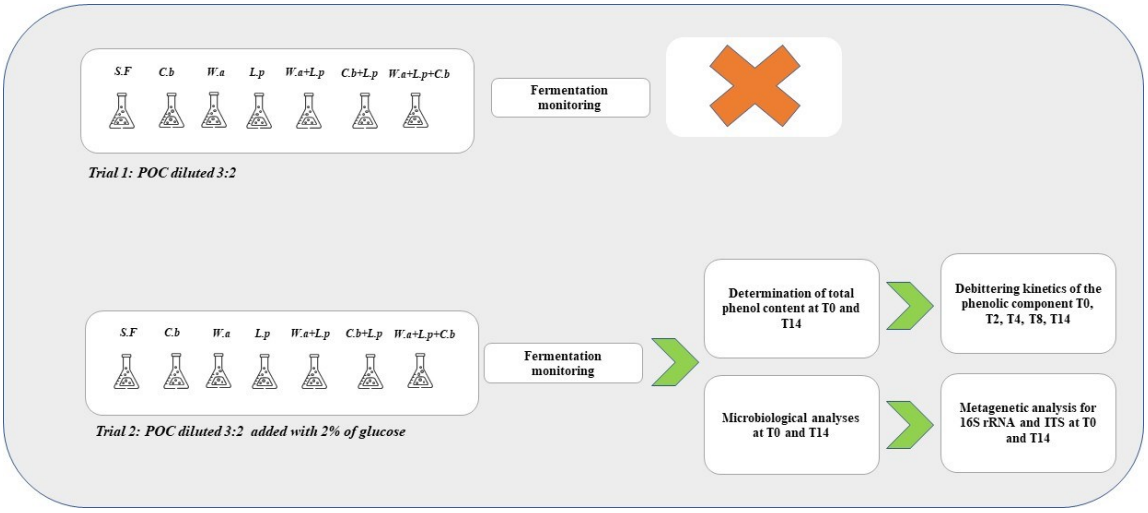


Figure 1. Experimental design.

2.4 Chemical analyses

During fermentation, pH was monitored using a Mettler DL25 pH meter (Mettler Toledo International Inc., Columbus, OH, USA) at T0, T8 and T14. The total phenol content in POC samples, at initial (T0) and final fermentation (T14), was determined according to the Folin–Ciocalteu's (FC) colorimetric method. The fermented POC samples, after filtering through Miracloth (Merck), were mixed with 5 mL of FC commercial reagent (Labochimica, Padova, Italy), diluted with water 1:10 (v/v) and blended with 7.5% sodium carbonate solution. After 2 h at room temperature (20 ± 1 °C) in the dark, the solution absorbance was measured spectrophotometrically at 765 nm (Cary 100 Scan UV–Visible, Agilent, Santa Clara, CA, USA). The total phenol content was expressed as mg of gallic acid equivalents (GAE)/L of sample (Romeo et al., 2021; Squillaci et al., 2022). All tests were performed in duplicate.

2.5 Microbiological analyses

POC samples were analyzed by serial dilution method in 0.9% (w/v) of sodium chloride sterile solution (Sigma Aldrich, Darmstadt, Germany), by using different selective agar media, and plates were incubated at different conditions. In details, LAB counts were detected into MRSA added with cycloheximide (at a final concentration of 2 mg/mL, Oxoid), and plates incubated at 32 ◦C for 48 h, under anaerobic conditions; mesophilic aerobic bacteria were detected into Plate Count Agar (PCA, Oxoid), incubated at 32 ◦C for 48 h; yeasts and moulds were counted into Sabouraud Agar added with chloramphenicol (Bio-Rad, Milan, Italy), incubated at 25 ◦C for 48 h; staphylococci into Mannitol Salt Agar (MSA, Oxoid) incubated at 32 ◦C for 72 h; acetic acid bacteria were counted in GYCA (10.0% glucose, 1.0% yeast extract, 1.5% agar) (Oxoid) with 2.0% calcium carbonate, incubated at 30 ◦C for 3–4 days; coliform bacteria on Chromogenic Coliform Agar Base (Bibby Scharlau, Milan, Italy) incubated at 37 ◦C for 24 h; Enterobacteriaceae on Violet Red Bile Glucose Agar, aerobically incubated at 37 ◦C for 24 h, and *Clostridium perfringens* on Sulphite-Polymyxin-Sulphadiazine Agar (SPSA, Oxoid) incubated at 35 ◦C− 37 ◦C for 18–48 h under anaerobic conditions, by using an Anaerogen kit (Oxoid). The analyses were performed in duplicate and results expressed as Log CFU/g.

2.6 Phenolic component in POC samples during fermentation

POC samples were collected at different fermentation times, at initial time (T0) after 2 (T2), 4 (T4), 8 (T8) and 14 (T14) days, in order to evaluate phenolic fraction dynamic through HPLC. The samples were directly filtered into vials with a 0.45 μm filter (PTFE filters, Merk). The instrument used was a Waters Alliance 2695 HPLC liquid chromatography system with a Waters 996 photodiode array (PDA) detector set at 280 nm associated with Waters Empower software (Waters Corporation, Milford, MA, USA). The column maintained at a temperature of 40 °C was a Luna C18 (250 mm × 4.6 mm i.d., 5 μm, 100 Å; Phenomenex, Torrance, CA, USA). Chromatographic separation was achieved by gradient elution using an initial composition of 90% solution A (water acidified with 2% acetic acid) and 10% solution B (methanol) (Merck). Solution B was increased to 30% in 15 min and 70% in 25 min and then, after 2 min in isocratic condition, the mobile phase was set to the initial conditions for 8 min at a flow rate of 1 mL/min. A

50 mM pure gallic acid (Fluka, Losanna, Switzerland) solution was used as internal standard (IS). Identification of phenolic compounds was obtained by comparing the retention time with standard compounds, as: hydroxytyrosol (HT), tyrosol (TYR), chlorogenic acid, vanillic acid, caffeic acid, syringic acid, p-coumaric acid, ferulic acid, verbascoside, luteolin-7-o-glucoside, o-coumaric acid, rutin, oleuropein, apigenin-7-o-glucoside, luteolin-4- glucoside, quercetin, luteolin, apigenin (Extrasynthése, Genay, France). All analyses were performed in triplicate.

2.7 DNA extraction and high-throughput sequencing by Illumina MiSeq

Total DNA was extracted from different POC samples by using FastDNA spin kit (MP Biomedicals, USA) and the DNA concentration and quality were detected by a Qubit spectrophotometer (Thermo Scientific, USA) and by electrophoresis on 1% agarose gel, respectively. The fresh un-inoculated sample was used as control, while inoculated samples were tested at the end of fermentation (T14). DNA samples were sequenced on an Illumina HiSeq 2500 platform. The V3-V4 hypervariable regions of 16S rRNA gene for bacteria and the ITS1-ITS2 region of 18S rRNA gene for fungi were amplified by a T100 thermal cycler (Bio-Rad, Hercules, CA, USA). In details the V3/V4Fw (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG - 3') and V3/V4Rv (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACT ACHVGGGTATCTAATCC – 3') and ITSFw (5'-TCGTCGGCAGCGTCAGATGTG TATAAGAGACAGTCCGTAGGTGAACCTGCGG - 3') and ITSRv (5'- GTCTCGT GGGCTCGGAGATGTGTATAAGAGACAGTCCTCCGCTTATTGATATGC - 3'), respectively. The NGS metagenomic analysis was performed at the Nucleotide Sequencing Service at University of Salamanca, Spain.

2.8 Bioinformatic data processing

The sequencing quality of the individual samples was validated by the FASTQC software, version 0.11.9, (Andrews, 2010). The adapters used in sequencing were removed from the readings using the cutadapt program (Martin, 2011). A reading filter was performed based on its parameters of quality with the DADA2 program (Callahan et al., 2016): for bacteria the left and right chain was cut at 230 and 220 bases respectively; for fungi a minimum length of 50 bases was established. Biodiversity characterization was carried out using the DADA2 package and the SILVA database, version 138 (Callahan et al.,

2016; Quast et al., 2012), for bacteria and UNITE, version 10.05.2021, for fungi (Nilsson et al., 2019). Results were represented by bar diagram R package and Krona software (McMurdie & Holmes, 2013; Ondov et al., 2011). In addition, the Chao1 and Simpson indices were used to assess microbial diversity in POC samples.

2.9 Statistical analysis

Statistical analysis was performed using a one-way analysis of variance (ANOVA), and Tukey's HSD post hoc test for means separation (significance level at $P \leq 0.05$), using the statistical software IBM SPSS Statistics for Windows, version 20 (IBM Corp., N.Y., USA).

3. Results and Discussion

3.1 Antimicrobial activity on yeasts and acid lactic bacteria and fermentation test

The inhibitory activity of POC, at different dilutions, against the used strains (*L. plantarum, W. anomalus* and *C. boidinii*), was preliminary evaluated and any inhibitory activity was observed (data not shown). This result, in agreement with Tufariello et al. (2019), confirms that the selected strains, isolated from table olive brines, a matrix rich in interfering factors (as polyphenols, and salt), were able to survive and grow in POC without any inhibiting effect. A potential explanation for the ability of *L. plantarum* to grow in presence of HT was suggested by Reverón et al. (2020), who, through a transcriptomic study, demonstrated that *L. plantarum* changes its metabolism to better adapt to the related stressed conditions. Indeed, according to the authors, the microorganism induces antioxidant mechanisms to counteract oxidative damage (including the induction of genes known to be part of the ROS resistome) by expressing genes involved in the response to oxidative stress and genes encoding for H_2S generating enzymes by decreasing the load of copper, a metal that promotes oxidative damage. According to previous reports, *W. anomalus* and *C. boidinii* were frequently detected in olive brines, where they constitute a stable dual species consortium (Pino et al., 2019). In the present study, the fermentation trials were monitored at fixed intervals (T0, T8 and T14) by measuring the pH. As shown in Table 1, different trend in pH values was detected in the two fermentation trials.

Table 1. pH values detected in trial 1 and trial 2 at different fermentation days.

Trial 1	**Time of fermentation**		
	T0	**T8**	**T14**
S. F	5.21 ± 0.01	5.00 ± 0.01^{a}	4.98 ± 0.01
C.b	5.25 ± 0.07	4.94 ± 0.06^{abc}	4.84 ± 0.08
W.a	5.28 ± 0.01	4.80 ± 0.02^{c}	4.78 ± 0.01
L.p	5.26 ± 0.01	5.00 ± 0.01^{a}	4.88 ± 0.02
W.a + L.p	5.38 ± 0.11	4.98 ±0.01^{ab}	4.97 ± 0.02
C.b + L.p	5.27 ±0.10	4.97 ± 0.02^{ab}	4.96 ± 0.01
W.a + L.p + C.b	5.33 ± 0.05	4.84 ± 0.08^{bc}	4.83 ± 0.11
	n.s.	*	n.s.
Trial 2			
S. F	5.18 ± 0.04	4.95 ± 0.07^{a}	4.79 ± 0.01^{a}
C.b	5.26 ± 0.06	4.81 ± 0.01^{a}	4.33 ± 0.01^{c}
W.a	5.21 ± 0.16	4.77 ± 0.03^{b}	4.36 ± 0.01^{c}
L.p	5.11 ± 0.13	4.97 ± 0.02^{a}	4.60 ± 0.01^{b}
W.a + L.p	5.18 ± 0.11	4.87 ± 0.03^{ab}	4.60 ± 0.07^{b}
C.b + L.p	5.20 ± 0.10	4.77 ± 0.01^{b}	4.41 ± 0.03^{c}
W.a + L.p + C.b	5.25 ± 0.07	4.84 ± 0.04^{ab}	4.64 ± 0.05^{b}
	n.s.	*	**

Data are expressed as means ± SD. Mean values with different letters within the same column are statistically different. n.s. not significant, *Significance at P ≤ 0.05; **Significance at P ≤ 0.01.

In details, the pH, starting from a mean value of 5.14, reached, in trial 1, the lowest mean value of 4.93, after 8 days (T8) to remain slight constant until the 14th day. The initial pH values, detected in the present study, were similar to those described by Lanza et al. (2020) for POC obtained from Leccino and Carboncella cultivar. Regarding the trial 2, a significant constant decrease of pH values was observed in all samples (Table 1). In particular, the pH dropped to 4.33 and 4.36 in samples inoculated with the monoculture of *C. boidinii* and *W. anomalus*, respectively. In the latest case, the inoculation of yeast combined with glucose addition enables the lowering of pH to a safe value, between 4.3 and 4.5 (Perricone et al., 2010). These results highlighted that in the present study the only addition of glucose was able to support the fermentation process, in contrast to Tufariello et al. (2019) that, to start the fermentation process, added yeast extract, peptone and glucose at 0.5% (w/v) into POC obtained from cultivar Cellino di Nardó and Leccino. Based on these results, only POC samples of trial 2 were used for further analyses.

3.2 Chemical and microbiological analyses

Through a circolar economy approach, the possible use of olive mill wastewater as plant pre-treated liquid waste and source of water, has been already tested by a few companies (Foti, Occhipinti, et al., 2022). The diluted POC samples subjected to fermentation through selected strains were characterised at the beginning and at the end of fermentation for total phenol content and for the main microbiol groups. At any sampling points the samples were filtered to remove solid residues, such as stone or oil residues, and results are reported in Table 2. The total phenol content at the beginning of fermentation ranged from 1757.30 to 2560.76 mg/L. At the end of fermentation, the total phenol concentration decreased in all samples, except in sample inoculated with *W. anomalus* in single culture, where even an increase was observed, reaching a concentration of 2426.15 mg/L. This result can probably be associated with the strong β-glucosidasic activity of *W. anomalus*, especially at pH close to 5.5 (Bautista-Gallego et al., 2011; Pino et al., 2019; Restuccia et al., 2011; Romeo et al., 2021). Different researchers showed that *W. anomalus*, together with *Debaryomyces hansenii* and *Pichia membranifaciens*, is mostly related to olive matrices thanks to its ability to tolerate extreme environmental conditions such as oxidative, salt, osmotic stress, as well as pH (Padilla et al., 2018). Whereas *C. boidinii* has been frequently exploited for its strong lipase and esterase activities, that positively affect the table olives sensorial traits (Bautista-Gallego et al., 2011).

Table 2. Total phenol content and microbiological counts in POC samples at initial and at the end of fermentation.

Sample	Time (days)	Total phenolic content (mg/L)	Lactic Acid Bacteria (Log CFU/g)	Yeasts and moulds (Log CFU/g)	Mesophilic aerobic bacteria (Log CFU/g)
S. F	T0	1858.07 ± 2.71^{d}	3.00 ± 0.01^{bc}	3.06 ± 0.08^{d}	3.80 ±0.02^{b}
C.b	T0	2046.53 ± 0.54^{b}	3.88 ± 0.57^{ab}	5.15 ± 0.21^{a}	3.50 ± 0.04^{bc}
W.a	T0	1757.30 ± 0.57^{e}	3.43 ± 0.21^{ab}	4.57 ± 0.02^{ab}	5.63 ± 0.04^{a}
L.p	T0	2560.76 ± 14.14^{a}	3.57 ±0.02^{ab}	3.23 ± 0.33^{d}	3.06 ± 0.08^{bc}
W.a + L.p	T0	1730.38 ± 31.00^{e}	2.10 ± 0.02^{c}	3.35 ± 0.49^{bc}	3.65 ± 0.52^{bc}
C.b + L.p	T0	1941.15 ± 1.63^{c}	4.30 ± 0.42^{a}	4.04 ± 0.06^{abc}	3.26 ± 0.37^{bc}
W.a + L.p + C.b	T0	2083.07 ±0.01^{b}	4.04 ±0.06^{ab}	3.39 ±0.55^{bc}	2.04 ± 0.06^{c}
		**	*	*	*
S. F	T14	1402.30 ± 1.08^{f}	3.04 ± 0.03^{c}	2.06 ± 0.08^{f}	4.80 ± 0.10^{a}
C.b	T14	1853.84 ± 2.17^{e}	3.04 ± 0.05^{c}	3.13 ±0.01^{e}	3.13 ± 0.13^{b}
W.a	T14	2426.15 ± 52.21^{a}	3.45 ± 0.01^{abc}	3.72 ± 0.04^{d}	3.14 ± 0.07^{b}
L.p	T14	1516.53 ± 3.80^{e}	3.12 ± 0.18^{bc}	2.24 ± 0.02^{f}	3.10 ± 0.02^{b}
W.a + L.p	T14	1651.92 ± 0.54^{d}	3.54 ± 0.02^{ab}	4.79 ± 0.05^{a}	2.50 ± 0.04^{c}
C.b + L.p	T14	1805.38 ± 2.17^{c}	3.71 ± 0.02^{a}	4.05 ± 0.08^{c}	2.71± 0.01^{c}
W.a + L.p + C.b	T14	2007.69 ± 6.52^{b}	3.15 ± 0.21^{bc}	4.41± 0.13^{b}	2.41 ± 0.10^{c}
		**	*	**	**

Data are expressed as means ± SD for total phenolic content and as Log CFU/g ± SD for microbial counts. Mean values with different letters within the same column are statistically different. *Significance at $P \leq 0.05$; **Significance at $P \leq 0.01$.

Overall, microbiological data, obtained by dependent culture techniques, showed low microbial cell density. In details, *Staphylococcus* spp., acetic acid bacteria, coliforms, *E. coli*, enterococci, or *Clostridium perfringens* were undetectable in all samples at both initial and final times, according to Tufariello et al. (2019). The flasks were maintained under shaking during fermentation to allow the growth of LAB and yeasts and different trends for LAB, yeasts and moulds, and mesophilic aerobic bacteria were observed in the differently treated samples at the end of fermentation (Table 2). Regarding LAB, constant densities were observed in all samples at the end of fermentation, except in the samples inoculated with *W. anomalus* and *L. plantarum* in mixed culture, where 1.0 Log unit increase was observed. In details, although the *L. plantarum* F 3.5 (DSM 34190) was inoculated both in single and in mixed cultures in POC samples at an initial density of 8 Log CFU/g any different concentrations were detected in all samples. Different behavior was observed for yeast counts at both initial and final fermentation, for which higher values were detected in samples inoculated with the two yeast strains, *C. boidinii* F3 30.1 and *W. anomalus* F5 60.5. For these samples 5.15 and 4.57 Log CFU/g initial concentrations were detected, respectively (Table 2). Different values were detected at

the end of fermentation, when the higher increases were observed in samples inoculated with *W. anomalus* and *L. plantarum* (1.4 Log unit), followed by samples inoculated with *W. anomalus* and *L. plantarum* and *C. boidinii* (1.0 Log unit). These data confirmed that certain combination of yeasts and LAB promote the lactobacilli growth, as yeasts produce substances, such as vitamins (B1 and B6), amino acids or break down complex carbohydrates that are essential for bacterial growth (Corsetti et al., 2012). In addition, the total mesophilic aerobic bacteria count decreased in all samples during fermentation, except in un-inoculated S.F sample which showed an increase of 1 Log unit at the end of fermentation.

3.3 POC phenolic component during fermentation

The dynamic of phenolic profile of POC differently treated at different times during fermentation: T0, T2, T4, T8 and T14 was assessed. The HPLC analysis revealed that at the beginning of the fermentation, different phenols, such as hydroxytyrosol (HT), tyrosol (TYR), oleuropein, vanillic acid, p-coumaric acid and apigenin were found in all samples. In details, while vanillic acid, p-coumaric acid, apigenin and oleuropein were found only at the beginning of fermentation (Table 3), at T2 and T14, the dominance of the two most important phenols of olive by-products, as HT and TYR (Table 4) were found, confirming that the main hydrolysis products of oleuropein include HT and TYR (Charoenprasert & Mitchell, 2012). Moreover, as recently reported, the effect of microbial enzymatic activity in table olives or in olive oil by-products can affect the phenolic profile (Romeo et al., 2021; Vaccalluzzo, Pino, De Angelis, et al., 2020) and the observed increase of HT content can be related to the used strains. These phenols have been recognised for their antioxidant properties. In detail, the HT (3,4-dihydroxyphenylethanol) is a phenolic alcohol to which multiple activities, including cardioprotective, anticarcinogenic, antimicrobial have been attributed (Parkinson & Cicerale, 2016; Robles-Almazan et al., 2018).

Table 3. Main phenolic compounds detected at the beginning of fermentation.

Sample	Vanillic acid (mg/L)	p-Cumaric acid (mg/L)	Oleuropein (mg/L)	Apigenin (mg/L)
SF	68.54 ± 0.73^{bc}	90.10 ± 0.96^{abc}	259.79 ± 2.78^{c}	90.10 ± 0.96^{abc}
C.b	96.17 ± 1.14^{abc}	119.84 ± 11.06^{a}	409.07 ± 40.53^{a}	119.84 ± 11.00^{a}
W.a	100.00 ± 7.24^{ab}	123.25 ± 10.35^{a}	394.08 ± 11.22^{ab}	123.25 ± 10.35^{a}
L.p	106.73 ± 10.87^{a}	124.21 ± 10.01^{a}	370.58 ± 37.46^{ab}	124.21 ± 10.01^{a}
W.a + L.p	43.18 ± 0.01^{c}	47.25 ± 0.02^{c}	108.29 ± 0.52^{d}	47.25 ± 0.01^{c}
C.b + L.p	70.64 ± 3.23^{bcd}	69.90 ± 3.80^{bc}	202.20 ± 14.73^{cd}	69.90 ± 3.80^{bc}
W.a + L.p + C.b	87.56 ± 15.98^{abc}	106.71 ± 26.53^{ab}	302.51 ± 33.43^{bc}	106.71 ± 26.53^{ab}
	*	*	**	*

Data are expressed as means ± SD. Mean values with different letters within the same column are statistically different *Significance at $P \leq 0.05$; **Significance at $P \leq 0.01$.

Furthermore, the TYR (2-(4-hydroxyphenyl)-ethanol), a stable compound less prone to autoxidation than other phenols, had shown cellular antioxidant activity and its effect seems to be associated with intracellular accumulation despite its weak antioxidant activity (Di Benedetto et al., 2007; Markovi'c et al., 2019). In the present study, in all inoculated samples, HT concentration decreased at the first two sampling times (T2 and T4), to increase at the last two sampling times (T8 and T14), with the exception of the un-inoculated sample (S.F). A significant increase occurs at the 8th day in sample inoculated with *C. boidinii* in single culture and in association with *L. plantarum*, reaching, after 8 days, concentrations of 275 mg/L and 261 mg/L, respectively. The increase of the HT concentration during fermentation driven by yeasts could be explained, according to Fernandez de Ullivarri, Mendoza, and Raya (2018), which demonstrated that in wine yeasts can produce higher concentrations of HT, as a secondary metabolite from tyrosine, and not only from the degradation of other polyphenolic compounds, such as anthocyanins or oleuropein (Charoenprasert & Mitchell, 2012; Motilva et al., 2016).

Table 4. Hydroxytyrosol (HT) and tyrosol (TYR) content in POC samples during fermentation.

Sample					
			Time		
HT (mg/L)	**T0**	**T2**	**T4**	**T8**	**T14**
S. F	297.79 ± 3.19^{f}	215.95 ± 0.50^{d}	198.63 ± 4.13^{c}	99.54 ± 2.29^{d}	93.83 ± 1.52^{d}
C.b	466.65 ± 0.69^{b}	267.01 ± 2.66^{b}	196.05 ± 0.76^{c}	275.83 ± 6.43^{a}	199.25 ± 4.55^{b}
W.a	415.25 ± 4.25^{c}	264.42 ± 2.61^{b}	258.07 ± 0.53^{b}	213.61 ± 0.53^{b}	132.86 ± 0.96^{d}
L.p	462.38 ± 2.00^{b}	341.86 ± 0.39^{a}	296.63 ± 5.31^{a}	215.08 ± 8.27^{b}	178.74 ± 3.63^{c}
W.a + L.p	351.85 ± 0.01^{e}	211.81 ± 5.23^{d}	155.21 ± 2.11^{d}	112.27 ± 3.97^{d}	96.44 ± 1.13^{e}
C.b + L.p	387.85 ± 0.69^{d}	153.98 ± 3.5^{e}	131.19 ± 2.60^{e}	261.18 ± 6.60^{a}	229.74 ± 1.06^{a}
W.a + L.p + C.b	478.47 ± 0.95^{a}	241.24 ± 0.95^{c}	119.03 ± 0.76^{f}	166.01 ± 1.48^{c}	123.93 ± 1.72^{e}
	**	**	**	**	**
TYR (mg/L)					
S. F	136.38 ± 1.40^{d}	147.58 ± 3.70^{bcd}	152.98 ± 1.87^{b}	103.64 ± 3.74^{d}	122.06 ± 2.47^{ab}
C.b	188.94± 13.50^{ab}	140.37± 14.40^{cd}	129.34 ± 7.70^{c}	206.04 ± 7.57^{a}	142.87 ± 0.83^{a}
W.a	187.04 ± 6.94^{abc}	162.79 ± 4.93^{abc}	210.47 ± 7.05^{a}	182.02 ± 7.04^{b}	106.89 ± 7.44^{bc}
L.p	203.10 ± 2.85^{a}	198.16 ± 16.73^{a}	202.80 ± 0.30^{a}	159.32 ± 1.70^{c}	123.26 ± 12.89^{ab}
W.a + L.p	162.85 ± 0.01^{c}	161.58 ± 10.20^{abc}	154.71 ± 2.43^{b}	109.3 ± 2.47^{d}	91.04 ± 1.84^{c}
C.b + L.p	171.42 ± 4.38^{bc}	107.05 ± 11.07^{d}	132.47 ± 0.61^{c}	157.13 ± 9.64^{c}	138.51 ± 1.68^{a}
W.a + L.p + C.b	206.81 ± 4.67^{a}	191.90 ± 0.67^{ab}	118.90 ± 3.47^{c}	149.13 ± 2.45^{c}	93.87 ± 8.10^{c}
	**	*	**	**	**

Data are expressed as means ± SD. Mean values with different letters within the same column are statisticallydifferent. *Significance at P ≤ 0.05; **Significance at P ≤ 0.01.

These data confirm that selected yeasts can promote LAB growth as well as the phenolic compounds degradation thanks to their β-glucosidasic activity (Corsetti et al., 2012). It is interesting to note that the association of the two yeasts together with *L. plantarum* slightly increased the concentration of HT. This phenomenon is probably due to competition for the carbon source between the two yeasts during fermentation. Indeed, authors claim that *W. anomalus* possesses a 'killer activity' against several yeasts, exhibiting a biocontrol action against both spoilage non-Saccharomyces yeasts and *Saccharomyces cerevisiae* strains (Csutak et al., 2017; Fernandez de Ullivarri, Mendoza, & Raya, 2018; Liu et., 2015). Regarding TYR, the highest concentrations were reached in the *C. boidinii* single-culture samples, after 8 days, and in *W. anomalus* and *L. plantarum*, at T4, reaching concentrations of 206.04 mg/L, 210.47 mg/L and 202.80 mg/L, respectively. However, a similar TYR concentration (206.81 mg/L) was reached at the first sampling time in sample co-inoculated with the three cultures (Table 4). Recently, to calculate olive oil polyphenol for applying the EFSA health claim, some authors have stressed the need to quantify aside from the HT, also the total TYR content (Bellumori et al., 2019). In the case of TYR, a higher concentration in the samples could also be related to the presence of yeasts. In fact, several studies use yeasts, such as *S. cerevisiae*, or modify the biosynthesis of TYR (via shikimate and the L-tyrosine branch) to increase the production of this compound (Guo et al., 2020; Liu et al., 2021).

3.4 Diversity and change of microbial community during fermentation

POC samples, both controls and inoculated, were subjected to sequencing of the ITS region and 16S rRNA. The control sample (S.F) was sequenced at initial time (T0) in order to mark the shift in microbial population at the end of fermentation. The Chao1 index showed, for the ITS profile, a total number of 61 determined genera, with an average number of 7.625 genera per sample. In particular, for the ITS region, both Chao1 and Simpson indices highlighted that the *C. boidinii* + *L. plantarum* sample showed the highest number of genera (13 and 0.82, respectively) at the end of fermentation (Fig. S1). As far as 16S rRNA gene is concerned, on the other hand, several species, in a range between 500 and 1050, were detected. Using the Chao1 and Simpson indices, the total number of found species was 5819 with an average per sample of 727.375 species (data not shown), and highest number of species was found in samples inoculated with *W. anomalus* + *L. plantarum* + *C. boidinii* (Fig. S2). Concerning the ITS biota, the results showed the dominance of *Dipodascus geotrichum*, a common yeast-like fungus, found in all samples both before and after the fermentation process (data not shown). *D. geotrichum* is a ubiquitous fungus commonly isolated form different habitats rich in sugars or other nutrients. The species has been isolated from soil, air, water, milk, silage, plant tissue and the digestive tract of humans and other mammals. Thermophilic species may be found colonizing the intestinal and respiratory tracts of mammals and may provoke disorders in immunocompromised patient (Pottier et al., 2008). In the food industry, *D. geotrichum* is a dominant yeast in dairy products and appears to exert a functional action related to sugars, milk fat and proteins (Kacániová et al., 2021) and flavour production (Grondin et., 2017). Only in the samples inoculated with *C. boidinii* + *L. plantarum* and in samples inoculated with the mix of the three strains, a slight change in fungal population occurred at the end of fermentation. Indeed, in samples inoculated with *C. boidinii* + *L. plantarum* the presence of Basidiomycota (2%), and specifically Agaricomycetes, was found. These fungi include the industrially cultivated for edible and medicinal mushrooms (Merényi et al., 2022). While in the sample inoculated with the three-strain combo, 0.6% of Metschnikowiaceae was found. This microbial group is known to be present in fermented foods and species belonging to *Metschnikowia* genus have shown to actively affect the aromatic profile of wines, as they induce a higher production of ethyl esters and long-chain fatty acids (Blanco et al., 2021; Wang et al., 2022). The metagenomic analysis performed highlighted in which way the inocula

influenced also the indigenous microbial community of the POC analyzed. These results showed that species, as *D. geotrichum*, already present in the initial matrix remain dominant until the end of fermentation. The bacterial microbioma showed a higher variable profile, compared to fungi (Fig. S3), although the highest relative abundance (at 0.3) occurred in the sample at the beginning of fermentation (S.F at T0). The NSG analyses also revealed between 32% and 42% of unidentified genera. The detected phyla in POC samples, showed with KRONA software, before fermentation, are shown in Fig. 2.

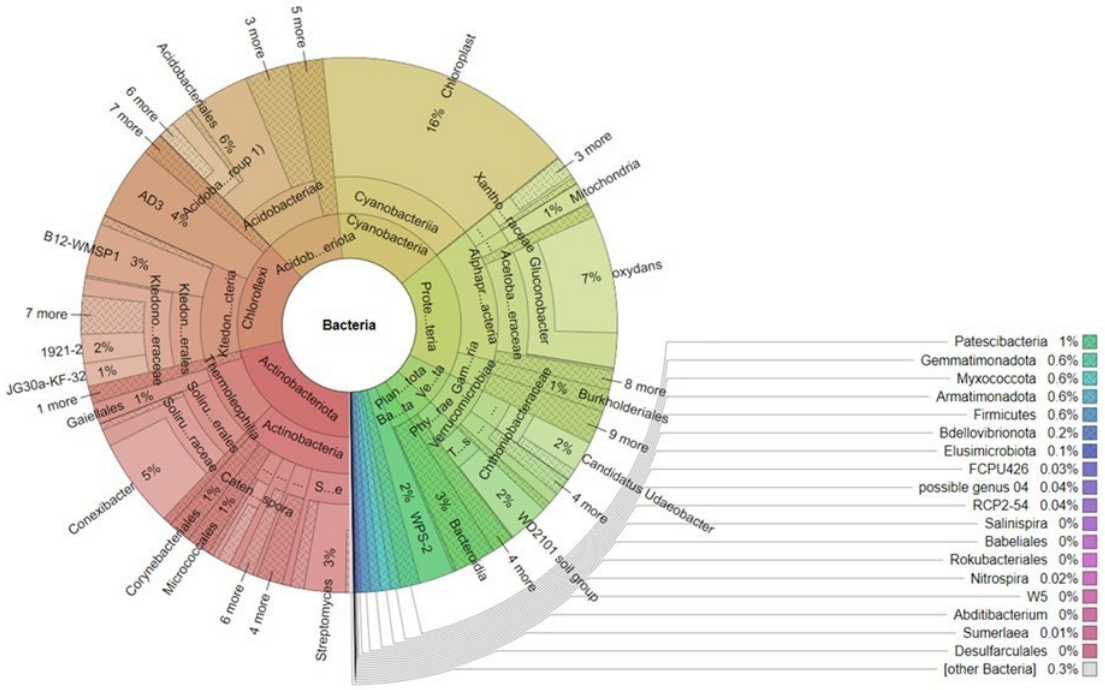


Figure 2. Krona graphical representation of taxonomic abundance in S.F sample at initial time.

In details, Actinobacteriota (21%), Proteobacteria (18%), Chloroflexi (16%), Acidobacteriota (11%), Cyanobacteria (16%) were detected. These phyla have been found as naturally present both in soil and in aquatic ecosystems (Mutoti et al., 2022; Wang et al., 2019). Interestingly, before the addition of selected microbial cultures, within the Acetobacteriaceae family, *Gluconobacter oxydans* was found as a dominant species (7%). Different strains of the species have been used for long time in biotechnology industry for their ability to oxidise various compounds such as sugars, polyols and alcohols, as well as ketones and aldehydes and for the production of bioactive compounds, including L-ascorbic acid (vitamin C) (Da Silva et al., 2022). At the end of fermentation (at T14), in all samples, including control sample (S.F), a decrease in Acetobacteriaceae

occurred, with the highest decrease detected for *G. oxydans*, that reached 0.02% in sample inoculated with *C. boidinii* + *L. plantarum*. Although difficult to cultivate, isolate and identify, acetic acid bacteria represent promising starter cultures to better control food fermentation processes but most importantly to produce new fermented foods and beverages (De Roos & De Vuyst, 2018). Zooming on Firmicutes phylum profile, only the 0.6% was found in samples at the beginning of fermentation (Fig. 2) while no Lactobacillaceae was revealed (Fig. 3A). This percentage remained almost unchanged up to the end of fermentation, although a slight different composition in Firmicutes was detected at the end of fermentation. Indeed, at the end of fermentation, the S.F sample showed the presence of 3% of *Lactiplantibacillus* spp., 4% of *Lacticaseibacillus* and 2% of *Lactobacillus jensenii* (Fig. 3B). *Lactiplantibacillus* was mainly found (5%) in samples inoculated with *L. plantarum* (Fig. 3C) and in the samples inoculated with the combination of *W. anomalus* and *L. plantarum* (9%) Fig. 3D), indicating the survival of *L. plantarum* selected strain in this matrix. Zooming on the Lactobacillaceae composition, in latter two samples, an increase on *Lactiplantibacillus* community was riveled, up to 80% and 87%, respectively (Fig. 3C and D). Although the low abundance of LAB in plant microbiota, as in the case of POC, there is a growing interest in the scientific community in use LAB in plant by-products to obtain functional products, rich in bioactive compounds, thanks the ability of LAB to grow and adapt to different plant niches (Sabater, Ruiz, Delgado, Ruas-Madiedo, & Margolles, 2020).

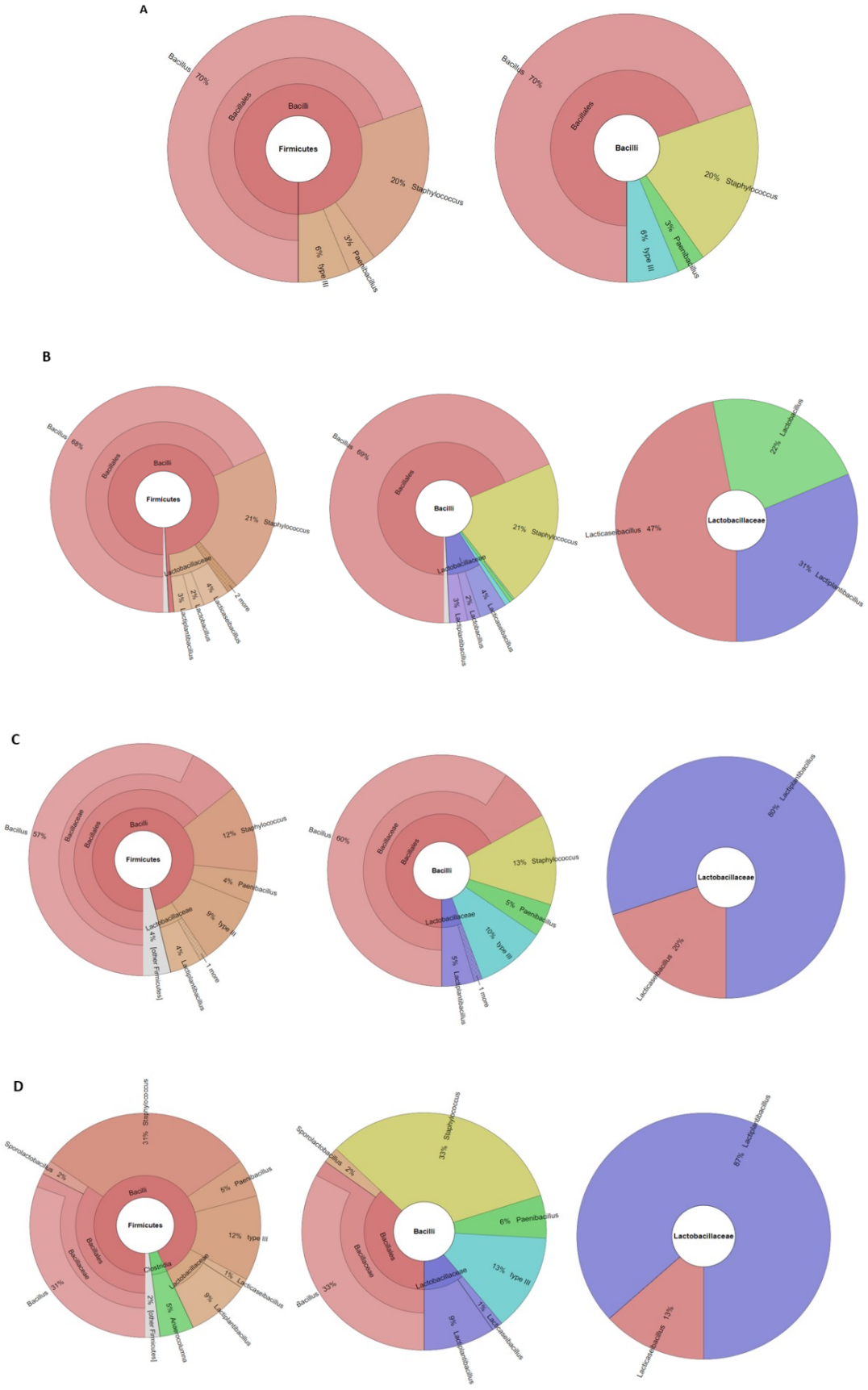


Figure 3. Krona graphical representation of taxonomic Firmicutes abundance A: S.F T0 (Spontaneus fermentation); B: S.F T14 (Spontaneus fermentation); C: sample inoculated with *L. plantarum* T14; D: sample inoculated with *W. anomanlus* and *L. plantarum*.

4. Conclusion

The aim of this study was to monitor the fermentation process of POC inoculated with microbial strains in different combinations in order to propose a strategy to improve the healthy value of such a by-product. Results on phenolic component showed that the use of microbial monocultures or mixed cultures can influence the profile of bioactive compounds during fermentation. Moreover, NGS analyses applied to in depth explore and better understand the function of microbial community, revealed a shift on bacterial population whereas lower effect were detected for yeast population. Moreover, results of the present study confirmed that POC can be considered microbiologically safe and a relevant source to be used as food and/or ingredient. Further studies will be conducted on a larger volume in order to standardise and replicate an industrial scale-up process to obtain new environmentally friendly foods or functional ingredients.

Supplementary material

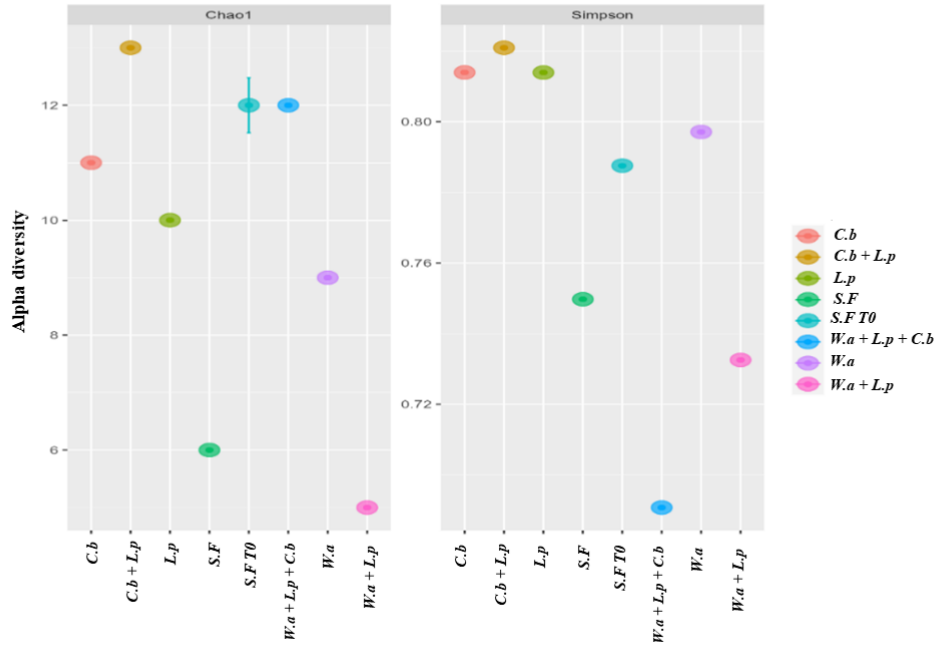


Figure S1. Alpha diversity of ITS, using the Chao1 and Simpson indices/measures.

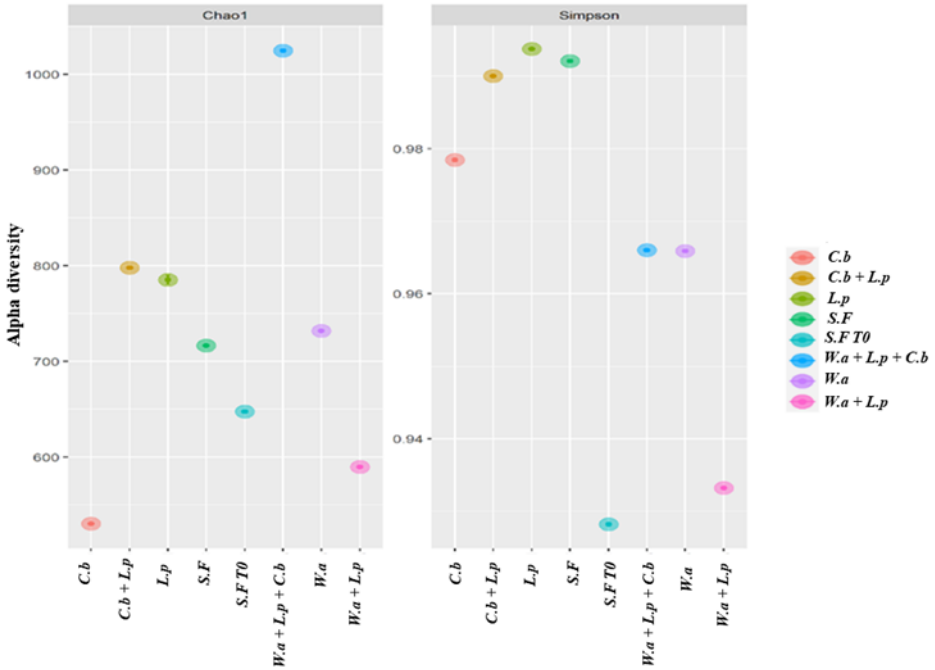


Figure S2. Alpha diversity 16S rDNA gene, using the Chao1 and Simpson indices/measures.

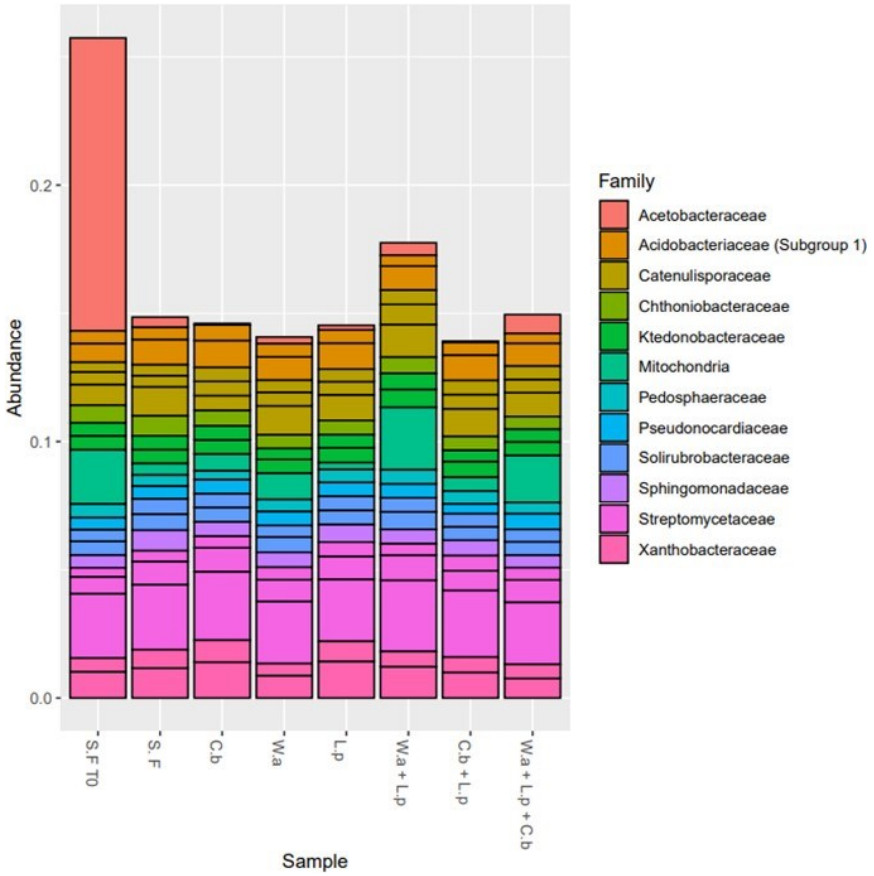


Figure S3. Bar-plot of 16S profile expressed as relative frequency (expressed as a decimal between 0 and 1).

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Effect of microbial fermentation of functional traits and volatiloma profile of pâté olive cake

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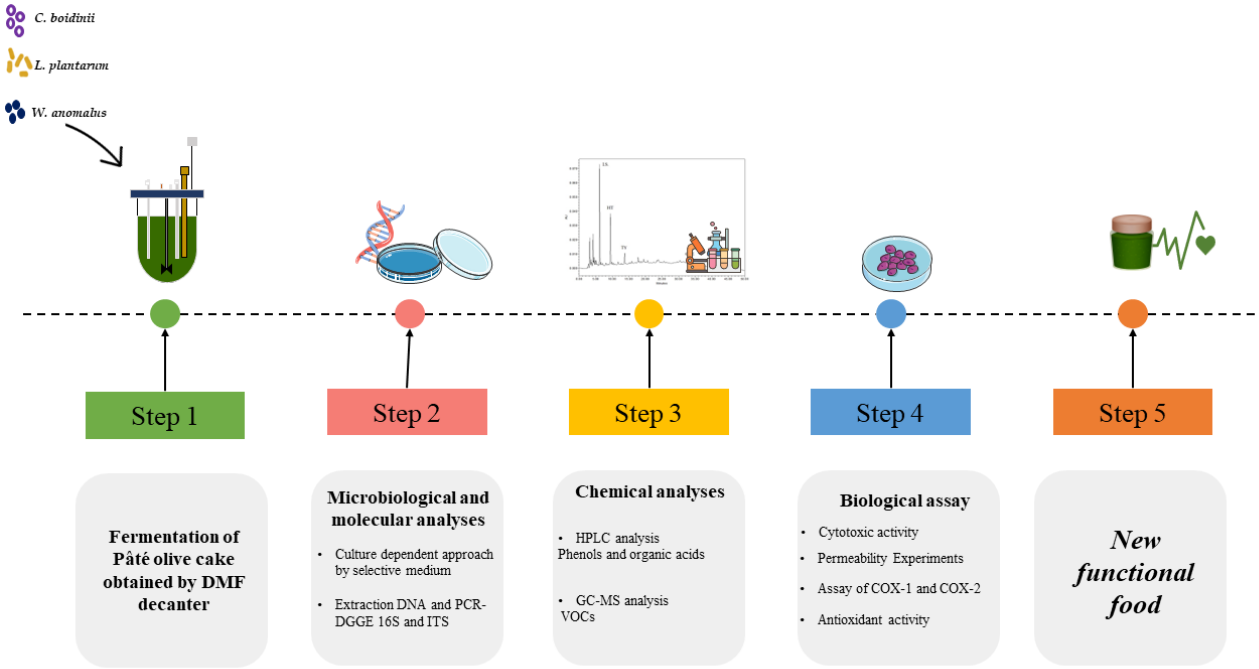
Abstract

In this study, the pâté olive cake (POC), a by-product of the olive oil industry, was subjected to fermentation in a bioreactor using three microbial strains, *Lactiplantibacillus plantarum*, *Wickerhamomyces anomalus* and *Candida boidinii,* previously isolated from fermented table olive brines. Chemical, microbiological and molecular analyses were carried out at the beginning and at the end of fermentation. The lowest pH value (4.09) was reached after 10 days in sample inoculated with *C. boidinii.* Microbiological analyses exhibited the dominance of yeasts throughout the whole process (from 5.5 to 7.80 Log_{10} CFU/g), as confirmed by PCR-DGGE analysis. The microbial cultures affected both phenolic and volatile organic compound profiles. Moreover, the POC samples treated with different microbial strains were investigated for biological assays. The sample fermented with *W. anomalus* showed the greatest diffusion speed of transepithelial transport through Caco-2 cell, the highest inhibitory activity towards the tested cyclooxygenases and the highest antioxidant activity.



1. Introduction

Fermentation, a process driven by microorganisms, applied on agro-food by-products can represent a strategy to valorize by-products and can be used to replace conventional extraction techniques. Fermentation process being characterized by low costs and low energy consumption can be considered a 'green technology' to generate high value-added products from organic waste (Azmir et al., 2013; Tlais et al., 2020). Indeed, several authors recently propose to recover value-added products, such as antibiotics, pigments, enzymes, plastics, and bioactive compounds from agro-food waste (Hadi Saadoun et al., 2021; Romeo et al., 2021). Moreover, the pâté obtained from the DMF (Leopard, Pieralisi, Italy) two-phase extraction, being a by-product composed of olive pulp and wastewater, is rich in bioactive compounds and may represent a promising matrix potentially suitable for the formulation of new functional foods (Foti et al., 2022). Several beneficial activities have been attributed to phenols present in olive oil products and by-products, including antioxidant and antimicrobial activity. In addition, recent studies suggest a possible anticancer effect, mainly against ovarian cancer (Moscatello et al., 2018; Benot-Dominguez et al., 2021). Through fermentation, desired changes can be achieved, including improved microbiological safety, nutritional and sensory properties, and extension of shelf life. Fermentations obtained with starter cultures are usually driven by both single and mixed microbial cultures. In food and beverage production, lactic acid bacteria (LAB) are widely used since they are classified as 'recognised as safe' (GRAS) and most of them have received the Qualified Presumption of Safety (QPS) status, the result of safety assessment procedure for microorganisms to be used in the food chain (EFSA, 2022). Furthermore, several LAB are able to produce antimicrobial compounds such as organic acids (lactic, acetic, etc.), diacetyl, bacteriocins and other metabolites positively affecting the stability of the final products (Ricci et al., 2019). The most used LAB include strains belonging to *L. plantarum* species, used in single culture or in co-culture with other bacteria or yeasts (Lanza et al., 2021). Despite the high number of studies focused on the effect of LAB on sensory traits of foods matrices, the effect of LAB in aroma production from waste and by-products has been rarely studied (Spaggiari et al., 2020). LAB are often used in combination with specific selected yeasts able to promote the LAB growth and the aroma and flavour developing. In addition, yeasts can synthesize several antioxidant compounds that act as biocontrol agents by producing

glycoproteins, known as killer toxins, that inhibit the growth of fungi and other undesirable yeast species (Leventdurur et al., 2016). Among yeasts, the most commonly used as starters are *Saccharomyces cerevisiae*, *Wickerhamomyces anomalus* and *Candida boidinii* (Rodríguez-Gómez et al., 2012).

The aim of the present study was to evaluate the effect of different strains on the bioactive molecules and on volatile organic compounds (VOCs) of pâté olive cake, through driven fermentations. For this purpose, trials were set up using three single-culture strains in a bioreactor: *L. plantarum*, *W. anomalus* and *C. boidinii*. The biotechnological aptitude of each single strain and their biological activity were evaluated.

2. Materials and Methods

2.1 Pâté olive cake sampling

The pâté samples were kindly provided by '*Frantoio Oleario Consoli di Consoli Pasquale e Fratelli s.n.c*' an olive oil production company located in Catania, Italy, during the 2020 oil processing season. The by-product is obtained using a multi-phase decanter (Leopard, Pieralisi, Italy), a two-phase centrifugal extractor. Once collected, the samples were stored at -20 °C until further analyses.

2.2 Microbial cultures

The *Candida boidinii* F3 30.1 and *Wickerhamomyces anomalus* F5 60.5 strains belonged to the culture collection of the Department of Agriculture, Food and Environment (Di3A) of the University of Catania, whereas the *Lactiplantibacillus plantarum* F 3.5 (DSM 34190) belonged to the ProBioEtna srl, Spin off of University of Catania. All the strains were previously isolated from naturally fermented table olives (Pino et al., 2019).

2.3 Fermentation test

For each fermentation trial, the pâté was diluted with sterile water in a ratio of 3:2 and 2 % of glucose (Biolife, Italy) was added. Four trials were set up: one spontaneous fermentation (control trial) and the other three trials were set up using the strains in single culture. In detail, *L. plantarum*, *W. anomalus* and *C. boidinii* strains were inoculated at 1 %, at a cell density of 10^8 CFU/mL and 10^7 CFU/mL for *L. plantarum* and the two yeasts, respectively. All trials were performed in a total volume of 3 L in a Biostat A®

bioreactor using a 5 L UniVessel® (Sartorius Stedim, Italy). The bioreactor allows to set up different process parameters essential for assessing the fermentation, such as pH, pO_2, temperature and foam formation, that were monitored over time (Figure 1). Regarding stirring and air parameters, the same conditions were set for all samples, equal to 10-20 cm3 for air and 650 rpm for stirring. The temperature was set up to 28 °C for yeast and to 30 °C for LAB. In all fermentations, the data acquisition was set every 30 seconds and showed as means for each day. The samples were taken at the start and at the end of fermentation and stored at -20 °C for further analyses.

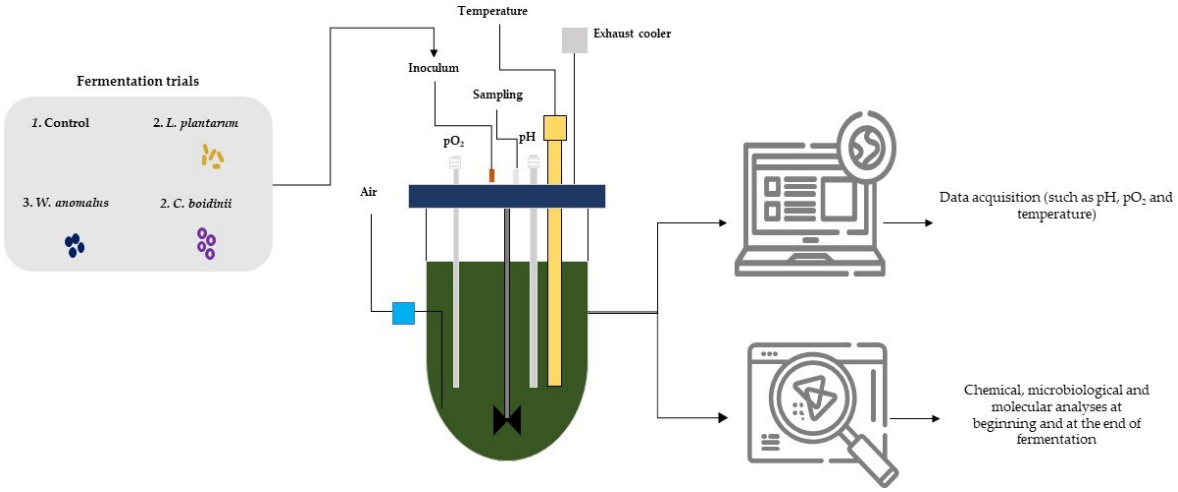


Figure 1. Experimental design of fermentation process.

2.4 Chemical analyses

The content of total soluble solids (TSS) and total phenols, at the beginning and at the final time of fermentation were monitored for each trial. Specifically, the TSS value was determined with a refractometer (Atago, RX-5000, Italy) and the value was expressed as °Brix. The total phenolic content detected in all samples was determined according to the Folin-Ciocalteu's (FC) colorimetric method. The samples, after filtering through Miracloth paper (Merck, Germany) were mixed with 5 mL of FC commercial reagent (Labochimica, Italy), 1:10 v/v diluted with water, and added with 7.5% sodium carbonate solution. After 2 h at room temperature in the dark, the absorbance of the solution was measured spectrophotometrically at 765 nm (Cary 100 Scan UV-Visible, Agilent, USA). The total phenolic content was expressed as mg gallic acid equivalents (GAE)/L of sample.

2.5 Microbiological analyses

The POC samples were subjected to microbiological analyses at the beginning and at the end of fermentation. The samples were transferred into sterile stomacher bags with sterile Ringer's solution and then pumped in a Stomacher (BagMixer 400, Interscience, France) at medium speed for 2 minutes. The decimal dilutions were then aseptically prepared and plated on the following selective media and conditions. De Man, Rogosa and Sharpe Agar (MRS, Oxoid, UK) for lactobacilli at 32 °C for 48 h under anaerobic conditions; Sabouraud Chloramphenicol Agar (SAB, Bio-Rad, Italy) for yeasts at 25 °C for 48 h; Mannitol Salt Agar (MSA, Oxoid) for *Staphylococcus* spp. At 32 °C for 72 h; Plate Count Agar (PCA, Oxoid) for mesophilic aerobic bacteria incubated at 25 °C for 48 h; Chromogenic Coliform Agar Base (Bibby-Scharlau, Italy) for coliform bacteria incubated at 37 °C for 24 h; and Sulphite-Polymyxin-Sulphadiazine Agar (SPS, Oxoid) for the detection of *Clostridium perfringens*, incubated at 35-37 °C for 18-48 h, under anaerobic conditions. Microbiological analyses were performed in triplicate and results expressed as Log10 CFU/g.

2.6 DNA Extraction, 16S and ITS PCR-DGGE analyses

All POC samples, at the beginning and at the end fermentation, were subjected to total DNA extraction using the Dneasy® Plant Mini Kit (Qiagen, Italy). To check DNA extraction, electrophoresis on agarose gel was carried out and the DNA concentration was determined using the fluorimeter Qubit 4.0 (Invitrogen, United States). DNA samples were storage at -20° C until use. The microbial community present in samples was assessed by DGGE analysis and PCR products were obtained using the primers U968-GC (5′-ACGCGAAGAACCTTAC-3′) and L1401-r (5′-GCGTGTACAAGACCC-3′), to amplify the V6 to V8 region of the 16S eubacterial rDNA and primers NL1 (5′-CGCCCGCGCGCGGCGGCGGGCCATATCAATAAGC-3′) and LS2 (5′-ATTCCCAAACAACTCGACTC-3′) to amplify the D1/D2 domain of the 26S rDNA. PCR amplification was performed in a 50 µL volume, using a Thermal Cycler 2720 (Applied Biosystems, USA). The reaction mixtures consisted of 1.25 U of Taq DNA polymerase (Invitrogen, USA), 20 mM Tris HCl (pH 8.4), 50 mM KCl, 3 mM MgCl2, 50 mM dNTPs, 5 pmol primers each and 2 µL of cDNA. The following amplification cycle was used for 16S: initial denaturation of DNA for 5 min at 94 °C; 35 cycles of 30 s at 94

°C, 30 s at 56 °C and 40 s at 68 °C; and extension of in-complete products for 7 min at 68 °C according to Pino et al. (2018). While the amplification cycle for 26S rDNA was: 5 min at 95 °C; 30 cycles for 60 s at 95 °C, 2 min at 52 °C and 72 °C for 2 min and a final extension at 72 °C for 7 min according to Durand et al., (2013). In detail, DGGE analysis was performed on the Dcode System equipment (BioRad, Italy). Electrophoresis was performed in a 0.8 mm thick 8% [w/v], acrylamide:bisacrylamide gel [37.5:1]. For 16S, separation was achieved using 40-60% urea-formamide denaturant gradient, increasing in the direction of electrophoresis. Whereas for 26S, a denaturant gradient in the range of 30-60% was used. A 100% denaturant corresponds to 7 M urea and 40% (v/v) formamide. The 16S gel was subjected to a constant voltage of 85 V at a temperature of 60 °C for 15 h, while the 26S gel was subjected to a constant voltage of 80 V at 60 °C for 12 h. Both gels were run in 0.5 x TAE buffer. DNA was visualized by silver staining and developed as previously described (Sanguinetti et al., 1994; Pino et al., 2018).

2.7 HPLC analyses

For HPLC analysis, the liquid fraction of all samples was used. HPLC analysis was performed by direct injection of filtered samples (0.45 μm PTFE filters, Merk, Germany) into the HPLC chromatographic system. The system consisted of a Waters Alliance 2695 HPLC liquid chromatography equipped with a Waters 996 photodiode array (PDA) detector set at 210 nm for organic acids and 280 nm for phenols. The instrument was equipped with Waters Empower software (Waters Corporation, USA). For the determination of organic acids, each sample was filtered and then injected into HPLC. Isocratic elution with 5 mM sulfuric acid was performed on a Rezex ROA Organic Acid H+ column (Phenomenex, Torrence, USA). The run time was set to 50 min at 0.6 mL/min. Pure standards at different concentrations were injected as external standards: lactic acid, citric acid, acetic acid, propionic acid, isobutyric acid, and butyric acid (all purchased from Sigma-Aldrich, Italy). All analyses were performed in triplicate for each sample. Regarding phenols, the chromatographic separation was performed according to Romeo et al. (2021). The column used was a Luna C18 (250 mm X 4.6 mm i.d., 5 m, 100 Å; Phenomenex, Torrence, USA) maintained at 40 °C in an oven. A flow rate of 1 mL/min was used. An internal standard (I.S.) of 50 mM of pure gallic acid (Fluka, Switzerland) was used to quantify phenolic compounds. Identification of phenolic compounds was obtained by comparing their retention time with pure hydroxytyrosol (HT), tyrosol

(TYR), chlorogenic acid, vanillic acid, caffeic acid, syringic acid p-coumaric acid, ferulic acid, verbascoside, luteolin-7-o-glucoside, o-coumaric acid, rutin, oleuropein, apigenin-7-o-glucoside, luteolin-4-glucoside, quercetin, luteolin, and apigenin (Extrasynthese, France). All analyses were performed in triplicate for each sample.

2.8 Volatile Organic Compounds analysis

A Teledyne Tekmar HT3 Headspace Analyser was used in the dynamic mode along with a Shimadzu GCMSQP2010 Ultra (Shimadzu Deutschland GmbH, Germany). An aliquot of 3.3 g of each sample was weighed into 20.0 mL headspace vial sealed with tops and Polytetrafluoroethylene (PTFE/silicon) Septa (Supelco, USA). The vials were then placed in the Headspace Analyser sample rack for analysis. Volatiles compounds (VOCs) were extracted under the conditions listed in Table S1 (supplementary material).

2.8.1 GC-MS analysis

All analyses were performed using a Shimadzu GCMSQP2010 Ultra (Shimadzu, Italy). Desorption of analytes was performed using a split/splitless injector, set at a temperature of 250 °C and equipped with a Restek split/splitless liner with the following dimensions: 3.5 mm x 5.0 mm x 95 mm (Restek, Italy). Analyte separation was accomplished on a Zebron ZB-5MS column (Phenomenex, USA), with an inner diameter of 0.25 mm, a length of 30 m, and a film thickness of 0.25 μm. The oven temperature was held at 40 °C for 3 min, then increased at 6 °C/min to 200 °C, and then increased at 15 °C/min to 250 °C for 10 min. The total run time was 43 min. The carrier gas was helium (purity > 99.999%) at a flow rate of 1.78 mL/min. The injection port temperature was 250 °C. The mass spectrometer (MS) was set to single ion monitoring (SIM) mode. Transfer line and ion source were both set to 280 °C. Total ion chromatograms and mass spectra were recorded in the electron impact ionization mode at 70 eV. Data were treated with full-scan methods. The compounds were identified by matching their mass spectra and to the US NIST (version 11) spectrum library.

2.9 Biological activity

2.9.1 Cytotoxic activity

Caco-2 cells were grown in Dulbecco's Modified Eagle Medium high glucose (DMEM high glucose) Caco-2 cells were a gift of Dr. Aldo Cavallini and Dr. Caterina Messa from the Laboratory of Biochemistry National Institute for Digestive Diseases "S. de Bellis", Bari (Italy). Cancer cell line representative of human hepatocellular liver carcinoma (Hep-G2) were purchased from American Type Culture Collection (ATCC). Hep-G2 cells were cultured in Eagle's Minimum Essential Medium (MEM,), supplemented with 1% Non-Essential Amino Acids (NEAA). Media were supplemented with 10% fetal bovine serum, 2 mm glutamine, 100 U mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin. Both cell lines were maintained in the logarithmic phase at 37 °C in a 5% CO_2-humidified air. Cell culture reagents were purchased from EuroClone (Milan, Italy). Cytotoxicity was performed by using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma-Aldrich, Italy). On the first day, 10.000 cells/well were seeded into 96-well plates in a final volume of 100 μL. Stock solutions were prepared just before their use. After 24 h, 100 μL of differently fermented POC samples, were added to each well at the appropriate dilution: as such, 1:10, 1:25, 1:50, and 1:100. After 72 h, MTT solution (10 μl, 0.5 mg/ml) freshly prepared was added to the cells, and incubated for 3-4 h at 37 °C. At the end of incubation, the supernatant was removed, and the formazan crystals were allowed to solubilize by using 100 μL of DMSO/Ethanol (1:1). Absorbance values (λ = 570 nm) were determined on a Victor 3 (PerkinElmer Life Sciences) microplate reader. Pure HT (Phytolab, Germany) was used as a positive control.

2.9.2 Permeability assay

Caco-2 cells were seeded onto a Millicell-96 assay system (Millipore, Italy) in which a cell monolayer was set in between a filter cell and a receiver plate, at a density of 20,000 cells/well. The culture medium was replaced every 48 h and the cells were kept in culture for 21 days. The Trans Epithelial Electrical Resistance (TEER) of the monolayers was measured daily before and after the experiment by using an epithelial voltohmmeter (Millicell-ERS). Generally, TEER values greater than 1000 Ω for a 21-day culture are considered optimal. After 21 days of Caco-2 cell growth, the medium was removed from the filter wells and the receiver plate, and they were filled with fresh Hank's balanced salt solution (HBSS) buffer (Invitrogen, Italy). This procedure was repeated twice, and the plates were incubated at 37 °C for 30 min. After the incubation time, the HBSS buffer was removed and POC samples (dilution 1:100) were added to the filter well whereas fresh HBSS was added to the receiver plate. The plates were incubated at 37 °C for 2 h. Afterward, samples were removed from the apical (filter well) and basolateral (receiver plate) side of the monolayer to measure the permeability. The apparent permeability (Papp) referred to HT in units of nm/second was calculated using the following equation (1):

$$P_{app} = \left(\frac{V_A}{Area \times time}\right) \times \left(\frac{[sample]acceptor}{[sample]initials}\right) \quad (1)$$

V_A = the volume (in mL) in the acceptor well; Area = the surface area of the membrane (0.11 cm^2 of the well); time = the total transport time in seconds (7200 s); [sample]acceptor = the concentration of the sample measured by U.V. spectroscopy; [sample]initial = the initial sample concentration (1 $x10^{-4}$ M) in the apical or basolateral wells.

2.9.3 Cyclooxygenase catalytic activity inhibition

The POC samples, differently fermented, were tested to evaluate their inhibitory activity and selectivity towards cyclooxygenase, a bifunctional enzyme exhibiting both cyclooxygenase and peroxidase activities, by using a colorimetric COX inhibitor screening assay kit (Catalog No. 7601050, Cayman Chemicals, USA) following the

manufacturer's instructions. The peroxidase activity of COXs that catalyzes the reduction of the endoperoxide PGG_2 into the corresponding alcohol (PGH_2), which is the precursor of PGs, thromboxane, and prostacyclin, was measured by monitoring the appearance of oxidized N, N, N', N'-tetramethyl-p-phenylenediamine (TMPD) at λ= 590 nm. Stock solutions of tested samples were solubilized in deionized distilled water.

2.9.4 Antioxidant activity

The DPPH absorbance values were evaluated at λ=517 nm by monitoring the kinetics for 5 min with a spectrophotometer (Shimadzu UV-1800, Denmark). Different dilutions of samples were added to the mixture of methanolic solution and 2,2-Diphenyl-1-picrylhydrazyl radical (DPPH) at 10^{-4} M. The radical scavenging activity was determined as % RSA (radical scavenging activity). The values were expressed by using the following equation (2):

$$RSA\ \% = \left(\frac{\text{Blank Absorbance} - \text{Sample Absorbance}}{\text{Blank Absorbance}}\right) \times 100 \quad (2)$$

Pure HT (Phytolab, Germany) was used as a positive control.

2.10 Data analyses

Statistical analysis of results was performed using a one-way analysis of variance (ANOVA), and Tukey's HSD post hoc test for means separation (significance level at $P \leq 0.05$), using the statistical software IBM SPSS Statistics for Windows, version 20 (IBM Corp., USA).

3. Results

3.1 Fermentation parameters

Fermentation was monitored in all samples until the stabilization of both pH and pO_2 was achieved. The monitoring of parameters was performed every 30 seconds in real time and recorded through the software connected to the fermenter (Figure 2). Regarding the pH, at the beginning of fermentation, the values ranged from 5.30 to 5.25 (Figure 2 A). In control sample, the pH reached the stability after 12 days of fermentation, when the value

of 4.48 was achieved. Whereas POC samples inoculated with *L. plantarum* exhibited the stable value of 4.33 after 9 days of fermentation. Similar trend was observed in samples fermented with *C. boidinii* and *W. anomalus*, for which the lowest pH values were reached after 10 days, as 4.09 and 4.60, respectively.

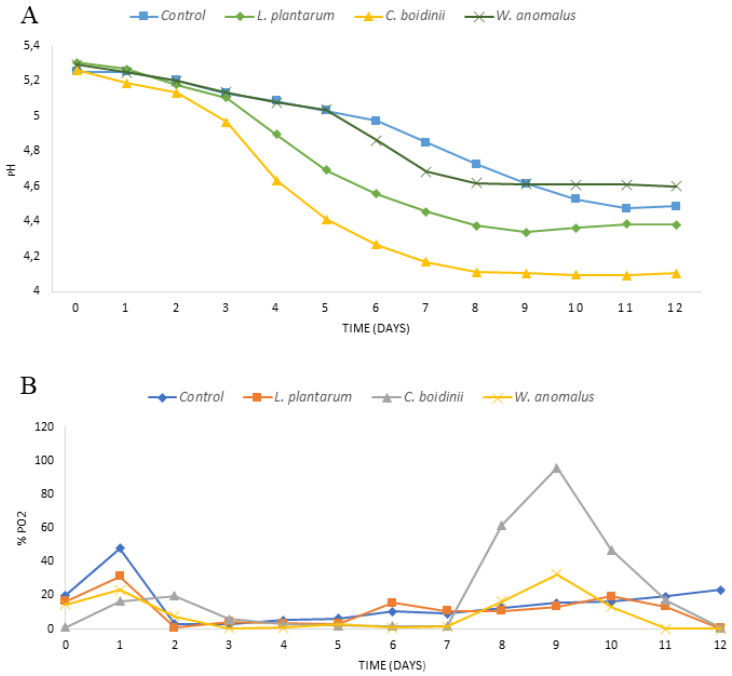


Figure 2. Data are expressed as means of pH and pO_2 (%) values obtained for each day of fermentation.

The pO_2 showed a variable trend, as reported in Figure 2 B. In details, during the first days of fermentation the control sample showed a higher increase of pO_2 value comparing to inoculated POC samples, to decrease until the 7th day of fermentation. Starting from the 7th day, a different trend was observed among samples, with POC fermented with *L. plantarum* similar to control. Regarding samples fermented with *C. boidinii* and *W. anomalus*, the pO_2 values exhibited a sudden increase after 9 days of fermentation when pO_2 reached values of 95.8% and 32.6% respectively. The pO_2 values dropped in samples inoculated with *C. boidinii* and *L. plantarum* after 12 days, and in sample inoculated with *W. anomalus* after 11 days.

3.2 Chemical and microbiological parameters

At the beginning and at the end of fermentation, TSS and total phenols were monitored. In addition, microbiological analyses were carried out using conventional methods (Table 1). Regarding TSS, at the beginning of fermentation, the detected average value was 8.85 °Brix. At the end of the process, in all samples, a significant decrease to values between 5.63 and 5.43 °Brix where detected, with the lowest values obtained in samples fermented with the two yeasts. For phenolic content, the range of initial values spanned from 2146.1 to 1215.0 mg/L. At the end of fermentation, a decrease in total phenols was observed in all samples, except in the sample inoculated with *C. boidinii*, where a slight increase, of 376.1 mg/L, was observed.

Table 1. Chemical and microbiological parameters in POC samples differently inoculated at initial and final fermentation.

Sample	Time (days)	TSS (°Brix)	Total phenol (mg/L)	LAB (Log10 CFU/g)	Yeasts (Log10 CFU/g)	Mesophilic aerobic bacteria (Log10 CFU/g)
Control	0	9.26±0.01^{a}	1744.2±0.54^{c}	2.84±0.01^{a}	5.63±0.04^{b}	2.02±0.03^{b}
L. plantarum	0	9.16±0.04^{a}	2146.1±1.09^{a}	2.73±0.38^{a}	4.02±0.03^{d}	3.09±0.12^{a}
C. boidinii	0	8.44±0.01^{b}	1215.0±0.52^{d}	0.00±0.00^{b}	6.77±0.01^{a}	3.04±0.06^{a}
W. anomalus	0	8.52±0.01^{b}	1772.7±0.54^{b}	0.00±0.00^{b}	4.49±0.02^{c}	3.15±0.03^{a}
		**	**	**	**	*
Control	EP	5.57±0.01^{a}	1316.5±0.51^{c}	3.14±0.20^{a}	6.78±0.17^{b}	3.23±0.17^{a}
L. plantarum	EP	5.63±0.02^{a}	1363.5±3.81^{b}	3.54±0.06^{a}	6.98±0.03^{b}	2.04±0.03^{b}
C. boidinii	EP	5.49±0.01^{b}	1591.1±0.54^{a}	3.04±0.06^{a}	5.50±0.03^{c}	2.00±0.01^{b}
W. anomalus	EP	5.43±0.02^{b}	1370.4±0.44^{b}	0.00±0.00^{b}	7.80±0.04^{a}	2.19±0.16^{b}
		*	**	*	**	*

Data are expressed as mean ± standard deviations. Mean values with different letters within the same column at the same time interval is statistically different. *Significance at $P \leq 0.05$; **Significance at $P \leq 0.01$.

Microbiological analyses revealed a dominance of the yeast group both at the beginning and end of fermentation in all samples. Overall, yeast cell density increased in all samples reaching an average value of 7.18 Log10 CFU/g, except in sample inoculated with *C. boidinii*, where a 1 Log unit decrease was observed. Regarding LAB at the initial time, their densities were detected at low values in all samples, except in control and in samples inoculated with *L. plantarum*, where a 2.84 and 2.73 Log10 CFU/g were counted. Furthermore, their densities remained quite constant, until the end of fermentation, maintaining values between 3.54 and 3.04 Log10 CFU/g, respectively. However, at the end of fermentation, in samples with *C. boidinii* a 3 Log unit increase was observed, while in samples inoculated with *W. anomalus* they were never detected. Regarding mesophilic

aerobic bacteria, they showed an increase of 1 Log unit in control sample, while a decrease in all inoculated samples was observed. Finally, *E. coli*, *Staphylococcus* spp. and *Clostridium perfringens* were never detected in any samples.

3.3 Biodiversity and dynamics of yeasts and bacteria

The diversity of both bacterial and fungal communities was explored by PCR-DGGE analysis performed at the beginning and at the end of fermentation. The profiles obtained from POC differently fermented are reported in Figure S1 A and B, where the bacterial and fungal dynamics were shown, respectively. Overall, the bacterial profile showed a lower biodiversity compared to those detected for yeasts. However, comparing DGGE profile obtained at the end with profile obtained at the beginning of fermentation, a slight shift in bacterial community was observed in samples fermented with the two yeast strains *W. anomalus* and *C. boidinii* (Figure S1 A, lines 6 and 8, respectively). Zooming on yeast community, although in control samples the PCR-DGGE profiles obtained at beginning and at the end of fermentation were quite overlapping, a slight change was observed, in all inoculated samples (Figure S1 B, lines 4, 6, and 8).

3.4 Organic acids and phenol detection

At the beginning and at the end of fermentation, the content of organic acids and phenols were evaluated by HPLC (Table 2 and 3). At both sampling times the citric, propionic, acetic, lactic, and butyric acids were found (Table 2). Regarding citric acid, at the end of fermentation, an increase was observed in the POC sample inoculated with *C. boidinii*, which reached a value of 6552.5 mg/L. Zooming on lactic acid, the highest concentrations were detected in control and in samples inoculated with *L. plantarum*, while it decreased in samples inoculated with the two yeasts. The acetic acid content increased greatly in all samples. In detail, in the control sample it reached the highest concentration (13048.7 mg/L) while the lowest concentration was found in sample fermented *W. anomalus* (8551.0 mg/L). At the end of fermentation, isobutyric and propionic acids decreased in all samples, with a slight increase (244 mg/L) detected for propionic acid in samples fermented with *W. anomalus*. No butyric acid was found in any samples at each sampling time (Table 2).

Table 2. Organic acids detected by HPLC analysis in POC samples differently inoculated at initial and final fermentation.

Sample	Time (days)	Citric acid	Lactic acid	Acetic acid	Propionic acid	Isobutyric acid
Control	0	6965.0±81.83^{b}	3670.0±29.55^{a}	2267.2±3.02	3411.3±12.04^{a}	5527.4±59.84^{a}
L. plantarum	0	7047.7±74.62^{a}	3491.8±218.13^{ab}	3203.5±76.64	3491.6±180.65^{a}	5296.0±414.93^{a}
C. boidinii	0	5895.2±16.94^{c}	2672.7±6.27^{c}	2907.9±48.53	2745.5±24.07^{b}	4301.1±86.24^{b}
W. anomalus	0	6106.8±3.93^{c}	3048.9±6.44^{bc}	3284.0±8.62	2698.8±3.89^{b}	3210.3±2.73^{c}
		**	*	n.s.	*	*
Control	EP	5962.6±43.92^{b}	3636.1±76.96^{a}	13048.7±196.51^{a}	3271.2±46.70^{a}	1401.5±58.47
L. plantarum	EP	6735.1±69.67^{a}	3578.2±60.14^{a}	12685.1±281.81^{a}	3072.4±226.90^{a}	1970.2±370.55
C. boidinii	EP	6552.5±39.15^{a}	2536.9±13.28^{c}	12522.8±108.65^{a}	2202.5±138.35^{b}	1582.0±255.20
W. anomalus	EP	5692.6±21.22^{c}	2990.5±25.95^{b}	8551.0±101.34^{b}	2942.8±13.50^{a}	1953.4±20.90
		**	*	*	*	n.s.

Data are expressed as means in mg/L ± SD. Mean values with different letters within the same column at the same time interval are statistically different. n.s. not significant, *Significance at $P \leq 0.05$; **Significance at $P \leq 0.01$.

Results regarding the detected phenol content are reported in Table 3. At initial time, in all samples, HT, TYR, chlorogenic acid, vanillic acid, caffeic acid, rutin, luteolin and apigenin were detected (Table 3). In all samples the HT was the phenol highest detected at initial time, with values ranging between 355.4 and 254.5 mg/L. At the end of fermentation, a decrease in HT was observed in all samples, with the highest decrease in control sample, where its concentration dropped to 120.1 mg/L; TYR increased in all samples reaching the highest value (103.6 mg/L) in control sample. While caffeic acid showed a decreasing trend in all samples, except in sample inoculated with *C. boidinii* where its concentration remained quite unchanged. Chlorogenic acid, vanillic acid, rutin, luteolin, and apigenin were not detected at the end of fermentation in any sample. Oleuropein was never detected in any samples.

Table 3. Phenols detected by HPLC analysis in POC samples differently inoculated at initial and final fermentation.

Sample	Time	Hydroxytyrosol	Tyrosol	Chlorogenic acid	Vanillic acid	Caffeic acid	Rutin	Oleuropein	Luteolin	Apigenin
Control	0	331.6±0.59^{b}	20.88±0.04^{b}	15.48±0.16^{d}	23.02±0.24^{a}	54.75±0.06^{a}	70.39±0.01^{b}	n. d.	44.58±0.31^{c}	52.07±0.01^{a}
L. plantarum	0	379.3±5.43^{a}	46.42±0.77^{a}	47.14±0.25^{a}	23.09±0.44^{a}	56.88±0.37^{a}	55.86±0.66^{c}	n. d.	47.52±0.12^{b}	47.57±0.59^{b}
C. boidinii	0	254.5±7.36^{c}	17.71±1.02^{c}	29.85±0.33^{c}	19.65±0.55^{b}	33.81±0.09^{c}	52.38±1.54^{c}	n. d.	53.95±0.87^{a}	42.73±0.01^{c}
W. anomalus	0	355.4±1.56^{b}	23.45±0.17^{b}	38.52±0.42^{b}	19.95±0.54^{b}	45.10±1.46^{b}	80.85±0.58^{a}	n. d.	52.29±0.94^{a}	51.61±0.02^{a}
		**	**	**	*	**	**		**	**
Control	EP	120.1±1.55^{c}	103.6±1.34^{a}	n. d.	n. d.	33.62±1.53^{ab}	n. d.	n. d.	n. d.	n. d.
L. plantarum	EP	161.0±0.07^{a}	67.01±0.90^{c}	n. d.	n. d.	36.79±0.65^{a}	n. d.	n. d.	n. d.	n. d.
C. boidinii	EP	141.8±1.66^{b}	61.13±1.07^{d}	n. d.	n. d.	32.21±0.67^{b}	n. d.	n. d.	n. d.	n. d.
W. anomalus	EP	152.0±4.49^{ab}	84.68±0.79^{b}	n. d.	n. d.	32.43±0.98^{b}	n. d.	n. d.	n. d.	n. d.
		**	**			*				

Data are expressed as mg/L of means ± SD. Mean values with different letters within the same column at the same time interval are statistically different.
*Significance at $P \leq 0.05$; **Significance at $P \leq 0.01$; n.d. not detected.

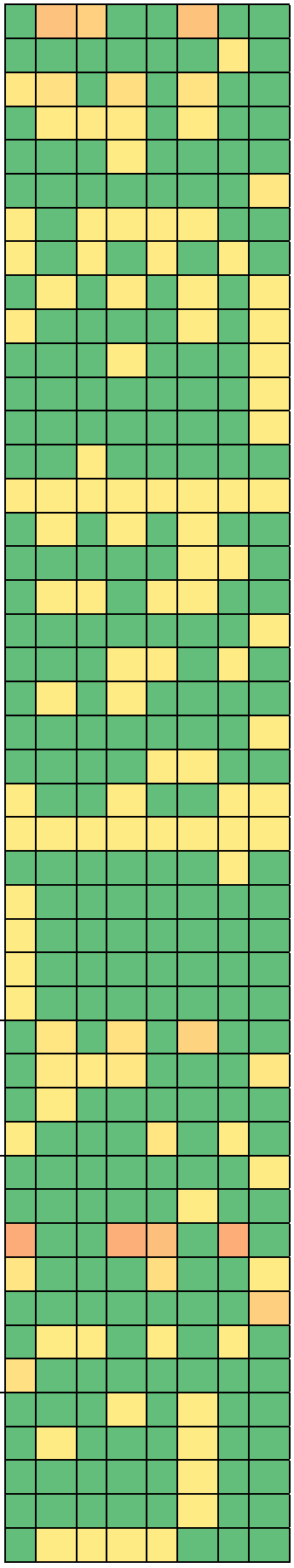
3.5 Volatile Organic Compounds Analysis

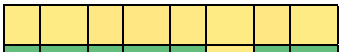
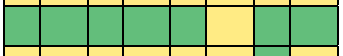
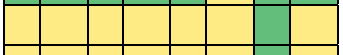
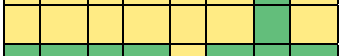
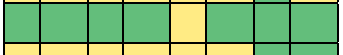
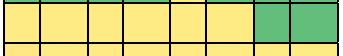
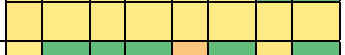
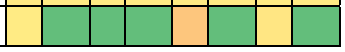
Volatile compounds detected at initial and at the end of fermentation in differently inoculated POC samples, revealed 131 identified volatile compounds, and their relative intensities are shown as a heatmap (Table 4). Fermentation process, performed by different strains, produced significant differences in terms of volatile profiles in POC samples. Furthermore, significant change in volatile profiles were detected in differently inoculated samples at the end of fermentation.

Table 4. Heatmap of POC samples showing area of compounds obtained by HS-GC/MS (green color means low peak area, red color is high peak area).

Chemical class	Compound	A	A1	B	B1	C	C1	D	D1
alcohols	1-Propanol, 2-methyl-								
	Ethanol								
	L-Threitol								
	1-Butanol, 2-methyl-								
	1-Pentanol								
	1-Butanol, 3-methyl-								
	1-Hexanol, 3-methyl-								
	2-Hexanol, 3-methyl-								
	1-Octen-3-ol								
	1-Pentanol, 2-methyl-								
	Phenylethyl Alcohol								
	Tyrosol								
	Homovanillyl alcohol								
	Glycidol								
	4-Hexen-1-ol, (Z)-								
aldehydes	Acetaldehyde								
	Propanal, 2-methyl-								
	Butanal, 3-methyl-								
	Butanal, 2-methyl-								
	Hexanal								
	2-Hexenal								
	Heptanal								
	2-Heptenal, (Z)-								
	Benzaldehyde								
	Octanal								
	Benzeneacetaldehyde								
	2-Octenal, (E)-								
	Nonanal								
	2-Nonenal, (E)-								
	2-Decenal, (E)-								
	2-Tridecenal, (E)-								

	2,4-Decadienal, (E,E)-	
	Vanillin	
carbon dioxide	Carbon dioxide	
esters	Methyl formate	
	Acetic acid, methyl ester	
	Ethyl Acetate	
	Propanoic acid, 2-methyl-, methyl ester	
	Propanoic acid, ethyl ester	
	Propanoic acid, 2-methyl-, ethyl ester	
	Acetic acid, butyl ester	
	Methyl isovalerate	
	Butanoic acid, ethyl ester	
	Pentanoic acid, methyl ester	
	Ammonium acetate	
	2-Penten-1-ol, acetate	
	2-Pentanol, acetate	
	Butanoic acid, 2-methyl-, ethyl ester	
	Ethyl isovalerate	
	Butanoic acid, 3-methyl-, ethyl ester	
	Formic acid, hexyl ester	
	1-Butanol, 3-methyl-, acetate	
	1-Butanol, 2-methyl-, acetate	
	Hexanoic acid, methyl ester	
	Sulfurous acid, 2-ethylhexyl isobutyl ester	
	Hexanoic acid, ethyl ester	
	Acetic acid, hexyl ester	
	Sulfurous acid, 2-ethylhexyl hexyl ester	
	Benzoic acid, methyl ester	
	Heptanoic acid, ethyl ester	
	Octanoic acid, methyl ester	
	Acetic acid, 2-ethylhexyl ester	
	Acetic acid, phenylmethyl ester	
	Octanoic acid, ethyl ester	
	Acetic acid, octyl ester	
	Nonanoic acid, methyl ester	
	Acetic acid, 2-phenylethyl ester	
	Acetic acid, nonyl ester	
	Decanoic acid, methyl ester	
	Benzoic acid, 4-formyl-, methyl ester	
	Hexadecanoic acid, methyl ester	
	Hexadecanoic acid, ethyl ester	
	9,12-Octadecadienoic acid, methyl ester	
	9-Octadecenoic acid (Z)-, methyl ester	
	9,12-Octadecadienoic acid, ethyl ester	
	(E)-9-Octadecenoic acid ethyl ester	
hydrocarbons	Pentane, 3-methyl-	

	Cyclopentane, methyl-	
	Propane, 2-fluoro-	
	Cyclohexane	
	1-Pentene, 2,4,4-trimethyl-	
	Pentane, 1-chloro-	
	3,4-Diethyl hexane	
	Toluene	
	1-Octene	
	Octane	
	Heptane, 2,4-dimethyl-	
	Pentane, 1-bromo-	
	2,4-Dimethyl-1-heptene	
	Octane, 4-methyl-	
	1-Hexene, 5-methyl-	
	Styrene	
	Hexane, 1-bromo-	
	Heptane, 2,3,5-trimethyl-	
	3-Ethyl-1,5-octadiene	
	Heptane, 3,3,5-trimethyl-	
	2-Octene, 2-methyl-6-methylene-	
	Octane, 1-chloro-	
	Decane, 3,6-dimethyl-	
	Naphthalene	
	Azulene	
	5-Octadecene, (E)-	
	Heptadecane, 2,6,10,15-tetramethyl-	
	Hexane, 1-methoxy-	
	Ethylbenzene	
	Benzene, 1,3-dimethyl-	
	Cyclopropane, 1,1-dimethyl-2-(2-propenyl)-	
ketones	2-Propanone, 1-hydroxy-	
	3-Pentanone	
	Acetophenone	
	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	
others	Propyl isopropyl ether	
	Formic acid hydrazide	
	Dimethyl sulfide	
	Dimethyl ether	
	Hydrazine, methyl-	
	Furan, 2-pentyl-	
	Allyl ethyl ether	
terpenes	o-Xylene	
	1R-(+)-alpha-Pinene	
	beta-Pinene	
	beta-Myrcene	
	o-Cymene	

	D-Limonene	
	Eucalyptol	
	beta-Ocimene	
	beta-Citronellol, chlorodifluoroacetate	
	(+)-4-Carene	
	(+)-Cyclosativene	
	alfa-Copaene	
volatile acids	Acetic acid	

Legend: A = control at initial time; A1 = control at the end of fermentation; B = sample inoculated with *C. boidinii* at initial time; B1 = samples inoculated with *C. boidinii* at the end of fermentation; C =sample inoculated with *L. plantarum* at initial time; C1 = sample inoculated with *L. plantarum* at the end of fermentation; D = sample inoculated with *W. anomalus* at initial time; D1 = sample inoculated with *W. anomalus* at the end of fermentation

At the end of fermentation, the control sample (A1) showed a significant increase of esters and hydrocarbons and a decrease of alcohols and aldehydes. Acetic acid methyl ester, ethyl acetate and propanoic acid 2-methyl- methyl ester were the major representative compounds among esters, while cyclopentane methyl- and cyclohexane were the major representative compounds within hydrocarbons. The sample inoculated with *C. boidinii* (B1) after fermentation showed an increase of alcohols and a decrease of aldehydes, esters and hydrocarbons. Ethanol was the major representative among alcohols. The sample fermented with *L. plantarum* (C1) showed an increase of esters, hydrocarbons and ketones and a decrease of alcohols, aldehydes and volatile acids. While 1-butanol 2-methyl- was the major representative compound within alcohols, while cyclopentane methyl- and cyclohexane were the major representative hydrocarbons and acetic acid methyl ester, ethyl acetate and propanoic acid 2-methyl- methyl ester were the major representative esters. Finally, the fermented sample inoculated with *W. anomalus* (D1) showed an increase of alcohols and a decrease of aldehydes. Ethanol and L-Threitol were the major representative alcohols. Identified volatiles belonged to the following chemical classes (Table S2): alcohols, esters, terpenes, hydrocarbons, aldehydes, ketones and volatile acids. As expected, the two yeasts increased the alcohol content during fermentation reaching the highest 66.04% in samples fermented with *W. anomalus*. On the contrary, *L. plantarum* showed a great lowering effect at the end of fermentation. Regarding esters, the highest increase, at the end of fermentation, was detected in control and in samples fermented with *L. plantarum*. However, the highest volatile acid value was found in the sample inoculated with *L. plantarum* immediately after the inoculum. In addition, the presence of the terpene D-Limonene was observed in all samples, and its content increased in control samples at the end of fermentation (A1) and in the sample

fermented with *C. boidinii* (B1) whereas decreased in samples inoculated with *L. plantarum* and with *W. anomalus* (C1 and D1, respectively) (data not shown).

3.6 Biological assay

3.6.1 Transepithelial POC samples transport through Caco-2 cell monolayers

To look into the physiological effects of bioactive POC samples, it has been necessary to understand how effectively they are absorbed from the intestine. Preliminary experiments proved that POC samples diluted at 1:25 can be safely used because no cytotoxic effect was observed after incubation with Caco2 and Hep-G2 cell lines (data not shown). The safe concentration (1:25) was used for all experiments. Apparent permeability (Papp) values for both directions, from basolateral to apical (BA) and reversed flow (AP), and efflux ratios (BA/AB ratio) are displayed in Table S3 (supplementary material) (Colabufo et al., 2013). The smaller is the BA/AB ratio value, the greater is the contribution of the active transport to the membrane crossing, which results in a significant involvement of the efflux pumps in controlling sample crossing since they are recognized. All the tested samples demonstrated preferential passive transport as showed by the high value of Papp AB (Table S3). This occurs mainly in samples inoculated with *W. anomalus* for which a Papp value of 634 nm/s was detected.

3.6.2 Activity on COX-1 and COX-2

POC samples potential involvement in anti-inflammatory processes through their capacity to inhibit cyclooxygenases (Calvello et al., 2017) in comparison with HT was investigated. All POC samples showed a low capacity to inhibit COXs isoforms as shown in Table S4. Only samples inoculated with *W. anomalus* determined a higher inhibition of COX-2 catalytic activity (11.34%), while the fermentation with *C. boidinii* exhibited a preferential COX-2 inhibitory activity since no inhibition was observed for COX-1 isoform.

3.6.3 Antioxidant activity

Both natural and synthetic antioxidants are therefore of great interest in preventing and treating diseases. In this context, differently fermented POC samples were evaluated for their reactivity with 1,1-diphenyl-2-picrylhydrazyl (DPPH), a stable violet-colored radical that absorbs strongly at $\lambda = 517$ nm, allowing to assess their potential efficacy as stable free radical scavengers. Antioxidant assay was performed to determine whether the known HT antioxidant activity could be comparable to that of fermented POC samples. The data showed that POC samples exhibited a lower antioxidant activity compared to pure HT. At all concentrations tested, the best antioxidant activity was obtained in the sample inoculated with *W. anomalus*, with a %RSA ranging from 34.87 and 48.53 (Figure S2 D and A, respectively).

4. Discussion

In this study, POC was subjected to a driven fermentation using different microbial strains, to investigate how microorganisms can affect qualitatively and quantitatively the bioactive molecules and the volatiloma profile. The POC is a by-product of considerable interest as it could be reused as a new fermented food, food additive but also in nutraceutical and pharmaceutical industry (Foti et., 2022). To date, however, there are few studies in literature in which the spontaneous or driven fermentation strategy were used. In this study, three microorganisms, *L. plantarum*, *W. anomalus* and *C. boidinii*, previously isolated from natural fermented olives, were used in single culture. The choice of using microorganisms isolated from complex food matrices such as wine, olive wastewater and brines, substrates characterized by the presence of interfering compounds (polyphenols, salt, alcohols), allows a higher survival and adaptation to similar matrices (Tufariello et al., 2019). Fermentation was conducted in a bioreactor, which allowed the process parameters to be set and monitored in order to be replicable. Indeed, the possibility of using a kinetic model of the treatment systems plays a key and fundamental role in developing and controlling more stable processes for industrial scale-up (Ripoll et al., 2022). At the start of fermentation, the pH value of the different POC samples was between 5.30 and 5.25 in agreement with the results reported by Lanza et al. (2021). In detail, the POC pH values were very similar to those reported in the Leccino and Carboncella cultivars of 5.28 and 5.13 respectively.

In this study, the partial oxygen pressure showed a different variation in the fermentation theses and a greater increase was recorded in the theses fermented with the two yeasts. In detail, the thesis inoculated with *C. boidinii* showed a significant increase from time 7 to the end of fermentation, reaching a maximum value of 95.80% pO_2, while the thesis inoculated with *W. anomalus* reached a value of 32.62%. According to Dai et al. (2022) during alcoholic fermentation, molecular oxygen is an essential component for yeast cells to produce those compounds, including sterols and unsaturated fatty acids, which significantly influence ethanol tolerance and fermentation capacity. Microbiological analyses showed a low presence of microorganisms. In general, there was an increase in LAB, yeasts, and a partial decrease in the total aerobic mesophilic load in all inoculated samples. In detail, the most abundant microbial population was represented by yeasts with a cell density at the end of fermentation always higher than 5.50 Log CFU/g. These results were confirmed by PCR-DGGE which showed a higher biodiversity in yeast community compared to bacteria population. However, the slight shift in microbial dynamics suggests that the indigenous yeast microbiota is dominant in fresh POC matrix. These results agreed with data reported by Lanza et al. (2020) in fresh POC of different cultivars, spontaneously fermented, where a variable but low LAB densities were detected at the end of fermentation, whereas a high density of yeast were found. Furthermore, in the present study *E. coli*, *Staphylococcus* spp. and *Clostridium perfringens* were never detected in any samples, according to Tuffariello et al. (2019) which, unlike our study, sterilized POC. The phenolic profile of the different samples varied during the fermentation with a general lowering at the end of process. In detail, at the end of fermentation, the most representative phenols were HT, TYR, and caffeic acid. The HT were detected at a slightly higher concentration in inoculated samples, while the TYR were detected at higher concentration in control sample. These results suggest that microbial strains perform a protective effect on HT oxidation. Moreover, the change in organic acid contents suggest the simultaneously sugar consumption, and the increase in lactic, citric and acetic acid have been already reported in inoculated black and green table olives (Tufariello et al., 2015; Nychas et al., 2022). Furthermore, in the present study, their increasing production resulted in a constant decrease in pH, during fermentation. Specifically, it is interesting to highlight that the concentration of acetic acid increased in all samples, above all in control sample, where 13048.7 mg/L were detected. It is known that among microbial groups involved in

fermented foods, acetic acid bacteria, as *Acetobacter aceti*, *Acetobacter xylinum*, *Acetobacter peroxidans* and *Acetobacter pasteurianus*, play a key role in oxidize ethyl alcohol and other organic compounds into acetic acid, considerably affecting the aroma and the VOCs profiles of final products (Seesaard et al., 2022). In the present study, a significant increase in esters was obtained in POC samples fermented with *L. plantarum* where their concentration was triplicated from 13.35% to 65.07%. These results are in accordance with those reported by Tufariello et al. (2019) in sterilized POC fermented with *Leuconostoc mesenteroides*. Moreover, similar results were reported by Randazzo et al. (2014) and Pino et al. (2019), in naturally fermented table olives, where the use of LAB cultures affected the aromatic profile, in terms of VOCs abundance, related to esters. Regarding aldehydes the decrease observed in the present study, is in accordance to results recently reported by Yang et al. (2022) who, working on different fermented fruits (peach), suggested that the aldehydes decrease affect mainly affect the total volatile organic compounds, right after 24 h of fermentation. Based on their instability, aldehydes are easily reduced to alcohols or oxidized to acids in food matrices, which lead to a general decrease at later fermentation (Cagno et al., 2017). This trend was confirmed in the present study in all samples since starting from an initial value of about 20% aldehydes dropped to a range between 0.10 and 1.64%. Yeast metabolic activities usually induce an increase of some volatile classes such as alcohols, esters, terpenes, hydrocarbons (Tufariello et al., 2019). In this study, a great increase of alcohols was obtained in samples inoculated with the two yeasts, whereas esters, terpenes and hydrocarbons decreased during fermentation. Moreover, *L. plantarum* showed a great lowering effect, as obtained by Vaccalluzzo et al. (2022) in table olives fermented with a mix of LAB. In vitro biological characterization of POC samples showed no cytotoxic effect on Caco-2 and Hep-G2 cell lines, used as models of the human intestine and liver, respectively. Moreover, it should be highlighted that POC fermented with *W. anomalus* exhibited the best performances, showing the highest COX-2 inhibition activity, Papp AB (nm/s) and antioxidant effect. This is probably due to the ability of the *W. anomalus* strain to produce certain metabolites or activities that modify the functional profile of fermented POC. It has been already reported that some yeasts isolated from table olives are able to produce like-glucosidase enzymes and to exert probiotic activity (Porru et al., 2018; Bonatsou et al., 2018). *W. anomalus* (formerly known as *Pichia anomala* or *Hansenula anomala*) exhibits several probiotic characteristics, such as the ability to

remove cholesterol (Zullo et al., 2019), and it has been pictured as a fascinating source of various enzymes relevant for biotechnology applications (Padilla et al., 2018). Moreover, it has been found that the antioxidant effect of HT is not only related to the scavenging of oxidant chemical species, but also to simultaneously stimulation of the activities or synthesis of antioxidant enzymes (Forman et al., 2014). Antioxidants can in vivo generate reactive species (oxidants) that promote the activation of signaling pathways involved in recognizing the presence of free radicals. Consequently, low concentrations of antioxidants in food and beverages may positively affect the nutritional food value (Forman et al., 2014). The results of the present study agree with Cecchi et al. (2018) that proposed POC as a green source of bioactive compounds, comparable to that of pure HT.

5. Conclusions

The POC, obtained from the DMF two-phase extraction, is a by-product of olive oil process still rich in bioactive compounds. For this reason, it may represent a promising matrix potentially suitable for the formulation of new functional foods and/or supplements or ingredients. However, to improve its taste and the consumer's acceptability the POC needs to be treated to reduce its bitterness. Therefore, the fermentation with selected microbial strains appears as a relevant strategy to drive in a standardized way the debittering process avoiding abnormal fermentations. In this study, the used strains successfully fermented products with an effect on VOCs profiles. *L. plantarum* positively affect the ester concentration whereas the two yeasts mostly affect the alcohols. Moreover, the driven fermentation showed an improvement of the biological activity, as the antioxidant and anti-inflammatory potential. In conclusion, the results reported in this study provide insights to explore how other microbial strains could enhance nutritional and sensorial traits of this matrix.

Supplementary material

Table S1. Conditions used for Volatiles compounds (VOCs).

Teledyne Tekmar HT3 Headspace Analyzer Parameters	
Variable	Value
Valve Oven Temp	150°C
Transfer Line Temp	160°C
Standby flow rate	50 mL/min
Trap Standby Temp	30°C
Trap Sweep Temp	0°C
Platen/Sample Temp	40°C
Sample Preheat Time	30.00 min
Preheat Mixer	On
Preheat Mixing Level	Level 5
Preheat Mixing Time	5.00 min
Preheat Mixer Stabilize Time	2.00 min
Sweep Flow Rate	50 mL/min
Sweep Flow Time	30.00 min
Dry Purge Time	3.00 min
Dry Purge Flow	100 mL/min
Dry Purge Temp	25°C
Desorb Preheat	200°C
Desorb Temp	220°C
Desorb Time	1.00 min
Trap Bake Temp	220°C
Trap Bake Time	5.00 min
Trap Bake Flow	450 mL/min

Figure S1. DGGE profiles of bacteria (A) and yeasts (B) in differently inoculated POC samples at initial and final fermentation.

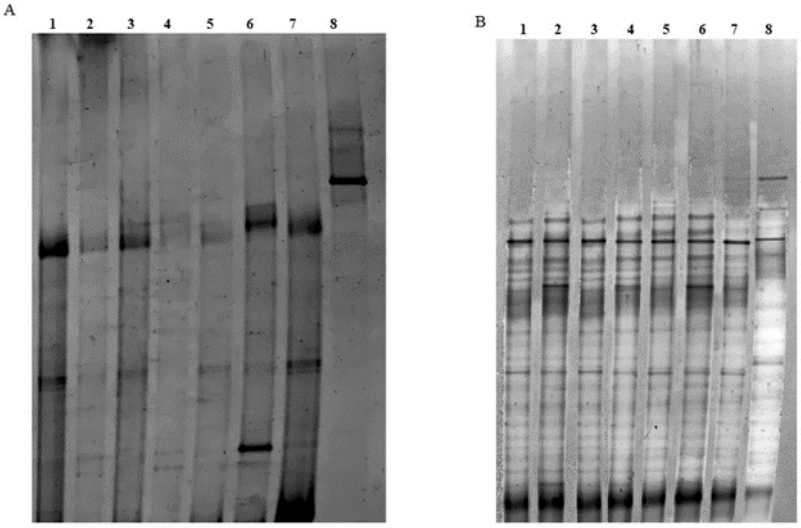


Table S2. Chemical classes of volatiles (as %) identified in POC samples differently inoculated at initial and final fermentation.

Samples	Alcohols	Aldehydes	Hydrocarbons	Esters	Ketones	Terpenes	Volatile acids	Carbon dioxide	Others
A	34.36±4.23^{b}	15.02±3.32^{b}	0.64±0.40^{bc}	2.29±0.34^{e}	0.15±0.02^{c}	2.62±0.29^{a}	0.17±0.01^{b}	4.49±0.43^{a}	34.87±4.35^{a}
A1	2.23±0.18^{d}	0.13±0.04^{c}	24.34±4.20^{a}	73.85±2.42^{a}	3.43±0.42^{bc}	0.46±0.03^{b}	0.00±0.00^{b}	0.63±0.03^{de}	0.05±0.00^{d}
B	6.53±0.34^{d}	25.55±4.14^{a}	26.71±2.91^{a}	34.38±2.57^{c}	0.64±0.03^{c}	2.31±0.24^{a}	0.00±0.00^{b}	2.56±0.32^{b}	0.18±0.01^{d}
B1	34.61±4.12^{b}	1.39±0.27^{c}	8.20±0.13^{b}	13.69±4.49^{d}	6.57±0.36^{b}	2.55±0.38^{a}	0.00±0.00^{b}	1.35±0.34^{cd}	25.39±2.64^{b}
C	24.22±3.47^{c}	14.40±3.48^{b}	2.71±0.35^{bc}	13.74±1.37^{d}	2.44±0.30^{c}	2.43±0.35^{a}	14.34±2.66^{a}	2.38±0.38^{b}	22.53±2.07^{b}
C1	1.55±0.38^{d}	0.13±0.01^{c}	25.53±2.87^{a}	64.08±3.05^{b}	12.72±3.37^{a}	0.65±0.04^{b}	0.00±0.00^{b}	0.66±0.02^{cde}	0.09±0.00^{d}
D	26.57±3.12^{bc}	25.32±2.17^{a}	2.46±0.35^{bc}	13.57±3.76^{d}	0.44±0.05^{c}	2.35±0.30^{a}	2.30±0.30^{b}	1.49±0.38^{c}	24.27±1.86^{b}
D1	66.04±2.83^{a}	0.24±0.04^{c}	1.50±0.33^{c}	16.13±3.14^{d}	1.28±0.37^{c}	0.25±0.02^{b}	0.00±0.00^{b}	0.27±0.01^{e}	14.11±3.65^{c}

Legend: A = control at initial time; A1 = control at the end of fermentation; B = sample inoculated with *C. boidinii* at initial time; B1 = samples inoculated with *C. boidinii* at the end of fermentation; C =sample inoculated with *L. plantarum* at initial time; C1 = sample inoculated with *L. plantarum* at the end of fermentation; D = sample inoculated with *W. anomalus* at initial time; D1 = sample inoculated with *W. anomalus* at the end of fermentation. Data are expressed as mean ± standard deviations. Mean values with different letters within the same column are statistically different ($P \leq 0.05$).

Table S3. Apparent permeability of POC samples fermented with the three strains.

Samples	HT (mg/L)	Papp BA (nm/s) Passive transport	Papp AB (nm/s) Active transport	BA/AB ratio	λ (nm)	ε
Control	1.20	2507	392	5.25	285	1.23
L. plantarum	1.60	1823	582	3.13	285	1.64
C. boidinii	1.41	2140	416	5.14	285	1.67
W. anomalus	1.52	2850	634	4.49	285	1.73

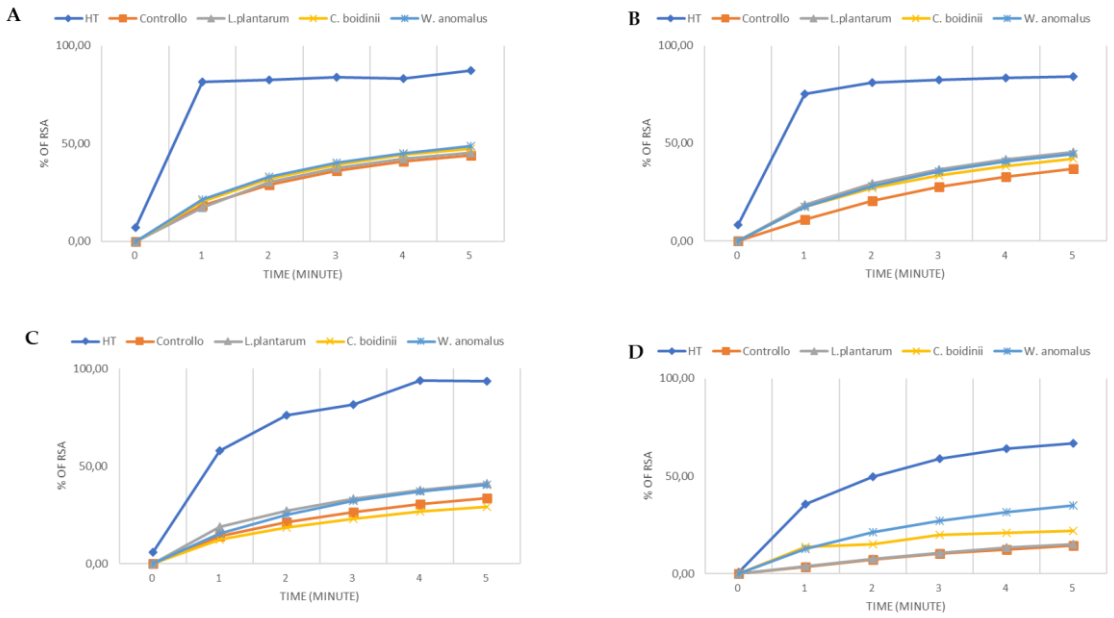
All samples were tested at a dilution of 1:100. In the table BA indicates basolateral to apical transport; AB indicates apical to basolateral transport; BA/AB values are from Papp AP−BL/Papp BL−AP.

Table S4. Cyclooxygenase percentage inhibition of POC samples fermented with the three strains.

Sample	mg/L of HT	% Inhibition	
		COX-1	COX-2
Control	4.80	1.14	6.28
L. plantarum	6.44	5.09	6.55
C. boidinii	5.57	0.00	9.27
W. anomalus	6.8	4.28	11.34

All samples were tested at a dilution of 1:25, calculated from the HT content found in HPLC.

Figure S2. POC samples antioxidant effect expressed as % RSA. Each graph corresponds to a volume (µL) used for each sample: A) 50 µL of samples; B) 37.5 µL of samples; C) 25 µL of samples; D) 12.5 µL of sample.



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Food Chemistry

Effect of microbial fermentation on functional traits and volatiloma profile of pâté olive cake

--Manuscript Draft--

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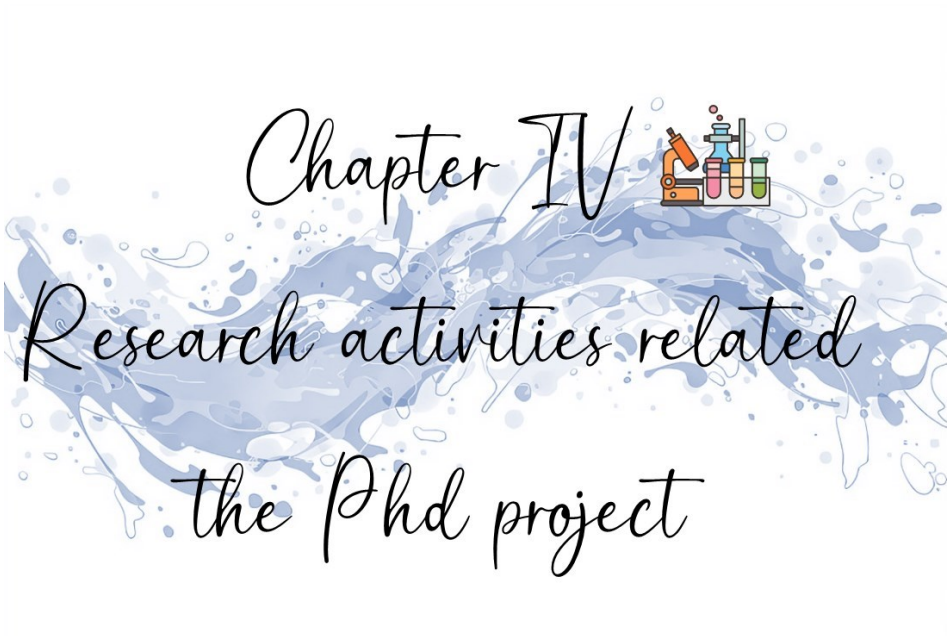
Abstract:	In this study, the pâté olive cake (POC), a by-product of the olive oil industry, was subjected to fermentation in a bioreactor using three microbial strains, Lactiplantibacillus plantarum, Wickerhamomyces anomalus and Candida boidinii, previously isolated from fermented table olive brines. Chemical, microbiological and molecular analyses were carried out at the beginning and at the end of fermentation. The lowest pH value (4.09) was reached after 10 days in sample inoculated with C. boidinii. Microbiological analyses exhibited the dominance of yeasts throughout the whole process (from 5.5 to 7.80 Log10 CFU/g), as confirmed by PCR-DGGE analysis. The microbial cultures affected both phenolic and volatile organic compound profiles. Moreover, the POC samples treated with different microbial strains were investigated for biological assays. The sample fermented with W. anomalus showed the greatest diffusion speed of transepithelial transport through Caco-2 cell, the highest inhibitory activity towards the tested cyclooxygenases and the highest antioxidant activity.

CHAPTER IV.
RESEARCH ACTIVITIES RELATED TO THE PHD PROJECT



Microbial Application to Improve Olive Mill Wastewater Phenolic Extracts

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Abstract

Olive mill wastewater (OMW) contains valuable and interesting bioactive compounds, among which is hydroxytyrosol, which is characterized by a remarkable antioxidant activity. Due to the health claims related to olive polyphenols, the aim of this study was to obtain an extract from OMW with an increased level of hydroxytyrosol by means of microbial enzymatic activity. For this purpose, four commercial adsorbent resins were selected and tested. The beta-glucosidase and esterase activity of strains of *Wickerhamomyces anomalus*, *Lactiplantibacillus plantarum*, and *Saccharomyces cerevisiae* were also investigated and compared to those of a commercial enzyme and an *Aspergillus niger* strain. The *W. anomalus* strain showed the best enzymatic performances. The SP207 resin showed the best efficiency in selective recovery of hydroxytyrosol, tyrosol, oleuropein, and total phenols. The bioconversion test of the OMW extract was assessed by using both culture broths and pellets of the tested strains. The results demonstrated that the pellets of *W. anomalus* and *L. plantarum* were the most effective in hydroxytyrosol increasing in phenolic extract. The interesting results suggest the possibility to study new formulations of OMW phenolic extracts with multifunctional microorganisms.

1. Introduction

The Mediterranean diet is becoming more and more popular due to its nutritional and health benefits, which are associated with lower incidences of atherosclerosis, certain cancers, and cardiovascular and neurodegenerative diseases [1–4]. These health benefits can be strongly related to the high content of antioxidant molecules present in food products widely consumed within the Mediterranean diet, such as extra virgin olive oil and table olives. Olives and their industrial derived products are rich in unsaturated fatty acids, tocopherols, and phenols. However, the olive oil industry generates large amounts of high-polluting by-products, such as a solid residue and an effluent commonly named olive mill wastewater (OMW) [5], which is a mix of olive vegetation water and water added during oil extraction, above all with the three-phase centrifugation system [6]. Olive waste still contains most of the valuable and interesting compounds. During the olive oil processing, most of the phenolic compounds are found in the aqueous phase, with a hydroxytyrosol concentration up to 100 fold higher than that found in olive oil [7]. OMW is rich in phenols, such as hydroxytyrosol, tyrosol, oleuropein, flavonoids, and other compounds. These molecules have great potential in the pharmaceutical and nutraceutical fields [8]. In particular, hydroxytyrosol is characterized by remarkable antioxidant activity, which is similar to that of the main synthetic antioxidants [7,9]. In 2011 [10], the scientific opinion on the substantiation of health claims related to olive polyphenols, standardized by their content of hydroxytyrosol and its derivatives (e.g., oleuropein complex), was published by European Food Safety Authority (EFSA). Substantial interest has been given to the production of hydroxytyrosol both from natural sources and by synthetic procedures [11]. Several techniques have been reported in literature aiming at recovering phenolic compounds from OMW, such as liquid–liquid extraction [12], membrane filtration [13], adsorption on selective polymeric resins [14,15], and enzymatic reactions [11,16,17]. Compared to chemical treatments, enzymatic treatment is advantageous because it requires mild operating conditions, such as pH, temperature, and the absence of toxic organic solvents [18]. To overcome the high costs of enzymes and their poor operational stabilities, new research has focused on enzyme immobilization strategies [19]. Among microorganisms, *Aspergillus* spp. has been extensively studied for its ability to produce extracellular enzymes that can be easily applied to food processing, because they are recognized as Generally Regarded as Safe

(GRAS) by the Food and Drug Administration (FDA) [20]. Within the *Aspergillus genus*, *A. niger* is the most efficient producer of β-glucosidase [21]. However, few reports focused on the effect of fungal enzyme addition on OMW phenols have been published, but even less on the direct application of live microorganisms into an OMW extract to improve antioxidant activity. In particular, yeasts and lactic acid bacteria (LAB) appear particularly interesting as they are commonly used as starter cultures in fermented food and widely isolated by OMW or spontaneous fermentation of table olives. During the last two decades, several *Lactiplantibacillus plantarum* strains have been extensively described for their ability to degrade oleuropein and for their probiotic potential [22,23].Aponte et al. [24] for the first time tested the in vivo bioconversion of oleuropein into hydroxytyrosol by oral granules containing probiotic *L. plantarum* and an olive leaves standardized extract. Their results showed that co-administration of live *L. plantarum* bacteria with the extract provides for higher amounts of bioavailable hydroxytyrosol, compared to the extract alone. Several yeasts isolated from table olives have been foundable to produce noticeable enzymes, such as β-glucosidase, and to exhibit probiotic traits [25,26]. Among yeasts, *Saccharomyces cerevisiae* and *Wickerhamomyces anomalus* havebeen described to produce enzymes useful in the wine making process and their application has been extensively investigated. In addition, *W. anomalus* (formerly knownas *Pichia anomala* or *Hansenula anomala*) shows several probiotic traits including cholesterol removal capability [27], and it is an interesting source of different enzymes and a biotechnologically relevant microorganism [28]. In light of this knowledge, this study aimed to investigate the use of live microorganisms together with OMW extracts. In fact, a selection of different types of resins was performed to obtain a phenol-rich extract with the main aim of evaluating the bioconversion activity of different microbial species. The hydroxytyrosol increase in the OMW extract provided by cultures already known as starters is the first step to design innovative formulations of new nutraceuticals supplemented with probiotics.

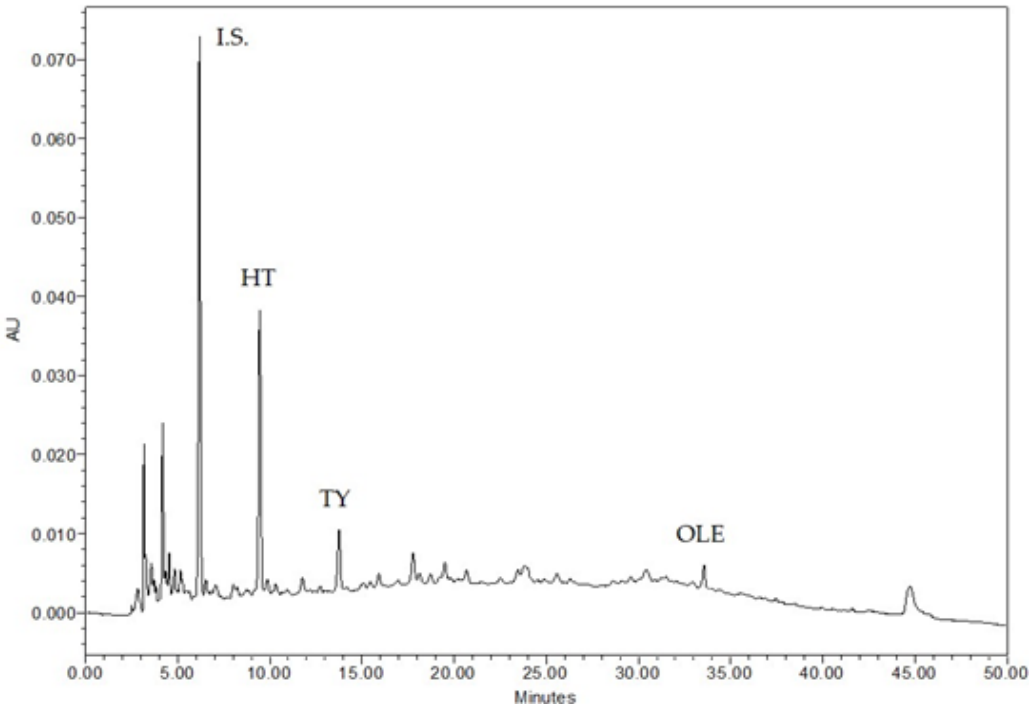


Figure 1. HPLC phenolic profile (at 280 nm) of OMW sample (I.S. = Internal Standard).

2. Results

2.1 Extraction of Phenols from OMW

2.1.1 OMW Analyses

Raw OMW was centrifuged and filtered to remove solid soil residues, olive oil and fragments of olive fruit and stone. The obtained sample was analyzed for the values of pH, dry matter, and total phenols, while hydroxytyrosol (HT), tyrosol (TY), and oleuropein (OLE) concentrations were detected by high performance liquid chromatography (HPLC) (Table 1). These results confirmed the acidic trait of the sample, which showed 6.24 g/100 mL of dry matter value, although subjected to centrifugation, and a high content of total phenolic compounds, for which it is considered a polluting effluent. As expected, among the three quantified peaks, HT showed the highest concentration.

Table 1. Chemical parameters of OMW after pretreatment (centrifugation and filtration).

Parameters	Means ± SD
pH	4.80 ± 0.06
Dry matter (g/100 mL)	6.24 ± 0.02
Hydroxytyrosol (HT)(mg/L)	218.29 ± 4.94
Tyrosol (TY)(mg/L)	67.24 ± 5.57
Oleuropein (OLE)(mg/L)	207.49 ± 56.37
Total phenols (mg/L)	1,343.26 ± 0.54

Moreover, HT was the most abundant phenol in OMW, as shown in Figure 1. OLE was quantified as gallic acid too, but this was done by calculating the response factor of the instrument in respect to the Internal Standard (I.S.) under the same HPLC operating conditions. HT, TY, and OLE were analyzed as principal markers of OLE enzymatic degradation. They were identified by comparing their retention times and absorption spectra with those of pure commercial standards.

2.1.2 Resins Adsorption

The results of OMW adsorption on resins are shown in Table 2. The results confirmed a different adsorbing resins' capacity for both polyphenols and individual bioactive compounds. The four tested resins can be divided into three groups. The most efficient were the S-DVB copolymers resins (namely SP207 and XAD16), which adsorbed the highest concentration of total phenolic compounds, followed by the C18 and the PAD900C resins, respectively. Concerning the capacity to adsorb the individual bioactive compounds, the SP207 resin showed the highest efficiency in concentrating HT, TY, and OLE from OMW, whereas the three others (XAD16, PAD900C, and C18 resins) showed a lower efficiency. The extract obtained by the most effective resin (SP207) was then selected for bioconversion experiment.

Table 2. Phenols detected in OMW extracts obtained with the different tested resins.

Adsorbent resin	Hydroxytyrosol (HT)	Tyrosol (TY)	Oleuropein (OLE)	Total phenols
SP207	3239.7 ± 57.34 a	2016.8 ± 49.36 a	11,336.6 ± 199.65 a	16,447.9 ± 16.31 a
XAD16	1381.7 ± 66.45 b	1000.3 ± 120.75 b	10,047.8 ± 120.29 ab	13,144.6 ± 21.75 b
PAD900C	294.3 ± 2.67 c	90.0 ± 10.59 c	4443.8 ± 176.32 c	4880.3 ± 10.87 d
C18	191.1 ± 16.59 c	50.4 ± 10.34 c	8092.3 ± 218.15 b	8763.6 ± 43.50 c

Data are expressed as mg/L of means ± standard deviations. Different letters indicate statistical differences within the same column (Significance at $p < 0.001$).

2.2 Enzymatic tests

The enzymatic activity of three strains was compared to that obtained by the commercial enzyme (Lallazyme beta), and to the activity of germinated conidia of *A. niger* strain. The *A. niger* DSM 2466 was selected among other commercial strains for its proven enzymatic activity (data not shown) and here used as positive control. As shown in Table 3, the strain, after germination in culture broth, produced the highest value (IU/mL) of both beta-glucosidase and esterase activities. The *A. niger* activity was also higher than that highlighted by the commercial tested enzyme, which is widely used in oenological applications for its known poly-enzymatic activity. Among *W. anomalus*, *L. plantarum* and *S. cerevisiae* tested strains, the *W. anomalus* was found as the most effective for beta-glucosidase, together with the commercial enzyme and followed by the *L. plantarum*, while, for esterase activity, results obtained from *W. anomalus* and *S. cerevisiae* were not statistically different.

Table 3. Beta-glucosidase and esterase activities detected for the commercial enzyme and the different microorganisms tested under their specific growth culture conditions

Samples	Beta-glucosidase	Esterase
Lallazyme beta enzyme	6,979.9 ± 3.22 b	316,713.9 ± 1,032.48 c
W. anomalus	7,066.4 ± 5.36 b	405,417.6 ± 516.24 b
L. plantarum	2,387.6 ± 3.22 c	316,348.9 ± 1,540.62 c
S. cerevisiae	1,437.9 ± 78.31 d	399,577.0 ± 1,548.72 b
A. niger	314,313.8 ± 107.28 a	4,506,229.2 ± 4,646.15 a

Data are expressed as IU/mL of broth sample (means ± standard deviations). Different letters indicate statistical differences within the same column (Significance at $p < 0.001$).

2.3. Bioconversion Efficiency by W. anomalus and L. plantarum

In the bioconversion test, the two microorganisms showing the best performances at the enzymatic tests (Table 3) were used to evaluate their ability to increase the HT and TY concentrations in the OMW extract. The SP207 extract was used as substrate for both pellets and filtered supernatants. The kinetic analysis of bioconversion was done through HPLC quantification of single phenols up to 6 h. The treatment with the commercial enzyme highlighted a slow and constant increasing of both HT and TY, whereas the treatment with the two tested microorganisms showed the highest value of phenols, reaching the plateau after 2 h of reaction. The only exception was detected after 6 h in the sample treated with pellets, when a further increase of HT value was registered (Table S1, on-line Supplementary Materials). Overall, the highest phenolic values were obtained using the microbial pellets rather than the filtered broths.

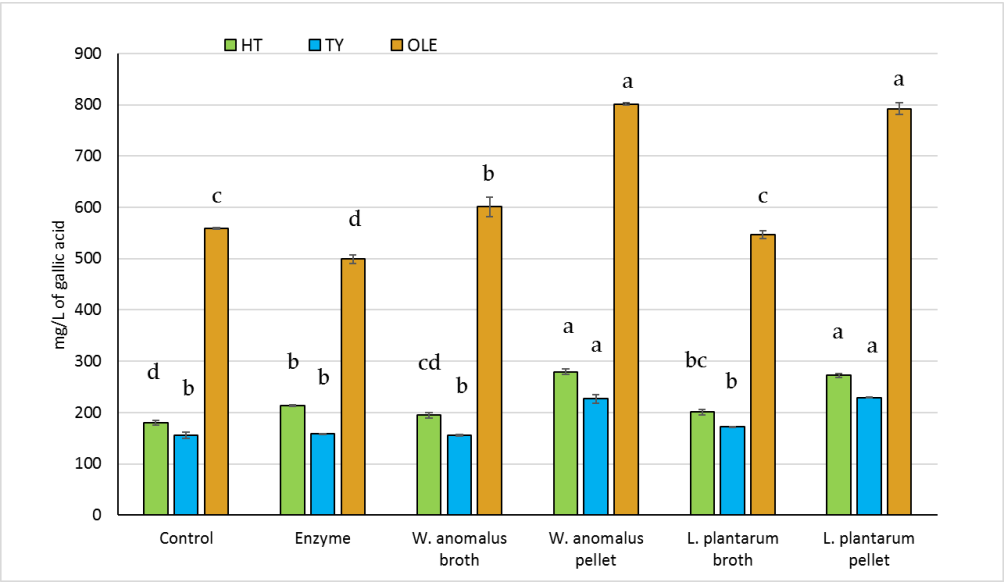


Figure 2. Concentration of hydroxytyrosol, tyrosol, and oleuropein after bioconversion of diluted OMW extract by Lallzyme, extracellular broths and pellets of the two tested strains after 2 h at 50 ◦C. Different letters indicate statistical differences among the columns of the same compound (Significance at $p < 0.001$).

The mean values of HT, TY, and OLE obtained after 2 h are shown in Figure 2. The highest valuesof HT were reached using *W. anomalus* and *L. plantarum* pellets (+35.5% and +33.7%, respectively), whereas no statistical difference between the control sample and the *W. anomalus* broth was observed. The TY content increased significantly only after contactwith both pellets (+32%). Regarding the OLE amount, although a decrease was registeredafter 2 h by using the commercial enzyme, its concentration remained almost constant upto 6 h (Table S1). On the contrary, the OLE concentration increased in the presence of both strains' pellet (+30.2% and +29.5%) and to a lesser degree with *W. anomalus* broth(+7%).

3. Discussion

Crude OMW was sampled from a collection tank during a continuous oil extraction from different olive cultivars. The sample showed a pH value of 4.8, within the range of 2.2–5.9 as reported by Dermeche et al. [5], and close to those reported by Aggoun et al. [6] and Dammak et al. [18] who found values between 4.5 and 5.16 for OMW from the threephase extracting system. Here, the dry matter value was slightly lower than those reported by the previous authors, as a consequence of sample pre-treatment, namely centrifugation and filtration. The total phenols were also lower than those discussed by

Belaqziz et al. [29] who found value of 6.46 g/L in effluent generated from the three-phase extraction system. The amount of 1.34 g/L detected in the present work was comparable to 1.8 g/L reported by Dammak [18] and within the range 0.03–6.13 g/L reported by Davies et al. [30]. The variability in total phenol concentration may be attributed to several factors, such as the olive cultivars and their degree of ripening. Moreover, the agronomic conditions and treatments applied to extract the olive oil and to treat the OMW, may also significantly impact the quantitative and qualitative phenolic content and solid residues of OMW [5,31]. The major compounds detected in OMW are HT and TY, with HT known for its high antioxidant activity [32]. Dermeche et al. [5] in their review reported that in OMW, HT is found in the range of 20–1224 mg/L, while TY is between 145–208 mg/L. Regarding OLE, it has not been detected in OMW deriving from late-harvest olives [31], and its content has been described as highly variable [11,29,33]. The solid phase recovery of polyphenols from OMW has been successfully proposed using adsorbent resins, particularly with the S-DVB-based resins [14,15,34]. In the present work, in order to optimize the process, different resins, including the SP207, never employed before in the recovery of phenolic compounds from OMW, were tested. All the tested resins were capable of successfully adsorbing polyphenols [34,35], with the SP207 resin showing a high and selective affinity for OMW polyphenols. This non-polar resin, generally used for the recovery of phenolic compounds from citrus, apple, and winery by-products and wastes [36–38], showed the best efficiency in terms of total phenols concentration and selective recovery of the main OMW bioactive compounds. This type of approach to the recovery of bioactive compounds from OMW is desirable for the scale-up. from laboratory to industrial production thanks to the simple and reproducible operating conditions and to the low costs of the process. The enzymatic activity, as expected, highlighted the absolute supremacy of the *A. niger* strain, confirming its successful employment at an industrial level to produce purified enzymes [11]. The hyphal mode of fungal growth and its robustness to a wide range of pH and water activity make fungi extremely efficient in the bioconversion of solid substrates [39]. In fact, live *A. niger* culture showed an enzymatic activity up to 45-fold higher than the commercial enzyme. The *W. anomalus* and *L. plantarum* tested strains gave interesting results. In particular, *W. anomalus* showed a beta-glucosidase activity statistically equal to that of the commercial enzyme, and an esterase value higher than that of the commercial enzyme. Despite its potential to produce enzymes of technological

importance, *W. anomalus* has been scarcely explored in food and byproduct applications. The interest in microbial enzymes for biotechnology applications has grown above all in the bioconversion of industrial by-products [40]. The enzymatic test assessed in the present work highlighted the importance of testing microbial cultures already known for their technological or probiotic properties. A considerable amount of research has been conducted to determine the enzymatic properties of LAB [41], while other studies reported a strong β-glucosidase activity for *W. anomalus* yeast [42–46]. The bioconversion test showed that *W. anomalus* and *L. plantarum* were able to produce more HT than the commercial enzyme after 2 h testing. Moreover, the bioconversion test highlighted that microbial live cells were more effective in increasing the HT and TY content than their filtered broths, probably because the phenol extract, generating stressful conditions, could induce a stimulating effect on microbial metabolism. The statistically significant increase in TY (the TY recently highlighted and quantified to apply the EFSA health claim on olive oil polyphenols) obtained using the single pellet of both *W. anomalus* and *L. plantarum* cultures, is also valuable [47]. In the present work, the OLE value was detected as increased, but this may be due to a depolymerizing effect that microbial enzymes may have had on OMW polyphenols.

4. Materials and Methods

4.1. Raw Material Treatment

Fresh OMW was sampled in October 2018 from an olive oil producing plant of a factory located in Catania, Italy, which used a three-phase continuous oil extraction system. Samples were centrifuged at 10,000 rpm for 5 min at +4 ◦C, then filtered (Miracloth paper, Calbiochem, Canada) and stored at −20 ◦C until further analyses.

4.2. Extraction of Phenols from OMW

Four commercial adsorbent resins, Amberlite XAD-16 (Sigma Aldrich, Milan, Italy), Sepabeads SP-207 (Mitsubishi Chem. Co., Tokyo, Japan), Purosorb PAD900C (Purolite, Milan, Italy), and Hamilton C18 (Thermo Fischer Scientific, San Jose, CA, USA) were selected and tested. XAD-16 and SP-207 are styrene-divinylbenzene (S-DVB) copolymers; PAD900C is polydivinylbenzene polymer; and finally, C18 having Poly (S-DVB) matrix was used as analytical reference. Before their employment, the adsorbent

resins were pretreated with 95% ethanol (food grade, Alcoolita, Italy), washed with water (HPLC grade, Carlo Erba, Italy), and then oven-dried at 70 ◦C up to constant weight. All adsorption experiments were conducted with determined quantities of activated dried resins. Then, 1 bed volume (BV) of each dry resin (about 20 mL) was loaded on a glass preparative column (length, 30 cm; i.d., 0.5 cm) connected with a peristaltic pump. After rinsing the resins with water, the OMW, pretreated as described in Section 4.1, was passed on resins until their saturation. Loading was stopped when the outflow OMW reached the same absorbance values and color of the loaded OMW. Before the desorption phase, each saturated resin was washed with 4 BV of water to remove water-soluble compounds. The adsorbed phenolic fraction of each resin was then recovered with 2 BV of a 95% ethanol/water solution (60:40, v/v). The collected desorbed fractions were finally concentrated after vacuum distillation of ethanol at 40 °C by using a rotary evaporator (Rotavapor RE111, Büchi, Cornaredo, Italy).

4.3. Physicochemical Characterisation

The pH value of OMW samples was measured using a Mettler DL25 pH meter (MettlerToledo International Inc., Columbus, OH, USA). Dry weight was determined by weighing the samples before and after drying in an oven at 105 ◦C up to constant weight. The total phenolic content of OMW and extracts was determined according to the Folin–Ciocalteu's (FC) colorimetric method. The OMW samples were mixed with 5 mL of FC commercial reagent (Labochimica, Italy) diluted with water 1:10, v/v, and 4 mL of a 7.5% sodium carbonate solution. After stirring for 2 h at room temperature away from light, the absorbance of the blue solution was measured spectrophotometrically at 765 nm (Cary 100 Scan UV-Visibile, Agilent, CA, USA). The total phenolic content was expressed as mg of gallic acid equivalents (GAE)/L of the sample.

4.4. HPLC Analysis

HPLC analyses of phenol fraction of OMW and extracts were obtained by directly injecting the filtered samples (0.45 µm Millipore filters) in the chromatographic HPLC system. The system consisted of a liquid chromatography Waters Alliance 2695 HPLC equipped with a Waters 996 photodiode array detector (PDA) set at 280 nm and with Waters Empower software (Waters Corporation, MA, USA). The column was a Luna C18 (250 mm × 4.6 mm i.d., 5 µm, 100 Å; Phenomenex, Torrence, CA, USA) maintained in an

oven at 40 ◦C. Chromatographic separation was achieved by elution gradient usingan initial composition of 95% of A solution (water acidified with 2% acetic acid) and 5%of B solution (methanol). Solvents were HPLC grade (Merck KGaA, Darmstadt, Germany). The B solution increased to 30% in 15 min and to 70% in 25 min and then, after 2 min in isocratic, the mobile phase was set at the initial conditions for 8 min. A flow of 1 mL/min was used [48]. The internal standard (I.S.) of 5 mM pure gallic acid (Fluka, Switzerland) was used to quantify the phenolic compounds. The identification ofphenolic compounds was obtained by comparing retention time with pure tyrosol, oleuropein, and hydroxytyrosol standards (Extrasynthese, Genay, France). All the analyses were carried out in triplicate for each sample.

4.5. Microorganisms

Lactiplantibacillus plantarum DSM 20205, *Saccharomyces cerevisiae* DSM 1333, and *Aspergillus niger* DSM 2466, were all purchased from Leibniz-Institute DSMZ, German collection. Moreover, *Wickerhamomyces anomalus* F6.05, a strain isolated from brine samples of naturally fermented table olives, was from the Culture Collection of the Department of Agricultural, Food, and Environment (Di3A), University of Catania, Italy. All the used cultures were maintained as stock solution in 20% (v/v) glycerol at −80 ◦C before use. *A. niger* stock suspension was obtained by growing the strain on Potato Dextrose Agar (PDA, Oxoid, UK) at 30 ◦C for 5 days and by harvesting the spores with 5 mL Tween 80, 2% (v/v) in sterile water. The spore solution was stored in 20% (v/v) glycerol at –20 °C before use.

4.6. Enzyme Production Test

The *L. plantarum* strain was cultured at 32 ◦C for 24 h in De Man, Rogosa, and Sharpe broth (MRS broth, Oxoid, Milan, Italy) while *S. cerevisiae* and *W. anomalus* strains were cultured at 30 °C for 24 h in Yeast Malt broth (YM broth, Merk, Italy). A cell density of 10^8 CFU/mL of each microorganism was used to inoculate test tubes containing media without a carbon source, MRS broth without Dextrose (Likson, Italy) for *L. plantarum*, and yeast nitrogen base (Likson, Italy) for *S. cerevisiae* and *W. anomalus* strains. After an overnight culture at the same temperature shown above, the samples were filtered with 0.45 µm paper filters (Minisart, Sartorius Stedim, France) to collect the filtrate containing the extracellular enzymes for subsequent enzymatic tests. The *A. niger* strain was used as

a positive control by using the method reported by Hamza et al. [11]. Flasks containing 100 mL of yeast nitrogen base (YNB, Likson, Italy) with 6.7 g of wheat bran (Ki, Italy) as carbon source were inoculated at 107 spores/mL concentration. The flasks were left in agitation at 120 rpm for 10 days at 25 °C. The culture broth was filtered through a muslin cloth to collect the filtrate containing the extracellular enzymes.

4.7. Beta-Glucosidase Activity

Beta-glucosidase activity was measured determining the quantity of p-nitrophenol released from the p-nitrophenyl-beta-D-glucopyranoside (p-NPG) by the enzymatic activity of the tested strains. The activity was determined according to the method of Khoufi et al. [16], slightly modified. For the enzymatic assay, the incubation mixture contained 0.9 mL of 5 mM p-NPG (Sigma-Aldrich, Milan, Italy) in 50 mM citrate buffer (pH 4.8), and 0.1 mL of centrifuged broth from each overnight culture. The reaction was maintained at 50 °C for 2 h and stopped by the addition of 2 mL of Na2CO3 buffer (1.0 M). The amount of p-nitrophenol (p-NP) released was spectrophotometrically determined at 400 nm and quantified by using p-NP (Glentham, Life Science, UK) to obtain a standard curve. One unit (IU) of enzyme activity was defined as the amount of enzyme that produced 1 µmol of p-NP/min under the same conditions. The enzymatic results were expressed as IU/mL of sample by means of the following equation: $IU/mL = Q/T \times C/V$, where Q is the quantity (µmole) of p-NP generated at the time T (min), V is the volume (mL) of the sample, and C is the total reaction volume (mL).

4.8. Esterase Activity

Esterase activity was determined according to the method of Mackness et al. [49]. The reaction mixture contained 50 µL of centrifuged broth from each overnight culture, 50 µL of 150 mM p-nitrophenyl-acetate (p-NPA, Acros Organics, Fair Lawn, NJ, USA) in ethanol, and 2.9 mL of Tris-HCl buffer (pH 7.5). The solution was incubated at 25 °C for 4 min. The amount of p-NP released was spectrophotometrically determined at 400 nm and quantified by using p-NP to obtain a standard curve. The enzymatic results were expressed as described in Section 4.7.

4.9. Bioconversion of Phenolic Compounds

The OMW extract obtained by the SP207 resin was used for the proposed enzymatic hydrolysis aimed at hydroxytyrosol increase. The procedure described in the enzyme production test above mentioned (Section 4.6) was repeated. In this case, only *L. plantarum* and *W. anomalus*, the two strains that showed the highest enzymatic activity, were tested. The two overnight cultures were centrifuged to collect separately the pellets and supernatants. The pellets were centrifuged twice at 5000 rpm for 5 min at +4 °C in sterile water and standardized at the same cell density of 10^8 CFU/mL in sterile water. Bioconversion reactions were assessed according to Hamza et al. [11], with modifications. The reactions were conducted in Erlenmeyer flasks by adding 5 mL of cultured samples (both pellet and filtered supernatant were tested separately) and 15 mL of OMW extract (diluted 1:10 in water). The enzymatic reaction was performed at 50 °C for 6 h under static conditions. Samples were collected every hour up to 6 h, and then filtered for subsequent analyses for phenols by HPLC. Moreover, the enzymatic activity of all the samples was compared to the activity of Lallzyme Beta (Lallemand, Blagnac, France) a commercial enzyme produced by *A. niger*, with β-glucosidase and polygalacturonase activity. The enzyme was used as a positive control (5 mL) at a dose of 5 mg/100 mL sterile water, in accordance with the manufacturer's instructions. The negative control was incubated with 5 mL of sterile water.

4.10 Statistical Analysis

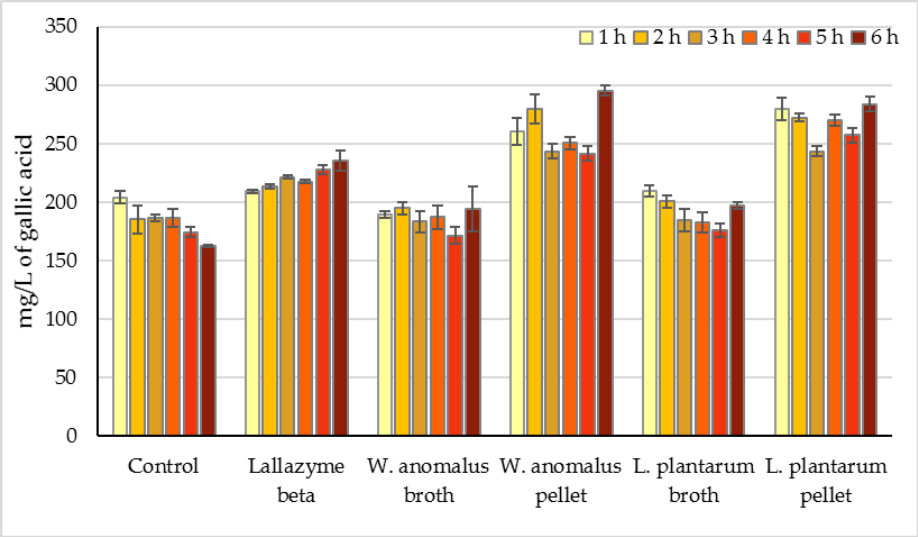
All analyses carried out were performed in triplicate. SPSS software (version 21.0, IBM Statistics, Armonk, NY, USA) was used for data processing. Statistical analysis of the results was performed using one-way analysis of variance (ANOVA), and Tukey's HSD post hoc test for means separation at a significance level of $P \leq 0.05$.

5. Conclusions

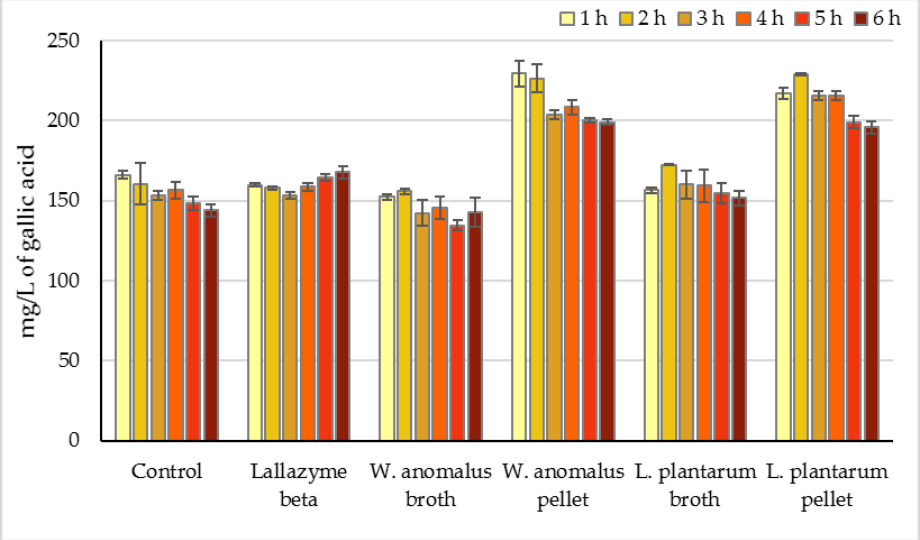
The effects of different adsorbent resins were tested to obtain an OMW extract rich in bioactive compounds. The OMW extract obtained from the SP207 resin showed the best efficiency in selective recovery of the main OMW bioactive compounds. The betaglucosidase and esterase activity of different microorganisms, already known for their technological or probiotic properties, were investigated and compared to those of a commercial enzyme and an *A. niger* strain. Remarkably, the results demonstrated that the *W. anomalus* and *L. plantarum* tested strains were the most effective in increasing HT and TY, especially when they were used as pellets in contact with the OMW extract. The results of the present study are promising and suggest the possibility to further explore liquid or solid formulations for antioxidant/nutraceutical supplements containing health promoting compounds and microorganisms.

Supplementary material

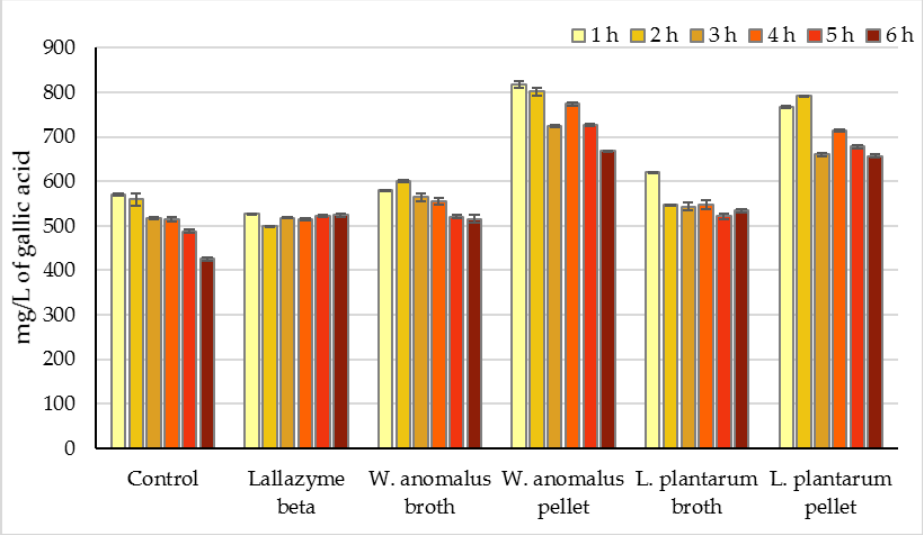
Table S1. Kinetics of bioconversion of single phenols analyzed through HPLC.



A) Hydroxytyrosol



B) Tyrosol



C) Oleuropein

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GENERAL DISCUSSION & FUTURE PERSPECTIVES

The main aim of the present industrial PhD doctorate was to valorise olive oil by-products through environmental friendly techniques that can be easily reproduced at industrial level, by the implementation of a circular economy strategy. It is relevant to underline that the reuse of by-products can lead to the creation of a virtuous recycling system, in accordance with the objectives of Global Food 2030, representing a concrete opportunity to recover competitiveness in the supply chain and relaunch the whole olive oil sector.

Chapter I is represented by two reviews, that discuss the state of the art of the two olive oil by-products, object of the PhD thesis. The reviews ***Olive mill wastewater asrenewable raw materials to generate high added-value ingredients for agro-foodindustries*** and ***Olive Pomace and Pâté Olive Cake as Suitable Ingredients for Food andFeed*** provide a general overview of the chemical and microbiological characteristics of matrices and discuss the use of these by-products in different fields of application. In particular, the reviews focused on the possible use of these matrices in the food sector, exploring them as food ingredients or novel functional foods, reporting on ongoing researches and potential new applications in different food products.

Chapter II includes three papers ***Phenols recovered from olive mill wastewater as a natural booster to fortify blood orange juice, Olive mill wastewater fermented with microbial pools as a potential new functional beverage*** and ***Protective Effect of Treated Olive Mill Wastewater on Target Bacteria and Mitochondrial Voltage-Dependent Anion-Selective Channel 1*** which deal with different OMWW valorisation strategies.

In the first paper, the aim was to use a tangential membrane filtration system, present at the company, to recover phenols from olive mill wastewater. The concentrates obtained through ultrafiltration and reverse osmosis were characterised for their physico-chemical traits, antioxidant activity and antimicrobial effects. In addition, the obtained permeates were evaluated for reuse for irrigation purposes. The best concentrate was subsequently added, in different ratios, into a commercial blood orange juice. The juice fortified with the addition of the concentrate, up to a ratio of 2:250 v/v, showed no off-flavour and off-odour compared to the control. Furthermore, after 60 days of refrigerated storage, the fortified juice still presents a hydroxytyrosol content in accordance with the EFSA-

recommended daily intake. The results obtained may be useful at industrial level for the production of orange juice fortified with a concentrate of natural antioxidants as a 'clean label' ingredient.

In the second study, the aim was to enhance OMWW through a driven fermentation using selected microbial pools. In particular, strains such as *Lactiplantibacillus plantarum*, *Candida boidinii* and *Wickerhamomyces anomalus* were selected to drive the fermentation process. Results showed that in all OMWW samples subjected to microfiltration, fermentation stopped after 21 days. Furthermore, in all inoculated samples a significant increase in phenols and organic acids were detected. Moreover, biological assays highlighted that fermentation determines an increase in the antioxidant and anti–inflammatory activity of OMWW. Lastly, an increment in the active permeability on Caco-2 cell line was also revealed. The results confirmid that fermented OMWW can be proposed as a new beverage and/or functional ingredient with nutraceutical effects.

In the third study, two strategies were used to concentrate bioactive components from OMWW: tangential membrane filtration (ultrafiltration and reverse osmosis) and selective resin extraction. The concentrates were evaluated for physico-chemical characteristics and antioxidant and antimicrobial activity. Furthermore, the effect on the mitochondrial voltage-dependent anion selective channel 1 was evaluated. The chemical results showed that the highest concentration of hydroxytyrosol was revealed in the sample obtained by reverse osmosis, while the highest concentration of oleuropein was detected in the sample obtained by resin extraction. The latter sample showed the highest antimicrobial effects against *Listeria monocytogenes*, *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Both samples showed a high impact on the electrophysiological parameters of VDAC1 activity. These results demonstrated that both enhancement techniques, reproducible at an industrial scale, provided phenolic concentrates with antioxidant and antimicrobial activity useful for various perspectives. These results are important to guide the choice of the most suitable technique for OMWW treatment, considering the criteria of cost-effectiveness and productivity.

Chapter III includes two studies ***Profiling of phenol content and microbial community dynamics during pâté olive cake fermentation*** and ***Effect of microbial fermentation of functional traits and volatiloma profile of pâté olive cake***. In both studies, the pâté olive cake was subjected to driven fermentation through microbial pools. In the first study, the

objective was to investigate the change in phenolic profile during fermentation and the microbial community dynamics through both dependent and independent culture techniques. Seven flask trials were set up with different strain combinations: *Lactiplantibacillus plantarum*, *Candida boidinii* and *Wickerhamomyces anomalus*. The results showed that during fermentation, the sample inoculated with *C. boidinii* in single culture and in combination with *L. plantarum* increased the hydroxytyrosol content by 275 and 261 mg/L, respectively. Furthermore, metagenetic analysis confirmed the results obtained through dependent culture techniques revealing low abundance for both 16S and ITS profiles. In the second study, three trials were set up using three monoculture strains: *L. plantarum*, *W. anomalus* and *C. boidinii* in order to study the biotechnological aptitude of the strain. In addition, fermentation was conducted in a bioreactor in a larger volume, which made possible to set and control various process parameters in order to monitor the fermentation process. Results showed that the three starters exhibited a different behavior. The lowest pH value (4.09) was reached after 10 days of fermentation in the sample inoculated with *C. boidinii*. Microbiological analyses revealed a dominance of the yeast group at both the beginning and the end of fermentation in all samples reaching a range value between 5.5 and 7.80 Log10 CFU/g. These results were confirmed by PCR-DGGE analyses. The starters affected in a different way the phenolic and VOCs profiles. Regarding the biological assays, the POC fermented with *W. anomalus* reached the highest inhibition of 11.34% for COX-2 and thehighest permeability value on Caco-2 cell monolayer. This study highlighted the possibility to improve the POC value to obtain an innovative healthier food or supplement.

Chapter IV is represented by the paper ***Microbial Application to Improve Olive Mill Wastewater Phenolic Extracts***, carried out in collaboration with CREA-OFA in Acireale. This research was funded by the Ministry of Agriculture, Food and Forestry Policies. (MiPAAF), grant number D.M. no. 12479 of the Project "Informed traceability and process/product innovations in the olive oil and table olive sector". Product innovations in the olive oil and table olive sector' - INFOLIVA. The aim of this study was to obtain an OMW extract with a higher level of hydroxytyrosol, through microbial enzymatic activity. Four commercial adsorbent resins were selected. The beta-glucosidase and esterase activity of *Wickerhamomyces anomalus, Lactiplantibacillus plantarum* and *Saccharomyces cerevisiae* strains were analysed and compared with those of a commercial enzyme and *Aspergillus niger*. The *W. anomalus* strain showed the best

enzymatic capabilities. The selective recovery of hydroxytyrosol, tyrosol, oleuropein and total phenols was higher using the SP207 resin. Using both culture and laboratory tests, the bioconversion test of OMW extract in the broths and pellets of the studied strains was evaluated. The results showed that *W. anomalus* and *L. plantarum* pellets were the most effective in increasing hydroxytyrosol in the phenolic extract. These interesting results suggest the idea of studying new formulations of OMW phenolic extracts with multifunctional microorganisms.

According to the Food and Agriculture Organisation (FAO), a significant amount of food is wasted or lost throughout the production chain, negatively affecting the global economy. In the context of agri-food sustainability, the implementation of projects that aim to create a balance between environmental, economic and social factors is of paramount importance (Ekren et al., 2022). Agri-food companies aim to develop various innovation systems in order to apply new strategies for sustainable management along the food production chain. In this scenario, a primary objective for sustainability and supply chain competitiveness is the valorisation of by-products generated during food production. Vegetable by-products are rich in bioactive compounds, and therefore are used as food additives, in active food packaging or as food ingredients to preserve food quality and increase nutraceutical characteristics. Many of these bioactive compounds, with a high antioxidant activity, have been studied for the treatment of chronic degenerative diseases induced by oxidative stress or inflammation-based diseases and their effect has been closely related to the change of gut microbiota composition (Maruca et al., 2019; Merra et al., 2022). For this reason, Mediterranean diet is confirmed as a recognised health food model based on its nutritional and health benefits related to the consumption of products containing antioxidant molecules, such as extra virgin olive oil and table olives (Romeo et al., 2010). Consumer awareness of foods and ingredients of natural origin "*specialty*" or "*value-added ingredients*", to which functional properties are attributed, is growing rapidly (Servili et al. 2020). Despite, the possibility to consume such bioactive compounds through the use of primary products such as table olives and/or extra virgin olive oils, the reuse of secondary products, specifically OMWW and POC, may represent a new way of acquiring compounds with health properties quite similar to primary products.

In this overview, the industrial PhD project was aimed to valorise by-products of olive oil supply chain. In detail, OMWW and POC were proved to be suitable matrices for the formulation of new additives or fermented foods for human consumption. Through the application of various green and environmental friendly valorisation technologies, new products with a high nutraceutical value can be obtained from these matrices, currently considered waste with a high environmental impact. In order to create a new, continuous production flow sheet, many efforts must be led by the collaboration between research institute and company, as only a multidisciplinary approach can lead to a beneficial and effective result to promote the expansion by-products and promote their production at industrial level.

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Pomegranate table (*Punica granatum* L.) and *Citrus* table (*Malus citria*) from Phytanthoza Iconographia, Johann Wilhelm Weinmann, Vol . III, 1737-1745.
The olives table (*Olea europea)* from Atlante di botanica popolare , V. Tenore & G.A Pasquale, Vol. III, 1881-1886.
All tables were photographed at the Acireale library "Biblioteca Zelantea".

Multi-functional potential of lactic acid bacteria strains and antimicrobial effects in minimally processed pomegranate (Punica granatum L. cv Jolly Red) arils

Article published in Microorganisms 2022, 10(10), 1876.

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Abstract

This study aimed to evaluate the antimicrobial activity of both cells, and cell-free supernatants (CFS) of 7 selected lactic acid bacteria (LAB) strains belonging to *Limosilactobacillus fermentum* (4 strains), *Lacticaseibacillus paracasei* (1 strain), *Lacticaseibacillus rhamnosus* (1 strain), and *Enterococcus faecium* (1 strain) species, against *Listeria monocytogenes*, *Escherichia coli, Salmonella* Typhimurium, *Pseudomonas aeruginosa* and *Staphylococcus aureus,* by both the agar-well diffusion and co-culture methods. In addition, probiotic and safety traits were also detected. Great variability was detected on antimicrobial effects, whereas all tested strains were found sensitive to most of the tested antibiotics, and without any DNase, gelatinase, or hemolytic activity. Moreover, strains showed excellent survival in acidic conditions and exhibited tolerance to pepsin and bile salts. Based on the in vitro results, the CFSs of two selected *L. fermentum* strains were applied, in a mixed solution, as bio-preservative into minimally processed pomegranate arils, inoculated with a cocktail of *L. monocytogenes* and *E. coli*. Samples, packaged in an ordinary atmosphere, were analyzed during refrigerated storage, for up to 12 days, for physicochemical (as weight loss, texture, color,

pH, total soluble solids and organic acid content) and for microbiological traits. Results revealed the effectiveness of CFS, up to 12 days, in reducing weight loss and microbial growth, without any significant effect on texture, total soluble solid content and color, found comparable to the acid citric treatment, highlighting the multi-functional potential of selected probiotic strains.

Metagenetic and Volatilomic Approaches to Elucidate the Effect of Lactiplantibacillus plantarum Starter Cultures on Sicilian Table Olives

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Abstract

The present study aimed to evaluate the effect of selected *Lactiplantibacillus plantarum* strains on both microbiota composition and volatile organic compound profile of Sicilian table olives. Two mixed cultures, named O1 and O2, were set up for pilot-plan scale fermentations at 5% of NaCl. Uninoculated table olives at 5 and 8% (C5 and C8) of salt were used as control. The fermentation process was monitored until 80 days through a dual approach, which included both classical microbiological and 16S amplicon-based sequencing and volatilomics analyses. Compared with control samples (C5 and C8), experimental samples, inoculated with starter cultures (O1 and O2), exhibited a faster acidification with a more pronounced drop in pH. Metagenetics data revealed significant differences of microbiota composition among samples, highlighting the dominance of lactobacilli in both experimental samples; a high occurrence of Enterobacter genus only in control samples with 5% of NaCl; and the presence of Bacteroides, Faecalibacterium, Klebsiella, and Raoultella genera only in control samples with 8% of NaCl. Furthermore, microbiota composition dynamics, through the fermentation process, significantly affected the volatile organic compounds of the final products, whereas no compounds involved in off-odors metabolites were detected in all samples investigated. In conclusion, the addition of the proposed starter cultures and the use of low concentrations of sodium chloride positively affected the microbiota and volatile organic compounds, ensuring the microbiological safety and the pleasant flavors of the final product.

Prebiotic effects of citrus pectic oligosaccharides

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Abstract

Citrus peel wastes, one of the major by-products of the agri-food industry, are a source of value-added compounds. In this work, a commercial pectin (PEC1) and one obtained by direct extraction from citrus fibre (PEC2), were hydrolysed and ultrafiltered at different cut-off (100, 50 and 30 kDa) and then tested in broth with four different probiotic strains: *Lacticaseibacillus paracasei*, *Limosilactobacillus fermentum*, *Lacticaseibacillus rhamnosus* and *Bifidobacterium animalis* subsp. *lactis*. In addition, the probiotic strains selected for their growth rate with the fractions of the two pectins were tested for tolerance to different pH values and bile salts. This study demonstrated that POS2 (product from the enzymatic hydrolysis by PEC2) showed greater prebiotic activity than POS1 (product from the enzymatic hydrolysis by PEC1). The results confirmed that citrus pectic oligosaccharides could be used as emerging prebiotics with improved properties due to their antimicrobial and modulating-microbiota ability.

Assessing the Effect of Ozonated Water on Microbial Load and Quality of Nocellara Etnea Table Olives

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Abstract

The effect of ozonated water on quality and peel microbial load reduction of Nocellara Etnea (NE) olives was evaluated. Different ozone concentrations in water and different time of contact were tested. Moreover, two different ozonated water washing methods, dipping, and continuous shower, were compared. Color, texture, chemical, and microbiological analyses were performed before and after treatments and after 20 days of fermentation in brine with and without *Lactiplantibacillus plantarum* starter culture at 10^8 CFU/mL. The continuous shower with 6.5 ppm ozonated water for 10 min led to a reduction of 1 logarithmic unit for mesophilic aerobic bacteria and 1.47 log unit for yeasts and mold population compared to water washing. Olive firmness and color were affected differently.

Effect of ozonated water combined with sodium bicarbonate on microbial load and shelf life of cold stored clementine (Citrus clementina Hort. ex Tan.)

Article published in Scientia Horticulturae 2021, 276, 109775.

Authors: Maria C. Strano [a], Nicolina Timpanaro [a]*, Maria Allegra [a], **Paola Foti [a],** Sonia Pangallo [b], Flora V. Romeo [a]

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Abstract

The effect of 3 mg L^{-1} ozonated water (O_3) in combination with 3% sodium bicarbonate (SBC), on the microbial control and the postharvest quality of cold stored Clementine fruit (*C. clementina* Hort. ex Tan.), compared to the single treatments and the water wash, was evaluated. After treatments fruits were stored for 30 days at 5 ± 1 °C and relative humidity (RH) 90 % followed by seven days at 20 ± 2 ◦C and RH 75 %, to simulate retail conditions (shelf life). Microbial reduction, decay incidence, physiological disorders, weight loss, rheological properties (deformation and firmness) physical-chemical parameters (colour, total soluble solids, titratable acidity, ascorbic acid) and sensory quality were evaluated soon after treatments during fruit cold storage (T10, T20, T30) and after 7 days of shelf-life (T30 + 7). The results showed that integrated treatments (O_3+SBC) greatly reduced the total viable count (more than 1 log unit), during the first 10 days of storage (T10), if compared to the other treatments. Moreover, O3+SBC reduced significantly the decay incidence during the whole storage (2.6 % at T30; 10.9 % at T30 + 7) with respect to the control (27.3 % at T30; 45.5 % at T30 + 7). In particular, the control of sour rots (*Galactomyces citri-aurantii* E.E. Butler) in treated fruits was observed. Our findings did not highlight noticeable changes among treatments concerning fruits weight loss, physiological disorders, chemical composition and sensory analysis.

Effects of Different Stress Parameters on Growth and on Oleuropein-Degrading Abilities of Lactiplantibacillus plantarum Strains Selected as Tailored Starter Cultures for Naturally Table Olives

Article published in Microorganisms 2020, 8(10), 1607.

Authors: Amanda Vaccalluzzo[1], Alessandra Pino[1,*], Maria De Angelis[2] Joaquín Bautista-Gallego[3], Flora Valeria Romeo[4], **Paola Foti[1]**, Cinzia Caggia[1] and Cinzia L Randazzo[1].
[1] Department of Agricultural, Food and Environment, University of Catania, 95123 Catania, Italy;[2] Department of Soil, Plant and Food Science, University of Bari Aldo Moro, 70121 Bari, Italy; [3] Faculty of Science—University of Extremadura, 06006 Badajoz, Spain; [4] Research Centre for Olive, Fruit and Citrus Crops (CREA), 95024 Acireale, Italy.

Abstract

The use of β-glucosidase positive strains, as tailored-starter cultures for table olives fermentation, is a useful biotechnological tool applied to accelerate the debittering process. Nowadays, strains belonging to *Lactiplantibacillus plantarum* species are selected for their high versatility and tolerance to stress conditions. The present study investigated the effect of different stress factors (pH, temperature and NaCl) on growth and on oleuropein-degrading abilities of selected *L. plantarum* strains. In addition, the presence of the beta-glucosidase gene was investigated by applying a PCR based approach. Results revealed that, overall, the performances of the tested strains appeared to be robust toward the different stressors. However, the temperature of 16 °C significantly affected the growth performance of the strains both singularly and in combination with other stressing factors since it prolongs the latency phase and reduces the maximum growth rate of strains. Similarly, the oleuropein degradation was mainly affected by the low temperature, especially in presence of low salt content. Despite all strains displayed the ability to reduce the oleuropein content, the beta-glucosidase gene was detected in five out of the nine selected strains, demonstrating that the ability to hydrolyze the oleuropein is not closely related to the presence of beta-glucosidase. Data of the present study suggest that is extremely important to test the technological

performances of strains at process conditions in order to achieve a good selection of tailored starter cultures for table olives.

LIST OF CONFERENCES

ORAL PRESENTATION

- V Convegno Nazionale dell'Olivo e dell'Olio. **"Impiego di pool microbici per la valorizzazione del sottoprodotto pâté di oliva". Paola Foti,** Flora V. Romeo, Cinzia L. Randazzo, Altino Branco Choupina, Cinzia Caggia. Alghero, 26-28 ottobre 2022.

- Speaker Eunice industrial doctorate workshop online. **"Olive Oil By-products as A New Functional Food and Source Of Nutritional Food Ingredients".**
  Paola Foti, Wednesday October the 20th 2021.
  https://eunice-university.eu/en/streamings/

POSTER PRESENTATION

- V Convegno Nazionale dell'Olivo e dell'Olio. ***Monitoring the hygienic quality of commercial table olives.***
  Enrica Pistorio*, Nicolina Timpanaro, **Paola Foti**, Solidea Mangiameli, Martina Bacceli, Barbara Lanza, Flora V. Romeo. Alghero, 26-28 ottobre 2022.

- 9th International Conference of MIKROBIOKOSMOS. ***Olive mill wastewater as a resource of biologically active phenols for food and beverages.***
  Paola Foti, Amanda Vaccalluzzo, Flora V. Romeo, Alessandra Pino, Nunziatina Russo, Cinzia L. Randazzo, Paride. S. Occhipinti, Cinzia Caggia. Atene, 16-18 dicembre 2021.

- XIII Convegno Nazionale sulla Biodiversità. ***Valorizzazione di acque di vegetazione olearie attraverso fermentazioni guidate.***
  Paola Foti*, Paride Occhipinti, Flora Valeria Romeo, Nunziatina Russo, Marco Finocchiaro, Cinzia Randazzo, Cinzia Caggia. 7-9 settembre 2021.

- XIII Convegno Nazionale sulla Biodiversità. ***Isolamento e caratterizzazione di ceppi non Saccharomyces in mosti siciliani.***
  Paola Foti*, Cristian Bua, Alessandra Pino, Cinzia Lucia Randazzo, Cinzia Caggia. 7-9 settembre 2021.

- 6th International Conference on Microbial Diversity (MD21). ***Novel biotechnological strategy to increase hydroxytyrosol content in olive mill wastewater phenolic extracts.***
  Paola Foti, Cinzia Caggia, Cinzia L. Randazzo, Flora V. Romeo. 14-15 Dicembre 2021.

- 6th International Conference on Microbial Diversity (MD21). ***Microbial dynamics in olive mill wastewater during fermentation with selected microbial strains.***
  Paola Foti, Amanda Vaccalluzzo, Alessandra Pino, Nunziatina Russo, Cinzia L. Randazzo, Flora V. Romeo, Cinzia Caggia. 14-15 Dicembre 2021.

- XIII Giornate Scientifiche SOI "I traguardi di Agenda 2030 per l'ortoflorofrutticoltura italiana". ***Efficacia del trattamento con acqua ozonizzata sulcontrollo microbico e sulla qualità di diverse specie di agrumi in frigoconservazione.***
  Strano M.C.*, Timpanaro N., Allegra M., **Foti P**., Romeo F.V. 22-23 Giugno 2021.

POST

- Post for scientific blog.
  Olive mill wastewater as a functional resource of bioactive compounds for Agro-Food Industries. **Paola Foti** & Cinzia Caggia, January 9, 2022. Science & Wine (ciencia-e-vinho.com) **https://www.ciencia-e-vinho.com/2022/01/09/olive-mill-wastewater-as-a-functional-resource-of-bioactive-compounds-for-agro-food industries/#:~:text=Olive%20mill%20wastewater%20as%20a%20functional%20resource%20of,Industries%20%E2%80%93%20Science%20%26%20Wine%20January%209%2C%202022**.

PATENT

Brevetto nr. 102022000008573 del 29 aprile 2022 - Ns Rif.: (I0197281) Proprietor: Consiglio per la ricerca in agricoltura e l'analisi dell'economia agraria **Inventors: Paola Foti**, Flora Valeria Romeo, Gabriele Ballistreri, Paolo Rapisarda.**Title:** ***"Citrus industry by-products formulation for a new nutraceutical with prebiotic, probiotic and antioxidant properties"***

The development of a new supplement containing pectins and antioxidant molecules bothextracted from citrus industry by-products was proposed together with lactic acid bacteriaprobiotic strains. The supplement showed a synergistic potential that places the innovation within the market segment of integration for human health.

TRAINING ACTIVITY

ATTENDED SUBJECTS

- ***Biotecnologie molecolari*** (8 CFU) Prof.ssa Roberta Angela Lo Piero, Di3A.
- ***Genetica dei microrganismi*** (6 CFU) Prof.ssa Cinzia Caggia, Di3A.

ATTENDED COURSES

- ***The Human Microbiome***.
  EIT FOOD, UNIVERSITY OF TURIN AND EUROPEAN INSTITUTE OF INNOVATION AND TECHNOLOGY (EIT).
- ***Food for Thought: The Relationship Between Food, Gut and Brain***.
  EIT FOOD, UNIVERSITY OF TURIN AND EUROPEAN INSTITUTE OF INNOVATION AND TECHNOLOGY (EIT).
- ***Bacterial Genomes: Disease Outbreaks and Antimicrobial Resistance.***
  EIT FOOD, UNIVERSITY OF TURIN AND EUROPEAN INSTITUTE OF INNOVATION AND TECHNOLOGY (EIT).
- ***Management and interpretation of complex biological data: theoretical foundations and use of analysis software. Excel (advanced) and R***.
  University of Catania. Prof. Mario di Guardo.

ATTENDED WORKSHOPS

- ***Progetto olgenome: i risultati. Completamento del sequenziamento del genoma dell'olio e annotazione dei geni***. Crea OFA, 30-04-2021.
- ***Il microbiota dell'olivo e il futuro dei prodotti biologici.*** Crea, 4-05-2021.
- ***Software per il design of experiment della sartorius*** (MODDE). 6-05-2021.
- ***Valorizzazione degli scarti delle produzioni agroalimentari: sfide e opportunità.***
  Crea Centro di ricerca Alimenti e Nutrizione, 17-06-2021.
- ***"Trasformazione delle olive da tavola: i metodi"***. Crea, 14-09-2021.

- ***"Batteri acetici e applicazioni industriali".*** Prof.ssa Maria Gullo, Università degli Studi di Modena e Reggio Emilia, Dipartimento di Scienze della Vita. 3 giugno 2020.
- ***"Le piante come bioreattori per la produzione di molecole ad alto valore aggiunto".*** Prof.ssa Linda Avesani, Università degli Studi di Verona Dipartimento di Biotecnologie. 29 maggio 2020.
- ***"Ruolo delle collezioni microbiche e dei networks dei centri di risorse Biologiche nello sviluppo delle biotecnologie".*** Dott.ssa Luciana De Vero, Università degli Studi di Modena e Reggio Emilia, Dipartimento di Scienze della Vita. 25 maggio 2020.

TEACHING ACTIVITY

MASTER'S THESIS CO-TUTOR

Student: Giovanni Russo

Title of thesis: *"Olive mill wastewater as a renewable raw material to generate a new functional product"* (Academic year 2021)

Relators: Chiar.ma Prof.ssa Cinzia Caggia and Dott.ssa Paola Foti

OTHER EDUCATIONAL ACTIVITIES

Didactic laboratory class for the subject 'Genetics of Microorganisms' of the LM7 (Agricultural Biotechnology) master course. Laboratory activities included:

- Preparation of selective media and microbiological analysis
- Extraction of DNA from fresh cultures
- Amplification by PCR
- Agarose gel preparation and electrophoretic run
- Antimicrobial activity
- Antibiotic resistance.

Progetto Alternanza scuola-lavoro (PCTO-2022). Esplorando i microrganismi di interesse alimentare. Istituto Regionale di Istruzione Secondaria Superiore «F. Morvillo» Catania.

Treated topics:

- Microbiological analyses of different food products (2h)
- Analyses of olive pâté (2h)
- Microbiological analysis of table olives and olive brines (2h).

TRAINING AT THE COMPANY

During the PhD period, 6 months were spent at the olive oil company "Consoli Pasquale & Fratelli s.n.c." according the PON RI 2014-2020 project.

During this period, the valorization of olive mill wastewater, obtained from the three-phase system, and the pâté olive cake, obtained from the multiphase decanter (DMF), was planned and discussed with the company tutor.

The main objective was to design a low-cost process rapidly and easily set up at industrial level (at the company) in order to validate new products for human consumption, according to the criteria set out in the Waste Framework Directive (2008/98/EC).

The experience carried out in the company enable to:

- establish a multidisciplinary collaboration between the university (Di3A), the research centre (CREA-OFA) and the company in order to exploit research results in competitive advantages for the company's production system;
- select the simplest and most economical valorisation techniques for an industrial scale-up;
- identify and schematise production flowsheet in collaboration with the company tutor;
- apply a problem-solving strategy to identify corrective actions and find innovative solutions during the various experiments;
- evaluate possible turnover and income recovery through the formulation of novel or functional foods in food sector.

In attachment the certification of the period spent at the company.

CONSOLI
dal 1960

Il sottoscritto Sig. Pasquale Consoli amministratore dell'Azienda Olearia "Consoli Pasquale & Fratelli s.n.c." Adrano CT) e tutor aziendale del progetto di dottorato innovativo a caratterizzazione industriale PON RI 2014-2020, dal titolo "Olive oil by-products as a new functional food and source of nutritional food ingredients",

Dichiara

che la Dott.ssa Paola Foti ha svolto il periodo di attività aziendale previsto dal progetto dal 1/01/2021 al 30/06/2021.

In questo periodo sono state eseguite differenti prove per la valorizzazione dei sottoprodotti oleari, acque di vegetazione olearie e patè di oliva, oggetto della tesi di dottorato, al fine di ottenere nuovi prodotti ad alto valore aggiunto da proporre al mercato del "foods", attraverso un approccio ecocompatibile, caratterizzato da una elevata efficienza di produzione e facile applicazione industriale.

Sono state svolte prove di filtrazione delle acque di vegetazione olearie attraverso l'utilizzo di cartoni filtranti a differente porosità al fine di ottenere una matrice limpida e priva di residui. Inoltre, un impianto di filtrazione tangenziale a membrana (ultrafiltrazione ed osmosi inversa) per ottenere dei concentrati fenolici ad elevato valore nutraceutico è stato messo a punto e i prodotti ottenuti sono stati oggetto di studio.

Per quanto riguarda l'impianto DMF (decanter-multifase, Leopard, Pieralisi), una volta ottenuto il patè sono disposte diverse prove di fermentazione in lab-scale con l'uso di pool microbici (batteri lattici e lieviti selezionati) al fine di deamarizzare e monitorare il processo fermentativo.

Tutte le prove sono state eseguite in concomitanza alle analisi microbiologiche e chimiche al fine di ottimizzare le prove per la produzione di nuovi prodotti funzionali apportando così una nuova possibile fonte di reddito all'azienda.

Adrano 30/06/2021

Firma

Azienda Olearia Consoli Pasquale & F.lli s.n.c.
C.da Giordano S.S. 284 Km 28,500 – 95031 Adrano (CT) Tel. 095 760 1517 e-mail: info@olioconsoli.com Sito web: www.olioconsoli.com

INTERNATIONAL TRAINING

The PhD project was planned to spend a 8 month-period abroad. The international experience was carried out in dual mode (one part in presence and one part in virtual mode) at the Polytechnic Institute of Braganza (IPB, Portugal) under the supervision of the international tutor Prof. Branco Altino Choupina.

The aim of the period abroad was to study the microbiome present in pâté olive cake undergoing a driven fermentation using microbial pools. In detail, during such a period, several tests were carried out to extract total DNA from fermented pâté samples in order to explore the microbial population by NGS from the beginning to the end of fermentation. Indeed, samples were sequenced on an Illumina HiSeq for the V3-V4 hypervariable regions of the bacterial 16S rRNA gene and the ITS1-ITS2 region of the fungal 18S rRNA gene. NGS metagenomic analysis was performed at the Nucleotide Sequencing Service of the University of Salamanca, Spain. Subsequently, the data were processed using different bioinformatics databases.

In attachment the certification of the period spent at the IPB.

INSTITUTO POLITÉCNICO DE BRAGANÇA
Escola Superior Agrária

Altino Branco Choupina, Professor and Chair of Molecular Biology and Genetic Engineering at the Department of Biology and Biotechnology, Polytechnic Institute of Bragança
E-mail: albracho@ipb.pt,
Movil and WhatsApp - + 351 938473565
Tel: (+351) 273 303 200

Recommendation letter for **Paola Foti**
To whom it may concern

I am international director of the Doctoral thesis of **Paola Foti** for which she developed research within the Molecular Biology, Genetic Engineering, Bioinformatics and Metagenomics, in my laboratory from 1/04/2002 to 24/06/2022 at the Polytechnic Institute of Bragança- Specifically: DNA extraction, purification and quantification; sequencing on the Ilumina miseq platform and bioinformatics analysis of results.

The monitoring of **Paola Foti** work during their academic and professional path, allows me to evaluate their performance in terms of research:

Develop and coordinate advanced scientific projects.
Overtaking, effectively, the experimental difficulties encountered that are natural in research work;
Ability to adapt to new situations;
Critical sense and great reflection in their interpretations;
Working well as a team and ease of relationship with other researchers working in the same laboratory;
Intelligent, determined and persevering.

In conclusion, **Paola Foti**, have initiative, autonomy, and demonstrated great rigor, dedication, commitment to carry out research work and has a scientific and technical profile with the capacity to carry out excellent scientific work and integration into any work team.

Bragança-Portugal, 25 June 2022
Prof. Doctor Altino Branco Choupina

Prof. Doctor Altino Branco Choupina

NATIONAL TRAINING

During the doctorate period, 3 months were spent at the Department of Pharmaceutical Sciences, University Aldo Moro (Bari), under the supervision of Prof.ssa Maria Grazia Perrone.

The research activities were aimed at evaluating the biological activity of fermented olive oil mill wastewaters and pâté olive cakes, based on the already published studies on both antioxidant and anti-inflammatory effects of oil phenols, as hydroxytyrosol, which may play a key role in the prevention and modulation of several pathologies, including cancer.

Several assays were carried out during this period, such as:

- Activity on COX-1 and COX-2
- Transepithelial Transport of samples through Caco-2 Cell Monolayers
- Cytotoxic activity
- Antioxidant activity.

In attachment the certification of the period spent at the Department of Pharmaceutical Sciences.

UNIVERSITÀ DEGLI STUDI DI BARI ALDO MORO

Dipartimento di
Farmacia-Scienze del farmaco

Prof. Maria Grazia Perrone
Dipartimento di Farmacia-Scienze del Farmaco
Università degli Studi di Bari-Italy
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Recommendation letter for **Paola Foti**

To whom it may concern

I am the head of the Biopharmaceutical laboratory where **Paola Foti** carried out research activities from 4/07/2022 to 29/07/2022 and from 5/09/2022 to 21/10/2022., in order to test samples obtained during her PhD program. In my laboratory **Paola Foti** cultivated human cell lines, performed cyclooxygenase inhibitor activity assay, she performed permeability tests through cell monolayers and tested her samples to verify their antioxidant activity.

In this period, she acquired the sufficient skills to be able to work independently and to carry out a critical evaluation of the results obtained, as well as a conscious use of the instrumentation used.

Paola Foti immediately established a peaceful atmosphere with my research group, demonstrating that she knows how to work in a team with perseverance and stubbornness. He has shown great dedication and passion for her work and above all excellent scientific skills.

Working with Paola was very stimulating.

BARI, 28/10/2022

FIRMA
Maria Grazia Perrone

ACKNOWLEDGEMENTS

I would like to thank Prof. Vito De Pinto for supervising me during my doctoral studies. I sincerely thank my tutor Prof.ssa Cinzia Caggia and my co-tutor Dott.ssa Flora Valeria Romeo who have always believed in me, supported, and driven me with patience and faith during my PhD course. For me, both are an example of strength, unity and perseverance, which are essential factors for reaching the set aims. Thanks to them, I have been highly motivated, and they have also given me the opportunity to get to know and work in various laboratories contributing to my professional and personal progress. I also thank my co-tutor Prof.ssa Cinzia Randazzo for always being there for motivating me. Furthermore, I thank all members of the research group at the Di3A: Alessandra, Nunzia, Amanda, Rosamaria, Fabrizio, Paride, Nunzio, Gigi, Andressa and Marco who have been there for me in happy but also difficult moments. I thank my international tutor Prof. Altino Choupinafor welcoming me into his research group in Portugal and making me an active part of all his experimental activities. Muito obrigada!

I thank Sig. Pasquale Consoli, the company tutor, for always believing in me and supporting my ideas. Together we slowly succeeded in having a 'problem-solving' outlook in order to achieve the desired results.

I would like to thank Prof.ssa Maria Grazia Perrone and Morena Miciaccia, for welcoming me to their team at the University of Bari, and for their great helpfulnessand kindness in transferring me new knowledge useful for my professional growth.

I would like to thank all my friends at CREA-OFA in Acireale, and in particular, Solidea and Federica, who supported me and were always by my side. Furthermore, I sincerely thank Dott.ssa Nicolina Timpanaro.

I thank all my friends, especially Gina and Luca who have been always my right arm and my greatest supporters. Thanks to my friend Francesco, my companion of adventure and happiness during my time in Portugal and in Bari.

Finally, I thank my family, who with love and affection has always helped and supported me. Thanks to them, I was able to find my way, passing on to me values such as the pleasure of work, grit against challenges and the defence of my ideas.

Finally, I conclude with good luck to myself ... ready to start a new chapter!