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Study of stress adaptation, versatility and functionality of selected Lactiplantibacillus plantarum strains used as starter cultures for table olives fermentation

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"To know how to wonder and question is the first step of the mind toward discovery"

-Luis Pasteur

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Research highlights

- Table olives are a source of microorganisms, genes, proteins and metabolites;
- Omics approaches as a new frontier to in-depth investigate table olives ecosystem;
- Dual approach to understand microbial variability and metabolites;
- Selected *Lactiplantibacillus plantarum* strains with a ß-glucosidase enzyme are promising tailor-made starter cultures for table olives;
- ß-glucosidase genes have the potential to encode enzymes such as glycoside hydrolases and glycosyl hydrolases;
- Candidate primers for β-glucosidase genes involved oleuropein degradation were designed;
- Metagenetics to reveal composition and dynamics of table olives microbiota;
- Volatilomics to detect VOCs profile related to pleasant flavour of table olives.

PREFACE

In recent decades, fermented products have acquired increasingly value as an essential source of antioxidant and nutritional compounds, and for these reasons are strongly recommended for the human diet. Among the fermented products known and consumed worldwide, table olives are an essential part of the Mediterranean Diet and part of the culinary tradition in many countries around the world. Due to their high nutritional value in terms of fibre, vitamins, minerals, polyphenol compounds and polyunsaturated fatty acids, table olives have long been considered as a functional product. This food matrix is and has been the subject of study for many years, both at the chemical and biological level, with particular attention on understanding its complexity at the microbiological level. Research studies have always highlighted how microbial variability can be strongly influenced by techniques, cultivars and territory, making often difficult the reproducibility of the fermentation process. Different methods to produce table olives (from Sevillian style, with chemical treatment, to Greek and traditional spontaneous styles, with and without the addition of starter cultures) are available, with the common aim to reduce the glucosidic compounds, such as oleuropein, responsible for the bittering of table olives, which make olives inedible for human consumption. The fermentation process is always carried out by indigenous microbial population or deliberately added starter cultures, mainly composed by lactic acid bacteria (LAB) and yeasts, which are responsible both of bitterness process and of organoleptic compounds formation in the final product. The selection of LAB and/or yeast strains, is one of the biotechnological tools needed for choosing a starter culture tailored for table olives fermentation. Current studies have identified several species, belonging to the Lactobacillaceae family, that are capable of performing this arduous duty. In particular, species belonging to

Lactiplantibacillus genus, often isolated from fermented vegetables, has been widely used as a model species for metabolic, ecological, and genetic studies. In addition, it is employed as a starter culture for table olives for their high versatility, tolerance to stress brine conditions, ability to cooperate with endogenous yeasts until the end of the fermentation process, ability to cope with the inhibitory phenolic compounds and to degrade, thought the enzymatic pathway, bitter substances of table olives. In this regard, the investigation of genes encoding for enzymes of the β-glucosidase family, involved in oleuropeinolytic degradation, is not always included in the selection criteria for a starter culture; and only few studies were conducted in this field.

Table olives are a traditional food of Mediterranean diet, for containing bioactive compounds, with antimicrobial and anti-inflammatory properties. However, the high level of salt content still limits their consumption and the reduction of daily salt intake, as already suggested by WHO/FAO, requires the design of new products or the re-formulation of this traditional Mediterranean food. In this scenario, the production of lowered salt table olives of Sicilian cultivars, through the use of selected starter cultures with functional properties, is seen as an effective way to extend their consumption, promoting their high nutritional value. However, in order to develop functional table olives, a snapshot of microbial population involved in the fermentation process and responsible of the organoleptic traits of the final products is needed. Recently, the spread of high-throughput approaches has allowed a more sophisticated level of investigation of microbes, genes, proteins and metabolites with enormous potential for integrating the composition of table olives with functional assessments. These culture-independent techniques have helped to understand microbial ecology and the development of fermentations but also to comprehend microbial interactions that drive a higher quality process.

OUTLINE OF THE THESIS

This thesis meanly deals with the selection of starter culture, with oleuropenolytic activity, tailored to produce Sicilian table olives at low salt content. In detail, the aim of this study project was to: (i) develop a selected starter cultures for table olives fermentation at low salt content; (ii) investigate gene expression of β-glucosidase enzyme of selected strains, under stress condition such as brine; and (iii) elucidate on the effect of the selected starter cultures on microbial composition and function during table olives fermentation through an omics approach. In detail, this thesis is composed by: An Introduction and three Chapters describes as following;

Introduction: is an extensive literature review, which provides an overview of the critical literature of culture-independent techniques, in particular omics approaches, applied on fermented table olives in the last decade, highlighting weaknesses and strengths related to their application. This work focused on the implementation of omics approaches, which make possible to investigate, in depth, the microbial composition and metabolic processes driving the fermentation process of table olives. In addition, the review highlights the role of omics techniques to elevate investigations of microbes, genes, proteins and metabolites to a more sophisticated level, with a huge potential for integrating table olive composition with functional assessment. The application of a multi-omics approach to the study of the table olive ecosystem can be useful to increase the knowledge of the mechanisms that may influence the organoleptic and safety characteristics of the final product.
This work presented here has been published in Food Microbiology. https://doi.org/10.1016/j.fm.2020.103606

Chapter 1: is a study article, which describes the application of the selection criteria applied for starter cultures, tailored for table olive fermentation. In detail, the present study investigated the effect of different stress factors (pH, temperature and NaCl) on growth and on oleuropein-degrading abilities of selected *Lactiplantibacillus 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 the importance to test the technological performances of strains at process conditions in order to achieve a good selection of tailored starter cultures for table olives.
This work presented in Chapter 1 has been published in Microorganims. https://doi.org/10.3390/microorganisms8101607

Chapter 2: is a study article focuses on the evaluation of the metabolomic and transcriptional profiles of the gene encoding for the β-glucosidase enzyme, in *Lactiplantibacillus plantarum* species. In detail, the study aims to elucidate the mechanisms responsible for the bioconversion of oleuropein into low molecular weight phenolic compounds in two selected *L. plantarum* strains, under stress brine conditions. For this purpose, an experimental strategy, combining high-resolution mass spectrometry, *in silico* functional analysis of

GH1 candidates and gene expression study was adopted. Oleuropein hydrolysis products and underlying enzymatic steps were identified, as well as a novel putative *bgl* gene, responsible for the β-glucosidase activity, under low temperature conditions. According to biochemical analysis, both strains showed the ability to hydrolyse oleuropein and release hydroxytyrosol through the formation of an intermediate compound Hy-EDA. The strain C11C8 showed a more pronounced activity at 16 °C. At the genomic level, the presence of the β-glucosidase gene was detected by using the primer pairs proposed in this work (CS400_14770) and by primer pairs developed by Zago et al. (2013) (CS400_14765) and Spano et al. (2005) (CS400_15205). Both strains were able to transcribe the 14770 gene under all tested conditions. In addition, the gene was expressed also with m-bgl primers, with the exception of strain C11C8 at 16 °C. The bglu gene was not transcribed, although it was detected genomically. The strain C11C8 showed a more pronounced activity at 16 °C, suggesting the involvement of the 14770 gene as the only transcribed gene in that stress condition. In conclusion, strains C11C8 and F3.5 have different metabolite patterns at 16 °C and their genomes harbour different variants of the CS400_14770 gene. The difference in alleles of the gene locus responsible for differential β-glucosidase activity under low temperature conditions could have an important practical implication in brine fermentation of table olives and could guide future selection criteria for new oleuropein-free *L. plantarum* starter cultures. This work presented in Chapter 2 will be submitted in Applied and Environmental Microbiology

Chapter 3: is a study article, which discusses the setting of low-salt Sicilian table olives with β-glucosidase positive strains, and the use of Metagenetics and Volatilomics approaches, in order to provide a better understanding of the microbial and metabolic profiles between inoculated and non-inoculated table olives. In detail, this study aimed

to investigate the effect of previously selected *Lactiplantibacillus plantarum* strains, able to grow and degrade oleuropein under stress conditions, on both the microbiota composition and the profile of volatile organic compounds of Sicilian table olives. Two starter cultures were set up and four pilot-scale fermentations were carried out at 5% (O1, O2 and C5) and 8% (C8) salt content. The fermentation process was monitored for up to 80 days using a dual approach that included both classical microbiological and metagenetic and volatilomic analyses based on 16S rRNA gene. Compared to the control samples (C5 and C8), the fermentations conducted with the addition of starter cultures (O1 and O2) showed a better acidification of the brine with a more pronounced drop in pH. Metagenetic data revealed, during the fermentation process, the dominance of the *Lactobacillus* genus in inoculated samples with a concomitant drastic reduction of *Enterobacter* sp. In contrast, the dominance of *Enterobacter* sp (57%) and *Weissella* sp was recorded in the control samples treated at 5% and 8% salt, respectively. The dual approach applied clearly demonstrated the dominance of *L. plantarum* species in inoculated samples, and the ability of the selected strains to adhere to the drupe surface and inhibit spoilage bacteria. According with volatilomics data, in the inoculated samples, the presence of volatile organic compounds responsible for pleasant taste were revealed.
This work in Chapter 3 has been submitted in Frontiers in Microbiology. Manuscript ID: 771636

Other activities: is as a collection of research articles, projects and conference participations carried out during the PhD cycle.

The research article "*Effect of Sequential Inoculum of Beta-Glucosidase Positive and Probiotic Strains on Brine Fermentation to Obtain Low Salt Sicilian Table Olives"* deals the application of β-glucosidase positive strain *Lactiplantibacillus plantarum* F3.3 as

starter during the fermentation of Sicilian table olives (Nocellara Etnea cultivar) at two different salt concentrations (5 and 8%), in order to accelerate the debittering process. The latter was monitored through the increase of hydroxytyrosol compound. In addition, the potential probiotic *Lactobacillus paracasei* N24 strain was added after 60 days of fermentation. Un-inoculated brine samples at 5 and 8% of salt were used as control. The fermentation was monitored till 120 days through physico-chemical and microbiological analyses. In addition, volatile organic compounds and sensorial analyses were performed during the process and at the end of the fermentation, respectively. Lactic acid bacteria and yeasts were, in depth, studied by molecular methods and the occurrence of the potential probiotic N24 strain in the final products was determined. Results highlighted that inoculated brines exhibited a higher acidification and debittering rate than control ones. In addition, inoculated brines at 5% of salt exhibited higher polyphenols (hydoxytyrosol, tyrosol, and verbascoside) content compared to samples at 8% of NaCl, suggesting a stronger oleuropeinolytic activity of the starter at low salt concentration. Lactobacilli and yeasts dominated during the fermentation process, with the highest occurrence of *L. plantarum* and *Wickerhamomyces anomalus*, respectively. Moreover, the potential probiotic *L. paracasei* N24 strain was able to survive in the final product. Hence, the sequential inoculum of beta-glucosidase positive and potential probiotic strains could be proposed as a suitable technology to produce low salt Sicilian table olives.

This work has been published in Frontiers in Microbiology https://doi.org/10.3389/fmicb.2019.00174

The review article "*Olive mill wastewater as renewable raw materials to generate high added-value ingredients for agro-food industries"* deals the olive oil production as an agro-industrial activity of vital economic importance for many Mediterranean countries. It is associated with the generation of a huge amount of byproducts, 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, anti-hyperglycemic activities. Therefore, the increasing interest in natural bioactivecompounds 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. The next page shows a figure illustrating the study design of the thesis project described above.
This work has been published in Applied Science. https://doi.org/10.3390/app11167511

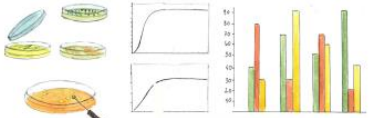
The research article *"Characterization of cell-envelope proteinases from two Lacticaseibacillus casei strains isolated from Parmigiano Reggiano cheese"* deals the characterization of two cell-envelope proteinases (CEP) from *Lacticaseibacillus casei* strains PRA205 and

2006 at both the biochemical and genetic levels. The genomes of both the L. casei strains included two putative CEP genes prtP2 and prtR1, but only prtR1 was transcribed. The extracted PrtR1 proteinases were serine proteinase with optimal activity at 40°C and pH 7.5 and activated by Ca2+ ions. Interestingly, PrtR1 from L. casei PRA205 exhibited high residual activity at pH 4 and at 5°C, suggesting its possible exploitation for fermented food production. The caseinolytic activity against αS1- and β-casein indicated that both the PrtR1 belong to PI/PIII type. These PrtR1 cleaved β-casein peptide bonds preferentially when amino acid M or N were present at the P1 sub-site and amino acid A and D at the P1' sub-site. Several bioactive peptides were found to be released from PrtR1 after αs1- and β-casein hydrolysis.

This work in Other Activities has been submitted in Food Chemistry em.foodchem.0.754986.752005d5@editorialmanager.com.

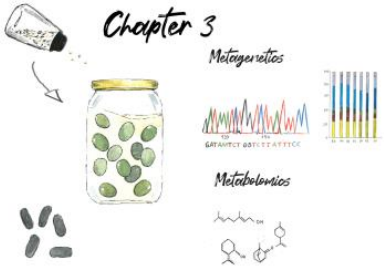
Introduction

Chapter 1



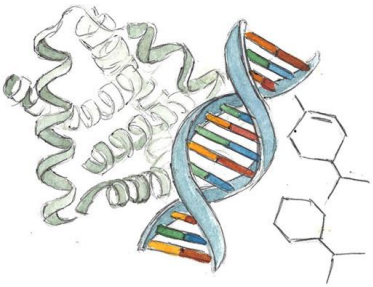
Chapter 2

Chapter 3



Other activities

Introduction



INTRODUCTION: Review Article

FoodOmics as a new frontier to reveal microbial community and metabolic processes occurring on table olives fermentation

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This review article was written in collaboration with Professor Maria De Angelis of the Department of Soil, Plant and Food Sciences at the University of Bari.

1 Introduction

1.1 Table olives: a fermented food with a complex microbial consortium

Fermented foods, such as table olives, bread, cheese, and wine have been prepared in rural households and small village communities for thousands of years and are strongly linked to culture and tradition (Botta and Cocolin 2012). At first, fermented foods were obtained through a spontaneous and unpredictable process, and over the years, many practices, such as back slopping, have been developed to improve the quality and the safety of the final product. Table olives are considered the most largely diffused fermented vegetables in the Mediterranean area and their consumption is expanding worldwide thanks to the nutritional and functional value of drupes, related to the presence of polyphenols, vitamins, minerals, and fatty acids. The content of the latter compounds changes according to both olive drupe maturity and cultivar (Lavermicocca et al. 2005). Overall, the olive drupe contains a low concentration of sugar (2.6–6.0%), a high oil content (12–30%) (Botta and Cocolin, 2012) and a polyphenols fraction, which is characterized by the presence of oleuropein, responsible for the intense bitter taste. During the fermentation, this compound is hydrolysed by the activity of the β-glucosidase enzyme of indigenous microorganisms, with the release of glucose and aglycones, which, in turn, are completely degraded by esterase in the no-bitter phenols hydroxytyrosol and elenolic acid (Bianchi 2003). Hence, the presence of these compounds is strongly influenced by microbial consortium of table olives, which is strongly affected by both cultivar and technological process. Generally, table olives microbiota includes members of lactic acid bacteria (LAB) and yeasts, which are the dominant microbial groups throughout the fermentation, whereas *Enterobacteriaceae*, *Clostridium*, *Pseudomonas*,

Staphylococcus strains and, occasionally, molds (Bonatsou et al. 2017) may occur at the beginning of the process (Abriouel, Benomar, Lucas, et al. 2011; Abriouel, Benomar, Pulido, et al. 2011; Panagou, Tassou, and Katsaboxakis 2003; Randazzo et al. 2012). Among LAB, members of the genera *Lactobacillus*, *Streptococcus*, *Leuconostoc*, and *Pediococcus* and of the new genera recently proposed by Zheng et al. (Zheng et al. 2020) were the main detected during the fermentation process (Abriouel, Benomar, Lucas, et al. 2011; Abriouel, Benomar, Pulido, et al. 2011; Corsetti et al. 2012). To large extent, LAB, by fermenting sugars to organic acids, decrease pH of brine and stabilize the final product. When olive drupes are subjected to lye treatment (2.0–3.5% of NaOH) LAB dominate at the end of the fermentation process. On the contrary, in directly brined olives, LAB occur during the whole process, but their dominance is subjected to the low sugars concentration and to the presence of molecules released from olive flesh. In addition, LAB development can be influenced by phenolic compounds releasing from drupe to brine as well as by both sodium chloride concentration and temperature of fermentation (Bautista-Gallego et al. 2011; Tassou, Panagou, and Katsaboxakis 2002). In fact, when the fermentation is carried out at low temperature (under 18 ◦C) and in presence of high salt content (over than 8.0%), LAB are overpowered by yeasts (Arroyo-López et al. 2008; Tassou et al. 2002), which determine mild taste and less self-preservation of final products (Panagou et al. 2008). However, when yeasts are present in a balanced proportion with LAB, they contribute to improve flavour and texture of the final product (Arroyo-López et al. 2008). Hence, LAB and yeast population create a microbial consortium, which acts for the success of the fermentation process. The most frequent LAB, and yeast species detected in table olive are summarized in Tables 1 and 2, respectively. Among LAB, *Lactiplantibacillus plantarum*, *Lactiplantibacillus pentosus*,

Lacticaseibacillus casei, *Levilactobacillus brevis* and *Leuconostoc mesenteroides* are the species most frequently isolated from different cultivars (Panagou et al. 2008; Randazzo et al. 2004; Sánchez et al. 2001), while *Wyckerhamomyces anomalus*, *Candida diddensiae*, *Candida boidinii*, *Debaryomyces hansenii* and *Pichia membranifaciens* are the main yeast species (Campus, Degirmencioglu, and Comunian 2018). During the last twenty years, food microbiologists have introduced considerable changes in the study of table olives microbial ecosystem, traditionally relied on cultivation, isolation and phenotypic and (or) genotypic characterization of the microbial isolates. In particular, the advent of the DNA-based approaches (such as single-strand conformation polymorphism, terminal-restriction fragment length polymorphism, denaturing gradient gel electrophoresis, temperature gradient gel electrophoresis, etc.) (Nocker, Burr, and Camper 2007), has enabled a clear picture of the microbiota of table olives, revealing microbial taxa previously overlooked. Thus, the application of culture-independent techniques has considerably changed the way to study food microbial ecology, leading to consider microbial populations as a consortium (Cocolin and Ercolini 2015). Given the great importance of the microbiota in determining the quality of table olives, considerable efforts are being made to identify the microbial species and their dynamics deepening the composition and functionality of table olives microbiota, through the application of omics approach mainly consisting in high-throughput methods and sophisticated bioinformatics tools. The term omics encompasses a set of approaches, which include metagenetics, metagenomics, metatranscriptomics, metaproteomics and metabolomics. The omics techniques can be considered the most powerful tool to study biological systems in terms of composition, activity, and function. The present review aims at illustrating the potentiality of omics approaches

as a new frontier to deepen the knowledge about microorganisms, enzymes, and metabolites involved in table olives fermentation and to discover new biomarkers of olives fermentation.

Table 1. Lactic acid bacteria species detected in fermented table olives of different cultivars.

Species detected	Table olives cultivar	Country	References
Lactiplantibacillus plantarum	Aloreña	Spain	(Abriouel, Benomar, Lucas, et al. 2011; Abriouel, Benomar, Pulido, et al. 2011; Bautista-Gallego et al. 2013; Benítez-Cabello, Calero-Delgado, et al. 2019)
	Arbequina	Spain	(Hurtado et al. 2008, 2009, 2010)
	Bella di Cerignola	Italy	(De Bellis et al. 2010; Bevilacqua et al. 2010)
	Conservolea	Greece	(Argyri et al. 2013; Bleve et al. 2015; Doulgeraki et al. 2013; Tassou et al. 2002)
	Edincik black olives	Turkey	Borcakli et al. 1993.
	Gemlik black olives	Turkey	Borcakli et al. 1993.
	Halkidiki	Greece	(Argyri et al. 2013; Doulgeraki et al. 2013)
	Kalamata	Greece	(Bleve et al. 2015; Doulgeraki et al. 2013)
	Galega	Portugal	(van den Berg et al. 1993; Oliveira et al. 2004)
	Giarraffa	Italy	(Randazzo et al. 2012)
	Gordal	Spain	(Bautista-Gallego et al. 2013; Benítez-Cabello, Calero-Delgado, et al. 2019)
	Grossa di Spagna	Italy	(Randazzo et al. 2012)
	Jijelian black olives	Algeria	(Idoui et al. 2009)
	Leccino	Italy	(Ercolini et al. 2006)
	Manzanilla	Spain	(Bautista-Gallego et al. 2013; Benítez-Cabello, Calero-Delgado, et al. 2019)
	Moroccan table olives	Morocco	(Abouloifa et al. 2020)
	Nocellara del Belice	Italy	(Guantario et al. 2018)
	Nocellara Etnea	Italy	(Botta et al. 2014; Pino et al. 2018, 2019; Randazzo et al. 2018)
	Oblica table olives	Croatia	(Kulišiae, Berkoviae, and Paviae 2004)
	Picholine	Morocco	(Asehraou et al. 2002; Ghabbour et al. 2011)

Lactiplantibacillus pentosus	Aloreña	Spain	(Abriouel et al. 2012; Bautista-Gallego et al. 2013; Benítez-Cabello, Calero-Delgado, et al. 2019; López-López et al. 2018)
	Arbequina	Spain	(Hurtado et al. 2008, 2009, 2010)
	Bella di Cerignola	Italy	(De Bellis et al. 2010; Campaniello et al. 2005)
	Conservolea	Greece	(Argyri et al. 2013; Doulgeraki et al. 2013; Panagou et al. 2008; Tassou et al. 2002)
	Gordal	Spain	(Bautista-Gallego et al. 2013; Benítez-Cabello et al. 2015; Benítez-Cabello, Calero-Delgado, et al. 2019; Domínguez-Manzano et al. 2012; Ghabbour et al. 2011)
	Halkidiki	Greece	(Argyri et al. 2013; Doulgeraki et al. 2013)
	Kalamata	Greece	(Doulgeraki et al. 2013)
	Manzanilla	Spain	(Francisco N. Arroyo-López et al. 2012; Bautista-Gallego et al. 2013; Benítez-Cabello, Calero-Delgado, et al. 2019; López-López et al. 2018)
	Moroccan green olives	Morocco	(Abouloifa et al. 2020)
	Nocellara del Belice	Italy	(Aponte et al. 2012; Guantario et al. 2018)
	Nocellara Etnea	Italy	(Botta et al. 2014; Pino et al. 2018, 2019).
	Picholine	Morocco	(Ghabbour et al. 2011).
Lactiplantibacillus paraplantarum	Aloreña	Spain	(Bautista-Gallego et al. 2013)
	Arbequina	Spain	(Hurtado et al. 2008, 2009)
	Conservolea	Greece	(Doulgeraki et al. 2013)
	Gordal	Spain	(Bautista-Gallego et al. 2013)
	Halkidiki	Greece	(Doulgeraki et al. 2013)
	Kalamata	Greece	(Doulgeraki et al. 2013)
Lacticaseibacillus casei	Bella di Cerignola	Italy	(De Bellis et al. 2010)
	Jijelian black olives	Algeria	(Idoui et al. 2009)
	Nocellara Etnea	Italy	(Asehraou et al. 2002; Mourad and Nour-Eddine 2006; Pino et al. 2018; Randazzo et al. 2018)
	Sigoise	Algeria	(Mourad and Nour-Eddine 2006)
Lacticaseibacillus rhamnosus	Bella di Cerignola	Italy	(De Bellis et al. 2010)
	Nocellara Etnea	Italy	(Kulišiae et al. 2004)
	Sigoise	Algeria	(Mourad and Nour-Eddine 2006)
Levilactobacillus brevis	Conservolea	Greece	(Tassou et al. 2002)
	Gemlik	Turkey	(Kumral et al. 2009).
	Jijelian	Algeria	(Idoui et al. 2009)
	Moroccan green olives	Morocco	(Abouloifa et al. 2020)
	Picholine	Morocco	(Ghabbour et al. 2011)
Lactococcus lactis	Bella di Cerignola	Italy	(De Bellis et al. 2010)

	Sigoise	Algeria	(Mourad and Nour-Eddine 2006)
Leuconostoc mesenteroides	Conservolea	Greece and Italy	(Argyri et al. 2013; Bleve et al. 2015; Doulgeraki et al. 2013; Tassou et al. 2002)
	Halkidiki	Greece	(Argyri et al. 2013; Doulgeraki et al. 2013)
	Kalamata	Greece and Italy	(Bleve et al. 2015; Doulgeraki et al. 2013)
	Nocellara del Belice	Italy	(Guantario et al. 2018)
	Nocellara Etnea	Italy	(Botta et al. 2014)
Enterococcus faecium	Cypriot	Cyprus	(Anagnostopoulos, Bozoudi, and Tsaltas 2018)
	Sigoise natural green olives	Algeria	(Mourad and Nour-Eddine 2006)

Table 2. Yeasts species detected in fermented table olives of different cultivars

Species detected	Table olives cultivar	Country	References
Candida boidinii	Aloreña	Spain	(Arroyo-López et al. 2006)
	Arbequina	Spain	(Hurtado et al. 2008)
	Bosana	Italy	(Porru et al. 2018)
	Galega	Portugal	(Pereira et al. 2008)
	Hojiblanca	Spain	(Arroyo-López et al. 2006)
	Kalamata	Greece	(Bonatsou, Paramithiotis, and Panagou 2018)
	Manzanilla	Portugal	(Alves, Gonçalves, and Quintas 2012)
	Negrinha de Freixo	Portugal	(Pereira et al. 2015)
	Nocellara Etnea	Italy	(Pino et al. 2019)
	Nocellara Messinese	Italy	(Sidari, Martorana, and De Bruno 2019)
Candida diddensiae	Aloreña	Spain	(Arroyo-López et al. 2006; Bautista-Gallego et al. 2011)
	Arbequina	Spain	(Hurtado et al. 2008; Romo-Sánchez et al. 2010)
	Cornicabra	Spain	(Romo-Sánchez et al. 2010)
	Gordal	Spain	(Bautista-Gallego et al. 2011)
	Hojiblanca	Spain	(Porru et al. 2018)
	Leccino	Greece	(Doulgeraki et al. 2013)
	Manzanilla	Portugal	(Alves et al. 2012; Bautista-Gallego et al. 2011)
	Nocellara Etnea	Italy	(Pino et al. 2019)
Wickerhamomyces anomalus	Aloreña	Spain	(Bautista-Gallego et al. 2011)
	Ascolana	Spain	(Ruiz-Moyano et al. 2019)
	Azeitera	Spain	(Ruiz-Moyano et al. 2019)
	Bella di Cerignola	Italy	(Bevilacqua et al. 2013)

	Bosana	Italy	(Porru et al. 2018)
	Gordal	Spain	(Bautista-Gallego et al. 2011; Ruiz-Moyano et al. 2019)
	Leccino	Italy	(Ciafardini and Zullo 2019)
	Nocellara Etnea	Italy	(Pino et al. 2019)
	Nocellara Messinese	Italy	(Sidari et al. 2019)
Saccharomyces cerevisiae	Aloreña	Spain	(Arroyo-López et al. 2006)
	Arbequina	Spain	(Romo-Sánchez et al. 2010)
	Ascolana	Spain	(Ruiz-Moyano et al. 2019)
	Azeitera	Spain	(Ruiz-Moyano et al. 2019)
	Bosana	Italy	(Porru et al. 2018)
	Conservolea	Italy	(Bleve et al. 2015)
	Cornicabra	Spain	(Romo-Sánchez et al. 2010)
	Gemlik	Turkey	(Mujdeci et al. 2018)
	Gordal	Spain	(Ruiz-Moyano et al. 2019)
	Kalamata	Greece	(Bleve et al. 2015; Bonatsou et al. 2018)
	Manzanilla	Portugal	(Alves et al. 2012; Hernández et al. 2007)
	Negrinha de Freixo	Portugal	(Pereira et al. 2015)
	Nocellara Messinese	Italy	(Sidari et al. 2019)
Debaryomyces hansenii	Manzanilla	Spain	(Hernández et al. 2007)
	Thassos	Greece	(Panagou, Tassou, and Katsaboxakis 2007)
Pichia kluyveri	Arbequina	Spain	(Hurtado et al. 2008)
	Nocellara Etnea	Italy	(Pino et al. 2018)
Pichia galeiformis	Manzanilla	Spain	(Francisco N. Arroyo-López et al. 2012)
Pichia membranifaciens	Arbequina	Spain	(Hurtado et al. 2008)
	Ascolana	Spain	(Ruiz-Moyano et al. 2019)
	Azeitera	Spain	(Ruiz-Moyano et al. 2019)
	Conservolea	Greece and Italy	(Bleve et al. 2015; Nisiotou et al. 2010)
	Gordal	Spain	(Benítez-Cabello et al. 2015)
	Negrinha de Freixo	Portugal	(Pereira et al. 2015)

1.2 Why do we need biomarkers for table olives fermentation?

Table olives fermentation involves the transformation of bitter inedible olives into an edible foodstuff. Numerous table olive processing methods are known, the choice of which mainly depends on olive variety, degree of ripeness, and on available process technology. The main fermentation processes are reported in Fig. 1. The fermentation of table olives is hard to control because the raw material cannot be thermally treated and abnormal phenomena could occur (Bonatsou et al. 2017; Heperkan 2013; Iorizzo et al. 2016). For this reason, salt is added in order to reduce the water activity, prevent the growth of spoilage microorganisms, and improve taste and textures of the final product (Bautista-Gallego et al. 2013). Recently, according to World Health Organization (WHO, 2012) which recommends to reduce the daily salt intake (5 g salt per day), several authors proposed the partial substitution of NaCl with calcium and potassium salts, such as KCl, CaCl2, and ZnCl2 (Ambra et al. 2017; Bautista-Gallego et al. 2010, 2013; Bautista Gallego et al. 2011; Mateus et al. 2016; Zinno et al. 2017). Nevertheless, the replacement affected the microbiota of table olives (Mateus et al. 2016) modifying the sensorial quality of the final product (Zinno et al. 2017). Recently, Pino and co-workers (Pino et al. 2018, 2019) demonstrated that the reduction of NaCl content, without the addition of other salts, resulted in a successful fermentation of Nocellara Etnea table olives, suggesting the possibility to formulate low salt table olives.

The use of starter cultures is largely applied to Spanish-style fermentation, where their addition is still considered a useful biotechnological strategy in order to prevent the development of pathogenic and/or spoilage bacteria, accelerating the brine acidification (Romeo 2012). The use of starter cultures has been the

subject of many studies and nowadays the interest in multifunctional starters, with adequate technological properties, has strongly increased (Ciafardini and Zullo 2019). Many studies have recently demonstrated the synergic effects between LAB and yeast starters, with a significant improvement of sensory quality of the final products (Benítez-Cabello et al. 2019a). However, further efforts are needed to appropriately select and design the starter inoculum in a cultivar dependent manner, including functional and sensorial profiles, and to elucidate the role of different table olives microbial populations and their relationship with specific metabolites. The exploitation of a specific microbiota and how its metabolism impacts on sensorial traits of table olives could help to identify biomarkers linked to certain table olives flavours, texture, and bioactive metabolites with potential effects on human health. Another reason to identify biomarkers is related to the development of a technology able to guarantee the reproducibility of table olives fermentation, achieving a final product with unique characteristics related to cultivar, process and geographical origin.

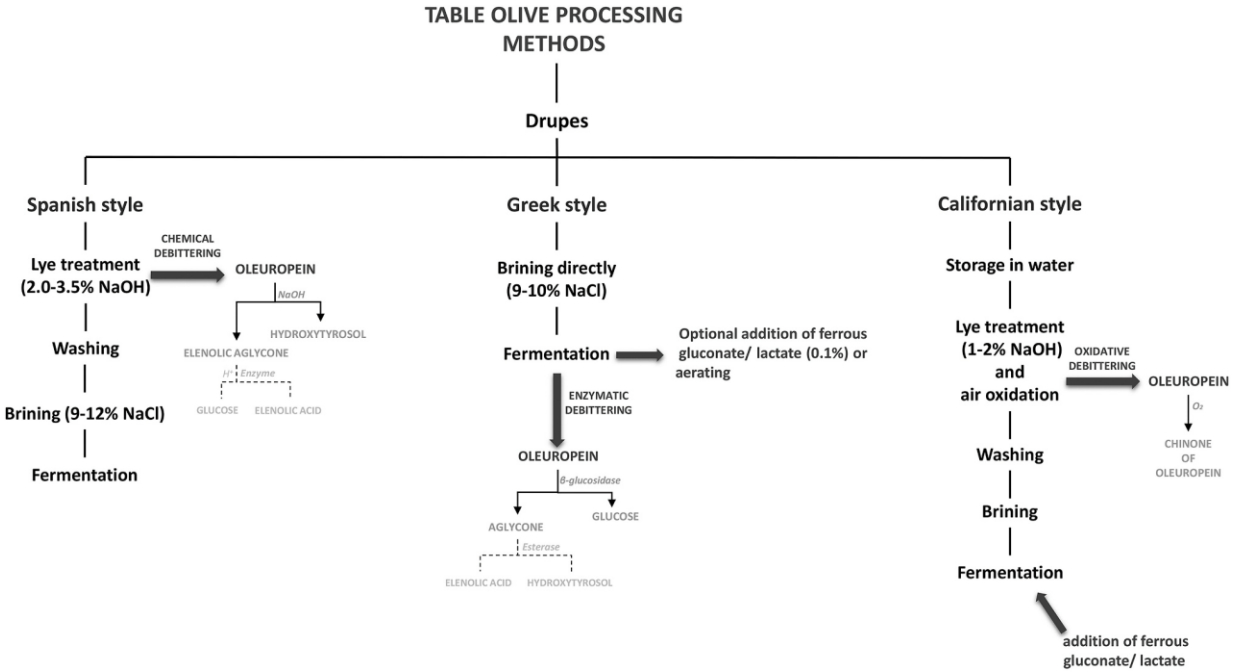

Figure 1 - Table olives fermentation processes.

2 Omics-based approach for molecular profiling of table olives microbial consortium

It has been already established that in food ecosystems, omics technologies have revolutionized our understanding of complex microbial population composition and functions, underlying what the microbial community is doing in terms of gene expression, protein production, and metabolism (Turnbaugh and Gordon 2008). As reported in Table 3, only metagenetics, metabolomics and proteomics approaches have been applied to table olives ecosystem and, overall, the number of the cultivars that have been studied is still limited. The majority (64%) of the studies reported in Table 3 was focused on the characterization of the metabolite produced during the fermentation process; others (28%) revealed table olives microbial composition; only few available studies (8%) have explored the proteomic profile of indigenous LAB. Zooming in each omics approach, the cultivars of table olives investigated, expressed as percentage, are shown in Fig. 2. Manzanilla and Hojiblanca were the main cultivars studied through metabolomics; Aloreña de Malaga, Manzanilla and Nocellara Etnea cultivars through metagenetics and only two studies on Aloreña de Malaga applied a proteomic approach (Fig. 2).

2.1 Metagenetics

The metagenetics approach is based on the analysis of a single gene such as the 16 S rRNA encoding gene, which is the most powerful marker for the identification of phylogenetic studies and bacterial species identification. The development and the application of metagenetics approach, by capturing a broad range of bacterial population, have deepened the knowledge about the composition and the dynamics of food ecosystems revealing greater microbial richness

than expected. In the field of table olives fermentation, the metagenetics approach has been used to gain information on communities less explored by classical cultural methods revealing the complexity of the LAB and yeast microbial consortium (Ferrocino and Cocolin 2017). Evaluating the literature, we can assert that overall metagenetics approach has been more in depth exploited for studying bacterial community that yeast/fungal ones. Presumably, this could be due to an overall lower interest in the yeast composition by the researchers or to (i) the lack of an inclusive, reliable public reference data set; (ii) the lack of means to refer to fungal species, for which no Latin name is available in a standardized stable way and (iii) to a non-standard workflow associated with yeast metagenetic analysis.

Although the ITS barcoding became a reliable taxonomical tool for fungal species identification, within the frame of a curated copy of the public fungal ITS sequences (Kõljalg et al. 2013), the intrinsic multicopy nature of ITS regions may lead to erroneous attribution of the reads to the right species, especially in complex microbial mixtures, like table olives, if intra-genomic variability occurs within single individuals (Colabella et al. 2018; Dakal, Giudici, and Solieri 2016). Table 3 summarizes the main genera and species detected thought the metagenetics approach. Cocolin and co-workers (Cocolin et al. 2013) applied for the first time a high-throughput sequencing approach to determine the bacterial ecology and dynamics occurring during the fermentation of Nocellara Etnea table olives, subjected or not to NaOH treatment. The authors in-depth studied the microbiota composition of brine and olive samples confirming the selective effect of NaOH treatment on bacterial ecology. In particular, the halophilic population (such as *Marinilactibacillus, Halomonas* and *Chromohalobacter*) and *Enterobacteria* resulted dominant on olive surface of untreated and treated samples, respectively. Similarly, Medina and co-workers (Medina et al. 2018), evaluating the microbial

diversity of Spanish style olives darkened by oxidation, revealed, through MiSeq sequencing of the 16 S rRNA, the presence of *Pseudoalteromonas, Alteromonas, Marinomonas* and *Oenococcus* genera. In addition, investigating fungi population though ITS region sequencing, *Pichia membranifaciens, Magnusiomyces capitatus, Kregervanrija fluxum, Dekkera anomala,* and *Dipodascaceae* spp. Were detected for the first time in Spanish-style table olives. de Castro and co-workers (de Castro et al. 2018a), focusing on spoilage bacterial and fungal biota responsible for the unpleasant cheesy and zapatera odors, highlighted the presence of unexpected bacterial taxa, such as *Cardiobacteriaceae* and *Ruminococcus* families. The fungal community of raw material, brines and drupes, during the fermentation of natural Aloreña de Málaga table olives, was studied by Arroyo-López and co-workers (Arroyo-López et al. 2016). Through high-throughput barcoded pyrosequencing analysis of ITS1-5.8 S-ITS2 region, the authors highlighted the existence of a complex fungal consortium which included phytopathogenic, saprofitic, spoilage and fermentative genera. In particular, *Penicillium, Cladosporium, Malassezia,* and *Candida* were identified as the main important genera in raw material. After 4 months of fermentation, *Zygotorulaspora* and *Pichia* were found predominant in brine whereas *Candida, Penicillium, Debaryomyces* and *Saccharomyces* were mainly detected in drupes. The fungal genera *Penicillium, Pichia,* and *Zygotorulaspora* were considered as the core fungal population. The phylogenetic analysis of the ITS sequences allowed to assign the operational taxonomic units (OTUs) to *Pichia manshurica, Candida parapsilosis/C. tropicalis, Candida diddensiae,* and *Citeromyces nyonensis* clades (Arroyo-López et al. 2016). For table olives fermentation, it is already established that the use of selected starter cultures comes out to be promising in order to standardize the process, to reduce the growth/survival of pathogenic and/or spoilage

microorganisms, to accelerate the hydrolysis of bitter compounds, to improve the aroma and stabilize the final product. By adopting an "omics" approach, these effects have been pointed out on Greek-style Bella di Cerignola (De Angelis et al. 2015) and Sicilian-style Nocellara Etnea (Randazzo et al. 2017) table olives. The bacterial tag-encoded FLX amplicon pyrosequencing showed the dominance of *Hafnia alvei* and *Methylobacterium* in un-inoculated Bella di Cerignola samples at the first day of fermentation. Differently, after 90 days of fermentation the vast majority of the OTUs were identified as *Lactiplantibacillus plantarum* and *Lactiplantibacillus pentosus*, followed by *Loigolactobacillus coryniformis*, *Levilactobacillus brevis*, *Lacticaseibacillus paracasei*, *Secundilactobacillus paracollinoides*, *Paucilactobacillus vaccinostercus* and *Lactococcus lactis* in all samples. On the contrary, *Proteobacteria*, including Enterobacteriacea, *Lactococcus lactis*, *Propionibacterium acidipropionici* and *Clostridium*, showed low abundance (De Angelis et al. 2015). Similar results were obtained on Nocellara Etnea brine samples through Ion Torrent PGM Sequencing of V3 region of the 16 S rRNA gene (Randazzo et al. 2017). The dominance of starter was highlighted at the initial stage of fermentation in all brine samples. At the end of the process (60 and 120 days of fermentation), a turnover on bacterial ecology and an increase of biodiversity was observed in all samples, with the detection of *S. paracollinoides*, *Pediococcus parvulus* and *Ligilactobacillus acidipiscis*, not found by culturing. Despite the central role of *Lactobacillaceae* in table olives fermentation, their low abundance has been reported in directly brined Aloreña de Málaga olives by Medina and co-workers (Medina et al. 2016), who investigated the bacterial biota composition and dynamic during fermentation through high-throughput barcoded pyrosequencing analysis of the V2 and V3 regions of the 16 S rRNA gene. The authors revealed a high abundance of members of

Celerinatantimonas, an undesirable genus, of spoilage microorganisms (*Pseudomonas* and *Propionibacterium*) and of halophilic bacteria (*Modestobacter, Rhodovibrio, Salinibacter*) during the whole fermentation, confirming the low presence of *Lactobacillaceae* and *Enterobacteriaceae*. These results were partially denied by a subsequent study conducted on heat-shocked Aloreña de Málaga table olives (Rodríguez-Gómez et al. 2017a). In this case, the metagenomic analysis conducted at the end of the fermentation revealed the dominance of *Lactobacillus*, *Pediococcus*, and *Celerinatantimonas* genera. The metagenetic studies above reported allowed to gain a comprehensive view of table olives microbiota at different taxonomic levels, revealing the presence of unexpected bacteria involved in table olives fermentation. However, the technique is not able to determine the activity of the genetic elements sequenced, providing a static image of genes of interest. For this reason, a metagenomics analysis, studying the entire metagenome of a sample, could deepen knowledge and strengthen the available information about table olives microbiota and its function. Indeed, whole-metagenome sequencing could help us to understand the taxonomic and functional composition of the table olives microbial communities. This approach could predict which strains, from a complex population, are involved in the flavour formation and the conditions affecting their metabolic pathway, shedding light on the complementary interactions at the species or strain level. One limitation, by targeting DNA, is the overestimation of the active portion of the microbiota because the technique gives information on live and dead cells. Hence, the RNA-based methods provide a dynamic microbial snapshots by exploring the activation of pathways and regulatory systems along with detection of the expression of the main genes involved in the ecosystem (De Filippis, Parente, and Ercolini 2017).

2.1.1 Whole genome sequence of table olives isolates

Whole genome sequencing (WGS) has become an important tool to investigate the information contained in the genome sequence of bacteria and yeasts. Nowadays, sequencing cost and high-throughput data generation are no longer limiting factors and for these reasons the WGS is increasingly used to address various questions in microbiology (He, 2015). Indeed, as reported in Table 4, the complete genome sequence of few bacteria strains isolated from brine and table olives surface is now available. Overall, among bacteria the vast majority of the sequenced strains are ascribed to the *Lactiplantibacillus pentosus* showing total genome sizes ranging from 3,591,251 to 4,033,890 bp. Similar G + C content, which varied from 45.00% to 46.32%, was achieved. In addition, several plasmids per strain, ranging from 13 to 5, were found (Table 4). Based on our knowledge, no information about the genome sequence of yeasts isolated from table olives is now available.

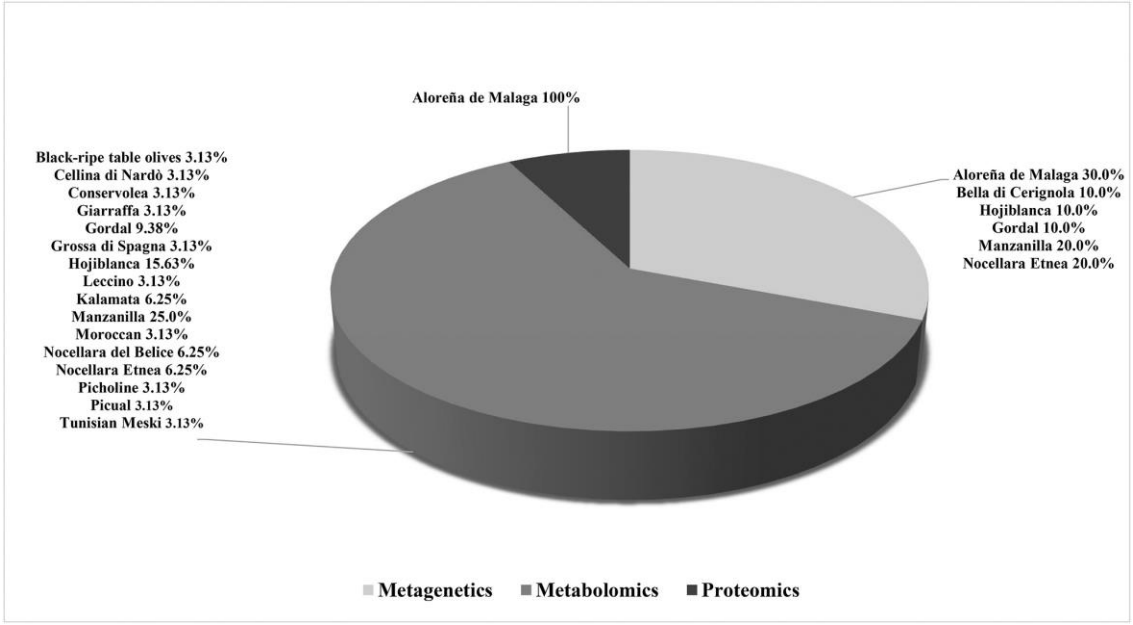

Figure 2 - Pie chart of omics techniques applied to table olives ecosystem

3.1 Transcriptomics and Metatranscriptomics

Transcriptomics is a cost-effective technology enabling the quantification of several thousands of defined mRNA species in a miniaturized presentation (Hegde, White, and Debouck 2003). While transcriptomics reveals valuable information on bacterial activities, the metatranscriptomic approach, by using high-throughput sequencing technologies, allows to understand the active microbes and their gene expression under different environmental niches (Sirén et al. 2019). The first studies, adopting a metatranscriptomic approach, were focused on freshwater and marine microbial communities (Frias-Lopez et al. 2008; Gilbert et al. 2008; Poretsky et al. 2005) demonstrating that, similarly to DNA, the microbial RNA could represent a valuable target to profile community structure, function and diversity. Metatranscriptomics approach, focusing on sequencing the entire complementary DNA (cDNA) converted from messenger RNA (mRNA), has been applied to fermented food (Chen et al. 2017; De Filippis et al. 2016, 2017; Jung et al. 2013) to explore the complex interaction network among microbial communities. Studies conducted on cheeses, sourdough and wine, highlighted the usefulness of this approach in revealing microbial dynamics that could be applied for technological purposes (De Filippis et al. 2016; Weckx, Van Kerrebroeck, and De Vuyst 2019). Despite the increase of metatranscriptomic studies on different food matrices, table olives are still unexplored with high success potentiality for shedding light on cell growth and stress response fluctuation during the fermentation process. In addition, such approach could help to underline the mechanism driven the debittering process for reduced fermentation rate in naturally fermented table olives.

3.2 Proteomics and Metaproteomics

Proteomics is defined as the study of the whole set of proteins encoded by a genome while the term metaproteomics is referred to the characterization of all proteins synthetized by a metagenome or present in an ecosystem in a given time (Wilmes and Bond 2004). The metaproteomic approach provides information related to all the metabolic pathways that are active during a food process (Ferrocino and Cocolin 2017) and allows the identification of new functions involved in complex biological pathways (Maron et al. 2007). Working on proteins, and more precisely on enzymes, involved in biotransformation processes, the proteomic and metaproteome analyses can be used to characterize the dynamics of microbial functions linking, directly *in situ*, genotype to phenotype (Chen et al. 2017; Wilmes and Bond 2006). The interpretation of proteomic data can be enough straightforward if the genome sequence or a partial genome sequence of an organism is available while it can be a challenge in the analysis of mixtures of organisms, as in fermented foods (Armengaud 2016). Currently, based on our knowledge, no metaproteomic studies have been conducted on fermented table olives and the available data have focused on the proteomic profile of LAB (Abriouel et al. 2017; Pérez Montoro et al. 2018; Pessione et al. 2015) Pessione and co-workers (Pessione et al. 2015), through the two-dimensional gel electrophoresis (2-DE) and the matrix-assisted laser desorption ionization source and tandem time-of-flight (MALDI-TOF/TOF) mass spectrometry, characterized the extracellular proteomes of *L. plantarum* S11T3 E and *L. pentosus* S3T60C strains both isolated from fermented olives and brine samples. The applied approach allowed to identify different isoforms of six and seven proteins, with extracellular location, from *L. pentosus* S3T60C and *L. plantarum* S11T3 E, respectively. The majority of the identified

proteins showed adhesive functions suggesting the strains' ability to adhere to the gut mucosa. Adhesion properties of *L. pentosus* strains, isolated from naturally fermented Aloreña green table olives, were also studied by Abriouel and co-workers (Abriouel et al. 2017) and by Montoro and co-workers (Pérez Montoro et al. 2018). The *L. pentosus* MP-10 strain, beyond the presence of several genes putatively involved in the adaptation to the human gastro-intestinal tract (such as those related to carbohydrate metabolism as well as proteins implicated in the interaction with host tissues), harbored enzymes related to carbohydrate modification and complex-carbohydrate metabolism. As reported by the authors, this layout influences the survival, the competitiveness, and the persistence of the MP-10 strain in the gastro-intestinal tract niche. In addition, the presence of genes encoding mucus-binding proteins and moonlighting proteins was also highlighted predicting the attractiveness of this bacterium as a potential probiotic for human and animal hosts. Montoro and co-workers ((Pérez Montoro et al. 2018)), by using an immobilized mucin model, studied the adhesion ability of thirty-one *L. pentosus* strains. Based on the exhibited mucus adhesion abilities, the strains were classified as highly adhesive (*L. pentosus* CF1-43 N, 73.49% of adhesion ability), moderately adhesive (*L. pentosus* CF1-37 N, 49.56% of adhesion ability) and poorly adhesive (*L. pentosus* CF2–20 P, 32.79% of adhesion ability). In addition, it was pointed up that the highly adhesive *L. pentosus* CF1-43 N strain overproduced four moonlighting proteins involved in the glycolytic pathway (phosphoglycerate mutase and glucosamine-6-phosphate deaminase), stress response (small heat shock protein) and transcription (transcription elongation factor GreA). Based on such evidence, the centrality of the metaproteomic approach to understand the link between microbial community composition and function is clearly highlighted. Nevertheless, the proteomic and metaproteomic

approaches remain underexploited on table olives ecosystem, although they represent a valuable and efficient tool in strain typing

Table 3. Omics approaches and methodology to reveal genera, volatile organic compounds (VOCs) and proteins on table olives of different cultivars.

Omics	Methodology	Olive Variety	Techniques	Genera/Compounds	References
Metagenetics	OTUs, QUIIME and SILVA 108 database, α-diversity estimator, ANOVA Tukey-Kramer post hoc test (STATISTICA 7.1software).	Aloreña de Malaga	Illumina	*Lactobacillus*[a], *Pediococcus*, *Marinilactibacillus*, *Celerinatantimonas*, *Salinicola*, *Marinobacter*, *Pseudomonas*, *and Vibrio*	(Rodríguez-Gómez et al. 2017)
	OTUs, QUIIME and SILVA 108 database, α and β-diversity indexes, *t*-test (999 Monte Carlo permutations) Krona hierarchical data browser, PCoA (KiNG graphic program).	Aloreña	Pyrosequencing	*Celerinatantimonas*, *Pseudomonas*, *Propionibacterium*, *Salinibacter*, *Staphylococcus*, *Rhodovibrio*, *Streptococcus*, *and Alicyclobacillus*	(Medina et al. 2016)
	OTUs, QUIIME, USEARCH, α and β-diversity indexes, rarefaction analysis, nonparametric two sample *t*-test with Monte Carlo permutations, PCoA (R, KiNG graphics program)	Aloreña de Malaga	Pyrosequencing	*Penicillium*, *Cladosporium*, *Malassezia*, *Candida Zygotorulaspora*, *Pichia*, *Debaryomyces*, *Saccharomyces*, *and Citeromyces*	(Arroyo-López et al. 2016)
	OTUs, QUIIME, USEARCH, α and β-diversity indexes and RDP (RDP v10.28, STATISTICA 7.0 software)	Bella di Cerignola	FLX pyrosequencing	*Hafnia*, *Methylobacterium*, *Clostridium*, *Propionibacterium*, *Lactiplantibacillus*, *Loigolactobacillus*, *Levilactobacillus*, *Lacticaseibacillus*, *Secoundilactobacillus*, *Paucilactobacillus*, *Lactococcus*	(De Angelis et al. 2015)
	OTUs, RDP, UNITE Fungal Classification database, α-diversity estimator, (ad-hoc pipeline RStatistics and STATISTICA 7.0 software).	Manzanilla and Hojiblanca	Miseq Illumina	*Pichia*, *Kregervanrija*, *Acetobacter*, *Lactobacillus*[a], *Oenococcus*, *Enterococcus*, *Lactococcus*, *Weisella*, *Pseudoalteromonas*, *Alteromonas*, *Marinomonas*,	(Medina et al. 2018)

	OTUs, ad-hoc pipeline RStatistics, RDP, UNITE Fungal Classification database, α-diversity estimator, PCA (XLSTAT v.2016 software)	Manzanilla and Gordal	Miseq Illumina	*Suttonella, Dekkera, Ruminococcus, Pichia, Candida,*	(de Castro et al. 2018)
	OTUs, QUIIME, USEARCH, α and β-diversity indexes (STATISTICA 7.1 software).	Nocellara Etnea	Pyrosequencing	*Marinilactibacillus, Halomonas, Chromohalobacter, Lactiplantibacillus*	(Cocolin et al. 2013)
	OTUs, QUIIME, UPGMA, α and β-diversity indexes (PMG software and	Nocellara Etnea	Ion Torrent PGM sequencing	*Secundilactobacillus, Ligilactobacillus, Pediococcus*	(Randazzo et al. 2017)
Metabolomics	Compounds identification.	Black-ripe. Table olives	GC-MS	Aldehydes, alcohols, esters, ketones, phenols, terpenes, norisoprenoids, pyridines, β-damascenone, nonanal, (E)-dec-2-enal,3- methylbutanal, ethyl benzoate, octanal, 2-methoxyphenols, 2-methylbutanal and 2- methoxy-4-methylphenol.	(Sansone-Land, Takeoka, and Shoemaker 2014)
	ANOVA (Duncan *post-hoc* test); PCA (STATISTICA 7.0 software).	Conservolea and Kalamata	HS-SPME-GC/MS	Ethanol, citric acid, phenol, aldehydes, ketones, esters, alcohols, terpenes, guaiacol, styrene.	(Bleve et al. 2015)
	ANOVA (Duncan *post-hoc* test); PCA (STATISTICA 7.0 software).	Cellina di Nard`o and Leccino	HS-SPME-GC/MS	Aldehydes, alcohols (2-methyl-1-propanol, 3- methyl-1-butanol), styrene, o-cymene, acetate esters	(Bleve et al. 2014)
	ANOVA (Duncan *post-hoc* test); PCA (XLSTAT PRO 5.7 software).	Giarraffa and Grossa di Spagna	SPME-GC-MS	Ethanol, isoamylalcohol, phenylethyl alcohol, esters, phenols, ethyl-acetate, ethylbutanoate, propionic acid, aldehydes, 3-octanal, 3- octanone.	(Randazzo et al. 2014)
	ANOVA (Tukey HSD *post-hoc* test); PCA, biplot (STATISTICA 7.0 software).	Kalamata, Picual and Manzanilla	HPLC and HS-SPME-GC/MS	Esters, alcohols, acids, hydrocarbons, terpenes and volatile phenol.	(Tufariello et al. 2019)
	ANOVA (Fisher's LSD *post-hoc* test), PLS analysis, biplot and bicluster graph (XLSTAT v2018 and R package Multbiplot v2018 software).	Manzanilla	GC-MS	Acetic acid, geraniol, 2-dodenal, 1,4-dimethoxy-benzene, 4,8-dumethyl-1,3,7-nonatriene, ketones, alcohols, aldehydes, volatile phenols.	(Benítez-Cabello, Rodríguez-Gómez, et al. 2019b)
	Variation array; tertiary graph; biplots; codadendrogram; Cluster and PCA (CoDaPack v.2.01.14 and XLSTAT, 2014 software).	Manzanilla and Hojiblanca	GC-MS	Propionic acid, 1-propanol, isopropanol, 2-heptenal, propyl acetate, (E)-2-decenal, methyl hexanoate, 1-heptanol, isobutanol, 1-butanol.	(Garrido-Fernández et al. 2017)

	ANOVA (test of Student-Newman-Keuls; Duncan *post hoc* test) Levene test; Shapiro-Wilk test; Welch test and Games-Howell *post hoc* test); HCA; PCA; PLS regression model (Microsoft Excel, 2010 and Statistica 7.0 software; XLSTAT v.2016 and SIMCA 14.1 software; Microsoft Excel and SPSS v.23.0 software)	Manzanilla, Gordal and Hojiblanca	SPME-GC-MS	Esters, alcohols, terpenes, aldehydes, phenols, hydrocarbons, sulphur compounds, ketones and lactone, carbonyl compounds, (E)-2-decenal and (E,E)-2,4-decadienol.	(Cortés-Delgado et al. 2016; López-López et al. 2018; Sánchez et al. 2018)
	Compounds identification	Moroccan	GC-MS	Guaiacol, 3-methylthioproprionaldehyde, α-farnesene, *trans*-nerolidol, nerol acetate, limonene, α-,β-,γ-terpineol, linalool and β-myrcene	(Montaño, Sanchez, and Rejano 1990)
	Compounds identification	Nocellara del Belice	GC-MS	Ethanol, acetic acid, 2-butanol, 1-propanol, propyl-acetate, ethyl propanoate, ethyl acetate, propionic acid, *cis*-3-hexen1-ol, 2-butanone, 1- hexanol, isopentanol, 3-pentanol, 2-pentanol, ethyl propanoate, 2-butanone.	(Sabatini and Marsilio 2008)
	GLM based on ANOVA (Tukey's *post hoc* test); HMCA (STATISTICA v.10 and XLSTAT 7.5.2 software)	Nocellara del Belice	HS-SPME-GC-MS	Homoguaiacol, 2-butanol, 4-ethylphenol, phenylethyl alcohol.	(Martorana et al. 2017)
	ANOVA (Tukey's *post-hoc* test), PCA, Permutation analysis (XLSTAT PRO 5.7, MATLAB, PermutMatrix software and STATISTICA 7.0; MATLAB and XLSTAT V.2016.1).	Nocellara Etnea	SPME-GC-MS	Isoamyl and phenyl-ethyl alcohol, esters, ethyl acetate, butanoic-acid-2-methylester, nonanal and cresol.	(Pino et al. 2018, 2019)
	PCA (SPSS 11.0)	Tunisian Meski, Picholine and Manzanilla	GC-MS	Aldehydes, ketones, alcohols and esters.	(Dabbou et al. 2012)
Proteomics	PacBio RS II technology; PROKKA pipeline; BlastKOALA (KEGG tool)	Alore˜na de Malaga	WGS and *in silico* analysis	Hexosyltransferases, pentosyltransferases, phosphotransferases, glycosylases (glycosyl hydrolases), isomerases.	(Abriouel et al. 2017)
	PEAKS 8.0; FASTA format	Aloreña de Malaga	2-DE-gel electrophoresis and nanoLC-MS/ MS	Phosphoglycerate mutase and glucosamine-6- phosphate deaminase, small heat shock protein, transcription elongation factor GreA.	(Pérez Montoro et al. 2018)

Table 4. Whole-genome sequencing of bacteria isolated from brine and table olives surface.

Species	Strain	Isolation source	Accession no.	Size (bp)	No. of contings	G + C content (%)	No. of protein-coding genes	No. of plasmids	No. of tRNAs	No. of rRNAs	References
Lactiplantibacillus pentosus	MP-10	Brines of naturally fermented Aloreña green table olives	GCA_900092635.1	3,698,214	108	46.32	3109	5	71	16	(Abriouel et al. 2016; Abriouel, Benomar, Lucas, et al. 2011; Abriouel, Benomar, Pulido, et al. 2011)
Lactiplantibacillus pentosus	BGM48	Sicilian-style green olive fermentation 118 days after the start of fermentation	PRJNA329412	3,591,251	NA	45.00	3179	5	81	NA	(Golomb et al. 2013)
Lactiplantibacillus plantarum	S2T10D	Brine from table olives after 10 days of fermentation	MQNK00000000	3,165,258	92	44.48	2871	0	45	6	(Botta et al. 2017)

	S11T3 E	Brine from table olives after 11 days of fermentation	MQNL00000000	3,168,693	58	44.49	2884	0	61	8	
	O2T60C	Surface of table olives after 60 days of fermentation	MPLC00000000	3,311,558	68	44.41	3005	0	65	11	
Lactiplantibacillus pentosus	IG2	Brines of traditional non-inoculated Spanish-style green table olive	PVOB00000000	4,033,890	460	45.70	3946	7	85	8	(Calero-Delgado et al. 2018)
	IG3		PVOA00000000	3,919,445	111	45.80	3639	13	92	3	
	IG4		PVNZ00000000	3,806,728	166	45.97	3522	6	84	5	
	IG5		PVNY00000000	3,768,924	96	45.98	3449	10	88	3	
	IG6		PVNX00000000	3,882,104	187	45.79	3631	11	88	3	
	IG7		PVNW00000000	3,802,404	352	45.79	3675	10	92	8	
Lactiplantibacillus pentosus	IG8	Biofilms on the skin of traditional fermented olives	RDCL00000000	3,791,593	99	45.91	3450	6	79	24	(Calero-Delgado et al. 2019)
	IG9		RDCK00000000	3,787,967	99	45.91	3447	6	81	16	
	IG10		RDCJ00000000	3,811,295	121	45.95	3432	7	78	12	
	IG11		RDCI00000000	3,790,820	107	45.91	3448	6	78	20	
	IG12		RDCH00000000	3,796,685	81	45.90	3459	6	80	16	

3.3 Metabolomics

Over the past few years, headspace solid phase microextraction (HS-SPME) and gas chromatography–mass spectrometry (GC–MS) have been extensively used to in-depth study the metabolic profile of Spanish, Greek, Castelvetrano and Tunisian styles table olives. In addition, as showed in Table 3, several studies have been conducted to determine the volatile organic compounds (VOCs) profile of table olives differently treated (spontaneous or pilot fermented), as well as belonging to different cultivars. In this context, Sabatini and Marsilio (Sabatini and Marsilio 2008) studied, by GC-MS analysis, the VOCs profile of spontaneously fermented Nocellara del Belice table olives, processed according to Spanish, Greek and Castelvetrano styles. The results revealed that the applied process technologies and the fermentation time significantly affected the VOCs profile of table olives. The VOCs profile of Spanish-style Nocellara del Belice table olives, fermented by using the *L. pentosus* OM13 starter, was also evaluated by Martorana and co-workers (2017). Head Space followed by Gas Chromatography/Mass Spectrometry (HS-SPMEGC/MS) applied to table olives at the end of fermentation (195 days) allowed to identify twenty-seven VOCs. Homoguaiacol, 2-butanol, 4-ethylphenol, phenylethyl alcohol and 4- ethylphenol were the compounds detected at highest concentrations in all experimental trials. Among Spanish-style green table olives, the volatile profile of Manzanilla, Gordal and Hojiblanca was identified by SPME and GC–MS (Cortés-Delgado et al., 2016; López-López et al., 2018). The metabolomics approach revealed the presence of more than one-hundred VOCs, including esters, alcohols, terpenes, aldehydes, phenols, hydrocarbons, sulphur compounds, ketones, and lactone, as previously reported (Cano-Lamadrid et al. 2015; de Castro et al. 2018a; Cortés-Delgado et al. 2016; Iraqi et al. 2005; Montaño et al.

1992, 1990; Sabatini and Marsilio 2008; Sánchez et al. 2017), highlighting that sampling time affects in a more pronounced way VOCs composition than olive cultivar. On the contrary, Garrido-Fernández and co-workers (Garrido-Fernández et al. 2017) by GC-MS analysis, differentiated Spanish-style Manzanilla and Hojiblanca green table olives, from different parts of Spain, by VOCs profiles in relation to both cultivars and production area. Benítez-Cabello and co-workers (Benítez-Cabello, Rodríguez-Gómez, et al. 2019b)investigated, for the first time, the volatile profile of Manzanilla Spanish-style green table olives, fermented by different starter cultures. Based on the VOCs profile, the authors concluded that the use of LAB and yeast as starters, singularly or in mixture, improve the aromatic profile of the final product. The ability of starters to influence the volatile profile of fermented table olives was confirmed by Tufariello and co-workers (Tufariello et al. 2019) who focused the attention on Kalamàta, Picual and Manzanilla Greek-style olives type. Twenty-one compounds belonging to esters, alcohols, acids, hydrocarbons, terpenes and volatile phenol were identified and the starter-fermented olives exhibited a more complex profile in esters, alcohols, and volatile phenols compared to non-inoculated samples. Similarly, even if in directly brined Sicilian table olives, Randazzo et al. (Randazzo et al. 2014) and Pino et al. (Pino et al. 2018, 2019) evaluating the influence of lactobacilli starter cultures on the volatile profile, demonstrated a significant change in the VOCs pattern. Greek-style fermented table olives were also investigated by Bleve and co-workers (Bleve et al. 2015) who found, between the natural fermented Conservolea and Kalamàta black olives, pronounced differences in the volatile profile. In addition, Bleve and co-workers (Bleve et al. 2014) characterized the volatile compounds generated during the fermentation process of Cellina di Nardò and Leccino cultivars. Through HS-SPME-GS/MS technique, the authors disclosed that, in

both cultivars, aldehydes were closely related to the first stage of fermentation (30 days); alcohols (2-methyl-1-propanol and 3-methyl-1-butanol), styrene, and o-cymene to the middle stage (90 days) whereas acetate esters were linked to the final stage fermentation (180 days). Commercial black-ripe table olives processed in United States, Spain, Egypt and Morocco were analysed by GC–MS (Sansone-Land et al., 2014). A variety of aldehydes, alcohols, esters, ketones, phenols, terpenes, norisoprenoids, and pyridines were isolated and among these β-damascenone, nonanal, (E)-dec-2-enal, 3-methylbutanal, ethyl benzoate, octanal, 2-methoxyphenol, 2-methylbutanal and 2-methoxy-4-methylphenol were identified as the major contributors to table olives' aroma. The metabolomics approach allowed to discriminate the imported olives from the domestic ones. Along with Spanish and Greek style, worthy of attention is the Tunisian-style olive processing, which was investigated by Dabbou and co-workers (Dabbou et al. 2012). The authors evaluated the changes in VOCs using three different cultivars: the autochthonous Tunisian Meski cultivar and two introduced table Picholine and Manzanilla. Sixty-six volatile compounds were identified by GC with the dominance of aldehydes while the percentages of total ketones, alcohols, and esters differed according to the cultivar. Table olives flavor develops by the combined metabolic activity of microbial community on drupes, carbohydrates, accompanied by further enzymatic and chemical conversions during fermentation. The identification of active VOCs compounds, using metabolomics, has led to create a library that can be used to associate desirable flavor or defects to specific molecules. In addition, the integration of metagenetics and metabolomics can be considered a valuable approach to reveal the existence of positive and/ or negative correlations between the microbiota composition and the produced microbial metabolites. In this context, De Angelis et al. (De Angelis et al. 2015)revealed that lactobacilli and *W. anomalus* strains

markedly affect the content of free fatty acids, phenolic compounds and VOCs in directly brined Bella di Cerignola table olives, highlighting differences between un-inoculated and inoculated samples. Randazzo and co-workers (Randazzo et al. 2017) showed the influence of the microbiota on metabolic profile of Nocellara Etnea table olives during controlled and spontaneous fermentation. The authors observed that *Proteobacteria* were positively correlated to aldehydes and octanal, yeasts with alcohols and ethanol, *S. paracollinoides* with esters, and *L. acidispicis* with acetic acid. Similarly, as suggested by de Castro al. (de Castro et al. 2019), the development of microbiota involved in olive spoilage is directly correlated to the development of VOCs responsible of off-odor. In particular, the genus *Propionibacterium* was positively correlated with acetic, propionic and succinic acids, and methyl propanoate while the genus *Ruminococcus* showed significant positive correlation with propionic and butyric acids

3.4 Data management and processing of information

Table 5 shown the applications, weakness, and challenges of omics techniques applied in table olives ecosystem, generating a vast amount of data, which need adequate management to ensure the quality of information maximizing knowledge-gleaning and protecting the data from loss or misuse (Schneider and Orchard, 2011). In amplicon-based metagenetics studies, conserved regions of a phylogenetic marker are amplified by PCR, sequenced, and assigned to an operational taxonomic unit (OTU). In detail, in metagenetics and metagenomics studies, the generated data must be subjected to: quality control of the sequences, elimination of the chimeric sequences, grouping of the sequences on the basis of similarity and clustering, and taxonomic assignation. Appropriate analytic pipelines are able to screen, trim and filter the raw sequences. Among these, the

Quantitative Insights into Microbial Ecology (QIIME) pipeline, which combines original published tools and algorithms directly into the pipeline, is a widely used analytical tool (De Angelis et al. 2015; Cocolin et al. 2013; Medina et al. 2016; Nilakanta et al. 2014; Randazzo et al. 2017; Rodríguez-Gómez et al. 2017). QIIME is an open-source software pipeline able to manage the sequencing data supporting a wide range of microbial community analyses and visualizations that allow users to interact with the data (Caporaso et al. 2010). To guarantee an appropriate and high level of accuracy, in terms of OTUs detection, sequences that pass the quality filters are subjected to denoising and chimera checking. The detection and removal of chimeras is of critical importance since they may be misinterpreted determining an inaccurate estimation of diversity and generating spurious inferences of differences between populations. For these purposes, different softwares are available such as UCHIME (Bautista-Gallego et al. 2013), Black Box Chimera Check (B2C2) (De Angelis et al. 2015), ChimeraSlayer (Rodriguez-Gomez et al., 2017), and prinseq-lite program (de Castro et al. 2018b; Medina et al. 2018). To grouping the sequences, based on similarity, and to clustering them, the sequence analysis tool USEARCH, is widely used for combining different algorithms into a single package (De Angelis et al. 2015; Bautista-Gallego et al. 2013; Medina et al. 2016). The Ribosomal Database Project (RDP), which is a Bayesian-type classifier, allows classifying up to genus-level sequences of bacterial and archaeal 16 S rRNA as well as intergenic ribosomal sequences (ITS) (De Angelis et al. 2015; de Castro et al. 2018; Medina et al. 2018; Randazzo et al. 2017). To in depth study the microbiota composition, the results are expressed as relative abundance of the different phyla, classes, orders, families, genera, and rarely species. The microbial community is evaluated in terms of richness and diversity through alpha and beta diversity indices (rarefaction, Good's

coverage, Chao1 richness and Shannon diversity indexes) (De Angelis et al. 2015; de Castro et al. 2018; Cocolin et al. 2013; Medina et al. 2016; Randazzo et al. 2017; Rodríguez-Gómez et al. 2017). Data analysis of metabolic compounds is intended to classify, discriminate and/or predict the metabolome of complex food matrices, such as table olives, during fermentation. Applying a discriminative analysis, is possible to evaluate the different metabolic profile among samples, without the support of statistical models or/and the implementation of metabolic pathways that may elucidate such differences (Cevallos-Cevallos et al. 2009). The use of internal standards, in VOCs analysis, has allowed the simultaneous, reproducible and accurate detection of the main metabolic compounds present in fermented green olives (Montaño et al. 1990; Sabatini and Marsilio 2008). In addition, volatile compounds can be identified by comparing the component's mass spectrum and experimental Kováts retention index (I) with an authentic reference standard (Montaño et al. 1990; Sabatini and Marsilio 2008; Sansone-Land et al. 2014). Moreover, statistical models, such as the one-way analysis of variance (ANOVA) and the multivariate data analysis (MVDA) can help to maximize the VOCs classification, highlighting the relations among these measurements (Cevallos-Cevallos et al. 2009). To underline the quantitative differences among samples, the Principal Components Analysis (PCA) is the most used statistical tool. In addition, variation array, tertiary graphs, biplots, or codadendrogram can be applied to discriminate among samples (Garrido-Fernández et al. 2017). In proteomics analysis, protein sequence databases, such as SEQUEST, UniprotKB, and MASCOT, allow the identification of proteins. Automatic annotation servers, such as BlastKOALA, perform KO (KEGG Orthology) assignments to characterize individual gene functions and reconstruct KEGG pathways (Abriouel et al. 2017). Different software platforms, such as PEAKS Studio, can be used to

discover proteomics, including protein identification and quantification, analysis of post-translational modifications (PTMs) and sequence variants (mutations), and peptide/protein *de novo* sequencing (Pérez Montoro et al. 2018). Integrate multi-omics approaches could increase the yield of information from genomics, transcriptomics, proteomics and metabolomics. For instance, MixOmics (R based software), presents a recent example of a modified concatenation-based approach. Software approaches for managing multi-omics data sets were also recently discussed for other environmental niches (O'Donnell, Ross, and Stanton 2020) and they also could be applied for table olive ecosystem.

Table 5. Applications, weakness and challenges of omics approaches on table olives.

Omics	Target	Application	Weakness	Challenges
Metagenetics	Analysis of a single type of 16s rRNA encoding gene or ITS region.	✓ Identification of spoilage or foodborne pathogens; ✓ Study of microbial diversity; ✓ Identification of non-cultivable microorganisms of table olives; ✓ Application to various fermented food matrix to identify spatial and temporal variations during fermentation process.	✓ Inability to distinguish between metabolically active cells and inactive; ✓ Lye treatment of table olives can compromise the DNA and the sequencing detection.	✓ Examine in depth table olives microbiota through the identification and characterization of VBNC (viable but non culturable) microorganisms and to allow their isolation and cultivation.
Transcriptomics and Metatranscriptomics	Analysis of mRNA or total RNA of microbial cells and ecosystem.	✓ Identification of RNA transcripts of microbial cells under specific conditions; ✓ Identification of whole gene expression and functions of microbial ecosystem; ✓ Measurement and evaluation of microbial gene expression. ✓ Identification of key genes involved in metabolisms of biofilm formation, VOCs production, proteins or specific metabolites.	✓ Difficulty on manipulation and extraction of mRNA; ✓ Environmental factors can compromise the gene expression of the microbiota; ✓ High estimated costs.	✓ Comprehension of microbial gene dynamics involved in fermentative processes; ✓ Separation of bacterial RNA from the matrix; ✓ Separation and preservation of microbial mRNA from the food matrix; ✓ Training of qualified personnel for the processing of bioinformatics data.

Proteomics and Metaproteomics	Analysis of the whole set of proteins (proteome) encoded by genome, including their structure and function.	✓ Study of metabolic, physiological state and environment effects on gene expression and proteins of the microorganisms; ✓ Comprehension of total proteins, expressed at a certain time by microorganisms; ✓ Improvement, validation and quality control of bioprocess; ✓ Characterization and detection of biofilm-forming microorganisms and bacteria, responsible of undesired effects; ✓ Investigation of a multitude of bacterial process through the proteome-guided optimization of strains for biotechnological use.	✓ Ineffective extraction method could compromise the amount and structure of isolated proteins; ✓ Interference of food-matrix proteins; ✓ Impossibility to identify less abundant proteins with gel-based method (2-DE, SDS-PAGE	✓ Improvements in extraction and separation techniques, identification and data searches of proteins; ✓ Comprehension of specific mechanisms of proteomic adaptation involved in microbial performances (carbohydrates utilization, energy metabolism, osmotic stress resistance).
Metabolomics	Analysis of endogenous and exogenous small molecules (VOCs and/or non-VOCs).	✓ Study of metabolome, able to reflect the microbial cell's biochemical state; ✓ Comprehension of small molecules generated from metabolic pathways; ✓ Detection of specific group of metabolites, identified and quantified in a sample under certain conditions; ✓ Study of the metabolite profile of unknown compounds responsible for changes in microbial systems.	✓ Difficulty in identifying metabolic compounds in complex food matrices; ✓ Low level of purification and selective extraction of metabolites; ✓ The use of unsuitable solvent can compromise the extraction of metabolic compounds.	✓ Utilization of specific techniques able to reveal different biological molecules of metabolome; ✓ Efficiency on biomarkers identification for the quality and authenticity of food; ✓ Efficiency on biomarkers identification with positive effect on human health;

✓ Possibility to discriminate, inform and predict metabolic processes of microorganisms, involved in table olives fermentation.

4 Conclusion and future prospectives

Table olives fermentation is an essential process by which the bitter phenolic compounds are removed by drupes, enhancing sensorial properties of the final product. Methods adopted for the investigation of table olives associated microbiota changed from the classical cultivation-based approach to the most recent omics sciences. For decades, table olives community have been investigated through culture-dependent techniques, revealing only the most adaptable microorganisms as responsible of fermentation. Subsequently, in the previous investigations, several achievements have been reached through the application of a polyphasic approach, combining traditional methods to culture-independent, especially in understanding the microbial species variation among olives cultivars and the widely applied process technologies (Spanish and Greek style). In this review, we presented a large-scale genetic, proteomic and metabolomic analyses that have taken place in table olives field, helping to change the way to study table olives ecology. With the spread of high throughput methods, a high level of association and interaction of microbial population was revealed in table olives ecosystem. However, the potential application of omics techniques, clearly emerge and is still limited on the combination of microbiota and metabolomics, letting possible the discover of new biological markers with high specificity to fully understand the molecular mechanisms at stake in this complex food ecosystem. The application of a multi-omics approach to table olives ecosystem can be considered suitable to obtain a comprehensive view of the mechanisms that can affect sensorial traits and safety aspects of final product. In this way, it could be possible to improve the knowledge about what happens in a complex process, such as in table olives fermentation, and what the microbiota does in this matrix, shedding light to the importance of how

"omics" approaches may lead to novel table olives biomarker molecules or molecular signatures with potential value in human health.

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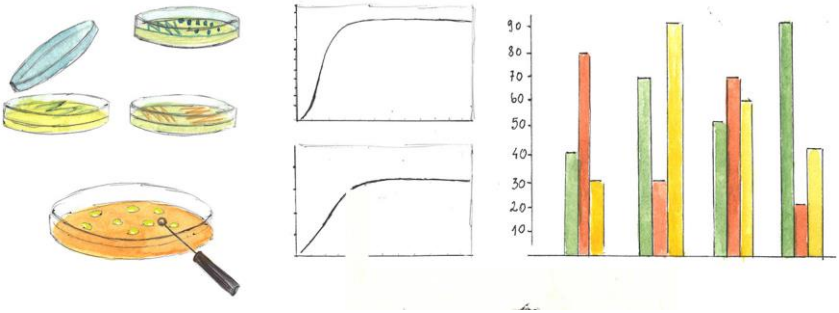
Chapter 1



CHAPTER 1: Experimental Activity

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

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1 Introduction

Table olives are the most widespread fermented vegetables in the Mediterranean area and their production and consumption are expanding worldwide, thanks to the nutritional and functional components of drupes, such as polyphenols, vitamins, fiber, minerals, and short chain fatty acids. Olive drupe contains low concentrations of sugar (2.6–6.0%) and high oil (12–30%) and polyphenols content (Botta and Cocolin 2012; Lavermicocca et al. 2005). The ladders are mainly represented by oleuropein, which is responsible for the bitterness taste (Othman et al. 2009) and for inhibiting a range of bacteria, especially lactic acid bacteria (LAB) (Castro, Romero, and Brenes 2005; Medina et al. 2008, 2009; Perpetuini et al. 2018). Nowadays, depending on the applied industrial process (i.e., Spanish style or Greek style), different debittering methods, enzymatic or chemical, are currently applied (Vaccalluzzo et al. 2020). In Sicilian style green table olives, the debittering process is exclusively relied on microorganisms naturally present on the drupes, through the activity of two enzymes, the beta-glucosidase, which leads to the release of two intermediates (glucose and aglycone), which are completely degraded by an esterase into tasteless phenols (hydroxytyrosol and elenolic acid) (Bianchi 2003). Therefore, in order to shorten the debittering step in naturally fermented table olives, tailored-starter cultures, with enhanced debittering ability, are required (Tofalo et al. 2012). It is well known that the use of selected starter cultures, besides to control the fermentation process, should possess the ability to survive in the fermentation environment (low pH, high concentrations of salts, and low fermentation substrates) and to exert acidifying activity (through organic acid production). In addition, they should be able to hydrolyze phenolic compounds (such as oleuropein) and to produce volatile molecules that positively contribute to the

development of the sensory profile of the final product (Alves, Gonçalves, and Quintas 2012). For these reasons, the choice of starter culture tailored for table olive fermentation is a complex assignment, which requires the evaluation of different features since the matrix is very complex and several compounds counter the metabolic activity of the selected strains. Furthermore, the selection of unfitting strains may lead to the production of undesirable metabolites, compromising the final product (Bonatsou et al. 2017). Among LAB species, strains belonging to Lactiplantibacillus plantarum species are often selected as starters (Corsetti et al. 2012; Hurtado et al. 2012) for their high versatility, tolerance to stress brine conditions, capacity to cooperate with autochthonous yeasts until the end of the fermentation process, and for their ability to cope with the inhibitory phenolic compounds (Ghabbour et al. 2011; Kaltsa et al. 2015; Landete et al. 2008; Marsilio and Lanza 1998; Marsilio, Lanza, and Pozzi 1996; Rozés and Peres 1996). In addition, the presence of genes involved in phenolic-compound degradation has been already demonstrated in some *L. plantarum* strains (Gury, Barthelmebs, and Ngoc Phuong Tran, Charles Divie`s 2004; Jiménez et al. 2014; Marasco et al. 1998; Spano et al. 2005). This featured is directly correlated with the reduction of the debittering time. In fact, as previously demonstrated by Pino and co-workers (Pino et al. 2019), a beta glucosidase-positive strain, ascribed to the *L. plantarum* species and used as starter in low salt fermentation, reduced the processing time and positively affected the polyphenol content and sensory profile of the final product (Pino et al. 2019). Up to now, different strains were tested for the ability to degrade the oleuropein under optimal growth conditions (Ghabbour et al. 2011; Iorizzo et al. 2016; Zago et al. 2013) or using MRS medium added with different salt concentrations (Kaltsa et al. 2015; Marsilio and Lanza 1998; Marsilio et al. 1996). In this context, Yao et al. (2020) demonstrated that the tolerance of *L. plantarum* D31 and T9 strains,

to 5% and 8% of salt, is related to the presence of specific salt tolerance-related genes. Recently, Ghabbour and co-workers (Ghabbour et al. 2020) have evaluated the oleuropein biodegradation ability of *the L. plantarum* FSO175 strain under stress conditions (pH and salt contents), addressing that multi-factor parameters should be taken into account. In addition to tolerance to pH and salt content, the ability to grow at low temperatures represents another key feature for the selection of starter cultures. Only few studies have been conducted to evaluate the capability of the strains to grow under combined pH and salt conditions at low temperatures (Aponte et al. 2012; Bevilacqua et al. 2010) and, up to now, no data are reported on the impact of technological stress factors on the oleuropein-degrading ability of starter cultures tailored for table olives fermentation.

Therefore, the in order to select tailored starter cultures for table olives fermentation, the objective of the present study was to investigate the effect of olive fermentation-related stress factors (pH, temperature, and NaCl) on growth and on oleuropein-degrading abilities of selected *L. plantarum* strains.

2 Materials and Methods

2.1 Microorganisms and Culture Conditions

A total of nine *Lactiplantibacillus plantarum* strains, belonging to the Culture Collection of the Department of Agricultural, Food, and Environment (Di3A), University of Catania, Italy were used in this study. The strains derived from a pool of *L. plantarum* isolates from brine samples of naturally fermented Sicilian table olives set up at 5% of NaCl (Pino et al. 2019), and were selected based on their fermentative abilities on laboratory-scale olives fermentation trials (data not shown). Cultures were maintained as stock solution in 20%

(vol/vol) glycerol at –80 °C and routinely propagated at 30 °C for 24 h in De Man, Rogosa and Sharpe (MRS) broth (Oxoid, Milan, Italy).

2.2 Growth Ability of L. plantarum Strains under Specific Stress Conditions

The growth ability of the *L. plantarum* strains was tested inoculating each strain at final concentration of 7 log colony forming unit (CFU)/mL in MRS (Oxoid) broth, by using different single and combined stress conditions, such as pH (4.5 and 5.5), salt (NaCl 5% and 6%) and temperatures (16 °C and 32 °C). In detail, MRS broth was acidified adding HCl (0.5 N) and supplemented with 5% or 6% of NaCl, to simulate the salt concentration used during low salt olives fermentation (Pino et al., 2018). Growth ability was evaluated after 72 h of incubation at both 16 °C and 32 °C, through the measurement of optical density at 620 nm (iMark. Microplate Absorbance Reader, Biorad, Milan, Italy) and by plating on MRS agar medium. Each experiment was conducted in triplicate and results were expressed as log CFU/mL standard deviation. The condition of pH 6.0 and incubation at 32 °C was used as control, since the specie *L. plantarum* exhibit the best growth optimum at that condition. In order to detect the time required to reach the stationary phase, the *L. plantarum* strains were previously tested in modified MRS medium acidified at pH 6.0 and added with 6% of NaCl, after incubation at 16 °C and 32 °C. The cell density was determined by plating onto MRS agar and growth data ($\boldsymbol{\mu_{max}}$ and $\boldsymbol{\lambda}$) were modelled according to the following Gompertz equation:

$$\boldsymbol{y = k + A exp\,\{-exp[(\mu_{max}\,e/A)(\lambda - t) + 1]\}}$$

In the Gompertz equation y is the extent of growth as log CFU/mL at the time $\boldsymbol{t}$; $\boldsymbol{k}$ is the initial cell density expressed as log CFU/mL; $\boldsymbol{A}$ represents the difference, in cell density, between the stationary phase and the inoculation; $\boldsymbol{\mu_{max}}$ is the maximum growth rate (Δlog

CFU/mL/h); λ is the length of the latency phase of growth expressed in hours, and $\boldsymbol{t}$ is the time.

2.3 L. plantarum Beta-Glucosidase Gene Detection

For each *L. plantarum* strain, the presence of the beta-glucosidase gene, encoding for the beta-glucosidase enzyme, was investigated, according to the method proposed by Spano et al. (Spano et al. 2005). PCR reactions were carried out in a final volume of 25 µL containing 0.5 U of Taq polymerase, 0.2 mM of dNTPs MIX, 1xPCR buffer, 1.5 mM MgCl2, and 0.25 mM of the primer pairs bgluF (50GTGACTATGGTAGAGTTTCC30) and bgluR (50TCAAAACCCATTCCGTTCCCCA30). The amplification program was as follows: 30 cycles at 94 °C for 1 min, 60 °C for 40 s, and 72 °C 1.2 min, with an initial denaturation at 94 °C for 5 min and a final extension at 72 °C for 10 min. PCR reactions were carried out in a GeneAmp PCR System 2400 (Applied Biosystems, Norwalk, CT, USA). The PCR products were resolved by electrophoresis using 1.2% agarose gel in TAE buffer (0.004 M Tris/acetate, EDTA 1 mM) for about 40 min at 90 V and visualized after staining with Gel Red Nucleic Acid Stain (Biotium, Merck Life Science S.r.l., Milan, Italy). A 200 bp ladder was used as a standard marker.

2.4 Oleuropein-Degrading Ability Test

Culture conditions: The degradation of oleuropein was tested by inoculating *L. plantarum* strains (7 log CFU/mL) in modified MRS broth medium, acidified at pH 6.0 and supplemented with 0.1% (w/v) (1.0 g/L) of oleuropein, (Sigma, Merck, Life Science S.r.l., Milan, Italy). The MRS medium was modified as follow: 10.0 g/L peptospecial; 10.0 g/L beef extract; 5.0 g/L yeast extract; 20.0 g/L glucose; 2.0 g/L triammonium citrate; 5.0 g/L sodium acetate; 0.2 g/L magnesium sulfate; 0.05 g/L manganese sulfate, and dipotassium

phosphate 2.0 g/L (Liofilchem, Roseto degli Abruzzi, Italy). Based on the growth performance of each strain, the assay was carried out at different multi-stress conditions of salt (NaCl 6% and 5%) and temperature (16 °C and 32 °C). After incubation for 72 h, the cultures where centrifuged (8.000 rpm, for 10 min, at 4 °C) and supernatants stored at -20 °C prior to further analyses. Un-inoculated medium was used as control. HPLC determination of oleuropein: Supernatants were filtered through 0.45 μm PTFE filters (Merck-Millipore, Milan, Italy) and injected into the chromatographic system for HPLC analysis. The HPLC apparatus consisted of a liquid chromatography Water Alliance 2695 HPLC equipped with a Waters 996 photodiode array detector (PDA) set at 280 nm and Waters Empower software. The instrument was provided with a Luna C18 column (250 mm×4.6 mm i.d., 5 μm, 100 Å, Phenomenex, Torrence, CA, USA) which was maintained at 40 °C in an oven. The flow rate was 1 mL/min. Separation was obtained by elution gradient using an initial composition of 95% of A solution (water acidified with 2% of acetic acid) and 5% of B solution (methanol). The concentration of B solution was 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 in 8 min. For quantification in the medium the oleuropein standard (Product Code: 0228 S, Purity for HPLC ≥98%) was purchased from Extrasynthese (Genay Cedex, France) (Sorrentino et al., 2016). All analyses were performed in triplicate for each sample analysed and results expressed as mg/L ± standard deviation of oleuropein.

2.5 Statistical Analysis

One-way ANOVA followed by Tukey's multiple comparison test was applied to the data from three biological replicates, using the Statistica software (version 7.0 for Windows, TIBCO Software, Palo

Alto, CA, USA) and differences were considered statistically significant at $p < 0.05$.

3 Results and Discussion

The increasing demand for healthy food imposes to table olives industry to develop new biotechnological strategies in order to reduce the salt content and to shorten the debittering process, besides reducing chemical treatments. In this contest, the use of *L. plantarum* beta-glucosidase positive strains, as starter cultures, is a promising choice to accelerate the fermentation and to obtain a stable and safety final product (Ghabbour et al. 2020). It is well established *that L. plantarum* strains closely fulfil the role of tailored starter culture thanks to its high versatility, adaptation ability, acidic and salt tolerance, and ability to hydrolyse bitter compounds present in olive fruits (Kaltsa et al. 2015; Landete et al. 2008; Randazzo et al. 2011; Rozés and Peres 1996) One of the main evolutionary strengths of microorganisms is their ability to adapt to changing environments and to tolerate different stress conditions. These abilities are mainly due to the adaptive response of microbial cells through the activation of genes encoding for General Stress Proteins, such as Dnak, DnaJ, GroES, and GroEL (Carvalho et al. 2004; De Angelis and Gobbetti, 2011). In the present study, nine selected strains belonging to *L. plantarum* specie were investigated for their growth behaviour at single and multiple stress conditions, similar to those occurring during olives fermentation, in order to pin point further key factors to be considered for the selection of starters for table olives fermentation.

3.1 Growth Performances Exhibited by the Tested Strains

In order to study the growth performance of the tested strains under stress conditions, the Gompertz's model was applied

(Zwietering et al. 1990). Table 1 shows the Gompertz parameters calculated by OD620 value collected until 80 h of incubation. However, the growth performances were stopped at 72 h in accordance with the reaching of stationary phase. In detail, when the strain performance (MRS broth medium, pH 6.0) was evaluated at 32 °C and 16 °C, the final average values of cell density were 9.20 and 8.50 log CFU/mL, respectively. According to the final cell density, the **A** and $\boldsymbol{\mu_{max}}$ values were higher at 32 °C than at 16 °C, whereas the lag phase (**λ**) was higher at 16 °C than 32 °C, except for the strains F1.16 and F3.8, for which a slightly decrease was observed at the highest tested temperature (32 °C) (Table l). In addition, in Table 1 growth parameters under combined stress conditions (MRS pH 6.0, added with NaCl 6%, at 32 °C and 16 °C) were reported. Results showed an evident reduction of **A** and $\boldsymbol{\mu_{max}}$ values, compared to control condition (MRS broth medium, pH 6.0).

Table 1. Growth Parameters, Calculated by Gompertz's Equation of *L. plantarum* Strains under Control Conditions (MRS Broth Medium pH 6.0) and under Stress Condition (MRS Broth Medium pH 6.0 and NaCl 6%) at 32 °C and 16 °C.

L. plantarum strains	Control condition (MRS broth medium pH 6.0)						Stress condition (MRS broth medium pH 6.0 and NaCl 6%)					
	A		μ_{max}		λ		A		μ_{max}		λ	
	32°C	16°C	32°C	16°C	32°C	16°C	32°C	16°C	32°C	16°C	32°C	16°C
F1.8M	2.63±0.02	0.43±0.02	0.16±0.02	0.01±0.01	13.57±0.08	20.02±0.02	0.51±0.04	0.46±0.02	0.03±0.02	0.03±0.02	29.99±0.07	32.91±0.07
F1.10	2.79±0.02	0.38±0.03	0.15±0.03	0.02±0.02	13.20±0.05	20.80±0.15	0.14±0.02	0.12±0.03	0.03±0.02	0.03±0.03	22.82±0.06	24.15±0.04
F1.16	1.15±0.03	0.45±0.02	0.05±0.02	0.02±0.01	21.42±0.04	19.96±0.02	0.32±0.03	0.29±0.02	0.02±0.02	0.02±0.02	20.92±0.06	22.77±0.03
F3.2	2.26±0.02	0.26±0.02	0.09±0.02	0.02±0.02	16.06±0.03	20.23±0.03	0.75±0.03	0.59±0.02	0.02±0.01	0.01±0.01	23.73±0.05	26.22±0.04
F3.5	2.25±0.02	0.50±0.02	0.09±0.02	0.01±0.01	16.07±0.02	18.76±0.02	0.51±0.03	0.49±0.02	0.03±0.03	0.07±0.02	30.03±0.03	31.48±0.08
F3.6	1.47±0.05	0.38±0.05	0.05±0.04	0.02±0.02	19.11±0.02	24.29±0.04	0.28±0.02	0.25±0.02	0.01±0.01	0.01±0.01	30.03±0.05	24.16±0.05
F3.7	2.87±0.02	0.44±0.04	0.18±0.02	0.08±0.07	14.12±0.03	15.50±0.05	0.58±0.02	0.49±0.03	0.02±0.01	0.02±0.02	23.80±0.06	25.29±0.06
F3.8	1.17±0.04	0.43±0.03	0.05±0.04	0.02±0.01	21.29±0.03	19.83±0.03	0.74±0.04	0.52±0.04	0.02±0.02	0.02±0.02	22.98±0.11	24.47±0.07
C11C8	2.39±0.03	0.38±0.02	0.16±0.04	0.02±0.02	13.35±0.04	24.26±0.06	0.80±0.04	0.60±0.06	0.03±0.01	0.02±0.01	21.22±0.03	22.89±0.06

However, the temperature of 32 °C positively affected the growth performances, in comparison with 16 °C, while **λ** was higher at 16 °C, except for the strain F3.6, which was negatively affected by 32 °C. The temperature of 16 °C is a key factor for strain's growth performance since it prolongs the latency phase and reduces the maximum growth rate of strains. By predictive modelling, it was possible to reveal that 72 h is the time required to achieve the maximum exponential phase under stress conditions.

3.2 Growth Ability under Stress Conditions

Different studies, conducted on the growth ability of *L. plantarum* species, confirmed the high versatility, adaptation ability, acidic, and salt tolerance as well as ability to decrease the bitter compounds naturally present in olive drupes (Kaltsa et al. 2015; Landete et al. 2008; Marsilio and Lanza 1998; Marsilio et al. 1996; Rozés and Peres 1996). In the present study, nine (9) selected *L. plantarum* strains were investigated for their growth behaviour at single and combined stress conditions simulating the table olives fermentation. The growth ability of the tested strains at 32 °C and 16 °C, as reported above, was monitored up to 72 h and results are displayed in Supplementary Figures S1 and S2. At control condition (pH 6.0 and incubation at 32 °C) all tested strains, starting from an average value of 7 log CFU/mL, increased their cell density more than 2 log units. Overall, all strains appeared more resistant toward both single and multi-stress conditions at 32 °C (Figure S1), showing a mean increase of cell densities higher than 1 log unit. In order to evaluate the growth ability of each tested strains under both single and multi-stress conditions, the survival rate percentage (SR %) was calculated based on viable cells under control condition (pH 6.0 and incubation at 32 °C) and under each stressor condition. The viable cells were enumerated by plate count on MRS agar. Figure 1 and Table

S1 show the SR % and the viable count (log CFU/mL) of the tested strains under each stressor at 32 °C, respectively. Overall, all strains showed the ability to survive under the tested stress conditions with SR % more than 85%. Zooming on each stress factor, out of the nine strains, 2 (F1.16 and F3.8) showed the highest SR % under all the tested stress conditions. Similar behaviour was exhibited by the F3.2 strain except at NaCl 6% and pH 6.0. The performances of the tested strains appeared to be robust toward the different stressors, in accordance with previous studies, which validated the aptitude of *L. plantarum* species to tolerate acidic environments, for the intrinsic ability to maintain an internal pH gradient that allows the survival at a much lower external pH (Zago et al. 2013). A recent study (Ma et al. 2020), carried out on *L. plantarum* KLOS 1.0328 strain, showed its greater inclination to tolerate acidic stress rather than osmotic stress, according to a previous work reporting that pH values from 5.0 to 9.0 did not significantly affect the growth of *L. plantarum* strains isolated from Italian Bella di Cerignola table olives (Bevilacqua et al. 2010). However, the same authors asserted that salt concentrations higher than 4% negatively influenced the strain growth. On the contrary, other studies (Randazzo et al. 2004; Romeo and Poiana 2007; Servili et al. 2006) reported the ability of *L. plantarum* strains to tolerate high (> 8%) NaCl concentrations. The tolerance to high salt concentrations and the ability to control osmotic stress have been recently confirmed (Yao et al. 2020), showing excellent growth performance of *L. plantarum* strains at 5% and 8% of NaCl. The authors observed that *L. plantarum* strains are equipped, at genomic level, with a complex molecular regulatory network involving genes associated with salt resistance through the recovery of the intracellular ion balance (Yao et al. 2020). Our study clearly highlights that the growth behaviour under stress conditions is strain-specific, especially at 16 °C (Figure 2 and Table S2). By including the temperature of 16 °C, as an additional

stressor, data revealed that the growth performance detected among strains, was highly variable, appearing to be hampered by the multi-stress conditions. Moreover, comparing the behaviour of each strain at different stress conditions, the growth performance, expressed as viable counts, was highly variable. Based on ANOVA results the strains F3.8, F3.7, and F1.16 showed the higher significant differences (Table S2). As expected, when incubated at 16 °C, the viable count at pH 6.0 was significantly lower than those observed at 32 °C (Table S2). Indeed, the low temperature significantly affected the growth performance of the strains both singularly and in combination with other stressing factors. Moreover, it is interesting to point out that at 16 °C under acidic conditions the growth of the *L. plantarum* strains was significantly affected. Our data were slightly in discordance with previous reports (Aponte et al. 2012; Bevilacqua et al. 2010) that revealed high growth ability of *L. plantarum* strains. at both 12 °C and 15 °C. Although both table olives process conditions and storage temperature may vary in relation to annual climate fluctuations, to type of containers and to industrial sheds, the most common temperature conditions measured during the fermentation process and storage period is about 20 °C. Hence, it is extremely important to select *L. plantarum* strains with enhanced technological performances at process parameters applied during table olives process production.

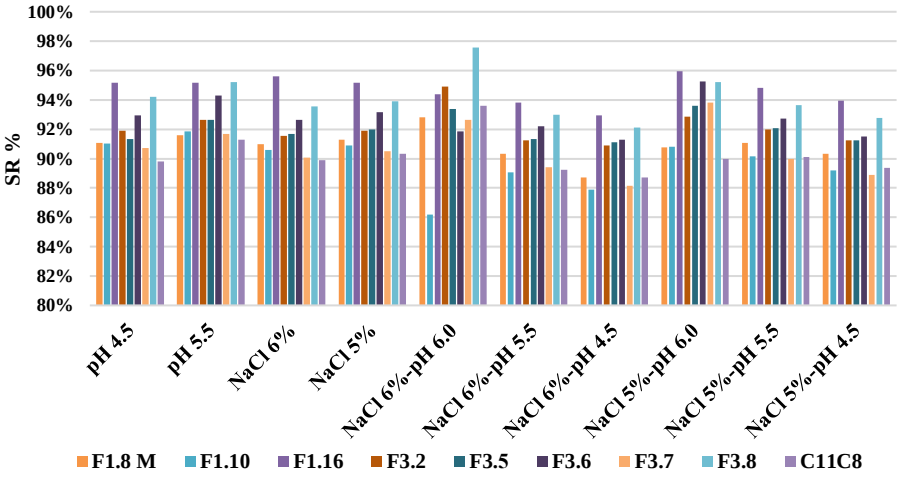

Figure 1. Survival rate plot of the strains, expressed in percentage, under single and combined stress conditions at 32 °C.

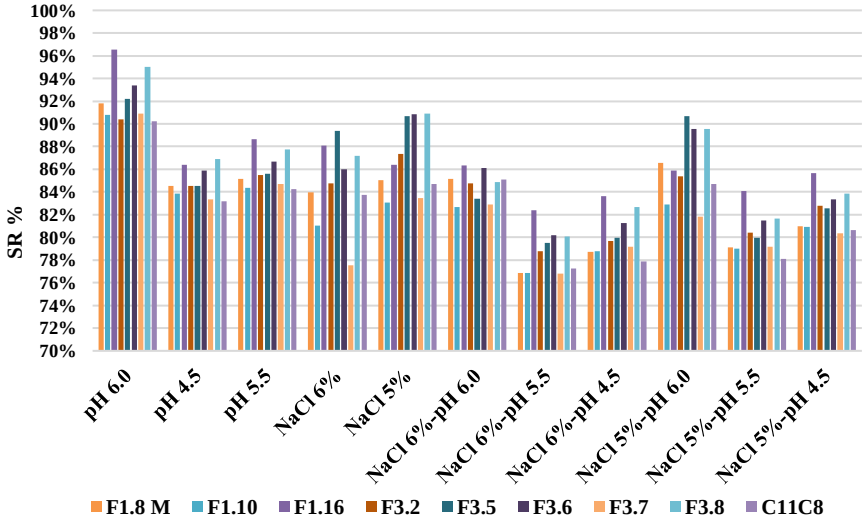

Figure 2. Survival rate plot of the strains, expressed in percentage values, under single and combined stress conditions at 16 °C.

3.3 Molecular Detection of Beta-Glucosidase Gene in L. plantarum Strains

According to the PCR protocol proposed by Spano et al. (Spano et al. 2005), for each *L. plantarum* strains the presence of the beta-glucosidase gene was investigated. The gene, encoding for a beta-glucosidase enzyme, has been selected for the relevance of oleuropeinolytic activity, towards phenolic glucosides in table olives, such as oleuropein, demethyl-oleuropein, verbascoside, and luteolin-7-glucosides (Kara, Sinan, and Turan 2011; Marasco et al. 1998, 2006; Zago et al. 2013). PCR reactions were performed with either degenerated primers, deduced from the nucleotide sequences of beta-glucosidase genes identified for *L. plantarum* species. A single PCR-product (of about 1400 bp) was obtained using the primer pairs designed on the putative beta-glucosidase gene of the *L. plantarum* WCFS1 strain. High identity value was observed between nucleotide sequences of *L. plantarum* tested strains. The beta-glucosidase gene is a ubiquitous gene detected on strains isolated from both vegetable and dairy products (Zago et al. 2013). Among the nine strains tested for the beta-glucosidase activity, five strains (F1.8M, F3.2, F3.5, F3.8, and C11C8) demonstrated the presence of the gene encoding for the beta-glucosidase activity. However, for four tested strains (F1.10, F1.16, F3.6, and F3.7) the beta-glucosidase gene was not detected, despite their displayed the ability to reduce the oleuropein content, under stress conditions. Similar results were obtained by Carrasco and co-workers (Carrasco et al. 2018) who demonstrated that the *Lactiplantibacillus pentosus* CECT4023 strain, although in absence of the gene encoding for the beta-glucosidase activity, is able to metabolize the oleuropein. This observation can be explained taking into account that the ability to hydrolyze the oleuropein is not closely

related to the presence of beta-glucosidase and could be led to the activity of tannases and esterases enzymes (Kaltsa et al. 2015; Rodríguez-Gómez et al. 2017a; Yuan et al. 2015).

3.4 Oleuropein Degrading Test

In selection process of tailored-starter cultures for table olives fermentation, the ability to degrade the oleuropein, present in drupes, is one of the key characteristic to be considered in order to shorten the debittering stage. In the present work, based on the growth performances, nine *L. plantarum* strains were subjected to the oleuropein degrading test at the following combined conditions: (1) pH 6.0 and NaCl 6%, (2) pH 6.0 and NaCl 5%, and both incubated at 32 °C and 16 °C. In Table 2 shows data on detected concentrations of oleuropein (OLE), expressed as mg/L, and on the OLE degradation, expressed as percentage. Overall, different degradation ability was detected among the tested strains, indicating a strain-specific behavior. In particular, at 32 °C, in presence of both 5% and 6% of NaCl, all strains showed the ability to degrade the oleuropein albeit the highest OLE degradation percentages were detected in presence of 6% of NaCl. In detail, as reported in Table 2, the strain F1.16 exhibited the highest OLE degradation percentage (97.8%). Zooming on the results obtained at 16 °C, low OLE degrading activity was revealed for almost all the tested strains at both 5% and 6% of NaCl with the exception of the F3.2 strain. In detail, at 5.0% of NaCl and at pH 6.0, the F1.10 strain, maintained the highest OLE degradation activity (88.8%), followed by F1.16, F3.2, and C11C8 strains, whereas the F1.8M, F3.7, and F3.8 strains showed lower OLE degradation performances, with degradation percentage values of 83.2%, 78.3%, and 84.7%, respectively. The lowest tested temperature (16 °C) negatively affected the OLE degrading ability exhibited by the F3.5 and F3.6

strains, with percentage values of 18.7% and 20.3%, respectively. The OLE degrading ability of *L. plantarum* strains, has been already confirmed at 30 °C by several authors (Ghabbour et al. 2011; Zago et al. 2013) and only recently Ghabbour et al. (Ghabbour et al. 2020) investigated the performance of *L. plantarum* strains under combined stress factors (such as pH and salt concentrations), revealing a good degrading ability at pH 4.5 and in presence of 5% of NaCl. However, Iorizzo et al. (Iorizzo et al. 2016) showed that the use of nutrient medium at pH 5.0, supplemented with oleuropein, shorten the OLE degradation time. Our results indicated a high degrading ability of the tested strains on modified MRS medium supplemented with oleuropein at pH 6.0. In addition, the salt content could improve the ability of the strains to degrade the oleuropein, when multi-stress conditions are occurring. Up to now, there is no scientific evidence on the ability of *L. plantarum* strains to degrade oleuropein at low temperature. The present study clearly revealed that the temperature is a key parameter, which could be proposed for the selection of tailored starter cultures for table olives fermentation.

Table 2. Detected Values of Oleuropein (OLE) (Expressed as mg/L) and Percentage of Degraded OLE from Strains Inoculated in Modified MRS Medium and Incubated at Different Temperatures (32 °C and 16 °C).

	MRS broth, 0.1% (w/v) of OLE, NaCl 5.0% (w/v) and pH 6.0				MRS broth, 0.1% (w/v) of OLE, NaCl 6.0% (w/v) and pH 6.0			
	32°C		16°C		32°C		16°C	
	OLE (mg/l)	OLE (%)	OLE (mg/l)	OLE (%)	OLE (mg/l)	OLE (%)	OLE (mg/l)	OLE (%)
Control *L. plantarum* strains	975.0±2.71^{a}	00.0	985.0±3.00^{a}	00.0	978.0±2.71^{a}	00.0	983.0±3.00^{a}	00.0
F1.8M	39.8±0.04^{h}	95.9	165.7±11.98^{d}	83.2	44.9±2.83^{gh}	95.4	93.7±6.47^{d}	90.5
F1.10	45.6±1.60^{h}	95.3	110.3±13.60^{fg}	88.8	66.9±2.56^{ef}	93.2	93.9±13.31^{d}	90.4
F1.16	82.7±0.25^{g}	91.5	123.7±1.05^{ef}	87.4	21.7±0.51^{i}	97.8	38.8±0.91^{ghi}	96.0
F3.2	129.8±0.64^{ef}	86.7	125.9±0.55^{ef}	87.2	46.8±1.19^{g}	95.2	49.6±1.57^{fg}	95.0
F3.5	102.3±0.18^{fg}	89.5	801.2±10.52^{b}	18.7	46.8±0.77^{g}	95.2	752.8±2.54^{b}	23.4
F3.6	84.8±2.66^{g}	91.3	785.0±7.72^{b}	20.3	26.9±1.57^{i}	97.3	723.0±3.28^{c}	26.4
F3.7	41.2±3.57^{h}	95.8	213.4±21.63^{c}	78.3	69.1±0.26^{e}	92.9	94.7±0.11^{d}	90.4
F3.8	44.8±1.85^{h}	95.4	150.8±1.51^{de}	84.7	35.5±4.11^{ghi}	96.4	96.8±8.34	90.2
C11C8	88.1±0.13^{g}	91.0	126.6±2.26^{ef}	87.1	27.9±0.61^{hi}	97.2	49.3±1.37^{g}	95.2

4 Conclusion

In the present study, nine selected *L. plantarum* strains were evaluated for the ability to growth and to degrade the oleuropein under stress conditions. Our data demonstrated that the behaviour of the selected strains was strain-dependent for all the tested stressors. The low temperature was the main stress factor affecting the survival rate, under simulated brine conditions. Regarding to the oleuropein degradation ability, it is interesting to highlight that out of the nine strains, 3 (F1.10, F1.16, and F3.7) showed high percentage of degraded OLE, even the beta-glucosidase gene was not detected. Therefore, further proteomics and genomics studies are ongoing to reveal gene loci related to oleuropein degradation. Based on the challenging brine environment factors, considered in the present study, the F1.16 and F3.8 strains are promising candidate as tailored starter culture for table olives.

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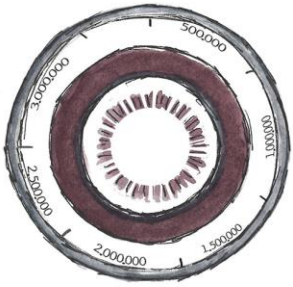
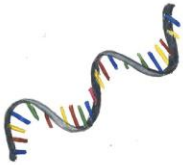
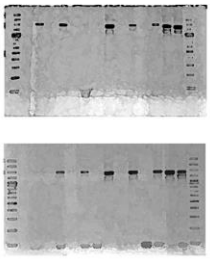
Chapter 2







Lactiplantibacillus plantarum

CHAPTER 2: Experimental activity

Metabolomic and transcriptional profiling of oleuropein bioconversion into hydroxytyrosol during table olive fermentation by *Lactiplantibacillus plantarum*

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1 Introduction

The β-glucosidase enzymes are glycosidic hydrolases (GHs), which catalyse the transfer of the glycosyl group between nucleophiles, enabling the release of monomers such as β-D-glucose from various disaccharides, oligosaccharides, alkyl- and aryl-β-D-glucosides (Bhatia, Mishra, and Bisaria 2002; Li et al. 2018; Zhang et al. 2021). Based on amino acid similarity, they are classified as glycosyl hydrolase families 1 and 3, respectively (Coutinho and Henrissat 1999). They can be isolated from plants, animals, and microorganisms. In food biotechnology, these enzymes are present in almost all microorganisms involved in fermentation processes of plant-derived substrates as these microbes utilize plant glycosides as a source of energy and require acid-stable β-glucosidases to release the sugar fraction at low pH (Barbagallo et al. 2004; Deutscher, Francke, and Postma 2006; González-Pombo et al. 2011; Li et al. 2018). In the production of table olives, the microbial β-glucosidase enzyme is responsible of the hydrolysis of the bitter compound oleuropein, releasing low molecular weight phenolic compounds, such as hydroxytyrosol and tyrosol. It's noteworthy that strains belonging to *Lactiplantibacillus plantarum* species are often used as starter culture for table olives, for their versatility to better adapt to fermentation conditions and to accelerate the debittering process thanks to β-glucosidase activity (Iorizzo et al. 2016; Vaccalluzzo et al. 2020.; Zago et al. 2013). However, few information is available on the genes encoding for the β-glucosidase enzymes (Marasco et al. 1998; Spano et al. 2005; Zago et al. 2013; Vaccalluzzo et al. 2020). For example, two different genes were reported to be responsible for β-glucosidase activity in *Lactiplantibacillus plantarum* (Spano et al. 2005; Zago et al. 2013). In a recent study, 9 *L. plantarum* strains were proven to hydrolyse oleuropein in vitro, and only 5 strains possessed the gene

putatively encoding for β-glucosidase enzyme (Vaccalluzzo et al. 2020). This genotype-phenotype inconsistency suggest that other genes could contribute to β-glucosidase activity. These genes have the potential to encode enzymes from several closely related hydrolase families, such as glycoside hydrolases and glycosyl hydrolases ((Davies and Henrissat 1995; Henrissat et al. 1996). Indeed, candidate primers often identify regions where β-glucosidase genes are allocated, that are not strictly involved in encoding the enzyme of interest. However, the ability to enzymatically degrade phenolic compounds is a strain-specific characteristic, which allows the discrimination of starter strains within the same species. Genetically, the discrimination, at the strain level, is much more difficult. Primers designed to detect β-glucosidase genes are not strain-specific, and, therefore, amplify a region where, for some strains of the same species, the candidate gene may not be present, because it is located at another gene locus. This work aims at elucidating the mechanisms responsible of oleuropein bioconversion into low molecular weight phenolic compounds in two previously characterized oleuropein-degrading *L. plantarum* strains (Vaccalluzzo et al. 2020), under conditions mimicking the brine olive fermentation. For this purpose, we adopted an experimental strategy, which combine high-resolution mass spectrometry, in silico functional analysis of GH1 candidates and gene expression study. We identified oleuropein hydrolysis products and the underpinning enzymatic steps, as well as a new putative bgl gene responsible for the observed β-glucosidase activity under low temperature condition.

2 Materials and Methods

2.1 Bacterial strains and culture conditions

Two oleuropein-degrading *Lactiplantibacillus plantarum* strains, namely F3.5 and C11C8, were considered in this study. In

addition, seven *L. plantarum* strains (F1.8M, F1.10, F1.16, F3.2, F3.6, F3.7 and F3.8) were used as control for the genomic detection of the candidate β-glucosidase genes. All strains belong to the Collection of the Department of Agriculture, Food and Environment (Di3a), University of Catania (Italy) and were propagated statically in de Man-Rogosa-Sharpe (MRS) broth (Oxoid, Basingstoke, Hampshire, UK) under aerobic conditions or on MRS agar plates (1.5% [wt/vol]); under anaerobic conditions at 30 °C. The strains were maintained in MRS medium supplemented with 0.7% (w/v) agar for the duration of the experiments.

2.2 Olive brine fermentation assay

Bacterial cells were harvested by centrifugation (10,000 rpm for 10 min, 4 °C) in the stationary phase (OD600nm 1.7 to 2.5), washed in saline solution (0.9% of NaCl) and used to inoculate filtered (0.22 μm) table olive brine medium (brine solution at 7% of NaCl of cracked Nocellara Etnea table olives, at 120 days of fermentation) at the final concentration of log 8 CFU/mL. After 72 h of incubation both at 30°C and 16 °C, cells at density of 8 Log unit CFU/mL were collected by centrifugation (10.000 rpm x 5 min) and used for the RNA extraction, while the supernatant was used for the high-resolution mass spectrometry analysis. Table olive brine medium without inoculum was used as control. The assay was carried out in triplicate.

2.3 High-resolution mass spectrometry analysis

Two mL of collected supernatant were freeze-dried and the obtained power re-suspended in 2 mL of dimethylsulfoxide (Merck KGaA, Darmstadt, Germany). After centrifugation (10,000 rpm; 4 °C), samples were filtered at 0.22 μm to remove insoluble material and subjected to UHPLC/HR-MS analysis for phenolic and related compounds identification and relative quantification. For each sample,

10 µL were injected in an UHPLC Ultimate 3000 separation module outfitted with a C18 column (Acquity UPLC HSS C18 reversed phase, 2.1 × 100 mm, 1.8 µm particle size, Waters, Milan, Italy). MS and MS2 experiments were carried out on a high-resolution Q Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Scientific, San Jose, CA, USA). The flow rate was fixed a 0.3 mL/min. The chromatographic conditions and the MS and MS/MS parameters are fully described in Martini et al. (2020). The relative quantification of the phenolic and related compounds was performed by integrating the area under the curve (AUC) by using the Genesis algorithm function in the Thermo Xcalibur Quantitative Browser. AUCs were calculated from the extracted ion chromatograms (EIC) achieved for each compound mass-to-charge ratio with the tolerance set at ± 3 ppm.

2.4 *In silico analysis and primers design for the β-glucosidase gene*

Amino acid sequences used in the present study were extracted from RefSeq CDD/SPARCLE database Arch ID 10006560 (Marchler-Bauer et al. 2017). The sequences were selected in an effort to prepare as representative dataset of *L. plantarum* GH1 members as possible. Multiple alignment of protein sequences was performed using the COBALT tool (Papadopoulos and Agarwala, 2007) with default settings and phylogenetic tree was built using the Kimura 2-parameter (K2P) model and the neighbour joining (NJ) method. Tree was visualized using Interactive Tree of Life (ITOL) (Letunic and Bork 2019) and rooted at outgroup reference strain Streptococcus thermophilus. Experimentally validated bgl genes were retrieved from Brenda database (Jeske et al. 2019).

2.5 Bacterial gDNA extraction and PCR gene-specific screening

Bacterial DNA was extracted from the overnight culture incubated at 37 °C according to the protocol proposed by Gala et al. (2008) The extracted DNA was suspended in TE buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 8.0) and quantified spectrophotometrically using Nanodrop Nd 1000 (Nano-drop Technologies, Wilmington, DE, USA). DNA quality was confirmed by electrophoretic running on 0.8% (w/v) agarose gels in 0.5X TBE buffer (45.0 mmol/L Tris-borate and 1.0 mmol/L EDTA, pH 8.0) and subsequent UV visualisation after staining with ethidium bromide (0.5 µg/mL). All primers used in this study was detailed in Table 1. Primer pair 14770_F/14770_R were designed with Primer 3 software (Kõressaar et al. 2018), using *L. plantarum* ATCC8014 as reference genome (BioProject PRJNA415899). All PCR reactions were performed in 20 µL volumes with 0.4-0.8 µmol/L of each primer, 0.1 U of Dream Taq (Thermo Scientific Waltman, MA, USA), 0.2 mmol/L dNTPs (Thermo Scientific Waltman, MA, USA), 2 µL of Dream Taq Buffer 1X (Thermo Scientific Waltman, MA, USA) and 50 ng of gDNA. PCR reactions were performed in a T100 thermal cycler (Bio-Rad, Hercules, CA, USA) and the thermal conditions were detailed in Table 1.

Table 1. Primers and cycling conditions used in this study.

Target	Sequences	Amplification program	Expected length (bp)	References
CS400_15205	**bglu_F** 5-GTGACTATGGTAGAGTTTCC-3 **bglu_R** 5-TCAAAACCCATTCCGTTCCCCA-3	95 °C for 5 min; 35 cycles (95 °C for 30 s; 54 °C for 30 s; 72 °C for 1 min); 72 °C for 10 min.	1485	Spano et al. (2005)
CS400_14765	**m-bgl F** 5-TGATTATACTTGTTGTAAGGGCTATCATTATTAGCTAACT -3 **m-bgl R** 5-CATTATGCTTAATCACATCTTGATACCAGTAGAACGACTT-3	95 °C for 5 min; 35 cycles (95 °C for 30 s; 62 °C for 30 s; 72 °C for 1 min); 72 °C for 10 min.	1485	Zago et al. (2013)
CS400_14770	**14770_F** 5-CAACTGGCTTTCCAAAGAAC-3 **14770_R** 5-CTGAATATCAATTATTAATAACTATCCCAA-3	95 °C for 5 min; 35 cycles (95 °C for 30 s; 56 °C for 30 s; 72 °C for 1 min); 72 °C for 10 min.	1490	This work

16S rRNA	**27F** 5'-CTGGGATCCATTTACTCGAGAGTTTGATCCTGGCTCAG- 3 **1490R** 5'-GGTTCCCCTAAGCTTACCTTGTTACGACTTC-3'	95 °C for 5 min; 30 cycles (95 °C for 1 min; 58 °C for 2,5 min; 72 °C for 2 min); 72 °C for 5 min.	1500	Tagliazucchi et al. (2020)

2.6 Sequencing and phylogenetic analysis

PCR products were purified with DNA Clean & Concentrator™-5 Kit (Zymo Research, Orange, CA, USA) and sequenced on both strands through a DNA Sanger dideoxy sequencing process. Sequences were assembled in DNAStar (DNASTAR, Inc. Madison, Wisconsin USA) and trimmed on both ends to remove primer sequences. Alignment was carried out with Muscle program (Edgar 2004) in MEGA X software (Patel, Kumar, and Shah 2018) and the resulting alignment was subjected to a DNA substitution model analysis to select the best-fitting model. Phylogenetic relationships were inferred using the Poisson correction model (Zuckerkandl and Pauling 1965) and the Neighbour Joining method. Among sites rate variation was modelled by a gamma distribution (shape parameter = 1). Bootstrap support values were obtained from 1,000 random resamplings. Alignments were visualized using JalView v2.11 (Waterhouse et al. 2009; Patel et al. 2018), while trees using the interactive tree of life (iTOL) v5.2 as reported above.

2.7 RNA extraction and RT-PCR assays

Bacterial cells collected from table olive brine assay were submitted to RNA extraction as previously reported (Solieri et al. 2021). Briefly cells were washed twice with DEPC-treated TE buffer (100 mmol/L Tris-HCl, 50 mmol/L EDTA, pH 8.0) and cell pellets were maintained at −80 °C until thawed with 1 mL of Tri-reagent using the Zymo Direct-zol RNA MiniPrep Kit (Zymo Research, Irvine, CA). Mechanical lysis was performed using a Vortex Genie 2 (Mo Bio Laboratories) for two rounds of 20 min at highest speed alternated with 3 min on ice. The quantity of total RNA was measured spectrophotometrically using Nanodrop Nd 1000 (Nano-drop Technologies, Wilmington, DE, USA), while the integrity was

checked by denaturing gel electrophoresis on a 0.9% (w/v) agarose gel with formaldehyde (10 mL of 10× MOPS running buffer) and 18 mL of 37% formaldehyde (12 mol/L) on a pH 7.0 1× MOPS running buffer (0.4 mol/L MOPS, 1 mol/L sodium acetate, and 0.01 mol/L EDTA), after RNA treatment at 65 °C for 10 min. PCR reactions were carried as reported above using cDNA as template instead of gDNA. 16S rRNA gene was used as reference gene and amplified according to Tagliazucchi et al. (2020). Cells grown in MRS medium at pH 5.5 supplemented with 5% (w/v) of NaCl were used as control.

3 Results and Discussions

3.1 Hydrolysis of oleuropein in table olive brine medium and identification of the reaction products

The *L. plantarum* strains C11C8 and F3.5 were previously selected for the different ability to hydrolyze oleuropein and for the presence of the β-glucosidase gene, as reported previously by Vaccalluzzo et al., 2020. However, no data are available regarding the oleuropein-degrading ability of the L. planatarum strains in brine olive-like environment. In the present study, the ability of *L. plantarum* strains C11C8 and F3.5 was investigated by incubating the two selected strains for 72 h in table olive brine medium at two different temperatures (16 °C and 30 °C). The data were compared with a control table olive brine medium without inoculated strains and incubated in the same conditions as reported above.

Phenolic and related compounds identified by high-resolution mass spectrometry in the medium were oleuropein, oleuropein aglycone, hydroxytyrosol, OME (oleoside-methyl ester), HyEDA (decarboxymethyl dialdehydic form of oleuropein aglycone), eleanolic acid (elenolic acid-methyl ester) and EDA (dialdehydic form

of decarboxymethyl eleanolic acid). The mass spectrometry data and the relative quantification data, expressed as area under the curve (AUC), are reported in Supplementary Table 1 and SY, respectively. As can be observed in Figure 1A (see also Supplementary Table 2), both the strains were able to hydrolyze oleuropein, as evidenced by the recorded decrease in the relative amount of this compound compared to the control. The highest decrease was detected for *L. plantarum* C11C8 after incubation in table olive brine medium at 30 °C (~33% decrease respect to the control). No significant differences in oleuropein degrading ability were found between the two tested temperature for *L. plantarum* C11C8. Differently, *L. plantarum* F3.5 showed a significantly lower oleuropein hydrolytic activity at 16 °C (15% decrease respect to the control) then at 30 °C (~30% decrease respect to the control). Different reaction products may arise from oleuropein hydrolysis depending on the type of enzymatic activity. Several previous studies highlighted the ability of *L. plantarum* strains to hydrolyze oleuropein thanks to the action of bacterial β-glucosidase and esterase (Ciafardini et al. 1994; Zago et al. 2013; De Leonardis et al. 2016; Ramírez et al. 2017). β-glucosidase activity results in the hydrolysis of the glucose moiety from oleuropein releasing oleuropein aglycone and/or HyEDA (De Leonardis et al. 2015; Ramírez et al. 2016; Guggenheim et al. 2018). Furthermore, esterase activity hydrolyze the ester bond of oleuropein resulting in the release of OME and hydroxytyrosol (Charoenprasert and Mitchell 2012; Ramírez et al. 2017). As reported in Figure 1B, the relative amount of both the β-glucosidase activity products, oleuropein aglycone and Hy-EDA, increased in inoculated media respect to the control one (see also Supplementary Table 2). The highest increase for both the reaction products was found in *L. plantarum* C11C8 inoculated medium at 30 °C (28% and 56% of increase for oleuropein aglycone and HyEDA, respectively). No differences were found in the oleuropein aglycone

relative amount between in *L. plantarum* C11C8 inoculated medium at 16 °C and 30 °C, whereas the amount of HyEDA was significantly higher in sample incubated at 30 °C. Once again, significantly higher amount of oleuropein aglycone and Hy-EDA were found in *L. plantarum* F3.5 inoculated medium at 30 °C respect to the medium incubated at 16 °C. The concentration of the esterase hydrolysis products, OME and hydroxytyrosol, also increased in inoculated media respect to the control medium (Figure 1C and 1D). The highest increase in hydroxytyrosol was found in *L. plantarum* C11C8 inoculated medium incubated at 30 °C (28% increase respect to the control). For both the strains, incubation at 16 °C resulted in a lower release of hydroxytyrosol respect to the sample incubated at 30 °C. On the contrary, the highest OME increase was recorded in table olive brine media incubated at 16°C for both the strains. OME still contain a bound glucose moiety and can be further hydrolyzed by bacterial β-glucosidase in eleanolic acid and glucose. This pathway was further confirmed by the recorded increase in eleanolic acid amount observed in the inoculated medium (Figure 1A). The decrease in OME concentration and the increase in HyEDA amount observed after incubation at 30 °C respect to the medium incubated at 16 °C for both the strains suggested a higher β-glucosidase at 30 °C than at 16 °C. This effect was not seen in the case of oleuropein aglycone probably because this compound was further hydrolyze by esterase in hydroxytyrosol and eleanolic acid. Finally, no significant differences were found for EDA concentration between the control medium and the media inoculated with the two strains (see also Supplementary Table 2). Overall, these results confirmed the presence of oleuropein degrading β-glucosidase and esterase activities in both the tested strains. The proposed pathway of oleuropein degradation by bacterial β-glucosidase and esterase is reported in Figure 2.

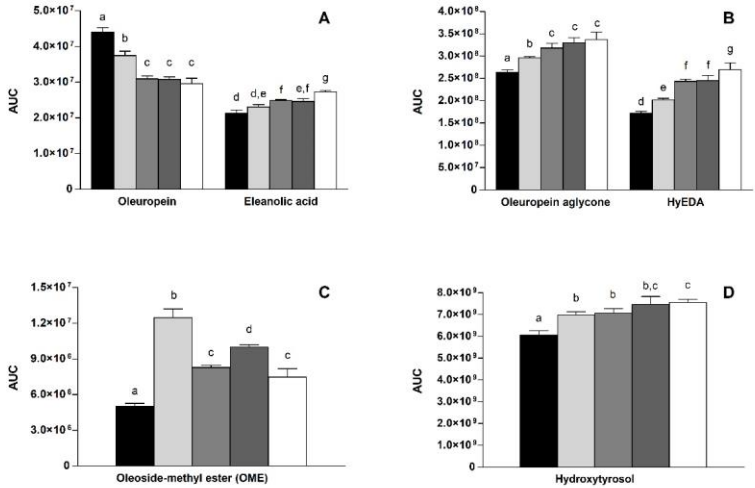


Figure 1. Relative quantification data for phenolic and related compounds identified in control and inoculated table olive brine medium. AUCs were calculated from the extracted ion chromatograms (EIC) obtained for each compound mass-to-charge ratio (tolerance ± 3 ppm). Black bars represent the control table olive brine medium without inoculum. Light grey bars represent table olive brine medium inoculated with *L. plantarum* F3.5 and incubated for 72 h at 16 °C. Grey bars represent table olive brine medium inoculated with *L. plantarum* F3.5 and incubated for 72 h at 30 °C. Dark grey bars represent table olive brine medium inoculated with *L. plantarum* C11C8 and incubated for 72 h at 16 °C. White bars represent table olive brine medium inoculated *with L. plantarum* C11C8 and incubated for 72 h at 30 °C. HyEDA means decarboxymethyl dialdehydic form of oleuropein aglycone. Raw data are reported in Supplementary Table 2.

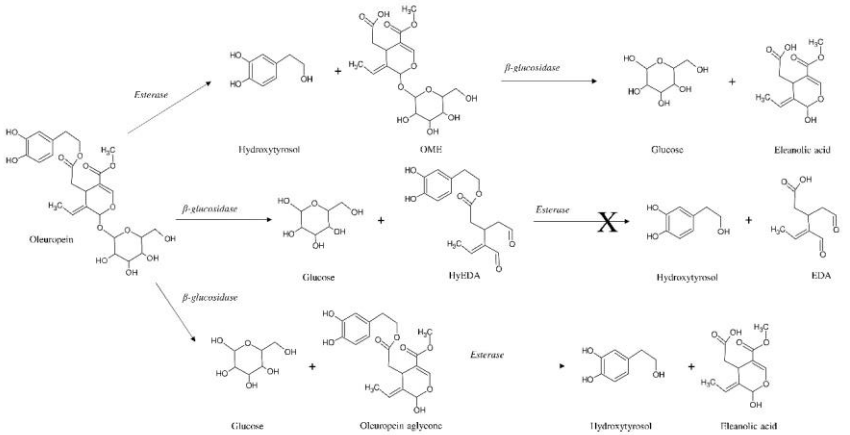


Figure 2. Proposed metabolic pathways for oleuropein degradation by *L. plantarum* F3.5 and C11C8. The symbol X means that the specific pathway was not active in both the strains. Abbreviations are: HyEDA: decarboxymethyl dialdehydic form of oleuropein aglycone; OME: oleoside-methyl ester; EDA: dialdehydic form of decarboxymethyl.

3.2 Identification of putative bgl genes in ATCC8014 genome

The proposed pathways highlight β-glucosidase as key enzymatic activity in order to degrade brine compounds and to increase the concentration of hydroxytyrosol in the final product. However, different information is reported in literature about candidate genes encoding for β-glucosidase (Spano et al., 2005; Zago et al., 2013).

The ability to hydrolyse β-glucosides is widespread among the lactic acid bacteria, although up to now little is known about the genes responsible for this phenotype. Glycoside hydrolase family 1 (GH1) comprises enzymes with a number of known activities, including both β-glucosidase (EC 3.2.1.21) and 6-phospho-β-glucosidase (EC 3.2.1.86). The most common pathway to hydrolyse β-glucosides relies on the direct hydrolytic cleavage by extracellular or cell wall associated glucosidases (EC 3.2.1.21) (Weber, Klein, and Henrich 1998). However, several evidences demonstrated that intracellular phospho-β-glucosidases (EC 3.2.1.86) are responsible for hydrolysing C6-phosphorylated β-glucosides releasing glucose-6-phosphate in several species including Lactobacillus acidophilus (Theilmann et al. 2017), *Streptococcus mutans* (Cote and Honeyman), and *Leuconostoc pseudomesenteroides* (Acin-Albiac et al. 2021a; Acin-Albiac et al. 2021b; Hernández et al. 2007). β-glucosides are generally transported by β-glucoside-specific phosphotransferase systems (PTS) whereby the substrate is vectorially phosphorylated as it is taken up, and subsequently cleaved by a phospho-β-glucosidase (Vadeboncoeur and Pelletier 1997). In CDD/SPARCLE database 185 RefSeq proteins with a BglB conserved domain (COG2723) were annotated as GH1 in *L. plantarum* genomes. Phylogenetic analysis inferred by Cobalt alignment showed that bgl proteins clustered in three major groups (Figure 3). Groups 1 and 2 included proteins highly homologous to the

only two *L. plantarum* bgl proteins with proven β-glucosidase, such as CS400_15205 (Spano et al. 2005) and CS400_14765 (Zago et al. 2013) in ATCC8014 genome, respectively (Figure 3). While CS400_15205 is annotated as GH1 protein, CS400_14765 encodes a 6-phospho-beta-glucosidase, which differs from the other 6-phospho-beta-glucosidases present in ATCC8014 genome for the BglB domain instead of the glyco_hydro super family domain (Supplementary Table S1). Interestingly a third gene in ATCC8014, namely CS400_14770, showed a BglB superfamily domain like CS400_14765 and clustered in Group 3 (Figure 3). CS400_14770 gene is downstream to CS400_14765 and upstream to two genes coding for a permease of the PTS (BglP) and a transcriptional antiterminator (BglG), respectively. This synteny resembles that described for the polycistronic operon bglGFB in *E. coli* (Boss et al. 1999; Chen and Amster-Choder 1999), bglP in *B. subtilis* (le Coq et al. 1995) and bglGPT in *L. plantarum* strain B21 (Marasco et al. 1998; Zago et al. 2013). Based on these evidences, we chosen CS400_15205, CS400_14765 and CS400_14765 as putative target candidates responsible for β-glucosidase activity in *L. plantarum* C11C8 and F3.5.

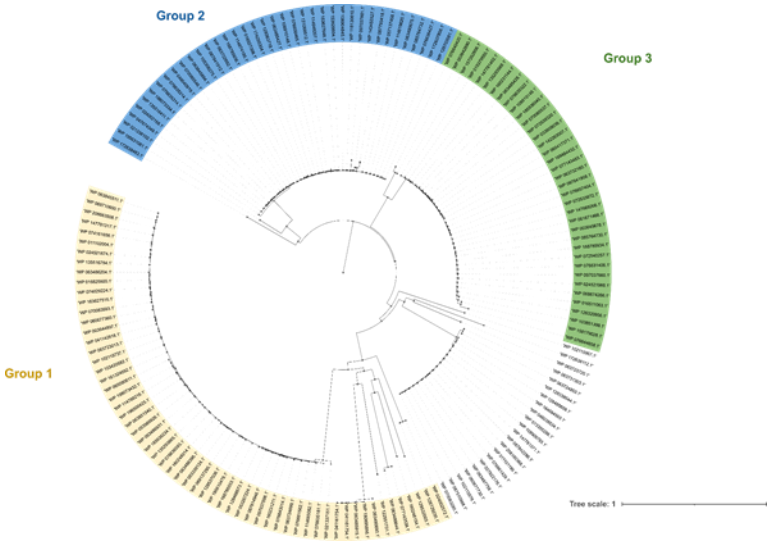


Figure 3. Neighbour-Joining tree of *Lactiplantibacillus plantarum* glycoside hydrolase family 1 proteins. The analysis includes 185 Refseq proteins (Arch ID 10006560), which were aligned with the constraint-based alignment tool Cobalt (Papadopoulos and Agarwala, 2007). The tree was rooted using Streptococcus thermophilus WP_220023437.1 as outgroup. The distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. Scale bar represents sequence divergence.

3.3 Screening and phylogenetic analysis of candidate bgl genes in L. plantarum wild strains

PCR assay targeting homologous genes of CS400_15205, CS400_14765 and CS400_14770 was carried out on 9 *L. plantarum* strains isolated from brine olive niche (Vaccalluzzo et al. 2020) and with different β-glucosidase activity against oleuropein, in order to establish the gene distribution at inter-strain level. Figure 4 shows that all tested strains have the amplicons of expected length for CS400_15205 and CS400_14770, respectively, while 4 out of 9 strains tested gave no PCR products with the primer pair targeting CS400_15205 (Spano et al. 2005). Even if we cannot exclude that SNPs and/or indel prevented correct amplification of gene CS400_15205 in these wild strains, the data suggest that either loss or mutations of CS400_15205 did not affect β-glucosidase activity against oleuropein in the set of tested strains. According to mass spectrometry analysis, we chosen strains C11C8 and F3.5 for sequencing of homologous genes of CS400_15205, CS400_14765 and CS400_14770. While *L. plantarum* C11C8 and F3.5 encode two proteins 100% homologous to CS400_15205 (99,89%) and CS400_14765 (99,66%), they significantly differed in CS400_14770 homologous gene nucleotide sequences (Supplementary Figure 1). Functional predictions based on analyses performed with CDD/SPARCLE database revealed that the inferred amino acid sequences of both allelic variants have a BglB super family domain configuration (Arch. ID 10006560; E-value 0e +00 for both C11C8 and F3.5). However, they displayed 2 substitutions and 1 indel. Phylogenetic analysis confirmed that genome of strains C11C8 and F.5 contains three genes homologous to CS400_15205 (Group 1), CS400_14765 (Group 2) and CS400_14770 (Group 3), respectively (Figure 5).

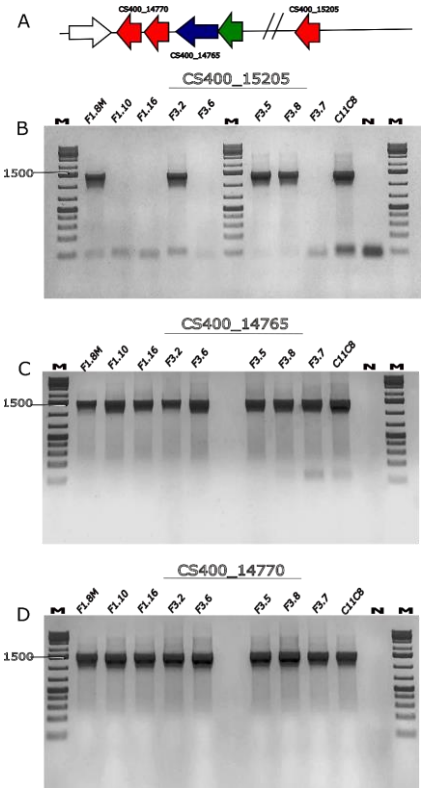


Figure 4. PCR assays targeting putative bgl genes in *Lactiplantibacillus plantarum* wild strains isolated from brine olive. A) Cartoon representing synteny of candidate bgl genes in ATCC8014 genome. Genes are represented as arrows and are not in scale. Red arrows indicate candidate bgl genes, blue arrow PTS beta-glucoside transporter subunit encoding gene, green arrow ORF encoding for antitermination protein BlgG, and white arrow flanking genes not involved in glucoside hydrolysis. B) PCR screening of CS400-1505 gene carried out with primer pair bglu_F/bglu_R B. Expected amplicon length is of 1,485bp. C) PCR screening of CS400-14765 gene carried out with primer pair m-bgl F/m-bgl R. Expected amplicon length is of 1,490bp; D) PCR screening of CS400-14765 gene carried out with primer pair 14770_F/14770_R. Expected amplicon length is of 1,490bp.

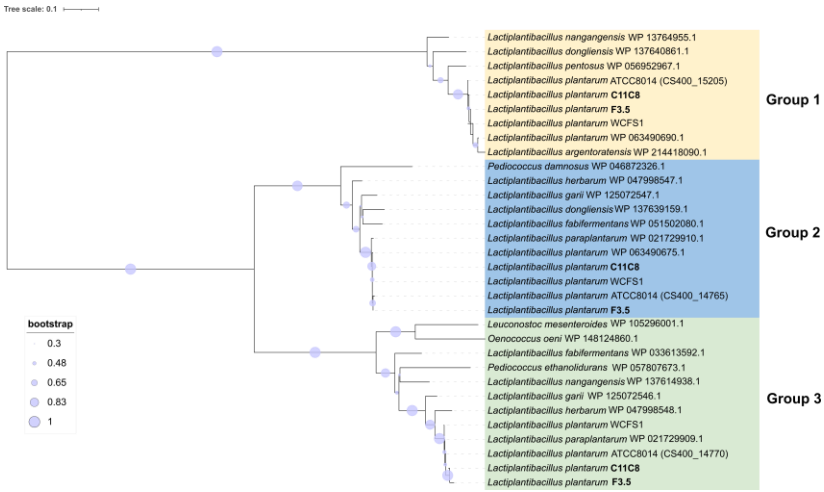


Figure 5. Neighbour-Joining tree showing the phylogenetic position of *bgl* candidate genes in *Lactiplantibacillus plantarum* strain C11C8 and F3.5. Amino acid sequences were aligned with Muscle. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of base substitutions per site. The rate variation among sites was modelled with a gamma distribution (shape parameter 1). Branch lengths are proportional to the numbers of nucleotide substitutions and are measured by the scale bar of sequence divergence. Bootstrap values (1,000 replicates) are shown as symbol at the nodes. Values lower than 0.3 were omitted. The analysis involved 38 amino acid sequences. All ambiguous positions were removed for each sequence pair. There were a total of 494 positions in the final dataset.

3.4 β-glucosidase gene expression profile under table olive brine medium

In order identify the genes responsible for β-glucosidase activity towards oleuropein in strains C11C8 and F3.5, cells were collected after 72 h of incubation in table olive brine medium at both 16 °C and 30 °C. Figure 6 shows that both strains did not actively transcribe CS400_15205 homologous gene regardless the temperature of incubation. Considering that C1C8 and F3.5 exhibited oleuropein degradation activity at this stage of table olive brine assay, the lack of CS400_15205 gene expression suggests that this gene could be not responsible for the βG activity. Remarkably, Acebrón et al. (2009) cloned and heterologously expressed this gene in E. coli and proved that the resulting recombinant enzyme has galactosidase but not glucosidase activity.

RT-PCR assay targeting CS400_14765 homologous gene showed that F3.5 actively transcribed this gene both at 16 and 30 °C, while C11C8 switched off the gene expression under cold condition (Figure 6). Comparison of gene expression profile with mass spectrometry data suggested that in strain C11C8 CS400_14765 gene could contribute to βG activity towards oleuropein at 30 °C but not at 16°C. By contrast, *L. plantarum* C11C8 increased transcription signal of CS400_14770 gene both at 16 °C and 30 °C when grown in olive brine medium (Figure 6). Further qPCR studies should be required to quantitatively confirm this gene transcription profile, but overall, these data demonstrated that, differently from previously reported (Spano et al. 2005), CS400_15205 gene is not involved in hydrolysis of oleuropein into aglycone under table olive brine conditions and that a new candidate gene, namely CS400_14770, could be related to βG activity at 16 °C, in addition to CS400_14765 (Zago et al. 2013).

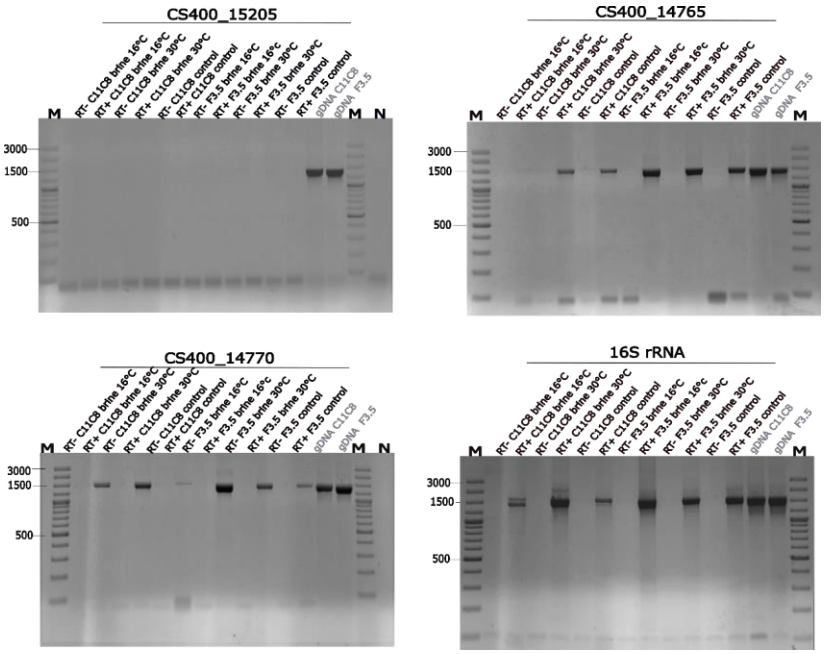


Figure 6. RT-PCR assays targeting putative bgl genes in *L. plantarum* C11C8 and F3.5. cDNA was amplified from total RNA extracted from cells growing both in table olive brine medium and control condition (MRS medium supplemented with 5% of NaCl, pH 5.5 and glucose). Target genes are indicated near to the corresponding picture of electrophoretic gel. Expected lengths of PCR amplicons were 1,490 bp for genes homologous to CS400_15205; 1,485 bp for CS400_14765; and 1,485 bp for CS400_14770. Plus or minus indicates with or without reverse transcriptase in cDNA synthesis reaction, respectively. gDNA amplification was used as positive PCR control. 16S rDNA gene was used as housekeeping gene. Abbreviation: M, molecular weight marker.

4 Conclusion

β-glucosidase enzyme is involved in several biological processes, but a few studies investigated glycoside hydrolysis mechanisms in GRAS species belonging to *L. plantarum*. In this study, mass spectrometry, in silico protein analysis and gene expression profile allowed to reveal that oleuropein bioconversion into hydroxytyrosol compound is a complex phenotype, which takes place by at least three different metabolic pathways and involves concerted action of both β-glucosidase and esterase enzymatic activities. Even if further quantitative analyses are required to corroborate these data, this is the first study that identifies specific metabolic pathways in *L. plantarum* that mediate the enzymatic degradation of oleuropein through the analysis of the variations of specific metabolites. We also demonstrated that the set of bgl genes responsible for β-glucosidase activity could be more complicated than that previously supposed and identified a new candidate, namely CS400_14770, for future functional study. Interestingly, this gene is annotated as 6-phospho-β-glucosidase, which links β-glucosidase activity with the requirement for *L. plantarum* strain to survive under glucose depletion in table olive brine. Similarly, *L. plantarum* and *L. pseudomesenteroides* grown on brain spent grain increased expression of gene 6-phospho-beta-glucidase encoding genes as major metabolic route for carbon catabolism during plant-based fermentation (Acin-Albiacet al. 2020). Remarkably, strains C11C8 and F3.5 have different pattern of metabolites at 16 °C and their genomes harbour different variants of gene CS400_14770. If different alleles of this locus are probably responsible for differential β-glucosidase activity under low temperature conditions, they could have an important practical implication in table olive brine fermentation and could drive future

selection criteria for new oleuropein-depredating *L. plantarum* starter cultures.

Supplementary Materials

Supplementary Table 1. High-resolution mass spectrometry data for phenolic and related compounds identified in control and inoculated table olive brine medium.

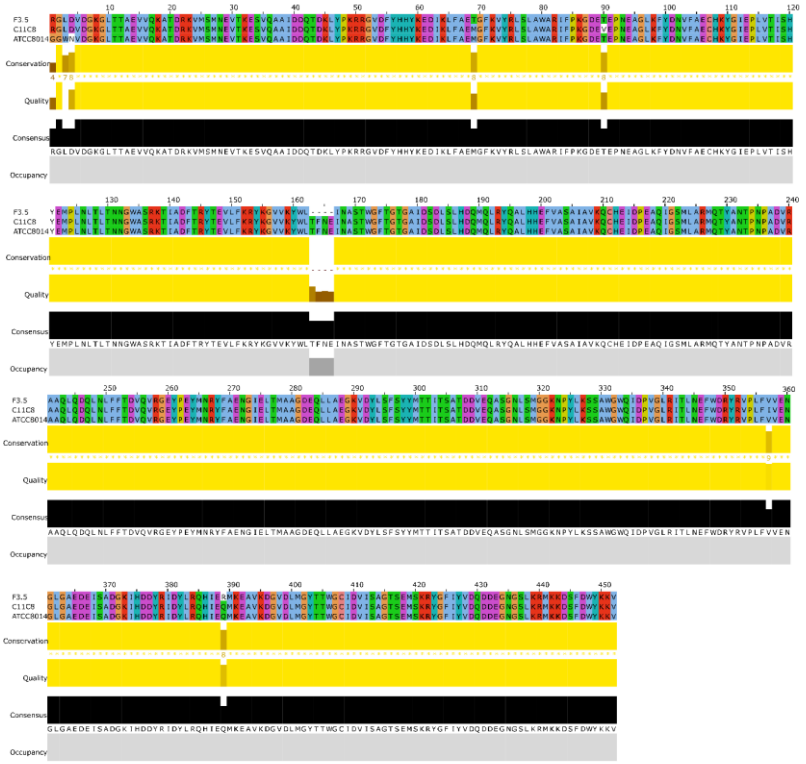
Compound	*Rt*	*Formula*	*Observed m/z*	*Calculated m/z*	*ppm*	*MS^2 fragment ions*
Hydroxytyrosol	5.19	$C_8H_{10}O_3$	153.0557	153.0557	0.0	123.0440
Eleanolic acid	7.50	$C_{11}H_{14}O_6$	241.0716	241.0718	-0.83	209.0452; 165.0548; 137.0597
EDA	7.51	$C_9H_{12}O_4$	183.0659	183.0663	-2.18	139.0754
OME	10.57	$C_{17}H_{24}O_6$	403.1248	403.1246	0.50	223.0617
Oleuropein	14.47	$C_{25}H_{32}O_{13}$	539.1774	539.1770	0.74	307.0826; 275.0953; 241.0717
Oleuropein aglycone	16.76	$C_{19}H_{22}O_8$	377.1247	377.1242	1.33	345.0981; 275.0943
HyEDA	17.04	$C_{17}H_{20}O_6$	319.1189	319.1187	0.63	183.0656; 139.0754

Abbreviation are: Rt: retention time; EDA: dialdehydic form of decarboxymethyl elenolic acid; OME: oleoside-methyl ester; HyEDA: decarboxymethyl dialdehydic form of oleuropein aglycone

Supplementary Table 2. Relative quantification data, expressed as AUC, for phenolic and related compounds identified in control and inoculated table olive brine medium.

Compound	*Control*	*F3.5 16 °C*	*F3.5 30 °C*	*C11C8 16 °C*	*C11C8 30 °C*
Hydroxytyrosol	$6.073x10^{9}$ ± $1.834x10^{8}$	$6.981x10^{9}$ ± $1.579x10^{8}$	$7.063x10^{9}$ ± $2.180x10^{8}$	$7.463x10^{9}$ ± $30.643x10^{8}$	$7.559x10^{7}$ ± $1.533x10^{8}$
Eleanolic acid	$2.131x10^{7}$ ± $8.858x10^{5}$	$2.301x10^{7}$ ± $7.153x10^{5}$	$2.488x10^{7}$ ± $2.103x10^{5}$	$2.457x10^{7}$ ± $8.251x10^{5}$	$2.729x10^{7}$ ± $4.128x10^{5}$
EDA	$4.250x10^{8}$ ± $5.960x10^{6}$	$4.250x10^{8}$ ± $4.214x10^{6}$	$4.149x10^{8}$ ± $3.966x10^{6}$	$4.173x10^{8}$ ± $4.113x10^{6}$	$4.112x10^{8}$ ± $7.4286x10^{6}$
OME	$4.996x10^{6}$ ± $2.550x10^{5}$	$1.245x10^{7}$ ± $7.375x10^{6}$	$8.271x10^{6}$ ± $1.887x10^{5}$	$9.911x10^{6}$ ± $1.931x10^{5}$	$7.472x10^{6}$ ± $7.024x10^{5}$
Oleuropein	$4.406x10^{7}$ ± $1.176x10^{6}$	$3.743x10^{7}$ ± $1.2426x10^{6}$	$3.092x10^{7}$ ± $8.409x10^{5}$	$3.076x10^{7}$ ± $7.233x10^{5}$	$2.957x10^{7}$ ± $1.541x10^{6}$
Oleuropein aglycone	$2.638x10^{8}$ ± $5.437x10^{6}$	$2.961x10^{8}$ ± $2.628x10^{6}$	$3.181x10^{8}$ ± $1.081x10^{7}$	$3.297x10^{8}$ ± $1.176x10^{7}$	$3.373x10^{7}$ ± $1.653x10^{6}$
HyEDA	$1.723x10^{8}$ ± $4.022x10^{6}$	$2.016x10^{8}$ ± $4.019x10^{6}$	$2.435x10^{8}$ ± $4.751x10^{6}$	$2.450x10^{8}$ ± $1.171x10^{7}$	$2.697x10^{8}$ ± $1.547x10^{7}$

Abbreviation are: AUC: under the curve; EDA: dialdehydic form of decarboxymethyl elenolic acid; OME: oleoside-methyl ester; HyEDA: decarboxymethyl dialdehydic form of oleuropein aglycone



Supplementary Figure Sx. Multiple sequence alignment of amino acid sequences of CS400_14770 of *Lactiplantibacillus plantarum* C11C8 e F3.5 with reference strain ATCC8014. Amino acid sequences were aligned using Muscle program [Edgar 2004] in MEGA X software [Patel et al. 2018] and the resulting alignment was visualized using JalView v2.11 [Waterhouse et al. 2009].

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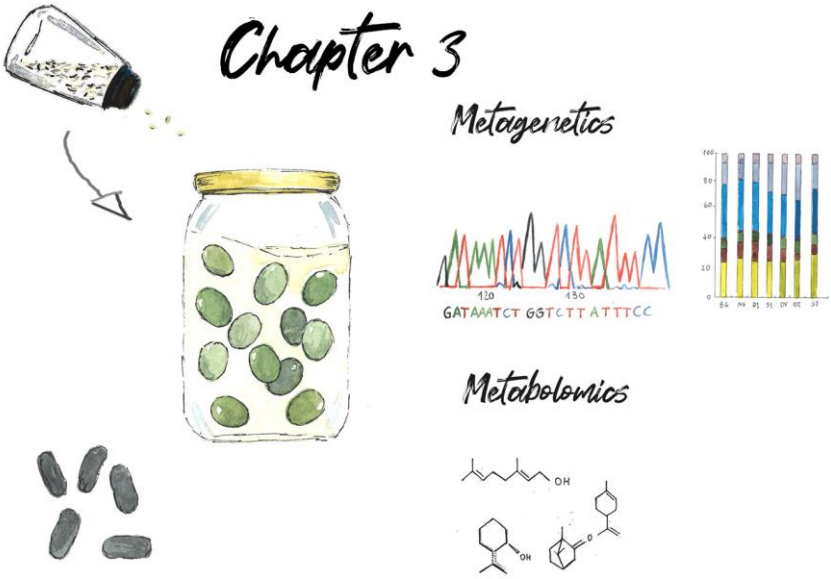
Chapter 3
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CHAPTER 3: Experimental activity

Dual approach based on culture dependent and omics techniques to elucidate the effect of *Lactiplantibacillus plantarum* strains on microbiota and volatile compounds of Sicilian table olives

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1 Introduction

Among fermented foods, table olives are one of the most well known and produced fermented vegetables, especially in the Mediterranean area, with a large consumption worldwide (Vaccalluzzo et al., 2020a). The high content of vitamins, minerals, dietary fibers, short-chain fatty acids, and bioactive compounds, such as polyphenols, contribute to the high nutritional and functional value of such a relevant fermented product (Argyri et al., 2020). Based on data recently reported by the International Olive Council (IOC), the table olives production is currently close to 2.9 million tonnes/season. The fermentation of table olives involves the conversion of inedible compounds into edible organic biomolecules, due to the metabolic activity of autochthonous or deliberately added starter cultures. It is already well established that in table olives a complex and variable microbial consortium, mainly composed of lactic acid bacteria (LAB) and yeasts, is present. This microbial consortium is mainly responsible of the debittering process, which occur spontaneously in the Sicilian table olives fermentation, through the activity of β-glucosidase and esterase enzymes. In recent years, the approach to study the complex microbiota of table olives has been completely revolutionized, contributing to better understand its dynamism in composition and functionality. Amplicon-based metagenomics analysis targeting either 16S rRNA gene or internal transcribed spacers (ITS) DNA region is the most widely used technique to reveal the complexity of microbial consortium of LAB and yeasts/fungal communities, respectively, in food matrices, as well as in table olives (Ferrocino and Cocolin 2017; De Filippis et al., 2017; Vaccalluzzo et al., 2020a). Indeed, metagenetic studies allowed to gain a comprehensive view of table olives microbiota at different taxonomic levels, revealing the presence of unexpected bacteria during fermentation. The presence of

halophilic species and soil-related bacteria, belonging to Ralstonia and Roultella genera, has been revealed in Nocellara Etnea and in Nyons black table olives, respectively (Cocolin et al., 2013; Penland et al., 2020). Through high-throughput bar-coded pyrosequencing analysis of ITS1-5.8 S-ITS2 region, the presence of a complex fungal consortium, including phytopathogenic, saprofitic, spoiling and fermentative genera, never detected by using culture-dependent techniques, has been recently revealed (Vaccalluzzo et al., 2020a). Furthermore, volatilomic approaches can detect biomolecules and aromatic compounds, generated during the fermentation process. According to previous reports on fermented table olives, HS-SPME gas chromatography-mass spectrometry is the most widely used technique to investigate the volatilome. Studies conducted by Martorana et al. (2017) and De Angelis et al. (2015) showed that the use of starter culture positively influenced the VOCs profile of fermented table olives, increasing the pleasant compounds in the final product. Cultivar and fermentation process, together with the microbial dynamism strongly influence the profile of volatile compounds in table olives. Indeed, Nanou et al. (2020) reported a significant difference in the VOC profile between the two varieties Halkidiki and Conservolea, fermented through the same process, and Bleve et al. (2014, 2015) highlighted differences in volatile compounds between two varieties. In addition, De Castro et al. (2019), found a correlation between metagenetics and volatilomics, identifying microbial species positively correlated with off-odours of table olives.

The aim of the present study was to elucidate the effect of two different starter cultures containing *Lactiplantibacillus plantarum* strains on the microbiota composition and organic compound profile of Sicilian table olives, processed at 5% and 8% of salt content. The study was conducted using a dual approach that includes both

conventional and amplicon-based metagenetics analyses and volatilomics analyses.

2 Materials and Methods

2.1 Olives processing method

In the present study, olives of Nocellara Etnea cultivar, kindly provided by local companies, situated in Adrano and Paternò, Catania, Sicily, were processed at industrial scale, following the Sicilian style method, without the addition of sodium hydroxide solution. After harvesting (September-October, 2019), drupes were pre-treated according to Pino et al. (2018a) and directly placed into brines, containing 5% and 8% of marine salt.

2.2 Inoculum of selected Lactiplantibacillus plantarum strains

The *Lactiplantibacillus plantarum* C11C8, F1.16 and F3.5 strains, previously isolated from brine samples of natural Sicilian table olives at 5% of NaCl (Pino et al., 2019) and characterized for the ability to grow and to degrade the oleuropein under stress conditions (Vaccalluzzo et al., 2020b), were used as starter culture. In detail, two starter cultures were set up: starter 1: with *L. plantarum* F1.16 and F3.5 strains and, starter 2: with *L. plantarum* C11C8, F1.16 and F3.5 strains. The starter cultures were inoculated to a final cell density of 7 log cfu/ml, directly after brining. As displayed in Figure 1, four different fermentations were carried out: O1: 5% of NaCl, with the addition of starter 1; O2: 5% of NaCl, with the addition of starter 2; C5 (5% of NaCl) and C8 (8% of NaCl) without the addition of any starters, both used as control. All fermentations were carried out at room temperature (18±2 °C) and monitored till 80 days. In addition, salt was periodically added into the samples, in order to maintain the

initial sodium chloride concentration. Each fermentation was carried out in triplicate.

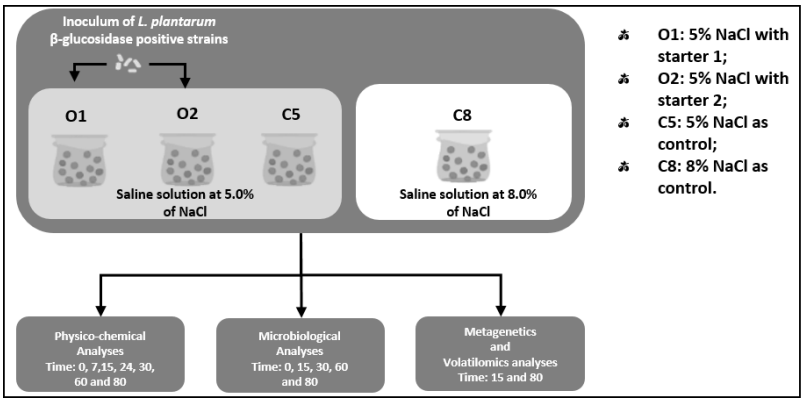


Figure 1. Study design of experimental table olives of Nocellara Etnea cv.

2.3 Chemical and microbiological analyses

The pH of brine was measured, during the fermentation process at times 0,7,15, 24, 30 60 and 80 days, using a MettlerDL25 pHmeter (MettlerDL25, Mettler-Toledo International Inc.). The NaCl salt content was monitored following the method proposed by Benítez-Cabello et al. 2020. Microbiological analyses were performed on both brine and olives samples after 0, 15, 30, 60 and 80 days of fermentation following the method reported by Pino et al. (2018-2019). Sulphite Polymyxin Suphadiazine (S.P.S) Agar, anaerobically incubated at 37°C for 24-48 hours, was used for the detection of sulfite-reducing clostridia, as Clostridium perfringens species. All media were purchased from Liofilchem (Roseto degli Abruzzi, Italy). All analyses were performed in triplicate. Results were expressed as log10 CFU/mL for brine and log10 CFU/g for olive samples.

2.4 16S rRNA gene metagenetics analysis of olive fruits

Olives from all samples, at 15 and 80 days of fermentation, were subjected to total DNA extraction using the Dneasy Mericon Food Kit (Quiagen, Milan, Italy) with some modifications. In detail, 12 g of olive samples were diluted with 30 mL of sterile Ringer's solution and incubated for 2h at 37 °C under constant shaking. After incubation, samples were homogenized in a stomacher apparatus for 3 min at room temperature. The suspension was collected, centrifuged at 10,000 g for 10 min at 20°C, and the pellet was washed twice with 30 mL of phosphate-buffered saline (PBS) pH 7.4. The pellet was re-suspended in 400µL of Tris-EDTA (TE) buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) 1X and the suspension was transferred into tube containing 0.3 g of zircon beads, added with 150 µL of phenol solution, and homogenized with Precellys Evolution Homogenizer (Bertin Technologies) at 10.000 rpm for 5 min. The obtained suspension was subjected to DNA extraction, following the kit manufactures' instructions. DNA concentration was determined using the fluorimeter Qubit 4.0 (Invitrogen, Carlsbad, CA, United States) before storage at -20°C until use.

Partial 16S rRNA gene sequences were amplified from extracted DNA using the primer pair Probio_Uni and Probio_Rev, targeting the V3 region of the 16S rRNA gene sequence (Milani et al., 2013). 16S rRNA gene amplification and amplicon checks were carried out as previously described (Milani et al., 2013) and 16S rRNA gene sequencing was performed using a MiSeq (Illumina) according to Milani et al., (2013).

2.5 Volatile organic compounds (VOCs) analysis by gas chromatography-mass-spectrometry (GC-MS)

The volatile organic compounds (VOCs) profile of all samples at 15 and 80 days of fermentation was investigated by gas

chromatography-mass-spectrometry (GC-MS). In detail, olives (approximately 100 g), from three different replicates, were pitted, homogenized, and 3.3 g of pulp were placed in a 20-mL glass vial. After the addition of 10 mL NaCl solution (300 g/L) and 10 μL of 2-methyl-4-pentanol (final concentration 75 μg/g) as internal standard, the vial was closed and extraction by HS-SPME was performed with subsequent analysis by GC–MS, according to method reported by de Castro et al. 2019. Compound identification was based on mass spectra matching with the standard NIST 08 MS library, on the comparison of retention indices (RI) sourced from the NIST Standard Reference Database and from authentic reference standards when available. All analyses were made in triplicate. A PAL COMBI-xt autosampler (CTC combiPAL, CTC Analysis AG, Zwingen, Switzerland) was used to standardize the extraction procedure. The olive samples were kept at 60 °C for 15 min. The divinylbenzene/carboxen/polydimethylsiloxane (DVB/CARB/ PDMS) (Supelco, Bellefonte, USA) fiber was exposed to the sample headspace for 60 min (Sánchez et al., 2018). The VOCs injection was made under splitless mode into the port at 230 °C, equipped with a Merlino sealed. A Clarus 680 (PerkinElmer, Beaconsfield UK) gas chromatography equipped with a Rtx-WAX column (30 m × 0.25 mm i. d., 0.25 μm film thickness) (Restek Superchrom, Milano, Italy) was used to thermally desorbed and to separate the head space volatile organic compounds. The column temperature was set initially at 35 °C for 8 min, then increased to 230 °C at 4 °C/min and held for 15 min (Montemurro et al., 2020). Helium was used as carrier gas at flow rate of 1 mL/min. A single-quadrupole mass spectrometer Clarus SQ8MS (PerkinElmer) was used to detect the different compounds the source and transfer line temperatures were 250 and 230 °C, respectively. The MS detector system operated in scan mode with mass-to-charge ratio interval 30–350 Da.

2.6 Statistical Analyses

One-way ANOVA followed by Tukey's post hoc multiple comparison test was applied to the pH values, microbiological and volatile organic compounds data from three biological replicates and differences were considered statistically significant at $p < 0.05$. In order to correlate the experimental and control samples with the volatile compounds, the data obtained at 15 and 80 days of fermentation were subjected to principal component analysis (PCA). Similarities between the volatile profiles of the inoculated and control olive samples were carried out using the PermutMatrix software. The permutation analysis of the significantly different VOCs was evaluated in the drupe samples with (O1-O2) and without (C5-C8) addition of starter during the fermentation process (T15-T80). All statistical analyses were performed using STATISTICA software (version 7.0 for Windows, TIBCO Software, Palo Alto, CA, USA). Following sequencing, the fastq files were processed using a custom script based on the QIIME software suite (Caporaso et al., 2010). Paired-end reads pairs were assembled to reconstruct the complete Probio_Uni / Probio_Rev amplicons. Quality control retained sequences with a length between 140 and 400 bp and mean sequence quality score >20 while sequences with homopolymers >7 bp and mismatched primers were omitted. In order to calculate downstream diversity measures (alpha and beta diversity indices, Unifrac analysis), 16S rRNA Operational Taxonomic Units (OTUs) were defined at ≥ 99 % sequence homology using uclust (Edgar, 2010) and OTUs with less than 10 sequences were filtered. All reads were classified to the lowest possible taxonomic rank using QIIME (Caporaso et al., 2010) and a reference dataset from the SILVA database (Quast et al., 2013). The bacterial profile at phylum, family and genus level was represented through Bar plot and reported as relative abundance. In detail, only taxa with relative abundance > 0.5% were shown. The microbial

richness of the samples (alpha-diversity) were calculated with Chao1 and Shannon indexes evaluated for 10 sub-samplings of sequenced read pools and represented by rarefaction curves. Similarities between samples (beta-diversity) were calculated by weighted uniFrac (Lozupone and Knight, 2005). In addition, Principal coordinates analysis (PCoA) was performed on the UniFrac distance matrices to show the differences among samples. Correlation analysis between cell density in different microbial groups and VOCs evaluated at 15 and 80 days of fermentation was performed. The Spearman rank correlation was computed in R by using the cor test package (https://www.rdocumentation.org/packages/stats/versions/3.6.2/topics/cor) and plotted by using the corrplot package (Wei T, Simko V (2021)). R package 'corrplot': Visualization of a Correlation Matrix. (Version 0.90), https://github.com/taiyun/corrplot). Results for species showing significant correlation ($p<0.05$) were visualized as a correlation matrix (R package 'corrplot' -Version 0.90) (https://github.com/taiyun/corrplot).

3 Results

3.1 Physico-chemical analyses

Table 1 shows pH values of O1, O2, C5, and C8 brine samples measured during the fermentation process. At the beginning of the fermentation, significant differences were found between the C5 and the C8 samples, with values of 6.04 and 6.24, respectively, and the inoculated samples (O1 and O2) exhibited a value of about 6.14. As expected, during the fermentation, the pH value decreased significantly, reaching a value of about 4.5 in the inoculated samples after 24 days, and in the controls after 60 days of fermentation. With the exception of the control at 5% of NaCl (C5), a pH < 4.3 was detected at the end of fermentation in all samples, falling within the recommended critical threshold, which would guarantee the

microbiological safety of the final product.

3.2 Microbiological data

Tables 2 and Supplementary Table 1, show microbial counts detected both in olives and brines, respectively, after 0, 15, 30, 60 and 80 days of fermentation. Overall, in all olive samples (Table 2) a significant decrease of enterobacteria counts was observed starting from the 30th day of fermentation, in inoculated samples, especially in O2 sample. Similar trend was revealed by coagulase negative staphylococci, while coagulase positive staphylococci counts were below the threshold limit of detection starting from the 15th day of fermentation, in both inoculated samples (Table 2). Viable mesophilic

bacteria exhibited different count levels among samples up to 30 days of fermentation, while at the 60th day of fermentation, the inoculated samples registered count values of 4.5 with the O2 sample reaching the value of 4.3 log CFU/g, at the end of fermentation. Higher mesophilic bacteria counts were detected in control samples, which showed, at the 60th and 80th day of fermentation, mean values of 6.20 and 5.5 log CFU/g, respectively. Regarding LAB count, no significant difference was found among samples at the beginning of fermentation, whereas a decrease and an increase of about 1 log unit, in control samples and in inoculated samples, respectively were observed at the end of fermentation. Regarding the yeast population, a significant increase (about 6 log units) was observed at the 15th of fermentation, in all samples, especially in C8 samples, reaching a value of 8 log CFU/g. Starting from the 60th day of fermentation, the yeast population decreased, reaching a mean value of 3.9 log CFU/g and 5.2 CFU/g in inoculated and control samples, respectively. Furthermore, the presence of sulphite-reducing species and E. coli was never detected in any samples at any times.

Regarding microbiological analyses of brine samples, at the

same sampling times mentioned above, results are reported in the Supplementary Table 1. Enterobacteriaceae counts showed an initial mean value of 3.6 log CFU/mL, with a significant decrease to a mean value of 2.6 log CFU/mL at the 30th day of fermentation. From an initial value of about 5.0 log CFU/mL, coagulase-negative staphylococci decreased significantly through the fermentation, reaching a value below the threshold limit of detection from the 60th day of fermentation in inoculated samples. With the exception of C5 sample, low values of coagulase-positive staphylococci were detected in all samples at the beginning of fermentation, which decreased significantly starting from the 30th day. Mesophilic bacteria decreased through the fermentation in all samples, reaching a mean final value of 6.1 log CFU/mL. LAB counts were higher in inoculated samples than in controls, showing a constant trend up to the end of fermentation. Yeast population showed a reduction of almost 3 log units, with the exception of inoculated sample O1, where a 2 log units' decrease was observed. The presence of E. coli was never detected in sample O2; its complete inhibition was revealed in O1 and C8 samples starting from the 30th day, and from 60th day in C5 sample. Sulphite-reducing species was never detected in any tested samples.

Table 1. Results of pH values in olive brines expressed as means and standard deviations at different times of fermentation

Samples	Days of fermentation						
	T0	T7	T15	T24	T30	T60	T80
				pH			
O1	6.17±0.08^{aA}	5.74±0.05^{bB}	4.85±0.05^{cC}	4.50±0.1^{bdD}	4.42±0.06^{cD}	4.33±0.07^{bDE}	4.23±0.07^{bcE}
O2	6.11±0.05^{abA}	5.07±0.08^{cB}	4.82±0.08^{bC}	4.49±0.1^{bD}	4.40±0.10^{cD}	4.14±0.06^{cE}	4.10±0.06^{cE}
C5	6.04±0.10^{bA}	5.91±0.05^{aAB}	5.70±0.09^{aB}	5.43±0.06^{aC}	5.40±0.09^{aC}	5.04±0.07^{aD}	5.04±0.09^{aD}
C8	6.24±0.06^{aA}	5.66±0.06^{bB}	5.61±0.08^{aB}	4.70±0.09^{bC}	4.70±0.07^{bcC}	4.41±0.08^{bD}	4.36±0.06^{bD}

a–c: different letters within the same column indicate significant differences at $p < 0.05$
A-E: different letters within the same row indicate significant differences at $p < 0.05$

Table 2. Microbial counts expressed as log10 CFU/g of 3 replicates ± standard deviation of the main microbial groups detected in O1, O2, C5 and C8 drupe samples during the fermentation.

Microbial groups	Days of fermentation				
	T0	T15	T30	T60	T80
Enterobacteriaceae					
O1	4.14 ± 0.06^{cA}	3.41 ± 0.10^{bB}	2.10 ± 0.16^{bC}	<1	<1
O2	3.35 ± 0.10^{dA}	2.61 ± 0.07^{cB}	<1	<1	<1
C5	4.83 ± 0.08^{aA}	4.63 ± 0.06^{aA}	3.56 ± 0.10^{aB}	2.43 ± 0.12^{C}	<1
C8	4.53 ± 0.06^{bA}	4.78 ± 0.09^{aB}	3.38 ± 0.09^{aC}	1.89 ± 0.05^{D}	<1
LAB					
O1	6.50 ± 0.06^{aC}	7.59 ± 0.09^{bA}	7.37 ± 0.08^{aB}	7.33 ± 0.05^{bB}	7.62 ± 0.11^{bA}
O2	6.45 ± 0.08^{aC}	7.91 ± 0.08^{aA}	7.33 ± 0.07^{aB}	7.65 ± 0.05^{aB}	7.85 ± 0.07^{aA}
C5	6.32 ± 0.14^{aA}	6.30 ± 0.08^{cA}	6.14 ± 0.09^{bA}	5.65 ± 0.05^{cB}	5.19 ± 0.09^{cC}
C8	6.40 ± 0.15^{aA}	5.78 ± 0.09^{dB}	5.61 ± 0.09^{bB}	5.70 ± 0.06^{cB}	5.28 ± 0.08^{cB}
Yeasts					
O1	2.32 ± 0.08^{bD}	7.52 ± 0.07^{bA}	7.37 ± 0.08^{cA}	5.70 ± 0.10^{cB}	4.00 ± 0.07^{cC}
O2	2.90 ± 0.10^{aE}	6.85 ± 0.05^{dA}	6.41 ± 0.08^{dB}	5.23 ± 0.07^{dC}	3.85 ± 0.07^{cD}
C5	2.48 ± 0.07^{bE}	7.10 ± 0.08^{cB}	7.75 ± 0.05^{bA}	6.79 ± 0.07^{aC}	5.58 ± 0.14^{aD}
C8	2.33 ± 0.07^{bD}	8.08 ± 0.11^{aA}	7.96 ± 0.09^{aA}	6.08 ± 0.07^{bB}	5.00 ± 0.09^{bC}
Mesophilic Bacteria					
O1	5.48 ± 0.08^{cA}	5.48 ± 0.10^{cA}	5.19 ± 0.09^{cB}	4.51 ± 0.08^{bC}	4.52 ± 0.07^{bC}
O2	5.70 ± 0.09^{bA}	4.36 ± 0.07^{dBC}	4.23 ± 0.06^{dC}	4.49 ± 0.08^{bB}	4.35 ± 0.09^{bBC}
C5	6.30 ± 0.09^{aB}	6.85 ± 0.06^{bA}	6.81 ± 0.08^{bA}	6.18 ± 0.08^{aB}	5.78 ± 0.08^{aC}
C8	5.00 ± 0.08^{dD}	8.08 ± 0.09^{aA}	8.26 ± 0.07^{aA}	6.30 ± 0.09^{aB}	5.30 ± 0.09^{aC}
Coagulase positive staphylococci					
O1	2.31 ± 0.07^{c}	<1	<1	<1	<1
O2	2.11 ± 0.21^{c}	<1	<1	<1	<1
C5	3.59 ± 0.09^{aB}	4.91 ± 0.08^{A}	4.95 ± 0.05^{A}	3.70 ± 0.10^{B}	<1
C8	3.23 ± 0.11^{bB}	4.90 ± 0.08^{A}	4.70 ± 0.08^{A}	3.33 ± 0.05^{B}	<1
Coagulase negative staphylococci					
O1	2.85 ± 0.06^{cB}	4.20 ± 0.09^{aA}	<1	<1	<1
O2	2.63 ± 0.07^{bA}	2.30 ± 0.09^{cA}	<1	<1	<1
C5	2.91 ± 0.06^{aC}	3.33 ± 0.06^{bB}	4.70 ± 0.08^{aA}	2.60 ± 0.09^{C}	<1
C8	2.45 ± 0.09^{aA}	1.54 ± 0.07^{dB}	2.55 ± 0.06^{bA}	<1	<1

a–d: different letters within the same column indicate significant differences at $p < 0.05$
A-E: different letters within the same row indicate significant differences at $p < 0.05$

3.3 Taxonomy analysis of table olives microbiota

Inoculated and control table olives samples both at 15 days and 80 days of fermentation were subjected to sequencing of the V3 region of the 16S rRNA gene. Number of reads, number of operational taxonomic units (OTUs), Shannon and Chao 1 indices, and Good's coverage values are reported in Table 4. Overall, the analysis allowed to obtain a total of 449654 bacterial sequences with an average of 56207 sequences for each analysed sample. The total number of OTUs assigned ranged from 14 to 68 with an average value of 29 detected OTUs per sample. The Chao1 index increased from 15 to 80 days of fermentation except in the control sample C8 in which a decrease of Chao1 index was observed during the fermentation process (Table 3). A satisfactory coverage of the bacterial diversity was found for all the analysed samples with Good's coverage values above 99% (Table 3), which was confirmed by rarefaction curves analysis (Supplementary Figure 1, only Richness data are shown). The bacterial biota of the analysed olives samples was covered by 3 phyla (Figure 2A), 7 families (Figure 2B), 10 genera (Figure 2C), and 12 species (Figure 2D), which were identified as the predominant bacterial OTUs (relative abundance >0.5%). In detail, Firmicutes and Proteobacteria were the phyla mainly detected. Among these, Firmicutes showed the highest relative abundance in all samples, with the exception of C5 sample at 80 days of fermentation. The Bacteroidetes phylum, was found, even at low abundance, only in C8 sample at the beginning of fermentation (Figure 2A). Zooming on the microbiota profile at family level (Figure 2B), Lactobacillaceae family was mainly detected in inoculated samples at both 15 and 80 days of fermentation, whereas Leuconostocaceae family was revealed in control samples. Enterobacteriaceae family showed the highest occurrence in C5 sample at 80 days of fermentation (Figure 2B). The prevalence of 10 genera and of 12 most abundant species (relative abundance > 0.5%) within table olives samples is depicted in Figures 3A and 3B. At the

genus level (Figure 2C), *Lactobacillus* dominated the O1 and O2 olive samples at both 15 and 80 days of fermentation. *Weissella* was mainly detected in C8 sample both at the beginning of fermentation (85.09%) and at the end of the process (78.34%), and in C5 sample at 15 days of fermentation (49.05%). High occurrence of Enterobacter genus was revealed only in C5 sample. The genera *Bacteroides, Faecalibacterium, Klebsiella,* and *Raoultella* were detected only in C8 sample at 15 days of fermentation (Figure 2C). At the species level (Figure 2D), *Lactiplantibacillus plantarum* was the dominant species detected in all inoculated samples at both 15 and 80 days of fermentation, and in the C5 sample at 80 days. *Weissella hellenica* was found at high occurrence in C5 and C8 samples at 15 days of fermentation and in C8 at 80 days. In addition, *Enterobacter sp.* BAB-1513 was revealed in C5 sample at both 15 and 80 days of fermentation (Figure 2D). *Kosakonia cowanii* and U.m. of *Raultella* genus were only found in O1 and in C8 at 15 days, respectively. Figure 3 shows the unweighted UniFrac analysis based in Principal Coordinates Analysis (PCoA) of 16S sequences. The PCoA allowed to group samples based on both treatment and fermentation time along the Axes 1 (PC1) that explained more than 75% of total variance. High similarity among C8 olive samples at 15 and 80 days of fermentation was recorded. In addition, based on the PCoA, olive samples O2, at both 15 and 80 days of fermentation, and olive sample O1 at 80 days of fermentation grouped together.

Table 3. Number of sequences analysed, observed OTUs, and biodiversity measures of total 16S rRNA gene of O1, O2, C5, and C8 olives samples at 15 and 80 days of fermentation.

Sample	Number of reads	Number of OTUs	Shannon	Chao1	Good coverage (%)
O1 T15	46554	22	1.62	22	99.54
O1 T80	73888	30	1.08	31	99.53
O2 T15	63960	23	0.98	24.31	99.62
O2 T80	53669	27	1.03	27.63	99.60
C5 T15	42679	12	1.75	11.9	99.70
C5 T80	52016	14	1.38	15.1	99.72
C8 T15	52726	68	1.59	69.16	99.84
C8 T80	64162	37	1.52	38.53	99.82

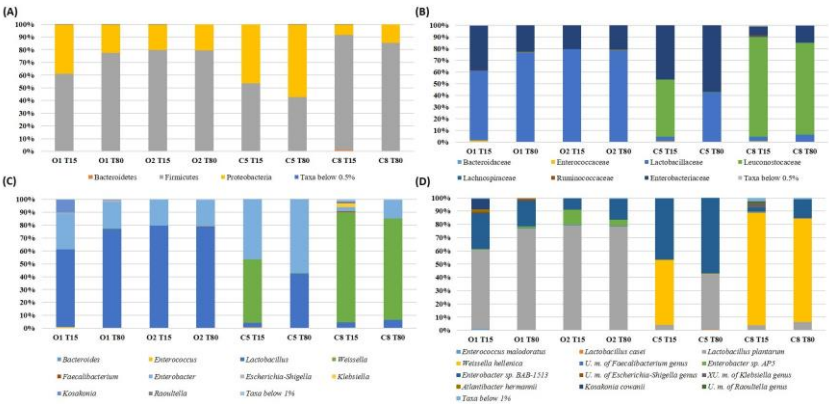


Figure 2. Relative abundance (%) of bacterial phyla (A), family (B), genera (C) and species (D) found on O1, O2, C5, and C8 olivs samples at 15 and 80 days of fermentation.

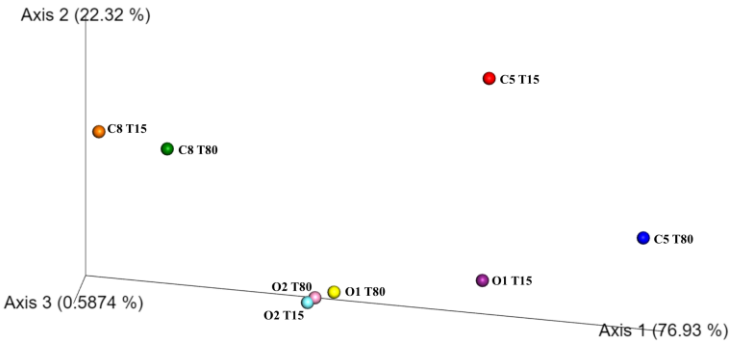


Figure 3. Principal Coordinates Analysis (PCoA) plot of 16S sequences

3.4 Evaluation of volatile organic compounds in olives samples

An objective comparison of volatile metabolic profile in table olive samples at 15 and 80 days of fermentation was performed based on qualitative and quantitative differences in VOCs using HS-SPME GC–MS methodology. Fifty-two volatile compounds were identified, and grouped according to chemical classes, i.e., alcohols (9), esters (21), aldehydes (7), phenols (5), ketones (2), organic acids (2), terpenes (3), and others (3). The two principal factors of PCA analysis (PC1 and PC2), explaining 63% of the total variance, showed that drupes were clearly distributed according to the time of fermentation (Supplementary Figure 2). Thirty-one out of fifty-two volatile compounds showed a significant difference among the un-inoculated and inoculated table olives during the fermentation (Table 4). The significant different compounds, resulting from ANOVA analysis, were used for the permutation analysis (Figure 4). The permutation analysis clearly showed that table olives were grouped into three clusters according to the fermentation process and time. Indeed,

cluster I grouped all inoculated table olives at 15 days, cluster II included the un-inoculated samples at the same time, while cluster III encompassed samples at 80 days of fermentation. It should be noted that O2 sample at 15 days of fermentation was characterized by the highest content of 2-butanone-3-hydroxy (acetoin), ethyl acetate and lactic acid ethyl ester. The same sample after 80 days of fermentation was characterized by the highest content of acetic acid, 3-methyl-1-butanol and their derivative esters as well as phenylethyl alcohol, acetic acid 2-methyl ester, 2-heptanal, benzene propanoic acid methyl ester. It is interesting to note that salt content did not discriminate the VOCs profile between control samples at the end of fermentation. In detail, compared to inoculated samples (O1 and O2) at 80 days of fermentation, C5 and C8 were characterized by the higher content (p-value < 0.05) of 4-ethyl-phenol and the 2-methoxy-phenol. Focusing on alcohols, it is possible to assert that after 80 days of fermentation, a significant decrease of this class of compounds, except for phenylethyl alcohol, 3-methyl-1-butanol, and benzyl alcohol, was observed. A similar trend was detected for aldehydes, with butanal-3-methyl and hexanal showing a significant decrease during the fermentation. An overall increase for acetic acid and derivative esters in all samples was detected during the fermentation. Of note ethyl acetate decrease in the inoculated olives (O1 and O2) after 80 days of fermentation, whereas an opposite trend was assessed in the un-inoculated (C5 and C8) samples.

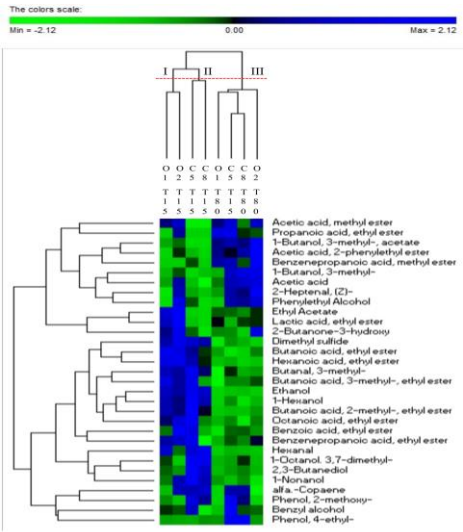


Figure 4. Permutation analysis of significantly different VOCs (ANOVA test corrected with Tukey) evaluated in drupe samples with (O1-O2) and without (C5-C8) addition of starters during fermentation (T15-T80).

Table 4. Statistically different VOCs of drupe samples, expressed as µg/g, evaluated at different salt concentrations (NaCl 5 and 8 %) under controlled (O1 and O2) and spontaneous (C5 and C8) fermentation after 15 and 80 days.

	O1		O2		C5		C8	
	T15	**T80**	**T15**	**T80**	**T15**	**T80**	**T15**	**T80**
Ethanol	248.76±43.66^{a}	56.87±13.95^{b}	219.53±29.30^{a}	66.92±4.28^{b}	317.88±80.53^{a}	75.37±35.84^{b}	260.36±45.05^{a}	84.21±30.38^{b}
1-Butanol, 3-methyl-	29.73±10.25^{bc}	31.57±8.77^{bc}	41.77±9.80^{b}	71±16.75^{a}	22.71±4.21^{c}	61.13±29.22^{ab}	28.31±21.07^{bc}	69.34±24.34^{a}
1-Octanol. 3,7-dimethyl-	12.52±1.66^{ab}	5.63±9.76^{b}	2.95±5.11^{b}	4.88±8.46^{b}	32.03±5.69^{a}	7.33±12.69^{b}	29.81±2.93^{a}	13.12±4.36^{ab}
1-Hexanol	5.47±2.48^{ab}	2.2±0.18^{b}	4.82±0.60^{ab}	2.79±0.78^{ab}	7.58±3.11^{a}	3.41±0.92^{ab}	5.55±2.58^{ab}	1.83±1.58^{b}
3-Hexen-1-ol (Z)-	10.59±18.34^{a}	8.63±8.41^{a}	16.76±2.09^{a}	12.73±16.64^{a}	24.53±11.28^{a}	4.12±7.14^{a}	nd	6.82±7.20^{a}
1-Octanol	4.16±1.77^{a}	2.99±0.43^{a}	4.63±1.69^{a}	5.55±1.39^{a}	4.73±2.38^{a}	5.5±1.48^{a}	1.89±1.67^{a}	2.58±0.74^{a}
1-Nonanol	3.84±3.34^{bc}	3.02±2.64^{bc}	7.86±0.92^{ab}	nd	12.4±3.12^{a}	1.83±3.17^{bc}	7.84±3^{ab}	3.39±3.56^{bc}
Benzyl alcohol	15.49±6.05^{ab}	12.06±2.22^{b}	15.01±0.86^{ab}	14.69±3.84^{ab}	19.43±3.13^{ab}	24.05±5.09^{a}	9.67±1.92^{b}	15.09±4.14^{ab}
Phenylethyl Alcohol	79.8±36.07^{c}	98.78±34.56^{bc}	255.9±116.41^{a}	237.59±43.68^{a}	153.45±61.66^{b}	205.31±84.67^{a}	84.85±18.03^{c}	195.22±77.54^{a}
Total Alcohols	**410.36**	**221.75**	**569.23**	**416.15**	**594.74**	**388.05**	**428.28**	**391.6**
Butanal, 3-methyl-	55.5±3.57^{ab}	nd	50.78±10.75^{b}	27.87±7.08^{c}	73.99±12.52^{a}	38.98±7.81^{bc}	44.8±3.17^{bc}	25.3±0.35^{c}
Hexanal	46.07±36.08^{ab}	1.48±0.14^{c}	17.63±7.09^{bc}	4.02±2.33^{c}	47.76±0.63^{ab}	7.61±6.08^{c}	57.86±8.73^{a}	6.4±1.33^{c}
2-Heptenal, (Z)-	0.81±1.41^{b}	4.04±0.99^{ab}	8.8±4.53^{ab}	12.91±4.7^{a}	3.58±3.11^{b}	8.33±3.57^{ab}	2.53±2.33^{b}	7.23±3.34^{ab}
Nonanal	32.69±17.80^{a}	13.52±1.06^{a}	17.44±2.31^{a}	23.1±7.72^{a}	28.15±3.59^{a}	26.48±8.49^{a}	19.66±5.21^{a}	17.63±4.32^{a}
Benzaldehyde	24.73±9.52^{a}	15.44±6.29^{a}	29.62±4.23^{a}	18.86±1.07^{a}	34.87±8.74^{a}	21.61±10.47^{a}	23.64±3.34^{a}	18.44±8.99^{a}
Benzeneacetaldehyde	27.83±10.91^{a}	10.43±0.31^{a}	31.6±13.11^{a}	14.57±5.16^{a}	24.85±5.89^{a}	12.31±4.57^{a}	15.5±4.30^{a}	5.81±5.15^{a}
2-Decenal, (Z)-	nd	0.81±1.40^{a}	nd	5.18±0.70^{a}	3.79±6.57a	3.89±1.20^{a}	2.01±1.90^{a}	4.09±1.28^{a}
Total Aldehydes	**187.63**	**45.72**	**155.87**	**106.51**	**216.99**	**119.21**	**166**	**84.9**
Acetic acid, methyl ester	14.86±2.75^{ab}	14.7±4.57^{ab}	19.24±3.46^{a}	18.97±5.06^{a}	nd	20.64±2.05^{a}	nd	9.57±1.43^{b}
Ethyl Acetate	255.42±74.82^{ab}	116.23±20.80^{bc}	351.38±137.06^{a}	130.74±28.3^{bc}	17.87±4.20^{c}	111.17±41.53^{bc}	11.16±3.23^{c}	90.3±45.48^{bc}
Propanoic acid, ethyl ester	nd	7.95±3.99^{a}	5.15±8.92^{ab}	2.92±5.06^{c}	nd	8.27±0.59^{a}	nd	3.3±5.72^{c}
Butanoic acid, ethyl ester	6.2±1.45^{a}	1.6±0.28^{cd}	5.68±1.85^{ab}	2.07±0.38^{cd}	3.87±1.85abc	nd	2.7±0.49^{bcd}	0.62±1.08^{cd}
Butanoic acid, 2-methyl-, ethyl ester	10.2±4.52^{ab}	2.78±0.77^{b}	8.75±3.07^{ab}	4.8±2.46^{b}	17.08±6.53^{a}	3.37±5.84^{b}	7.36±1.79^{ab}	3.24±2.81^{b}

Butanoic acid, 3-methyl-, ethyl ester	11.39±4.44^{a}	2.47±2.31^{c}	9.07±3.13^{ab}	7.18±3.31^{b}	11.87±6.44^{a}	6.43±6.21^{b}	5.61±1.57^{bc}	6.37±2.71^{b}
1-Butanol, 3-methyl-, acetate	7.57±2.93^{cd}	20.68±8.66^{bc}	12.66±5.30^{bcd}	41.49±13.02^{a}	nd	24.62±0.44^{ab}	nd	20.25±2.85^{bc}
Hexanoic acid, ethyl ester	18.24±10.40^{a}	3.43±2.99^{b}	19.78±5.16^{a}	3.24±3.18^{b}	14.02±3.49^{ab}	4.6±1.26^{b}	9.21±1.711^{ab}	2.14±1.95^{b}
Lactic acid, methyl ester	nd	1.59±1.50^{a}	0.9±1.57^{a}	2.73±2.62^{a}	nd	nd	nd	nd
Lactic acid, ethyl ester	62.21±12.10^{ab}	35.67±13.62^{bc}	99.42±40.83^{a}	34.34±5.06^{bc}	7.21±8.66^{c}	12.11±17.88^{c}	nd	33.34±6.68^{bc}
Octanoic acid, ethyl ester	6.29±1.99^{abc}	2.8±1.98^{bc}	9.48±2.54^{ab}	4.11±1.95^{bc}	12.08±3.43^{a}	4.02±3.61^{bc}	4.03±4.12^{bc}	0.4±0.70^{c}
Pentanoic acid, 2-hydroxy-4-methyl-, ethyl ester	3.52±3.62^{a}	3±0.92^{a}	6.39±3.24^{a}	1.74±1.51^{a}	3.8±1.3^{a}	nd	0.59±1.02^{a}	1.71±1.49^{a}
Benzoic acid, ethyl ester	5.88±2.69^{ab}	4.06±2.00^{b}	9.88±1.40^{a}	5.48±1.18^{ab}	8.74±1.77^{ab}	6.01±2.99^{ab}	5.14±1.37^{ab}	6.06±0.71^{ab}
Butanedioic acid, diethyl ester	19±8.20^{a}	7.26±8.40^{a}	21.83±7.28^{a}	4.4±2.43^{a}	22.7±25.98^{a}	1.26±2.19^{a}	2.63±2.38^{a}	2.24±2.65^{a}
Benzeneacetic acid, ethyl ester	4.21±3.84^{a}	3.22±2.54^{a}	1.83±1.60^{a}	2.1±1.97^{a}	3.11±2.87^{a}	3.67±3.18^{a}	2.13±1.92^{a}	4.02±2.08^{a}
Benzoic acid, 2-hydroxy-, ethyl ester	6.84±2.35^{a}	1.38±1.25^{a}	4.04±1.26^{a}	1.67±1.52^{a}	6.59±4.32^{a}	2.5±2.43^{a}	3.57±1.09^{a}	0.85±1.47^{a}
Acetic acid, 2-phenylethyl ester	nd	5.01±2.18^{ab}	3.59±3.81^{b}	10.48±0.64^{a}	1.12±1.93^{b}	3.77±3.27^{b}	nd	5.72±2.92^{ab}
Benzenepropanoic acid, methyl ester	nd	1.41±0.35^{ab}	nd	2.61±0.96^{a}	0.78±1.35^{ab}	1.54±1.42^{ab}	nd	0.76±1.31^{ab}
Benzenepropanoic acid, ethyl ester	11.52±3.74^{a}	6.76±2.71^{ab}	12.86±2.19^{a}	9.64±2.38^{ab}	15.32±2.87^{a}	8.82±5.10^{ab}	2.16±1.92^{b}	7.93±2.01^{ab}
2-Propenoic acid, 3-phenyl-, ethyl ester	1.3±1.15^{a}	nd	1.35±1.18^{a}	nd	nd	nd	0.66±1.15^{a}	nd
Hexadecanoic acid, ethyl ester	0.6±1.04^{a}	0.74±0.69^{a}	1.04±0.90^{a}	1.18±1.04^{a}	0.98±0.93^{a}	0.8±1.39^{a}	0.84±0.75^{a}	1.49±1.34^{a}
Total Esters	**445.25**	**242.74**	**604.32**	**291.89**	**147.14**	**223.6**	**57.79**	**200.31**
Acetic acid	31.88±6.48^{cde}	37.82±14.52^{bcd}	58.59±20.65^{abc}	72.17±16.16^{a}	25.36±4.86^{de}	61.44±2.23^{abc}	3.83±3.42^{e}	67.38±6.48^{ab}
Butanoic acid, 2-methyl	20.5±8.72^{a}	8.2±3.71^{a}	16.25±7.15^{a}	22.18±5.67^{a}	13.21±8.28^{a}	22.74±4.19^{a}	2.6±2.49^{a}	11.82±11.32^{a}
Total Organic Acids	**52.38**	**46.02**	**74.84**	**94.35**	**38.57**	**84.18**	**6.43**	**79.2**
2-Butanone-3-hydroxy	29.3±3.72^{ab}	19.17±2.39^{bc}	45.54±13.17^{a}	42.59±1.55^{a}	21.37±4.78^{bc}	18.5±2.43^{bc}	6.32±6.15^{c}	10.94±5.49^{c}
6-Methyl-5-hepten-2-one	5.36±5.59^{a}	5.39±1.28^{a}	7.05±0.72^{a}	7.68±1.38^{a}	8.86±8.61^{a}	6.64±3.33^{a}	4.43±3.93^{a}	4.37±3.88^{a}
Total Ketones	**34.66**	**24.56**	**52.59**	**50.27**	**30.23**	**25.14**	**10.75**	**15.31**
Phenol, 2-methoxy-	66.23±12.31^{cde}	33.18±4.92^{e}	42.85±14.83^{de}	33.76±10.58^{e}	143.75±0.42^{ab}	163.08±27.65a	107.64±22.53^{abc}	101.38±43.80^{bcd}
Phenol	8.87±3.82^{a}	4.43±2.68^{a}	5.7±2.207^{a}	4.05±0.84^{a}	10.38±4.77^{a}	16.5±10.12^{a}	6.17±4.71^{a}	7.19±7.51^{a}
Phenol, 4-ethyl-2-methoxy-	nd	nd	nd	nd	nd	nd	2.06±1.793^{a}	2.4±2.74^{a}
Phenol, 4-ethyl-	15.75±2.18^{b}	23.06±8.17^{b}	8.01±2.52^{b}	21.35±1.98^{b}	5.04±1.34^{b}	256.55±105.70^{a}	28.1±10.40^{b}	283.9±106.00^{a}
Vanillin	nd	1.27±1.12^{a}	nd	2.2±2.14^{a}	nd	1.85±1.66^{a}	nd	2.84±0.78^{a}

Total Phenols	**90.85**	**61.94**	**56.56**	**61.36**	**159.17**	**437.98**	**143.97**	**397.71**
alfa.-Copaene	54.42±22.9^{ab}	45.15±22.36^{ab}	103.62±23.79^{a}	24.6±34.38^{b}	101.56±23.94^{a}	99.54±6.21^{a}	109.66±20.25^{a}	98.97±41.71^{a}
α-Muurolene,	12.99±4.20^{a}	7.85±0.23^{a}	14.47±2.84^{a}	10.77±1.85^{a}	17.75±3.89^{a}	11.56±2.71^{a}	14.38±3.47^{a}	14.31±6.15^{a}
α-Farnesene	2.05±3.55^{a}	0.91±0.79^{a}	7.23±0.20^{a}	1.4±2.42^{a}	8.08±0.39^{a}	3.36±5.81^{a}	3.58±4.90^{a}	1.78±2.33^{a}
Total Terpenes	**69.46**	**53.91**	**125.32**	**36.77**	**127.39**	**114.46**	**127.62**	**115.06**
Dimethyl sulfide	13.47±1.45^{abc}	4.74±2.07^{c}	16.93±6.12^{a}	6.31±2.65^{bc}	14.99±1.81^{ab}	8.73±4.52^{abc}	13.45±1.21^{abc}	7.71±1.38^{bc}
2,3-Butanediol	24.57±42.55^{bc}	2.42±4.20^{c}	13.87±24.02^{bc}	4.6±4.73^{bc}	112.41±18.49^{a}	12.25±0.94^{bc}	65.28±13.33^{ab}	18.83±29.77^{bc}
Benzofuran, 2,3-dihydro-	2.66±0.45^{a}	3.76±2.47^{a}	4.89±2.10^{a}	3.54±1.07^{a}	3.16±1.61^{a}	2.77±0.90^{a}	2.55±1.11^{a}	3.42±0.37^{a}
Total Others	**40.7**	**10.92**	**35.69**	**14.45**	**130.56**	**23.75**	**81.28**	**29.96**

3.5 Correlation between VOCs profile and viable microbial groups

Statistically significant correlations between microbial groups and VOCs are shown in Figure 5. LAB cell density values positively correlated with acetic acid (p= 0.017) and its derivative ethyl and methyl esters (acetic acid methyl ester, ethyl acetate, 1-butanol 3-methyl-acetate) as well as with lactic acid ethyl ester (p=0.009) and 2-butanone-3-hydroxy (p=0.006). Looking at mesophilic bacteria group, a positive correlation was detected with phenol-2-methoxy and phenol 4-ethyl, which was negatively correlated with Enterobacteriaceae group (p=0.011). Moreover, the latter microbial group showed a positive correlation with alcohols (ethanol, 1−hexanol) esters (butanoic acid ethyl ester, hexanoic acid ethyl ester, benzoic acid 2-hydroxy ethyl ester) aldehydes (hexanal and benzeneacetaldehyde), and dimethyl sulphide and a negative correlation with acetic acid (p=0.012), 1-butanol 3-methyl- (p=0.015), and phenylethyl alcohol (p=0.039). Regarding yeasts, a positive correlation with benzoic acid 2−hydroxy−ethyl ester was revealed whereas 1-butanol-3-methyl-acetate, acetic acid−2−phenylethyl ester, and 6−methyl−5-hepten−2−one negatively correlated.

Figure 5. Spearman correlation matrix between VOCs (black font characters) and microbial groups (red font characters) detected values after 15 and 80 days of fermentation in olive drupes. The normalized scaled matrices were merged and used for correlation computing. Only statistically significant correlations ($p<0.05$) were plotted. The colour graduated scale ranges from -1 (green - negative correlations) to 1 (orange - positive correlations). Blue delimited square marked the outgroup comparisons.

4 Discussion

Table olives are an integral part of the Mediterranean diet, and their production is mainly based on the fermentation process using starter cultures able to reduce the debittering time and the risk of survival/growth of spoiling or pathogenic microorganisms (Panagou et al., 2008; Bevilacqua et al., 2013; Bonatsou et al., 2015; Pino et al., 2019). Studies conducted on table olive highlighted the importance of selection strains with beta-glucosidase activity and able to grow at low salt content as promising strategy to produce safe and healthy products (Tataridou and Kotzekidou 2015; Pino et al. 2018b, 2019; Anagnostopoulos et al., 2020; Perpetuini et al., 2020), in accordance with WHO recommendations. In this contest, to understand the composition, diversity and functioning of microbial ecosystems was the relevant challenge of the present study. In detail, the effect of two different starter cultures obtained using three selected β glucosidase positive *L. plantarum* strains (C11C8, F1.16 and F3.5) was investigated on microbial composition and on volatile organic compound of Sicilian table olives, processed under 5% and 8% of salt, during fermentation up to 80 days. Our results revealed that inoculated table olives exhibited a more pronounced drop in pH, reaching values ≤ 4.5 starting from the 15th day, indicating a faster brine acidification respect to control samples. The data confirm the importance of starter cultures for ensuring the microbiological safety of final product, in accordance to previous studies (Corsetti et al., 2012; Martorana et al., 2017; Pino et al, 2019). When spontaneous fermentation was conducted, the salt content exerts a selective pressure on microbial composition. Accordingly, metagenetics data revealed an abundance of Enterobacter sp (57%) and a dominance of Weissella sp (78%) in control samples at 5 % and at 8% of NaCl, respectively. Within the Weissella genus, Weissella hellenica species, recently identified as

Weissella paramesenteroides (Teixeira 2021) was the most abundant detected species, according to results reported by Lucena-Padrós and collaborators (2014), who describe the presence of this species, for the first time, in treated Manzanilla table olives. Enterobacter sp, often found in fresh fruits and associated to Spanish-style and to spontaneous fermentation table olives (Cocolin et al. 2013; De Angelis et al., 2015), was drastically reduced in inoculated samples, while persisted mainly in control samples processed at lower salt content. This finding is not in line with microbiological data, which highlighted the complete inhibition of Enterobacteriaceae in final product. This incongruity could be explained by the fact that culture-independent techniques applied on total bacteria could not distinguish truly active from dead or compromised microbial cells (Kazou et al., 2020), supporting the importance to apply an integrated approach, coupling culture-dependent and independent methods, for such a complex microbial ecosystem. Based on the dual approach, Lactobacillus genus was found dominating the fermentation of samples inoculated with *L. plantarum* strains, preventing the spoilage microbial growth, and positively contributing to pleasant VOCs formation in the final product, as previously reported (Perpetuini et al. 2020). The presence of high metabolically active lactobacilli on the drupes confirms the ability of *L. plantarum* to adhere and colonize the fruit surface, according to previously reported data (Lavermicocca et al. 2005; Arroyo-López et al. 2012; Domínguez-Manzano et al. 2012; Blana et al. 2014; Benítez-Cabello et al. 2015; De Angelis et al. 2015; Grounta et al., 2015; Faten et al. 2016; Grounta et al. 2016; Perpetuini et al., 2016; Pérez Montoro et al., 2018). According to Anagnastopoulos and co-workers (2020) and to De Angelis et al. (2015), in the present study the clear dominance of *L. plantarum* in inoculated samples confirms that the used strains were able to withstand the competition with microorganisms naturally present in the drupes and to persist up to the

end of fermentation (Benitez et al., 2020; Randazzo et al., 2014; Hurtado et al., 2012). Both coagulase positive and negative staphylococci were strongly reduced from the 30th day of fermentation both in experimental olive and brine samples. In addition, Kosakonia genus, frequently found in environmental sources, such as soil, plants and trees, enclosing nitrogen-fixing species (Brady et al., 2013), was revealed (9.9 %) only in inoculated sample at the 15th day of fermentation, and disappeared at the end of the process. Gram-negative bacteria such as, Raoultella genus, frequently recovered from water, soil and plants, was detected in un-inoculated samples, corroborating its presence in naturally fermented table olives, as reported by other authors (Ercolini et al., 2006; Penland et al., 2020). The detection of this bacterial genus in olives is not un-common. Indeed, Maza-Márquez and co-workers (2017) revealed the presence of species belonging to Raoultella, capable to degrade phenolic compounds in olive wastewater.

In the present study the volatilomics approach revealed the presence of fifty-two volatile organic compounds, including alcohols, esters, aldehydes, phenols, ketones, organic acid and terpenes, typically found in table olives (Vaccalluzzo et al., 2020a). According to Randazzo et al. (2014) and Pino et al. (2018-2019), our data disclosed that the use of lactobacilli culture affects the profile of Sicilian table olives in terms of VOCs abundances. Among VOCs, ester compounds were the most abundance especially in inoculated samples. In particular, acetate esters, which are synthesized by an alcohol-acyl-transferase that catalyzes the esterification of volatile alcohols with acetyl CoA molecules to produce volatile esters and free CoAeSH (Salas, 2004). The 3-methyl-1-butanol acetate, and the 2-methyl ester compounds, mainly detected in inoculated samples, have been reported as responsible of pleasant flavours (Sabatini and Marsilio 2008). The occurrence of off-flavour may be attributed to the

formation of malodorous compounds, like 4-ethylphenol (Brenes et al., 2004). This phenolic compound is generally formed by microorganisms via the decarboxylation of p-coumaric acid to form 4-vinylphenol and reduction of the latter compound (Dias et al., 2003; Rodríguez et al., 2009). One of the most outstanding differences between inoculated and uninoculated samples was assessed for the 4-ethylphenol concentration. The highest concentration of this phenolic compound was detected in control samples at 80 days of fermentation. Although, the 4-ethylphenol concentration is related to storage time (Manthos et al., 2021), in the present study the addition of starter cultures seemed to inhibit its production as off-flavor. It is also interesting to point out that butyric, propionic and cyclohexanoic acids, which are responsible for zapatera off-odours (de Castro et al., 2018), were never found in tested samples, confirming the inhibition of Clostridium and Propionibacterium species by selected starter cultures. From a qualitative perspective, no major differences were observed regarding VOCs profiles among different fermentation processes, so the use of starter does not appear to favor any specific aroma compound. According to Penland et al. (2020), VOCs profile may probably be related main to cultivar rather than changes in the microbial community during the fermentation process.

5 Conclusion

The present study confirmed that a dual approach based on culture dependent, metagenetics and volatilomics techniques allowed to in depth explore the microbial composition and functioning of Sicilian table olives. Metagenetics revealed that *L. plantarum* strains selected for the oleuropein-degrading activity were able to drive the Sicilian table olives fermentation at low salt content, reducing the main undesirable bacteria. Furthermore, although no differences in VOCs profile were found between inoculated and control samples, it

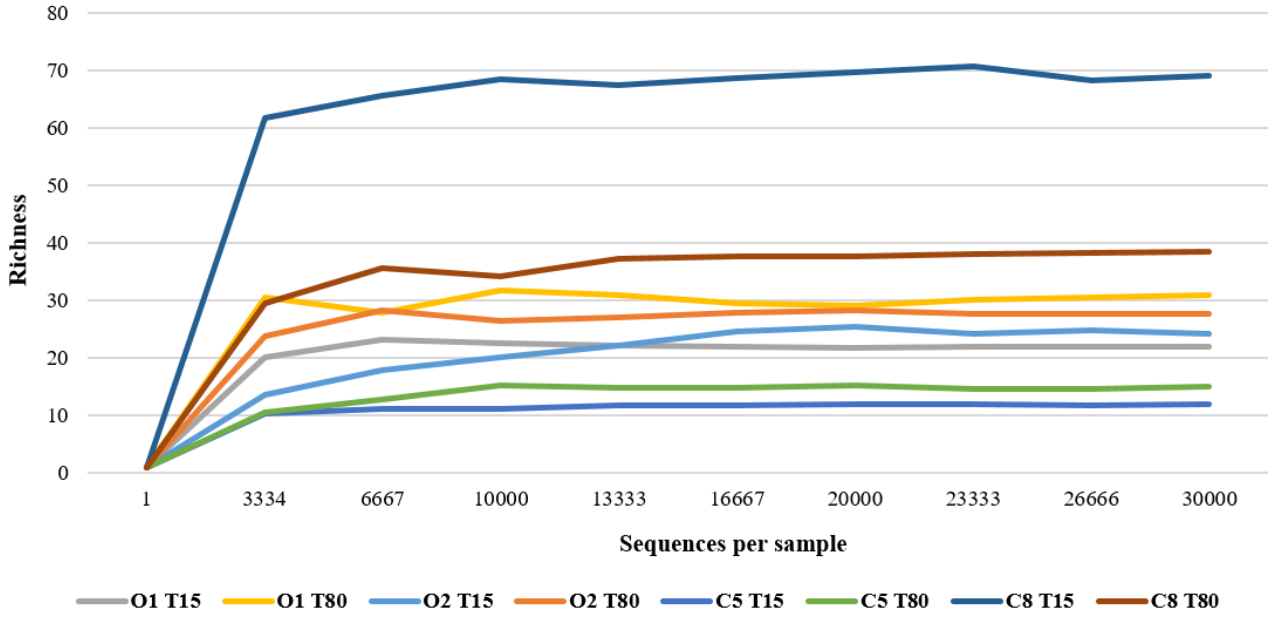
is relevant to highlight that the two selected starter cultures did not affect the formation of off-flavour compounds in final products.

Supplementary Materials

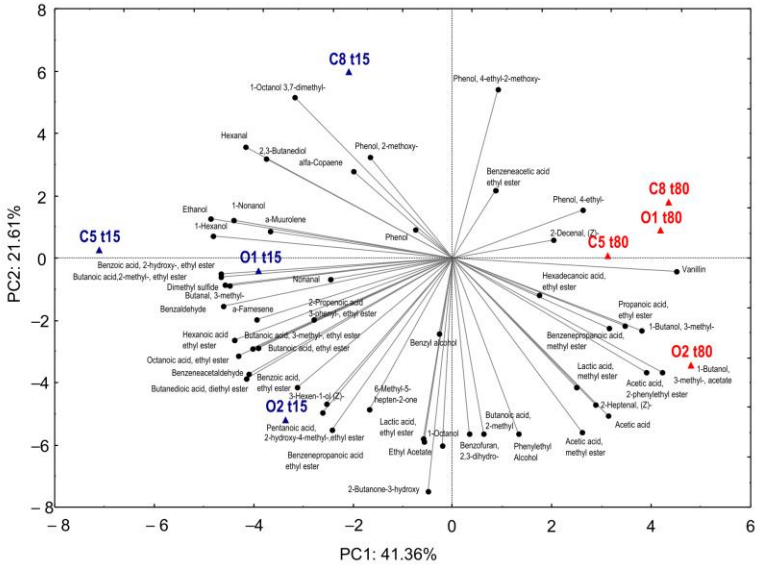
Supplementary Table 1. Microbial counts expressed as log10 CFU/ml of 3 replicates ± standard deviation of the main microbial groups detected in O1, O2, C5 and C8 brine samples during the fermentation process.

	Days of fermentation				
Microbial groups	T0	T15	T30	T60	T80
Enterobacteriaceae					
O1	3.54 ± 0.06^{aB}	4.49 ± 0.10^{bcA}	2.44 ± 0.06^{bC}	<1	<1
O2	3.53 ± 0.12^{aB}	4.38 ± 0.10^{cA}	2.35 ± 0.05^{bC}	<1	<1
C5	3.72 ± 0.07^{aB}	4.72 ± 0.07^{aA}	2.76 ± 0.10^{aC}	<1	<1
C8	3.63 ± 0.07^{aB}	4.61 ± 0.07^{abA}	2.68 ± 0.10^{aC}	<1	<1
LAB					
O1	7.83 ± 0.05^{cA}	7.58 ± 0.07^{aB}	7.50 ± 0.09^{aB}	7.23 ± 0.07^{aC}	7.20 ± 0.09^{aC}
O2	8.20 ± 0.07^{aA}	7.58 ± 0.08^{aB}	7.25 ± 0.08^{bC}	7.04 ± 0.17^{aC}	7.26 ± 0.10^{aC}
C5	7.52 ± 0.10^{bA}	7.01 ± 0.16^{bC}	7.30 ± 0.08^{abAB}	7.20 ± 0.08^{aBC}	6.23 ± 0.06^{bD}
C8	7.50 ± 0.09^{cA}	6.34 ± 0.11^{cB}	7.30 ± 0.09^{abA}	6.34 ± 0.06^{bB}	5.78 ± 0.11^{cC}
Yeasts					
O1	7.40 ± 0.09^{bA}	7.40 ± 0.13^{bA}	7.22 ± 0.06^{bA}	7.21 ± 0.08^{aA}	5.23 ± 0.07^{aB}
O2	7.37 ± 0.08^{bA}	7.36 ± 0.06^{bA}	6.32 ± 0.08^{cC}	6.88 ± 0.07^{bB}	4.85 ± 0.07^{bD}
C5	7.80 ± 0.09^{aA}	7.77 ± 0.13^{aA}	7.40 ± 0.09^{bB}	6.93 ± 0.07^{bC}	4.70 ± 0.09^{bcD}
C8	7.54 ± 0.06^{bAB}	7.45 ± 0.11^{bB}	7.74 ± 0.08^{aA}	7.37 ± 0.08^{aB}	4.61 ± 0.08^{cC}
Mesophilic Bacteria					
O1	7.50 ± 0.08^{bA}	6.80 ± 0.09^{cB}	6.20 ± 0.09^{dC}	6.11 ± 0.09^{cC}	5.67 ± 0.20^{cD}
O2	7.00 ± 0.10^{cA}	6.78 ± 0.07^{cB}	6.54 ± 0.05^{cC}	6.04 ± 0.06^{cD}	5.77 ± 0.08^{cE}
C5	8.20 ± 0.07^{aA}	8.15 ± 0.08^{bA}	7.30 ± 0.07^{bB}	6.74 ± 0.12^{bC}	6.20 ± 0.16^{bD}
C8	8.33 ± 0.08^{aAB}	8.58 ± 0.08^{aA}	8.08 ± 0.16^{aB}	7.00 ± 0.07^{aC}	6.90 ± 0.09^{aC}
Coagulase positive staphylococci					
O1	3.70 ± 0.07^{cA}	<1	<1	<1	<1
O2	2.33 ± 0.12^{d}	<1	<1	<1	<1
C5	5.32 ± 0.08^{aB}	6.81 ± 0.08^{aA}	3.90 ± 0.09^{C}	3.48 ± 0.10^{D}	<1
C8	4.30 ± 0.09^{b}	5.95 ± 0.10^{b}	<1	<1	<1
Coagulase negative staphylococci					
O1	4.55 ± 0.09^{cA}	4.50 ± 0.09^{cAB}	2.31 ± 0.08^{cC}	<1	<1
O2	5.00 ± 0.15^{bA}	4.11 ± 0.12^{dB}	2.45 ± 0.06^{cC}	<1	<1
C5	5.70 ± 0.09^{aA}	5.76 ± 0.08^{aA}	6.84 ± 0.11^{aB}	4.44 ± 0.12^{C}	<1
C8	5.10 ± 0.09^{bA}	5.00 ± 0.09^{bA}	4.94 ± 0.10^{bA}	2.95 ± 0.12^{B}	<1
Escherichia coli					
O1	2.10 ± 0.20^{c}	1.91 ± 0.08^{c}	<1	<1	<1
O2	<1	<1	<1	<1	<1
C5	5.71 ± 0.16^{aA}	5.71 ± 0.21^{aA}	3.63 ± 0.06^{B}	<1	<1
C8	4.20 ± 0.10^{b}	4.63 ± 0.06^{b}	<1	<1	<1

Supplementary Figure 1. Richness data of Chao1 index.



Sepplementary Figure 2. Principal Component Analysis of VOCs at T15 and T80 time of fermentation.



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GENERAL CONCLUSION

This thesis contributes to provide further insights into the field of research concerning the selection and use of starter cultures suitable for the natural fermentation of table olives, through the application of dependent and independent culture techniques. In detail, the review article provides an overview of the omics techniques currently applied to the study of the table olive microbiota. It also highlights the possibility of using an integrated approach capable of understanding in detail the dynamics and variability of this complex microbial consortium.

Chapter 1 showed how the in vitro study of selection criteria revealed that the ability to grow at different pH, salt concentration and temperature is a strain-dependent condition, and that low temperature is the parameter that most negatively affected the survival ability of strains under simulated process conditions. The comprehension of these capabilities allows the discrimination at strain level and the appropriate selection of strains tailored for fermentation processes.

Although subsequent transcriptomic and proteomic studies are required to validate the use of selected b-glucosidase-positive strains, the results of Chapter 2 showed that, through transcriptomic profiling, the designed primers were able to detect the gene encoding for the b-glucosidase enzyme directly involved in oleuropein hydrolysis. In addition, the use of mass spectrometry techniques made it possible to detect intermediate molecules (Hy-EDA) and thus to propose an alternative metabolic pathway for the formation of the final hydrolysis compounds.

The application of β-glucosidase-positive starter strains was further validated in the study reported in Chapter 3. By applying a dual approach based on culture-dependent, metagenetic and volatilomics, it was possible to detect differences in microbial varibility and VOC

profiles between table olive samples with and without the addition of starter cultures. Besides sharply reducing the pH values in the medium, making it safer, the use of starter cultures contributes to the development of pleasant compounds in the final product, as well as preserving it from being spoilage microorganisms responsible for the appearance of unpleasant substances.

Other activities

OTHER ACTIVITIES: Study Article

Effect of Sequential Inoculum of Beta-Glucosidase Positive and Probiotic Strains on Brine Fermentation to Obtain Low Salt Sicilian Table Olives

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Part of the activities of the research article were carried out during a research traineeship, under supervision of Dr. Joaquín Bautista-Gallego and Dr Noé Arroyo-López, at Instituto de la Grasa, Food Biotechnology Department, Instituto de la Grasa, Consejo Superior de Investigaciones Científicas, Universidad Pablo de Olavide, Seville, Spain.

1 Introduction

Among fermented vegetables, table olives are widespread in the Mediterranean area with increasing consumption in both European and non-European countries (International Olive Council, 2016). Olives are intrinsically health thanks to the high content of fiber, vitamins, and polyphenols which play a very important role, exhibiting pharmacological properties and antioxidants effects. In particular, hydroxytyrosol scavenges free radicals, inhibits human low-density lipoprotein (LDL) oxidation, inhibits platelet aggregation, and discloses anticancer activity by means of pro-apoptotic mechanisms (Allouche et al. 2011; Buckland and Gonzalez 2015; Raederstorff 2009). In Sicily, table olives fermentation is mainly performed under traditional methods exploiting the fermentative action of the autochthonous microbiota. Olives are directly brined without previous debittering treatment; therefore, the indigenous microorganisms and the effect of the physico-chemical conditions of brine (pH, salt, presence/absence O2, etc.) are mainly responsible of the hydrolysis of the oleuropein and of other b-glucosides. The oleuropein, a b-glucoside compound lending the strong “bitterness” aroma to the olive fruit, is hydrolyzed by b-glucosidases enzymes with the release of glucose and aglycones which are degraded, by an esterase, in the no-bitter phenols hydroxytyrosol, and elenolic acid (Bianchi 2003). The spontaneous debittering is time consuming and not predictable, and it is strongly influenced by physico-chemical parameters, by the presence of fermentable substrates, and by the autochthonous microbiota. Starter cultures with oleuropein degrading activity were extensively applied in order to reduce the debittering time and to control the fermentation process (Bevilacqua et al. 2013; Bonatsou et al. 2015; Marsilio et al. 2005; Panagou et al. 2008, 2003; Servili et al. 2006), and, among them, the use of b-glucosidase positive

strains could be promising (Ghabbour et al. 2011; Tataridou and Kotzekidou 2015). Recently, salt intake consumption hypertension and cardiovasular diseases [U.S. Dept. of Agriculture and U.S. Dept. of Health and Human Services, 2010; World Health Organization (WHO), 2012]. Recently, high attention was paid to salt intake since its overmuch intake is considered a risk factor for the onset of hypertension and cardiovascular diseases [U.S. Dept. of Agriculture and U.S. Dept. of Health and Human Services, 2010; World Health Organization (WHO), 2012]. Indeed, the setup of table olives with low NaCl content is an issue of great interest for the sector (Bautista-Gallego et al., 2013a). However, a complete removal of salt may lead to an increased risk in the survival/ growth of spoilage or food pathogen microorganisms and may also alter food flavor, causing important economic losses. Hence, several studies evaluated the use of KCl, CaCl2, and ZnCl2 as NaCl replacers. It is well-demonstrated that table olives dealt with NaCl reduction and partial substitution with other salts have a more equilibrated mineral composition, enhancing the consumers' acceptance (Bautista-Gallego et al. 2010, 2013b; Bautista Gallego et al. 2011a). Nevertheless, the effect of NaCl replaces on sensorial aspects is still controversial and strongly influenced by the concentration of the salt mixture used (Zinno et al. 2017). Recently, Pino et al. (Pino et al. 2018a) demonstrated that the reduction of NaCl content to 5%, without any NaCl replacers, did not negatively affect the Nocellara Etnea table olives fermentation, obtaining a microbiologically safe product with appreciate sensorial traits. Another challenge for vegetable product industry is to satisfy the increasing consumer demand for healthier products. Numerous studies demonstrated that table olives are promising carrier for probiotic strains being able to support their survival, probably thanks to the release of prebiotic substances from fruits. Additionally, their microstructure, in terms of roughness of olive surface, promotes the

formation of biofilm that seem to protect probiotic bacteria from stressful conditions (such as acidic environment), favoring their survival through the human gastrointestinal tract (F. N. Arroyo-López et al. 2012; De Bellis et al. 2010; Blana et al. 2014; Randazzo et al. 2017; Rodríguez-Gómez et al. 2017a) According to that, the present study was aimed to evaluate the effect of a sequential inoculum of a b-glucosidase positive strain and probiotic bacteria on brine fermentation in order to set up a low salt Sicilian table olives.

2 Materials and Methods

2.1 Pilot Scale Olives Processing

Traditionally Sicilian–style table olives from Nocellara Etnea cultivar, provided from a local company, located in Paternò (Sicily), were processed without any lye treatment. Olives were pre-treated as previously reported (Pino et al. 2018a) and directly immersed in sterilized brine, containing 5 or 8% (w/v) of NaCl. The b-glucosidase positive strain Lactobacillus plantarum F3.3, previously isolated from fermented table olives was used as starter. The strain was previously characterized for the presence of bglH gene according to Marasco et al. (Marasco et al. 1998) and its b-glucosidase activity was evaluated by enzymatic assay, according to Sestelo et al. (Sestelo, A. B. F., Poza, M., and Villa 2004). To set-up probiotic table olives, the potential probiotic *Lactobacillus paracasei* N24, belonging to the Di3A microbial collection was added. This strain was selected according to its technological and probiotic features (Pitino et al. 2010; Randazzo et al. 2010) and for its good ability to survive in table olives (Pino et al. 2018; Randazzo et al. 2017). Both microorganisms were applied as lyophilized strains. The experimental fermentation design comprised 8 treatments: 4 fermentations at 5% of NaCl with (F5A; F5B) and without (F5C; F5D) the addition of *L. plantarum* F3.3 strain, and 4 fermentations at 8% of NaCl with (F8A; F8B) and without (F8C; F8D)

the addition of *L. plantarum* F3.3. Brine samples F5A; F5B; F8A; F8B were inoculated with the *L. plantarum* starter culture, to a final cell density of 7 log cfu/ml, directly after brining. The potential probiotic L. paracasei N24 strain was inoculated in F5B; F5D; F8B; F8D samples after 60 days of brining (at final cell density of 9 log cfu/ml). All fermentations were done at room temperature (18 ± 2◦C), and followed up to 120 days. Marine salt was periodically added to maintain the initial concentration and fresh brine was supplied to keep olives totally dipped. Each fermentation was carried out in triplicate.

2.2 Physico-Chemical Analysis

The pH of the brines was detected by using a MettlerDL25 pHmeter (MettlerDL25, Mettler-Toledo International Inc.). Titratable acidity was determined by titring brine samples with 0.1N NaOH and was expressed as lactic acid (g/100ml). The olive brines were filtered through PTFE filters (Millipore, 0.45μm) and injected in the chromatographic system to analyse the phenol fraction. The HPLC instrument consisted of a chromatography Waters Alliance 2695 HPLC equipped with a Waters 996 photodiode array detector (PDA) set at 280 nm. The column used was a Luna C18 (250mm × 4.6mm i.d., 5μm, 100 Å, Phenomenex, Torrence, CA) which was maintained at 30◦C in an oven. The flow rate was 1 mL/min. Separation was obtained by elution gradient using an initial composition of 95% of A solution (2% acetic acid in water) and 5% of B solution (methanol). The concentration of B solution was increased to 30% in 15min and to 70% in 25min and then, after 2min in isocratic condition, the mobile phase was set at the same initial concentration in 8min. Phenolic compounds were identified by injecting the pure standards of oleuropein, verbascoside, tyrosol, and hydroxytyrosol and by comparing their retention time and UV-Vis spectra. All the analyses

were performed in triplicate.

2.3 Microbiological Analyses

Brine samples at 1, 30, 60, 90, and 120 days of fermentation were subjected to microbiological analysis as previously described (Pino et al. 2018a). Mannitol Salt Agar (MSA), incubated at 32◦C for 48 h, was used to isolate both coagulase positive and negative staphylococci. All media were provided from Oxoid (Italy) with the exception of YM provided from Difco (Italy). Microbiological analyses were performed in triplicate and results were expressed as log cfu/ml ± standard deviation.

2.4 LAB Isolation and Identification

For each brine sample (F5A, F5B, F5C, F5D, F8A, F8B, F8C, and F8D) and each sampling time (1, 30, 60, 90, and 120 days), 20% of the total number of colonies, recovered on MRS agar plate, were randomly selected, purified, checked for catalase activity and Gram reaction, and microscopically examined before storing in liquid culture using 20% (v/v) glycerol at −80◦C. Six-hundred (600) LAB isolates were purified and subjected to total genomic DNA (gDNA) extraction following the method described by Pino et al. (Pino et al. 2018b). gDNA concentration and quality were evaluated using the Fluorometer Qubit (Invitrogen, Carlsbad, 278 CA, USA). Multiplex RecA and Tuf gene species-specific PCR were performed as previously described (Torriani, Felis, and Dellaglio 2001; Ventura et al. 2003), respectively. Strains not identified at species level with species-specific PCR were subjected to 16S rRNA gene PCR-RFLP analysis according to Pino et al. (Pino et al. 2018b). For each PCR-RFLP cluster, the 16S rRNA gene PCR amplicon of one representative

strain was purified using the Qiaquick PCR purification kit (Qiagen Hilden, Germany) and was subjected to sequencing and Blast analysis.

2.5 Isolation and Genotypic Identification of Yeasts

From each brine samples at each sampling time, as previously reported, 200 colonies were randomly isolated from YM medium, purified, and microscopically examined prior to storing in liquid culture using 20% (v/v) glycerol at −80◦C. For the yeasts characterization, DNA was extracted according to Ruiz-Barba et al. (Ruiz-barba et al. 2005) and subjected to repetitive element palindromic (rep)-PCR analysis by using GTG5 primer. The PCR reaction was carried out in a final volume of 25 µl, containing: 5 µl of DNA, 5 µl 5X PCR Buffer, 1 µl of primer GTG5 (5-GTGGTGGTGGTGGTG-3), 13.9 µl of filtered water on 0.1 µl of Taq polymerase (Invitrogen, Italy). The amplification program was as follows; an initial denaturation (95◦C, 5min) followed by 30 cycles of denaturation (95◦C, 30 s), annealing (40◦C, 1min), and extension (65◦C, 8min) with a single final extension (65◦C, 16min). PCR products were electrophoresed in a 2 % agarose gel in 1X TAE buffer, stained with ethidium bromide (30min) and visualized under ultraviolet light. The resulting fingerprints were digitally captured and analyzed with the Bionumerics 6.6 software package (Applied Maths, Kortrijk, Belgium). Dendrogram for clustering comparison was built with UPGMA (Unweighted Pair Group Method) method and Pearson correlation. To validate the clustering analysis and for identification of strains, the 26S rDNA gene of all isolates was further sequenced. The gDNA amplification was performed according to Porru et al. (Porru et al. 2018). PCR products were resolved by electrophoresis on agarose gel (1% w/v) stained with ethidium bromide. DNA ladder plus (Invitrogen, USA) was used to evaluate the molecular weight of

amplified DNA. PCR products were purified using Isolate DNA kit (Bioline, USA) according to the manufacturer's instructions and quantified by agarose gel electrophoresis (1% w/v) in 0.5X TBE buffer (89mMTris-borate, 2mM EDTA pH 8). An amount of 10 µl of purified product with forward primer NL1 was used for sequencing by Stab Vida (Lisbon, Portugal). Nucleotide sequences were aligned with the software Molecular Evolution Genetic Analysis (MEGA).

2.6 Rep-PCR for Detecting the Presence of Probiotic L. paracasei N24 Strain

Rep-PCR genomic fingerprinting was performed on 79 L. paracasei strains, isolated from F5B, F5D, F8B, and F8D brine samples at 120 days of fermentation, using the (GTG)5-primer, as described by Versalovic et al. (1994). PCR was carried out in a 20 µl reactionmixture containing 1x Thermo Green buffer (Thermo Scientific, Waltman, MA, USA), 3.0mM MgCl2, 200µM of each dNTP (Fermentas), 1U of Taq polymerase (Thermo Scientific, Waltman, MA, USA), 2µM (GTG)5 primer and 50 ng gDNA. Amplifications were performed in a MyCycler thermal cycler (BioRad, Hercules, CA). The PCR cycling parameters and gel running conditions were set according to Solieri et al. (Solieri, Bianchi, and Giudici 2012). The only modification was the change of annealing temperature from 40 to 45◦C. The GeneRuler 100 bp Plus DNA Ladder (Thermo Scientific, Waltman,MA, USA) was used as amolecular size marker. BioDoc Gel Analyzer device (Biometra GmbH, Germany) was used to capture DNA fingerprint images which were then processed through the BioNumerics software v3.0 (Applied Maths, Sint-Martens-Latem, Belgium). Repeatability of rep-PCR was assessed using the inoculated strain N24 as internal control. Pearson's correlation similarity coefficient was chosen to calculate bands

patterns similarity matrix with optimization and curve smoothening values at 1%. Unweighted pair group method with arithmetic mean (UPGMA) analysis was exploited to build the $(GTG)_5$-based dendrogram.

2.7 Analysis of Volatile Organic Compounds (VOCs)

VOCs analysis was performed on brine samples at 1, 60, and 120 days of fermentation following method and conditions previously described (Pino et al. 2018a; Randazzo et al. 2017)) using a gas chromatography-mass-spectrometry (GC-MS). All analyses were performed in triplicate and the results were expressed as means in µg/l of brine.

2.8 Sensory Evaluation of Table Olives

Table olives at 120 days of fermentation were subjected to sensory evaluation by trained panelists (6 females and 4 males, aged from 22 to 40 years). Sensory panel was conducted according to the International Olive Council method (International Olive Council, 2011). Descriptors related to negative sensations, gustatory, and kinaesthetic perceptions were evaluated as previously described (International Olive Council, 2011, 2016; Pino, et al. 2018a). In addition, the overall acceptability descriptor, such an indication of the overall quality, was also scored. Sensory data were acquired by a direct computerized registration system (FIZZ Biosystemes. Couternon, France).

2.9 Statistical Analysis

Microbiological and chemical (i.e., single phenol compounds, pH and acidity) data were analyzed by ANOVA (One-way Analysis of Variance) using Tukey's post-hoc test, in order to assess the overall

differences among treatments. The reference level of significance was 0.01 for chemical assay and 0.05 for VOCs and microbiolgical assays. All statistical analyses were performed using MATLAB software (MathWorks, version 8.5.0), while sensory data were analyzed using the software package Statgraphics R Centurion XVI (Statpoint Technologies, INC.) setting samples as treatments. Data correlations between brine samples differently treated and VOCs were computed using XLStat software (version 2016.1).

3 Results

3.1 Physico-Chemical Data

In Table 1, the results of pH and titratable acidity detected in brine samples analyzed thought the fermentation are shown. At the beginning of fermentation, pH values ranged from 5.8 to 6.3, and then they decreased after 30 days. Differences among samples become more appreciable after 60 days of fermentation with the lowest values showed by F5B and F8C samples. At the end of fermentation (120 days) the pH fitted the hygienic limit of 4.3 in all samples (Table 1). The titratable acidity values exhibited an increasing trend in all samples with the exception of F8A and F8B brines, which slightly decreased in acidity at 120 days. The highest acidity values were detected in brines at 5% of NaCl (F5A, F5B, F5C, and F5D). In addition, a significant increase in acidity was recorded after the addition of N24 strain (90 days) mainly in F5B and F5D samples (Table 1). Results of hydroxytyrosol, tyrosol, oleuropein, and verbascoside quantification are shown in Table 2. Overall, all polyphenols analyzed showed an increasing trend during the fermentation mainly in 5% NaCl brine samples inoculated with starter culture. The highest values of hydroxytyrosol, tyrosol, and verbascoside were mainly recorded in brines inoculated with *L.*

plantarum F3.3 starter culture already at 30 days. Similar behavior was observed for oleuropein.

3.2 Microbial Count

Table 3 shown microbial counts of brine samples at both 5 and 8% of NaCl, which is expressed as log cfu/ml. Viable mesophilic bacteria showed different trend among samples. In detail, brines at 5% of NaCl (F5A–F5D), from an initial average value of 7.11 log unit, exhibited a steady trend during the fermentation with slight decrease of cell density after 60 days. Similar behavior was observed for brine samples at 8% of NaCl (F8A–F8D) which showed a mean initial value of 6.73 log unit and a final mean value of 5.56 log unit (Table 3). Regarding LAB population, all brine samples inoculated with starter culture (F5A, F5B, F8A, F8B) presented, at the beginning of fermentation, higher cell density than spontaneous ones (F5C, F5D, F8C, F8D). From60 to 120 days, LAB reached the highest values in samples inoculated with the potential probiotic N24 strain (F5B, F5D, F8B, F8D). Yeasts were present at an initial level of about 3 log cfu/mlin all experimental brines with the exception of F5A and F5B samples which exhibited initial value of 4.04 and 4.37 log cfu/ml, respectively (Table 3). The yeasts cell densities significantly increased thought the fermentation process, achieving, at 120 days, an average value of 7.37 log unit and 6.64 log unit in brine samples at 5 and 8% of NaCl, respectively. Regarding the staphylococci count, only coagulase negative staphylococci, forming red colonies in the medium, were enumerated and their level, at the beginning of the fermentation, was quite similar among all samples. After a slight increase till 60 days, a decrease to final average values of 3.0 and 3.8

Table 1. Results of pH and titratable acidity values in olive brines expressed as means and standard deviations at different time of fermentations.

	Days of fermentation				
	1	**30**	**60**	**90**	**120**
pH					
F1	5.9 ± 0.02^{ab}	4.6 ± 0.01^{bc}	4.4 ± 0.01^{bc}	4.4 ± 0.03^{c}	4.2 ± 0.03b
F2	6.3 ± 0.02^{c}	4.5 ± 0.01^{ab}	4.3 ± 0.01^{ab}	4.2 ± 0.02^{a}	4.2 ± 0.01^{b}
F3	6.1 ± 0.01^{abc}	4.4 ± 0.03^{a}	4.4 ± 0.02^{bc}	4.3 ± 0.01^{b}	4.3 ± 0.03^{b}
F4	5.9 ± 0.02^{ab}	4.4 ± 0.02^{a}	4.4 ± 0.03^{bc}	4.2 ± 0.01^{a}	4.0 ± 0.02^{a}
F5	5.9 ± 0.01^{ab}	4.6 ± 0.01^{bc}	4.5 ± 0.07^{c}	4.4 ± 0.01^{c}	4.3 ± 0.03^{b}
F6	6.1 ± 0.02^{abc}	4.7 ± 0.07^{c}	4.5 ± 0.03^{c}	4.4 ± 0.03^{c}	4.2 ± 0.02^{b}
F7	6.2 ± 0.03^{bc}	4.5 ± 0.04^{ab}	4.2 ± 0.01^{a}	4.5 ± 0.03^{d}	4.3 ± 0.04^{b}
F8	5.8 ± 0.03^{a}	4.6 ± 0.04^{bc}	4.4 ± 0.01^{bc}	4.3 ± 0.02^{b}	4.3 ± 0.08^{b}
ACIDITY (g LACTIC ACID 100/ml)					
F1	0.0225 ± 0.003^{a}	0.356 ± 0.001^{bc}	0.401 ± 0.001^{ab}	0.436 ± 0.015^{bc}	0.413 ± 0.016^{abc}
F2	0.1010 ± 0.015^{b}	0.367 ± 0.017^{c}	0.430 ± 0.007^{b}	0.526 ± 0.016^{d}	0.528 ± 0.013^{d}
F3	0.0135 ± 0.002^{a}	0.307 ± 0.007^{a}	0.368 ± 0.020^{a}	0.379 ± 0.032^{ab}	0.436 ± 0.015^{bc}
F4	0.0082 ± 0.002^{a}	0.329 ± 0.008^{ab}	0.363 ± 0.003^{a}	0.458 ± 0.016^{c}	0.458 ± 0.017^{c}
F5	0.0133 ± 0.003^{a}	0.374 ± 0.008^{c}	0.396 ± 0.007^{ab}	0.419 ± 0.008^{bc}	0.385 ± 0.007^{a}
F6	0.0067 ± 0.001^{a}	0.385 ± 0.007^{c}	0.408 ± 0.08^{ab}	0.419 ± 0.008^{bc}	0.396 ± 0.007^{ab}
F7	0.0082 ± 0.002^{a}	0.318 ± 0.008^{a}	0.372 ± 0.005^{a}	0.352 ± 0.008^{a}	0.396 ± 0.008^{ab}
F8	0.0077 ± 0.001^{a}	0.318 ± 0.008^{a}	0.363 ± 0.008^{a}	0.396 ± 0.007^{abc}	0.420 ± 0.007^{abc}

Data in the same column with different letters are significantly different at P< 0.01.

Table 2. Results of the analyzed phenols in olive brines expressed as means (mg/l) and standard deviations at different time of fermentations.

	Days of fermentation				
	1	**30**	**60**	**90**	**120**
HYDROXYTYROSOL					
F5A	4.77 ± 0.01^{c}	104.13 ± 0.78^{e}	128.14 ± 0.36^{c}	199.81 ± 0.69^{c}	267.34 ± 5.56^{f}
F5B	5.24 ± 0.11^{d}	95.68 ± 0.34^{d}	101.38 ± 3.86^{a}	166.81 ± 0.79^{b}	180.12 ± 0.63^{d}
F5C	3.14 ± 0.04^{b}	86.86 ± 0.16^{c}	112.14 ± 0.23^{b}	137.87 ± 1.38^{a}	174.32 ± 1.37^{cd}
F5D	3.25 ± 0.06^{b}	84.93 ± 0.17^{bc}	113.47 ± 0.41^{b}	151.95 ± 0.49^{ab}	160.50 ± 3.87^{ab}
F8A	4.97 ± 0.01^{c}	94.16 ± 2.28^{d}	121.63 ± 1.75^{c}	191.93 ± 0.11^{c}	192.66 ± 0.30^{e}
F8B	4.98 ± 0.02^{c}	89.57 ± 1.67^{cd}	112.21 ± 1.99^{b}	153.63 ± 0.47^{ab}	172.98 ± 0.15^{cd}
F8C	3.05 ± 0.08^{b}	56.89 ± 3.17^{a}	112.21 ± 0.49^{ab}	142.40 ± 0.47^{a}	167.71 ± 2.05^{bc}
F8D	2.54 ± 0.07^{a}	80.65 ± 0.88^{b}	102.57 ± 2.00^{a}	147.98 ± 0.69^{ab}	152.78 ± 0.90^{a}
TYROSOL					
F5A	0.0 ± 0.0	8.89 ± 0.01^{d}	10.29 ± 0.06^{b}	14.77 ± 0.80^{e}	19.56 ± 0.16^{d}
F5B	0.0 ± 0.0	8.32 ± 0.37^{d}	10.28 ± 1.12^{b}	14.17 ± 0.09^{de}	13.52 ± 0.01^{bc}
F5C	0.0 ± 0.0	7.36 ± 0.02^{c}	9.37 ± 0.05^{ab}	11.29 ± 0.16^{a}	13.62 ± 0.14^{c}
F5D	0.0 ± 0.0	7.45 ± 0.01^{c}	9.66 ± 0.02^{ab}	12.58 ± 0.04^{bc}	12.66 ± 0.30^{abc}
F8A	0.0 ± 0.0	6.79 ± 0.27^{bc}	9.35 ± 0.06^{ab}	13.56 ± 0.24^{cd}	13.51 ± 0.35^{bc}
F8B	0.0 ± 0.0	6.62 ± 0.21^{b}	8.03 ± 0.36^{a}	10.98 ± 0.03^{a}	12.08 ± 0.06^{a}

F8C	0.0 ± 0.0	4.80 ± 0.06^{a}	8.58 ± 0.08a	11.29 ± 0.04^{a}	12.57 ± 0.45^{ab}
F8D	0.0 ± 0.0	6.78 ± 0.04^{bc}	8.32 ± 0.05^{a}	11.80 ± 0.04^{ab}	11.66 ± 0.24^{a}
OLEUROPEIN					
F5A	0.0 ± 0.0	16.07 ± 2.85^{b}	21.97 ± 0.39^{e}	35.60 ± 1.61^{c}	50.62 ± 0.59^{d}
F5B	0.0 ± 0.0	15.41 ± 2.12^{b}	11.51 ± 0.71^{bc}	20.47 ± 0.93^{ab}	21.41 ± 0.18^{ab}
F5C	0.0 ± 0.0	9.08 ± 0.22^{a}	13.51 ± 0.36^{d}	19.86 ± 2.14^{ab}	23.43 ± 0.62^{abc}
F5D	0.0 ± 0.0	8.30 ± 0.11^{a}	12.01 ± 0.33^{bc}	15.09 ± 0.45^{a}	18.81 ± 1.30^{a}
F8A	0.0 ± 0.0	9.06 ± 0.51^{a}	12.26 ± 0.05^{bcd}	26.99 ± 3.80^{b}	28.48 ± 1.22^{bc}
F8B	0.0 ± 0.0	8.32 ± 0.18^{a}	9.99 ± 0.15^{a}	22.38 ± 2.03^{ab}	24.64 ± 0.50^{abc}
F8C	0.0 ± 0.0	7.55 ± 0.10^{a}	12.79 ± 0.03^{cd}	19.22 ± 0.56^{a}	31.94 ± 3.60^{c}
F8D	0.0 ± 0.0	9.13 ± 0.24^{a}	11.19 ± 0.01^{b}	18.84 ± 1.29^{a}	24.02 ± 1.27^{abc}
VERBASCOSIDE					
F5A	0.0 ± 0.0	31.69 ± 2.39^{cd}	32.36 ± 1.95^{ab}	50.73 ± 0.17^{bc}	65.39 ± 0.41^{d}
F5B	0.0 ± 0.0	31.61 ± 1.10^{cd}	28.91 ± 3.11^{a}	54.53 ± 0.63^{cd}	58.57 ± 1.78^{c}
F5C	0.0 ± 0.0	30.03 ± 0.21^{cd}	31.72 ± 0.06^{ab}	33.32 ± 0.42^{a}	44.04 ± 0.16^{a}
F5D	0.0 ± 0.0	29.41 ± 0.25^{bc}	37.59 ± 0.71^{bc}	55.40 ± 0.93^{de}	52.57 ± 0.68^{b}
F8A	0.0 ± 0.0	32.32 ± 0.91^{cd}	36.45 ± 3.38^{bc}	59.66 ± 0.90^{e}	57.66 ± 1.39^{c}
F8B	0.0 ± 0.0	33.91 ± 1.27^{d}	40.85 ± 1.22^{c}	59.75 ± 1.56^{e}	56.92 ± 1.75^{bc}
F8C	0.0 ± 0.0	18.37 ± 0.40^{a}	32.29 ± 0.23^{ab}	35.25 ± 2.31^{a}	42.53 ± 1.64^{a}
F8D	0.0 ± 0.0	25.26 ± 0.41^{b}	31.93 ± 0.18^{ab}	47.92 ± 0.81^{b}	45.75 ± 0.74^{a}

a–f: for each phenols, data in the same column with different superscript letters are significantly different $P < 0.01$.

log cfu/ml was achieved in 5 and 8% brine samples, respectively. Similar behavior was observed for enterobacteria counts, which significantly decreased through the fermentation process. At the end of fermentation (120 days) this microbial group was detected, at value below 2 log, in brine samples at 8% of NaCl and below the detection limit in samples at 5% of NaCl, with the exception of the F5D sample. *E. coli* was never detected in any brine samples analyzed (data not shown).

Table 3. Microbial counts expressed as log10 CFU/ml of 3 replicates ± standard deviation of the main microbial groups detected in experimental brine samples during the fermentation process.

Microbial groups	Days of fermentation				
	1	30	60	90	120
MESOPHILIC BACTERIA					
F5A	7.34 ± 0.03^{f}	7.14 ± 0.03^{d}	7.44 ± 0.03^{f}	6.79 ± 0.05^{e}	5.86 ± 0.04^{c}
F5B	7.28 ± 0.02^{bd}	6.99 ± 0.10^{d}	7.95 ± 0.03^{g}	7.43 ± 0.02^{f}	6.43 ± 0.01^{e}
F5C	6.90 ± 0.07^{f}	6.57 ± 0.10^{c}	6.35 ± 0.03^{d}	5.82 ± 0.03^{c}	5.63 ± 0.02^{d}
F5D	6.93 ± 0.02^{be}	6.44 ± 0.07^{c}	6.12 ± 0.06^{c}	6.15 ± 0.08^{d}	6.94 ± 0.06^{f}
F8A	6.72 ± 0.07^{acd}	5.84 ± 0.08^{ab}	5.76 ± 0.06^{b}	5.35 ± 0.03^{ab}	4.95 ± 0.03^{a}
F8B	6.87 ± 0.03^{bc}	5.72 ± 0.03^{a}	6.86 ± 0.02^{e}	6.94 ± 0.03^{e}	6.47 ± 0.09^{e}
F8C	6.58 ± 0.10^{a}	5.95 ± 0.02^{b}	5.38 ± 0.07^{a}	5.26 ± 0.05^{a}	5.21 ± 0.05^{b}
F8D	6.74 ± 0.03^{acde}	5.64 ± 0.11^{a}	5.51 ± 0.06^{a}	5.51 ± 0.08^{b}	5.61 ± 0.06^{d}
LACTOBACILLI					
F5A	7.11 ± 0.07^{eg}	7.73 ± 0.11^{h}	7.44 ± 0.06^{c}	6.92 ± 0.05^{d}	6.26 ± 0.13^{a}
F5B	7.20 ± 0.06^{fg}	7.74 ± 0.11^{h}	8.83 ± 0.07^{ef}	8.49 ± 0.09^{h}	8.29 ± 0.04^{c}
F5C	6.09 ± 0.06^{a}	6.97 ± 0.06^{cfg}	6.78 ± 0.06^{b}	6.21 ± 0.42^{ac}	6.02 ± 0.11^{a}
F5D	6.22 ± 0.06^{ab}	6.73 ± 0.08^{deg}	8.64 ± 0.10^{d}	7.86 ± 0.06^{ef}	7.16 ± 0.09^{b}
F8A	7.68 ± 0.18^{h}	6.69 ± 0.05^{bcd}	7.03 ± 0.08^{ab}	6.38 ± 0.09^{bc}	6.18 ± 0.06^{a}
F8B	7.02 ± 0.08^{def}	6.74 ± 0.01^{bef}	8.14 ± 0.09^{fg}	7.98 ± 0.09^{fg}	7.10 ± 0.10^{b}
F8C	6.59 ± 0.15^{bc}	6.25 ± 0.10^{a}	6.56 ± 0.06^{a}	6.00 ± 0.07^{ab}	5.87 ± 0.03^{a}

F8D	6.68 ± 0.07^{cd}	6.12 ± 0.05^{a}	8.58 ± 0.12^{eg}	7.63 ± 0.38^{eg}	6.94 ± 0.10^{b}
YEASTS					
F5A	4.04 ± 0.04^{de}	4.53 ± 0.05^{ce}	5.03 ± 0.04^{b}	7.22 ± 0.10^{d}	8.39 ± 0.12^{c}
F5B	4.37 ± 0.12^{e}	4.56 ± 0.10^{c}	6.80 ± 0.04^{d}	7.14 ± 0.12^{d}	7.07 ± 0.13^{b}
F5C	3.77 ± 0.10^{cd}	4.11 ± 0.14^{be}	6.12 ± 0.12^{c}	6.42 ± 0.09^{c}	7.11 ± 0.14^{b}
F5D	3.47 ± 0.10^{bc}	6.06 ± 0.08^{f}	5.79 ± 0.04^{c}	5.55 ± 0.05^{a}	6.93 ± 0.09^{b}
F8A	3.04 ± 0.04^{a}	3.16 ± 0.12^{a}	4.37 ± 0.03^{a}	5.64 ± 0.03^{a}	7.06 ± 0.12^{b}
F8B	3.05 ± 0.03^{ab}	4.03 ± 0.03^{b}	6.13 ± 0.14^{c}	6.51 ± 0.03^{bc}	6.04 ± 0.04^{a}
F8C	3.35 ± 0.14^{abc}	4.64 ± 0.04^{c}	6.06 ± 0.06^{c}	6.65 ± 0.03^{bc}	7.22 ± 0.10^{b}
F8D	3.24 ± 0.12^{ab}	3.42 ± 0.13^{a}	4.66 ± 0.13^{ab}	5.33 ± 0.03^{a}	6.24 ± 0.05^{a}
STAPHYLOCOCCI					
F5A	4.08 ± 0.07^{a}	4.40 ± 0.13^{c}	4.62 ± 0.03^{a}	4.25 ± 0.11^{c}	3.74 ± 0.20^{bc}
F5B	4.03 ± 0.09^{a}	4.70 ± 0.13^{d}	4.80 ± 0.10^{ab}	3.42 ± 0.21^{ab}	3.04 ± 0.06^{a}
F5C	4.15 ± 0.20^{a}	4.30 ± 0.24^{a}	4.41 ± 0.15^{a}	3.68 ± 0.52^{ab}	3.21 ± 0.08^{a}
F5D	4.06 ± 0.14^{a}	4.80 ± 0.05^{e}	4.79 ± 0.20^{ab}	3.27 ± 0.27^{a}	3.07 ± 0.11^{a}
F8A	4.85 ± 0.08^{b}	5.28 ± 0.22^{f}	4.83 ± 0.16^{ab}	4.81 ± 0.10^{d}	4.28 ± 0.14^{d}
F8B	4.21 ± 0.10^{a}	4.85 ± 0.07^{b}	5.03 ± 0.15^{b}	4.77 ± 0.17^{d}	3.98 ± 0.03^{cd}
F8C	4.80 ± 0.23^{b}	5.38 ± 0.10^{b}	5.01 ± 0.09^{b}	3.76 ± 0.14^{b}	3.51 ± 0.29^{ab}
F8D	4.31 ± 0.10^{a}	4.71 ± 0.24^{b}	5.08 ± 0.12^{b}	4.47 ± 0.20^{cd}	3.48 ± 0.19^{ab}
ENTEROBACTERIA					
F5A	2.66 ± 0.31^{a}	1.19 ± 0.15^{a}	<1	<1	<1
F5B	2.29 ± 0.09^{a}	1.49 ± 0.31^{a}	<1	<1	<1
F5C	2.28 ± 0.17^{a}	1.36 ± 0.41^{a}	<1	<1	<1
F5D	4.01 ± 0.11^{cd}	3.38 ± 0.29^{c}	2.18 ± 0.09^{a}	1.63 ± 0.15^{a}	1.55 ± 0.19^{a}

					Other Activities
F8A	3.31 ± 0.21[b]	3.06 ± 0.10[bc]	2.16 ± 0.15[a]	1.38 ± 0.27[a]	1.21 ± 0.15[a]
F8B	3.88 ± 0.16[bc]	3.32 ± 0.19[bc]	2.57 ± 0.23[a]	1.14 ± 0.10[a]	1.63 ± 0.14[a]
F8C	3.41 ± 0.15[bd]	2.72 ± 0.39[b]	2.00 ± 0.18[a]	1.31 ± 0.17[a]	1.27 ± 0.17[a]
F8D	3.43 ± 0.11[bd]	2.75 ± 0.11[b]	2.04 ± 0.06[a]	1.28 ± 0.20[a]	1.24 ± 0.14[a]

a–h: for each medium, data in the same column with different superscript letters are significantly different ($P < 0.05$).

3.3 Molecular Identification of LAB

Six hundred isolates from MRS agar plates were considered LAB based on their positive Gram reaction, non-motility, absence of catalase activity and spore formation, as well as rod or coccal shape. RecA and Tuf gene species-specific PCRs revealed the presence of strains belonging to *L. plantarum, Lactobacillus pentosus, L. paracasei*, and *Lactobacillus casei* species. The distribution of lactobacilli at different sampling times is reported in Figure 1A and their occurrence in the different brine samples is showed in Figure 1B. Isolates not identified at species level were indicated as "others." Evaluating the distribution of LAB strains through the fermentation, results indicated that *L. plantarum* and *L. pentosus* represented the dominant species at the beginning of the process (till 30th days) and a high occurrence of *L. paracasei* species was detected up to 60 days. Whereas, the highest occurrence of *L. casei* strains was achieved at 120 days (Figure 1A). Zooming on the fermentation at different salt content (5 and 8%) differently inoculated, results showed the dominance of *L. plantarum* strains in samples inoculated with starter, the highest occurrence of *L. casei* and *L. pentosus* in spontaneous samples (F5C, F8C, F5D, F8D) and of *L. paracasei* in samples inoculated with the probiotic strain N24 (Figure 1B). Isolates not identified initially with recA and Tuf gene primer pairs were subjected to PCR-RFLP analysis of the 16SrDNA and clustered into four different groups (data not shown). One representative isolate for each cluster was identified by 16S rRNA gene sequencing and were deposited in the GenBank database. The species attribution and the accession numbers of the sequenced strains were as follows (isolates code in parentheses): *Leuconostoc mesenteroides* MK085109 (F5C.1), *Lactococcus lacti*s MK085110 (F5B.38), *Lactobacillus brevis* MK085111 (F5D.44), *and Enterococcus faecium* MK085112 (F5A.21).

3.4 Molecular Identification of Yeasts

Two-hundred yeast isolates were randomly obtained during the fermentation process. The dendogram generated by rep-PCR with primer GTG5 showed that the isolates formed 17 groups clearly differentiated. The most numerous groups belonged to the *Wickerhamomyces anomalus* and *Candida boidinii* species, although representatives of *Candida diddensiae*, *Pichia kluyveri* and *Meyerozyma guillermondii* were also identified (Figure 2A). The evolution of the different yeast species throughout the fermentative process is presented in Figure 2B. At this regard, *W. anomalus* and *C. boidinii* formed a stable dual species consortium through the fermentation, since they were both detected more frequently than others species, with a mean frequency of 49 and 37%, respectively. Indeed, these species were dominant in all brine samples differently treated (Figure 2C). The rest of the species were isolated at very low mean frequencies; in particular, *M. guillermondii* (1.97%) was detected only in samples at 8% of NaCl till 30th days of fermentation, whereas, *P. kluyveri* (2.98%) in samples F5A, F5D, and F8D till 60 days and C. diddensiae (5.87%) was detected starting from the 30th day of fermentation only in brines at 8% of NaCl (Figure 2C).

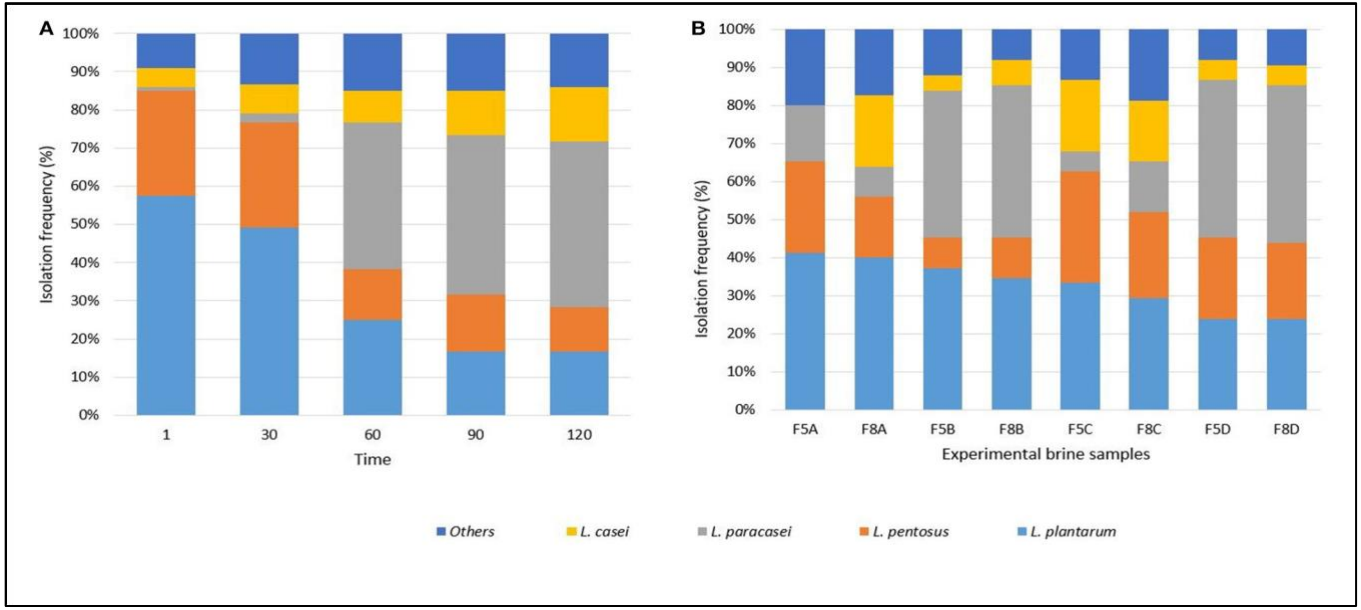

Figure 1. Isolation frequency, expressed in percentage, of LAB species throughout the fermentation (A) and in the experimental brine samples (B).

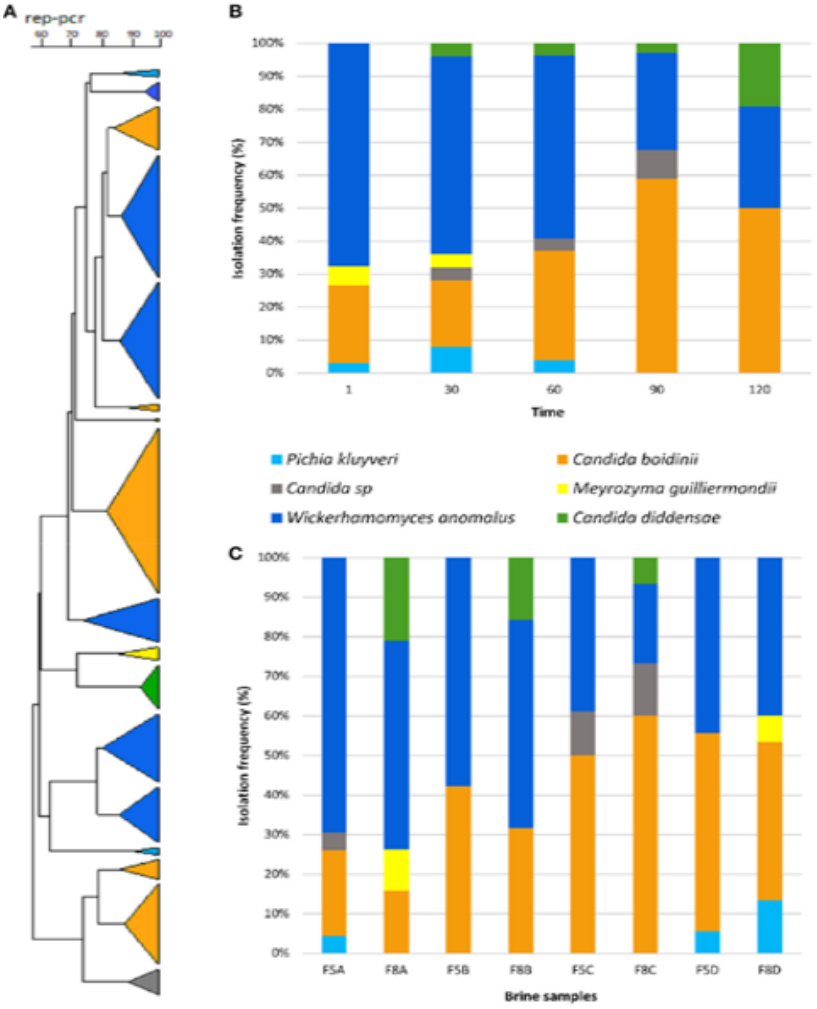

Figure 2. Dendrogram generated after bioinformatics analysis with Bionumerics 6.6 software package of the rep-PCR profiles obtained with GTG5 (A). Isolation frequency (%) of yeasts during the fermentation time of Nocellara Etnea table olives (B). Distribution of yeast species in different experimental brine samples (C).

3.5 Detection of L. paracasei N24 Strain at 120 Days of Fermentation

The presence of inoculated strain N24 was assessed at 120 days of fermentation by rep-PCR with primer GTG5 on a pool of 79 *L. paracasei* isolates from samples F5B, F5D, F8B, and F8D. Preliminarily, the rep-PCR repeatability was evaluated using gDNA from strain N24 as internal control in four different gels, obtaining a similarity of 74.3% (data not shown). Accordingly, this value was retained as similarity threshold to establish the identity of isolates compared to the rep-PCR profile of promising probiotic strain N24. The dendrogram generated using the GTG5-based patterns of *L. paracasei* isolates revealed the presence of four major clusters (from A to D) and four singleton *L. paracasei* isolates below 74.3% similarity (Figure 3; Supplementary Figure 1). The cluster analysis showed that the major cluster A grouped both the inoculated strain N24 and 48 out of 79 isolates, indicating that these isolates were assimilated to the N24 strain profile. The majority of them were isolated from samples F5B and F8B, over the indigenous L. paracasei isolates. In detail, out of 48, 18 strains were isolated from F5B, 12 from F8B, 10 from F5D, and 8 from F8D. The remaining isolates belonged to clusters B, C, and D.

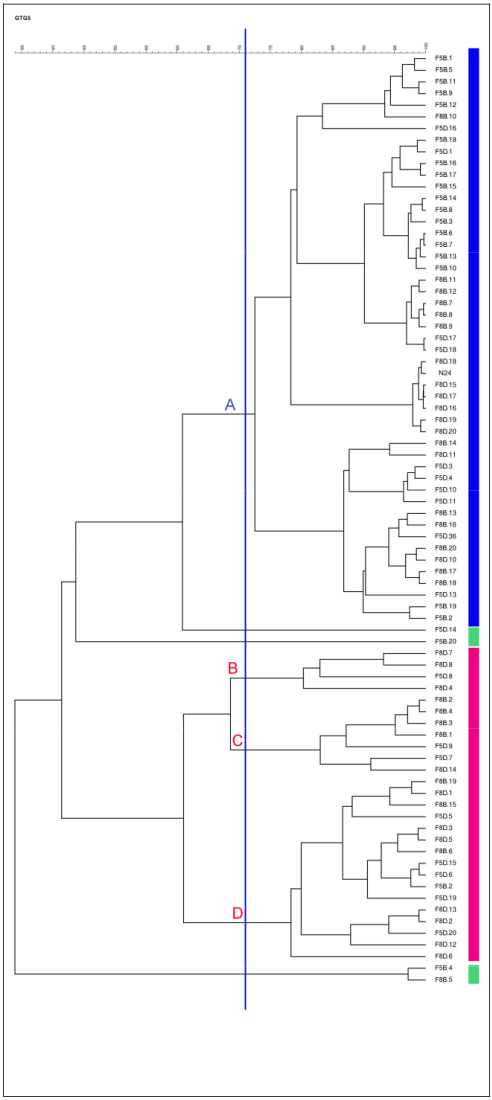

Figure 3. Dendrogram generated after cluster analysis of the digitized GTG_5-PCR fingerprints of the Lactobacillus paracasei strains isolated from F5B, F5D, F8B, and F8D brine samples at 120 days of fermentation.

3.6 Volatile Organic Compounds (VOCs)

Results of VOCs of different brine samples at 60 and 120 days of fermentation are reported in Table 4. Overall, 46 compounds as acids, alcohols, esters, aldehydes, and phenols were identified, exhibiting a growing trend through the fermentation, with the exception of samples F5A, F5D, F8A, and F8D. The highest value was registered in samples F8C, with a value of 2,739.17 µg/l. Alcohols were the most abundant compounds, followed by esters and acids, whereas aldehydes and phenols were detected at lower concentrations. Focusing on each compound, among alcohols, ethanol dominated the fermentation process showing an increase only in spontaneous samples (F5C and F8C), followed by isoamylalcohol and phenylethylalcohol, which registered a variable trend through the fermentation (Table 4). Ethyl-acetate and methyl 2-methylbutanoate were the main detected esters. Among acids, the acetic acid was the most abundant compound, with the highest value in F5C and F8C samples. The most abundant aldehydes and phenols were nonanal, benzaldehyde and creasol, respectively (Table 4). Figure 4 shows correlation between VOCs and brine samples differently treated. Overall, it is possible to point out that the salt concentration did not influence the VOCs formation through fermentation in brine samples, which were mainly grouped based on the treatment (starter and/or probiotic addition and spontaneous). In detail, samples inoculated with starter (F5A and F8A) were clustered together, showing a negative correlation with alcohol and ester compounds; spontaneous brine samples inoculated with the probiotic strain N24 (F5D and F8D) were negatively correlated to phenols, aldehydes and alcohols. Different correlations were detected for samples inoculated with both starter and probiotic strains (F5B and F8B). In particular, sample F8B at 60 and 120 days of fermentation were grouped together, exhibiting a positive correlation with alcohols and acids and a negative correlation with

Table 4. Volatile organic compounds (VOCs) expressed as µg/l of experimental brine samples at 60 and 120 days of fermentation.

Compounds	rt	T0	F5A_60	F5A_120	F5B_60	F5B_120	F5C_60	F5C_120	F5D_60	F5D_120	F8A_60	F8A_120	F8B_60	F8B_120	F8C_60	F8C_120	F8D_60	F8D_120
Acids		**0.00**	**48.46**	**92.24**	**70.48**	**150.75**	**74.29**	**124.08**	**49.57**	**40.69**	**57.77**	**35.47**	**81.06**	**111.28**	**58.14**	**135.11**	**35.60**	**50.30**
Acetic acid	24.78	0.00.^{a}	26.43.^{b}	58.74.^{e}	46.26.^{d}	77.75.^{f}	72.30.^{f}	96.15.^{g}	44.02.^{d}	39.67.^{c}	29.76.^{b}	35.47.^{c}	50.02.^{de}	62.01.^{e}	58.14.^{e}	106.25.^{h}	35.60.^{c}	34.45.^{c}
Propionic acid	29.74	0.00.^{a}	4.15.^{d}	3.62.^{d}	5.22.^{e}	32.50.^{i}	1.01.^{b}	2.31.^{c}	0.00.^{a}	1.02.^{b}	5.84.^{e}	0.00.^{a}	2.06.^{c}	0.65.^{b}	0.00.^{a}	3.14.^{d}	0.00.^{a}	0.96.^{b}
Isobutyric acid	31.43	0.00.^{a}	0.91.^{b}	0.25.^{b}	1.28.^{bc}	3.10.^{c}	0.98.^{b}	1.98.^{c}	0.00.^{a}	0.00.^{a}	1.02.^{b}	0.00.^{a}	6.56.^{d}	2.01.^{c}	0.00.^{a}	2.26.^{c}	0.00.^{a}	0.00.^{a}
Butanoic acid	35.01	0.00.^{a}	0.32.^{b}	0.62.^{b}	0.90.^{b}	4.37.^{c}	0.00.^{a}	0.15.^{b}	5.55.^{c}	0.00.^{a}	0.62.^{b}	0.00.^{a}	1.05.^{b}	10.60.^{d}	0.00.^{a}	0.96.^{b}	0.00.^{a}	0.00.^{a}
Hexanoic acid	36.19	0.00.^{a}	8.96.^{b}	27.89.^{f}	7.61.^{b}	31.99.^{f}	0.00.^{a}	13.67.^{cd}	0.00.^{a}	0.00.^{a}	10.54.^{c}	0.00.^{a}	18.21.^{e}	34.12.^{f}	0.00.^{a}	12.63.^{c}	0.00.^{a}	14.89.^{a}
2-Ethyleptanoic acid	57.20	0.00.^{a}	7.69.^{d}	1.12.^{b}	9.21.^{e}	1.04.^{b}	0.00.^{a}	9.82.^{e}	0.00.^{a}	0.00.^{a}	9.99.^{e}	0.00.^{a}	3.16.^{c}	1.89.^{b}	0.00.^{a}	9.87.^{e}	0.00.^{a}	0.00
Alcohols		**63.03**	**519.08**	**287.57**	**494.50**	**437.32**	**914.38**	**1662.74**	**276.30**	**152.56**	**539.05**	**312.90**	**962.57**	**995.99**	**960.57**	**1842.36**	**229.37**	**191.88**
Ethanol	3.33	6.77.^{a}	290.25.^{c}	151.13.^{b}	315.24.^{c}	162.54.^{b}	582.12.^{d}	1325.14.^{e}	164.48.^{b}	83.96.^{b}	305.17.^{c}	164.48.^{b}	639.44.^{d}	669.21.^{d}	615.98.^{d}	1485.32.^{e}	152.36.^{b}	125.87.^{b}
Isoamylalcohol	11.58	0.00.^{a}	79.84.^{d}	2.61.^{a}	48.91.^{c}	69.71.^{d}	78.83.^{d}	118.13.^{e}	22.14.^{b}	20.16.^{b}	83.78.^{d}	3.01.^{a}	75.12.^{d}	78.65.^{d}	78.01.^{d}	129.58.^{e}	1.03.^{a}	23.04.^{b}
1-Hexanol	19.23	0.00.^{a}	9.52.^{bc}	8.44.^{b}	12.47.^{c}	15.86.^{d}	13.15.^{c}	20.14.^{e}	13.52.^{c}	11.33.^{c}	10.03.^{c}	7.55.^{b}	18.39.^{e}	15.19.^{d}	13.84.^{c}	21.06.^{e}	3.69.^{a}	3.52.^{a}
cis Hexen 1 ol	20.84	0.00.^{a}	21.01.^{d}	22.63.^{d}	29.82.^{e}	28.56.^{e}	59.62.^{g}	83.22.^{i}	14.25.^{c}	7.86.^{b}	22.97.^{d}	31.24.^{e}	71.98.^{h}	52.12.^{f}	61.24.^{g}	89.61.^{i}	5.12.^{b}	1.14.^{a}
3-Octenol	25.13	0.00.^{a}	0.00.^{a}	0.96.^{a}	0.86.^{a}	1.99.^{b}	2.05.^{b}	2.63.^{bc}	0.00.^{a}	0.00.^{a}	0.00.^{a}	0.62.^{a}	3.92.^{d}	1.84.^{b}	1.16.^{a}	2.98.^{c}	0.00.^{a}	0.00.^{a}
1-Eptanol	25.79	2.98.^{b}	3.10.^{b}	3.54.^{b}	5.01.^{c}	5.03.^{c}	12.13.^{e}	5.14.^{c}	1.07.^{a}	0.93.^{a}	2.44.^{b}	2.58.^{b}	8.52.^{d}	3.01.^{b}	9.84.^{d}	6.23.^{c}	0.00.^{a}	0.00.^{a}
1-Octanol	31.12	19.58.^{f}	68.11.^{i}	34.99.^{h}	2.84.^{b}	11.84.^{d}	23.14.^{g}	3.21.^{c}	0.86.^{a}	0.51.^{a}	65.28.^{i}	37.37.^{h}	9.68.^{c}	8.72.^{c}	23.69.^{g}	3.02.^{b}	14.32.^{e}	0.00.^{a}
1-Nonanol	35.91	4.11.^{c}	0.00.^{a}	1.05.^{b}	0.00.^{a}	0.00.^{a}	23.96.^{d}	0.00.^{a}	0.00.^{a}	0.00.^{a}	0.00.^{a}	0.00.^{a}	0.00.^{a}	0.00.^{a}	22.10.^{d}	0.00.^{a}	0.00.^{a}	0.00.^{a}
Benzyl Alcohol	47.77	0.00.^{a}	7.42.^{c}	14.26.^{d}	9.63.^{c}	13.65.^{d}	13.68.^{d}	1.01.^{a}	4.42.^{b}	3.63.^{b}	8.12.^{c}	9.08.^{c}	29.63.^{g}	22.13.^{f}	16.54.^{e}	0.00.^{a}	0.00.^{a}	0.00
Phenylethyl alcohol	50.96	0.00.^{a}	39.83.^{c}	47.96.^{cd}	69.72.^{d}	128.14.^{ef}	102.56.^{e}	103.89.^{e}	55.56.^{d}	24.18.^{b}	41.26.^{c}	56.97.^{d}	105.89.^{e}	145.12.^{f}	114.21.^{e}	104.56.^{e}	52.85.^{d}	38.31.^{a}
1-U<1ecanol	53.54	27.49.^{c}	0.00.^{a}	0.00.^{a}	0.00.^{a}	0.00.^{a}	3.14.^{b}	0.23.^{a}	0.00.^{a}	0.00.^{a}	0.00.^{a}	0.00.^{a}	0.00.^{a}	0.00.^{a}	3.96.^{b}	0.00.^{a}	0.00.^{a}	0.00.^{a}
Esters		**4.09**	**181.46**	**200.51**	**283.60**	**421.49**	**334.35**	**448.16**	**284.96**	**363.63**	**274.22**	**358.87**	**287.75**	**433.17**	**378.62**	**579.82**	**306.71**	**308.86**
Ethyl acetate	2.75	0.00.^{a}	140.11.^{h}	145.91.^{h}	129.71.^{h}	149.52.^{h}	147.22.^{h}	248.78.^{d}	136.82.^{h}	298.72.^{e}	205.62.^{c}	244.42.^{d}	132.71.^{h}	178.12.^{c}	205.61.^{c}	325.16.^{e}	222.53	195.93
Ethyl propanoate	3.56	0.00.^{a}	5.71.^{b}	1.49.^{a}	0.00.^{a}	7.02.^{b}	0.00.^{a}	0.00.^{a}	0.00.^{a}	0.00.^{a}	7.69.^{b}	7.71.^{b}	0.00.^{a}	0.00.^{a}	0.00.^{a}	0.00.^{a}	0.00.^{a}	0.00.^{a}
Ethyl butanoate	4.07	0.00.^{a}	0.00.^{a}	2.50.^{a}	1.88.^{a}	9.02.^{c}	3.96.^{b}	1.02.^{a}	0.00.^{a}	0.00.^{a}	7.53.^{c}	0.00.^{a}	2.48.^{a}	10.86.^{c}	8.88.^{c}	0.00.^{a}	0.00.^{a}	0.00.^{a}
Methyl 2-methylbutanoate	5.44	0.00.^{a}	0.00.^{a}	8.37.^{b}	15.12.^{c}	91.13.^{g}	12.33.^{c}	61.20.^{e}	11.72.^{c}	0.00.^{a}	0.00.^{a}	22.94.^{d}	6.42.^{b}	83.25.^{g}	25.84.^{d}	70.58.^{f}	10.83.^{bc}	14.18.^{c}
Methyl 3-methylbutanoate	5.85	0.00.^{a}	0.00.^{a}	0.72.^{a}	12.09.^{e}	61.05.^{g}	1.04.^{a}	8.52.^{d}	0.00.^{a}	1.14.^{a}	0.00.^{a}	16.24.^{f}	6.23.^{c}	16.14.^{f}	2.51.^{b}	10.58.^{e}	0.00.^{a}	15.42.^{f}
Isoamylacetate	7.47	0.00.^{a}	8.52.^{c}	0.44.^{a}	8.13.^{c}	10.28.^{c}	105.31.^{i}	39.18.^{f}	96.21.^{i}	25.47.^{e}	16.18.^{d}	38.55.^{f}	5.62.^{b}	18.12.^{d}	3.46.^{b}	44.01.^{g}	54.99.^{h}	57.92.^{h}
Methyl hexanoate	9.90	0.00.^{a}	0.00.^{a}	2.43.^{a}	0.00.^{a}	0.00.^{a}	0.00.^{a}	0.21.^{a}	0.00.^{a}	0.47.^{a}	14.83.^{b}	0.00.^{a}	0.00.^{a}	0.00.^{a}	0.00.^{a}	0.00.^{a}	0.00.^{a}	0.00.^{a}
Ethyl hexanoate	12.29	0.00.^{a}	0.00.^{a}	0.46.^{a}	7.58.^{c}	4.02.^{b}	3.65.^{b}	3.68.^{b}	0.00.^{a}	0.00.^{a}	0.00.^{a}	0.00.^{a}	11.13.^{e}	9.06.^{d}	9.01.^{d}	5.12.^{b}	0.00.^{a}	0.00.^{a}
Ethyl lactate	18.49	0.00.^{a}	0.00.^{a}	6.14.^{b}	29.34.^{e}	39.42.^{f}	10.97.^{c}	31.74.^{e}	0.00.^{a}	5.11.^{b}	0.00.^{a}	0.00.^{a}	32.67.^{e}	52.36.^{h}	23.12.^{d}	45.49.^{g}	0.00.^{a}	0.00.^{a}
Methyl 2-hydroxy-3-methylbutanoate	23.10	0.00.^{a}	0.00.^{a}	0.00.^{a}	0.00.^{a}	3.96.^{b}	0.21.^{a}	7.76.^{c}	0.00.^{a}	0.00.^{a}	0.00.^{a}	11.09.^{d}	0.00.^{a}	4.05.^{b}	0.00.^{a}	8.23.^{c}	0.00.^{a}	0.00.^{a}
Ethyl octanoate	23.44	0.00.^{a}	0.00.^{a}	2.95.^{c}	1.87.^{b}	0.26.^{a}	1.48.^{b}	1.02.^{b}	0.00.^{a}	4.60.^{c}	0.00.^{a}	0.00.^{a}	3.02.^{c}	0.75.^{b}	9.09.^{d}	1.81.^{b}	0.00.^{a}	0.00.^{a}
Ethyl-3-hydroxybutyrate	28.92	0.00.^{a}	18.04.^{d}	9.20.^{c}	12.47.^{c}	6.99.^{b}	0.00.^{a}	0.80.^{a}	0.00.^{a}	8.17.^{b}	0.00.^{a}	0.00.^{a}	3.82.^{b}	6.31.^{b}	0.00.^{a}	1.63.^{b}	0.00.^{a}	10.85.^{c}
Ethyl 2-hydroxy-4-methylpentanoate	30.00	0.00.^{a}	1.50.^{a}	10.49.^{e}	6.15.^{c}	7.54.^{d}	3.96.^{b}	7.14.^{d}	9.38.^{e}	4.67.^{c}	3.60.^{b}	5.06.^{c}	11.36.^{f}	12.12.^{f}	6.77.^{cd}	14.75.^{g}	3.54.^{b}	5.60.^{c}
Methyl decanoate	32.95	2.95.^{b}	0.00.^{a}	0.00.^{a}	0.00.^{a}	0.00.^{a}	0.00.^{a}	0.00.^{a}	0.00.^{a}	0.00.^{a}	0.00.^{a}	0.00.^{a}	0.00.^{a}	0.00.^{a}	0.00.^{a}	0.23.^{a}	0.00.^{a}	0.00.^{a}
Ethyl decanoate	35.77	0.00.^{a}	0.00.^{a}	0.00.^{a}	1.24.^{b}	0.33.^{a}	1.97.^{b}	2.23.^{b}	0.00.^{a}	0.00.^{a}	0.00.^{a}	0.00.^{a}	1.68.^{b}	1.46.^{b}	1.86.^{b}	4.96.^{c}	0.00.^{a}	0.00.^{a}
Ethyl benzoate	36.74	0.00.^{a}	0.00.^{a}	0.95.^{b}	13.82.^{d}	3.68.^{c}	1.45.^{b}	1.88.^{b}	0.00.^{a}	0.00.^{a}	0.00.^{a}	0.00.^{a}	14.58.^{d}	5.02.^{c}	2.41.^{b}	5.12.^{c}	0.00.^{a}	0.00.^{a}
Acetic acid, 2 phenylethyl	43.01	0.00.^{a}	0.00.^{a}	1.68.^{b}	7.28.^{d}	2.13.^{b}	0.68.^{a}	1.63.^{b}	0.00.^{a}	0.00.^{a}	0.00.^{a}	0.00.^{a}	1.73.^{b}	2.36.^{b}	1.72.^{b}	4.16.^{c}	0.00.^{a}	0.00.^{a}
Methyl hydrocinnamate	45.91	1.14.^{b}	0.00.^{a}	3.65.^{c}	0.00.^{a}	7.61.^{e}	5.23.^{d}	2.22.^{b}	0.00.^{a}	0.00.^{a}	0.00.^{a}	0.00.^{a}	10.03.^{f}	12.13.^{f}	10.36.^{f}	5.16.^{d}	0.00.^{a}	0.00.^{a}
Ethyl dodecanoate	46.20	0.00.^{a}	0.00.^{a}	0.00.^{a}	8.36.^{c}	0.48.^{a}	0.71.^{a}	9.04.^{c}	6.03.^{b}	0.00.^{a}	8.55.^{c}	0.00.^{a}	12.12.^{d}	1.58.^{a}	1.12.^{a}	21.85	0.00	0.00
Ethyl hydrocinnamate	48.97	0.00.^{a}	7.58.^{c}	3.12.^{b}	28.56.^{i}	17.05.^{g}	34.18.^{m}	20.11.^{h}	24.80.^{i}	15.28.^{f}	10.22.^{cd}	12.86.^{e}	32.15.^{m}	19.48.^{h}	66.86.^{n}	10.98.^{d}	14.82.^{f}	8.96.^{c}

Aldheydes		**149.90**	**87.40**	**62.52**	**36.83**	**72.14**	**44.29**	**50.71**	**32.71**	**1.40**	**120.28**	**117.41**	**53.09**	**34.44**	**42.84**	**71.32**	**45.34**	**16.55**
Octanal	14.81	14.12.^{e}	10.58.^{d}	6.70.^{c}	6.52.^{c}	5.98.^{bc}	6.48.^{c}	5.12.^{b}	0.00.^{a}	0.61.^{a}	9.21.^{d}	10.64.^{d}	13.58.^{e}	5.86.^{b}	4.47.^{b}	9.36.^{d}	0.00.^{a}	0.00.^{a}
Nonanal	20.78	65.32.^{i}	40.78.^{h}	4.25.^{b}	11.93.^{d}	31.85.^{g}	14.56.^{d}	18.17.^{e}	13.25.^{d}	0.00.^{a}	32.76.^{g}	52.07.^{i}	19.63.^{e}	9.86.^{c}	14.02.^{d}	25.12.^{f}	24.46.^{f}	1.76.^{b}
3-Octanal	21.64	0.00.^{a}	0.00.^{a}	1.90.^{b}	1.58.^{b}	1.96.^{b}	2.36.^{b}	2.36.^{b}	1.74.^{b}	0.79.^{a}	0.00.^{a}	26.47.^{e}	2.56.^{b}	1.02.^{a}	2.36.^{b}	2.15.^{b}	12.78.^{d}	5.27.^{c}
Decanal	26.85	68.82.^{h}	30.75.^{f}	41.89.^{g}	6.78.^{b}	18.44.^{d}	9.57.^{c}	9.99.^{a}	7.46.^{b}	0.00.^{a}	24.09.^{e}	0.00.^{a}	5.69.^{b}	5.68.^{b}	11.00.^{c}	18.96.^{d}	0.00.^{a}	7.47.^{b}
Benzaldehyde	28.08	1.64.^{b}	5.29.^{c}	7.78.^{d}	10.02.^{e}	13.91.^{f}	11.32.^{e}	15.07.^{f}	10.26.^{e}	0.00.^{a}	54.22.^{h}	28.23.^{g}	11.63.^{e}	12.02.^{e}	10.99.^{e}	15.73.^{f}	8.10.^{d}	2.05.^{b}
Phenols		**0.00**	**102.59**	**49.90**	**21.94**	**144.69**	**116.02**	**146.22**	**26.95**	**72.45**	**137.38**	**147.21**	**7.80**	**43.19**	**112.46**	**110.56**	**5.61**	**2.24**
Guaiacol	47.25	0.00.^{a}	0.00.^{a}	2.99.^{b}	0.00.^{a}	29.08.^{d}	57.16.^{e}	61.12.^{f}	12.85.^{c}	3.58.^{b}	0.00.^{a}	0.00.^{a}	0.00.^{a}	11.02.^{c}	56.23.^{e}	53.01.^{e}	0.00.^{a}	0.00.^{a}
Creosol	52.57	0.00.^{a}	102.59.^{h}	43.69.^{e}	10.50.^{b}	98.65.^{g}	17.89.^{bc}	44.32.^{e}	12.54.^{a}	52.36	95.11	106.62	3.16	23.14	19.67	28.15	0.00	0.00
Phenol	55.36	0.00.^{a}	0.00.^{a}	0.63.^{a}	1.33.^{b}	3.56.^{c}	5.69.^{d}	9.75.^{e}	1.56.^{b}	2.58.^{c}	0.00.^{a}	0.00.^{a}	0.23.^{a}	1.02.^{b}	4.50.^{d}	10.42.^{e}	0.00.^{a}	0.00.^{a}
4-Ethyl phenol	63.20	0.00.^{a}	0.00.^{a}	2.59.^{b}	10.11.^{d}	13.40.^{e}	35.28.^{g}	31.03.^{h}	0.00.^{a}	13.93.^{e}	42.27.^{h}	40.59.^{h}	4.41.^{c}	8.01.^{d}	32.06.^{g}	18.98.^{f}	5.61.^{c}	2.24.^{b}
Total		**217.02**	**938.99**	**692.74**	**907.35**	**1226.39**	**1483.33**	**2431.91**	**670.49**	**630.72**	**1128.70**	**971.86**	**1392.27**	**1618.07**	**1552.63**	**2739.17**	**622.63**	**569.83**

a-h for each compound different superscript letters in the same raw are significantly different (P<0.05)

Table 5. Sensory data obtained for the evaluation of fruits in the different treatments assayed.

Samples	Descriptors													
	Abnormal fermentation							Gustatory sensations			Kinaesthetic sensations			Overall acceptability
	Musty	Rancid	Cooking Effect	Soapy	Metallic	Earthy	Winey-Vinegary	Acidity	Saltiness	Bitterness	Hardness	Fibrousness	Crunchiness	
F5A	1.1±0.18^{a}	0.6±0.29^{a}	0.6±0.22^{a}	0.2±0.14^{a}	0.6±0.26^{a}	1.6±0.12^{a}	1.3±0.24^{a}	2.7±0.18^{a}	5.4±0.11^{a}	2.3±0.09^{a}	6.4±0.24^{b}	2.1±0.06^{a}	7.2±0.26^{a}	7.5±0.04^{a}
F5B	1.1±0.37^{a}	0.5±0.43^{a}	0.6±0.16^{a}	0.3±0.11^{a}	0.8±0.21^{a}	1.5±0.29^{a}	1.2±0.28^{a}	2.5±0.26^{a}	5.3±0.33^{a}	2.0±0.14^{a}	6.0±0.21^{a}	2.8±0.45^{a}	7.5±0.19^{a}	9.8±0.41^{b}
F5C	1.3±0.21^{a}	0.6±0.34^{a}	0.8±0.24^{a}	0.4±0.21^{a}	0.6±0.09^{a}	1.6±0.26^{a}	1.0±0.31^{a}	7.9±0.12^{b}	5.4±0.25^{a}	6.7±0.24^{b}	5.9±0.33^{a}	2.2±0.28^{a}	7.6±0.21^{a}	6.9±0.11^{a}
F5D	1.1±0.38^{a}	0.5±0.26^{a}	0.7±0.26^{a}	0.3±0.04^{a}	0.7±0.19^{a}	1.6±0.61^{a}	0.8±0.21^{a}	2.9±0.21^{a}	5.8±0.34^{a}	6.9±0.29^{b}	6.3±0.25^{a}	2.5±0.36^{a}	7.7±0.28^{a}	6.7±0.32^{a}
F8A	1.1±0.26^{a}	0.4±0.22^{a}	0.5±0.18^{a}	0.3±0.04^{a}	0.5±0.24^{a}	1.4±0.58^{a}	1.2±0.39^{a}	2.7±0.61^{a}	5.4±0.10^{a}	2.5±0.09^{a}	6.2±0.18^{a}	2.7±0.29^{a}	7.6±0.35^{a}	7.6±0.09^{a}
F8B	1.3±0.24^{a}	0.6±0.28^{a}	0.8±0.23^{a}	0.2±0.35^{a}	0.5±0.22^{a}	1.4±0.29^{a}	0.9±0.15^{a}	2.6±0.86^{a}	5.6±0.06^{a}	2.6±0.32^{a}	5.9±0.24^{a}	2.1±0.43^{a}	7.5±0.32^{a}	9.3±0.45^{b}

Other Activities

F8C	1.4±0.27[a]	0.4±0.21[a]	0.5±0.26[a]	0.4±0.25[a]	0.7±0.13[a]	1.5±0.54[a]	1.3±0.44[a]	8.8±0.37[c]	5.7±0.21[a]	7.3±0.43[b]	6.2±0.05[a]	2.7±0.51[a]	7.6±0.33[a]	6.4±0.42[a]
F8D	1.4±0.32[a]	0.3±0.35[a]	0.7±0.28[a]	0.4±0.23[a]	0.8±0.31[a]	1.4±0.61[a]	1.3±0.43[a]	2.9±0.53[a]	5.7±0.34[a]	7.2±0.32[b]	6.4±0.09[a]	2.5±0.44[a]	7.8±0.31[a]	6.8±0.38[a]

phenols and aldehydes (Figure 4). Evaluating sample F5B, it is possible to assert that VOCs formation was strongly influenced by the fermentation time. In fact, the sample F5B at 120 days revealed a distinct VOCs profile, displaying a positive correlation with esters, acids, and phenols. Similarly, spontaneous fermentation samples (F5C and F8C) were grouped based on fermentation time, showing a divergent VOCs profile through the fermentation.

3.7 Sensory Data

Table 5 shown results of sensory analysis. Overall, none negative sensation was perceived, as deduced by the low scores attributed by panelist to these descriptors. No statistically significant differences were achieved among samples for hardness, fibrousness, and crunchiness. Among gustatory descriptors, higher scores for acidity were attributed to uninoculated brine samples at both 5 and 8% of NaCl (F5C and F8C), while higher bitterness score was observed in samples without the addition of the b-glucosidase *L. plantarum* strain (F5C, F5D, F8C, and F8D). Finally, F5B and F8B samples received higher scores for the overall acceptability descriptor.

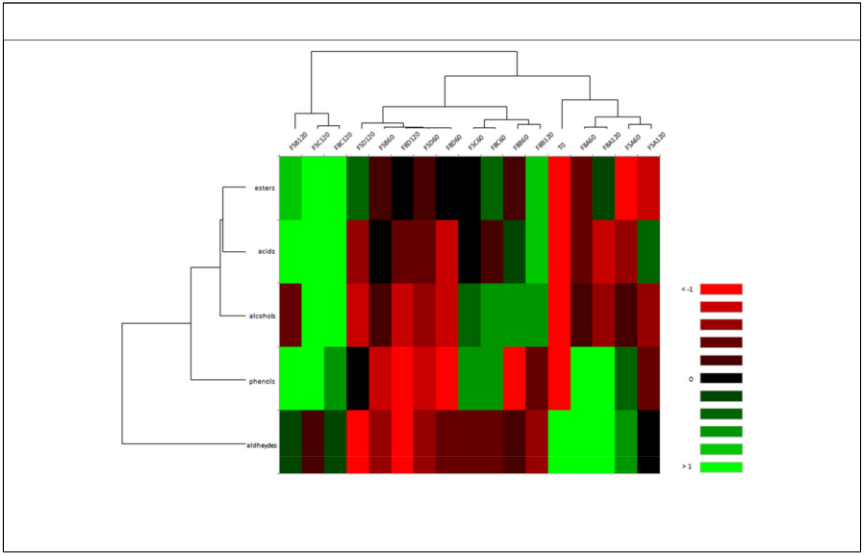

Figure 4. Heat map of correlation between VOCs and F5 (A-D) and F8 (A-D) brine samples at 60 and 120 days of fermentation. The colors of the scale bar denote the nature of the correlation, with 1 indicating a perfectly positive correlation (green) and −1 indicating a perfectly negative correlation (red) between VOCs and brine samples.

4 Discussion

A current challenge in the processing technology of table olives is the selection of starter cultures able to fasten and safely drive the fermentation process. In contrast to industrial starter cultures, autochthonous strains, that naturally dominate spontaneous fermentation, tend to have high metabolic capacities, which can beneficially affect the quality of the final product. In addition, it was already established that the microbial dynamics through fermentation is influenced by the technology applied (e.g., salt reduction). One of the most widely employed strategies to reduce sodium content in table olives is the use of NaCl substitutes, which can be added alone or in combination with other salts (Bautista-Gallego et al. 2013a). Few studies have evaluated the possibility to setup low NaCl table olives without any salt replacement. Based on our previously reported data (Pino et al. 2018a; Randazzo et al. 2017), in the present study a wild b-glucosidase positive strain was used both as debittering and as driven agent during olive fermentations at lowered salt content (5%). It is well-known that b-glucosidase enzyme is important for oleuropein hydrolysis and, among lactobacilli, *L. plantarum* species has been successfully used as starter for its strong ability to break the glycosidic bond of oleuropein (Ciafardini et al. 1994; Tataridou and Kotzekidou 2015) and for its high versatility. Overall, our data revealed that all brine samples reached a pH value ≤4.3 and exhibited a good acidification rate, indicating the success of the fermentation and ensuring the microbiological safety of the final product, in accordance to other researches (Corsetti et al. 2012; Martorana et al. 2017). In particular, samples inoculated with the b-glucosidase positive strain exhibited a more pronounced reduction of the fermentation time, with a higher content of hydroxytyrosol, tyrosol, and verbascoside compounds from 30 days of fermentation than un-

inoculated ones, according to other studies (Kaltsa et al. 2015; Othman et al. 2009; Pistarino et al. 2013; Romero et al. 2004). In addition, the autochthonous strain exhibited a better adaptation/growth rate in brine samples at 5% of salt. It is well-established that autochthonous strains are generally more adapted to harsh conditions of raw material than allochthonous ones, and, therefore, to dominate the microbiota, driving the fermentation and counteracting spoilage microorganisms (Bevilacqua et al. 2013; Di Cagno et al. 2008). Microbiological data indicated a significant reduction of Enterobacteriaceae starting from 30 days of fermentation, with an improvement of the safety of the final product, as previously reported (Randazzo et al. 2018a). It is interesting to point out that results revealed a high occurrence of yeasts as part of table olive natural microbiota, coexisting with LAB during the whole fermentation process (Arroyo-López et al. 2008; Arroyo-López et al. 2012a,c), which could be related to the geographic area, and cultivars (Bleve et al. 2014). Our data registered an unusual yeast count at the end of the process, higher than Spanish style and Sicilian-style table olives, which generally reached value of 4-5- log CFU/ml. This could be linked to the processing technology applied in the present study. Yeasts flavor bacteria growth, enhancing lactic acid production to inhibit spoilage microorganisms and affect flavor and texture of the final products (Arroyo-López et al. 2008, 2012b; Bevilacqua et al. 2013). As reported in a recent review, Candida boidinii, Debaryomyces hansenii, and Pichia membranifaciens were revealed as the most geographically diffused species (Campus et al. 2018). Evaluating yeast behavior, although it is noteworthy that yeast development is related to high salt level and phenolic compounds or low pH. In the present study, W. anomalus, and C. boidinii were the species mainly detected in brines processed at 5% of salt, whereas C. diddensae and M. guilliermondii were mainly revealed in brines at 8% NaCl. Several studies reported strong b-glucosidase activity for W.

anomalus species (F. N. Arroyo-López, Romero-Gil, et al. 2012; Bautista-Gallego et al. 2011; Bonatsou et al. 2015; Romero-Gil et al. 2013), and strong lipase and esterase activities for C. boidinii species, which positive impacts to fruity and olive flavor (F. N. Arroyo-López, Romero-Gil, et al. 2012; Bautista-Gallego et al. 2011; Hernández et al. 2007; Pereira et al. 2015). In contrast to previously published data (Bautista-Gallego et al. 2011b; Hurtado et al. 2008), C. diddensae, which is generally associated to the early stage of fermentation, was detected at the highest frequency at 120 days. Focusing on LAB population, *L. plantarum*, L. pentosus, and L. paracasei were the main species found in all brine samples, confirming both their key role in table olive fermentation and biofilm formation with yeasts. In addition, a high survivability of the potential probiotic L. paracasei N24 strain was depicted in the final products, mainly in samples at 5% of NaCl. This evidence confirms its suitability to growth in harsh environment, such as brines, and that table olives are able to support probiotic survival (Lavermicocca et al. 2005; Pino et al. 2018a; Rodríguez-Gómez et al. 2017). In fact, nutrients and prebiotics released into the brines flavor the biofilm formation, protecting bacteria from acidic environment and enhancing their passage through human gastrointestinal (GI) tract (F. N. Arroyo-López et al. 2012; De Bellis et al. 2010; Blana et al. 2014; Grounta, Doulgeraki, and Panagou 2015; Ranadheera, Baines, and Adams 2010; Rodríguez-Gómez et al. 2014a,b, 2017). It is interesting to point out that the addition of the potential probiotic strain at 60 days of fermentation in brine samples processed at low salt content and with starter, significantly modified the VOCs pattern. In particular, compounds responsible for floral and fruity notes, such as phenylethyl alcohol and methyl 2-methylbutanoate, highly increased, while ethanol and isoamyl-alcohol significantly deceased compared to un-inoculated samples. The high content of alcohols in un-inoculated brine samples

could be related to yeast metabolic activities (Bleve et al. 2014; Randazzo et al. 2017). This evidence was in accordance to sensory data since panelists attributed the higher score to the bitterness and acid descriptors in un-inoculated samples. Finally, data obtained from correlation between VOCs and brine samples differently treated revealed that the VOCs formation was mainly influenced by the starter and/or probiotic addition instead of salt content.

5 Conclusion

The effects of a sequential inoculum of b-glucosidase positive and potential probiotic strains on the fermentation of Sicilian table olives were investigated. Remarkably, results demonstrate that the technology applied, based on the sequential inoculum and the brines fermentation at low salt content, without any salt replacement, did not increase the risk of microbial spoilage, nor the overgrowth of foodborne pathogens. Indeed, the composition and the dynamics of brine microbiota, mainly constituted by LAB and yeasts consortium, significantly affected the composition of the VOCs and the sensorial traits of the final products, which were confirmed by a panel of trained assessors. Hence, the results of the present study are promising, suggesting the possibility to formulate table olives with reduced salt content.

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OTHER ACTIVITIES: Review Article

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

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1 Introduction

Olive growing is spread over 10 million and 800 thousand hectares in the world, 97% of which are concentrated in the Mediterranean area, where olive tree (Olea europaea L.) has always taken a central role in population life. Olive oil is one of the oldest food 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 (FaoStat, 2021). 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 (Roig, Cayuela, and Sánchez-Monedero 2006). The worldwide production of OMWW is estimated around 6x106 m3 and the 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 m3 of OMWW and 30 million m3 in the Mediterranean basin (Casa et al. 2003; Rinaldi, Rana, and Introna 2003). In recent years, technological innovations in the olive oil extraction have affected the whole supply chain, impacting the composition of OMWW, primarily composed by vegetation water, and water added both during malaxation and during pressing. In details, 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 presses consists of a mixture of olive juice and added water and residual oil. Finally, olive oil is separated from water by vertical centrifugation or decanting. The traditional process

is actually applied almost only in small olive 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 (Dermeche et al. 2013; Fernández-Bolaños et al. 2006). The two-phase system does not require the addition of water, other than during horizontal centrifugation, and results in olive oil and semi-solid olive cake. The three-phase decanter process requires the addition of hot water 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 less amount of olive cake but a significant higher production of OMWW. The management of liquid wastes in olive mills has always been challenging, and extensive efforts have been done to find an effective strategy. Nevertheless, the disposal of OMWW in soil or waterway continues to represent still a serious issue for Mediterranean countries due to its severe phytotoxicity and antimicrobial properties that can compromise the ecological balance, with a long-term environmental detrimental 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, various systems 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 (EU Directive 2018/815), “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 considered waste, introducing the concept of by-product, which is described in Article 183 bis of the Legislative Decree n. 152/06 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 presents in olives is found in OMWW (53%) and OP (45%), with only the 2% of initial content remaining in virgin olive oil (Di Nunzio et al. 2020). The phenolic compounds present in OMWW are hydroxytyrosol, tyrosol, verbascoside, acids (such as caffeic, gallic, vanillic and syringic) and polymeric substances (D'Antuono et al. 2014; Obied et al. 2009). 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 membrane processes, such as microfiltration, ultrafiltration, nanofiltration and reverse osmosis.

Therefore, OMWW could be considered as a potential low cost starting matrix for extraction of antioxidants to be applied in several fields, included food industry, where they could be used for both fortifying and prolonging the shelf-life of final products (De Marco et al. 2007; Obied, Prenzler, and Robards 2008; Zbakh and El Abbassi 2012). In the present work, the literature survey was carried out taking into account a fixed timeline, between 1996 and 2020, and the keyword "olive mill wastewater". Searching on ScienceDirect, 794 records were found. Most of them falls within the scope of environmental science, such as chemical engineering, energy fuels and agricultural, with a quite constant increasing numbers in the last years, from 2 papers published in 1992 to 54 in the last five years (Fig. 1a). To confirm the increasing interest in biotechnologic approach of OMWW treatment, 298 records have been found in the field of biotechnology and microbiology (Fig. 1b, 1c) (WOS, 2021).

The aims of this review is to provide a summary of updated information on research that has been conducted in using OMWW as renewable raw materials to generate high added-value ingredients/products for agro-food industries, including functional food sector.

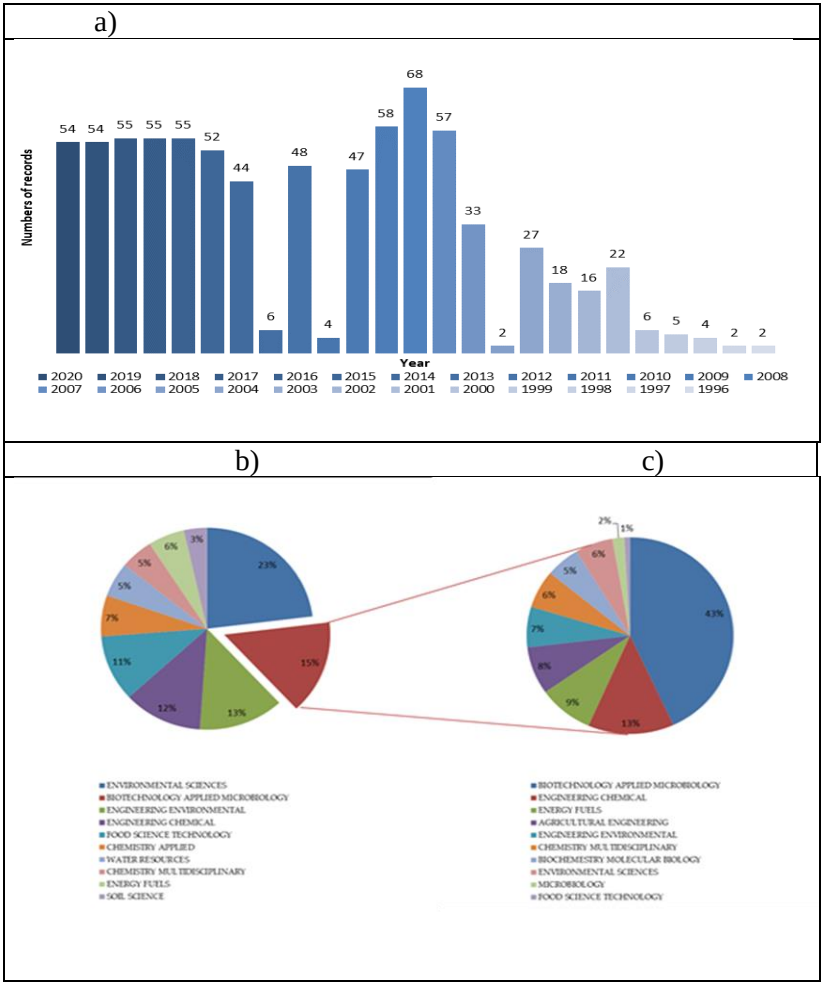

Figure 1. a) Records on olive mill wastewater found on Pub med; b) Distribution in different application areas of general records on OMWW; c) Specific records on OMWW focused on applied biotechnology and microbiology.

2 Characteristics of OMWW

2.1 Sensory Evaluation of Table Olives

The OMWW is a mixture of vegetation water and soft tissues (mucilage, pectin) of olive fruits and water used in the various stages of extraction process, i.e., water added during centrifugation, and water from equipment washing (Paredes et al. 1999). The physico-chemical traits of OMWW are strongly influenced by soil and climate conditions of growing area, olive cultivar, ripeness state and, above all, by the oil extraction system. The OMWW are dark, as far as black, and characterized by a typical, rather intense, odor. Due to the content of organic acids, namely malic and citric acids, they present 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 to other organic wastes, OMWW presents a higher concentration of potassium and considerable levels of nitrogen, phosphorus, calcium, magnesium and iron (Peri, 2014), deriving from the contact with oil during the extraction phase, and due to the high hydrophilic nature of phenols (Rodis, Karathanos, and Mantzavinou 2002).

2.2 Microbiological traits of OMWW

The microbial community presents in OMWW is strongly influenced by several parameters, among which the ripeness state and the olive variety are the most influent (Kavroulakis and Ntougias 2011; Tsiamis et al. 2012). The microbial density in OMWW varies between 10^5 and 10^6 (coloy forming unity: CFU) CFU/mL and it is mainly composed of yeasts, bacteria and moulds (Bleve et al. 2011; Kavroulakis and Ntougias 2011; Ben Sassi et al. 2008; Tsiamis et al. 2012)]. Yeast population includes species belonging to *Pichia*, *Candida*, and *Saccharomyces* genera (Bleve et al. 2011; Ben Sassi et

al. 2008). 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* (Ntougias, Bourtzis, and Tsiamis 2013).

Table 1. Physico-chemical characteristics of OMWW, adapted by Demerche et al., (2013)

Parameters	Values	Reference
pH	2.2 - 5.9	(Akar et al. 2009; Baeta-Hall et al. 2005)
Water (%)	80 - 96	(Pisante et al. 2009)
Chemical oxygen demand (g/L)	30 - 320	(Al-Malah et al. 2000; Caporaso et al. 2018; Galiatsatou et al. 2002; Niaounakis et al. 2006)
Biological oxygen demand (g//L)	35 - 132	(Al-Malah et al. 2000; Caporaso et al. 2018; Niaounakis et al. 2006)
Dry matter (%)	6.3 - 7.2	(Sierra et al. 2001; Vlyssides, Loizides, and Karlis 2004)
Ash (%)	1.0	(Lafka et al. 2011; Martín García et al. 2003; Sierra et al. 2001)
Electrical conductivity (ds/m)	5.5 - 10	(Baeta-Hall et al. 2005; Paredes et al. 1999)
Organic matter (%)	57 – 62	
Total carbon (%)	2.0 - 3.3	(Garcia-Castello et al. 2010; Di Giovacchino et al. 2001; Sierra et al. 2001)
Total nitrogen (g/L)	2.0 – 2.4	(Pisante et al. 2009)
Total sugar (g/L)	5.0-12.0	(García García et al. 2000; Paredes et al. 1999; Sierra et al. 2001; Vlyssides et al. 2004; Pisante et al. 2009)
Total fat (%)	1.0-23	(Alburquerque et al. 2004)
Total suspended solids (g/L)	25 – 30	(Azbar et al. 2004; Fiestas Ros De Ursinos and Borja-Padilla 1996)
Polyalcohol (%)	9.0 - 15	(Alburquerque et al. 2004; Pisante et al. 2009; Khoufi, Hamza, and Sayadi 2011)
Total phenols (g/L)	0.5 – 6.1	(Dermeche et al. 2013; Lafka et al. 2011; Obied et al. 2005; Sierra et al. 2001; Vlyssides et al. 2004; Tsagaraki et al. 2007;Yangui et al. 2009)

Although many studies report that the culturable microbial population is represented by only few bacterial communities, such as: *Firmicutes, Actinobacteria, Alphaproteobacteria, Betaproteobacteria,*

Gammaproteobacteria, recently, microarray analyses have revealed high-density of a larger microbial population, including *Proteobacteria, Bacteroidetes, Chloroflexi, Cyanobacteria* and *Actinobacteria*. Howewer, the most commonly reported microbial communities, representing the 50% of the 16S rRNA gene sequences deposited in GenBank, includes *Gammaproteobacteria (Enterobacteriaceae, Moraxellaceae, Xanthomonadaceae* and *Pseudomonadaceae*) with a percentage of almost 30%, and *Betaproteobacteria (Oxalobacteraceae* and *Comamonadaceae*) with a percentage of 21.5% (Ntougias et al. 2013). The *Alphaproteobacteria* and the *Actinobacteria (Micrococcaceae, Microbacteriaceae,* and *Propionibacteriaceae)*, together covered the 20%, whereas the *Firmicutes* (*Bacillaceae, Clostridiaceae, Lactobacillaceae,* and *Paenibacillaceae*) and the *Bacterioides (Prevotellaceae, Porphyromonadaceae,* and *Sphingobatteriaceae)* phyla, accounted for approximately the 6.8%, respectvely. Furthermore, differences in microbial population have been detected, highlighting that only the 15% of operational taxonomic units (OTUs) are commonly detected (El-Abbassi et al. 2017). 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 (Venieri et al. 2010).

3 Resuse 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 resulting in a reduction

of cost management and environmental impact.

According to Tsagaraki and co-workers (2007), 1 m3 of OMWW corresponds to 100-200 m3 of domestic wastewater. The COD and BOD5 values of OMWW are very consistent and even higher when obtained by conventional system (150 g O2/L COD and 90 g O2/L BOD5 versus 90 g O2/L COD and 30 g O2/L BOD5 for conventional and two-phase extraction system, respectively).

According to the European Directive 2000/60/CE, 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, as altering the color of natural water sources and exercises toxic effects on aquatic life and soil quality. OMWW is characterized by a high content in components with low biodegradability (e.g., long-chain fatty acids, lipids, 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 (Caporaso et al. 2018).

A plethora of physico-chemical 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 colour, 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 like 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 metals and mineral salt contents (Ahmed et al. 2019; Said-Pullicino and Gigliotti

2007). In addition to traditional settling (conducted in tanks called "hell"), various treatments have been proposed: physico-chemical, biological, or a combination of them. Physico-chemical systems include different methods, based on the use of flocculant, coagulant, membrane filtration and reverse osmosis (Paraskeva et al. 2007) or applying oxidation cryogenesis, electrocoagulation (Adhoum and Monser 2004; Ochando-Pulido et al. 2017) or photochemical system (Cermola et al. 2004). 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 (Belaqziz et al. 2016). 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 (Ahmed et al. 2019). 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 (Hachicha et al. 2009; Hamdi 1991). The main interest in anaerobic digestion is the production of energy and reuse of the effluent for irrigation purposes (Koutsos et al. 2018). However, the leading limitation is the inhibition of methanogenic bacteria by both phenolic and organic acids compounds (Hamdi 1996). According to Azbar and co-workers (Azbar et al. 2004), anaerobic filters or upflow anaerobic sludge bed reactors are suitable systems to remove unwanted compounds from OMWW. Filidei et al. (2003) 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 both a certain biostatic and phytotoxic effects. Aerobic treatment has been also 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 (Tomati et al. 1991; Ramos-Cormenzana et al. 1996; Scioli and Vollaro 1997). 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 added with 6.0 g/L of (NH4)2SO4 at 10°C (Sinigaglia et al. 2010). OMWW has been proposed (Ehaliotis et al. 1999; Piperidou et al. 2000) 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 integrating pest management programs. Several studies have reported the use of microorganisms (as single or consortia) to degrade organic compounds in effluents (Cerrone et al. 2011; Maza-Márquez et al. 2017). 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. (Aissam, Penninckx, and Benlemlih 2007) treated OMWW with microrganismisms 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. (Bleve et al. 2011) 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. (2017) demonstrated that the use of a microalgal-bacterial consortium, in a photo-bioreactor, induces a decrease in pollutant load, by affecting COD, BOD5, 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, leding to their decomposition. In addition, the effect of *Lactiplantibacillus plantarum* strains on decolorization and biodegradation of phenolic compounds has evaluated (Maza-Márquez et al. 2017), highlithing 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 in a significant reduction in total phenols (Lamia and Moktar 2003). 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.

Futhermore, OMWW has been also proposed for biopesticide and compost production. The OMWW application on soil and crops resulted in a growth suppression of most of phytopathogens bacteria and 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 (El-

Abbassi et al. 2017).

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 (Ntougias et al. 2013) demonstrated that ligninolytic fungi are a useful source of phenoloxidase, polyphenoloxidase and peroxidase usefull 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 (Romo-Sánchez et al. 2010; Sinigaglia et al. 2010). Several yeasts have been described as able to reduce phenolics and sugars present in OMWW, although white-rot fungi appear to contribute more in discoloration (Sassi et al. 2010). Moreover, Giannoutsou and co-workers (Giannoutsou et al. 2004) 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 (Mann et al. 2010; Millan et al. 2000). 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 (Fenice et al. 2003; Koutrotsios et al. 2016; Zerva et al. 2017). Filamentous fungi, such as Aspergillus oryzae, A. niger, Aspergillus ibericus, Aspergillus uvarum, G. candidum, Rizhopus oryzae, Rhizopus arrhizus, and Penicillium citrinum, have been described as lipolytic reservoirs thanks to their ability to produce lipase (Crognale et al. 2006). These enzymes have been used in different industries, such as dairy and pharmaceutical (Cordova et al. 1998). 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 to other products on the market, showed a broad spectrum endopolygalacturonase activity (Federici 1985,1988; Petruccioli, Maurizio et al. 1988). Besides enzyme production, OMWWs have also been evaluated as a source of polysaccharides, especially exopolysaccharides (ESP) (Nadour et al. 2015) with glucose as the main monosaccharide, followed by galactose, arabinose, rhamnose, and galacturonic acid. The xanthan, a glucose-mannose and glucuronic acid repeating unit compound, is the main ESP used in different areas, such as in cosmetic formulations or as a supplement and thickening compound (Petri 2015). However, the EPS production 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 (Lopez and Ramos-Cormenzana 1996). Similarly, Paenibacillus jamilae sp. highilithed, on OMWW, the production of an EPS consisting of fucose, xylose, rhamnose, arabinose, mannose, galactose, and glucose. Morillo et al. (Morillo et al. 2007) reported that P. jamilae CECT 5266 strain (in a 80% v/v of OMWW) produced an EPS consisting of glucose, galactose, mannose, arabinose, rhamnose, hexosamine, and uronic acid, in agreenment with results previously reported by Ruiz-Bravo et

al. (Ruiz-Bravo et al. 2001) using the strain *P. jamilae* CP-7.

4.2 Production of bio-energy 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 (Hill, 2009). 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 (Ahmed et al. 2019; Dermeche et al. 2013). 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, CO2 and hydrogen. In the second step, through interactions between methanogenic and acetogenic microorganisms, these metabolites are transformed into CH4 and CO2 (Moraes, Zaiat, and Bonomi 2015). However due to the presence of oily residues or phenols responsible for antimicrobial activity, OMWW must be first treated or diluted (Lercker, 2014). 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. (Hamdi 1991) and Borja and co-wokers

(Borja et al. 1993), 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 the purpose, the authors examined the effects of dilution with water (in a 1:1 ratio), the heat treatment (at 100°C) and the treatment with H2O2. The results showed that the degradation of phenols by P. sajor-caju reached a level of 50% in heat-treated OMWW, of 53% in heat-treated OMWW pretreated with H2O2, and of 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. (Sarris et al. 2014) and Nikolaou et al. (Nikolaou and Kourkoutas 2018) 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. (Zanichelli et al. 2007) proposed a multiphase treatment using S. cerevisiae into OMWW added 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 (Taccari and Ciani 2011). Furthermore, Sarris et al. (Sarris et al. 2017) demonstrated the ability of Y. lipolytica strain ACA-DC 5029 to grow on media contaning low concentration of crude glycerol and OMWW, producing a significant amount of citric acid and erythritol. In 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 Olive oil by-products as feed

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. However, the addition of olive by-products must be monitored (up to 5 and 10% replacement) because the low protein concentration and the presence of phytosterols may have a negative effect, moreover, their high energy content (as they are rich in fat) may also reduce the total feed intake (Berbel and Posadillo 2018). 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. (Makri et al. 2018) 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 effectvness in reduction of oxidative stress and in stimulation of immune response of the same extract for pigs. Gerasopoulos et al. (Gerasopoulos et al. 2015) 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; proteincarbonyls: CARB; and reactive thiobarbituric acids: TBARS) in blood and tissues and a decrease in oxidative stress, with an overall increase of

productivity. In addition, Varricchio et al. (Varricchio et al. 2019) evaluated the antioxidant activity in piglet fed with phenol extracts, and results highlighted an increase of leukocytes and cyclooxygenase-2 (COX-2), known as markers of inflammation. Gerasopoulos et al. (2015) repeated the test in chicken, 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 (Obied et al. 2005). Ciriminna et al. (Ciriminna et al. 2016) investigated the relationship between phenolics and health benefits on food, pharmaceutical and cosmetic applications. Regarding food sector, the addtion 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 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 (FDA 2014; Galanakis, 2015). Commercial OMWW implementation in food and recovery of phenols is of great interest (Galanakis 2012; Rahmanian, Jafari, and Galanakis 2014)] and at least five companies worldwide recover phenols from OMWWs (Galanakis and Schieber 2014) to sell them as natural preservatives or bioactive additives in food products (Veneziani et al. 2017).

5.1 Olive oil by-products as feed

degenerative diseases, such as cancer and cardiovascular diseases (Pellegrini et al. 2003) are related to oxidative stress, which has also been identified as a causative agent for declining immune function and atherosclerosis (Meydani 1998). Several nutraceuticals aimed to reduce the oxidative stress are currently available on the market (Visioli et al. 2020). Phenols are recognized as the main responsible for the health effects of the Mediterranean diet in prevention of chronic diseases, 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 (2011) 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 (Aissa et al. 2017). Phenols are active ingredients of many medicinal plants and the mechanisms of their pharmacological activity are not yet fully understood. Beyond the mechanism of protection, based on antioxidant activities, phenols have highlighted: scavenger property against free radicals and reactive oxygen forms (ROS); ability to act as chelators of heavy metals (especially iron) and cability to inhibit lipoxygenase, involved in inflammatory processes. The main radical species, involved in diseases, responsible of cytotoxic effect and in damaging membranes' lipids, are the superoxide anion (O2-), the hydrogen peroxide (H2O2) and the hydroxyl radical (OH-) (Girotti 1998).

5.2 Antimicrobial properties

The main phenolic compounds present in OMWW are: oleuropein, hydroxytyrosol, tyrosol, caffeic acid, p-coumaric acid,

vanillic acid, syringic acid, gallic acid, luteolin, quercetin, cyanidin, verbascoside, and other polymeric compounds (D'Antuono et al. 2014; Obied et al. 2009). Marković et al. (Marković et al. 2019) 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 (Furneri et al. 2004) 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 (Boudet 2007), 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 of infections. Moreover, they have been evaluated for drug formulations to reduce the spread of antimicrobial resistance bacteria (Tafesh et al. 2011). Bisignano et al. (Bisignano et al. 1999)demonstrated that hydroxytyrosol possesses an *in vitro* antimicrobial property against respiratory and gastrointestinal infectious agents, such as *Vibrio parahaemolyticus*, *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 (Di Nunzio et al. 2020). Several studies have focused on replacing synthetic additives with natural substances, mainly derived from plants and agro-industry by-products (Farag, Mahmoud, and Basuny

2007; Jaber et al. 2012)] 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. In details, encapsulation protects them from degradation due to different factors reducing the amount of compounds needed to be efficient and controlling their release into food matrix (Mohammadi et al. 2016). Besides the therapeutic activities, the biophenols present in OMWW have been explored for their antimicrobial, antifungal and antiviral activities. Obied et al. (Obied et al. 2008) reported that the phenolic fraction of OMWW shows antibacterial activity against several species, particularly S. aureus, Bacillus subtilis, Escherichia coli and Pseudomonas aeruginosa. However, the antimicrobial activity was found higher when the whole phenolic content is used, compared to the activity of the single phenolic compound (Obied et al. 2007). In particular, Serra et al. (Serra et al. 2008) showed that natural OMWW extracts exhibited a higher antimicrobial activity compared to 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 both Gram-positive and Gram-negative bacteria (Tafesh et al. 2011). 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 (López de las Hazas et al. 2016). Hydroxytyrosol has found also active against foodborne pathogens such as *Listeria monocytogenes*, *S. aureus*, *Salmonella enterica*, and

Yersinia spp. (Medina et al. 2006) and against beneficial microorganisms, like L. acidophilus and Bifidobacterium bifidum. In addition, Fasolato et al., (Fasolato et al. 2015) confirmed the bactericidal effect of phenol extract purified from OMWWs. In particular, S. aureus and *L. monocytogenes* showed the lowest level of resistance (minimum bactericidal concentration MBC=1.5-3.0 mg/mL) while Gram negative bacteria (e.g. Salmonella Typhimurium and Pseudomonas spp.) showed higher resistance, with MBC 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 concentration 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 has been added as concentrates or ingredient in the formulation of novel foods in different agro-food supply chains (Table 2). In a review, Galanakis (Galanakis 2018) 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 final product. Olive phenols have shown better performance in raw meat treatment (Barbier, 2009) as they were able to hinder the lipid oxidation. To evaluate such an effect, the oxidation test with 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. (Chaves-López et al. 2015) and Veneziani et al. (Veneziani et al. 2017) have 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 a species -dependent activity both in vitro and in situ tests. In particular, the treatment with 2.5% of OMWW extract strongly inhibited the in situ growth of Cladosporium cladosporioides, Penicillium aurantiogriseum, Penicillium commune and Eurotium amstelodami. Veneziani et al. (2017) 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 g/kg and 1.50 g/kg) delayed the growth of mesophilic aerobic bacteria, highliting a dose-dependent behaviour, with a 24 h extension of shelf-life, compared to both control and sample treated with the lowest concentration. In addition, Fasolato et al. (Fasolato et al. 2015b), according to Servili et al. (Servili et al. 2011), 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 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 days increase of shelf-life, compared to the control. In addition, the treatments were shown to positively affect the odor of meat, decreasing the TBARS value. De Leonardis et al. (De Leonardis et al. 2007) 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. (Troise et al. 2014) tested the antioxidant activity of OMWW phenolic extract into UHT milk

samples, on inhibition of 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 (Servili et al. 2011). The addition of extract 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 (Mikdame et al. 2020), 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. (Roila et al. 2019) added biophenols 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. (Galanakis et al. 2018) tested the antioxidant effect of OMWW phenolic extracts in combination with other antioxidants, demostrating a reducing of 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. (Cedola et al. 2020) 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 sensory point of view. Ultrafiltered OMWW, have been 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. Results showed that the

addition into bread and pasta of OMWW slightly increased the chemical quality of bread and pasta without compromising their sensory traits. Zbakh et al. (Zbakh and El Abbassi 2012) 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, 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 is pointed out on new beverages, aqueous extracts obtained with olive leaves, characterized by a high concentration of biophenols and some 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 health through reducing the incidence of many diseases, especially cardiovascular and chronic degenerative diseases.

As prevously reported by Zbakh et al. (2012), which confirmed that OMWW phenolic compounds are highly bioavailable and safe, the potential application of OMWW for setting up a 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 as such for human consumption is still available.

7 Conclusion and future perspectives

According to The future of Food and Agriculture: trends and

challenges (2017), about one third of all produced food is still lost or wasted along the food chain, from production to consumption highlighting an inefficiency of current food systems. Furthermore, the valorization and re-use of by-products aimed to create a virtuous recycling system, in accordance with the Global Food 2030 objectives.

The chance for agro-food companies to implement a circular economy strategy has offered new choices in by-product valorization. Agro-food by-products are characterized by antioxidant and antimicrobial properties that may have various applications in several sectors of food industries, replacing synthetic food additives. Despite several chemical characterizations of olive oil products and olive oil by-products, further researches 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 biotechcnological processes, followed by further treatments, to utilize them as they are 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. Furthermore, the main objective remains to propose a cheaper and innovative industrial scale-up process and the use of microorganisms has shown to be an excellent strategy for the valorization of OMWW to further increase the nutritional value of OMWW to be proposed as ingredients for functional food.

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	(Chaves-López et al. 2015)
	White meat burgers	0.75-1.50 g/kg	Antimicrobial	Retarding the growth of aerobic mesophilic bacteria	(Veneziani et al. 2017)
	Lard	100-200 ppm	Natural Antioxidant	Stabilization in oxidative of lard	(De Leonardis et al. 2008)
	Meat	75 to 100 mg/L	Natural Antioxidant	Extension of shelf-life: colour retaining, inhibition of microbial growth and fat deterioration	(Barbier, 2009)
	Fresh chicken	38.6 g/L	Antimicrobial	Delay in Enterobacteriaceae and *Pseudomonas* spp. growth	(Fasolato et al. 2015a; Servili et al. 2011)
	Milk	0.1-0.05% w/v	Functional Ingredient	Increasing product stability	(Troise et al. 2014)
	Functional Milk, Fortified Beverage	100-200 mg/L	Beverage Fortification	Formulation of functional milk	(Servili et al. 2011)
	Butter	80 mg/kg	Natural Antioxidants	Confering resistance to oxidative stress	(Mikdame et al. 2020)
	'Fior di latte' cheese	250- 500 µg/mL	Antimicrobial	Increasing shelf-life	(Roila et al. 2019)
	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	(Galanakis 2018)

				during cooking	
	Bread and Pasta	900 g of OMWW (for bread) and 30% w/w (for pasta)	Antioxidant and Food Fortification	Food fortification: ehnancing chemical composition without compromising the sensory characteristics	(Cedola et al. 2020)

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OTHER ACTIVITIES: Study Article

Characterization of cell-envelope proteinases from two *Lacticaseibacillus casei* strains isolated from Parmigiano Reggiano cheese

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During my research activity at the Department of Life Sciences, University of Modena-Reggio Emilia, Reggio Emilia, I participated in the present research work, carrying out the activity related to the identification of Prt gene expression, by RT-PCR technique.

1 Introduction

Lactic acid bacteria (LAB) are a group of microorganisms generally found in nutrient-rich environments and commonly used in the manufacturing of fermented dairy foods. LAB are nutritionally fastidious and their growth is dependent on the presence of an external source of nitrogen (i.e. amino acids or short peptides), since they are auxotrophic for numerous amino acids (Christensen, Dudley, Pederson, and Steele, 1999). The low amount of amino acids and peptides in milk has caused the LAB to evolve a complex proteolytic system to achieve casein hydrolysis releasing amino acids and oligopeptides (Christensen et al. 1999). In addition to the well-studied starter LAB, also some non-starter LAB (NS-LAB) exhibit a proteolytic phenotype (Tagliazucchi et al. 2020). These NS-LAB typically colonize cheese during ripening, giving an important contribute to milk protein hydrolysis and to the formation of texture and flavour of the fermented milk products (Solieri, Bianchi, and Giudici, 2012). The LAB proteolytic system involves different components such as: (i) cell-envelope proteinases (CEPs) that are responsible of the first degradation of caseins into oligopeptides, (ii) specific transport system that internalizes peptides and (iii) a wide variety of internal peptidases such as specific endopeptidases, aminopeptidases, tri- and di-peptidases and proline-specific peptidases (Sadat-Mekmene et al. 2011; Savijoki, Ingmer, & Varmanen, 2006). To date, six major kinds of CEPs have been described among LAB strains, including PrtP from *Lacticaseibacillus paracasei* and *Lactococcus lactis*; PrtB typical of *Lactobacillus delbrueckii subsp. bulgaricus*; PrtH characteristic of *Lactobacillus helveticus*; PrtS from *Streptococcus thermophilus*; PrtR found in *Lacticaseibacillus rhamnosus* and PrtL typical of *Lactobacillus delbrueckii subsp. lactis* (Savijoki et al. 2006; Ji et al. 2021).

Moreover, CEPs have also been identified in other LAB species such as Lactobacillus acidophilus and (Chen et al. 2018; Fira et al. 2001). Most LAB are thought to have only one specific CEP but four unique types of CEPs, namely PrtH, PrtH2, PrtH3, and PrtH4, have been characterized in *Lactobacillus helveticus* (Sadat-Mekmene et al., 2011). As revealed by comparative genomics analysis, the quantity of CEP genes in LAB was comprised between one to four depending on the strain (Liu et al. 2010). CEPs are typically synthesized in the cytoplasmic compartment in the form of pre-pro-proteinases of about 2000 amino acids (Ji, et al., 2021; Savijoki et al. 2006). They are organized in six to eight functional domains comprising a signal sequence S (absent in PrtH2), a pro-domain PP, a catalytic domain PR, an insert domain I (absent in PrtR and PrtH2), the A- and B-domains, and helix domain H (only present in PrtP, PrtH and PrtH2), a cell-wall domain W and an anchored domain AN (only present in PrtP, PrtS and PrtR) (Ji et al., 2021; Savijoki et al. 2006). Besides the key role of CEPs for LAB growth in milk, they also play a pivotal contribution in developing the organoleptic properties of fermented dairy foods and impact on the health properties of fermented dairy food and probiotic LAB. Several studies demonstrated that many bioactive peptides might be generated after casein hydrolysis, both during fermentation and in vitro hydrolysis by purified CEPs (Ji et al. 2021; Tagliazucchi, Martini, and Solieri, 2019). Beyond their known nutritional value, these peptides can regulate important physiological functions since they displayed a plethora of activities such as anti-hypertensive, anti-microbial, anti-thrombotic, immunomodulatory, opioid, antioxidant, and mineral binding activities (Tagliazucchi et al. 2019). Indeed, CEPs were also able to degrade pro-inflammatory chemokine exerting in vivo physiologically significant anti-inflammatory effects at intestinal level (Von Schillde et al., 2012). Therefore, CEPs have been found several applications in functional food technology (reviewed by Ji et

al. 2021). The aim of this study was to characterize the genetic and biochemical features of CEPs from *Lacticaseibacillus casei* strains PRA205 and 2006, two highly-proteolytic non-starter LAB previously isolated from ripened Parmigiano Reggiano cheese, in order to verify their ability to produce bioactive peptides from milk proteins and their potential technological exploitation.

2 Materials and Methods

2.1 Reference strains and chemicals

The strains used in this study were *L. casei* PRA205 and 2006. These strains were isolated from ripened Parmigiano Reggiano cheeses and previously identified by 16S rRNA gene sequencing (Solieri et al. 2012; Tagliazucchi et al. 2020). Stocks of cultures were stored frozen at -80°C in de Man-Rogosa-Sharpe (MRS) medium (Oxoid, Basingstoke, Hampshire, UK) supplemented with 25% (w/v) glycerol. Before experimental use, all the strains were twice propagated in MRS broth at 37°C for 24 h under anaerobic conditions. For the entire duration of the experiments, the reference strains were maintained in MRS medium supplemented with 7% (w/v) agar at 4°C. All media and chemicals used in this study were purchased from Sigma Aldrich (St. Louis, MO, USA), unless otherwise indicated. Primers and sequencing service were provided by Bio-Fab Research (Rome, Italy).

2.2 DNA extraction

Genomic DNA (gDNA) was extracted from late exponential cultures grown in MRS according to Gala et al. (2008). Briefly, cells (1.5 mL) were centrifuged at 8,000 rpm for 10 min, washed with 500 µL of TE buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 8.0) and resuspended in 200 µL of TE buffer with glass beads (diameter 0.106 mm). Subsequently, cell suspension was vortexed with Vortex Genie

2 (MoBio, USA) for 4 min (two rounds of 2 min at the maximum speed, with 1 min in ice, and then 2 minutes at maximum speed). Fifteen µL of proteinase K (20 mg/mL) were added and the mixture was incubated at 60°C for 1 h. After incubation, 40 µL of 20% SDS were added and the samples were incubated at 65°C for 15 min. After cooling at room temperature, 90 µL of refrigerated 5 mol/L potassium acetate was added and the mixture was centrifuged at 10,000 rpm for 10 min. After performing phenol–chloroform extraction and ethanol precipitation, DNA samples were suspended in 50 µL of TE buffer. Then, the suspension was mixed with 1.5 µL RNAse (10 mg/mL) and incubated at 37°C for 2 h. Concentration and purity of gDNA samples were determined by using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), while gDNA quality was evaluated by electrophoresis on 0.8% (w/v) agarose gel containing ethidium bromide (0.5 µg/mL) in 0.5X TBE buffer (45 mmol/L Tris–HCl, 45 mmol/L boric acid, and 1 mmol/L EDTA, pH 8.0). gDNA samples were diluted to 50 ng/µL in ddH2O and stored at −20°C for subsequent analysis.

2.3 *In silico analysis, PCR screening, and phylogenetic tree construction*

The in silico search for putative prt genes in *L. casei* genomes was performed using BLASTp algorithm against NCBI database using *L. casei* PrtP (AFJ15093.1) as query sequences. A curated dataset containing 44 proteins from 23 strains was built and sequences were aligned with Constraint-Based Alignment Tool method (Cobalt) (Papadopoulos and Agarwala, 2007) using the default settings (Supplementary Table S1).). The Cobalt tool anchors the alignment using constraints derived from the conserved domain database (CDD) and PROSITE protein-motif database so that conserved residues of Prt proteins were accurately aligned. Conserved protein domains were

analysed using NCBI Batch CD-Search (https://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi; analysed on June 2021; E- Value threshold 0.01, max. hits 500) using the CDD database. Primers pairs were designed on the conserved regions within or surrounding the catalytic domains of prt genes using the Primer3 (Koressaar et al. 2018) (Supplementary Table S2). All PCR reactions were carried out in a T100 thermal cycler (Bio-Rad, Hercules, CA, USA) with Dream Taq DNA Polymerase (Thermo Scientific Waltman, MA, USA) following manufacturer's instructions. PCR products were resolved by 1.2% (w/v) agarose gel electrophoresis stained with ethidium bromide (5 µg/mL), and when required, PCR amplicons were digested with the endonuclease EcoRV (Thermo Scientific, Waltham, USA) in a 20 µL final volume, according to manufacturer's instructions. PCR products were purified with DNA Clean & Concentrator™-5 Kit (Zymo Research, Orange, CA, USA) and sequenced on both strands through a DNA Sanger dideoxy sequencing process using both external and internal primers (Supplementary Table S2). Sequences were assembled in DNAStar (DNASTAR, Inc. Madison, Wisconsin USA) and trimmed on both ends to remove primer sequences. The nucleic acid sequences of prtP and prtR partial genes have been deposited at GenBank database and can be retrieved by the respective accession numbers: MZ606853 to MZ606856. Phylogenetic relationships were inferred using the gamma distribution (shape parameter = 1) model and the neighbour joining (NJ) method. Bootstrap support values were calculated from 1,000 replicates in MEGA6 (Tamura et al. 2013). All trees were visualized using the interactive tree of life (iTOL) v5.2 (Letunic and Bork, 2019).

2.4 Cell-envelope proteinases (CEPs) induction

To induce CEPs expression, the strains were pre-cultured in 50

mL of MRS broth and incubated for 72 h at 37°C under anaerobic conditions. After centrifugation at 10,000 rpm for 20 minutes at 4°C, cells were re-suspended in physiological solution (0.9% NaCl) at the final OD600nm values of 1010 CFU/mL and then spread on Milk-Citrate-Agar (MCA) plates (4.4% resuspended skim milk, 0.1% Na-citrate, 0.1% yeast extract, 0.5% glucose and 1.5% agar) in triplicates. After incubation for 48 h at 37°C, cells were recovered from plates and re-suspended in physiological solution at the final concentration of 1010 CFU/mL. Samples were used for both RNA extraction and biochemical characterization.

2.5 RNA extraction and RT-PCR

For RNA extraction, glassware was baked at 180 °C for at least 4 h to degrade RNases and all the solutions were prepared with diethylpyrocarbonate (DEPC)-treated water. MCA-induced cells were washed twice with DEPC-treated TE buffer (100 mmol/L Tris-HCl, 50 mmol/L EDTA, pH 8.0) and cell pellets were maintained at −80°C until thawed with 1 mL of Tri-reagent using the Zymo Direct-zol RNA MiniPrep Kit (Zymo Research, Irvine, CA). Mechanical lysis was performed using a Vortex Genie 2 (Mo Bio Laboratories) for two rounds of 20 min at highest speed alternated with 3 min on ice. The quantity of total RNA was measured spectrophotometrically using Nanodrop Nd 1000 (Nano-drop Technologies, Wilmington, DE, USA), while the integrity was checked by denaturing gel electrophoresis on a 0.9% (w/v) agarose gel with formaldehyde (10 mL of 10× MOPS running buffer) and 18 mL of 37% formaldehyde (12 mol/L) on a pH 7.0 1× MOPS running buffer (0.4 mol/L MOPS, 1 mol/L sodium acetate, and 0.01 mol/L EDTA), after RNA treatment at 65°C for 10 min. Contaminating DNA was removed by treating 2 µg of RNA sample with dsDNase (Thermo Fisher Scientific, Waltham, MA) at 37°C for 2 min in a preheated thermocycler with lid

temperature adjusted to 37°C. After chilled on ice and briefly centrifuged, 12.5 µL of treated RNA samples were used as templates for first strand cDNA synthesis with random hexamers (Thermo Fisher Scientific, Waltham, MA) by using RevertAid Reverse Transcriptase (Thermo Fisher Scientific, Waltham, MA), according to the manufacturer's instructions. CEP-specific RT-PCR assay was performed with primers reported above (Supplementary Table S2). RT-PCR of 16S rRNA gene was carried out as previously reported (Tagliazucchi et al., 2020) and used as positive control.

2.6 CEPs extraction and protein concentration determination

MCA-induced-cells (app. 1010 CFU) were re-suspended in 50 mmol/L sodium-phosphate buffer (pH 7.5) for the CEPs extraction using Ca2+-free buffer. Cells were re-suspended in 100 µL of 50 mmol/L Tris-Cl (pH 7.5) and incubated at 37°C for 30 minutes. After centrifugation at 10,000 rpm for 10 minutes, the supernatants were collected, and the pellets were re-suspended in the same buffer for additional three cycles of incubation (Kojic et al. 1991). Collected supernatants containing the crude enzyme extracts were pooled and stored at -20°C, awaiting biochemical assays. The protein concentration was assessed by the Bradford assay. The release of lactate dehydrogenase was determined in order to monitor the membrane integrity and the cell lysis during the extraction phase as previously described (Guo et al. 2016).

2.7 Cell-envelope proteinases (CEPs) enzymatic activity assay

The proteinase activity of crude extracts was evaluated by a chromogenic assay. The activity was measured using the specific substrate succinyl-alanyl-alanyl-prolyl-phenylalanine-p-nitroanilide.

The assay mixture, containing 107 µL of 50 mmol/L sodium phosphate buffer (pH 7), 56 µL of 5 mol/L NaCl, 9.5 µL of 20 mmol/L of substrate and 15 µL of extract, was incubated at 40°C for 120 min in a covered water bath. The reaction was stopped by adding 94 µL of 80% acetic acid. The released p-nitroaniline was measured at 410 nm by using a microplate reader. Control reactions with the substrate but without CEPs were also prepared (Hebert et al., 2008). One unit of proteinase activity was defined as the amount required to liberate 1 µmol of p-nitroanilide per minute. Specific activity was expressed as units of proteinase activity per mg protein.

2.8 *Effect of temperature, pH, metal ions and inhibitors on CEPs activity*

The effect of temperature on the CEP activity was measured as described above, modifying the incubation temperature. The assay was carried out at four different temperatures of 5°C, 35°C, 40°C, and 45°C at a constant optimal pH value of 7. The effect of pH on enzyme activity was evaluated by modifying the pH of the reaction buffer keeping constant the temperature at 40°C. Sodium acetate buffer (50 mmol/L) was utilized for reaction carried out at pH from 4 to 6 whereas sodium phosphate buffer (50 mmol/L) for the reaction performed at pH 8. To analyse the influence of metal ions on proteinase activity, KCl or CaCl2 or MgCl2 were added to the reaction mixture at the final concentration of 2 mmol/L (pH 7, 40°C). Similarly, to evaluate the effects of inhibitors on proteinase activity, EDTA or PMSF were added to the mix at the final concentration of 0.5 mmol/L (pH 7, 40°C).

2.9 *Casein hydrolysis*

To test the caseinolytic activity of CEPs, the crude extracts were mixed with 5 mg/mL of αS1-or β-casein solution, dissolved in sodium

phosphate buffer (pH 7.0; 100 mmol/L) at a 1:1 volume ratio. The mixtures were incubated at 40°C and after various intervals (0, 8, 24, 30, 48 and 56 h), aliquots of samples were withdrawn and stored at -20°C for further analysis.

2.10 SDS-PAGE

The casein breakdown pattern was assessed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Samples collected during the caseinolytic test described above, were diluted in Laemmli buffer in order to load 2.5 µg of total protein per lane. Denaturation was completed by boiling the samples for 3 minutes. SDS-PAGE was carried out on 12% polyacrylamide gels on vertical electrophoresis cells for 1 h at 200V. Gels were stained with the Coomassie blue staining method (0.1% Coomassie brilliant blue in 100 mL of 40% methanol, 10% glacial acetic acid, 50% H2O) for 1 h under stirring. Subsequently, gels were de-stained with the de-staining solution (40% methanol, 10% glacial acetic acid, 50% H2O) for 30 minutes under stirring. The de-staining step was repeated four times.

2.11 Identification of peptides by ultra-high performance liquid chromatography/high resolution mass spectrometry (UHPLC/HR-MS)

Samples collected during casein hydrolysis assay were mixed with 1% trifluoroacetic acid at a 1:1 volume ratio and submitted to UHPLC/HR-MS analysis for peptide identification. Chromatographic separation was carried out with UHPLC (UHPLC Ultimate 3000 separation module, Thermo Scientific, San Jose, CA, USA) equipped with a C18 column (Acquity UPLC HSS C18 Reversed phase, 2.1 × 100 mm, 1.8 µm particle size, Waters, Milan, Italy). Mass spectrometry (MS) and tandem MS experiments were performed on a Q Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo

Scientific, San Jose, CA, USA). The full description of the gradient, flow rate, MS and MS/MS parameters is reported in Martini et al. (2021).

Peptide sequencing was carried out by using MASCOT (Matrix Science, Boston, MA, USA) protein identification software with the following search parameters: enzyme, none; peptide mass tolerance, ± 5 ppm; fragment mass tolerance, ± 0.12 Da; variable modification, oxidation (M) and phosphorylation (ST); maximal number of post-translational modifications permitted in a single peptide, 4. The assignment procedure was confirmed by the manual verification of MS/MS spectra.

2.12 Bioactive peptides identification

The peptides identified in mass spectrometry analysis were investigated in relation to bioactive peptides previously identified. The identification of bioactive peptides was carried out by using the Milk Bioactive Peptide Database (MBPDB), an online database of human milk and dairy-derived bioactive peptides (Nielsen et al. 2018).

2.13 Calculation of the cleavage specificity

The cleavage probability and the positive or negative influence on the cleavage of a specific amino acid in the P1 and P1' subsites were calculated according to Solieri et al. (2018). The amino acid residues are designated as P1 in the N-terminal direction (on left of the sequence) and P1' in the C-terminal direction (on right of the sequence) from the cleaved bond. The residue P1 interacts with the subsite S1 in the enzyme active site, whereas the residue P1' interact with the subsite S1' in the enzyme active site. The peptidic bond cleaved by the CEPs is defined as the P1-P1' bond. The amino acid residue in position P1 or P1' influenced the CEP cleavage probability. If the amino acid residue A is in the position n (P1 or P1' subsite), the

cleavage probability of the P1-P1' bond will be:

$$\%Pn = \frac{total\ amino\ acid\ A\ cleaved\ in\ position\ n}{total\ amino\ acid\ A\ in\ proteins} \times 100$$

The mean cleavage probability was defined by the formula:

$$\%\overline{Pn} = \sum_{\#=1}^{20} \frac{\%Pn}{20}$$

The positive or negative influence of an amino acid residue *A* in the P1 and P1' subsites was quantified by the coefficient *Kn*:

$$Kn = \frac{\%Pn}{\%\overline{Pn}} - 1$$

Kn values > 0 indicated a favourable influence of the amino acid *A* in the specific subsite on the cleavage of the P1-P1' bond, whereas *Kn* values < 0 suggested a negative effect on the cleavage.

2.14 Statistical analysis

Data are shown as mean ± standard deviation (SD) for three replicates. Univariate analysis of variance (ANOVA) with Tukey post-hoc test was applied using GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA). The differences were considered significant with $P < 0.05$.

3 Results and discussion

3.1 In silico survey of putative prt genes in L. casei genome

Most LAB are thought to possess only one type of CEP (Sadat-Mekmene et al. 2011), but it has been proved that more than one prt gene exist in the genome of several LAB species and that the pattern of prt genes is highly variable at inter-strain level. For instance, *L.*

helveticus possesses four unique types of CEPs, namely prtH1, prtH2, prtH3, and prtH4 (Sadat-Mekmene et al. 2011), while *L. rhamnosus* CGMCC11055 possesses both prtP and prtR genes (Guo et al. 2016), even if prtP was initially detected only in Lactococcus lactis (Kok. Leenhouts et al. 1988) and *L. paracasei/L. casei* species (Nikolić et al. 2009; Vukotić et al. 2016). Using *L. casei* PrtP (AFJ15093.1) amino acid sequence as query in Blastp search, we built a dataset consisting of 44 *L. casei* putative Prt-encoding gene. Protein alignment with Cobalt showed that twenty-two exhibited the insert domain and were categorized as PrtP (Supplementary Figure S1). The remaining 22 proteins lacked the insert domain and were categorized as PrtR with three different lengths of approx. 1,500, 1,800, and 2,200 amino acids, respectively (Supplementary Figure S1). This result suggested that both prtP and prtR paralogs exist in several *L. casei* genomes, including in the type strain ATCC 393T. Phylogenetic analysis showed two distinct clades for *L. casei* PrtP proteins, referred to as PrtP1 and PrtP2, respectively, while PrtR proteins grouped into three different clusters, referred to as PrtR1, PrtR2, and PrtR3, respectively (Figure 1A). "CD-search" of the Conserved Domain Database (CDD) predicted that PrtP1 and PrtP2 share the same functional domains except for FIVAR domain (pfam07554), which is present in PrtP2 but not in PrtP1, and CHB_HEX_C domain, which is present in PrtP1, but not in PrtP2 (Supplementary Figure S2). PrtR proteins shared the subtilisins S8 family domain annotated in the Pfam database (PF00082), but mainly differed each other at the C-terminus (Supplementary Figure S2).

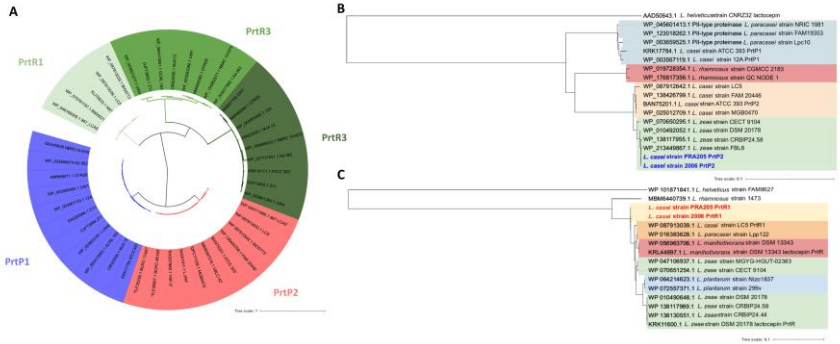

Figure 1. Fast minimum evolution tree of 44 putative S8 subtilisin proteases from *Lacticaseibacillus casei* genomes reported in GenBank (A) (last accession on April 2021) and phylogenetic trees of PrtP2 (B) and PrtR1 (C) amino acid partial sequences.

3.2 PCR screening and phylogenetic analysis

Based on the survey of putative prt genes in 44 *L. casei* genomes, CEP-specific primer pairs were designed across the putative active domain of 5 different Prt-encoding genes and used to assess the type and distribution of prtP and prtR paralogues in *L. casei* PRA205 and 2006. Both strains were positive to PrtP2 and PrtR1-specific PCR assays as they exhibited two bands with the expected length of approximately 2630 bp and 1723 bp, respectively, while no PCR products were obtained with the primer pairs targeting prtP1, prtR2, and prtR3 genes, respectively (Figure 2). This pattern of prt genes resembled that we scored in the genome of *L. casei* LC5 used as reference strain in the subsequent analyses. Recently, Asahina et al. (2020) also identified both prtR and prtP genes in a *L. paracasei* proteolytic strain used as adjunct starter in Gouda-type cheese production. To exclude that putative SNPs prevented the annealing of prtP1-specific primers, an additional primer set was designed upon the conserved regions of prtP1 and prtP2 genes. Restriction analysis of the resulting PCR amplicons with the diagnostic endonuclease EcoRV confirmed the presence of prtP2 gene (Supplementary Figure S3). We sequenced the prtP2 and prtR1 PCR amplicons from L. casei PRA205 and 2006 for comparative analysis. Blastn search showed that PRA205 and 2006 prtP2 nucleotide sequences were 93.75 and 93.71% identical to *L. casei* ATCC 393T prtP2 sequence, respectively, and more identical (> 99%) to S8 peptidase encoding genes in recently released genomes of *Lacticaseibacillus zeae* strains FBL8 and CECT 9104. *Lacticaseibacillus zeae* is a recently restored species closely related to *L. casei* and undistinguishable from *L. casei* based on 16S rRNA gene sequencing and mutL multiplex PCR assay (Huang et al., 2020; Liu, & Gu, 2020). BlastX results using the predicted protein sequences revealed that the most similar proteins to PRA205 and 2006 PrtP2 was

WP_213449867.1 (*L. zeae* strain FBL8; 99.81%). Furthermore, PRA205 and 2006 PrtP2 partial proteins diverged from *L. casei* LC5 PrtP2 due to thirteen substitutions, namely E120K, N122S, V143A, D229A, T237A, S454R, T551A, D652A, A771T, R776G, S837G, K860R, N874S (Supplementary Figure S4). Phylogenetic analysis showed that putative PrtPs from *L. zeae* strains grouped in a homogeneous cluster divergent from *L. casei* homologs and placed PRA205 and 2006 PrtP2 from strains more closely related to *L. zeae* than to *L. casei* ATCC 393T and LC5 PrtP2 proteins (Figure 1B). The prtR1 nucleotide partial sequences in *L. casei* PRA205 and 2006 were 94.98% identical to LC5 prtR1 gene and encoded PrtR1 proteins with six substitutions, namely V539I, T571S, S896T, A946T, V923I and S946T, compared with LC5 PrtR1 (Supplementary Figure S5). Phylogenetic tree revealed that PrtR1 protein sequences from *L. casei*, *L. paracasei* and *L. zeae* formed a heterogeneous group together with plasmid encoded PrtR1 proteins from *Lacticaseibacillus manihotivorans* and *Lactiplantibacillus plantarum* (Figure 1C). Proteinase genes (prtP and prtM) were proven to be plasmid-encoded also in *L. lactis* too (Kojic et al. 2005). In *S. thermophilus*, the prtS gene is part of a genomic island flanked by conserved insertion sequence (IS) elements (Dandoy et al. 2011; Seller et al. 2015). This mobile island has been shown to be involved in gene gain and loss recombination events, which could be responsible for the huge interspecies and intraspecies variability in proteolytic activity and prt gene pattern observed in lactobacilli. Remarkably, PRA205 and 2006 PrtR1 predicted proteins diverged from those of *L. casei* group and clustered separately (Figure 1C). Further experiments are required to establish whether prtR1 gene is plasmid encoded in *L. casei* PRA205 and 2006 or whether it belongs to a genomic island flanked by IS regions.

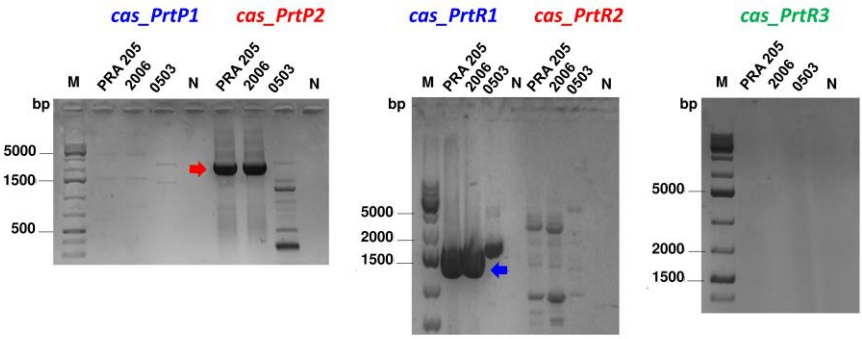

Figure 2. Screening of prtP and prtR genes in Lacticaseibacillus casei PRA205 and 2006.

3.3 CEP extraction and prt gene expression profile

Peptide-rich media generally repress proteinase activities in several lactobacilli, including *L. casei* (Alcantara et al. 2016; Hebert et al. 2002). Therefore, we decided to assess the CEP activities of strains PRA205 and 2006 grown until the stationary phase on the MCA medium. CEPs extraction was performed by using Ca2+-free buffer and the specific enzyme activity was determined with the chromogenic substrate succinyl-alanyl-alanyl-prolyl-phenylalanine-p-nitroanilide. Strain 2006 showed a specific CEP activity of 11.34 ± 0.45 U/mg, whereas PRA205 of 3.73 ± 0.03 U/mg. Lactate dehydrogenase activity assay was performed to check the membrane integrity during the extraction process. In the crude extracts, the LDH

activity was undetectable (data not shown), proving that the proteolytic activity reported was due to the action of CEPs rather than intracellular peptidases. To qualitative establish which prt gene is actively transcribed in MCA-grown cells, we carried out a RT-PCR assay. Figure 3A and 3B shows that both strains PRA205 and 2006 transcribed prtR1 but not prtP2 gene, suggesting that the CEP activity detected above mainly came from PrtR1 proteinase when strains were grown on MCA medium. This result differed from that found in *L. rhamnosus* strain CGMCC11055 which has both prtP and prtR genes but only prtP gene transcription was detected when grown in synthetic medium (Guo et al. 2016).

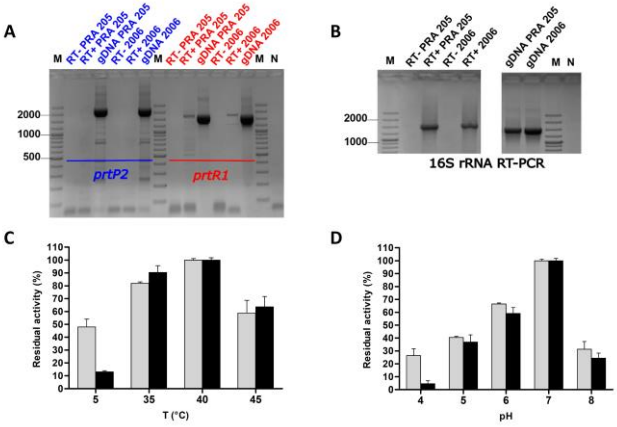

Figure 3. RT-PCR assay targeting *prtP2* and *prtR1* genes in *Lacticaseibacillus casei* PRA205 and 2006 cells grown on MCA and biochemical characterization of extracted PrtR1.

3.4 Biochemical characterization of L. casei PRA205 and 2006 cell-envelope proteinases PrtR1

The proteolytic activity of PrtR1 was analysed by modifying the incubation temperature and the pH of the reaction buffer in order to determine the best conditions of substrate hydrolysis. The PrtR1 extracted from each strain displayed the highest activity at 40°C and the enzymatic activity at 40°C was taken as reference (100%) for the calculation of residual (%) activity (Figure 3C). A slight decrease in the PrtR1 catalytic activity was observed at 35°C, whereas at the temperature of 5°C, the residual activity rapidly fell down for PrtR1 extracted from *L. casei* 2006, whereas the proteinase extracted from *L. casei* PRA205 retained the 48% of its activity. The high proteolytic activity at 5°C seems to be a peculiar feature of *L. casei* PRA205 PrtR1 since the majority of previously characterized CEPs lost almost completely the activity at temperature near to 20°C (Chen et al. 2018; Fernández de Palencia et al. 1997; Fira et al. 2001; Guo et al. 2016).

The effect of pH on the enzyme activity was analysed by varying the pH of the reaction buffer from 4 to 8 and the constant temperature of 40°C (Figure 3D). All the crude extracts achieved the highest activity at an optimum pH value of 7 that was used as reference (100%) for the calculation of residual (%) activity. In particular, the residual activity gradually decreased at pH below and above 7 for both the strains. However, when the PrtR1 activity was tested at pH 4, proteinase extracted from *L. casei* PRA205 showed a residual proteolytic activity significantly higher respect to that extracted from *L. casei* 2006. This property could be of paramount importance because suggest a possible exploitation of *L. casei* PRA205 in the production of bioactive peptides in fermented dairy foods such as cheese, yogurt and fermented milk, which are characterized by low pH values. This observation could also explain the fact that *L. casei* PRA205 whole cells presented higher proteolytic activity in fermented

milk respect to L. casei 2006 whole cells, despite the latter strains showed a highest CEP specific activity (Solieri et al. 2015; Tagliazucchi et al. 2020). Moreover, the fact that *L. casei* PRA205 PrtR1 retained almost the 50% of its activity at low temperature suggested that the enzyme might be active also during the cold storage of fermented dairy food promoting the continuous release of bioactive peptides from caseins during cold storage. The proteolytic activity of both the PrtR1 was inhibited by K+ (71.2 ± 1.5% and 67.0 ± 6.1% of residual activity for *L. casei* PRA205 and *L. casei* 2006 PrtR1, respectively) and Mg2+ (80.9 ± 2.4% and 70.5 ± 6.4% of residual activity for *L. casei* PRA205 and *L. casei* 2006 PrtR1, respectively) ions, when added at 2 mmol/L. Vice versa, PrtR1 activity was enhanced by 2 mmol/L Ca2+ ions (143.5 ± 5.3% and 183.0 ± 2.% of residual activity for *L. casei* PRA205 and *L. casei* 2006 PrtR1, respectively) as reported for numerous others extracted CEPs (Ji et al., 2021). Indeed, the activity of both the PrtR1 was almost completely abolished by 1 mmol/L of PMSF (86.5% and 99.9% of inhibition for *L. casei* PRA205 and *L. casei* 2006 PrtR1, respectively), suggesting that both the PrtR1 were members of the serine proteinases family. The chelator EDTA at 1 mmol/L concentration also inhibited the PrtR1 activity (31.5% and 39.2% of inhibition for *L. casei* PRA205 and *L. casei* 2006 PrtR1, respectively), indicating that cations (i.e. Ca2+ ions) are required for their activity.

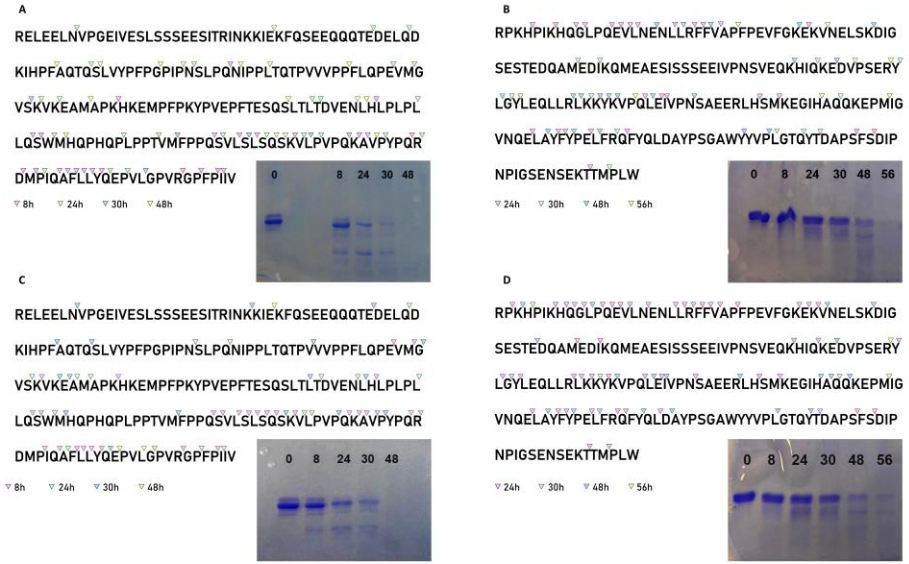

Figure 4. Time course hydrolysis of β-casein and αS1-casein and distribution of the cleavage sites by PrtR1 from Lacticaseibacillus casei PRA205 and 2006.

3.5 Caseinolytic specificity of extracted PrtR1 and peptidomics analysis of casein hydrolysates

The proteolytic activity of PrtR1 was assessed against αS1- and β-caseins as substrates. Aliquots of samples were taken after 0, 8, 24, 30, 48 and 56 hours and analysed by SDS-PAGE and mass spectrometry experiments. As shown in Figure 4, both the extracted PrtR1 completely degraded β-casein after 48 h of incubation at the optimal conditions, while αS1-casein was hydrolysed at a lower rate with the protein bands still present after 56 h (Figure 4). Based on the specific hydrolysis pattern of αS1-, β-, and κ-caseins, CEPs were classified in three different types. CEPs belonging to the PI type primarily hydrolyse β-casein that is cleaved into more than 100 different oligopeptides ranging from 4 to 30 amino acid residues (Ji et al. 2021). PIII-type CEPs can hydrolyse αS1-, β-, and κ-caseins equally well (Ji et al. 2021). There is also a mixed CEP type named PI/PIII that cleave β-casein in a similar manner to PI-type and is also able to cleave, to a lesser extent, αS1-casein (Tagliazucchi et al. 2019). The above reported results indicated that β-casein was the preferential substrate over αS1-casein, suggesting that PrtR1 extracted from *L. casei* PRA205 and *L. casei* 2006 belonged to the PI/PIII-type. These results disagreed with the outcomes of Kojic et al. (1991) that characterized a PI-type CEP in *L. casei* HN14, while it is consistent with the mixed PI/PIII type CEP isolated from *L. casei* IFLP 731 (Fernández de Palencia et al. 1997). Samples collected during caseins hydrolysis assay were submitted to UHPLC/HR-MS analysis for peptide identification to study the caseins breakdown pattern produced by the PrtR1 extracted from the selected strains. The full list of identified peptides at the different time points for both β- and αS1-caseins and the mass spectrometry data are reported in supplementary Tables S3-S6. Crude extracts were incubated with β-casein up to 48

hours and the peptidomic analysis revealed that a total of 116 and 119 peptides were released by PrtR1 extracted from *L. casei* PRA205 and *L. casei* 2006, respectively (Supplementary Table S3 and S5). In the αS1-casein hydrolysis, samples were incubated up to 56 hours and at the end of incubation time a total of 102 and 124 peptides were released by PrtR1 extracted from *L. casei* PRA205 and *L. casei* 2006, respectively (Supplementary Table S4 and S6). The number of identified peptides released from β-casein constantly increased during hydrolysis for both the PrtR1 (Supplementary Table S3 and S5 and Supplementary Figure S6A), whereas, in the case of αS1-casein, the number of peptides peaked after 48 hours of incubation (Supplementary Table S4 and S6 and Supplementary Figure S6B). As reported in the Venn diagrams (Supplementary Figure S7) the 57.7% of peptides released from β-casein and the 61.4% of peptides released from αS1-casein were commonly found in the hydrolysates obtained from the two PrtR1 suggesting a similar cleavage specificity.

3.6 Analysis of the β-casein cleavage site-specificity

The analysis of the β-casein cleavage site-specificity revealed the presence of 63 and 66 different cleavage sites in samples hydrolysed by PrtR1 extracted form *L. casei* PRA205 and *L. casei* 2006, respectively (Figure 9A and C). They represented the 30.3% and 31.7% of all peptide bonds present in the protein. A total of 54 cleavage sites were in common between the two PrtR1, representing the 85.7 and 81.8% of total sites found in *L. casei* PRA205 and *L. casei* 2006 CEPs, respectively, suggesting that these CEPs had almost the same cleavage specificity. The time-course analysis evidenced that the hydrolysis of β-casein began at the hydrophobic C-terminal region. After 8 hours of reaction, the 80% and 76% of the cleavage sites produced by *L. casei* PRA205 and *L. casei* 2006 PrtR1, respectively, were located between the residue 161 and the C-terminal amino acid

V in position 209. At this time point, no cleavage sites were found in the N-terminal region between the residues 1 and 72 and in the central region comprised between the amino acids in position 106 and 134. Furthermore, considering the long region between amino acids 1 and 134, only 2 and 4 cleavage sites were detected in L. casei PRA205 and *L. casei* 2006 PrtR1 hydrolysates. After 24 hours of hydrolysis, still the majority (59% for *L. casei* PRA205 PrtR1 and 62% for L. casei 20065 PrtR1) of the cleavage sites were in the C-terminal region. At the end of the hydrolysis, the cleavage sites were distributed throughout the entire β-casein sequence for both the PrtR1 activities, however, no cleavage sites were identified in the poly-phosphorylated region between residues 8 and 28. It is worth to note that majority of the previously characterized lactobacilli proteinases have a proven preference for hydrolysing the C-terminal region of β-casein (Ji et al. 2021; Lozo et al. 2011). Furthermore, the cleavage probability at sub-sites P1 and P1' and the Kn coefficients, which measure the positive or negative effect of amino acids on the P1-P1' cleavage probabilities, were calculated (Table 1). PrtR1 extracted from both the *L. casei* strains showed a marked preference for the amino acid M, for polar un-charged amino acids (S, Q and N) and for positively charged amino acids (R and K) in the P1 position. In particular, the amino acids M and N showed the highest Kn values for both the PrtR1 and therefore exerting the strongest positive effect on cleavage probability at the P1 sub-site. The only difference between the two PrtR1 was the strong positive effect of amino acid A for PrtR1 extracted from *L. casei* 2006. In position P1, the amino acids I, T, P, V and H as well as the negatively charged (D and E) and aromatic (F and Y) amino acids exhibited a strong negative effect on cleavage probability for both the extracted PrtR1. The strongest positive effect on cleavage probability at the sub-site P1' was found for the small aliphatic amino acids A and G and amino acids H and D for both the extracted PrtR1. In both the

cases, the amino acid D and A showed the highest Kn values. The strongest negative effect in position P1' was exhibited by the polar uncharged amino acids (especially P, Q and T) and by the amino acid E. Some of the identified preferred amino acids have been already reported in previous studies. For example, the positive effect of amino acids Q in position P1 have been already described for CEPs characterized from *L. delbrueckii* CRL581, *L. helveticus* BGRA43, *L. paracasei* BGHN14, *L. rhamnosus* BGT10 and PRA331 and *L. lactis* NCDO763 (Hebert et al. 2008; Lozo et al. 2011; Monnet et al. 1992; Solieri et al. 2018). The preference for this amino acid at the P1 subsite seemed a common features among the CEPs extracted from lactobacilli belonging to different species. In addition, the preference for N and M in position P1 was already described for L. lactis NCDO763 and *L. rhamnosus* PRA331, respectively (Monnet et al. 1992; Solieri et al. 2018). To the best of our knowledge, the strong positive effect exerted by the positively charged amino acids (R and K) in P1 position and by the residue D in P1' position has never been reported in lactic acid bacteria CEPs. The profile of cleavage-site specificity was slightly distinct from that previously reported for *L. casei* PRA205 (Solieri et al. 2018). These differences may be related to different experimental conditions, i.e. hydrolysis carried out with PRA205 whole cells and milk.

Table 1. Cleavage occurrence, cleavage probability (%*P*) and *Kn* coefficients for *Lacticaseibacillus casei* PRA205 and *Lacticaseibacillus casei* 2006 PrtR1 on β-casein at different amino acids in the P1 and P1'subsites after 48 hours of hydrolysis.

	L. casei PRA205 CEP				*L. casei* 2006 CEP			
	P1 subsite		**P1' subsite**		**P1 subsite**		**P1' subsite**	
Amino acid[a]	Number of cleaved bond[b] (%P1[c])	*Kn*	Number of cleaved bond[b] (%P1'[c])	*Kn*	Number of cleaved bond[b] (%P1[c])	*Kn*	Number of cleaved bond[b] (%P1'[c])	*Kn*
Aliphatic amino acids								
A (5)	2 (40.0)	0.08	4 (80.0)	**0.86**	3 (60.0)	**0.69**	5 (100.0)	**1.42**
G (5)	1 (20.0)	-0.46	3 (60.0)	**0.40**	2 (40.0)	0.13	3 (60.0)	**0.45**
V (19)	3 (15.8)	-0.58	9 (47.4)	0.10	3 (15.8)	-0.56	8 (42.1)	0.02
L (22)	10 (45.5)	**0.22**	10 (45.5)	0.06	9 (40.9)	0.15	8 (36.4)	-0.12
I (10)	1 (10.0)	-0.73	4 (40.0)	-0.07	1 (10.0)	-0.72	3 (30.0)	-0.27
M (6)	5 (83.3)	**1.24**	2 (33.3)	-0.22	4 (66.7)	**0.88**	2 (33.3)	-0.19
Polar un-charged amino acids								

							Other Activities	
T (9)	2 (22.2)	-0.40	2 (22.2)	-0.48	1 (11.1)	-0.69	1 (11.1)	-0.73
S (16)	8 (50.0)	**0.35**	7 (43.8)	0.02	7 (43.8)	**0.23**	7 (43.8)	0.06
Q (20)	10 (50.0)	**0.35**	2 (10.0)	-0.77	10 (50.0)	**0.41**	3 (15.0)	-0.64
N (5)	4 (80.0)	**1.15**	2 (40.0)	-0.07	3 (60.0)	**0.69**	1 (20.0)	-0.52
P (35)	8 (22.9)	-0.38	4 (11.4)	-0.73	5 (14.3)	-0.60	4 (11.4)	-0.72
Positively charged amino acids								
R (4)	2 (50.0)	**0.35**	1 (25.0)	-0.42	2 (50.0)	**0.41**	1 (25.0)	-0.40
K (11)	5 (45.5)	**0.22**	4 (36.4)	-0.15	6 (54.6)	**0.54**	5 (45.5)	0.10
H (5)	1 (20.0)	-0.46	3 (60.0)	**0.40**	1 (20.0)	-0.44	3 (60.0)	**0.45**
Negatively charged amino acids								
E (19)	3 (15.8)	-0.58	2 (10.5)	-0.76	5 (26.3)	-0.26	2 (10.5)	-0.75
D (4)	1 (25.0)	-0.33	4 (100.0)	**1.33**	0 (0)	-1.00	4 (100.0)	**1.42**
Aromatic amino acids								
F (9)	2 (22.2)	-0.40	4 (44.4)	0.03	2 (22.2)	-0.37	3 (33.3)	-0.19
Y (4)	1 (25.0)	-0.33	2 (50.0)	0.16	1 (25.0)	-0.30	2 (50.0)	**0.21**

3.7 Analysis of the αS1-casein cleavage site-specificity

Differently form β-casein, the hydrolysis of αS1-casein started at the N-terminal part of the protein. After 24 h of incubation, the 50% and 45% of the cleavage sites were located in the sequence between the residues 1 and 36 for PrtR1 extracted from *L. casei* PRA205 and *L. casei* 2006 (Figure 4B and D). Most of these cleavage sites were positioned in the fragment 1-23. Additional preferred cleavage sites at the beginning of the hydrolysis occurred in the sequence 90-110 for *L. casei* 2006 CEP and sequence 140-160 for both the extracted CEPs. At the end of hydrolysis, the cleavage sites were mainly concentrated in the fragments 1-40 and 90-160, as already reported for the majority of characterized CEPs (Ji et al. 2021).

3.8 Bioactive peptides identification using Milk Bioactive Peptide Database

The identification of bioactive peptides released by PrtR1 was carried out by searching all the peptides found in the mass spectrometry analysis experiments against the Milk Bioactive Peptide Database (MBPDB). The hydrolysis of β-casein by PrtR1 of *L. casei* PRA205 and *L. casei* 2006 produced respectively 14 and 18 functional peptides previously demonstrated to have several bioactivities (Table 2). The PrtR1 of both the *L. casei* strains commonly released a total of 14 bioactive peptides whereas 3 were uniquely produced by *L. casei* 2006 PrtR1 and one by *L. casei* PRA205 PrtR1. Instead, the hydrolysis of αS1-casein by PrtR1 of *L. casei* PRA205 and *L. casei* 2006 generated 9 and11 bioactive peptides, respectively, among which 8 were commonly released by the PrtR1 of both the strains. Identified bioactive peptides showed several bio-functional properties as 13 peptides were ACE-inhibitors, 13 were antimicrobial, 6 were antioxidant, 5 had immunomodulatory activity, one showed

dipeptidyl-peptidase IV (DPPIV) inhibitory activity and one anxiolytic activity. Most of the identified bioactive peptides were tested in vitro for their bioactivity, whereas some of them exhibited their activities also in vivo. For example, the tripeptide LLY demonstrated a positive influence in vivo in Swiss Albino mice against ethanol-induced oxidative stress (Sowmya et al. 2018). The β-casein fragment KVLPVPQ showed antihypertensive activity in vivo in spontaneously hypertensive rats (SHR) (Tagliazucchi et al. 2019). In addition, the bioactive peptide LYQEPVLGPVRGPFPIIV exerted its immunomodulatory activities by stimulating lymphocyte proliferation in rats (Coste et al. 1992).

With the only exception of the αS1-casein-derived peptides LGY and RPKHPIKHQGLPQEVLNENLLRFFVAPFPEVFGKEK, all the others identified bioactive peptides were found at the end of the hydrolysis, suggesting their resistance to further hydrolysis by PrtR1 (Table S3). In addition, 16 bioactive peptides were released only in the last periods of hydrolysis. To exert their physiological effect, bioactive peptides should be stable under gastro-intestinal conditions and resistant to the hydrolysis by gastro-intestinal proteases. In a recent study, it was demonstrated that most of peptides identified after in vitro gastro-pancreatic digestion, contained from one to four proline residues near to the carboxylic end of their sequences. In addition, the simultaneous presence of DPP-IV inhibitor peptides causes a strong decrease of the intestinal prolyl peptidases activity (Tagliazucchi et al. 2016). Among the identified peptides, LLYQEPVLGPVRGPFPIIV, YQEPVLGPVRGPFPIIV and QEPVLGPVRGPFPIIV were found after simulating gastric-pancreatic digestion of homogenized yogurt and in addition, KVLPVPQ, VLPVPQK, YQEPVLGPVRGPFPIIV, LPVPQ and EPVLGPVRGPFP were found in the human gastro-intestinal tract (Boutrou et al. 2013).

Table 2. Peptides with previously demonstrated bioactivity (100% homology) identified in the β-casein and αS1-casein hydrolysates produced by PrtR1 activity of Lacticaseibacillus casei PRA205 and Lacticaseibacillus casei 2006.

Sequence	*Fragment*	*Bioactivity*	*PrtR1*
VVPP	β-casein f(83-86)	ACE inhibition	*L. casei* PRA205
VKEAMAPK	β-casein f(98-105)	Antioxidant Anti-microbial	*L. casei* 2006
EAMAPK	β-casein f(100-105)	Anti-microbial	*L. casei* 2006
SQSKVLPVPQ	β-casein f(166-175)	ACE inhibition	*L. casei* PRA205 *L. casei* 2006
KVLPVPQ	β-casein f(169-175)	ACE inhibition	*L. casei* 2006
VLPVPQKAVPYPQR	β-casein f(170-183)	Anti-microbial	*L. casei* PRA205 *L. casei* 2006
VLPVPQK	β-casein f(170-176)	ACE inhibition Antioxidant Anti-microbial	*L. casei* 2006
LPVPQ	β-casein f(171-175)	DPP-IV inhibition	*L. casei* PRA205 *L. casei* 2006

		Other Activities	
PYPQ	β-casein f(179-182)	Antioxidant	*L. casei* PRA205 *L. casei* 2006
RDMPIQAF	β-casein f(183-190)	ACE inhibition	*L. casei* PRA205 *L. casei* 2006
LLY	β-casein f(191-193)	Immunomodulation	*L. casei* PRA205 *L. casei* 2006
LLYQEPVLGPVRGPFPIIV	β-casein f(191-209)	ACE inhibition	*L. casei* PRA205 *L. casei* 2006
LYQEPVLGPVRGPFPIIV	β-casein f(192-209)	Immunomodulation	*L. casei* PRA205 *L. casei* 2006
YQEPVLGPVR	β-casein f(193-202)	ACE inhibition Immunomodulation	*L. casei* PRA205 *L. casei* 2006
YQEPVLGPVRGPFPI	β-casein f(193-207)	Anti-microbial	*L. casei* PRA205 *L. casei* 2006
YQEPVLGPVRGPFPIIV	β-casein f(193-209)	ACE inhibition Immunomodulation Anti-microbial	*L. casei* PRA205 *L. casei* 2006
QEPVLGPVRGPFPIIV	β-casein f(194-209)	ACE inhibition	*L. casei* PRA205 *L. casei* 2006
EPVLGPVRGPFP	β-casein f(195-206)	ACE inhibition	*L. casei* PRA205 *L. casei* 2006
RPKHPIK	αS1-casein f(1-7)	Anti-microbial	*L. casei* 2006

RPKHPIKHQ	αS1-casein f(1-9)	ACE inhibition	*L. casei* 2006
RPKHPIKHQGLPQEVLNENLLRF	αS1-casein f(1-23)	Immunomodulation Anti-microbial	*L. casei* PRA205 *L. casei* 2006
RPKHPIKHQGLPQEVLNENLLRFF	αS1-casein f(1-24)	Anti-microbial	*L. casei* PRA205 *L. casei* 2006
RPKHPIKHQGLPQEVLNENLLRFFVAPFPE VFGKEK	αS1-casein f(1-36)	Anti-microbial	*L. casei* 2006
VLNENLLR	αS1-casein f(15-22)	Anti-microbial	*L. casei* PRA205 *L. casei* 2006
YLGYLEQLLR	αS1-casein f(91-100)	Anxiolytic	*L. casei* PRA205
LGY	αS1-casein f(92-94)	ACE inhibition Antioxidant	*L. casei* PRA205 *L. casei* 2006
LGYLEQLLRL	αS1-casein f(92-101)	Anti-microbial	*L. casei* PRA205 *L. casei* 2006
YLEQLLR	αS1-casein f(94-100)	Anti-microbial	*L. casei* PRA205 *L. casei* 2006
PEL	αS1-casein f(147-149)	Antioxidant	*L. casei* PRA205 *L. casei* 2006
GTQYTDAPSFSDIPNPIGSENSEKTTMPLW	αS1-casein f(170-199)	ACE inhibition Antioxidant	*L. casei* PRA205 *L. casei* 2006

4 Conclusion

In this work, two unique CEPs from the highly proteolytic strains *L. casei* PRA205 and L. casei 2006, previously isolated from ripened Parmigiano Reggiano cheese, have been characterized. Both the strains possessed two different prt genes in their genome but only one protein, PrtR1, was expressed in the MCS-induced cells. In both the *L. casei* strains, the predicted protein sequences of PrtR1 showed six amino acids substitutions, compared with the reference sequence, suggesting that these proteases were peculiar to the selected strains. In addition, PrtR1 identified in *L. casei* PRA205 and 2006 showed high similarity with PrtR1-like sequences, which are plasmid-encoded. The PrtR1 presence on plasmid could confer an important evolutionary advantage to these strains, but further analysis is required to understand the gene position. PrtR1 released from both the strains displayed the highest activity at 40°C, pH 7, and interestingly, PrtR1 extracted from PRA205 retained the 48% of its activity at 5°C and showed the highest activity at pH 4 among the tested strains. These important biotechnological features can be exploited to produce fermented dairy products with low pH and low storage temperature, such as fermented milk and yoghurt. Remarkable, peptidomics analysis assisted us to demonstrate that these CEPs are able to release β- and αS1-casein-derived bioactive peptides. Most of these peptides matched the sequences of previously reported bioactive peptides and some of them were resistant to gastro-intestinal hydrolysis. Overall, the results presented in this study provided new knowledge on the proteolytic system of two strains belonging *to L. casei* species, which is poorly explored in comparison with thermophilic lactobacilli or lactococci. Furthermore, PrtR1 from both the strains were able to release some bioactive peptides suggesting that *L. casei* can be a source of new proteases that can be exploited as enzymes for the formulation of dairy beverages or hydrolysates

enriched in bioactive peptides and with improved milk proteins digestibility. Thereby, NS-LABs can be considered as a source of new enzymes and the knowledge on CEP developed in this work as a tool for the selection of new proteinase proficient strains and for the improvement of functional lactobacilli performance, particularly in the *L. casei* species.

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OTHER ACTIVITIES: VERIFICO Project

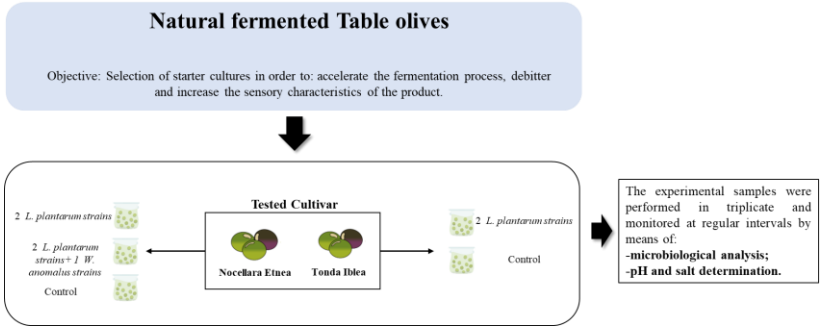
"*Approccio integrato per la valorizzazione e l'innovazione nella filiera olivicola attraverso la produzione di olive da tavola probiotiche*"

"*Integrated approach for valorisation and innovation in the olive supply chain through the production of probiotic table olives*"

Progetto_di_Investimento_144511020025, Azione 1.2.3 PO FESR Sicilia 2014-2020. 2019-2021.

Final Report of Verifico Project 113 (Department of Agriculture, Food and Environment, University of Catania).

In the first phase of the project, 2 cultivars (Nocellara Etnea and Tonda Iblea) were evaluated. The olives were placed in brine with 10% and 7% sodium chloride, respectively, and inoculated with selected strains of yeast and lactobacilli. Fermentation was monitored for 30 days.



Setting up of experimental brines and inoculum of starter strains

Starter strains belonging to the *Lactiplantibacillus plantarum* and Wickeramomyces anomalus species, taken from the microbial collection of the Department of Agriculture, Food and Environment (Di3A), University of Catania, were used for the experimental fermentations. The selected strains were previously isolated from table olive brines of the Nocellara Etnea cultivar and tested for the phenotypic, genotypic and technological characteristics. The technological aptitude of the strains was evaluated considering specific selection criteria, such as: the growth capacity under different acidic, osmotic and thermic stress conditions, identification of the gene encoding for the β-glucosidase enzyme, and finally, the capacity to degrade oleuropein. The selected strains for the characteristics reported above were inoculated at a cell density of 10^7 CFU/mL for lactobacilli and 10^5 CFU/mL for yeast. The table below shows the sample codes

and experimental conditions. All fermentations were maintained at room temperature (18±2°C). Table 1 showed experimental conditions of the tested samples.

Table 1. Experimental conditions of Nocellara Etnea cv. green olives and Tonda Iblea cv. black olives.

Samples	Cod.	Experimental condition
Nocellara Etnea cv. Green olive	C.NE	Brine at 10% NaCl
	LAB1.NE	Brine at 10% NaCl; inoculum of 2 *L. plantarum strains* (F1.16 e C11C8 1:1, 7 Log CFU/mL)
	LAB1.NEbis	Brine at 10% NaCl; inoculum of 2 *L. plantarum* strains (F1.16 e C11C8 1:1, 7 Log CFU/mL)
	Y1.NE	Brine at 10% NaCl; inoculum of 2 L. plantarum strains (F1.16 e C11C8 1:1, 7 Log CFU/mL) and *W. anomalus* strain (F1.60.5; 5 Log CFU/mL)
	Y1.NEbis	Brine at 10% NaCl; inoculum of 2 L. plantarum strains (F1.16 e C11C8 1:1, 7 Log CFU/mL) and *W. anomalus* strain (F1.60.5; 5 Log CFU/mL)
Tonda Iblea cv.	C.TI	Green olive at 7% of NaCl
	LAB.TI	Green olives at 7% of NaCl and inoculum of 2 *L. plantarum* strains (F1.16 e C11C8 1:1, 7 Log CFU/mL)
	LAB.TI n	Black olives at 7% of NaCl and inoculum of 2 *L. plantarum* strains (F1.16 e C11C8 1:1, 7 Log CFU/mL)

The experimental samples were monitored by microbiological analysis after 1, 15 and 30 days (T1, T15 and T30) of fermentation. The experimental brines were analysed by serial dilution in saline (9 g/L) and inoculated in selective media (Table 2) for the cultivation and enumeration of specific microorganisms, as shown in the table below:

Table2. Microbial groups researched, selective media used and incubation conditions

Microrganismi	Terreno selettivo	Condizione incubazione
Mesofili aerobi totali	Plate Count Agar *(PCA)*	30°C/ 48h
Enterobatteri	Violet Red Bile Glucose Agar (VRBGA)	30-35°C/ 18-24h
Enterococchi	Kanamicine Aesculine Agar (KAA)	37°C/ 72h
Stafilococchi	Mannitol Salt Agar (MSA)	37°C/ 18-24h
Lieviti e Muffe	Sabouraud Dextrose Agar (SAB)	25°C/48-72h
Batteri Lattici	MRS Agar	30°C/ 48-72h
Escherichia coli	ChromaticTM EC X- GLUC Agar	44°C/ 18-24h
C. perfringers	Sulphite Polymyxin Suphadiazine (S.P.S) Agar	37°C/ 24-48h
B. cereus	Bacillus cereus Agar Base	30°C/ 24h

Chemical analysis

The experimental brines were subjected to pH and salt concentration determination. The pH was measured using a bench-top pH meter (XS Instruments), the salt concentration was determined by Mohr titration.

Results

Microbiological analyses

The table below shows the results of the microbiological analyses, conducted at times T1, T15 and T30. The results are expressed in Log CFU/mL ± standard deviation (Table 3)

Table 3. Microbial counts (expressed as log CFU/mL) at different fermentation times (T1, T15 and T30).

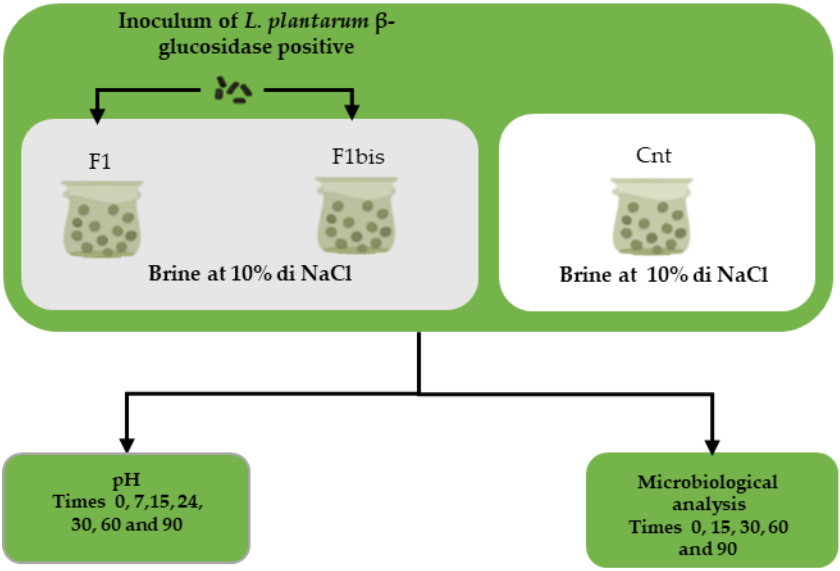
	T_1	T_{15}	T_{30}
Mesophilic bacteria			
C. NE	5.90±0.14	6.00±0.05	6.00±0.06
LAB1.NE	*6.67*±0.24	7.08±0.08	7.28±0.11
LAB1.NEbis	5.63±0.07	7.03±0.20	7.26±0.08
Y1.NE	5.51±0.05	7.86±0.05	4.95±0.05
Y1.NEbis	6.10±0.14	5.48±0.20	7.60±0.05
C.TI	4.60±0.14	7.97±0.10	6.90±0.06
LAB.TI	4.10±0.14	8.49±0.11	6.60±0.10
LAB.TI n	6.48±0.14	8.29±0.06	7.28±0.11
Lactobacilli			
C. NE	6.95±0.08	7.87±0.07	7.72±0.11
LAB1.NE	*7.00*±0.05	7.72±0.08	7.00±0.05
LAB1.NEbis	6.77±0.12	8.49±0.06	8.43±0.07
Y1.NE	6.30±0.05	7.62±0.07	5.18±0.06
Y1.NEbis	6.23±0.10	6.70±0.05	6.70±0.05
C.TI	4.70±0.09	7.54±0.05	6.61±0.05
LAB.TI	4.51±0.11	8.66±0.09	7.43±0.12
LAB.TI(n	7.23±0.06	7.97±0.10	7.38±0.10
Yeasts anf molds			
C. NE	6.92±0.10	8.00±0.07	6.70±0.06
LAB1.NE	*6.90*±0.11	8.15±0.05	7.78±0.12
LAB1.NEbis	4.90±0.06	9.00±0.05	7.43±0.09
Y1.NE	5.26±0.07	8.48±0.06	6.48±0.07
Y1.NEbis	6.00±0.20	6.00±0.10	6.30±0.11
C.TI	7.14±0.08	8.91±0.20	7.00±0.13
LAB.TI	7.63±0.06	9.26±0.07	6.70±0.07
LAB.TI n	5.79±0.13	8.90±0.07	7.85±0.06
Enterobatteriacee			

C. NE	3.35±0.06	<1	<1
LAB1.NE	*5.29*±0.07	<1	<1
LAB1.NEbis	3.35±0.06	<1	<1
Y1.NE	4.44±0.05	3.60±0.07	3.00±0.06
Y1.NEbis	4.53±0.05	<1	<1
C.TI	3.10±0.14	3.00±0.10	<1
LAB.TI	<1	<1	<1
LAB.TIn	3.64±0.20	3.00±0.06	<1
Enterococci			
C. NE	6.05±0.07	4.54±0.05	5.70±0.06
LAB1.NE	6.35±0.07	4.18±0.09	5.61±0.10
LAB1.NEbis	<1	<1	<1
Y1.NE	<1	<1	<1
Y1.NEbis	<1	<1	<1
C.TI	<1	4.04±0.20	5.71±0.12
LAB.TI	<1	4.11±0.05	4.91±0.09
LAB.TI n	<1	4.08±0.12	4.88±0.11
Coagulase positive staphylococci			
C. NE	*<1*	6.00±0.05	<1
LAB1.NE	*<1*	5.76±0.07	*<1*
LAB1.NEbis	<1	5.65±0.06	<1
Y1.NE	<1	5.48±0.012	<1
Y1.NEbis	<1	<1	<1
C.TI	<1	5.79±0.05	<1
LAB.TI	<1	3.00±0.06	<1
LAB.TIn	<1	5.86±0.07	<1
Coagulase negative staohylococci			
C. NE	*<1*	5.78±0.07	*<1*
LAB1.NE	*<1*	5.64±0.20	*<1*
LAB1.NEbis	<1	5.72±0.10	<1
Y1.NE	<1	5.53±0.07	<1
Y1.NEbis	<1	<1	<1
C.TI	<1	5.28±0.08	<1
LAB.TI	<1	<1	<1
LAB.TIn	<1	5.73±0.09	<1

A significant reduction in the total aerobic mesophilic load was achieved for all the samples examined, with the exception of the Nocellara Etnea control (Table 3). With regard to lactobacilli, an increase at 15 days of fermentation and a reduction at 30 days was recorded in all the samples analysed, with the exception of the Nocellara Etnea control, which presented an almost constant value (1 x 10^7 CFU/mL). A similar trend was recorded for yeasts, with an average decrease of approximately 2 log units, except for sample Y1. Enterobacteriaceae counts were below the detection sensitivity limit, while enterococci showed values of approximately 5 log units in samples C.NE, LAB1.NE, C.TI, LAB.TI n and below the limit of quantification in samples LAB1.NEbis, Y1.NE, Y1.NEbis. Coagulase negative and coagulase positive staphylococci, *Escherichia coli*, *Clostridium perfringens*, *Bacillus cereus* were below the quantification limit (data not shown) (Table 3).

Phase II

Based on results, a further trial was set up only treating the Nocellara del Belice cultivar, wich was inoculated with lactobacilli strains, selected for their technological performance. Fermentation was monitored for 90 days through microbiological and chemical analyses.



Preparation of experimental samples and inoculation of β-glucosidase positive starter strains

Starter strains belonging to *Lactiplantibacillus plantarum* species, from the microbial collection of the Department of Agriculture, Food and Environment (Di3A) of the University of Catania, were used for the experimental fermentations. The selected strains F1.16, F3.3 and C11C8 were previously isolated from table olive brines of the Nocellara Etnea cultivar and tested for phenotypic, genotypic and technological characteristics. The technological suitability of the strains was assessed

by considering specific selection criteria, such as: the ability to grow under different acid, osmotic and temperature stress conditions, the identification of the gene encoding for the β-glucosidase enzyme and, finally, the ability to degrade oleuropein, the bitter compound of table olives. The selected strains were inoculated at a cell density of 7 log CFU/mL. All fermentations were maintained and monitored at room temperature (18±2°C). The table 4 shows the code of the samples and the experimental conditions of the treated theses.

Table 4. Experimental conditions of Nocellara Etnea cv. green olives

Samples	Code	Experimental condition
Green olives Nocellara Etnea cv.	F1	Brine at 10% NaCl; inoculum of 3 *L. plantarum* strains (F1.16, F3.3 e C11C8 1:1:1, 7 log CFU/mL)
	F1bis	Brine at 10% NaCl; inoculum of 3 *L. plantarum* strains (F1.16, F3.3 e C11C8 1:1:1, 7 log CFU/mL)
	Cnt	Brine at 10% NaCl

Microbiological analysis of samples

The experimental fermentations were, to date, monitored by microbiological analysis after 1, 15, 30, 60 and 90 days (T0, T15, T30, T60 and T90). The experimental brines were analysed by serial dilution in physiological solution (9 g/L) and inoculated in selective media for the cultivation and enumeration of specific microorganisms, as reported below (Table 5).

Table 5. Microbial groups researched, selective media used and incubation conditions

Microrganismi	Terreno selettivo	Condizione incubazione
Mesphilic bacteria	Plate Count Agar *(PCA)*	30°C/ 48h

Enterobatteriaceae	Violet Red Bile Glucose Agar (VRBGA)	30-35°C/ 18-24h
Enterococci	Kanamicine Aesculine Agar (KAA)	37°C/ 72h
Staphylococci	Mannitol Salt Agar (MSA)	37°C/ 18-24h
Yeasts and Molds	Sabouraud Dextrose Agar (SAB)	25°C/48-72h
Lactobacilli	MRS Agar	30°C/ 48-72h
Escherichia coli	ChromaticTM EC X- GLUC Agar	44°C/ 18-24h
C. perfringers	Sulphite Polymyxin Suphadiazine (S.P.S) Agar	37°C/ 24-48h
B. cereus	Bacillus cereus Agar Base	30°C/ 24h

Chemical analysis

The experimental brines were subjected to pH and salt concentration determination. The pH was measured at times 0, 7, 15, 24, 30, 60, 90 using a bench-top pH meter (XS Instruments), the salt concentration was determined by Mohr titration.

Results

Microbiological analysis

The table shows the results of the microbiological tests, conducted at times T0, T15, T30, T60 and T90. The results are expressed in Log CFU/mL ± standard deviation (Table 6)

Table 6. Microbial counts (expressed as log CFU/mL) at different fermentation times (T1, T15 and T30).

	T_0	T_{15}	T_{30}	T_{60}	T_{90}
Mesophilic bacteria					
F1	6.20±0.14	6.00±0.05	5.90±0.06	5.85±0.09	5.67±0.20
F1bis	*6.17*±0.24	6.08±0.08	6.00±0.11	5.92±0.05	5.77±0.10
Cnt	6.50±0.06	7.03±0.20	7.26±0.08	7.02±0.07	6.94±0.05
Lactobacilli					
F1	6.00±0.14	7.10±0.05	7.02±0.06	7.00±0.05	6.92±0.07
F1bis	6.02±0.09	7.28±0.08	7.13±0.11	7.08±0.10	7.00±0.10
Cnt	5.63±0.07	6.03±0.20	5.86±0.07	5.76±0.08	5.16±0.11
Yeasts and Molds					
F1	5.90±0.14	6.00±0.05	6.30±0.06	6.30±0.06	5.00±0.06

F1bis	5.80±0.10	6.08±0.08	6.28±0.10	6.28±0.10	5.04±0.10
Cnt	5.97±0.07	6.64±0.20	7.00±0.08	7.00±0.08	6.30±0.07
Enterobatteriaceae					
F1	3.54±0.06	4.38±0.05	2.44±0.06	<1	<1
F1bis	3.53±0.12	4.42±0.08	2.50±0.11	<1	<1
Cnt	5.63±0.07	7.03±0.20	5.26±0.08	3.32±0.09	<1
Coagulase positive staphilococci					
F1	3.90±0.14	3.00±0.05	2.20±0.06	<1	<1
F1bis	3.87±0.24	3.08±0.08	2.28±0.11	<1	<1
Cnt	3.93±0.07	5.03±0.20	4.26±0.08	2.56±0.09	<1
Coagulase negative staphilococci					
F1	4.55±0.09	4.50±0.09	4.31±0.08	2.78±0.11	<1
F1bis	4.65±0.15	4.53±0.12	4.40±0.09	2.80±0.05	<1
Cnt	5.00±0.08	5.21±0.08	5.56±0.12	3.50±0.15	2.10±0.10
Enterococci					
F1	4.00±0.07	5.10±0.08	4.54±0.05	3.02±0.08	<1
F1bis	4.02±0.05	5.24±0.09	4.53±0.12	3.05±0.10	<1
Cnt	4.63±0.10	6.35±0.20	5.86±0.05	5.36±0.08	5.15±0.08

Chemical analysis

pH was monitored through the experimental fermentations, at times 0, 7, 15, 24, 30, 60, 90, and results showed a constant decrease in all the brine samples. In detail, in the inoculated samples, F1 and F1bis, it was possible to achieve values equal to or below 4.30, after 30 days of fermentation (Table 7)

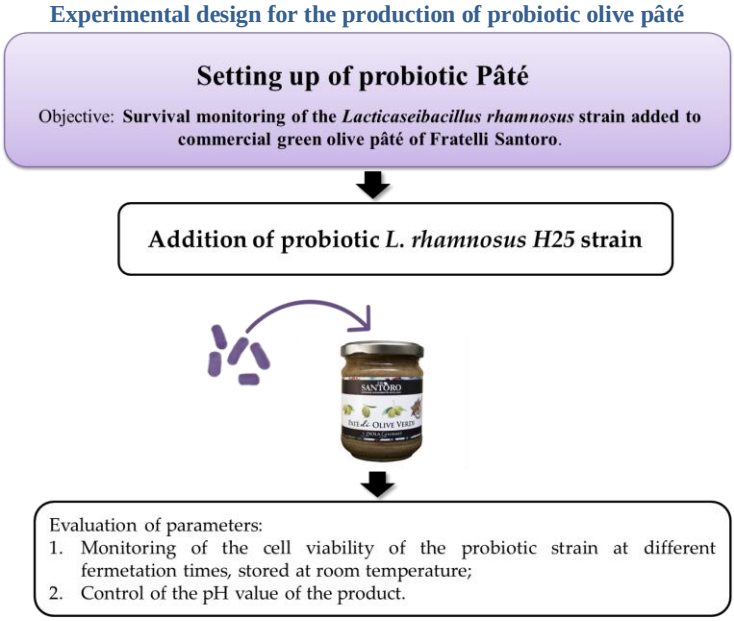
Table 7. pH values at different fermentation times 0, 7,15, 24, 30, 60, 90 days.

Sample	**pH**						
	T_1	T_5	T_{15}	T_{24}	T_{30}	T_{60}	T_{90}
F1	5.48	5.04	4.44	4.32	4.22	4.18	4.10
F1bis	5.51	5.10	4.56	4.36	4.20	4.18	4.12
Cnt	5.8	5.61	5.20	4.85	4.55	4.32	4.30

The second part of the project concerned the evaluation of survival of L. rhamnosus strain on Patè matrix.

Material and methods

In detail, the probiotic H25 strain, belonging to the *Lacticaseibacillus rhamnosus* species, previously characterized for probiotic characteristics and deposited at the Di3A microbial collection, was inoculated onto pate samples at a final concentration of 109 CFU/g.



Pâté samples inoculated with sterile saline were used as controls. The samples were placed at room temperature for 90 days and periodically analysed. To assess the survival of the probiotic strain, lactobacilli counts were performed in MRS agar medium, added with

cycloheximide. The plates were incubated anaerobically at 30 °C for 48-72h. Microbiological and chemical analyses were conducted in the pate samples.

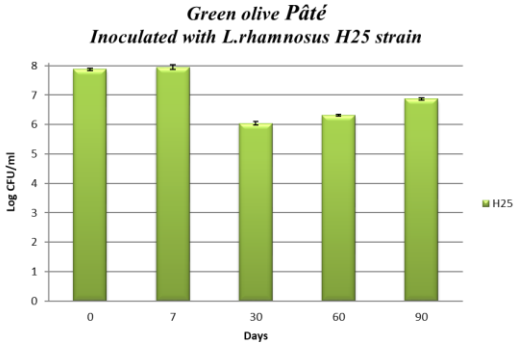
Results

The results of the microbiological analysis showed a good survival of the strain in the pate matrix. In detail, the cell density of the strain wa quite constant up to 7 days of storage at room temperature, while showed a low degree after 30 days of storage, reaching a degree of 3 log unit at 90^{th} day.

As far as pH monitoring is concerned, the values remain constant throughout the period considered in the control samples, while in the samples inoculated with the probiotic strain a slight increase was observed after 7 days of storage, and remains constant until the 60th day. A slight increase is observed at day 90. It is interesting to note that the pH values always remained below 4.5.

Microbiological analysis at different fermentation times

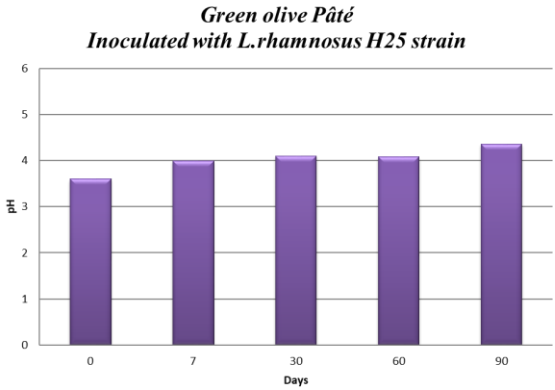
Inoculum at: 9 Log CFU/ml



Samples	Time	Log CFU/ml± dev.st
Controllo patè		<1
Patè Santoro	0	7,87±0,04
Patè Santoro	7	7,95±0,07
Patè Santoro	30	6,04±0,06
Patè Santoro	60	6,32±0,03
Patè Santoro	90	6,876±0,04

Chemical analysis

Monitoring of pH values at different fermentation times



During the fermentation process, the pH values were maintained below the threshold values of 4,5.

OTHER ACTIVITIES: Partecipation to Conferences

Partecipazione al XIII Convegno Nazionale sulla Biodiversità. Dipartimento di Scienze Agrarie, Alimenti, Risorse Naturali e Ingegneria (DAFNE) e Università degli Studi di Foggia. 7-9 settembre in modalità online. Poster sessione: 1c. Biodiversità microbica.

SELEZIONE DI CEPPI β-GLUCOSIDASI POSITIVI DA IMPIEGARE NELLA FERMENTAZIONE NATURALE DI OLIVE DA TAVOLA SICILIANE.

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Keywords: olive da tavola, Lactiplantibacillus plantarum, oleuropeina, β-glucosidasi.

Riassunto

L'impiego di ceppi starter β-glucosidasi positivi rappresenta un'innovazione biotecnologica utile per accelerare, controllare e condurre al meglio il processo fermentativo delle olive da tavola. Tali ceppi contribuiscono, inoltre, allo sviluppo delle caratteristiche sensoriali e all'incremento della sicurezza del prodotto finito. Ai fini dell'applicazione tecnologica, la scelta di colture starter, è basata sulla ricerca di criteri di selezione che mirano a valutare la sopravvivenza nelle condizioni stressanti di processo (temperatura, pH, pressione osmotica, ecc.). La specie *Lactiplantibacillus plantarum* è considerata una coltura starter d'eccellenza, per l'elevata versatilità e adattamento in diversi ambienti e matrici alimentari. In aggiunta, svolge un ruolo chiave nella fermentazione delle olive da tavola, principalmente per l'attività β-glucosidasica, necessaria per la deamarizzazione dell'oleuropeina, la cui presenza, in elevate concentrazioni, rende le

olive non edibili. L'obiettivo principale del presente lavoro è stato quello di valutare la crescita di ceppi ascritti alla specie *L. plantarum*, in diverse condizioni di pH, sale e temperature, al fine di stabilire le migliori condizioni di degradazione dell'oleuropeina, considerando i principali fattori di stress che caratterizzano il processo di produzione delle olive da tavola. ln dettaglio, nove ceppi di *L. plantarum* sono stati precedentemente isolati da olive da tavola siciliane, fermentate naturalmente, e identificati a livello di specie, mediante il gene *recA* PCR multiplex. Le performance di crescita dei ceppi sono state valutate nelle condizioni singole e combinate, a diverse concentrazioni di pH (4.5, 5.5 e 6.0), NaCl (5.0% e 6.0%) e temperature (32°C e 16°C), dopo 72 ore di incubazione. Inoltre, sulla base dei risultati conseguiti, è stata valutata la capacità di degradare l'oleuropeina, impiegando il terreno di coltura MRS modificato a pH 6.0 al 5.0% e 6.0% di NaCl e addizionato con oleuropeina pura (1g/L). La capacità degradativa è stata testata dopo 48 e 96 ore di incubazione, nelle rispettive temperature di 32 e 16°C. In aggiunta, per i ceppi in studio, è stata valutata la presenza del gene che codifica per l'enzima β-glucosidasi.

I risultati hanno evidenziato ottime performance di crescita nelle condizioni di stress singolo e multiplo, a 32°C, con un interessante incremento nella condizione combinata, MRS a pH 6.0 e NaCl 6.0%. Tuttavia, la diminuzione della temperatura di incubazione (16°C) ha influito in modo evidente sulle performance di crescita, evidenziando prestazioni significativamente inferiori da parte dei ceppi testati. Complessivamente la temperatura di 16°C sembra ostacolare le performance di crescita dei ceppi nelle condizioni combinate, con un tasso di crescita inferiore a 0.4 unità log nella condizione pH 5.5 e NaCl 6.0%. Inoltre, tutti i ceppi sono stati in grado di degradare l'oleuropeina nelle condizioni testate, a 32°C. In aggiunta, i ceppi F1.16, F3.2 e C11C8 hanno mostrato ottime performance degradative a 16°C, con capacità di riduzione del contenuto totale del 96, 95 rispettivamente,

nella condizione di NaCl 6.0% a pH 6.0. Inoltre, il gene che codifica per l'enzima β-glucosidasi è stato identificato nei ceppi F1.8M, F3.2, F3.3, F3.8 e C11C8. In conclusione, sulla base dei risultati ottenuti, i ceppi β-glucosidasi positivi F3.2 e C11C8, per i quali le performance di crescita e di degradazione dell'oleuropeina hanno dato esito positivo, nelle condizioni e temperature sopra citate, possono essere considerati ceppi promettenti da impiegare come colture starter nella fermentazione delle olive da tavola.

XIII Convegno Nazionale sulla Biodiversità. Dipartimento di Scienze Agrarie, Alimenti, Risorse Naturali e Ingegneria (DAFNE) e Università degli Studi di Foggia. 7-9 settembre in modalità online. Poster sessione: 4. Biodiversità e culture tradizionali.

STUDIO E CARATTERIZZAZIONE DEL MICROBIOTA DI IMPASTI ACIDI DI GRANI ANTICHI SICILIANI.

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Keywords: lieviti, LAB, PCR-DGGE, VOCs, sequenziamento

Riassunto

La pasta acida, un impasto di farina e acqua, spontaneamente fermentata da lieviti e batteri lattici (LAB) presenti nelle materie prime e nell'ambiente di lavorazione, rappresenta un ecosistema eterogeneo che ospita consorzi microbici molto complessi. Le interazioni tra i vari gruppi microbici che si instaurano nell'impasto e la cultivar di grano influenzano fortemente le caratteristiche nutrizionali e sensoriali del prodotto finito. Negli ultimi anni, la crescente attenzione dei consumatori verso i prodotti a filiera corta e la diffusione di attitudini salutistiche hanno suscitato grande interesse per i grani antichi. Tuttavia sono scarse le informazioni sulle paste acide ottenute con grano antico Maiorca (*Triticum vulgare* var. *albidum*), coltivato in Sicilia e recentemente apprezzato per il suo basso contenuto in glutine e la sua attitudine alla panificazione.

Lo scopo del presente lavoro è stato quello di analizzare e caratterizzare la biodiversità microbica di impasti acidi, prodotti con farina di Maiorca, presso 4 panifici situati in differenti zone della Sicilia, attraverso approcci fenotipici e molecolari e di valutarne gli effetti sulla composizione aromatica dei prodotti finiti. In dettaglio, i LAB, isolati mediante l'impiego di differenti terreni selettivi, sono stati clusterizzati

mediante 16S rDNA PCR-RFLP e tipizzati mediante $(GTG)_5$ fingerprinting. Ceppi rappresentativi di ciascun biotipo sono stati sottoposti al sequenziamento del gene 16S rRNA. I ceppi di lievito isolati sono stati caratterizzati fenotipicamente, e identificati mediante PCR-RFLP della regione ribosomiale includente lo spaziatore interno ITS1, il gene 5.8S rDNA e lo spaziatore interno ITS2 (genericamente riferita come ITS) e mediante sequenziamento della regione D1/D2 del gene 26S rRNA. Infine, il profilo aromatico dei campioni di pasta acida è stato determinato mediante analisi SPME-GC-MS.

I risultati ottenuti hanno evidenziato la presenza, in tutti i campioni analizzati, di poche specie di LAB eterofermentanti, con una netta dominanza (80%) della specie *Levilactobacillus brevis*, in accordo con quanto riportato in letteratura sui grani teneri italiani. Tra i lieviti, *Wickerhamomyces anomalus* è stata la specie maggiormente rilevata in tre dei quattro campioni analizzati, seguita da *Saccharomyces cerevisiae*. Solo su alcuni campioni è stata, inoltre, riscontrata la presenza di specie minoritarie, quali *Pichia kluyveri*, *Candida diddensiae*, e *Candida boidinii*, specie metilotrofica e xilosio-fermentante. Diversamente da quanto riportato in letteratura, non è stata riscontrata la specie *Kazachstania humilis* (syn. *Candida humilis*), generalmente associata alla presenza di *Fructilactobacillus sanfranciscensis*.

Sebbene gli impasti acidi analizzati abbiano presentato un profilo aromatico differente in termini quali e quantitativi, è stato possibile correlare la presenza di esteri e terpeni alla dominanza di specie di LAB e di lieviti, evidenziando l'importanza del microbiota degli impasti nello sviluppo dei composti aromatici del prodotto finito.

5th International Conference on Microbial Diversity. Microbial Diversity as a Source of Novelty. September 25-27, 2019. Catania, Italy.

Selection of β-glucosidase positive strains from naturally fermented table olives

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Introduction

Oleuropein is a β-glucosidase compound responsible for intense bitter flavour of drupes and its hydrolysis is necessary to make the final product edible for the consumer. The use of microbial starter cultures, able to degrade bitter compounds, is useful to accelerate, control and lead the fermentative process, maintaining the original organoleptic characteristic of the final product. *Lactobacillus plantarum* species is considered a potential starter culture in fermented table olives, for the high versatility, adaptation in different environments, ability to degrade oleuropein and for salt tolerance. The aim of the present study was to select β-glucosidase positive strains able to conduct the fermentation and to accelerate the debittering process of table olives. For this purpose, the oleuropein-degrading ability of *Lactobacillus plantarum* strains was tested under different stress conditions and the gene encoding for the β-glucosidase enzyme was detected.

Materials and Methods

The beta-glucosidase activity of *L. plantarum* strains, previously isolated from Nocellara Etnea table olives and identified by sequencing, was determined enzymatically by using p-nitrophenyl-β-D-glucoside substrate, under the following stress condition: salt concentration (4.0, 5.0, 6.0), pH (4.5, 5.5) temperature (16 °C and 32 °C). The enzymatic test was also performed in simulated brine medium at the same stress conditions described above. In addition, the ability to degrade

oleuropein was estimated using MRS broth added with pure oleuropein (1g/L), under the stress condition mentioned above. For each *L. plantarum* strain the presence of the gene encoding for the β-glucosidase enzyme was investigated through PCR assay.

Results

The results obtained showed good growth performances under different stress conditions for all tested strains. Furthermore, the strains demonstrated both good β-glucosidase activity and oleuropein-degrading ability. In particular, the strains C11C8, F3.6, F3.7, F3.8 and F3.9 exhibited the highest β-glucosidase activity in brine at 5% NaCl at 16°C, while the strains F1.8M and F1.10 in brine at 4% NaCl at 32°C. The presence of the gene encoding for β-glucosidase enzyme was revealed only in the strains F1.16, F3.2, F3.6, F3.9 and C11C8.

Conclusions

The present study allowed to select *L. plantarum* strains with promising oleuropein-degrading ability under stress conditions that could be considered as potential starter cultures for table olives fermentation.

5th International Conference on Microbial Diversity. Microbial Diversity as a Source of Novelty. September 25-27, 2019. Catania, Italy.

Isolation and characterization of promising probiotic lactobacilli and bifidobacteria from stools of breast-fed infants and honeybees' gut.

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Introduction

Members of the *Bifidobacterium* and *Lactobacillus* genera are widely recognized as health-promoting since they are able to exert nutritional or therapeutic benefits to the host. Recently, there is a growing interest in the isolation and identification of new potential probiotics to be used as feed supplements or to setup functional foods.

Materials and Methods

Honeybees' gut and fresh fecal samples of breast-fed Algerian infants, aged 3-6 months, were collected, homogenized and inoculated in a reducing medium containing Brain Heart Infusion (BHI) broth (Oxoid, Italy), 0.5% glucose, 0.5% yeast extract (Oxoid, Italy), 0.25% cysteine (Merck), 10 μg L^{-1} vitamin K1 (Sigma Aldrich, Italy), and 0.02 g L^{-1} hemin (Sigma Aldrich, Italy). Ten-fold serial dilutions were made and plated in duplicate on De Man, Rogosa and Sharpe agar (MRS, Oxoid, Italy) supplemented with 0.25 % (w/v) L-cysteine hydrochloride (MRSc) and on Bifidobacterium Selective Medium agar (BSM, Sigma Aldrich, Italy) and incubated under anaerobic conditions at 37°C for 24-72h. Forty-eight isolates were presumptively identified as *Bifidobacterium* sp. and *Lactobacillus* sp. based on morphological characteristics, physiological and biochemical properties such as

catalase, oxidase, spore formation, gelatinase activities, production of indole, NH_3 from arginine, and CO_2 from glucose. Molecular tools (genus-specific PCR and 16S rRNA gene sequencing) were applied for confirmation. The identified stains were screened for safety features (DNAse, gelatinase, haemolytic activity, antibiotic susceptibility, genes encoding for virulence factors and antibiotic resistance) and functional properties (resistance to low pH and bile salts, lysozyme tolerance, gastrointestinal survival, antagonistic activity against pathogens, hydrophobicity, auto-aggregation, and co-aggregation abilities). The strains, fulfilling the criteria described above, were evaluated for growth in camel milk and their viability, under refrigerated conditions, was monitored till 15 days.

Results

Seven stains ascribed to *Lactobacillus paracasei* and *Bifidobacterium asteroides* species beyond satisfying the safety requirements, were able to tolerate the harsh environmental condition occurring during the GIT passage and to antagonize both foodborne and intestinal pathogens. The selected stains were able to growth in camel milk and their viability was unaffected during refrigerated storage for 15 days.

Conclusion

The seven promising probiotic strains were able to grow and survive in camel milk suggesting their possible use for the formulation of new functional foods.

Ongoing research activities abroad

The interest in this complex food matrix is not only directed towards to the study of *Lactobacillus* strains as ideal starter cultures to best conduct the fermentation process, but also towards the yeasts population, which has always represented an important fraction of this complex microbial consortium. The relationship between these microbial populations has always been the subject of great interest, especially because they are responsible of the fermentation processes.

In this regard, the activity I am currently carrying out at the University of Extremadura, Badajoz, Spain, concerns the selection of yeast strains suitable for table olive fermentation. The strains under study were previously isolated from table olive brines, identified by sequencing and subjected to degrading-oleuropein activity. The strains will be further subjected to in vitro test, for technological properties, evaluating their growth performances at different salt and pH concentrations. In addition, mannoprotein will be isolated from the yeasts strains in order to evaluate the relationship with lactobacilli and how this mannoprotein can improve their survival under fermentation process.