NEW TRENDS IN TABLE OLIVE FERMENTATION, 2nd Edition

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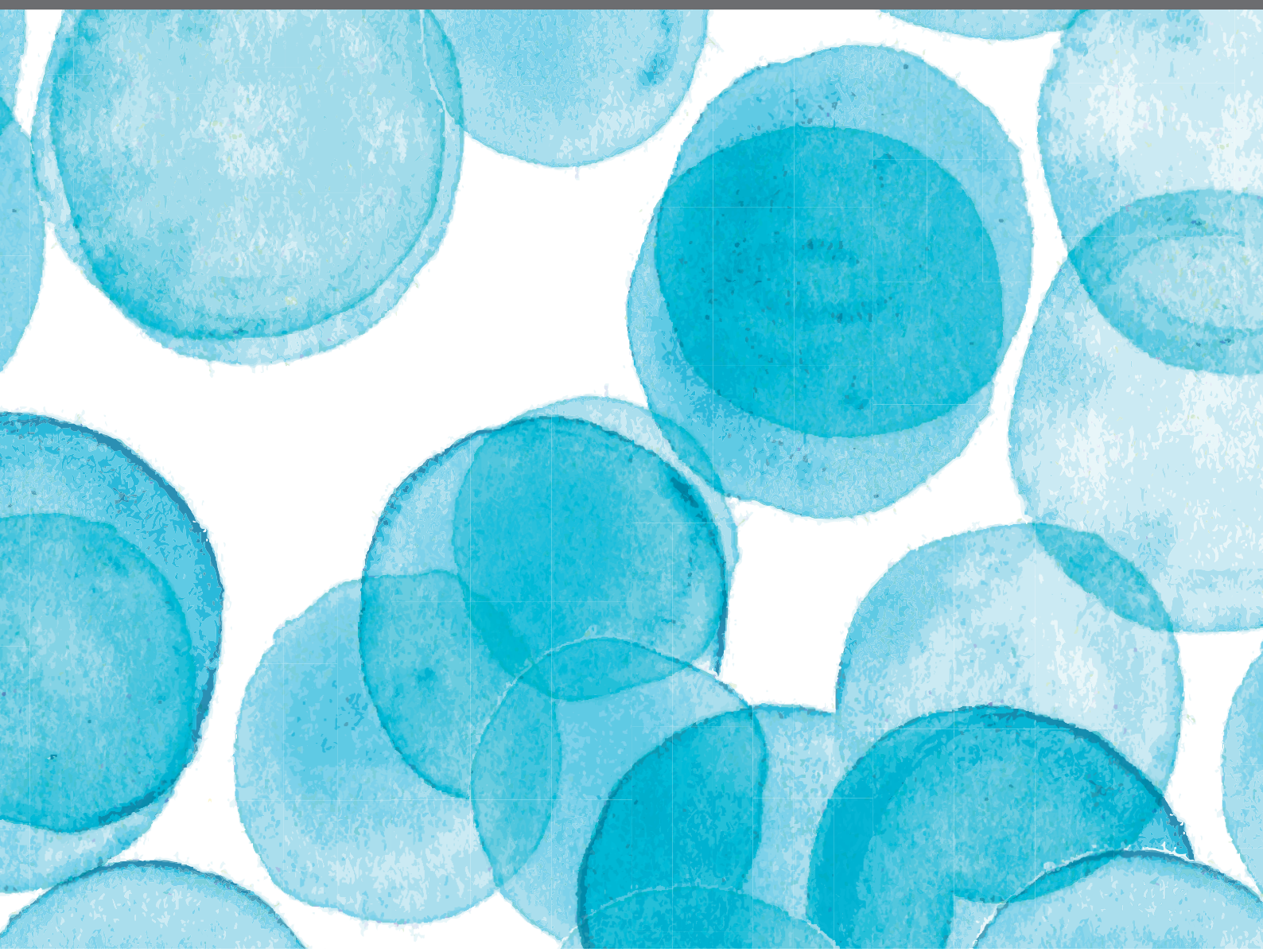

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NEW TRENDS IN TABLE OLIVE FERMENTATION, 2nd Edition

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Table olives are a traditional fermented vegetable with many centuries of history, particularly in the Mediterranean basin, where this food has had a great influence on the culture and diet of many countries. Moreover, this fermented food is prepared with fruits obtained from cultivated Olea eoropaea subsp. europaea var. europea trees and has been expanded for many countries all over the world. At present, the table olive is one of the major fermented vegetables, with an overall production above 2,500,000 tons/year. Thus, the table olive industry is increasingly demanding new biotechnological approaches, sensory characteristics and differentiation of the products. So scientists have to focus on solving problems and providing new tools in this fermented food process.

In recent years, there is an increased interest in different nutritional and microbial aspects related to table olives. During the last five years, new fields have been implemented or developed, such us new probiotic strains to produce an enriched product, study of pathogen survival, NaCl content reduction, microbial resistant to stress conditions, microbial biofilms, predictive microbiology, use of NGS and metagenomics, use of bioactive compounds derived from table olive processing and the treatment of effluents generated during olive processing.

The diversity of research displayed in this Research Topic demonstrates the important potential of this product and its impact on the fermented vegetables economy.

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Editorial: New Trends in Table Olive Fermentation

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Editorial on the Research Topic

New Trends in Table Olive Fermentation

Table olives are a traditional food of the Mediterranean Basin with many centuries of history. They are prepared with fruits obtained from cultivated *Olea europaea* subsp. *europaea* var. *europaea* trees and their production has been also expanded for many countries all over the world (South and North America, Australia, etc.). At present, table olives have an average annual worldwide production of 2.7 million tons (International Olive Oil Council (IOC), 2018), representing an important economic source in the producing countries.

This fermented vegetable has several interesting characteristics. From one side, table olives contain several attractive nutritional compounds such as monounsaturated and polyunsaturated fat, fiber, vitamins, or minerals; and from the other they have important concentrations of well-known bioactive compounds (Garrido-Fernández et al., 1997; Preedy and Watson, 2010). Thus, these properties have led scientists to propose table olives as a functional food. However, there are some drawbacks which have limited the use of this claim: (i) the high caloric content (ii) the sodium level (5–8%, except black olives by oxidation with 2% NaCl). However, because of the high presence of other healthy compounds, the olives could be considered as a healthy food, given the usual composition and proportion of some of its components (oleic acid, vitamin E, vitamin A, calcium, maslinic acid or hydroxytyrosol, among others). Moreover, table olives can be carrier of beneficial microorganisms to consumers (Peres et al., 2012), which is another exciting property from the functional point of view.

Finally, one of the most important challenge of the industry is the treatment of effluents generated during olive processing, because of their high volume of production, conductivity, and chemical oxygen demand (COD), especially in countries with scarce water resources.

The Research Topic "New Trends in Table Olive Fermentation" belongs to the Food Microbiology section in the Frontiers in Microbiology journal. It covers a total of 15 contributions divided in two reviews and 13 original research papers. Many of the most relevant researchers in the field have collaborated in its elaboration.

We present an overview of these papers which can be grouped under different research themes as follows: (i) microbial stability and safety conditions; (ii) table olives as carrier and source of probiotic lactic acid bacteria (LAB); (iii) the effect of new procedures on the sensory profile of table olives; and (iv) the management of table olive wastewater. The diversity of research displayed in this Research Topic demonstrates the important potential of this product and its impact in the fermented vegetables economy.

In the first group of papers, Perpetuini et al. have evaluated the impact of different irrigation regimes on the fermentation process of inoculated and spontaneous *Itrana* table olives. At harvest and fermentation, the concentration of phenolic compounds was always higher in olives from

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trees which had received less water. Furthermore, the use of a LAB starter positively influenced the fermentation process whereas the irrigation regime did not alter the microbial dynamics or brine acidification.

Bavaro et al. have described different molds isolated from Italian and Greek fermented black table olives. They carried out a technological and safety characterization and determined different strains belonging to *Penicillium roqueforti* (4) and *P. panem* (1) which could be used as co-starters with LAB and yeasts. Also, in black olives, Bonatsou et al. aimed at the yeast diversity during the *Kalamata* natural fermentation under different initial acidification procedures. The dominant yeast species at the beginning were *Aureobasidium pullulans* for control and acidified treatments, and *Candida naeodendra* for lactic acid treatment. At the end of the fermentation, the dominant species were *Candda boidinii* and *Candda molendinolei*. Thus, the different acidification agents, such as vinegar and lactic acid, affected the final composition of yeast species on olives.

Rodríguez-Gómez et al. have studied a high value green table olive variety *Aloreña de Málaga*, which has been the first variety in Spain under Protected Designation of Origin (PDO) recognized by the European Union (DOUE, 2012). Authors focused in the effects of a previous heat shock treatment on the fermentation and packing processes. Interestingly, the application of this treatment was beneficial since it favored the LAB growth, a better green appearance color of fruits and improved the stability of the packaged product. Regarding the same variety, Ruiz Bellido et al. deepened in the lack of standardization of production processes and HACCP systems. Authors based a decision-making scoring system on the identification of potential hazards or deficiencies in hygienic processes for the subsequent implementation of corrective measures to standardize production process. They found that corrective measures should be focused on reducing the microbial contamination of brines and fruits at primary steps and the implementation of novel treatments on olive dressings (irradiation, scalding, and ozonation). Furthermore, industry could reduce the levels of salt and preservatives in packaging producing a healthier product.

Regarding to the safety issues, Bevilacqua et al. have studied the effect of sugar, NaCl and temperature on the survival of *Staphylococcus aureus* and *Listeria monocytogenes* in synthetic brine. Thus, the addition of sugar (a widespread practice in producers) could be a challenge as it could increase the survival of some pathogens. In addition, Lavermicocca et al. delved in the bio-preservation of *Bella di Cerignola* table olives during refrigerated storage. Authors used *Lactobacillus plantarum* 5BG and showed a better performance by halving brining volumes and avoiding chemical stabilizers, and significantly reducing the salt concentration. In addition, the final product was also safely preserved for almost 5 months as suggested by the reduction of the survival rate of *L. monocytogenes*.

In their review, Campus et al. focused on current technologies and recent advances in the processing technology of table olives. They proposed the use of some pre-processing technologies (ionizing radiations, ultrasounds and electrolyzed water solutions), the use of LAB starter cultures, salt reduction strategies, table olives as carrier of probiotic LAB and new packaging technologies (modified atmosphere, high pressure processing and biopreservation).

With concern of table olives as source and carrier of probiotic lactic acid bacteria, four original researches overview different studies to their implementation. Guantario et al. have provided a first approach of the characterization and probiotic potential of LAB strains isolated from *Nocellara del Belice* table olives. One *Lactobacillus pentosus* and one *L. coryniformis* strains significantly induced prolongevity effects (*in vivo* study with the *Caenorhabditis elegans* model), protection from pathogen-mediated infection, adhesion to human intestinal epithelial Caco-2 cells and were able to outcompete foodborne pathogens for cell adhesion.

Pino et al. delved the fermentation of *Nocellara Etnea* table olives at low salt levels by the probiotic starter *Lactobacillus paracasei* N24, focusing on the volatile organic compounds (VOCs) formation. Even more, the starter was persistent until the end of the fermentation, revealing its promising perspectives as starter culture. Both microbial population and VOCs were slightly affected by salt content while a strong influence was determined by time of fermentation.

Rodríguez-Gómez et al. and have studied the factors that may affect the survival of a probiotic starter in large-scale fermentations of green Spanish-style olives. Authors proposed three important recommendations: inoculation after brining to reduce the presence of initial wild microorganisms, the use of re-inoculation to replace the possible initial died starter, and the starter survival is higher at the beginning of the season.

Abriouel et al. emphasized on the importance of the genetic base for the health promoting capacities of *L. pentosus* MP-10, isolated from *Aloreña de Málaga* table olives. Authors carried out *in silico* analysis of this strain's carbohydrate metabolism (as glycoside hydrolases, glycoside transferases, and isomerases) and the proteins (as mucus-binding) involved in the interaction with host tissues.

However, all modifications and starters used during table olive fermentations can alter the final product so researchers have to control their possible effect on the sensory profile. López-López et al. have evaluated the organoleptic profile by 200 consumers of traditional spontaneously fermented and potentially probiotic (inoculation of *L. pentosus* TOMC-LAB2) green table olives. Both final products were perceived similarly and they detected that the attributes with favorable influence on the *Overall score* and the *Buying predisposition* were *Appearance*, *Odor*, and *Crispness*; on the contrary, *Salty* had a marked adverse effect. Bautista-Gallego et al. have investigated the influence of the addition of zinc chloride in packaged natural black table olives. The presence of this salt in the packaging brine reduced the sugar diffusion and the bitter perception, while increased the overall acceptability, hardness and crunchiness.

Finally, Rincón-Llorente et al. have reviewed the problematic high quantity of wastewater produced by the table olive industry. At present, there is no standard treatment for these wastewaters with acceptable results. Twadahe most common treatment is the storage of wastewaters in large evaporation

ponds where disappear due to evaporation. However, this treatment also has a high number of problems (bad odors, insect proliferation, and the contamination of underground aquifers). Other alternative wastewater treatments could be ozonation, Fenton's reaction, TiO_2 photocatalysis, electro-coagulation, biological treatments (anaerobic or aerobic), and bioremediation technologies, among others. A promising alternative would be an integrated purification processes combining a first step of chemical oxidation (i.e., electrochemical treatments with boron-doped diamond), with a second biological step.

The varied contributions to this Research Topic are evidence of the study undertaken by researchers that provide an updated and high-quality overview of the current work on the table olive fermentation. We hope that this Research Topic informs readers properly about the benefit of this product and the challenges that have yet to be overcome in this field.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REFERENCES

DOUE (2012). Official Journal of the European Union Regulation N° 1068/2012. *L318/3*. 55, 3–4. doi: 10.3000/19770677.L_2012.318.eng

Garrido-Fernández, A., Adams, M. R., and Fernández-Díez, M. J. (1997). *Table Olives: Production and Processing*. Springer Science and Business Media.

International Olive Oil Council (IOC) (2018) *Updates Series of World Statistics on Production, Imports, Exports and Consumption*. Available online at: http://www.internationaloliveoil.org/estaticos/view/132-world-table-olive-figures (accessed October 23, 2018).

Peres, C. M., Peres, C., Hernández-Mendoza, A., and Malcata, F. X. (2012). Review on fermented plant materials as carriers and sources of potentially probiotic lactic acid bacteria–with an emphasis on table olives. *Trends Food Sci. Technol.* 26, 31–42. doi: 10.1016/j.tifs.2012.01.006

Preedy, V. R., and Watson, R. R. (Eds.). (2010). "Chemical composition of fermented green olives: fatty acids, sterols, and fatty alcohols," in *Olives and Olive Oil in Health and Disease Prevention* (Academic Press).

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Changes in Polyphenolic Concentrations of Table Olives (cv. Itrana) Produced Under Different Irrigation Regimes During Spontaneous or Inoculated Fermentation

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Irrigation is widely used for the production of table olives because it increases fruit size and yield. However, irrigation also determines less accumulation of total phenols, an increase in water content, a decrease of firmness, lower concentrations of soluble sugars in the mesocarp, thus positively or negatively affecting the fermentation process for the production of table olives. In this study we tested the hypothesis that green fruits of cultivar Itrana obtained by different irrigation regimes had different phenolic concentration that responded differentially to spontaneous or inoculated fermentation. Fruits were harvested from two orchards in the Latina province of Latium, Italy, which had been irrigated with different volumes of water during the growing season to compare the evolution of spontaneous and inoculated fermentation processes. We measured fruit characteristics at harvest, changes in the concentrations of secoiridoids and lignans, and main microbial groups abundance during fermentation. At harvest and during fermentation the concentration of phenolic compounds was higher in fruits sampled from trees that had received less water in the field. Differences were observed between spontaneous and inoculated fermentations, with a prevalence of lactic acid bacteria (LAB) in inoculated samples. In particular, oleuropein concentration completely disappeared only from samples inoculated with the two selected strains used as starters. The inoculum with selected LAB positively influenced the fermentation process of green olives, whereas the irrigation regime previously experienced by trees did not alter fermentation.

Keywords: table olive, irrigation, starter cultures, phenolic compounds, oleuropein, Itrana

Abbreviations: LAB, lactic acid bacteria; MRS, de Man, Rogosa and Sharpe; PAL, phenylalanine ammonia-lyase; 3,4-DHPEA-EDA, 2-(3,4-hydroxyphenyl)ethyl (3S,4E)-4-formyl-3-(2-oxoethyl)hex-4-enoate.

INTRODUCTION

Table olives are mainly produced in Spain, Turkey, Italy, Syria, and Greece, but the market for table olives is expanding well beyond the Mediterranean area. In 2017/18 the world production of table olives is estimated at 2951500 t confirming the increasing trend reported over the last few years (International Olive Council (IOC), 2017). This increase in production was due to good harvests in countries like Egypt, Turkey, Morocco, Argentina, and Tunisia (International Olive Council (IOC), 2017). Table olives are highly appreciated for their sensory characteristics and nutritional value. They also exert potential beneficial effects on human health since they are rich in antioxidant phenols (1–2% of fresh fruit), which are strong free-radical scavengers (Tataridou and Kotzekidou, 2015). Large differences in sensory and nutritional characteristics depend on the genotype and the processing method (Alagna et al., 2012; Kiai and Hafidi, 2014; Ambra et al., 2017). Italy is particularly rich in cultivars for table production, such as Ascolana Tenera, Cellina di Nardò, Itrana, Maiatica di Ferrandina, Nocellara del Belice, Nocellara Etnea (International Olive Council (IOC), 2000), that are widely appreciated as specialty foods. Many of these cultivars, including Itrana, can be actually considered dual purpose because they also produce excellent olive oils. The Itrana cultivar is the most widespread dual purpose (table and oil) cultivar in Latium (Central Italy). For table consumption it is commonly harvested green or black to undergo a process of natural fermentation (Tofalo et al., 2012). Commercially the cultivar Itrana is known as "Gaeta olive" since Gaeta was the port where these products were shipped from to reach far markets. Other factors affecting fruit characteristics include fruit development, crop load, climatic conditions, and cultural practices (Ryan and Robards, 1998; Gucci et al., 2007; Servili et al., 2007; Tura et al., 2008; Alagna et al., 2012).

In Mediterranean countries olive trees are usually grown under rainfed conditions because of the high resistance of *Olea europaea* L. to drought (Gucci and Fereres, 2012). However, exposure to long periods of water scarcity during the summer limits olive productivity (Gucci et al., 2007; Lavee et al., 2007; Gucci and Fereres, 2012; Torres et al., 2017). Irrigation, still seldom used in olive groves for oil production, is more common in plantations for table olive production, mainly because of the positive effects on fruit size, pulp-to-pit ratio, mesocarp cell size, chlorophyll content, productivity, and consequently, the commercial value of olive fruits (Costagli et al., 2003; Gucci et al., 2007, 2009; Lavee et al., 2007; Caruso et al., 2014; Torres et al., 2017). In addition, soil water availability reduces firmness and sugar content of the fruit and affects the concentrations of polyphenols, and secoiridoids in particular, in the oil (Gomez-Rico et al., 2006; Servili et al., 2007; Caruso et al., 2014). In particular, an abundant water supply during fruit development determines lower phenolic concentrations in the oil (Tovar et al., 2002; Gomez-Rico et al., 2006; Servili et al., 2007), probably because of changes both in the biosynthetic and catabolic pathways in the fruit (Alagna et al., 2012; Cirilli et al., 2017).

Water availability represents the main limiting factor for growth and yield of olive trees in the Mediterranean region. However, most papers focused on the evolution of phenolic compounds in olive oil (Gomez-Rico et al., 2006; Servili et al., 2007; Caruso et al., 2014), while no data are available regarding the changes in phenolic concentrations in cultivars for table production, despite the even more critical role played by irrigation on fruit quality and the effect of irrigation on microbial groups and starter cultures efficiency. For instance, it remains to be clarified whether different water regimes can have an impact on the fermentation process and the activity of starter cultures. The use of selected lactic acid bacteria (LAB) strains, associated or not with yeasts, is important since LAB are the main inducers of brine acidification which inhibits the growth of spoilage microorganisms and pathogens and are, therefore, fundamental for the stability of the final product (Corsetti et al., 2012). Moreover, LAB strains reduce debittering time and improve the sensorial and hygienic quality of the final product (Hurtado et al., 2012).

In this study a comparison between the spontaneous and inoculated fermentation processes of naturally fermented table olives from olive trees grown under different irrigation regimes was carried out. The objective of our study was to determine the effect of different irrigation regimes on the fermentation process of Itrana table olives. Two fermentation methods were compared whereby the process progressed spontaneously or was inoculated. In particular, two *L. pentosus* strains (C8 and C11) showing interesting (resistance to NaCl and oleuropein, short debittering time) table olive technological properties (Tofalo et al., 2014; Patent N0. 0001428559) were used as mixed starter cultures. Fermentations were monitored through the determination of pH and microbiological analyses and phenolic composition for 30 days.

MATERIALS AND METHODS

Plant Material and Fruit Sampling

Fruits were harvested on 22 October 2012 from olive (*Olea europaea* L.) trees subjected to different irrigation treatments established in two commercial olive orchards of cv. Itrana located at Fogliano (2 m a.s.l.) and Rocca Massima (375 m a.s.l.) in the Latina province of Latium, Italy. At the Fogliano orchard 6-year-old olive trees (cv. Itrana), spaced at 4 × 4 m, were used ($n = 3$). Water was supplied once a week or every 2 weeks, from July 1 through September 30, using drip lines (1.6 L h^{-1}, pressure compensated drippers spaced at 0.6 m). Each tree received about 375 and 750 L during the irrigation season (**Table 1**). At the Rocca Massima orchard 50-year-old olive trees, spaced at 5.5 × 6 m, were grown under either rainfed conditions (RF) or received two complementary irrigations (100 L/tree) on 28 July and 18 August ($n = 3$). Total precipitations from 1 June through 30 September were 202 mm (7, 16, 13, and 144 mm in June, July, August, and September, respectively) and 255 mm (0, 22, 89, and 166 mm in June, July, August, and September, respectively) at Fogliano and Rocca Massima, respectively.

About 5.5 kg of fruits per tree were harvested by hand from different zones of the canopy and carried in refrigerated boxes to the laboratory. An aliquot of 100 fruits was immediately weighed

TABLE 1 | Coding of treatments for the different locations, irrigation regimes, and starter type.

Treatment	Site	Irrigation	Water applied (L/tree)	Starter
A	Fogliano	Less	375	LAB
Ac		Less	375	Control
B		More	750	LAB
Bc		More	750	Control
C	Rocca Massima	Rainfed	0	LAB
Cc		Rainfed	0	Control
D		Complementary	200	LAB
Dc		Complementary	200	Control

for fresh weigh determination, then oven-dried at 70°C to constant weight, and the oil content in the mesocarp measured by nuclear magnetic resonance using an Oxford MQC-23 analyzer (Oxford /analytical Instruments Ltd., Oxford, United Kingdom) as reported in Caruso et al. (2013). About 3.5 kg of fruits were used for oil extraction and analytical determinations (Caruso et al., 2014).

Starter Strains and Preparation of Inocula

The two LAB strains (C8 and C11) used in this study were previously isolated from Itrana olive brine and technologically characterized. They were stored at −80°C in de Man, Rogosa and Sharpe (MRS) broth supplemented with glycerol (20% v/v final concentration). Strains belong to the collection of the Faculty of BioScience and Technology for Food, Agriculture and Environment of University of Teramo.

Before inoculation of the olive brine *L. pentosus* C11 and C8 were subcultured overnight at 30°C in MRS broth containing 4% NaCl (wt/vol) for adaptation to the saline environment. After preincubation C11 and C8 strains were centrifuged, washed in a saline solution, and resuspended in sterile brine (water containing 6% NaCl). Each strain was inoculated into the container of olives at a final cell count of approximately 6 log CFU/mL.

Olive Brining Procedure

The pilot-scale fermentations were performed in triplicate. Samples of healthy olives were washed, selected for a 10–12 mm caliber and processed according to the Greek-type protocol. Before processing, olives with mechanical or insect damage were discarded. Olives were put in sterile vessels, containing 1.5 kg olives and 1.5 L of brine (6% NaCl) and eventually inoculated. The olives were allowed to ferment at ambient temperature, in presence or absence of starter additions. Fermentations were carried out using fruits obtained from individual irrigation trials and the following codes were used (**Table 1**): A (Fogliano less irrigation + LAB); Ac (Fogliano less irrigation); B (Fogliano more irrigation + LAB); Bc (Fogliano more irrigation); C (Rocca Massima rainfed + LAB); Cc (Rocca Massima rainfed); D (Rocca Massima complementary irrigation + LAB); Dc (Rocca Massima complementary irrigation). Fermentations were monitored through the determination of pH and microbiological analyses at different times (0, 7, 15, and 30 days). Phenolic compounds in the olive mesocarp were determined before the beginning and at the end of the fermentation process.

Olive fermentation progress was considered ended when oleuropein disappeared from inoculated samples.

Microbiological Analysis

Microbiological analyses were performed on brines at different times. Aliquots of 25 mL of brine were diluted with sterile peptone water (0.1% w/v), homogenized with a Stomacher Lab-Blender 400 (Seward Medical, London, United Kingdom) for 2 min, serially diluted and plated in triplicate for microbial enumeration of the following microorganisms: total aerobic mesophilic bacteria (AMB) on Plate count agar (PCA) at 30°C for 2 days; LAB on MRS agar, at 30°C 2 days in microaerophilic conditions and yeasts on Yeast Peptone Dextrose Agar [YPD; 1% (wt/v) yeast extract, 2% (wt/v) peptone, 2% (wt/v) glucose and 2% (wt/v) agar] supplemented with chloramphenicol (150 mg/L) at 25°C for 3 days. The presence/absence of *Salmonella* spp., *Listeria monocytogenes* and *Escherichia coli* O157:H7 was determined according to standard methods (Association Française de Normalisation, 1997; International Organization for Standardization [ISO], 1998, 2002). All media and supplements were provided by Oxoid (Milan, Italy). The analyses were performed in triplicate.

Physico-Chemical Analysis of Olive Fruits and Brines

pH measurement was carried out on sample (10 mL) of brine using a pH meter MP 220 (Mettler, Toledo, Spain).

Phenolic compounds both olive and brine matrixes were extracted and evaluated by HPLC according to Servili et al. (2008). The concentration was expressed as mg of phenols/Kg of fruits and mg of phenols/L for brines. The dialdehydic form of elenolic acid linked to hydroxytyrosol (3,4-DHPEA-EDA), (+)-1-acetoxypinoresinol and (+)-pinoresinol were isolated from EVOO by semi-preparative HPLC according to Antonini et al. (2015); demethyloleuropein were isolated from the phenolic extract of olive fruit by semi-preparative HPLC according to Servili et al. (1999). Tyrosol (*p*-HPEA) was purchased from Fluka (Milan, Italy), hydroxytyrosol (3,4-DHPEA) was obtained from Cabru s.a.s. (Arcore, Milan, Italy), while the oleuropein and verbascoside were purchased from Extrasynthese (Genay, France). For each compounds were constructed calibration curves to obtain the real concentration.

Statistical Analysis

One-way analysis of variance followed by the Tukey test were performed by SigmaPlot software package, version 12.3 (Systat Software Inc., San Jose, CA, United States). Windows). Principal component analysis (PCA) was performed using the software XLStat 2014 (Addinsoft, New York, NY, United States).

RESULTS AND DISCUSSION

Effects of Irrigation on Fruit Characteristics

The use of irrigation was effective in determining changes in fruit characteristics from both sites. At the Fogliano location supplying more water resulted in higher fruit weight (both fresh and dry) (**Table 2**). Both the mesocarp and the endocarp were more developed in fruits from more irrigated trees, but the increase in mesocarp was more than proportional to that of the endocarp and, hence, the mesocarp-to-endocarp ratio of fruits sampled from more irrigated trees was greater. Similar results were obtained at the Rocca Massima site (**Table 2**) and confirmed the importance of irrigation to produce fruit of large size with a high pulp-to-pit ratio (Gucci et al., 2007, 2009; Lavee et al., 2007; Caruso et al., 2013). The different weights in fruits and fruit tissues harvested at the two orchards can be explained by the greater volumes of water received by all trees and the lower crop load at the Fogliano orchard. Interestingly, the mesocarp oil content of the more irrigated trees was significantly greater at both locations (**Table 2**). The oil content is relatively insensitive to soil water availability when the degree of water deficit experienced during fruit development is low, whereas it decreases if stress becomes severe (Gucci et al., 2007).

Acidification and Microbiological Analyses

All brine fermentations started with a mean pH value of 5. Within the 7th as apex day of fermentation the pH significantly decreased reaching values of about 4 in all inoculated samples regardless of the irrigation regime. At the end of fermentation, there were no differences among samples with pH values of about 3.7. This finding was consistent with other published data obtained using LAB during Greek- or Spanish-style processing (e.g., *L. plantarum, L. pentosus, L. paracasei*, and *L. rhamnosus*) (De Bellis et al., 2010; Aponte et al., 2012; Blana et al., 2014; Randazzo et al., 2014; Benincasa et al., 2015; De Angelis et al., 2015; Martorana et al., 2017). pH values below 4.5 inhibit the growth of Proteobacteria and other acid-sensitive bacteria avoiding olive spoilage and the development of pathogens during fermentation/storage (Perricone et al., 2010; De Angelis et al., 2015). The pH of uninoculated samples decreased more slowly as expected. After 30 days, control samples reached a value of 4.8 independently from the irrigation regime (**Figure 1**).

Microbial counts were not affected by the water regime, but showed a shift during fermentation (**Figure 2**). Pathogens were absent in all samples. The initial number of total AMB was about 7 log CFU/mL and then increased during the first 15 days of fermentation, while a decrease after 30 days was observed (about 6 log CFU/mL). This reduction could be related to the low pH values which probably determined a reduction/disappearance of

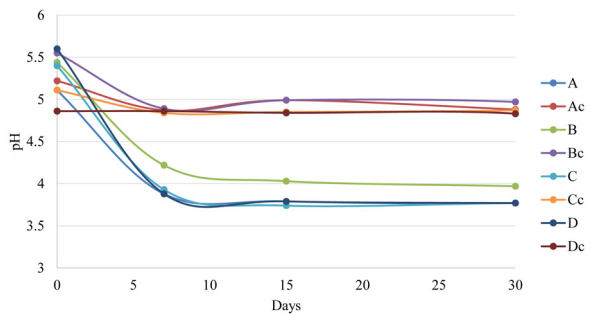

FIGURE 1 | pH evolution during table olive fermentation in inoculated and control samples. A (Fogliano less irrigation + LAB); Ac (Fogliano less irrigation); B (Fogliano more irrigation + LAB); Bc (Fogliano more irrigation); C (Rocca Massima rainfed + LAB); Cc (Rocca Massima rainfed); D (Rocca Massima complementary irrigation + LAB); Dc (Rocca Massima complementary irrigation).

TABLE 2 | Fruit parameters obtained from the different irrigation trials at two locations in 2012.

Fruit parameter	Fogliano		Rocca Massima	
	A	B	C	D
Mesocarp FW (g)	4.78 ± 0.08^a	3.24 ± 0.09^b	2.14 ± 0.23^a	1.30 ± 0.04^b
Endocarp FW (g)	0.98 ± 0.017^a	0.73 ± 0.001^b	0.65 ± 0.06	0.57 ± 0.02
Mesocarp DW (g)	1.16 ± 0.061^a	0.53 ± 0.004^b	0.64 ± 0.07^a	0.40 ± 0.03^b
Endocarp DW (g)	0.69 ± 0.011^b	0.82 ± 0.005^a	0.48 ± 0.03^a	0.42 ± 0.01^b
Fruit FW (g)	5.76 ± 0.09^a	3.97 ± 0.09^b	2.80 ± 0.30^a	1.87 ± 0.03^b
Fruit DW (g)	1.85 ± 0.058^a	1.35 ± 0.004^b	1.12 ± 0.10^a	0.81 ± 0.02^b
Mesocarp/Endocarp (FW)	4.87 ± 0.10^a	4.48 ± 0.12^b	3.30 ± 0.06^a	2.29 ± 0.14^b
Fruit DW/Fruit FW	0.32 ± 0.01^b	0.34 ± 0.01^a	0.44 ± 0.01^a	0.4 ± 0.01^b
Oil in mesocarp (% DW)	60.3 ± 1.54^a	56.2 ± 1.24^b	59.3 ± 0.78^a	51.6 ± 2.35^b

Values are means ± standard deviations of three trees for each irrigation treatment. Different letters indicate significant differences ($p < 0.05$) after analysis of variance within each location.

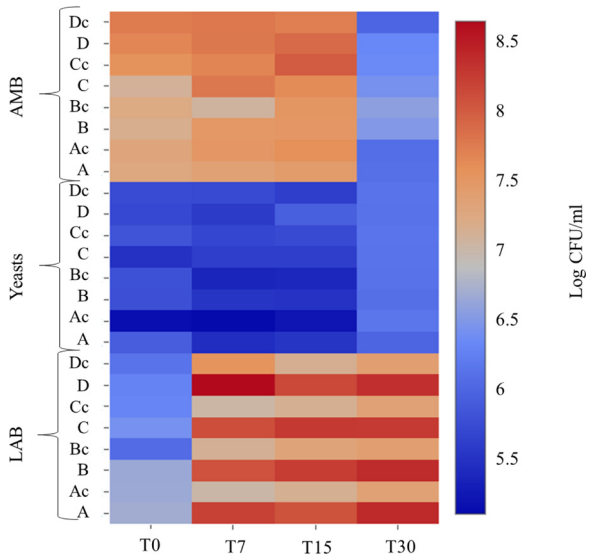

FIGURE 2 | Heatmap showing the distribution of some microbial groups (total aerobic mesophilic bacteria – AMB, LAB, yeasts) in inoculated and control samples during the fermentation process. After an adaptation step C11 and C8 strains were inoculated at a final cell count of approximately 6 log CFU/mL. *Salmonella* spp., *Listeria monocytogenes* and *Escherichia coli* O157:H7 were absent in all samples.

Enterobacteriaceae (Panagou et al., 2003; Abriouel et al., 2011). Yeasts populations showed an initial level of about 5 log CFU/mL and increased of about 1 log at the end of fermentation in all samples. Regarding LAB, the initial cell density was about 6 log CFU/mL at T0. In inoculated samples an increase of LAB counts was observed. The water regime did not influence their kinetics since in all inoculated samples the final counts were around 8 log CFU/mL, while in control fermentation vats a final value of 7 log CFU/mL was reached. The more rapid growth of LAB in inoculated samples and their higher count is in agreement with the better acidification rate of these samples compared to control ones. The highest concentration of LAB can be considered a guarantee for the quality of the final product (Corsetti et al., 2012). In fact, the monitoring of pH clearly shows the positive effect of the starter strains. Therefore, we can conclude that the irrigation regime did not influence the fermentation performance of starter strains and, in general, no differences in acidification dynamics and cultivable microbiota growth were evident between the samples obtained under different irrigation treatments.

Phenolic Compounds Evolution

The phenolic composition is influenced by many factors, such as cultivar, fruit development, climate conditions, and cultural practices including water regime (Servili et al., 2007; Alagna et al., 2012; Caruso et al., 2017; Cirilli et al., 2017). The majority of studies focused on the impact of irrigation system on phenolic compound profile of olive oil, while very few data are available about the impact of water regimes on table olives polyphenolic profiles.

The different irrigation regimes significantly affected the phenolic concentrations in the mesocarp (**Table 3**). Oleuropein was the most abundant phenolic compound and accounted for 48 and 63–68% of total phenolic fractions at the Fogliano and Rocca Massina trials, respectively. Significant differences were observed in the concentrations of the oleuropein, derivative of oleuropein (3,4-DHPEA-EDA) and verbascoside, whereas the lignans (+)-1-acetoxypinoresinol and (+)-pinoresinol were unaffected by the water supply. The irrigation treatments produced an average decrease of the verbascoside content of 36.2%, oleuropein of 9.0%, and 3,4-DHPEA-EDA of 6.3%. Results partially matched those obtained by other authors (Patumi et al., 2002; Tovar et al., 2002; Servili et al., 2007). It is generally accepted that phenolic compounds are more abundant in drought-stressed olive trees than in irrigated ones. These results confirm for cv. Itrana the typical response of secoiridoids concentrations to soil water availability already reported for several other cultivars: as the degree of water deficit becomes more severe phenolic concentrations increase in the fruit and the oil (Tovar et al., 2002; Servili et al., 2007; Caruso et al., 2014). In Greek cultivars, an increase in total phenol content, mainly due to a rise in oleuropein content has been observed under severe water stress (Petridis et al., 2012). A positive relationship between total phenol content and antioxidant activity has also been detected, suggesting that phenols could play a relevant role in the protection against the effects of drought (Petridis et al., 2012). This evidence could be related to drought-related variation in the enzymatic activity of phenylalanine ammonia-lyase (PAL), a key enzyme in the biosynthetic pathway of phenolic compounds, which is directly involved in the accumulation of polyphenols and o-diphenol contents in the olive fruit. It has been reported that the activity of the enzyme PAL in olive fruit decreased with increased irrigation (Patumi et al., 1999; Tovar et al., 2002). Modification of enzymatic activities could explain also other differences. The lower concentration of p-HPEA in drought-stressed samples may be a consequence of decreased activity of the endogenous esterase in the olive fruit that hydrolyzes the bond between p-HPEA and the elenolic acid of ligustroside (Servili et al., 2007).

The debitterig activity of LAB strains (C8 and C11) resulted in a strong decrease of oleuropein, demethyloleuropein, and 3,4-DHPEA-EDA in the olive pulp (**Table 4**). The reduction of oleuropein and 3,4-DHPEA-EDA of inoculated olives was also accompanied by an increase in their hydrolysis products (hydroxytyrosol), confirming the enzymatic activity of these strains affect the secoiridoid glucosides and their aglycon derivatives (3,4-DHPEA-EDA) (Servili et al., 2006, 2008). On the contrary, enzymatic activity did not influence the decrease of verbascoside, indeed in agreement with results reported previously (Servili et al., 2006, 2008) no hydrolytic products (caffeic acid) were found in the olive fruits or brine (data not shown). The reduction of the verbascoside observed in the olives after fermentation, seems to be due to its release in the brine after the process (**Table 5**). The results obtained showed that the microbial combination of the two bacterial strains used was able to carry out the debittering process starting from olives characterized by a different phenolic composition and

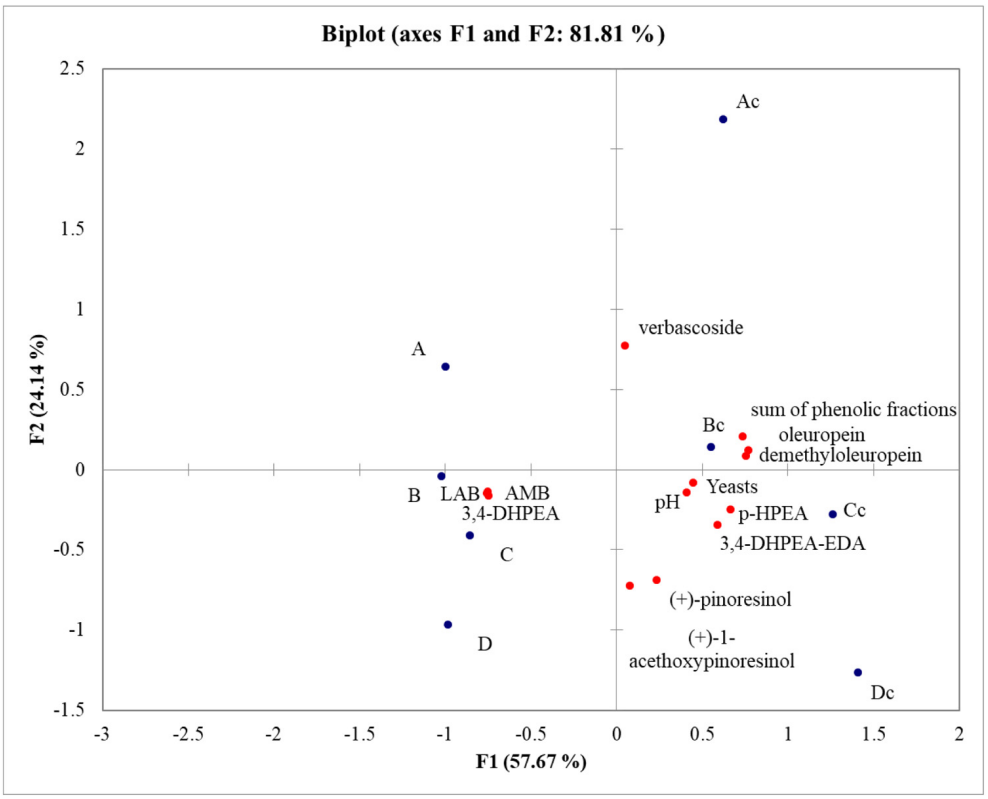

FIGURE 3 | PCA analysis based on brine acidification, microbial groups and amount of phenolic compounds in olives at the end of fermentation.

TABLE 3 | Phenolic concentration (mg/kg) of olive fruits of cv. Itrana obtained under different irrigation regimes.

Compound	Fogliano		Rocca Massima	
	A	B	C	D
3,4-DHPEA	495 ± 6.6[a]	479 ± 5.2[b]	175 ± 7.8[a]	400 ± 5.3[b]
p-HPEA	75 ± 5.1	68 ± 2.9	251 ± 21.2[a]	129 ± 8.5[b]
Demethyloleuropein	120 ± 10[a]	220 ± 19.4[b]	352 ± 16.2	345 ± 11.4
Verbascoside	1491 ± 53.5[a]	2498 ± 70.5[b]	908 ± 47.3[a]	1338 ± 69.5[b]
3,4-DHPEA-EDA	3088 ± 28.8[a]	3221 ± 75.7[b]	7300 ± 174.4[a]	8048 ± 231.4[b]
Oleuropein	10354 ± 128.9[a]	11177 ± 177.7[b]	8404 ± 210.1[a]	9408 ± 125.5[b]
(+)-1-acetoxypinoresinol	6 ± 0.1	6 ± 0.4	6.5 ± 0.7	6 ± 0.2
(+)-pinoresinol	1.3 ± 0.1[a]	0.34 ± 0.01[b]	2.2 ± 0.2	2.1 ± 0.1
Σ Phenolic fractions	15633 ± 136.9[a]	17670 ± 205.8[b]	17469 ± 278.5[a]	19677 ± 272.7[b]

Fruits were harvested by hand on 22 October 2012. Values are means ± standard deviations of three trees for each irrigation treatment. Different letters indicate significant differences ($p < 0.05$) after analysis of variance within each location.

water regimes. In the current study we also found that the concentrations of total phenols and ortho-diphenols in the oils of more irrigated trees were lower than in rainfed or less irrigated treatments (Supplementary Table S1).

Statistical Analysis

In order to understand the variability among samples PCA analysis was performed. PCA explained 81.81% of the total variance. F1 accounted for 57.67% of the variance, while F2 explained 24.14% of the variance. Samples were well differentiated and the main differences were observed between inoculated and not inoculated samples. Table olives obtained with the addition of starter cultures were characterized by a high concentration of LAB, AMB, and 3,4 DHPEA. Irrigation system did not influence the fermentation outcome and the main microbial groups, but water availability impacted the amount of phenolic compounds. In fact, Rocca Massima rainfed (Cc) and Rocca Massima complementary irrigation (Dc) samples clustered together and were characterized by a higher concentration of almost all phenolic compounds detected (**Figure 3**).

TABLE 4 | Phenolic composition (mg/kg) of table olives after 30 days of fermentation.

Compounds	Ac	A	Bc	B	Cc	C	Dc	D
3,4-DHPEA[a]	521.2 ± 33.9[a]	1018.8 ± 55.7[b]	538.3 ± 35[a]	1110.6 ± 60.7[b]	559 ± 37.8[a]	1149.7 ± 92[bc]	388.9 ± 24.2[a]	1296 ± 103.7[c]
p-HPEA	58.3 ± 3.8[a]	39.5 ± 1.4[bd]	51.2 ± 3.3[ab]	56.6 ± 4.8[a]	112 ± 7.9[c]	25.1 ± 2.5[d]	206.7 ± 10.9[e]	49.3 ± 2.4[ab]
demethyloleuropein	200.3 ± 11[a]	n.d.	98.2 ± 5.4[b]	n.d.	256.0 ± 10.3[c]	n.d.	226.3 ± 17.1[ac]	n.d.
Verbascoside	2128.9 ± 138.4[a]	1549.4 ± 71.4[b]	1187.1 ± 87.2[c]	1060.3 ± 63.1[ce]	965.7 ± 50.1[e]	846.9 ± 67.8[e]	610.4 ± 31.4[d]	463.2 ± 61.1[d]
3,4-DHPEA-EDA	2788.2 ± 181.2[a]	1323.8 ± 101.4[b]	2682.5 ± 174.4[a]	1216 ± 55.2[b]	6950.9 ± 401.3[c]	3225.1 ± 209.3[a]	6165.1 ± 402.1[d]	1496.5 ± 99.8[b]
Oleuropein	8979.6 ± 493.9[a]	5 ± 0.4[b]	8899.8 ± 489.5[a]	3.5 ± 0.3[b]	8721.9 ± 547.4[a]	n.d.	7502.2 ± 525.2[c]	n.d.
(+)-1-acetoxypinoresinol	4.1 ± 0.1[a]	5.0 ± 0.3[abc]	5.2 ± 0.4[bc]	4.8 ± 0.2[ab]	5.1 ± 0.2[bc]	5.4 ± 0.3[bc]	5.8 ± 0.6[c]	5.3 ± 0.3[bc]
(+)-pinoresinol	1.0 ± 0.1[a]	1.1 ± 0.1[a]	1.8 ± 0.1[bde]	1.7 ± 0.1[bd]	1.9 ± 0.1[bce]	1.5 ± 0.1[d]	2.1 ± 0.2[ce]	2.0 ± 0.1[be]
sum of phenolic fractions	14682 ± 545.2[ae]	3942.6 ± 136[b]	13464.1 ± 528.1[a]	3453.5 ± 103.6[b]	17572.5 ± 681.8[c]	5253.7 ± 238.5[d]	15107.5 ± 662.[9e]	3312.3 ± 156.4[b]

[a]*Data are the mean values of three independent evaluations. Values in each row followed by different letters (a–e) are significantly different from one another at $p < 0.05$. n.d. not detected.*

TABLE 5 | Phenolic composition (mg/L) of brines after 30 days of fermentation.

Compounds	Ac	A	Bc	B	Cc	C	Dc	D
3,4-DHPEA[a]	41.8 ± 2.7[a]	648.8 ± 29.7[b]	42.9 ± 2.8[a]	653.2 ± 29.8[b]	154 ± 9.2[c]	1252.3 ± 93[c]	523.6 ± 31.4[d]	916.7 ± 66.9[e]
p-HPEA	8.1 ± 0.4[a]	22.1 ± 1.3[b]	24.3 ± 1.2[b]	13.2 ± 0.7[a]	30 ± 1.2[b]	110.8 ± 6.5[c]	7.7 ± 0.3[a]	75.2 ± 5.3[d]
demethyloleuropein	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
Verbascoside	198.7 ± 14.9[a]	614.7 ± 39.2[b]	144.4 ± 9.4[c]	390.8 ± 5.9[d]	256.1 ± 17.4[e]	111.1 ± 8.9[c]	317.6 ± 21.6[f]	118.8 ± 6.5[c]
3,4-DHPEA-EDA	323 ± 21[a]	525.7 ± 27.9[b]	384.9 ± 20.2[a]	530.8 ± 30.2[b]	125 ± 6.3[c]	1043.3 ± 93.1[d]	293.7 ± 14.7[a]	1315.7 ± 65.8[e]
Oleuropein	1117.4 ± 66.5[a]	144.7 ± 10.2[b]	953.7 ± 55.5[c]	158.9 ± 6.9[b]	686.2 ± 44.6[d]	50.4 ± 6.9[e]	419.5 ± 31.8[f]	31.6 ± 2.3[e]
(+)-1-acetoxypinoresinol	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
(+)-pinoresinol	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
sum of phenolic fractions	1689.0 ± 71.4[a]	1956.0 ± 57.5[b]	1550.2 ± 59.9[a]	1746.9 ± 43.4[ab]	1251.3 ± 49.2[c]	2567.9 ± 132.2[d]	1562.1 ± 51.8[a]	2458.0 ± 94.2[d]

[a]*Data are the mean values of three independent evaluations. Values in each row followed by different letters (a–f) are significantly different from one another at $p < 0.05$. n.d. not detected.*

CONCLUSION

This study highlighted the effect of water availability on phenolic compounds profile and fermentation outcome in inoculated and spontaneous fermentation. Microbial dynamics and brine acidification were not influenced by irrigation but only by the inoculation of starter cultures. Selected starter cultures were able to complete the fermentation in 30 days regardless of the irrigation regime, suggesting their adaptation to this ecological niche. Irrigation affected the content of phenolic compounds, which were present in higher concentration in fruits and oils from less irrigated trees. It is important to develop tailored starter culture and optimize irrigation strategies considering some factors such as rainfall seasonality, soil water-holding capacity, and crop evapotranspiration which could influence table olives quality.

AUTHOR CONTRIBUTIONS

AlC and RT contributed to the conception and design of the work and supervised all activities. MaS and GP performed microbiological analysis, elaboration, and interpretation of data. AuC, NG-G, and RP prepared starter cultures. RG and GC managed the irrigation plan, fruit sampling and determinations, MuS, SE, and SU evaluated the phenolic content. AlC, RT, MuS, GC, and RG drafted the manuscript. All authors approved the final version of the manuscript to be submitted for publication and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy and integrity of any part of the work are appropriately investigated and resolved.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.01287/full#supplementary-material

REFERENCES

Abriouel, H., Benomar, N., Lucas, R., and Gálvez, A. (2011). Culture independent study of the diversity of microbial populations in brines during fermentation of naturally fermented Aloreña green table olives. *Int. J. Food Microbiol.* 144, 487–496. doi: 10.1016/j.ijfoodmicro.2010.11.006

Alagna, F., Mariotti, R., Panara, F., Caporali, S., Urbani, S., Veneziani, G., et al. (2012). Olive phenolic compounds: metabolic and transcriptional profiling during fruit development. *BMC Plant Biol.* 12:162. doi: 10.1186/1471-2229-12-162

Ambra, R., Natella, F., Bello, C., Lucchetti, S., Forte, V., and Pastore, G. (2017). Phenolics fate in table olives (*Olea europaea* L. cv. Nocellara del Belice) debittered using the Spanish and Castelvetrano methods. *Food Res. Int.* 100, 369–376. doi: 10.1016/j.foodres.2017.07.027

Antonini, E., Farina, A., Leone, A., Mazzara, E., Urbani, S., Selvaggini, R., et al. (2015). Phenolic compounds and quality parameters of family farming versus protected designation of origin (PDO) extra-virgin olive oils. *J. Food. Compos. Anal.* 43, 75–81. doi: 10.1016/j.jfca.2015.04.015

Aponte, M., Blaiotta, G., La Croce, F., Mazzaglia, A., Farina, V., Settanni, L., et al. (2012). Use of selected autochthonous lactic acid bacteria for Spanish-style table olive fermentation. *Food Microbiol.* 30, 8–16. doi: 10.1016/j.fm.2011.10.005

Association Française de Normalisation (1997). *Microbiologie des Aliments e Recherche des Salmonella e Me'thode de Routine NF V 08–052*. Saint-Denis: Association Française de Normalisation.

Benincasa, C., Muccilli, S., Amenta, M., Perri, E., and Romeo, F. V. (2015). Phenolic trend and hygienic quality of green table olives fermented with *Lactobacillus plantarum* starter culture. *Food Chem.* 186, 271–276. doi: 10.1016/j.foodchem.2015.02.010

Blana, V. A., Grounta, A., Tassou, C. C., Nychas, G. J. E., and Panagou, E. Z. (2014). Inoculated fermentation of green olives with potential probiotic *Lactobacillus pentosus* and *Lactobacillus plantarum* starter cultures isolated from industrially fermented olives. *Food Microbiol.* 38, 208–218. doi: 10.1016/j.fm.2013.09.007

Caruso, G., Gucci, R., Sifola, M. I., Selvaggini, R., Urbani, S., Esposto, S., et al. (2017). Irrigation and fruit canopy position modify oil quality of olive trees (cv Frantoio). *J. Sci. Food Agric.* 97, 3530–3539. doi: 10.1002/jsfa.8207

Caruso, G., Gucci, R., Urbani, S., Esposto, S., Taticchi, A., Di Maio, I., et al. (2014). Effect of different irrigation volumes during fruit development on quality of virgin olive oil of cv Frantoio. *Agric. Water Manage.* 134, 94–103. doi: 10.1016/j.agwat.2013.12.003

Caruso, G., Rapoport, H. F., and Gucci, R. (2013). Long-term evaluation of yield components of young olive trees during the onset of fruit production under different irrigation regimes. *Irrig. Sci.* 31, 37–47. doi: 10.1007/s00271-011-0286-0

Cirilli, M., Caruso, G., Gennai, C., Urbani, S., Frioni, E., Ruzzi, M., et al. (2017). The role of polyphenoloxidase, peroxidase, and β-Glucosidase in phenolics accumulation in *Olea europaea* L. fruits under different water regimes. *Front. Plant Sci.* 8:717. doi: 10.3389/fpls.2017.00717

Corsetti, A., Perpetuini, G., Schirone, M., Tofalo, R., and Suzzi, G. (2012). Application of starter cultures to table olive fermentation: an overview on the experimental studies. *Front. Microbiol.* 3:248. doi: 10.3389/fmicb.2012.00248

Costagli, G., Gucci, R., and Rapoport, H. (2003). Growth and development of fruits of olive 'Frantoio' under irrigated and rainfed conditions. *J. Hortic. Sci. Biotechnol.* 78, 119–124. doi: 10.1080/14620316.2003.11511577

De Angelis, M., Campanella, D., Cosmai, L., Summo, C., Rizziello, C. G., and Caponio, F. (2015). Microbiota and metabolome of un-started and started Greek-type fermentation of *Bella di Cerignola* table olives. *Food Microbiol.* 52, 18–30. doi: 10.1016/j.fm.2015.06.002

De Bellis, P., Valerio, F., Sisto, A., Lonigro, S. L., and Lavermicocca, P. (2010). Probiotic table olives: microbial populations adhering on olive surface in fermentation sets inoculated with the probiotic strain *Lactobacillus paracasei* IMPC2.1 in an industrial plant. *Int. J. Food Microbiol.* 140, 6–13. doi: 10.1016/j.ijfoodmicro.2010.02.024

Gomez-Rico, A., Salvador, M. D., La Greca, M., and Fregapane, G. (2006). Phenolic and volatile compounds of extra virgin olive oil (*Olea europaea* L. cv. Cornicabra) with regard to fruit ripening and irrigation management. *J. Agric. Food Chem.* 54, 7130–7136. doi: 10.1021/jf060798r

Gucci, R., and Fereres, E. (2012). "Fruit trees and vines. Olive," in *Crop Yield Response to Water. FAO Irrigation and Drainage Paper*, Vol. 66, (Rome: Food and Agriculture Organization of the United Nations), 300–313.

Gucci, R., Lodolini, E. M., and Rapoport, H. F. (2007). Productivity of olive trees with different water status and crop load. *J. Hortic. Sci. Biotechnol.* 82, 648–656. doi: 10.1080/14620316.2007.11512286

Gucci, R., Lodolini, E. M., and Rapoport, H. F. (2009). Water deficit-induced changes in mesocarp cellular processes and the relationship between mesocarp and endocarp during olive fruit development. *Tree Physiol.* 29, 1575–1585. doi: 10.1093/treephys/tpp086

Hurtado, A., Reguant, C., Bordons, A., and Rozes, N. (2012). Lactic acid bacteria from fermented table olives. *Food Microbiol.* 31, 1–8. doi: 10.1016/j.fm.2012.01.006

International Olive Council (IOC) (2000). *World Catalogue of Olive Varieties*. Madrid: International Olive Oil Council.

International Olive Council (IOC) (2017). *Market Newsletter No 119 – September 2017*. Available at: http://www.internationaloliveoil.org/news/view/697-year-2017-news/881-market-newsletter-september-2017

International Organization for Standardization [ISO] (1998). *Microbiology of Food and Animal Feeding Stuffs e Horizontal Method for the Detection and Enumeration of Listeria Monocytogenes e Part 2: Enumeration Method*. Geneva: International Organization for Standardization 11290-2.

International Organization for Standardization [ISO] (2002). *International Organization for Standardization 16654. Microbiology of Food and Animal Feeding Stuffs e Horizontal Method for the Detection and Enumeration of Escherichia coli O 157, H7*. Geneva: International Organization for Standardization.

Kiai, H., and Hafidi, A. (2014). Chemical composition changes in four green olive cultivars during spontaneous fermentation. *LWT - Food Sci. Technol.* 57, 663–670. doi: 10.1016/j.lwt.2014.02.011

Lavee, S., Hanoch, E., Wodner, M., and Abramovitch, H. (2007). The effect of predetermined deficit irrigation on the performance of cv. Muhasan olives (*Olea europaea* L.) in the estern coastal plain of Israel. *Sci. Hortic.* 112, 156–163. doi: 10.1016/j.scienta.2006.12.017

Martorana, A., Alfonzo, A., Gaglio, R., Settanni, L., Corona, O., La Croce, F., et al. (2017). Evaluation of different conditions to enhance the performances of *Lactobacillus pentosus* OM13 during industrial production of Spanish-style table olives. *Food Microbiol.* 61, 150–158. doi: 10.1016/j.fm.2016.08.007

Panagou, E. Z., Tassou, C. C., and Katsaboxakis, C. Z. (2003). Induced lactic acid fermentation of untreated green olives of the Conservolea cultivar by *Lactobacillus pentosus*. *J. Sci. Food Agric.* 83, 667–674. doi: 10.1002/jsfa.1336

Patumi, M., d'Andria, R., Fontanazza, G., Morelli, G., Giorio, P., and Sorrentino, G. (1999). Yield and oil quality of intensively trained trees of three cultivars of olive (*Olea europaea* L.) under different irrigation regimes. *J. Hortic. Sci. Biotech.* 74, 729–737. doi: 10.1080/14620316.1999.11511180

Patumi, M., d'Andria, R., Marsilio, V., Fontanazza, G., Morelli, G., and Lanza, B. (2002). Olive and olive oil quality after intensive monocone olive growing (*Olea europaea* L., cv. Kalamata) in different irrigation regimes. *Food Chem.* 77, 27–34. doi: 10.1016/S0308-8146(01)00317-X

Perricone, M., Bevilacqua, A., Corbo, M. R., and Sinigaglia, M. (2010). Use of *Lactobacillus plantarum* and glucose to control the fermentation of "Bella di Cerignola" table olives, a traditional variety of Apulian region (Southern Italy). *J. Food Sci.* 75, M430–M436. doi: 10.1111/j.1750-3841.2010.01742.x

Petridis, A., Therios, I., and Samouris, G. (2012). Genotypic variation of total phenol and oleuropein concentration and antioxidant activity of 11 Greek olive cultivars (*Olea europaea* L.). *Hortscience* 47, 339–342.

Randazzo, C. L., Todaro, A., Pino, A., Pitino, I., Corona, O., Mazzaglia, A., et al. (2014). Giarraffa and Grossa di Spagna naturally fermented table olives: effect of starter and probiotic cultures on chemical, microbiological and sensory traits. *Food Res. Int.* 62, 1154–1164. doi: 10.1016/j.foodres.2014.05.056

Ryan, D., and Robards, K. (1998). Phenolic compounds in olives. *Analyst* 123, 31–44. doi: 10.1039/a708920a

Servili, M., Baldioli, M., Selvaggini, R., Macchioni, A., and Montedoro, G. F. (1999). Phenolic compounds of olive fruit: one and two dimensional nuclear magnetic resonance characterization of nuzhenide and its distribution in the constitutive parts of fruit. *J. Agric. Food Chem.* 47, 12–18. doi: 10.1021/jf9806210

Servili, M., Esposto, S., Lodolini, E., Selvaggini, R., Taticchi, A., Urbani, S., et al. (2007). Irrigation effects on quality, phenolic composition, and selected volatiles of virgin olive oils cv. Leccino. *J. Agric. Food Chem.* 55, 6609–6618. doi: 10.1021/jf070599n

Servili, M., Minnocci, A., Veneziani, G., Taticchi, A., Urbani, S., Esposto, S., et al. (2008). Compositional and tissue modifications induced by natural fermentation process in table olives. *J. Agric. Food Chem.* 56, 6389–6396. doi: 10.1021/jf8007019

Servili, M., Settanni, L., Veneziani, G., Esposto, S., Massitti, O., Taticchi, A., et al. (2006). The use of *Lactobacillus pentosus* 1MO to shorten the debittering process time of black table olives (Cv. Itrana and Leccino): a pilot-scale application. *J. Agric. Food Chem.* 54, 3869–3875. doi: 10.1021/jf053206y

Tataridou, M., and Kotzekidou, P. (2015). Fermentation of table olives by oleuropeinolytic starter culture in reduced salt brines and inactivation of *Escherichia coli* O157:H7 and *Listeria monocytogenes*. *Int. J. Food Microbiol.* 208, 122–130. doi: 10.1016/j.ijfoodmicro.2015.06.001

Tofalo, R., Perpetuini, G., Schirone, M., Ciarrocchi, A., Fasoli, G., Suzzi, G., et al. (2014). *Lactobacillus pentosus* dominates spontaneous fermentation of Italian table olives. *LWT-Food Sci. Technol.* 57, 710–717. doi: 10.1016/j.lwt.2014.01.035

Tofalo, R., Schirone, M., Perpetuini, G., Angelozzi, G., Suzzi, G., and Corsetti, A. (2012). Microbiological and chemical profiles of naturally fermented table olives and brines from different Italian cultivars. *Antonie Leeuwenhoek* 102, 121–131. doi: 10.1007/s10482-012-9719-x

Torres, M., Pierantozzi, P., Searles, P., Rousseaux, M. C., García-Inza, G., Miserere, A., et al. (2017). Olive cultivation in the Southern hemisphere: flowering, water requirements and oil quality responses to new crop environments. *Front. Plant Sci.* 8:1830. doi: 10.3389/fpls.2017.01830

Tovar, M. J., Romero, M. P., Girona, J., and Motilva, M. J. (2002). L-Phenylalanine ammonia-lyase activity and concentration of phenolics in developing olive (*Olea europaea* L. cv Arbequina) fruit grown under different irrigation regimes. *J. Sci. Food Agric.* 82, 892–898. doi: 10.1002/jsfa.1122

Tura, D., Failla, O., Bassi, D., Pedo, S., and Serraiocco, A. (2008). Cultivar influence on virgin olive (*Olea europea* L.) oil flavor based on aromatic compounds and sensorial profile. *Sci. Hortic.* 118, 139–148. doi: 10.1016/j.scienta.2008.05.030

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Isolation, Characterization, and Selection of Molds Associated to Fermented Black Table Olives

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Table olives are one of the most important fermented food in the Mediterranean countries. Apart from lactic acid bacteria and yeasts that mainly conduct the olive fermentation, molds can develop on the brine surface, and can have either deleterious or useful effects on this process. From the food safety point of view, occurring molds could also produce mycotoxins, so, it is important to monitor and control them. In this respect, identification of molds associated to two Italian and two Greek fermented black table olives cultivars, was carried out. Sixty strains were isolated and molecularly identified as *Penicillium crustosum* (21), *P. roqueforti* (29), *P. paneum* (1), *P. expansum* (6), *P. polonicum* (2), *P. commune* (1). A group of 20 selected isolates was subjected to technological (beta-glucosidase, cellulolytic, ligninolytic, pectolytic, and xylanolytic activities; proteolytic enzymes) and safety (biogenic amines and secondary metabolites, including mycotoxins) characterization. Combining both technological (presence of desired and absence of undesired enzymatic activities) and safety aspects (no or low production of biogenic amines and regulated mycotoxins), it was possible to select six strains with biotechnological interest. These are putative candidates for future studies as autochthonous co-starters with yeasts and lactic acid bacteria for black table olive production.

Keywords: table olives, fermentation, molds, starter, mycotoxins

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INTRODUCTION

During fermentation of table olives, molds can develop on the brine surface and produce a thick layer on the top. Mold growth during storage in the market can result in appearance of visible mycelia. They are generally considered spoilage microorganisms responsible for product alterations, such as flesh softening and development of moldy taste, flavor, and appearance. In table olives, the most representative identified mold genera are *Aspergillus* and *Penicillium* (Fernandez et al., 1997). Their presence reduces product acceptance by the consumers, and it is also of relevant interest for the safety of table olives, since they can be responsible for mycotoxin production. The occurrence of *Penicillium citrinum* and *P. verrucosum* during fermentation, in particular in black olives, was linked to the production of ochratoxin A (OTA) and citrinin, while the contamination by aflatoxin B_1 (AFB) is mainly related to *Aspergillus flavus* on damaged olives during drying and storage (El Adlouni et al., 2006; Ghitakou et al., 2006; Heperkan et al., 2006, 2009).

A survey, performed in Turkey in 2000–2001, for the presence of molds in several commercial products revealed that 77% of table olives examined samples contained high amount of citrinin (Heperkan et al., 2006), whereas AFB was detected in all 30 samples of table olives and olive pasta from Athens market and OTA in two out of 30 samples. In black olives from the Marmara Region (Turkey), in addition to citrinin, aflatoxin, patulin, and penicillic acid were also detected (Korukluoğlu et al., 2000). Although, their concentrations are very low, in Greek style black table olives produced in Morocco, OTA was detected in seven of ten samples, five samples contained OTA and citrinin, whereas four out of ten samples contained AFB (El Adlouni et al., 2006). In another study, although their levels were very low, AFB was found in 4 loose and in 6 packed olive samples out of 40 commercial samples, whereas OTA was found in 12 loose and in 11 packed olive samples out of 40 samples (Franzetti et al., 2011).

Even if mycotoxin levels detected in table olives were too low to cause diseases (Medina-Pradas and Arroyo-López, 2015), green and black table olives could be a possible source of mycotoxins.

Thus, some measures have been suggested to reduce the presence of molds and mycotoxin production along the entire table olive chain by applying good handling procedure during harvest, controlling storage conditions (temperature, packaging, salinity).

On the other hand, molds can be also useful microorganisms in food production. On sausage surfaces they can lead to desirable effects mainly related to successful production or consumer appeal. Selection and production of industrialized mold starter cultures allow olive producers to reduce the risks for consumer safety and to improve sausage organoleptic traits and taste. In fact, molds can have a role in sausage maturation, in aroma and texture improvement, in shortening ripening time and/or shelf life expansion. They can produce enzymes responsible of lipids and proteins modification and degradation and they can help in reduction of lipid oxidation (Sunesen and Stahnke, 2003).

In this paper, the identification of molds associated to two Italian and two Greek fermented black table olives cultivars, was carried out. For the first time, a multi-step selection protocol, consisting in both analytical and biochemical tests, has been proposed in order to identify mold candidates to be tested as autochthonous co-starter (together with already selected yeasts and lactic acid bacteria) for black table olive production.

MATERIALS AND METHODS

Brine Samples

Brine samples were collected during season 2012–2013 and 2013–2014 from Italian (Apulia) and Greek (Epirus) lab-scale (Bleve et al., 2014, 2015) and industrial fermentations of Cellina di Nardò, Leccino, Kalamàta, and Conservolea table olives.

Isolation of Fungi

The isolation of fungi from brines was carried out by serial dilution of samples in sterile water solution with 0.01% Tween 80 (Sigma-Aldrich, Darmstadt, Germany) added to assist the dispersal of conidia and transferring them to agar. One hundred microliter were spread on Dichloran Rose Bengal Chlorotetracycline (DRBC; Oxoid Ltd., Hampshire, UK) agar medium (King et al., 1979), in 90-mm petri dishes in triplicate and incubated at 25°C for 5–7 days in the dark. After incubation, single-spore isolations were made according to Crous et al. (2009) for representative colonies on the basis of their morphological traits (shape, size, and color of the colony; shape of cells). All strains isolated in this study were deposited in the ITEM collection (ITEM collection: http://www.ispa.cnr.it/Collection/). In addition, a subset of representative strains of *Penicillium* subgenus *Penicillium* used by Frisvad and Samson (2004) and available in the ITEM collection were included in the analysis as reference strains for species identification.

Molecular Identification of Fungi

DNA was extracted from mycelium of isolates grown in Wickerham's medium (glucose, 40 g; peptone, 5 g; yeast extract, 3 g; malt extract, 3 g; and distilled water to 1 L) and incubated in an orbital shaker (150 rpm) for 48 h at 25°C. All components of Wickerham's medium were purchased by LabM Limited (Lancashire, UK). Following incubation, the mycelia were filtered and lyophilized for total DNA extraction. DNA was extracted starting from 10 mg of lyophilized mycelium, grinded with 5 mm iron bead in Mixer Mill MM 400 (Retsch, Germany), and processed with "Wizard® Magnetic DNA Purification System for Food" kit (Promega, Madison, WI, USA). The quality of genomic DNA was determined by electrophoresis and the quantification using a Spectrophotometer ND-1000 (Thermo Fisher, Waltham, MA, USA). Isolates recovered from DRBC agar medium were sequenced firstly in ITS region and only *fungal* spp. isolates were further sequenced in beta-tubulin gene. Amplification of the ITS and β-tubulin DNA regions was performed using primers ITS4/ITS5 and Bt2a/Bt2b, respectively, specific to filamentous ascomycetes (White et al., 1990; Glass and Donaldson, 1995), according to published protocols. PCR reactions were performed in 20 μL reaction mixtures containing 1 μL DNA template (20 ng/μL), 2 μL PCR buffer, 15.3 μL ultra pure sterile water, 0.4 μL dNTP (10 mM), 0.6 μL of each primer (10 pmol/μL), and 0.1 μL Hot Master Taq DNA Polymerase (2.5 U/μL, 5 *PRIME* GmbH, Germany). Amplifications were performed in a GeneAmp PCR system 9700 (AB Applied Biosystems, CA).

PCR amplicons were purified using the enzymatic mixture EXO/SAP (Exonuclease I, *Escherichia coli*/Shrimp Alkaline Phosphatase; Thermo Fisher Scientific, Waltham, MA, USA) and sequenced in both strands using standard conditions with BigDye™ Terminator v3.0 Ready reaction Kit (Applied Biosystems, Foster City, CA, USA). Sequence reactions were analyzed using an ABI- Prism model 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) after purification by gel filtration through Sephadex G-50 (Little Chalfont, UK).

Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011) and inferred using the UPGMA method (Sneath and Sokal, 1973). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the number of

differences method (Nei and Kumar, 2000) and are in the units of the number of base differences per sequence.

Biochemical Analyses

All chemicals for biochemical analyses were purchased from Sigma-Aldrich (Darmstadt, Germany). To perform the screening tests on the biochemical activity, *Penicillium* isolates were cultivated on basal medium (LBM) containing per liter: KH_2PO_4 1 g, ammonium tartrate 0.5 g, $MgSO_4$ $7H_2O$ 0.01 g, $CaCl_2$ $2H_2O$ 0.01 g, yeast extract 0.001 g; $CuSO_4$ $5H_2O$ 0.001 g; $Fe_2(SO_4)_3$ 0.001 g; and $MnSO_4$ 0.001 g, and incubated in the dark at 25°C for 5 days. After this period, agar disks (6 mm in diameter) of active mycelia were plated on solid media containing the different substrates for the detection of beta-glucosidase, lypolitic activities, cellulase, laccase, tyrosinase, xylanase, lignin modifying, pectolytic, protease (gelatin and milk agar) activities. For all enzyme activities a score 3 (intense brown), 2 (light brown), 1 (yellow-milky), 0 (white) was assigned.

Beta-Glucosidase Activity

The activity of β-glucosidase was detected by growing the test fungus on agar containing esculin (6,7- dihydroxycomarin-6-glucosidase) as the sole carbon source. Cellulolysis Basal Medium (CBM; $C_4H_{12}N_2O_6$ 5 g, yeast extract 0.1 g, KH_3PO_4 1 g, $MgSO_4{\cdot}7H_2O$ 0.5 g, $CaCl_2{\cdot}2H_2O$ 0.001 g, in 1 l distilled water) was supplemented with 0.5% esculin (w/v), 1.8% (w/v) agar and autoclaved. One ml of a sterile 2% (w/v) aqueous ferric sulfate solution was aseptically added for each 100 ml of CBM. The medium was dispensed into Petri dishes, allowed to solidify, inoculated and incubated at 25°C for 7 days in darkness. A black color developed in the medium by the colonies producing β-glucosidase (Pointing et al., 1999).

Hemicellulolytic (Xylanolytic) Enzyme Assays

This enzymatic activity was detected through use of the XBM medium ($C_4H_{12}O_6$ 0.5 g, KH_2PO_4 1 g, $MgSO_4{\cdot}7H_2O$ 0.5 g, yeast extract 0.1 g, $CaCl_2{\cdot}2H_2O$ 0.001 g in 1 L distilled water) with 4% (w/v) xylan and 1.6% (w/v) agar and autoclaved. The medium was inoculated with the test fungus and incubated at 22°C for 7 days in darkness. The plates were stained with iodine (0.25% w/v aqueous I2 and KI), xylan degradation around the colonies appeared as a yellow-opaque area against a blue/red dish purple color for under grads xylan indicated endoxylanase activity (Pointing et al., 1999).

Proteolytic Plate Assay

The extracellular proteases were detected on agar plates, using different substrates Milk Agar (Tryptone 5 g, yeast extract 2.5 g, Dextrose 1.0 g, Skim Milk powder 1 g, 1.5% agar) supplemented with 0.0015% Bromocresol green (BCG) reagent and MEA supplemented with 1% of gelatin (MEAG) at 25°C and in presence or absence of 2.5% of NaCl. On Milk Agar, the enzyme activity was detected as clearer areas surrounding the colony, indicating that hydrolysis of the substrate had occurred. Also, it was developed a method to detect proteolytic activity using MEA as basal medium supplemented with 1% of gelatin. In this case, the detection of extracellular proteases was done after staining with Coomassie Blue (0.25% w/v) in methanol–acetic acid–water (5:1:4 v/v/v) for 1 h at room temperature and destining with methanol–acetic acid (Vermelho et al., 1996). Enzyme activity was detected as clear regions surrounding the colony, indicating that hydrolysis of the substrate had occurred (Ludemann et al., 2004).

Pectinolytic Activity

The extracellular pectinolytic activity was assessed by medium contained 500 mL of mineral salt solution, 1 g yeast extract, 15 g of agar, 5 g of pectin, and 500 mL of distilled water. The mineral salts solution contained per liter: $(NH_4)_2SO_4$ 2 g, KH_2PO_4 4 g, Na_2HPO_4 6 g, $FeSO_4$. 7 H_2O 0.2 g, $CaCl_2$ 1 mg, H_3BO_3 l g, $MnSO_4$ l g, $ZnSO_4$ l g, $CuSO_4$ l g, MoO_3 l g, pH 7, or pH 5 as needed. This medium at pH 7 was used to detect pectate lyase production. For all tests, plates were incubated for 5–10 days and then flooded with 1% aqueous solution of hexadecyltrimethyl ammonium bromide. This reagent precipitates intact pectin in the medium and thus a clear zone around a colony in an otherwise opaque medium indicates degradation of the pectin (Hankin et al., 1975).

Cellulase Activity

The hydrolysis of cellulose into sugars was investigated using carboxymethylcellulose (CMC) plates. CBM medium was supplemented with 2% low viscosity CMC and 1.6% agar. The plates were incubated for 5–10 days in darkness at 25°C. When the colony diameters were ∼30 mm were flooded with 2% aqueous solution of Congo Red and leaved for 15 min. Removed the stain and washed the agar surface with distilled water, the plates were flood with 1 M NaCl and discolored for 15 min. The CMC degradation around the colonies appeared as yellow-opaque area against a red color for under graded CMC (Pointing et al., 1999).

Laccase Assay

Laccase activity was determined with 2,2′-azino-di-(3-ethylbenzothialozin-6-sulfonic acid) (ABTS) as the substrate. LBM medium was supplemented with 0.1% (w/v) glucose, 1.6% (w/v) agar and sterilized. Aseptically was added 1 mL of a sterilized solution of aqueous glucose 20% to each 100 mL of growth medium prepared. The medium was inoculated with the test fungus. The plates were incubated for 10 days in darkness at 25°C. The production of laccase was detected as the formation of green color in the growth medium (Pointing et al., 1999).

Tyrosinase Assay

The tyrosinase enzyme is implicated in the detoxification of lignin breakdown products (Eaton and Hale, 1993). The production of tyrosinase can be assayed by the well test procedure using p-cresol (4-methoxyphenol). LBM medium was supplemented with 1.6% (w/v) agar and sterilized. Aseptically was added 1 mL of a separately sterilized 20% (w/v) aqueous glucose solution to each 100 mL of growth medium prepared. Test microorganism were inoculated and incubated at 25°C in darkness for 5–10 days. Spot tests were carried out as follows. Wells of approximately 5 mm in diameter were done in the agar medium and few drops of 0.1% (w/v) p-cresol in 0.05% (w/v)

aqueous glycine solution were added inside them. Presence of a red-brown color around the well indicated a positive result (Pointing et al., 1999).

Lignin Modifying Enzymes

Decolorization of the Remazol Brilliant Blue R (RBBR) by fungi has been positively correlated with production of the polyphenol oxidases lignin peroxidase, Mn-dependent peroxidase (Boominathan and Reddy, 1992) and laccase (Pointing et al., 1999). The test foresees the use of LBM medium supplemented with 0.05% (w/v) RBBR and 1.6% (w/v) agar and sterilized. Aseptically was added 1 ml of a separately sterilized 20% (w/v) aqueous glucose solution to each 100 mL of growth medium prepared. The fungi were inoculated and incubated at 25°C in darkness and examined plates daily for 10 days.

Production of Biogenic Amines

To assess the ability of the colonies to decarboxylate aminoacids producing biogenic amines a specific media has been designed. About 0.1 g of glucose, 0.06 g of bromocresol purple, 1.5% (w/v) agar, and 10 g of each amino acid to be tested were dissolved in 900 mL of demineralized water. After sterilization, 100 mL of yeast nitrogen base (Difco Laboratories, Franklin Lakes NJ, USA) solution (6.7% w/v), previously sterilized by filtration, were aseptically added. Final pH was adjusted to 5.3 ± 0.02 using HCl. The amino acids tested were histidine, phenylalanine, tyrosine, ornithine, and lysine (Sigma-Aldrich, Darmstadt, Germany). The colonies were streaked on the surface of the agar plates and then incubated at 25°C for 4 d. At the pH of the plates the dye was yellow. Slight increase of pH turned this color to purple. The reaction was considered positive if a violet halo surrounded the colonies (Gardini et al., 2006).

Lipolytic Activity

For assaying total lipolytic activity olive oil and rhodamine B were used. Rhodamine B (1 mg/mL) was dissolved in distilled water and sterilized by filtration. Growth medium contained per liter: nutrient broth, 8 g; sodium chloride, 4 g; and agar, 10 g. The medium was adjusted to pH 7.0, autoclaved, and cooled to about 60°C. Then 2.5% (w/v) olive oil and 0.001% (w/v) of rhodamine B solution were added with vigorous stirring and emulsified by mixing for 1 min. After the medium was allowed to stand for 10 min at 60°C to reduce foaming, 20 mL of medium was poured into each plastic petri dish. Plugs of *Penicillium*, previously cultivated on LBM for 5 days, were transferred on the surface of lipolytic agar medium. Subsequently, the plates were incubated at 25°C for 48 h. Lipase activity was identified on the plate as an orange fluorescent halo around the colonies.

Secondary Metabolite Profile Determination

Growth Media and Conditions

Penicillium isolates were grown on two different media: Czapek yeast autolysate (CYA) agar (BD Biosciences, San Jose, CA, USA), and CYA agar modified with 5% NaCl and by having pH 5.5. All isolates were incubated in triplicates in both media for 12 days in darkness at 25°C. Five agar plugs (diameter 6 mm) were cut out of the colony from the center and in a radius toward the edge of the colony for the extraction of secondary metabolites was based on a standard method for cultures grown on solid medium.

Chemicals

LC—MS and analytical grade chemicals (Sigma-Aldrich, Darmstadt, Germany) were used. ESI—TOF tune mix was from (Agilent Technologies CA, USA). Approximately 1,500 mycotoxins and microbial metabolites used as reference standards derived from other studies (Frisvad and Thrane, 1987; Nielsen and Smedsgaard, 2003), commercial sources and from other research groups. Other standards were obtained by Sigma-Aldrich, Cayman (Ann Arbor, MI), Calbiochem, (San Diego, CA), and ICN (Irvine, CA). TebuBio (Le-Perray-en-Yvelines, France), Axxora (Bingham, UK), Biopure (Tulln, Austria). All standards were tested for original UV—VIS data, accurate mass, and relative RT from previous studies (Frisvad and Thrane, 1987). Agar plugs containing *Penicillium* colonies were extracted using a (3:2:1) (ethyl acetate:dichloromethane:methanol) mixture (Smedsgaard, 1997).

Fungal Metabolites Analysis (UHPLC—DAD—QTOFMS)

A UHPLC—DAD—QTOF method was set up for screening, with typical injection volumes of 0.1–2 μL extract. All chemicals were purchased from Sigma-Aldrich (Darmstadt, Germany). Separation was performed on a Dionex Ultimate 3,000 UHPLC system (Thermo Scientific, Dionex, CA, USA) equipped with a 100 × 2.1 mm, 2.6 μm, Kinetex C 18 column, held at a temperature of 40°C, and using a linear gradient system composed of A: 20 mmol L^{-1} formic acid in water, and B: 20 mmol L^{-1} formic acid in acetonitrile. The flow was 0.4 mL min^{-1}, 90% A graduating to 100% B in 10 min, 100% B 10–13 min, and 90% A 13.1–15 min. Time-of-flight detection was performed using a maXis 3G QTOF orthogonal mass spectrometer (Bruker Daltonics, Bremen, Germany) operated at a resolving power of ∼50,000 full width at half maximum (FWHM). The instrument was equipped with an orthogonal electrospray ionization source, and mass spectra were recorded in the range m/z 100–1,000 as centroid spectra, with five scans per second. For calibration, 1 μL 10 mmol L^{-1} sodium formiate was injected at the beginning of each chromatographic run, using the divert valve (0.3–0.4 min). Data files were calibrated post-run on the average spectrum from this time segment, using the Bruker HPC (high-precision calibration) algorithm. For ESI+ the capillary voltage was maintained at 4,200 V, the gas flow to the nebulizer was set to 2.4 bar, the drying temperature was 220°C, and the drying gas flow was 12.0 L min^{-1}. Transfer optics (ion-funnel energies, quadrupole energy) were tuned on HT-2 toxin to minimize fragmentation. For ESI—the settings were the same, except that the capillary voltage was maintained at −2,500 V. Unless otherwise stated, ion-cooler settings were: transfer time 50 μs, radio frequency (RF) 55 V peak-to-peak (Vpp), and pre-pulse storage time 5 μs. After changing the polarity, the mass spectrometer needed to equilibrate the power supply temperature for 1 h to provide stable mass accuracy.

Automated Screening of Fungal Samples

Target Analysis 1.2 (Bruker Daltonics, Bremen, Germany), was used to process data-files, with the following typical settings: (A) retention time (if known) as ±1.2 min as broad, 0.8 min as medium, and 0.3 min as narrow range; (B) SigmaFit; 1,000 (broad) (isotope fit not used), 40 (medium), and 20 (narrow); and (C) mass accuracy of the peak assessed at 4 ppm (broad), 2.5 ppm (medium), and 1.5 ppm (narrow). Area cut-off was set to 3,000 counts as default, but was often adjusted for very concentrated or dilute samples. The software DataAnalysis (DA) from Bruker Daltonics was used for manual comparison of all extracted-ion chromatograms (EIC) generated by Target Analysis to the base peak chromatograms (BPC), to identify non-detected major peaks.

RESULTS

Fungal Isolates

In the present study, molds were collected during fermentation from different lab- and industrial-scale table olive fermentations performed in Italy and Greece and from different Italian and Greek commercial products belonging to the four cultivars Leccino, Cellina di Nardò, Kalamàta, and Conservolea. A total of 60 fungal strains were isolated from DRBC medium plates. Molecular identification of all of those isolates was performed at species level by sequencing ITS and beta-tubulin gene. Species were identified using BLAST on the NCBI website (www.ncbi.nlm.nih.gov/BLAST/) and through comparison with the sequence database of *Penicillium* type strains sequenced at ISPA-CNR (**Figure 1**). The analysis revealed 21 *Penicillium crustosum* isolates from all the cultivars (>99% similarity to Acc. n. KJ410745.1), one *P. commune* isolate from Kalamàta (100% similarity to EF198566), six *P. expansum* from Cellina di Nardò and Kalamàta (>99% similarity to AY674400.1), one *P. paneum* (>99% similarity to AY674389.1), two *P. polonicum* isolates from Cellina di Nardò (>99% similarity to EU128563.1), 29 *P. roqueforti* isolates from all the cultivars (>99% similarity to AY674382.1).

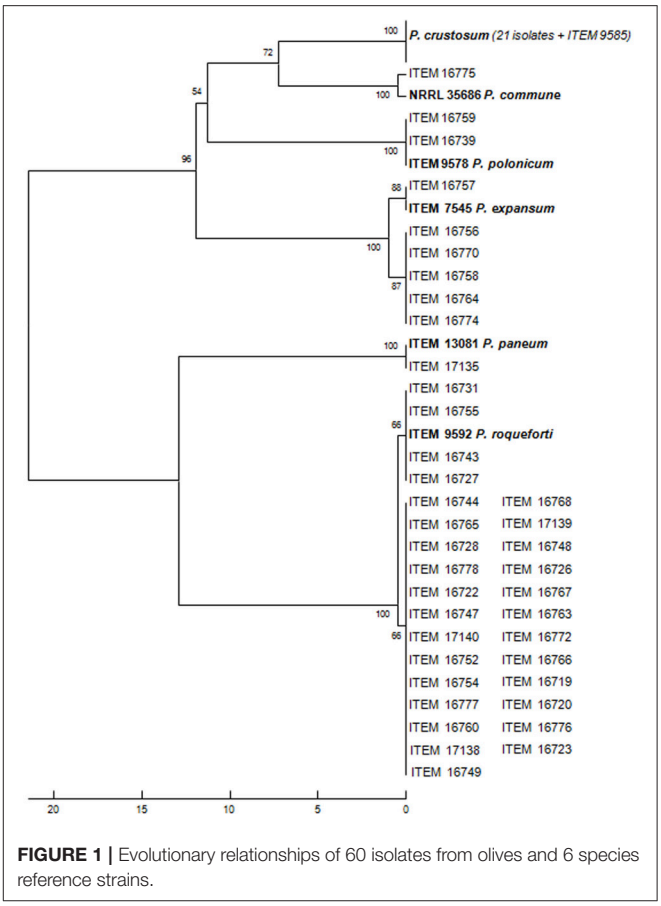

FIGURE 1 | Evolutionary relationships of 60 isolates from olives and 6 species reference strains.

Characterization of Fungi

A subgroup of 20 isolates (**Table 1**) was selected among the 60 fungal isolates, in order to perform biochemical characterization for their technological and safety traits (**Figure 2**). The subset included representative isolates for the different table olive cultivars, geographic localizations, morphotypes inside the same species. This subgroup consisted of 20 mold isolates in parallel subjected to:

1. Technological tests: (i) the presence of beta-glucosidase activity, required to degrade oleuropein and of lypolytic activity, involved in formation of several volatile compounds able to improve the flavor of olives; (ii) the absence of enzymatic activities (protease enzymes, cellulolytic, ligninolytic, pectolytic, and xylanolytic activities) with a possible negative effect on olive texture and quality.
2. Safety assessments for the production of biogenic amines and mycotoxins.

Concerning technological characterization, specific qualitative plate tests were used to determine the presence of extracellular enzymatic activities (beta-glucosidase, lypolitic activities, cellulase, laccase, tyrosinase, xylanase, lignin modifying, pectolytic, and protease) in the 20 selected mold isolates.

All the isolates showed beta-glucosidase activity (**Table 2**). The isolate ITEM 16756 revealed the highest lipolytic activity (score 3), whereas ITEM 16728, 16733, 16740, 16741, 16750, 16753, 16754, 16762, 16763, 16775, 17138, 17139, and 17134 showed slight activity (score 1). No lipolytic activity was revealed in the remaining isolates. Although, no isolates produced degradation activities on milk agar, some isolate showed ability to degrade gelatine (ITEM 16720 with score 3; ITEM 16727, 16743, 16750, 16753, 16762, 16763, and 17139 with score 2; ITEM 16728, 16744, 17138, and 17135 with score 1; **Table 2**). Moreover, all tested isolates revealed the presence of different levels of pectolytic activity, whereas the ability to degrade xylan is absent in many of them (ITEM 16720, 16728, 16744, 16754, 16756, 16762, 16763, 16775, 17138, 17139, 17133; **Table 2**). Concerning lignin modifying enzymes, they were detected at low and medium levels only for the isolates ITEM 16721, 16741, 16756. Many isolates did not produce detectable cellulolytic activities (ITEM 16720, 16727, 16728, 16733, 16740, 16741, 16743, 16744, 16750, 16756, 16762, 17134, and 17133; **Table 2**). Laccase and tyrosinase activities were undetectable in all the tested molds (**Table 2**).

TABLE 1 | Fungal isolates selected for biochemical characterization.

ITEM	Sample	Species identification (beta tubulin)	Country
16721	Leccino industrial fermentation 16	*Penicillium crustosum*	Italy
16750	Cellina di Nardò industrial fermentation 10		
17134	Cellina di Nardò commercial product		
16733	Cellina di Nardò industrial fermentation 4		
16720	Leccino industrial fermentation 16	*Penicillium roqueforti*	
16727	Leccino industrial fermentation 4		
16728	Leccino industrial fermentation 7		
16743	Cellina di Nardò industrial fermentation 13		
16744	Cellina di Nardò industrial fermentation 13		
16756	Cellina di Nardò industrial fermentation 10	*Penicillium expansum*	
17133	Cellina di Nardò commercial product	*Penicillium paneum*	
16775	Kalamata industrial fermentation	*Penicillium commune*	Greece
16741	Conservolea industrial fermentation A3 P2	*Penicillium crustosum*	
16753	Conservolea industrial fermentation A5 P2		
16762	Conservolea lab-scale fermentation 4		
16740	Kalamata industrial fermentation K3 P1		
17138	Kalamata lab-scale fermentation C	*Penicillium roqueforti*	
17139	Kalamata lab-scale fermentation C		
16754	Conservolea industrial fermentation A5 P2		
16763	Conservolea lab-scale fermentation 4		

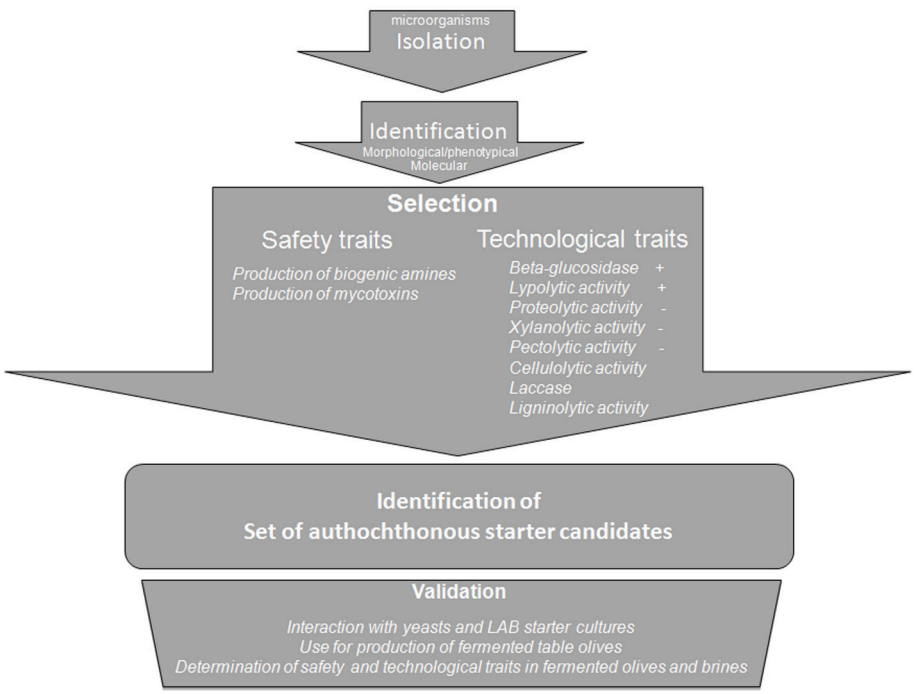

FIGURE 2 | Flow-sheet for the selection of a mold starter culture.

For the determination of the amino acids decarboxylation activities, the value 0 was used to indicate isolates that remained white, isolates surrounded by an intense purple halo were marked with the value 3, isolates producing intense blue halo with the value 2, isolates producing slight blue halo with the value 1 (**Table 2**). None of the isolates were able to decarboxylate histidine, tyrosine and phenylalanine, precursors of histamine, tyramine and 2-phenyl-ethylamine, respectively. Only the isolate

TABLE 2 | Enzymatic activities of the selected molds isolated from table olives.

ITEM	Beta glucosidase	Lypolytic activity	Cellulolytic activity	Laccase	Tyrosinase	Lignin modifying enzymes	Protease activity (Milk agar)	Protease activity (Gelatin)	Pectolytic activity	Xilanolytic activity	Aminoacid decarboxylation activities			
											Arginine	Phenylalanine	Tyrosine	Histidine
16720	++	−	−	−	−	−	−	+++	++	−	−	−	−	−
16721	+++	−	++	−	−	+	−	−	++	++	++	−	−	−
16727	++	−	−	−	−	−	−	++	+	+	-	−	−	−
16728	++	+	−	−	−	−	−	+	+++	−	−	−	−	−
16733	+++	+	−	−	−	−	−	−	++	++	++	−	−	−
16740	+++	+	−	−	−	−	−	−	++	+++	++	−	−	−
16741	+++	+	−	−	−	+	−	−	++	++	++	−	−	−
16743	+	−	−	−	−	−	−	++	+	+	-	−	−	−
16744	++	−	−	−	−	−	−	+	++	−	-	−	−	−
16750	++	+	−	−	−	−	−	++	+++	++	++	−	−	−
16753	++	+	+	−	−	−	−	++	++	+++	++	−	−	−
16754	+	+	+	−	−	−	−	−	++	−	-	−	−	−
16756	+++	+++	−	−	−	++	−	−	+++	−	-	−	−	−
16762	++	+	−	−	−	−	−	++	++	−	++	−	+++	−
16763	+	+	+	−	−	−	−	++	++	−	-	−	−	−
16775	++	+	+	−	−	−	−	−	+++	−	+	−	−	−
17133	++	−	−	−	−	−	−	+	+++	−	-	−	−	−
17134	+++	+	−	−	−	−	−	−	++	+	++	−	−	−
17138	++	+	+	−	−	−	−	+	++	−	−	−	−	−
17139	++	+	+	−	−	−	−	++	++	−	−	−	−	−

ITEM 16762 showed good decarboxylation activity (value 3) of tyrosine. Various levels of arginine decarboxylation activity were detected for the isolates ITEM 16721, 16733, 16740, 16741, 16750, 16753, 16762, 16775, 17134.

Fungal ability to produce secondary metabolites, included mycotoxins, was investigated by growing isolates in two different conditions: namely in CYA medium and CYA medium with the addition of the two constrains represented by salinity and acidity (5% NaCl and pH 5.5) (CYAS) in order to evaluate the role of salinity and acidity in affecting secondary metabolites production. The secondary metabolites were analyzed by the Target Analysis system (Klitgaard et al., 2014), able to screen each extract for 3,000 compounds, considering mass accuracy, isotope fit, and retention time (RT), producing a qualitative representation of all mycotoxins present in the sample (**Table 3**).

Aflatoxins, Ochratoxin, and Patulin were not detected in any mold isolates. Secondary metabolites produced by the isolate ITEM 16728 (*P. roqueforti*) are not affected by the salt and acidity in the medium. At the two conditions (CYA and CYAS) it produced mycophenolic acid and roquefortine C.

The isolate ITEM 16775 produced cyclopiazonic acid and sclerotigenin in the two tested conditions, but FKL-3389 was released only in the medium without salt and acidic conditions (CYA). The isolate ITEM 16756 (*P. expansum*), in addition to chaetoglobosin A, communesin A, and roquefortine C, in presence of salt and acidity produced also chaetoglobosin C and communesin B. Among isolates belonging to the species *P. roqueforti*, all of them produced mycophenolic acid in absence of salt and acidity stress, whereas the isolates ITEM 16727 and 16754 produced roquefortine C in the two tested conditions. This last metabolite was revealed in ITEM 16743 and 17138 grown on salt and acidic medium. The PR-toxin producers ITEM 16727 and 17138 did not release this toxin in salt and acidic conditions. The isolates ITEM 16743, 16744, 16754, and 17138 also produced agroclavine in presence of salt and acidic pH (CYAS). Finally, the ITEM 17138 released roquefortine C and andrastatin A in salt and acidic conditions, whereas it produced roquefortine X in absence of these stresses.

Considering *P. crustosum* isolates, all of them produced dehydrocyclopeptin in stress (salt and acid) conditions in addition to andrastin A, cyclopenol, penitrem A, roquefortine C, viridicatol. In CYAS medium, in presence of the isolates ITEM 16721 and 17134 there anacin and viridicatin were also detected, whereas viridicatin was produced by ITEM 16740 and 17134 and terrestric acid was produced only by ITEM 16740.

Summarizing, the presence of salt and low pH affects in different way all metabolites: in particular they stimulate production of dehydrocyclopeptin and in some case also viridicatin in all *P. crustosum* (ITEM 16740 and 17134) strains tested and stimulated terrestric acid production in ITEM 16740; in *P. roqueforti* isolates ITEM 16743 and 17138 produced roquefortine C and only ITEM 17138 produced andrastin A, whereas a roquefortine derivative (present in ITEM 17138 grown in no salt and no acidic conditions) and PR-toxin (revealed in ITEM 17138 and ITEM 16727 in absence of salt and low pH conditions) were not detected; *P. expansum* ITEM 16756 produced chaetoglobosin C and communesin B but not roquefortine C; marcfortine was not detected in *P. paneum* ITEM 17133. *P. roqueforti* ITEM 16743, 16744, 16754, and 17138 produced agroclavine.

Selection of Fungi

Combining qualitative tests for both technological (the presence of desired enzymatic activities, i.e., beta-glucosidase and/or lipase activities, the reduced or absence of undesired traits, i.e., proteases, pectolytic and/or xylanolytic activities) and safety assessments of tested fungal isolates, it was possible to select some interesting isolates that can be considered as putative candidates for future studies as co-starters with yeasts and lactic acid bacteria. At the end of this procedure, the set of candidate strains for standardized fermentation of table olives includes: *P. roqueforti* ITEM 16728 selected from Leccino cultivar, *P. paneum* ITEM 17133 and *P. roqueforti* ITEM 16744 selected from Cellina di Nardò cultivar, *P. roqueforti* ITEM 17138 selected from Kalàmata cultivar and *P. roqueforti* ITEM 16754 selected from Conservolea cultivar. It is to be considered, however, that table olives fermentations need to be performed in real conditions using the selected candidate strains in order to evaluate both safety and technological traits in olive and brine samples.

DISCUSSION

Molds belonging to *Penicillium* and other molds genera (*Aureobasidium, Aspergillus, Geotrichum*) are often associated to Black "Greek style" table olives. Their role in table olives production is not completely understood yet. At present, they are considered as contaminating microorganisms, responsible for spoilage (texture softening, production of moldy odor and taste) and potentially toxicity (Heperkan, 2013).

The interest on molds associated to table olives starts from the consideration that they are used as secondary starter cultures in Europe to process meat and cheese products, affecting positively their flavor, taste, texture, offering protection against spontaneous undesired microorganisms, delay of rancidity, stabilization of color, oxygen, and light protection, etc. On the other end, they can also release highly toxic secondary metabolites such as mycotoxins. Non-toxigenic and technological mold starters can be exploited for standardized fermentation, hindering the growth of undesired microorganisms, similarly to what have been done for dry-cured meat using *P. nalgiovense*, *P. chrysogenum,* and recently of *P. salamii* (Sunesen and Stahnke, 2003; Delgado et al., 2016; Magistà et al., 2016); for the production of white cheeses using *P. camemberti* (Brie and Camembert), and for the production of blue cheeses (Roquefort, Gorgonzola, Stilton, Gammelost, etc.) using *P. roqueforti* (Geisen, 1993).

In the present paper, for the first time we (i) identified and characterized mold isolates associated to four different black table olives cultivars and (ii) selected some of them for their potential use as co-starter for table olives production. It can be recommended to use species from series *Roquefortorum* (*P. roqueforti* and *P. paneum*) in olive fermentation,

TABLE 3 | Secondary metabolites produced in culture by molds isolates from table olives.

		ITEM 16775	ITEM 16721	ITEM 16740	ITEM 16741	ITEM 17134	ITEM 16756	ITEM 17133	ITEM 16727	ITEM 16728	ITEM 16743	ITEM 16744	ITEM 16754	ITEM 17138
		P. commune	*P. crustosum*	*P. crustosum*	*P. crustosum*	*P. crustosum*	*P. expansum*	*P. paneum*	*P. roqueforti*	*P. roqueforti*	*P. roqueforti*	*P. roqueforti*	*P. roqueforti*	*P. roqueforti*
Chaetoglobosin A	CYA						+							
	CYAS						+							
Chaetoglobosin C	CYA													
	CYAS						+							
Communesin A	CYA						+							
	CYAS						+							
Communesin B	CYA													
	CYAS						+							
Marcfortine	CYA							+						
	CYAS													
Cyclopiazonic acid	CYA	+												
	CYAS	+												
FKI-3389	CYA	+												
	CYAS													
Sclerotigenin	CYA	+												
	CYAS	+												
Anacine or similar compound	CYA		+			+								
	CYAS		+			+								
Andrastin A	CYA		+	+	+	+		+			+	+		
	CYAS		+	+	+	+		+			+	+		+
Cyclopenol	CYA		+	+	+	+								
	CYAS		+	+	+	+								
Dehydro cyclopeptin	CYA													
	CYAS		+	+	+	+								
Penitrem A	CYA		+	+	+	+								
	CYAS		+	+	+	+								

(Continued)

TABLE 3 | Continued

		ITEM 16775 *P. commune*	ITEM 16721 *P. crustosum*	ITEM 16740 *P. crustosum*	ITEM 16741 *P. crustosum*	ITEM 17134 *P. crustosum*	ITEM 16756 *P. expansum*	ITEM 17133 *P. paneum*	ITEM 16727 *P. roqueforti*	ITEM 16728 *P. roqueforti*	ITEM 16743 *P. roqueforti*	ITEM 16744 *P. roqueforti*	ITEM 16754 *P. roqueforti*	ITEM 17138 *P. roqueforti*
Roquefortine C	CYA		+	+	+	+	+		+	+			+	
	CYAS		+	+	+	+			+	+	+		+	+
Roquefortine X	CYA													+
	CYAS													
Viridicatol	CYA		+	+	+	+								
	CYAS		+	+	+	+								
Viridicatin	CYA													
	CYAS			+		+								
Mycophenolic acid	CYA								+	+	+	+	+	+
	CYAS								+	+	+	+	+	+
PR-toxin	CYA								+					+
	CYAS													
Terrestric acid	CYA													
	CYAS			+										
Agroclavine	CYA													
	CYAS										+	+	+	+

+*denotes the presence of a metabolite.*

because these are the only Penicillia that can tolerate higher concentrations of acetic acid and lactic acid produced by the lactic acid bacteria (Frisvad et al., 2004; Houbraken et al., 2010). In other lactic acid and acetic acid containing foods and feeds *Roquefortorum* species are also the dominating Penicillia for example cocoa, silage, rye bread, sauerkraut (O'Brien et al., 2006; Copetti et al., 2011; Gallo et al., 2015) etc., as long as the concentration of acetic acid is sufficiently high. If the concentration of these acids is low, toxigenic species such as *P. crustosum, P. commune,* and *P. expansum* may thrive. These toxigenic fungi were found in olives in this study, so it is recommended to secure that sufficiently high concentration of acetic acid and lactic acid is present in the table olives.

For the first time, a flow sheet has been proposed for selection of molds associated to table olives. After identification using morphology/phenotypic characteristics and molecular approaches, the mold isolates can be selected using two main criteria (**Figure 2**): presence/absence of enzymatic activities and of toxic compounds. This selection step can help to individuate candidates to be used in further validation step consisting in the study of their positive/negative interaction with already existing starter cultures for table olives and in the use of them alone or in combination with yeasts and LAB for table olive production.

Molds associated to Leccino, Cellina di Nardó, Kalamàta, and Conservolea fermented table olives were for the first time isolated and identified. Classical morphological identification combined with molecular approach was used to improve resolution and discriminating power inside *Penicillium* species (Baffi et al., 2012). The *P. commune*, *P. crustosum*, *P. expansum, P. roqueforti* were already reported in previous works as associated to table olives (Ghitakou et al., 2006; Heperkan et al., 2006; Baffi et al., 2012), whereas the species *P. paneum* and *P. polonicum* were not reported earlier in this product and they could represent an occasional contamination, considering the low number of colonies observed. Following the selection strategy proposed for yeasts and lactic acid bacteria, both technological and safety tests have been chosen in order to perform a selection of mold isolates.

A subgroup (20) of them, selected in order to represent the overall observed molecular biodiversity, was assayed for their positive trait to produce beta-glucosidase, responsible of polyphenols degradation (in particular oleuropein) and debittering of table olives together with impact on flavor, due to the production of secondary metabolites (Bevilacqua et al., 2013). Seventeen isolates showed good beta-glucosidase activity, representing promising candidates able to help other starters to degrade oleuropein, reducing time for debittering and NaCl content in table olive processing. Several isolates (14) revealed the presence of lipase activity, desired activity that could improve the aromatic profile of fermented olives (Savitha et al., 2007; Rodríguez-Gómez et al., 2012).

In this preliminary study, following the approach previously used for yeasts and LAB (Arroyo-López et al., 2012; Bevilacqua et al., 2013; Bleve et al., 2014, 2015), the presence of proteolytic activity was considered as a negative characteristic, since it is responsible for the release of aminoacids and ammoniacal nitrogen causing in turn a pH increase and the risk for the product to be unsafe (Tosi et al., 2008; Ledenbach and Marshall, 2009). Moreover, proteolytic activity could be also responsible of undesired impact on olive quality provoking olive softening. Although, no isolates produced detectable activity on milk agar, 12 of them metabolized gelatin at different levels (score 1–3). However, considering microbiological research performed so far and scientific data available, it is not possible to definitely establish the real impact of these enzymatic activities on table olives quality. In fact, the relative influence of fungal enzymatic activities could contribute to the maturation of aroma together with other parameters, such as raw materials and processing conditions (Meynier et al., 1999; Harkouss et al., 2012). Other enzymatic activities that have been described as undesired traits in yeasts, such as cellulases, xylanases, pectinases, need to be verified for molds when they are inoculated in table olives. Also in this case, scarce information is still available about the compounds and enzymes produced by the mold population during fermentation and their concentrations in packed olives. This is an important aspect since the presence of molds that are able to secrete these enzymes in table olives could modify in a not predictable manner the nutritional composition of table olives and of their organoleptic characteristics.

It is nevertheless worth noting that beta-glucosidase, cellulase, xylanase, pectinase, lignin modifying, lypolytic, and proteolityc enzymatic activities detected in many mold isolates can be of interest also for major industrial applications. Potential biotechnological uses of Penicillia, isolated from fresh olive fruits, olive paste and pomace, were suggested by Baffi et al. (2012). Analogously to the approach followed for yeasts (Bleve et al., 2011), mold isolates (as whole cells in free or immobilized forms) or enzymes purified from different mold species can be useful for treatment and/or bioconversion of agro-food by-products, such as wastewaters deriving from olive oil and table olives industry.

Table olives are one of the most contaminated fermented food by biogenic amines like putrescine, cadaverine, and tyramine, as reported in "Zapatera" spoiled olives (Hornero-Mendez and Garrido-Fernandez, 1994) and in naturally fermented (Greek-style) table olives (Tofalo et al., 2012).

Recently, in addition to LAB, also yeasts have been considered as a source of biogenic amines and preliminary assays have been included in selection programs to check their ability to produce these compounds "*in vitro*" conditions (Bevilacqua et al., 2013; Bleve et al., 2014, 2015). In order to reduce the risks for consumer, these tests have been introduced in this study also for molds, to select microbial sources unable to produce biogenic amines in fermented table olives.

In this paper a preliminary screening of mold isolates, on media with or without salt and acidic constraints, was also carried out for qualitative analysis of many secondary metabolites (including mycotoxins) in an attempt to identify isolates potentially low producer of mycotoxins.

The three regulated mycotoxins Aflatoxins, Ochratoxin A, and Patulin were not detected in any mold strains isolated from the fermented black olives.

P. expansum ITEM 16756 produced chaetoglobosin A and communesin A independently from the presence of absence of salt and low pH, whereas communesin B was revealed only in

presence of these conditions. The isolation of chaetoglobosin A, characterized by embryolethal but not teratogenic effects on chickens was reported in the genus *Penicillium* (Veselý and Jelínek, 1995). Besides patulin and roquefortine C, Andersen et al. (2004) described the presence of chaetoglobosin A and communesin B in *P. expansum* contaminated fruit juices and potato pulp. Oral toxicity in cockerels, embryonic chickens, rats, and mice, cytotoxic activity in HeLa cells, teratogenic activity in mice were demonstrated for chaetoglobosin A (Sekita et al., 1982; Cherton et al., 1994), whereas communesin B is cytotoxic to lymphocytic leukemia cells (Numata et al., 1993). However, despite their negative effects reported in literature, these mycotoxins are not regulated as food contaminants.

The presence of cyclopiazonic acid (CPA), an indol–tetramic acid mycotoxin produced by the nearly ubiquitous molds *Aspergillus* and *Penicillium*, was revealed in *P. commune* ITEM 16775.

Although, CPA is not considered to be a potent acute toxin, it has different target organs (hepatic tissue, and spleen). Its toxicity and symptoms are linked to its ability to alter normal intracellular calcium flux and potential immunomodulatory effect in "*in vitro*" studies. Several *Penicillium* species (*P. camamberti, P. cyclopium, P. viridictum, P. griseofulvum, P. crustosum,* and others) associated to cheese (Camembert, brie, cheddar, cream, cheese rind, etc.), acorn, barley, corn, peanuts, ham, and chicken meat were reported as producers of CPA (Burdock and Flamm, 2000).

The potent neurotoxin penitrem A has been detected in important agricultural commodities (maize, rice, wheat, oat, rye, barley, and sorghum) and in moldy cream cheese and it is produced by several species of fungi. According to the results reported by El-Banna and Leistner (1988), also all isolates of *P. crustosum* tested in this study were able to produce detectable levels of penitrem A.

The *P. expansum* ITEM 16756 and all isolates of *P. crustosum* and of *P. roqueforti*, with the exception of the isolate ITEM 16744, produced roquefortine C. This mycotoxin has neurotoxic properties (Wagener et al., 1980), but the data on its neurotoxicity are equivocal (Fog Nielsen et al., 2006). Several studies have revealed the presence of roquefortine C in blue-veined cheeses (Ohmomo et al., 1975; Scott and Kennedy, 1976; López-Díaz et al., 1996). It is always found in cheeses, since fungal strains (*P. camamberti* and *P. roqueforti*) used as starters in the dairy industry are able to produce this toxin *in vitro*. However, the relatively low toxicity and low concentrations of this toxin in blue cheese make this product safe to be consumed (Finoli et al., 2001).

According to data reported in this paper and by other authors (Frisvad et al., 2004), *P. roqueforti* is also known to produce mycophenolic acid (MPA) and PR-toxin (detected only in ITEM 16727 and 17138).

Although, MPA possess immunosuppressive effects (Bentley, 2000), compared to other mycotoxins (T-2 toxin, gliotoxin, DON, and patulin), roquefortine C and MPA showed low acute cytotoxicity on the human intestinal cell line Caco-2 (Rasmussen et al., 2010).

PR-toxin is instead the most toxic metabolite produced by *P. roqueforti* because of a high toxic or lethal effects in rats, mice, and cats and is mutagenic in the Ames test (Arnold et al., 1978; Fog Nielsen et al., 2006). However, PR-toxin is unstable because it quickly reacts with different salts, amino acids (especially Sulfur containing amino acids), amines, casein, and the decomposition products resulting in the less toxic PR imine (Erdogan et al., 2003).

Despite the detection of variable contents roquefortine C and MPA in several blue-veined cheeses, no cases of intoxications linked to the consumption of these products have ever been reported (Fontaine et al., 2015). Roquefortine C and MPA combined exposure was studied on human intestinal cells (Caco-2 cells) and on monocytes (cancer lineage model cell THP-1) by Fontaine et al. (2015). These authors demonstrated that roquefortine C and MPA may not create problems of acute exposure in food, since human are exposed at relatively low levels and these toxins show low acute cytotoxic effects in comparison to other regulated mycotoxins. Agroclavine produced by *P. roqueforti* is the precursor of isofumigaclavine A, and its hydrolysis product, isofumigaclavine B, are identical to Roquefortine A and B, respectively. Low levels or traces of Isofumigaclavine A and B were found in marbled and blue cheese. The toxicological effects of these substances are not well-known, but some neurotoxic data are described by several studies (Ohmomo et al., 1975; Scott and Kennedy, 1976; Scott, 1984). However, Agroclavine itself is probably not very toxic, although there are few available data on its toxicity.

Although, several isolates of *P. expansum* were shown to be able to produce patulin and citrinin (Andersen et al., 2004), and different isolates of *P. paneum* were responsible of production of patulin (O'Brien et al., 2006), none of these toxins were detectable for all the tested isolates.

Considering technological and safety data collected for all tested isolates, at the end of this procedure, it is possible to select some isolate, autochthonous for each table olive cultivar, suitable for future validation tests as co-starters with yeasts and lactic acid bacteria and for production of fermented table olives. Tests on an *ad hoc* optimized "olive agar" and then directly on fermented table olives and brine samples inoculated with selected molds might indicate the quantity and which biogenic amines and mycotoxins will be eventually produced on the final product during a fermentation process in real conditions.

AUTHOR CONTRIBUTIONS

Fundamental contributions to the conception and design of the work, acquisition, analysis and interpretation of data: GB, SB, AS, AL; Acquisition, analysis, elaboration, and interpretation of data: SB, AS, GB, JF, MT, AC; Drafting the work and revising it critically for intellectual content: GB, AS, GP, GM, AL. All authors approved the final version of the manuscript to be submitted for publication and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy and integrity of any part of the work are appropriately investigated and resolved.

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REFERENCES

Andersen, B., Smedsgaard, J., and Frisvad, J. C. (2004). *Penicillium expansum*: consistent production of patulin, chaetoglobosins, and other secondary metabolites in culture and their natural occurrence in fruit products. *J. Agric. Food Chem.* 52, 2421–2428. doi: 10.1021/jf035406k

Arnold, D. L., Scott, P. M., McGure, P. F., Harwig, J., and Nera, E. A. (1978). Acute toxicity studies of roquefortine and PR toxin, metabolites of *Penicillium roqueforti*, in the mouse. *Food Cosmet. Toxicol.* 16, 369–371. doi: 10.1016/S0015-6264(78)80009-1

Arroyo-López, F. N., Romero-Gil, V., Bautista-Gallego, J., Rodriguez-Gómez, F., Jiménez-Díaz, and R., García-García, P. (2012). Yeasts in table olive processing: desirable or spoilage microorganisms. *Int. J. Food Microbiol.* 160, 42–49. doi: 10.1016/j.ijfoodmicro.2012.08.003

Baffi, M. A., Romo-Sanchez, S., Ubeda-Iranzo, J., and Briones-Perez, A. I. (2012). Fungi isolated from olive ecosystems and screening of their potential biotechnological use. *New Biotechnol.* 29, 451–456. doi: 10.1016/j.nbt.2011.05.004

Bentley, R. (2000). Mycophenolic acid: a one hundred year odyssey from antibiotic immunosuppressant. *Chem. Rev.* 100, 3801–3825. doi: 10.1021/cr990097b

Bevilacqua, A., Beneduce, L., Sinigaglia, M., and Corbo, M. R. (2013). Selection of yeasts as starter cultures for table olives. *J. Food Sci.* 78, M742–M751. doi: 10.1111/1750-3841.12117

Bleve, G., Lezzi, C., Chiriatti, M. A., D'Ostuni, I., Tristezza, M., Di Venere, D., et al. (2011). Selection of non-conventional yeasts and their use in immobilized form for the bioremediation of olive oil mill wastewaters. *Bioresour. Technol.* 102, 982–989. doi: 10.1016/j.biortech.2010.09.059.

Bleve, G., Tufariello, M., Durante, M., Perbellini, E., Ramires, F. A., Grieco, F., et al. (2014). Physico-chemical and microbiological characterization of spontaneous fermentation of Cellina di Nardò and Leccino table olives. *Front. Microbiol.* 5:570. doi: 10.3389/fmicb.2014.00570

Bleve, G., Tufariello, M., Durante, M., Ramires, F. A., Grieco, F., Mita, G., et al. (2015). Physico-chemical characterization of natural fermentation process of Conservolea and Kalamàta table olives and developement of a protocol for the pre-selection of fermentation starters. *Food Microbiol.* 46, 368–382. doi: 10.1016/j.fm.2014.08.021

Boominathan, K., and Reddy, C. A. (1992). Fungal degradation of lignin: biotechnological applications. *Handbook Appl. Mycol.* 4, 763–822.

Burdock, W. G. A., and Flamm, G. (2000). Review article: safety assessment of the mycotoxin cyclopiazonic. *Acid. Int. J. Toxicol.* 19, 195–218. doi: 10.1080/10915810050074964

Cherton, J. C., Jallal, A., Lhommet, G., Loutelier, C., Dardoize, F., Lacoste, L., et al. (1994). Unexpected production of chaeto- globosins from maize incubated by *Phomopsis lepstromiformis*. I. Isolation and optimization of the production in liquid media by LC monitoring. *Analysis* 22, 210–216.

Copetti, M. V., Iamanaka, B. T., Frisvad, J. C., Pereira, J. L., Fungaro, M. H., and Taniwaki, M. (2011). Mycobiota of cacao. From farm to chocolate. *Food Microbiol.* 28, 1499–1504. doi: 10.1016/j.fm.2011.08.005

Crous, P. W., Verkley, G. J. M., Groenewald, J. Z., and Samson, R. A. (eds.). (2009). *Fungal Biodiversity*. [*CBS Laboratory Manual Series no.1.*]. Utrecht: CBS-KNAW Fungal Biodiversity Centre.

Delgado, J., Peromingo, B., Núñez, F., and Asensio, M. A. (2016). Use of moulds and their antifungal proteins for biocontrol of toxigenic moulds on dry-ripened cheese and meats. *Curr. Opin. Food Sci.* 11, 40–45. doi: 10.1016/j.cofs.2016.09.003

Eaton, R. A., and Hale, M. D. C. (1993). *Wood: Decay, Pests and Protection*. London: Chapman and Hall Ltd.

El Adlouni, C., Tozlovanu, M., Naman, F., Faid, M., and Pfohl-Leszkowicz, A. (2006). Preliminary data on the presence of mycotoxins (ochratoxin A, citrinin and aflatoxin B1) in black table olives "Greek style" of Moroccan origin. *Mol. Nutr. Food Res.* 50, 507–512. doi: 10.1002/mnfr.200600055

El-Banna, A. A., and Leistner, L. (1988). Production of penitrem A by *Penicillium crustosum* isolated from foodstuffs. *Int. J. Food Microbiol.* 7, 9–17. doi: 10.1016/0168-1605(88)90067-0

Erdogan, A., Gurses, M., and Sert, S. (2003). Isolation of moulds capable of producing mycotoxin from blue mouldy Tulum cheeses produced in Turkey. *Food Microbiol.* 85, 83–85. doi: 10.1016/S0168-1605(02)00485-3

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39, 783–791. doi: 10.1111/j.1558-5646.1985.tb00420.x

Fernandez, G. A., Adams, M. R., and Diez, M. J. F. (1997). *Table Olives: Production and Processing*. London: C*hapman et Hall.* doi: 10.1007/978-1-4899-4683-6

Finoli, C., Vecchio, A., Galli, A., and Dragoni, I. (2001). Roquefortine C occurrence in blue cheese. *J. Food Protect.* 64, 246–251. doi: 10.4315/0362-028X-64.2.246

Fog Nielsen, K., Nielsen, O. G., Sumarah, M. W., Frisvad, J. C., and Miller, J. D. (2006). Production of metabolites from the *Penicillium roqueforti* complex. *J. Agric. Food Chem.* 54, 3756–3763. doi: 10.1021/jf060114f

Fontaine, K., Passeró, E., Vallone, L., Hymery, N., Coton, M., Jany, J.-L. et al. (2015). Occurrence of roquefortine C, mycophenolic acid and aflatoxin M1 mycotoxins in blue-veined cheeses. *Food Control* 47, 634–640. doi: 10.1016/j.foodcont.2014.07.046

Franzetti, L., Scarpellini, M., Vecchio, A., and Planeta, D. (2011). Microbiological and safety evaluation of green table olives marketed in Italy. *Ann. Microbiol.* 61, 843–851. doi: 10.1007/s13213-011-0205-x

Frisvad, J. C., and Samson, R. A. (2004). *Emericella venezuelensis*, a new species with stellate ascospores producing sterigmatocystin and aflatoxin B1. *Syst. Appl. Microbiol.* 27, 672–680. doi: 10.1078/0723202042369910

Frisvad, J. C., Smedsgaard, J., Larsen, T. O., Samson, R. A., and Robert, A. (2004). Mycotoxins, drugs and other extrolites produced by species in *Penicillium* subgenus. *Penicillium*. *Stud. Mycol.* 49, 201–241.

Frisvad, J. C., and Thrane, U. (1987). Standardised High-Performance Liquid Chromatography of 182 mycotoxins and other fungal metabolites based on alkylphenone retention indices and UV-VIS spectra (Diode Array Detection). *J. Chromatogr.* 404, 195–214. doi: 10.1016/S0021-9673(01)86850-3

Gallo, A., Giuberti, G., Frisvad, J. C., Bertuzzi, T., and Nielsen, K. F. (2015). Review on mycotoxin issues in ruminants: occurrence in forages, effects of mycotoxin ingestion on health status and animal performance and practical strategies to counteract their negative effect. *Toxins* 7, 3057–3111. doi: 10.3390/toxins7083057

Gardini, F., Tofalo, R., Belletti, N., Iucci, L., Suzzi, G., Torriani, S., et al. (2006). Characterization of yeasts involved in the ripening of Pecorino Crotonese cheese. *Food Microbiol.* 23, 641–648. doi: 10.1016/j.fm.2005.12.005

Geisen, R. (1993). Fungal starter cultures for fermented foods: molecular aspects. *Trends Food Sci. Technol.* 4, 251–255. doi: 10.1016/0924-2244(93)90140-6

Ghitakou, S., Koutras, K., Kanellou, E., and Markaki, P. (2006). Study of aflatoxin B1 and ochratoxin A production by natural microflora and *Aspergillus parasiticus* in black and green olives of Greek origin. *Food Microbiol.* 23, 612–621. doi: 10.1016/j.fm.2005.12.008

Glass, N. L., and Donaldson, G. C. (1995). Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous Ascomycetes. *Appl. Environ. Microb.* 61, 1323–1330.

Hankin, L., Poincelot, R. P., and Anagnostakis, S. L. (1975). Microorganisms from composting leaves: ability to produce extracellular degradative enzymes. *Microb. Ecol.* 2, 296–308. doi: 10.1007/BF02011649

Harkouss, R., Mirade, P. S., and Gatellier, P. (2012). Development of a rapid, specific and efficient procedure for the determination of proteolytic activity in dry-cured ham: definition of a new proteolysis index. *Meat Sci.* 92, 84–88. doi: 10.1016/j.meatsci.2012.04.017

Heperkan, D. (2013). Microbiota of table olive fermentations and criteria of selection for their use as starters. *Front Microbiol.* 4:143. doi: 10.3389/fmicb.2013.00143

Heperkan, D., Dazkir, G. S., Kansu, D. Z., and Guler, F. K. (2009). Influence of temperature on citrinin accumulation by *Penicillium citrinum* and *Penicillium verrucosum* in black table olives. *Toxin Rev.* 28, 180–186. doi: 10.1080/15569540903084982

Heperkan, D., Meric, B. E., Sismanoglu, G., Dalkili,ç, G., and Güler, F. K. (2006). Mycobiota, mycotoxigenic fungi, and citrinin production in black olives. *Adv. Exp. Med. Biol.* 571, 203–210. doi: 10.1007/0-387-28391-9_13

Hornero-Mendez, D., and Garrido-Fernandez, A. (1994). Biogenic- amines in table olives analysis by high-performance liquid- chromatography. *Analyst* 119, 2037–2041. doi: 10.1039/AN9941902037

Houbraken, J., Frisvad, J. C., and Samson, R. A. (2010). Sex in *Penicillium* series *Roqueforti*. *IMA Fungus* 1, 171–180. doi: 10.5598/imafungus.2010.01.02.10

King, A. D. Jr., Hocking, A. D., and Pitt, J. I. (1979). Dichloran-rose bengal medium for enumeration and isolation of molds from foods. *Appl. Environ. Microbiol.* 37, 959–964.

Klitgaard, A., Iversen, A., Andersen, M. R., Larsen, T. O., Frisvad, J. C., and Nielsen, K. F. (2014). Aggressive dereplication using UHPLC-DAD-QTOF - screening extracts for up to 3000 fungal secondary metabolites. *Anal. Bioanal. Chem.* 406, 1933–1943. doi: 10.1007/s00216-013-7582-x

Korukluoğlu, M., Gürbüz, O., uylaser, V., Yildirim, A., and Sahin, I. (2000). "Gemlik tipi zeytinlerde mikotoksin kirliliginin arastirilmasi, Türkiye 1," in *Zeytincilik Sempozyumu Uludağ Üniversitesi 6–9 Haziran, Bildiri Kitabi* (Bursa), 218.

Ledenbach, L. H., and Marshall, R. T. (2009). "Microbiological spoilage of dairy products," in *Compendium of the Microbiological Spoilage of Foods and Beverages, Food Microbiology and Food Safety,* eds W. H. Sperber and M. P. Doyle (New York, NY: Springer Science+Business Media), 41–67. doi: 10.1007/978-1-4419-0826-1_2

López-Díaz, T. M., Román-Blanco, C., García-Arias, M. T., García-Fernández, M. C., and García-López, M. L. (1996). Mycotoxins in two Spanish cheese varieties. *Int. J. Food Microbiol.* 30, 391–395. doi: 10.1016/0168-1605(96)00957-9

Ludemann, V., Pose, G., Pollio, M. L., and Segura, J. (2004). Determination of growth characteristics and lipolytic and proteolytic activities of *Penicillium* strains isolated from Argentinean salami. *Int. J. Food Microbiol.* 96, 13–18. doi: 10.1016/j.ijfoodmicro.2004.03.003

Magistà, D., Ferrara, M., Del Nobile, M. A., Gammariello, D., Conte, A., and Perrone, G. (2016). Penicillium salamii strain ITEM 15302: a new promising fungal starter for salami production. *Int. J. Food Microbiol.* 231, 33–41. doi: 10.1016/j.ijfoodmicro.2016.04.029

Medina-Pradas, E., and Arroyo-López, F. N. (2015). Presence of toxic microbial metabolites in table olives. *Front. Microbiol.* 6:873. doi: 10.3389/fmicb.2015.00873

Meynier, A., Novelli, E., Chizzolini, R., Zanardi, E., and Gandemer, G. (1999). Volatile compounds of commercial Milano salami. *Meat Sci.* 51, 175–183. doi: 10.1016/S0309-1740(98)00122-3

Nei, M., and Kumar, S. (2000). *Molecular Evolution and Phylogenetics*. New York, NY: Oxford University Press.

Nielsen, K. F., and Smedsgaard, J. (2003). Fungal metabolite screening: database of 474 mycotoxins and fungal metabolites for de-replication by standardized liquid chromatography-UV-mass spectrometry methodology. *J. Chromatogr. A* 1002, 111–136. doi: 10.1016/s0021-9673(03)00490-4

Numata, A., Takahashi, C., Ito, Y., Takada, T., Kawai, K., Usami, Y., et al. (1993). Communesins, cytotoxic metabolites of a fungus isolated from a marine alga. *Tetrahedron Lett.* 34, 2355–2358. doi: 10.1016/s0040-4039(00)77612-x

O'Brien, M., Nielsen, K. F., O'Kiely, P., Forristal, P. D., Fuller, H. T., and Frisvad, J. C. (2006). Mycotoxins and other secondary metabolites produced *in vitro* by *Penicillium paneum* Frisvad and *Penicillium roqueforti* Thom isolated from baled grass silage in Ireland. *J. Agric. Food Chem.* 54, 9268–9276. doi: 10.1021/jf0621018

Ohmomo, S., Sato, T., Utagawa, T., and Abe, M. (1975). Isolation of Festuclavine and three new indole alkaloids, roquefortine A, B and C from cultures of *Penicillium roqueforti*. *J. Agr. Chem. Soc. Jpn.* 49, 615–623. doi: 10.1271/bbb1961.39.1333

Pointing, S. B., Vrijmoed, L. L. P., and Jones, E. B. G. (1999). Laccase is produced as the sole lignin modifying enzyme in submerged liquid culture by the white-rot fungus *Pycnoporus sanguineus*. *Mycologia* 91, 345–349.

Rasmussen, R. R., Binderup, M.-L., Larsen, T. O., and Rasmussen, P. H. (2010). *Mycotoxins in Maize Silage e Detection of Toxins and Toxicological Aspects*. Kgs, Lyngby:: Technical University of Denmark (DTU).

Rodríguez-Gómez, F., Romero-Gil, V., Bautista-Gallego, J., Garrido-Fernández, and A., Arroyo-López, F. N. (2012). Multivariate analysis to discriminate yeast strains with technological applications in table olive processing. *World J. Microbiol. Biotechnol.* 28, 1761–1770. doi: 10.1007/s11274-011-0990-1

Savitha, J., Srividya, S., Jagat, R., Payal, P., Priyanki, S., Rashmi, G. W.,., et al. (2007). Identification of potential fungal strain (s) for the production of inducible, extracellular and alkalophilic lipase. *Afr. J. Biotechnol.* 6, 564–568.

Scott, P. M. (1984). "Mycotoxins production, isolation, separation and purification," in *PR Toxin*, ed V. Betina (Amsterdam: Elsevier), 469–474.

Scott, P. M., and Kennedy, B. P. C. (1976). Analysis of blue cheese for roquefortine and others alkaloids from *Penicillium roqueforti*. *J. Agr. Food Chem.* 24, 865–868. doi: 10.1021/jf60206a028

Sekita, S., Yoshihira, K., Natori, S., Udagawa, S., Sakabe, F., Kurata, H., et al. (1982). Chaetoglobosins, cytotoxic 10-(indol- 3-yl)-[13]cytochalasans from *Chaetomium* spp. I. Production, isolation and some cytological effects of chaetoglobosin A-J. *Chem. Pharm. Bull.* 30, 1609–1617. doi: 10.1248/cpb.30.1609

Smedsgaard, J. (1997). Micro-scale extraction procedure for standardized screening of fungal metabolite production in cultures. *J. Chromatogr. A* 760, 264–270. doi: 10.1016/S0021-9673(96)00803-5

Sneath, P. H. A., and Sokal, R. R. (1973). *Numerical Taxonomy*. San Francisco, CA: Freeman.

Sunesen, L. O., and Stahnke, L. H. (2003). Mould starter cultures for dry sausages-selection, application and effects. *Meat Sci.* 65, 935–948. doi: 10.1016/S0309-1740(02)00281-4

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* 28, 2731–2739. doi: 10.1093/molbev/msr121

Tofalo, R., Schirone, M., Perpetuini, G., Angelozzi, G., Suzzi, G., and Corsetti, A. (2012). Microbiological and chemical profiles of naturally fermented table olives and brines from different Italian cultivars. *Anton. Leeuw.* 102, 121–131. doi: 10.1007/s10482-012-9719-x

Tosi, F., Sandri, S., Tedeschi, G., Malacarne, M., and Fossa, E. (2008). Variazioni di composizione e prorpietà fisico-chimiche del parmigiano-reggiano durante la maturazione e in differenti zone della forma. *Scienza e Tecnica Lattiero Casearia* 59, 507–528.

Vermelho, A. B., Meirelles, M. N., Lopes, A., Petinate, S. D., Chaia, A. A., and Branquinha, M. H. (1996). Detection of extracellular proteases from microorganism on agar plates. *Mem. Inst. Oswaldo Cruz.* 91, 755–760. doi: 10.1590/S0074-02761996000600020

Veselý, D., and Jelínek, R. (1995). *Penicillium aurantiogriseum* Dierckx produces chaetoglobosin a toxic to embryonic chickens. *Mycopathologia* 132, 31–33. doi: 10.1007/BF01138601

Wagener, R. E., Davis, N. D., and Diener, U. L. (1980). Penitrem A and roquefortine production by *Penicillium commune*. *Appl. Environ. Microbiol.* 39, 882–887.

White, T. J., Bruns, T., Lee, S., and Taylor, J. W. (1990). "Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics" in *PCR Protocols: A Guide to Methods and Applications*, eds M. A. Innis, D. H. Gelfand, J. J.Sninsky, and T. J. White (New York, NY: Academic Press, Inc.), 315–322.

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frontiers in Microbiology

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Evolution of Yeast Consortia during the Fermentation of Kalamata Natural Black Olives upon Two Initial Acidification Treatments

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The objective of this study was to elucidate the yeast consortia structure and dynamics during Greek-style processing of Kalamata natural black olives in different brine solutions. Olives were subjected to spontaneous fermentation in 7% (w/v) NaCl brine solution (control treatment) or brine acidified with (a) 0.5% (v/v) vinegar, and (b) 0.1% (v/v) lactic acid at the onset of fermentation. Changes in microbial counts, pH, acidity, organic acids, sugars, and alcohols were analyzed for a period of 187 days. Yeast consortia diversity was evaluated at days 4, 34, 90, 140, and 187 of fermentation. A total of 260 isolates were characterized at sub-species level by rep-PCR genomic fingerprinting with the oligo-nucleotide primer $(GTG)_5$. The characterization of yeast isolates at species level was performed by sequencing of the D1/D2 domain of 26S rRNA gene. Results showed that yeasts dominated the process presenting a relatively broad range of biodiversity composed of 11 genera and 21 species. No lactic acid bacteria (LAB) or *Enterobacteriaceae* could be enumerated after 20 and 10 days of fermentation, respectively. The dominant yeast species at the beginning were *Aureobasidium pullulans* for control and vinegar acidification treatments, and *Candida naeodendra* for lactic acid treatment. Between 34 and 140 days the dominant species were *Candida boidinii*, *Candida molendinolei* and *Saccharomyces cerevisiae*. In the end of fermentation the dominant species in all processes were *C. boidinii* and *C. molendinolei*, followed by *Pichia manshurica* and *S. cerevisiae* in lactic acid acidification treatment, *P. manshurica* in vinegar acidification treatment, and *Pichia membranifaciens* in control fermentation.

Keywords: black olives, Kalamata variety, fermentation, molecular identification, yeasts

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INTRODUCTION

Table olives are a well-known fermented vegetable of the Western world with a great impact on the economy of the Mediterranean countries, which have an outstanding contribution in the global production of processed olives amounting to *ca.* 30%, with Spain being the leading producer followed by Greece and Italy (International Olive Council [IOC], 2017). A renewed interest has been shown in the last years for the functional properties of table olives (Bevilacqua et al., 2012a; Kailis and Kiritsakis, 2017), which in relation to optimal nutrition as indicated by

the Mediterranean diet (Bach-Faig et al., 2017) resulted in increased consumption of table olives worldwide of *ca.* 2.5 million tons for the period 2015–2016 (International Olive Council [IOC], 2017). The Greek table olive sector has an important contribution in the economy of the country. According to the Interprofessional Association for Table Olives (DOEPEL, 2017), about 62,000 farmers and more than 100 companies are involved in the primary and secondary production of table olives. The annual production of processed table olives exceeds 200,000 tons, from which 85% is exported, representing 9.2% of the exports of Greek agricultural products.

Although all trade preparations of table olives are produced in the country, Greece has a long tradition in the production of natural black olives in brine, using olives from cv. Conservolea and Kalamata, which are two of the most economically important varieties for table olive processing in the country (Grounta et al., 2017). In the Greek processing system, olives are placed directly in brine solution with salt concentration of 8–10% (w/v) or even higher according to local practices, without any prior debittering pre-treatment. During the process, olives are subjected to spontaneous fermentation resulting in a characteristic final product with fruity aroma and a slightly bitter taste (Bleve et al., 2015). Diverse microbial populations are involved in olive fermentation including members of lactic acid bacteria (LAB) and yeasts which dominate the fermentation (Garrido-Fernández et al., 1997; Hurtado et al., 2008). These microorganisms through their metabolic activities in fermenting brines determine the organoleptic profile and the stability of the final product.

Taking into account that yeasts have a central role in the fermentation of natural black olives and the development of the final organoleptic characteristics, assessment of the biodiversity of yeast communities is fundamental for this trade preparation of table olives. Moreover, based on recent scientific findings, the contribution of yeasts in table olive processing has been reconsidered, giving emphasis on the beneficial aspects of yeasts due to their biotechnological and functional traits. For instance, yeasts have the ability to enhance the aroma and taste of fermented olives through the activity of specific enzymes such as lipases and esterases, that increase the free fatty acid content and thus result in the formation of several aromatic compounds such as ethanol, glycerol, higher alcohols and other desirable volatile compounds. Furthermore, biodegradation of polyphenols by specific yeast strains through the activity of β-glucosidase could lead to olive debittering without the use of any chemical treatment. In addition, the ability of specific yeast strains to produce enzymes such as phosphatases and phytases is an important technological feature as these enzymes can degrade phytic complexes and release inorganic phosphorous to the cells. Apart from the technological characteristics, yeasts exhibit probiotic potential such as tolerance through the gastrointestinal tract, inhibition of pathogens, adhesion to intestinal Caco-2 cell lines, and immune stimulation (Psani and Kotzekidou, 2006; Olstorpe et al., 2009; Arroyo-López et al., 2012b,c; Bevilacqua et al., 2012b; Tofalo et al., 2013; Bleve et al., 2015; Bonatsou et al., 2015, 2017b; Porru et al., 2018). Moreover, the ability of yeasts to co-aggregate on the surface of olives together with LAB and establish poly-microbial communities (biofilms) has been reported recently (Arroyo-López et al., 2012a; Grounta and Panagou, 2014; Benítez-Cabello et al., 2015) providing new perspectives for their use as multifunctional starters in Greek-style table olive processing.

In the past, identification of yeasts from Greek-style black olive fermentation has been reported (Kotzekidou, 1997) for industrially fermented olives, although no information was provided for the table olive varieties used. In another study (Nisiotou et al., 2010), the yeast succession and dominance in spontaneously fermented cv. Conservolea black olives in different brine solutions was reported at three different time points of fermentation. However, in the case of cv. Kalamata black olives the evolution of yeast microbiota during processing has been rarely explored. In a recent study (Bleve et al., 2015), molecular identification of LAB and yeast species with specific technological properties (i.e., presence of beta-glucosidase and inability for biogenic amine production) was reported during the spontaneous fermentation of cv. Conservolea and Kalamata natural black olives, in an attempt to develop a protocol for the pre-selection of fermentation starters. In another work (Tufariello et al., 2015), the dominance of inoculated yeast and LAB strains was reported for the same table olive varieties during Greek-style processing using molecular analyses. The aim of the present study was to assess the evolution of yeast community structure during the spontaneous fermentation of cv. Kalamata natural black olives upon different initial acidification treatments (with vinegar and lactic acid) by a multidisciplinary approach comprising chemical analyses and molecular profiling of yeast consortia.

MATERIALS AND METHODS

Olive Samples and Fermentation Procedures

Kalamata natural black table olives (*Olea europea* var. *ceraticarpa*) were harvested in early December 2015 at the appropriate stage of ripeness to be processed according to the Greek-style method. The raw material was kindly provided by Konstantopoulos S.A. table olive industry located in Katerini, Northern Greece, subjected to quality control by the provider and transported within 24 h to the Agricultural University of Athens. Fermentation was undertaken in 12 total volume screw-capped plastic vessels, containing 8 kg of olives and 4 L of freshly prepared 7.0% (w/v) NaCl brine (control treatment) or brine acidified at the onset of fermentation with (a) 0.5% (v/v) vinegar (*ca.* 6%, v/v, acetic acid), and (b) 0.1% (v/v) lactic acid (90%, Sigma) at the same salt concentration. The different acidification treatments with vinegar or lactic acid at these concentrations were selected since they are increasingly employed by processors of Kalamata black olives in Greece today. All treatments were performed in duplicate and the fermentation vessels were maintained at room temperature for an overall period of 187 days (*ca.* 6 months). During the process, salt concentration was adjusted to the initial value of 7.0% by periodic additions of coarse salt in the brine.

Microbiological Analyses

Olive samples were analyzed at 21 time points throughout fermentation (at days 1, 4, 7, 11, 15, 19, 22, 27, 34, 42, 50, 57, 66, 75, 90, 105, 118, 140, 153, 168, and 187) to determine the evolution of the indigenous microbiota on the surface of Kalamata olives. For this reason, four olives were randomly sampled at different depths from each fermentation vessel and the seed was removed using a sterile scalpel and forceps under aseptic conditions. Ten grams (10 g) of olive pulp were aseptically added in 90 mL sterile ¼ Ringer's solution and homogenized in a stomacher (LabBlender, Seward Medical, London, United Kingdom) for 60 s at room temperature. The resulting suspension was serially diluted in the same diluent and 1 or 0.1 mL of the appropriate dilutions were mixed or spread on the following agar media to enumerate the main microbial groups driving fermentation, namely: (i) Lactic acid bacteria (LAB) on de Man-Rogosa-Sharpe medium (MRS; 401728, Biolife, Milan, Italy) adjusted to pH 5.7 and supplemented with 0.05% (w/v) cycloheximide (AppliChem GmbH, Darmstadt, Germany), overlaid with the same medium and incubated at 25°C for 72 h; (ii) Yeasts and Molds on Rose Bengal Chloramphenicol agar (RBC; supplemented with selective supplement X009, Bury, United Kingdom), incubated at 25°C for 48 h; and (iii) *Enterobacteriaceae* on Violet Red Bile Glucose agar (VRBGA; Biolife, Milan, Italy), incubated at 37°C for 24 h. All plates were examined visually for typical colony types and morphological characteristics that were associated with each growth medium. Moreover, the selectivity of each medium was routinely checked by microscopic examination of smears prepared from randomly selected colonies obtained from the media and Gram staining. Results were expressed as log values of colony forming units per gram (log CFU/g) of olives.

Physicochemical Analyses

Physicochemical analyses were undertaken throughout the fermentation in the brines to monitor the changes of pH, titratable acidity, salt concentration, organic acids (lactic, acetic, malic, citric, tartaric, succinic), sugars (glucose, fructose), and alcohols (ethanol, glycerol). Specifically, pH values were monitored using a digital pH-meter (Russel Inc., Boston, MA, United States). Titratable acidity and salt concentration were determined according to Garrido-Fernández et al. (1997). Finally, organic acids, sugars and alcohols were analyzed by HPLC based on the protocol described by Bleve et al. (2015). All analyses were undertaken in duplicate and results are expressed as mean values ± standard deviation.

Yeast Isolation and Characterization

Yeast colonies were selected from the RBC plates according to Harrigan and McCance (1976) and purified by successive streaking on the same medium. Pure cultures were maintained at −80°C in Yeast Mold (YM) medium supplemented with 20% glycerol. Yeast species diversity was evaluated at five different sampling times, namely 4 (T_4), 34 (T_{34}), 90 (T_{90}), 140 (T_{140}), and 187 (T_{187}) days of fermentation in order to describe the evolution of yeast consortia at the beginning, middle and end of fermentation together with two additional intermediate points during the course of fermentation. A total of 271 isolates (i.e., 15–20 colonies per plate, sampling time and fermentation process) were subjected to sequencing of the D1/D2 domain of 26S rRNA gene for species assignment and microsatellite-primed PCR with the oligo-nucleotide primer $(GTG)_5$ for their characterization at sub-species level.

DNA extraction was performed according to Querol et al. (1992) modified by using lyticase (2.5 U/mL) (Lyticase from *Arthrobacter luteus*, Sigma–Aldrich, Germany) for yeast cell lysis. Amplification and sequencing of the D1/D2 domain of 26S rRNA gene was performed according to Pateraki et al. (2014). The taxonomic affiliation was assessed using the BLAST software in the GENBANK collection. Genotypic diversity at sub-species level was assessed in a final volume of 25 μl containing 3 mM $MgCl_2$, 2 mM $(GTG)_5$ primer, 2 U Taq polymerase (KAPA Taq PCR kit, KAPA Biosystems, United States), 0.2 mM dNTP's (Invitrogen) and 75 ng of template DNA. Amplification was carried out in a thermocycler (Applied Biosystems, Bedford, MA, United States) under the following conditions: initial denaturation at 94°C for 5 min; 30 cycles of 94°C for 30 s, 40°C for 1 min, and 72°C for 8 min; and a final extension at 72°C for 16 min. All PCR products were analyzed by electrophoresis in 1.5% agarose at 100 V for 1.5 h. Gels were scanned with the GelDoc system (Bio-Rad, Hercules, CA, United States). Conversion, normalization, and further analysis were performed using the Dice coefficient and the unweighted pair group method with arithmetic mean (UPGMA) cluster analysis with Bionumerics software version 6.1 (Applied Maths, Sint-Martens-Latem, Belgium). Results were expressed as isolation frequency (%) that was determined as the number of isolates of a particular yeast species divided by the total number of yeast isolates at a given sampling time.

Statistical Analysis

The data were subjected to one-way analysis of variance using Statistica 7.1 software (Statsoft Inc., Tulsa, OK, United States) to check for significant differences among microbiological and physicochemical characteristics according to the different fermentation treatments. Differences between means were determined by the statistical LSD test at $p \leq 0.05$.

RESULTS AND DISCUSSION

Population Dynamics and Physicochemical Changes

The population dynamics of the main microbial groups on olive drupes during the different fermentation processes is presented in **Figure 1**. Specifically, at the beginning of the process LAB population was 4.7 $\log_{10}$ CFU/g and began to actively grow until day 7 where the highest counts ($p \leq 0.05$) were observed in the acidified brines compared to control treatment. From this point, a progressive reduction in LAB population was observed until day 20 where no LAB could be detected on olive drupes. It needs to be noted that acidified brines favored higher growth profiles for LAB and this was more evident in the case of lactic

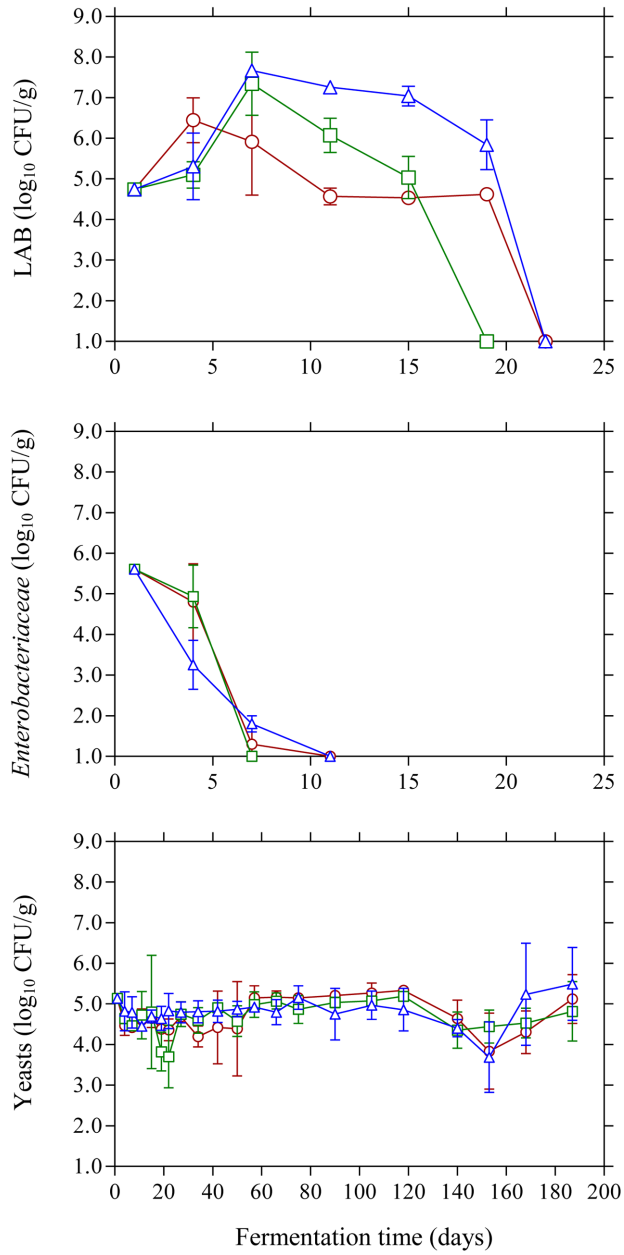


FIGURE 1 | Changes in the population of LAB, *Enterobacteriaceae*, and yeasts on olive drupes during the spontaneous fermentation of Kalamata natural black olives in brines with different initial acidification treatments. (○) without brine acidification (control); (□) brine acidified with 0.5 % (v/v) vinegar; (△) brine acidified with 0.1% (v/v) lactic acid. Data points are mean values of duplicate fermentations ± standard deviation.

acid where higher population ($p \leq 0.05$) of *ca.* 6.0 $\log_{10}$ CFU/g was observed at day 20 compared with the other two treatments, possibly due to the acid-tolerant features of this bacteria group. The changes in the population of *Enterobacteriaceae* were very similar (no statistical differences) between acidified and control treatments. Specifically, they were detected at *ca.* 5.6 $\log_{10}$ CFU/g but decreased rapidly and were below the enumeration limit (1.0 $\log_{10}$ CFU/g) after 11 days of fermentation. Regarding the effect of the different initial acidification treatments, the use of vinegar reduced the survival time by 4 days compared to control and lactic acid acidification. The survival period of this microbial group should be reduced to a minimum as prolonged persistence may result in spoilage of Kalamata olives in the form of gas pockets and fissures on the drupes (Sánchez-Gómez et al., 2006). Finally, yeasts coexisted with LAB for the first 15–20 days and their population was maintained 2–3 log cycles below LAB counts. However, from this time point onward, yeasts became the dominant microbial group that controlled fermentation. Their growth pattern was similar in all treatments (no statistical differences) regardless of initial acidification of the brine presenting an average initial population of 5.2 $\log_{10}$ CFU/g that was maintained around this value throughout the process.

The changes in pH and acidity were typical for Kalamata natural black olive fermentation. As expected, brine acidification affected the initial pH values (**Figure 2**). Thus, lactic acid acidified brine presented the lowest initial pH (3.04) followed by vinegar acidification (4.44) and finally the control (6.65) where no brine acidification was performed. In the case of brines acidified with lactic acid, pH presented a gradual increase until day 20 reaching a plateau at 4.52, whereas less variation in pH was observed for the vinegar acidification treatment. The final pH measured in the brines at the end of fermentation was similar in all treatments reaching a minimum value of 4.2. The effect of initial acidification was also evident in the initial values of titratable acidity in the brines, with control treatment presenting lower acidity values (0.05%) compared to lactic acid (0.12%) and vinegar (0.10%) treatments (**Figure 2**). However, the profile of acidity was similar in all cases presenting a gradual increase until the end of fermentation with final values of 0.38–0.44%. The obtained final values for pH and acidity differ from those reported in a previous work on Kalamata natural black olives (Bonatsou et al., 2017a), where the process was dominated by LAB instead of yeasts resulting in a vigorous lactic acid process with lower pH and higher acidity values. However, it must be underlined that the population dynamics of LAB is not fully comparable since in the latter work olives had been subjected to osmotic pre-treatment prior to processing and the brines had been supplemented with monosodium glutamate. In another study (Bleve et al., 2015) Conservolea and Kalamata olives were fermented naturally and the minimum pH values attained at the end of fermentation were 4.2–4.3 due to the dominance of yeasts over LAB, which is in good agreement with the final pH values reported in this work. It needs to be noted that despite the fact that yeasts were the dominant microbial group, the final values for pH and acidity are within the limits of the trade standard applying to table olives of the International Olive Council (International Olive Council [IOC], 2004) where for natural fermentations the maximum limit for pH and minimum acidity should be 4.3 and 0.3 %, respectively. Salinity in the brines was monitored throughout fermentation and adjusted to the initial value of 7% by periodic dry salt additions in the brines. Salt equilibrium was reached in *ca.* 2 months and until the end of the process salt concentration was maintained between 6 and 7%. It is

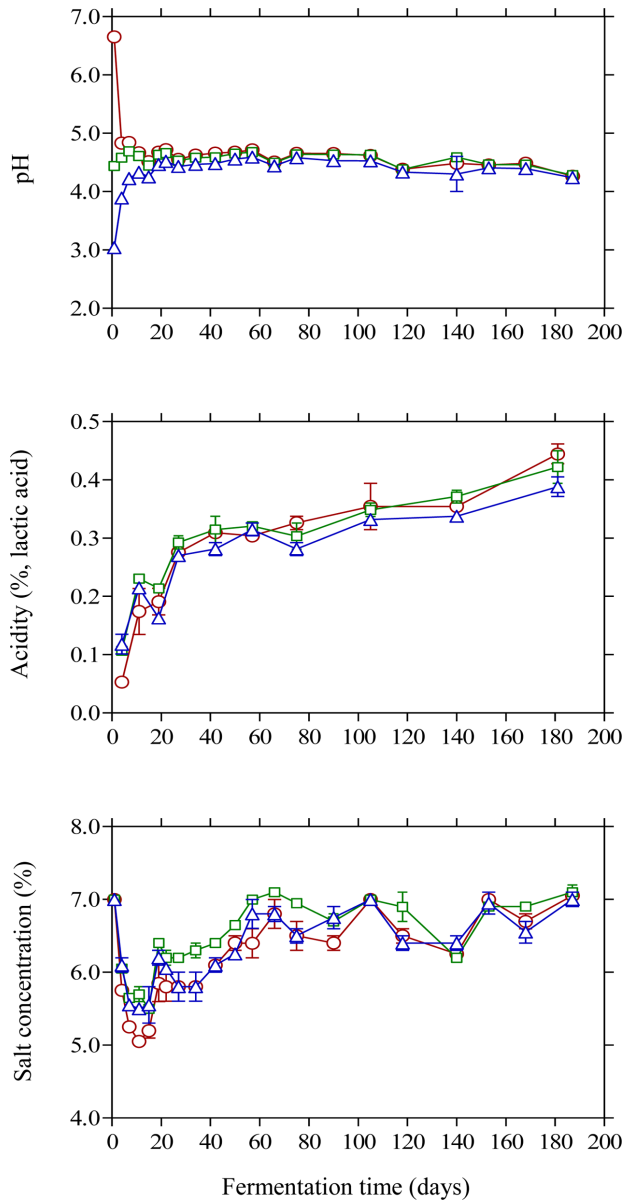


FIGURE 2 | Changes in pH, titratable acidity and salt concentration in the brines of Kalamata natural black olives during spontaneous fermentation with different initial acidification treatments. (○) without brine acidification (control); (□) brine acidified with 0.5% (v/v) vinegar; (△) brine acidified with 0.1% (v/v) lactic acid. Data points are mean values of duplicate fermentations ± standard deviation.

characteristic that within the first 10 days of fermentation the salt level was reduced by 1.5–2.0% and reached concentrations as low as 5% in control treatment (**Figure 2**) favoring thus the growth of LAB that became the dominant microbial group at the early stage of fermentation. It must be underlined that the maximum population of LAB was observed within this time period where salt concentration reached its lowest levels in the brines. The continuous salt addition in the brines from day 10 onward to reach the desired level of 7% could have possibly resulted in a potential stress on LAB that started to decline rapidly and became undetectable after 20 days of fermentation.

The changes in the concentration of organic acids in the brines are shown in **Figure 3**. Acetic acid was the main acid with considerable presence in the brines as detected by HPLC. Its concentration presented a gradual increase until day 105 and then remained unchanged until the end of the process. As expected, brines acidified with vinegar presented higher concentrations ($p \leq 0.05$) at the end of fermentation (3.6 g/L) compared with control (3.2 g/L) and lactic acid acidified brines (2.6 g/L). The presence of acetic acid could be attributed to yeast activity (Querol and Fleet, 2006; Bleve et al., 2014) although the contribution of LAB in acetic acid production could also be taken into consideration, especially in the early stage of fermentation (until day 20), due to the potential of homo- and heterofermentative LAB to generate acetic acid from fermentable material under particular conditions of environmental stress as well as from the metabolism of citrate (Bobillo and Marshall, 1991; Laëtitia et al., 2014). Lactic acid was also detected in the brines in concentrations not exceeding 2.0 g/L throughout the process. It presented a gradual increase until day 40 followed by a steady decline thereafter without statistically significant differences among the treatments. As LAB were practically absent from the process, the concentration of this organic acid was lower compared with previously published works on Kalamata olives fermentation (Bleve et al., 2015; Tufariello et al., 2015; Bonatsou et al., 2017a). Citric, malic and tartaric acids were also detected in the brines presenting a similar pattern with concentrations not exceeding 3.0 and 2.0 g/L, respectively, whereas succinic acid was presented in lower concentrations (<0.5 g/L) without statistical differences at the end of fermentations.

Glucose and fructose were the main sugars in the brines detected by HPLC (**Figure 4**). Glucose content rapidly increased at the early stage of fermentation and reached a maximum at day 20 and then decreased thereafter since it was consumed for microbial growth. Higher amounts of glucose were detected in the vinegar acidification treatment at this time compared with the other two fermentation procedures. This could be attributed to the fact that no LAB could be enumerated on olives at day 20, compared with the control and lactic acid acidification treatment where the counts of LAB were 4.6 and 5.8 $\log_{10}$ CFU/g, respectively (**Figure 1**). Thus, the different levels of glucose at day 20 reflect the different metabolic activity of bacteria in the brines at this particular time point. It is characteristic that at the end of fermentation glucose was not totally depleted but there was a remaining amount of *ca.* 0.5 g/L in the brines. A similar pattern was observed for fructose that was detected at lower levels in the brines compared with glucose. The concentration of this sugar in the brines never exceeded 0.5 g/L and it was undetectable after 60 days of fermentation. It has been reported that the prevalence of residual glucose over fructose could be attributed to the presence of some fructophilic non-*Saccharomyces* yeasts commonly found in the brine together with varietal characteristics of the fermented olives (Tufariello et al., 2015). Ethanol is a major metabolite which is produced by yeast fermentation which is important

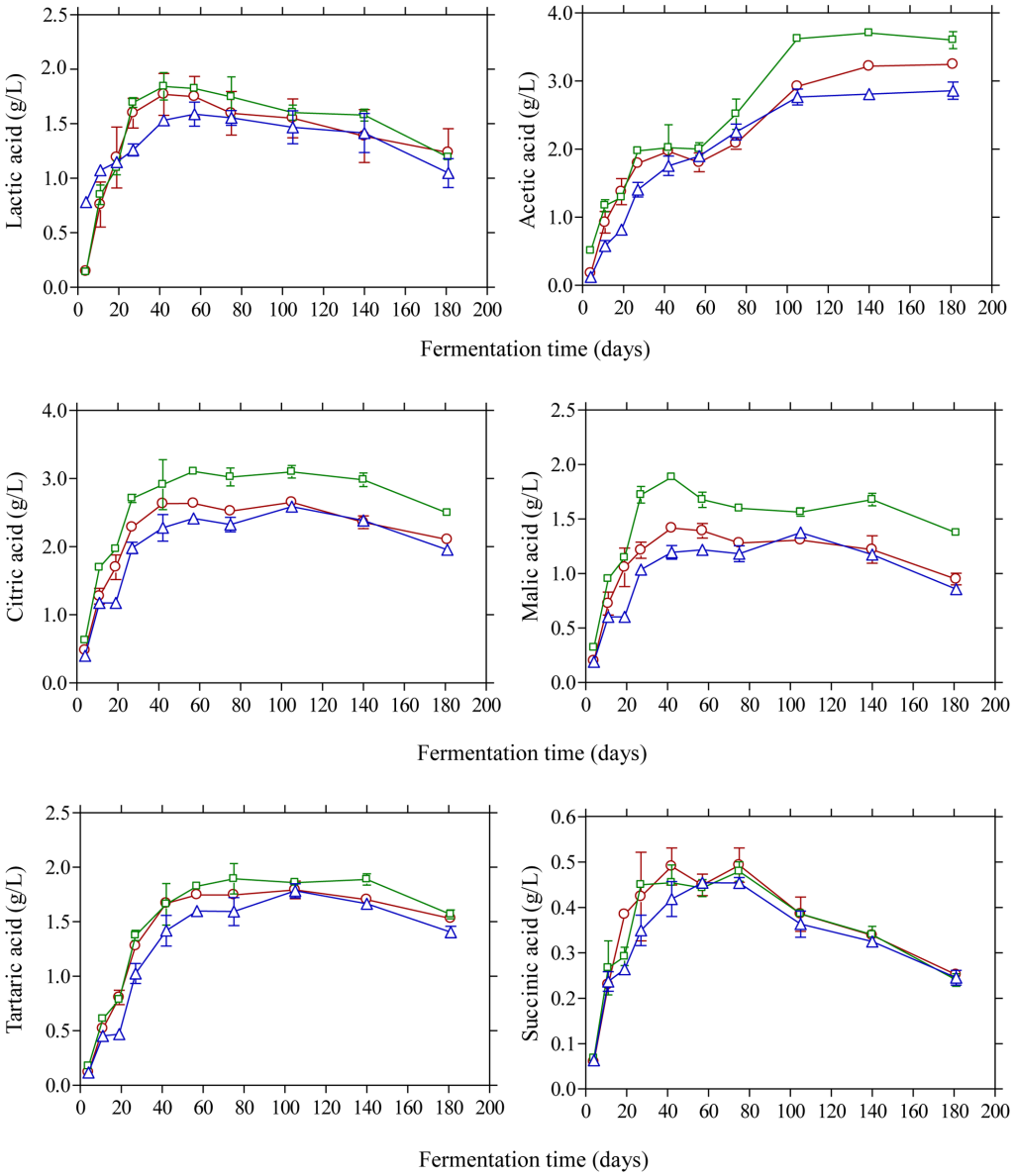


FIGURE 3 | Changes in the concentration (g/L) of organic acids (lactic, acetic, citric, malic, tartaric, and succinic) during the spontaneous fermentation of Kalamata natural black olives in brines acidified with 0.5% (v/v) vinegar (□), 0.1% (v/v) lactic acid (△), and without brine acidification (○). Data points are mean values of duplicate fermentations ± standard deviation.

for the sensory properties of natural black olives (Fleming et al., 1969). Its concentration increased gradually until day 60 of fermentation reaching values 4.9–5.1 g/L followed by a gradual decrease thereafter until the end of the process where ethanol concentration was maintained at *ca.* 3.5–3.8 g/L. No significant differences among the fermentation profiles could be established for ethanol concentration, indicating that yeast growth was not affected by the different acidification treatments in the brines. Glycerol is an important secondary product of yeast metabolism of sugars (Erasmus et al., 2004; Swiegers et al., 2005). It is also important to yeasts because it protects the cell from osmotic stress since it is an effective compatible solute (Dickinson and Kruckeberg, 2006). Its concentration increased gradually until the 60th day of fermentation in levels not exceeding 1.5 g/L followed by a decrease afterward reaching final values of *ca.* 1.0 g/L. The presence of this compound in Kalamata natural black olives has been reported previously by Italian researchers (Bleve et al., 2015; Tufariello et al., 2015) in amounts comparable with those reported in this work. The production of ethanol and glycerol due to yeast activity together with other volatile compounds has important contribution in flavor development and texture maintenance during table olive processing (Arroyo-López et al., 2008, 2012b,c).

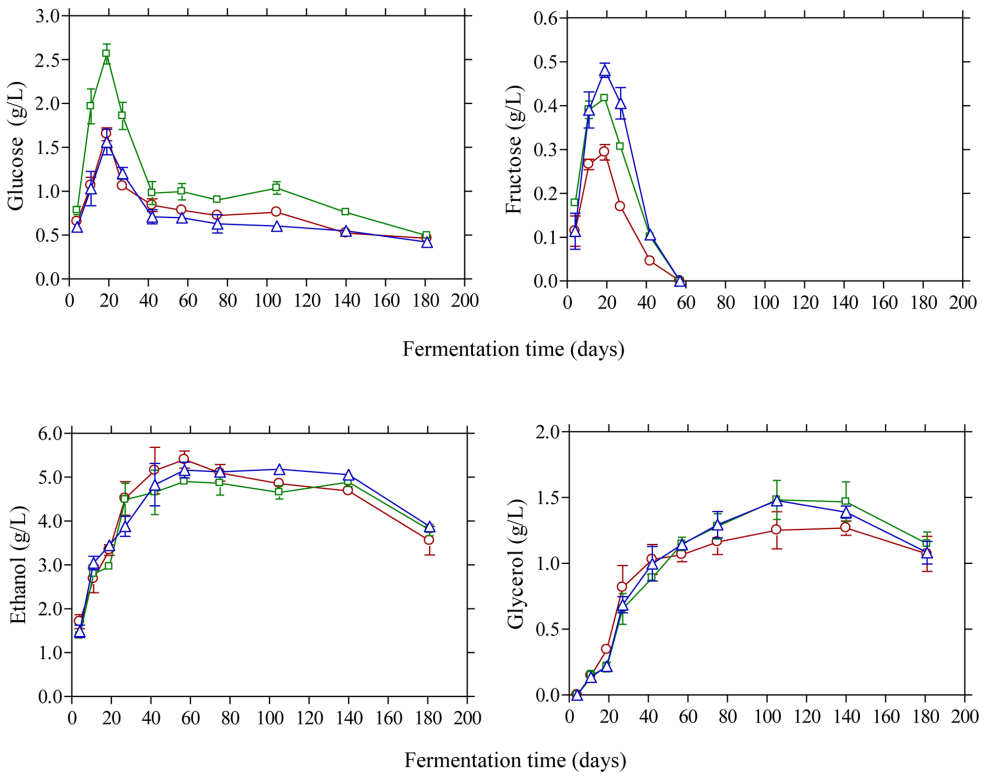


FIGURE 4 | Changes in the concentration of glucose, fructose, ethanol, and glycerol (g/L) in the brines during processing of Kalamata natural black olives with different initial acidification treatments. (□) Brine acidified with 0.5 % (v/v) vinegar; (△) brine acidified with 0.1 % (v/v) lactic acid; (○) without brine acidification (control).

Yeast Species Identification and Heterogeneity

A total of 260 isolates were subjected to sequencing of the D1/D2 region of 26S-rRNA gene for their taxonomic assignment at species level as well as to $(GTG)_5$ rep-PCR fingerprinting for their differentiation at subspecies level. The majority of the isolates were identified as *Candida molendinolei*, *Candida boidinii,* and *Saccharomyces cerevisiae*; another 18 species, namely *Aureobasidium pullulans*, *Barnettozyma californica*, *C. diddensiae*, *C. naeodendra*, *C. oleophila*, *C. silvae*, *Citeromyces matritensis*, *Cryptococcus laurentii*, *Debaryomyces hansenii*, *Metchnikowia pulcherrima*, *Pichia guilliermondii*, *Pichia kluyveri*, *P. manshurica*, *P. membranifaciens*, *Rhodotorula diobovatum*, *Rhodotorula glutinis*, *Rhodotorula mucilaginosa,* and *Zygoascus hellenicus* were also detected throughout fermentation. The yeast microecosystem composition during the three fermentation procedures assayed in this study is presented in **Figures 5A–C**. *C. molendinolei* and *C. boidinii* seemed to form a relatively stable dual species consortium since they were both detected from the 34th day of fermentation regarding the non-acidified brine and from the 4th day of fermentation regarding the acidified ones. A plethora of yeast species, namely *A. pullulans*, *B. californica*, *C. diddensiae*, *C. naeodendra*, *C. oleophila*, *C. silvae*, *C. matritensis*, *C. laurentii*, *C. bisporidii*, *D. hansenii*, *M. pulcherrima*, *P. guilliermondii*, *P. kluyveri*, *R. diobovatum*, *R. glutinis* and *R. mucilaginosa* was associated with the early stages of fermentation (until the 90th day) while significantly less, namely *P. manshurica*, *P. membranifaciens*, *S. cerevisiae* and *Z. hellenicus* in the final stages. This observation is consistent with a previous study undertaken on cv. Conservolea natural black olives to elucidate yeast diversity in different brine solutions (Nisiotou et al., 2010), in which a broader range of yeast species was also revealed at the beginning compared to the end of fermentation. This could be attributed to the fact that the species recovered at the beginning of fermentation originated from the initial microbiota adhered to the surface of olives and could be different to those present at the industrial environment where the resident yeast microbiota (e.g., in fermentation vessels) has an important contribution in fermentation (Botta and Cocolin, 2012).

The initial acidification of the brine with vinegar or lactic acid affected the heterogeneity of yeasts during the process. Thus, the dominant yeast species at the early stage of fermentation (T_4) were *A. pullulans* for control and vinegar acidification treatments, with 35.3 and 37.5% isolation frequency, respectively, and *C. naeodendra* for lactic acid treatment (26.7%). *A. pullulans* is an ubiquitous oxidative yeast like species encountered on food surfaces and the phyllosphere (Zalar et al., 2008), which is inhibited after the first fermentation days (Sabate et al., 2002). Its presence at the initial stage of fermentation has been reported recently in green (Valenčič et al., 2010; Alves et al., 2012) and black olives (Nisiotou et al., 2010). *C. naeodendra* has been

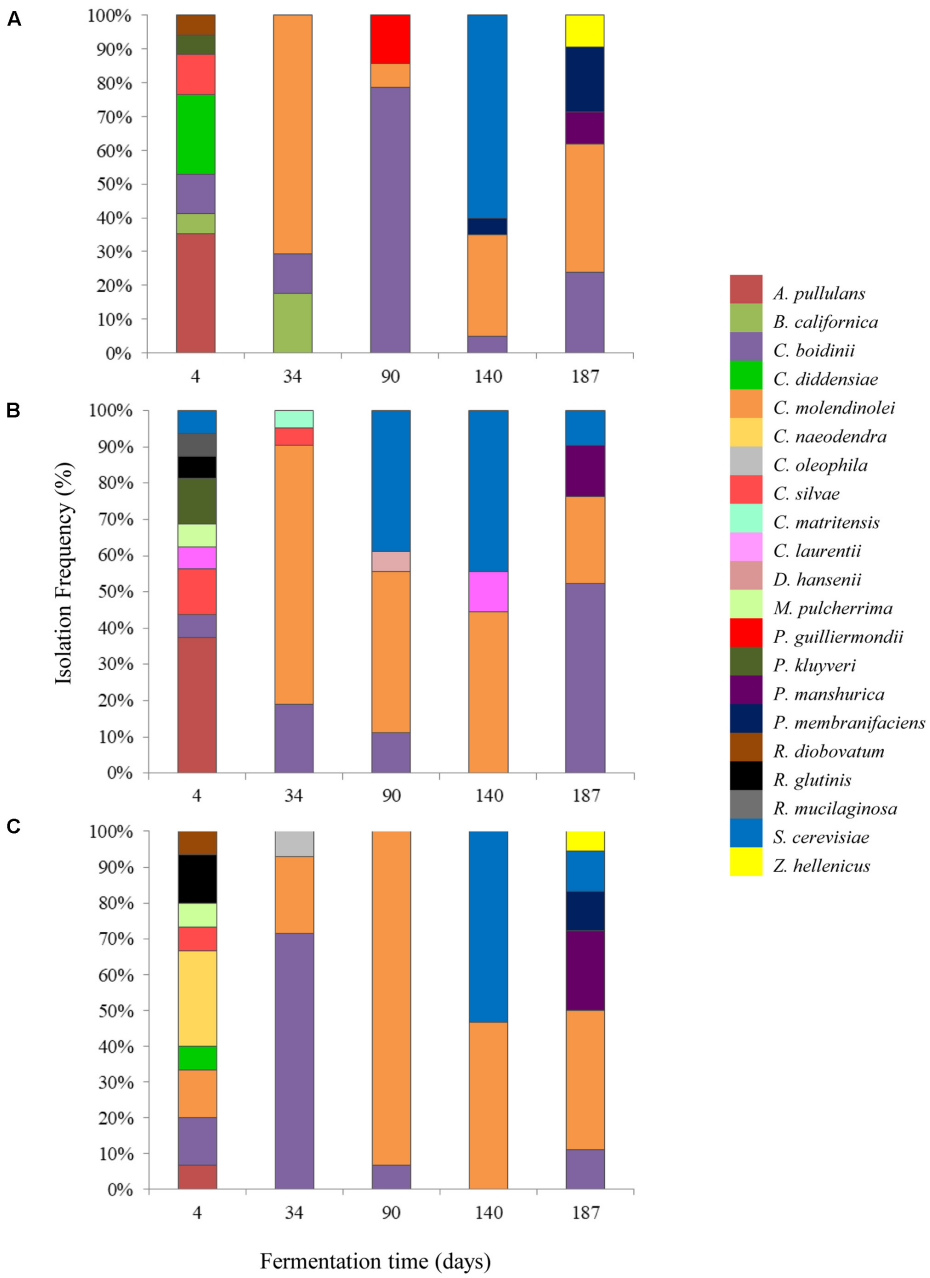


FIGURE 5 | Isolation frequency (%) of yeasts during the spontaneous fermentation of Kalamata natural black olives in brines with different initial acidification treatments. **(A)** Without brine acidification (control); **(B)** brine acidified with 0.5% (v/v) vinegar; **(C)** brine acidified with 0.1% (v/v) lactic acid.

described as a new species of the *C. diddensiae* group (van der Walt et al., 1973) and it is differentiated from most members of this group on the basis of its marked lipolytic activity. Its presence on olives is reported for the first time in this work. In the middle stage of fermentation (T_{90}) the dominant species were *C. boidini* in control fermentation (78.5% isolation frequency) and *C. molendinolei* in lactic acid and vinegar acidified brines with 93.3 and 44.4% isolation frequencies, respectively. *C. boidini* is very common in all table olives preparations (Nisiotou et al., 2010; Bautista-Gallego et al., 2011; Tofalo et al., 2013; Pereira et al., 2015; Porru et al., 2018), whereas *C. molendinolei* has been initially isolated from olive oil (Mari et al., 2016) and only recently from table olives (Mateus et al., 2016; Porru et al., 2018). Finally, at the end of fermentation (T_{187}), apart from the above mentioned yeast species, *P. membranifaciens* was also recovered from control fermentation (19.0%) as well as *P. manshurica* from lactic acid (22.2%) and vinegar (14.3%) acidified brines. Both yeasts have been widely identified in table olives (Hurtado et al., 2008; Nisiotou et al., 2010; Bleve et al., 2014; Pereira et al., 2015), whereas *P. membranifaciens* may also

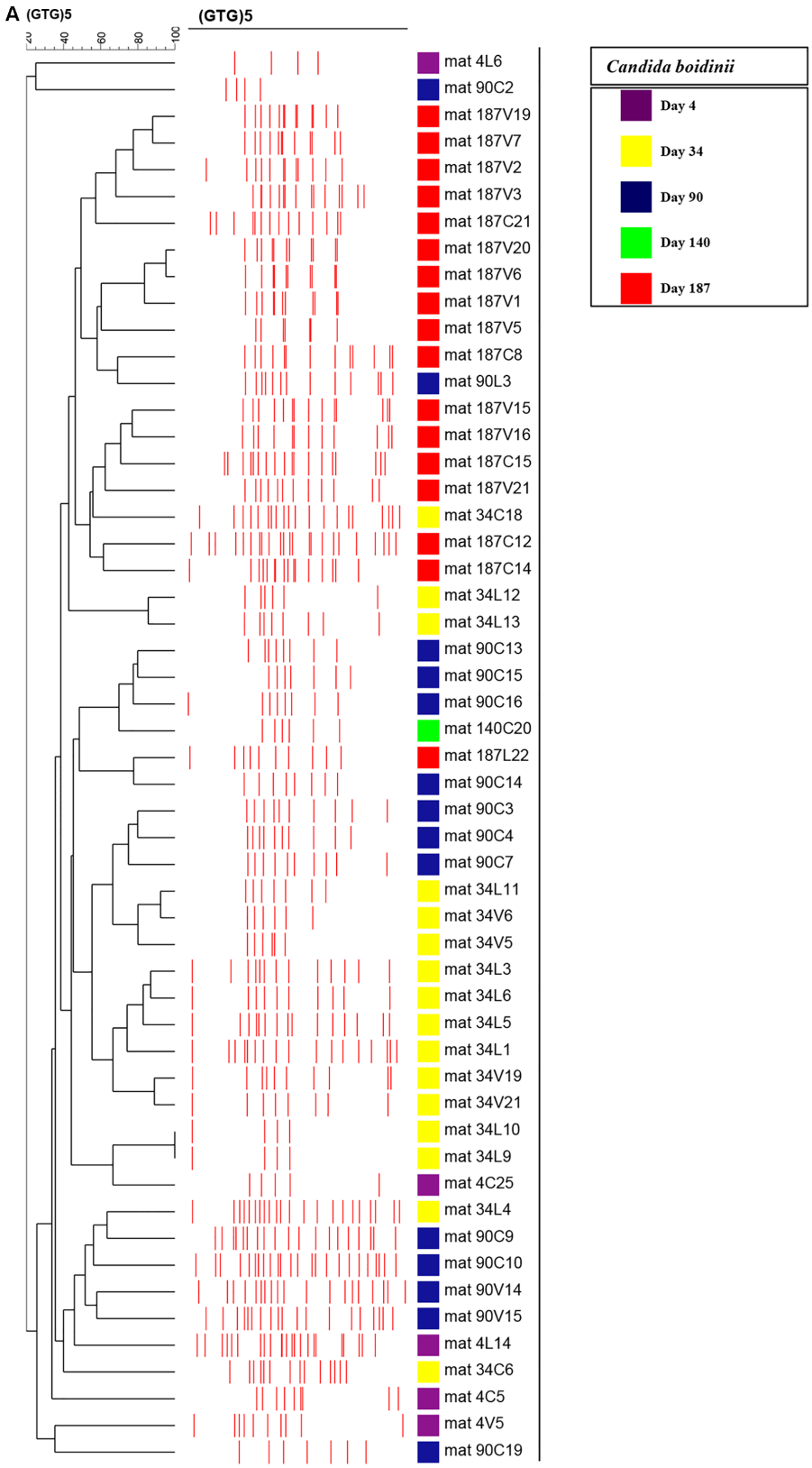
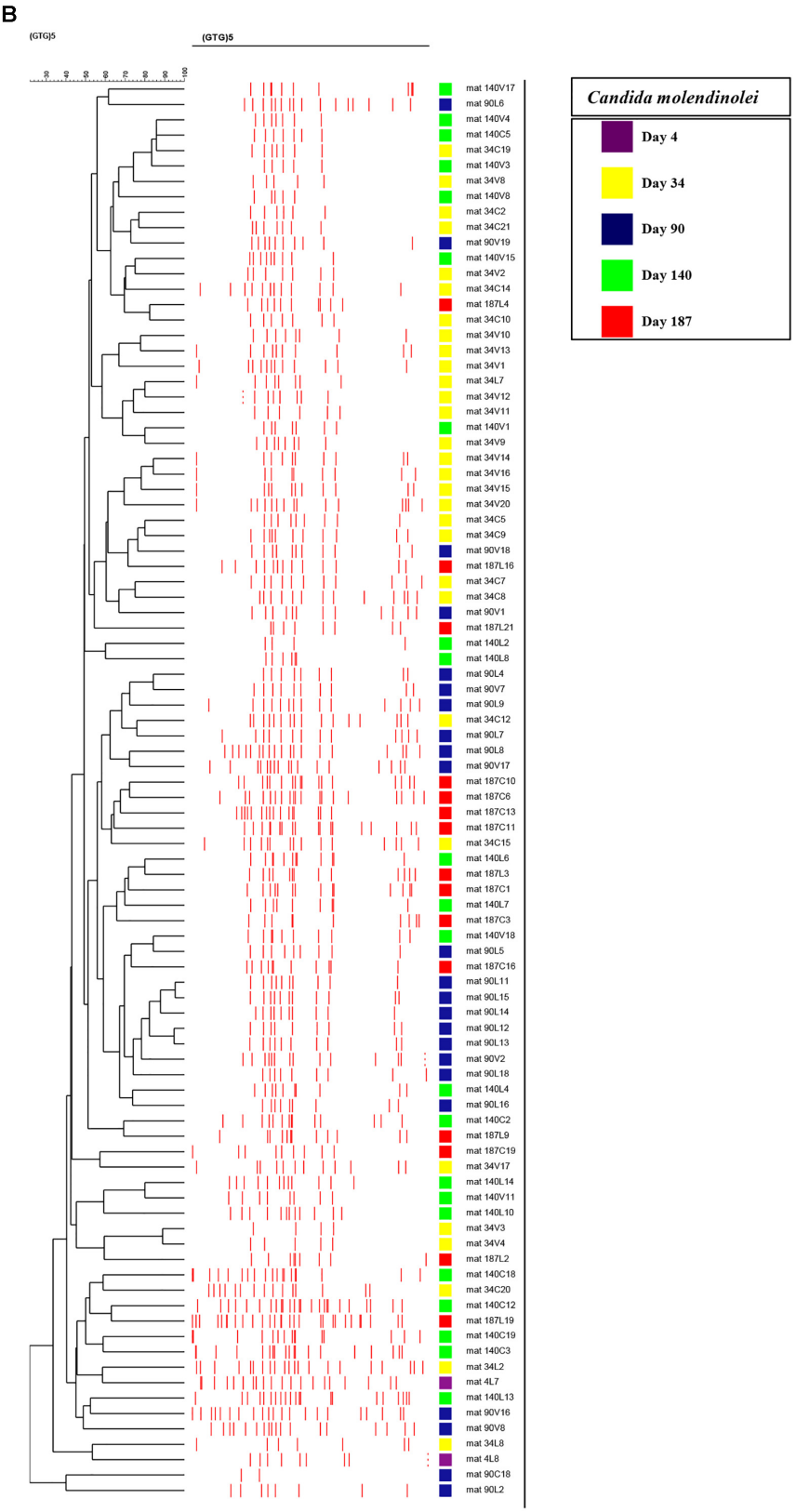


FIGURE 6 | Continued



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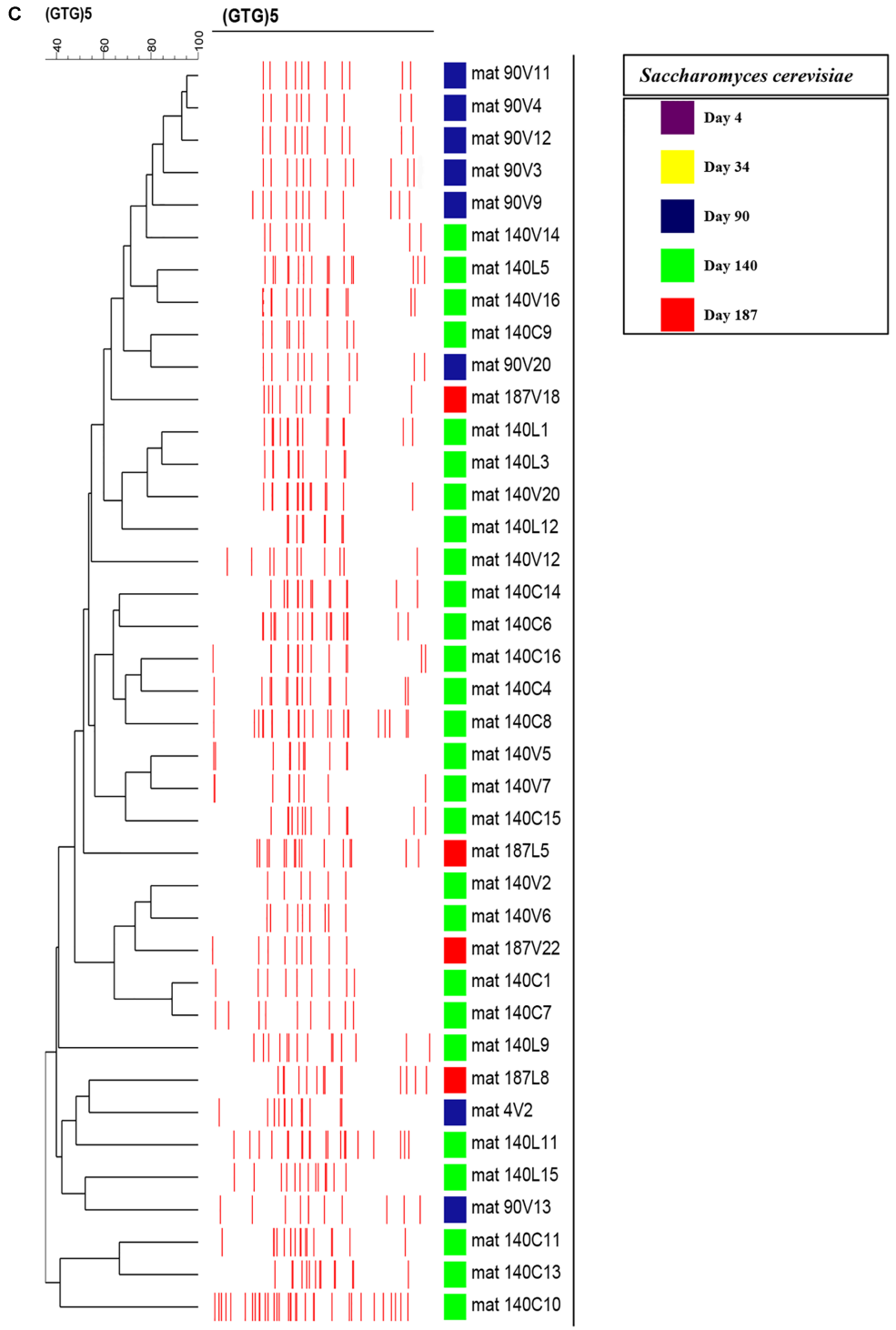

FIGURE 6 | Cluster analysis of rep-PCR patterns of the dominant yeast species obtained during the spontaneous fermentation of Kalamata natural black olives: **(A)** *Candida boidinii*, **(B)** *Candida molendinolei*, **(C)** *Saccharomyces cerevisiae*. Distance is indicated by the mean correlation coefficient [*r* (%)] and clustering was performed by UPGMA analysis.

influence fermentation by affecting yeast association through the production of killer toxins (Psani and Kotzekidou, 2006; Arroyo-López et al., 2008).

The cluster analysis of rep-PCR patterns of the yeast isolates obtained during fermentation without acidification (control) or with the addition of vinegar or lactic acid, respectively,

is exhibited in Supplementary Figures 1a–c. Moreover, cluster analysis of each of the main yeast species identified (i.e., *C. molendinolei*, *C. boidinii,* and *S. cerevisiae*) was created and presented in **Figures 6A–C**. In all cases, with two exceptions different genotypic profiles were obtained from isolates belonging to the same species, which indicates effective differentiation at subspecies level. The exceptions refer to *S. cerevisiae* strains $90V_{11}$ and $90V_{12}$, as well as *C. boidinii* strains $34L_9$ and $34L_{10}$, that were isolated from the vinegar acidification treatment at day 90 and lactic acid treatment at day 34, respectively. The ability of $(GTG)_5$ fingerprinting to differentiate yeasts at subspecies level has been adequately exhibited (Pateraki et al., 2014; Ramirez-Castrillon et al., 2014; Capece et al., 2016; Verspohl et al., 2017). Assessment of yeast population dynamics with this approach often reveals a succession at subspecies level, which is quite reasonable considering the spontaneous nature of fermentation.

CONCLUSION

This work described for the first time the evolution of yeast consortia at species and sub-species level associated with cv. Kalamata natural black olive fermentation under different initial acidification procedures commonly employed by Greek processors. Despite the fact that LAB dominated at the early stage of fermentation, they could not eventually survive in the brines and fermentation was undertaken by yeasts that became the dominant microorganisms, presenting a broad range of biodiversity including 11 genera and 21 species. The different acidification agents (vinegar and lactic acid) employed in the fermentation affected the final composition of yeast species on olives. Given the important contribution of yeasts in natural black olive fermentation, the results of this work could provide information on yeast heterogeneity for one of the most economically important Greek table olive varieties. In addition, table olive research has been focused on the beneficial effects of yeasts during olive processing due to their biotechnological and probiotic potential. Thus, further work is underway to elucidate the multifunctional features of selected yeast species from this consortium to be used as starter cultures in inoculated fermentations of Kalamata black olives to provide new perspectives for this particular trade preparation of table olives.

AUTHOR CONTRIBUTIONS

SB performed the experiments, analyzed the data, and contributed in the preparation of the paper. SP was involved in the molecular characterization of yeast species. EP was involved in the experimental design, interpretation of the results, and revised the paper.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2017.02673/full#supplementary-material

REFERENCES

Alves, M., Gonçalves, T., and Quintas, C. (2012). Microbial quality and yeast population dynamics in cracked green table olives' fermentations. *Food Control* 23, 363–368. doi: 10.1016/j.foodcont.2011.07.033

Arroyo-López, F. N., Bautista-Gallego, J., Domínguez-Manzano, J., Romero-Gil, V., Rodríguez-Gómez, F., García-García, P., et al. (2012a). Formation of lactic acid bacteria-yeasts communities on the olive surface during Spanish-style Manzanilla fermentations. *Food Microbiol.* 32, 295–301. doi: 10.1016/j.fm.2012.07.003

Arroyo-López, F. N., Querol, A., Bautista-Gallego, J., and Garrido-Fernández, A. (2008). Role of yeasts in table olive production. *Int. J. Food Microbiol.* 128, 189–196. doi: 10.1016/j.ijfoodmicro.2008.08.018

Arroyo-López, F. N., Romero-Gil, V., Bautista-Gallego, J., Rodríguez-Gómez, F., Jiménez-Díaz, R., García-García, P., et al. (2012b). Potential benefits of the application of yeast starters in table olive processing. *Front. Microbiol.* 3:161. doi: 10.3389/fmicb.2012.00161

Arroyo-López, F. N., Romero-Gil, V., Bautista-Gallego, J., Rodríguez-Gómez, F., Jiménez-Díaz, R., García-García, P., et al. (2012c). Yeasts in table olive processing: desirable or spoilage microorganisms? *Int. J. Food Microbiol.* 160, 42–49. doi: 10.1016/j.ijfoodmicro.2012.08.003

Bach-Faig, A., Berry, E. M., Lairon, D., Reguant, J., Trichopoulou, A., Dernini, S., et al. (2017). Mediterranean diet pyramid today. Science and cultural updates. *Public Health Nutr.* 14, 2274–2284. doi: 10.1017/S1368980011002515

Bautista-Gallego, J., Rodríguez-Gómez, F., Barrio, E., Querol, A., Garrido-Fernández, A., and Arroyo-López, F. N. (2011). Exploring the yeast biodiversity of green table olive industrial fermentations for technological applications. *Int. J. Food Microbiol.* 147, 89–96. doi: 10.1016/j.ijfoodmicro.2011.03.013

Benítez-Cabello, A., Romero-Gil, V., Rodríguez-Gómez, F., Garrido-Fernández, A., Jiménez-Díaz, R., and Arroyo-López, F. N. (2015). Evaluation and identification of poly-microbial biofilms on natural green Gordal table olives. *Antonie Van Leeuwenhoek* 108, 597–610. doi: 10.1007/s10482-015-0515-2

Bevilacqua, A., Corbo, M. R., and Sinigaglia, M. (2012a). Selection of yeasts as starter cultures for table olives: a step-by-step procedure. *Front. Microbiol.* 3:194. doi: 10.3389/fmicb.2012.00194

Bevilacqua, A., de Stefano, F., Augello, S., Pignatiello, S., Sinigaglia, M., and Corbo, M. R. (2012b). Biotechnological innovations for table olives. *Int. J. Food Sci. Nutr.* 66, 127–131. doi: 10.3109/09637486.2014.959901

Bleve, G., Tufariello, M., Durante, M., Grieco, F., Ramires, F. A., Mita, G., et al. (2015). Physico-chemical characterization of natural fermentation process of Conservolea and Kalamata table olives and development of a protocol for the pre-selection of fermentation starters. *Food Microbiol.* 46, 368–382. doi: 10.1016/j.fm.2014.08.021

Bleve, G., Tufariello, M., Durante, M., Perbellini, E., Ramires, F. A., Grieco, F., et al. (2014). Physico-chemical and microbiological characterization of spontaneous fermentation of Cellina di Nardò and Leccino table olives. *Front. Microbiol.* 5:570. doi: 10.3389/fmicb.2014.00570

Bobillo, M., and Marshall, V. M. (1991). Effect of salt and culture aeration on lactate and acetate production by *Lactobacillus plantarum*. *Food Microbiol.* 8, 153–160. doi: 10.1016/0740-0020(91)90008-P

Bonatsou, S., Benítez, A., Rodríguez-Gómez, F., Panagou, E. Z., and Arroyo-López, F. N. (2015). Selection of yeasts with multifunctional features for application as starters in natural black table olive processing. *Food Microbiol.* 46, 66–73. doi: 10.1016/j.fm.2014.07.011

Bonatsou, S., Iliopoulos, V., Mallouchos, A., Gogou, E., Oikonomopoulou, V., Krokida, M., et al. (2017a). Effect of osmotic dehydration of olives as pre-fermentation treatment and partial substitution of sodium chloride by monosodium glutamate in the fermentation profile of Kalamata natural black olives. *Food Microbiol.* 63, 72–83. doi: 10.1016/j.fm.2016.11.001

Bonatsou, S., Tassou, C. C., Panagou, E. Z., and Nychas, G.-J. E. (2017b). Table olive fermentation using starter cultures with multifunctional potential. *Microorganisms* 5:30. doi: 10.3390/microorganisms5020030

Botta, C., and Cocolin, L. (2012). Microbial dynamics and biodiversity in table olive fermentation: culture-dependent and -independent approaches. *Front. Microbiol.* 3:245. doi: 10.3389/fmicb.2012.00245

Capece, A., Granchi, L., Guerrini, S., Mangani, S., Romaniello, R., Vincenzini, M., et al. (2016). Diversity of *Saccharomyces cerevisiae* strains isolated from two Italian wine-producing regions. *Front. Microbiol.* 7:1018. doi: 10.3389/fmicb.2016.01018

Dickinson, J. R., and Kruckeberg, A. L. (2006). "Carbohydrate metabolism," in *Yeasts in Food and Beverages*, eds A. Querol and G. H. Fleet (Berlin: Springer-Verlag), 215–242.

DOEPEL (2017). *Interprofessional Association for Table Olives*. Available at: http://www.doepel.gr [accessed October 28, 2017].

Erasmus, D. J., Cliff, M., and van Vuuren, H. J. J. (2004). Impact of yeast strain on the production of acetic acid, glycerol and the sensory attributes of icewine. *Am. J. Enol. Vitic.* 55, 371–378.

Fleming, H. P., Etchells, J. L., and Bell, T. A. (1969). Vapor analysis of fermented Spanish-type green olives by gas chromatography. *J. Food Sci.* 34, 419–422. doi: 10.1111/j.1365-2621.1969.tb12794.x

Garrido-Fernández, A., Fernández Díez, M. J., and Adams, M. R. (1997). *Table Olives: Production and Processing*. London: Chapman & Hall. doi: 10.1007/978-1-4899-4683-6

Grounta, A., and Panagou, E. Z. (2014). Mono and dual species biofilm formation between *Lactobacillus pentosus* and *Pichia membranifaciens* on the surface of black olives under different sterile brine conditions. *Ann. Microbiol.* 64, 1757–1767. doi: 10.1007/s13213-014-0820-4

Grounta, A., Tassou, C. C., and Panagou, E. Z. (2017). "Greek-style table olives and their functional value," in *Olives and Olive Oil as Functional Foods*, eds A. Kiritsakis and F. Shahidi (Oxford: Wiley), 325–342. doi: 10.1002/9781119135340.ch16

Harrigan, W. F., and McCance, M. E. (1976). *Laboratory Methods in Food and Dairy Microbiology*. London: Academic Press.

Hurtado, A., Reguant, C., Esteve-Zarzoso, B., Bordons, A., and Rozès, N. (2008). Microbial population dynamics during the processing of Arbequina table olives. *Food Res. Int.* 41, 738–744. doi: 10.1016/j.fm.2009.05.010

International Olive Council [IOC] (2004). *Trade Standard Applying to Table Olives. DOI/OT/Nc no 1, Madrid, Spain*. Available at: http://www.internationaloliveoil.org/estaticos/view/222-standards

International Olive Council [IOC] (2017). *World Table Olive Figures*. Available at: http://www.internationaloliveoil.org/estaticos/view/132-world-table-olive-figures [accessed October 29, 2017].

Kailis, G., and Kiritsakis, A. (2017). "Table olives: processing, nutritional and health implications," in *Olives and Olive Oil as Functional Foods*, eds A. Kiritsakis and F. Shahidi (Oxford: Wiley), 295–324. doi: 10.1002/9781119135340.ch15

Kotzekidou, P. (1997). Identification of yeasts from black olives in rapid system microtitre plates. *Food Microbiol.* 14, 609–616. doi: 10.1006/fmic.1997.0133

Laëtitia, G., Pascal, D., and Yann, D. (2014). The citrate metabolism in homo- and heterofermentative LAB: a selective means of becoming dominant over other microorganisms in complex ecosystems. *Food Nutr. Sci.* 5, 953–969. doi: 10.4236/fns.2014.510106

Mari, E., Guerrini, S., Granchi, L., and Vincenzini, M. (2016). Enumeration and rapid identification of yeasts during extraction process of extra virgin olive oil in Tuscany. *World J. Microbiol. Biotechnol.* 32:93. doi: 10.1007/s11274-016-2049-9

Mateus, T., Santo, D., Saúde, C., Pires-Cabral, P., and Quintas, P. (2016). The effect of NaCl reduction in the microbiological quality of cracked green table olives of the Maçanilha Algravia cultivar. *Int. J. Food Microbiol.* 218, 57–65. doi: 10.1016/j.ijfoodmicro.2015.11.008

Nisiotou, A. A., Chorianopoulos, N., Nychas, G. J. E., and Panagou, E. Z. (2010). Yeast heterogeneity during spontaneous fermentation of black Conservolea olives in different brine solutions. *J. Appl. Microbiol.* 108, 396–405. doi: 10.1111/j.1365-2672.2009.04424.x

Olstorpe, M., Schnüren, J., and Passoth, V. (2009). Screening of yeast strains for phytase activity. *FEMS Yeast Res.* 9, 478–488. doi: 10.1111/j.1567-1364.2009.00493.x

Pateraki, C., Paramithiotis, S., Doulgeraki, A. I., Kallithraka, S., Kotseridis, G., and Drosinos, E. H. (2014). Effect of sulfur dioxide addition in wild yeast population dynamics and polyphenolic composition during spontaneous red wine fermentation from *Vitis vinifera* cultivar Agiorgitiko. *Eur. Food Res. Technol.* 239, 1067–1075. doi: 10.1007/s00217-014-2303-z

Pereira, L. E., Ramalhosa, E., Borges, A., Pereira, J. A., and Baptista, P. (2015). Yeast dynamics during the natural fermentation process of table olives (*Negrinha de Freixo* cv.). *Food Microbiol.* 46, 582–586. doi: 10.1016/j.fm.2014.10.003

Porru, C., Rodríguez-Gómez, F., Benítez-Cabello, A., Jiménez-Díaz, R., Zara, G., Budroni, M., et al. (2018). Genotyping, identification and multifunctional features of yeasts associated to Bosana naturally black table olive fermentations. *Food Microbiol.* 69, 33–42. doi: 10.1016/j.fm.2017.07.010

Psani, M., and Kotzekidou, P. (2006). Technological characteristics of yeast strains and their potential as starter adjuncts in Greek-style black olive fermentation. *World J. Microbiol. Biotechnol.* 22, 1329–1336. doi: 10.1007/s11274-006-9180-y

Querol, A., Barrio, E., Huerta, T., and Ramon, D. (1992). Molecular monitoring of wine fermentations conducted by active dry yeast srtains. *Appl. Environ. Microbiol.* 58, 2948–2953.

Querol, A., and Fleet, G. H. (eds). (2006). *Yeasts in Food and Beverages*. Berlin: Springer-Verlag.

Ramirez-Castrillon, M., Mendes, S. D. C., Inostroza-Ponta, M., and Valente, P. (2014). $(GTG)_5$ MSP-PCR fingerprinting as a technique for discrimination of wine associated yeasts? *PLOS ONE* 9:e105870. doi: 10.1371/journal.pone.0105870

Sabate, J., Cano, J., Esteve-Zarzoso, B., and Guillamón, J. M. (2002). Isolation and identification of yeasts associated with vineyard and winery by RFLP analysis of ribosomal genes and mitochondrial DNA. *Microbiol. Res.* 157, 267–274. doi: 10.1078/0944-5013-00163

Sánchez-Gómez, A. H., Garcia Garcia, P., and Rejano Navarro, L. (2006). Elaboration of table olives. *Grasas Aceites* 57, 86–94. doi: 10.3989/gya.2006.v57.i1.24

Swiegers, J. H., Bartowsky, E. J., Henschke, P. A., and Pretorius, I. S. (2005). Yeast and bacterial modulation of wine aroma and flavour. *Aust. J. Grape Wine Res.* 11, 139–173. doi: 10.1111/j.1755-0238.2005.tb00285.x

Tofalo, R., Perpetuini, G., Schirone, M., Suzzi, G., and Corsetti, A. (2013). Yeast biota associated to naturally fermented table olives from different Italian cultivars. *Int. J. Food Microbiol.* 161, 203–208. doi: 10.1016/j.ijfoodmicro.2012.12.011

Tufariello, M., Durante, M., Ramires, F. A., Grieco, F., Tommasi, L., Perbellini, E., et al. (2015). New process for production of fermented black table olives using selected autochthonous microbial resources. *Front. Microbiol.* 6:1007. doi: 10.3389/fmicb.2015.01007

Valenčič, V., Mavsar, D. B., Bučar-Miklavčič, M., Butinar, B., Čadež, N., Golob, T., et al. (2010). The impact of production technology on the growth of indigenous microflora and quality of table olives from Slovenian Istria. *Food technol. Biotechnol.* 48, 404–410.

van der Walt, J. P., Johannsen, E., and Nakase, T. (1973). Candida naeodendra, a new species of the *Candida diddensii* group. *Antonie Van Leeuwenhoek* 39, 491–495. doi: 10.1007/BF02578892

Verspohl, A., Solieri, L., and Giudici, P. (2017). Exploration of genetic and phenotypic diversity within *Saccharomyces uvarum* for driving strain improvement in winemaking. *Appl. Microbiol. Biotechnol.* 101, 2507–2521. doi: 10.1007/s00253-016-8008-4

Zalar, P., Gostinčar, C., de Hoog, G. S., Sudhadham, M., and Gunde-Cimerman, N. (2008). Redefinition of *Aureobasidium pullulans* and its varieties. *Stud. Mycol.* 61, 21–38. doi: 10.3114/sim.2008.61.02

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ORIGINAL RESEARCH
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Microbiological and Physicochemical Changes in Natural Green Heat-Shocked *Aloreña de Málaga* Table Olives

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Preserving the highly appreciated natural freshness of *Aloreña de Málaga* table olives and preventing their progressive darkening during processing is a major challenge. In this work, heat-shocked (60°C, 5 min) fruits were processed according to the three denominations referred to in the Protected Designation of Origen (cured, fresh green, and traditional) and their characteristics compared with those that followed the habitual industrial process (controls). The results revealed that the effects of the heat treatment on the evolution of pH, titratable acidity, salt, sugar, organic acid, ethanol content, texture, and color of fruits as well as on microbial populations (yeasts and lactic acid bacteria) were slight in the case of the fresh green and cured presentations. However, the differences between heat-shocked and its control were remarkable in the traditional process. Notably, the heat treatment favored lactic acid fermentation, retention of the green appearance of the fruits, stability during packaging, and led to the highest sensory evaluation. The metagenomic analysis carried out at the end of the fermentation revealed the presence in all samples of three genera (*Lactobacillus*, *Pediococcus,* and *Celerinatantimonas*) which encompassed most of the sequences. The number of *Lactobacillus* sequences was statistically higher ($p \geq 0.05$) in the case of traditional heat-shocked fruits than in its control.

Keywords: heat treatment, olive packaging, sensory evaluation, table olives, metagenomic analysis

INTRODUCTION

Table olives are a major component of the Mediterranean diet and culture. Nowadays, they constitute one of the most important fermented vegetables in the world, with a production which exceeds 2.4 million tons/year (International Olive Council [IOC], 2016). Green Spanish-style, Greek naturally black, and ripe Californian styles are among the most popular and well-known table olive commercial presentations in the world (Garrido-Fernández et al., 1997).

However, in the last years, consumers have demanded more traditional and natural homemade-style elaborations. This is the case of *Aloreña de Málaga*, a table olive speciality processed as natural green olives under a Spanish Protected Designation of Origin (PDO) recognized by the European Union (DOUE, 2012). Their peculiar characteristics are related

to the production area (climate, edaphology, and geographical location in the Guadalhorce Valley, Málaga, Spain). Therefore, their products are quite different from other green natural table olives. *Aloreña de Málaga* usually contains low-to-moderate concentrations of oleuropein (the main bitter compound of olives) and, for this reason, is not subjected to lye treatment for debittering. The speciality is seasoned with fennel, thyme, garlic, and pepper, which are frequently added during packaging, making the product rich in aroma. To preserve their typical organoleptic characteristics and highly valued freshness (green aspect), packages are not usually stabilized by pasteurization.

The PDO regulation includes three different denominations (López López and Garrido Fernández, 2006):

(i) Cured *Aloreña de Málaga* olives (CA). The harvested fruits are placed directly in brine (5–6% NaCl, 10,000 L fermentation vessels) where they undergo a full fermentation for a minimum of 90 days. Then, the olives are progressively cracked, seasoned and packaged according to demand.

(ii) Fresh Green (FG) *Aloreña de Málaga* olives. The product is characterized by the immediate cracking after harvesting. Then, the fruits are brined in a 10–11% NaCl solution in plastic drums (220 L volume), where they should remain for at least 3 days. After this period, the partially debittered olives are seasoned and packaged or, otherwise, stored in the same containers in chilled chambers (8°C). Under these conditions, the fruits retain their green appearance for several months.

(iii) Traditional *Aloreña de Málaga* olives (TA). In this case, just after harvesting, the fruits are cracked and brined in plastic drums (200 L volume) in a 10–11% NaCl solution. Then, the olives are stored for at least 20 days before commercialisation. During this period, the fruits undergo a partial fermentation, where progression and partial green color degradation depend on the storage time. Finally, the olives are seasoned and packaged according to demand using similar conditions to the previous process (FG).

In general, the freshness appearance is an attribute highly appreciated in this table olive speciality. However, greenness progressively decreases as the fermentation, storage or packaging time is prolonged. At the same time, brine and surface color gradually brown. Several factors may contribute to these changes. The loss in green color could be due to the degradation of chlorophyll in the acidic medium of the brines (Gallardo-Guerrero et al., 2013). The browning could also be caused by the oxidation and polymerisation of polyphenols by the polyphenol oxidase (PPO) activity (Segovia-Bravo et al., 2009). As demonstrated by Arroyo-López et al. (2007), most of these changes are produced during storage. Consequently, several strategies for mitigating these adverse effects have been tested, such as the application of washings and protective carbon dioxide atmosphere (Arroyo-López et al., 2007). Other alternatives recently studied are the use of antioxidant compounds (ascorbic acid and sodium metabisulfite) or various mineral salts ($MgCl_2$ and $ZnCl_2$) (Arroyo-López et al., 2008; Gallardo-Guerrero et al., 2013). However, an entirely satisfactory solution is not yet available.

Heat-shocked olives was a convenient procedure for ridding the fruits of naturally occurring interfering and competitive microbial groups, but also made the olives highly fermentable (Etchells et al., 1966). Balatsouras et al. (1983) also reported a slight improvement in fermentability by means of a heat-shock treatment applied to *Conservolea* green olives. Recently, the European project Probiolives (FP7-SME, ID-243471) also included heat-shock as a method for enhancing green olive fermentability and contribute to the predominance of the potential probiotic starter culture. Results showed that heat-shocked (80°C for 10 min) olives led to final products with high acceptability, although the inoculum predominance depended on the strain assayed.

The present work investigates the effects of a previous mild heat-shock treatment of the fruits on the fermentation and packaging processes of *Aloreña de Málaga* table olives. The objective is the production of a better product than the commercial commodity with improved fresh appearance and stability while maintaining similar sensory attributes.

MATERIALS AND METHODS

Raw Material and Experimental Design

Aloreña de Málaga fruits at the green ripening stage were provided by a local farmer (Manzanilla Aloreña S.C.A., Alora, Málaga, Spain) during the 2015/2016 season (140–260 fruits/kg

TABLE 1 | Summary of the experimental design applied in the study.

Acronym	PDO denomination	Heat-shock application	Storage temperature (°C)	Brining conditions*
CA-C	Cured *Aloreña* (whole fruits)	No (control)	25	6.7 Na, 0.54 AA
CA-H	Cured *Aloreña* (whole fruits)	Yes	25	6.7 Na, 0.54 AA
FG-C	Fresh Green *Aloreña* (cracked fruits)	No (control)	8	15.8 Na
FG-H	Fresh Green *Aloreña* (cracked fruits)	Yes	8	15.8 Na
TA-C	Traditional *Aloreña* (cracked fruits)	No (control)	25	15.8 Na
TA-H	Traditional *Aloreña* (cracked fruits)	Yes	25	15.8 Na

**Na, NaCl concentration (%, w/v); AA, acetic acid (%, v/v). The heat-shock treatment consisted of dipping the fruits into a water bath at 60°C for 5 min just before brining. All treatments were run in duplicate.*

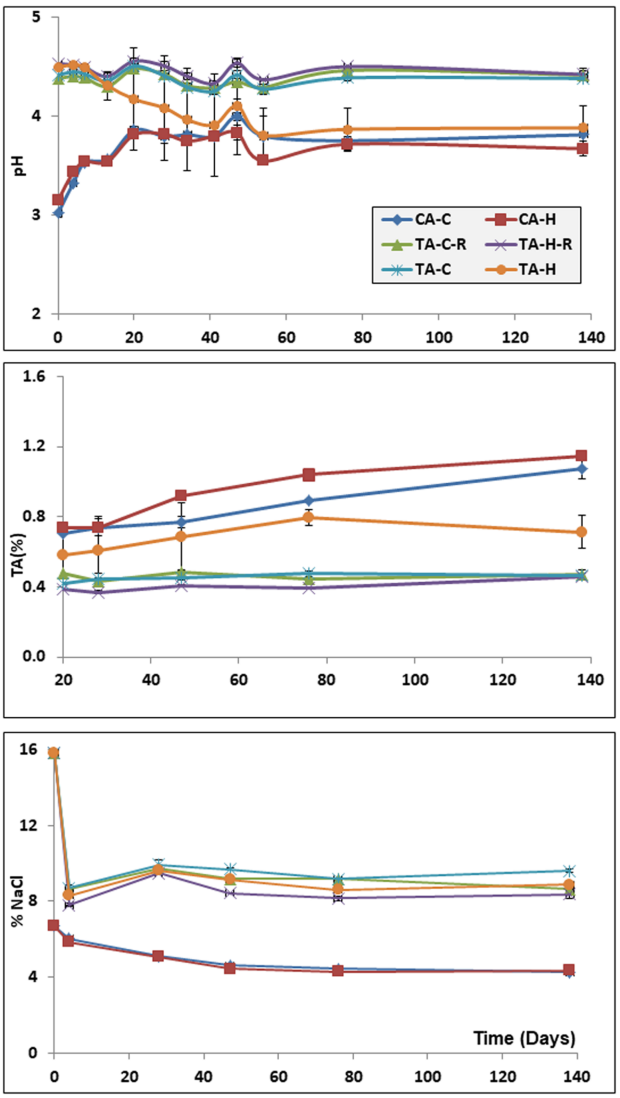

FIGURE 1 | Evolution of the pH **(upper)**, titratable acidity **(middle)** and salt content **(lower)** during fermentation in the diverse treatments. Error bars denote standard deviation calculated from duplicate fermentation vessels. CA-C, cured control; CA-H, heat-shocked cured olives; FG-C, refrigerated fresh green control; FG-H, heat-shocked refrigerated fresh green olives; TA-C, control of traditional olives; and TA-H, heat-shocked traditional olives. All treatments were applied to the *Aloreña de Malaga* cultivar.

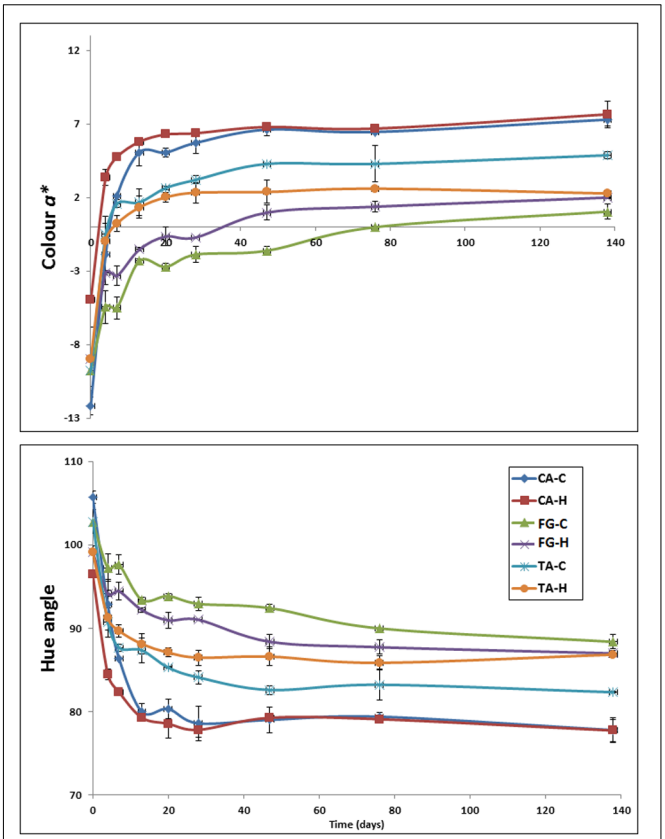

FIGURE 2 | Evolution of color parameters a^* **(upper)** and h_{ab} **(lower)** during fermentation in the diverse treatments. Error bars denote standard deviation calculated from duplicate fermentation vessels. CA-C, cured control; CA-H, heat-shocked cured olives; FG-C, refrigerated fresh green control; FG-H, heat-shocked refrigerated fresh green olives; TA-C, control of traditional olives; and TA-H, heat-shocked traditional olives. All treatments were applied to the *Aloreña de Malaga* cultivar.

size). The olives were processed at the pilot plant of the Instituto de la Grasa (CSIC, Seville) according to the three commercial denominations included in the PDO regulation. One part of them was prepared following the conditions applied by the industry (control treatments) while the rest were subjected to a mild-heat-shock treatment. **Table 1** summarizes the different treatments that constituted the experimental design. The heat-shock treatment was applied by dipping the fruits into a water bath at 60°C for 5 min just before brining. Then, the fruits were rapidly transferred into cool water and, after temperature equilibrium, placed in the fermentation vessels (5.3 kg of fruit and 3.8 L of brine). All treatments were run in duplicate, making a total of 12 containers. The fermentation process was monitored during 138 days.

Monitoring of the Fermentation Process

The analyses of the olive brine for pH, NaCl, titratable and combined acidity during the fermentation process were carried out by applying the usual methods described by Garrido-Fernández et al. (1997). The instrumental firmness and surface color of fruits analyses followed the methods described elsewhere (Chen et al., 2010; Bautista-Gallego et al., 2011). Color was measured using a BYKGardner Model 9000 Color-view spectrophotometer (MD, United States). Interference by stray light was minimized by covering the samples with a box with a matt black interior. Color was expressed as the CIE L^* (lightness), a^* (freshness, negative values indicate green while positive values are related to red tones), and h_{ab} (hue angle) parameters. The firmness of the olives was measured using a Kramer shear compression cell coupled to an Instron Universal Machine (Canton, MA, United States). The crosshead speed

TABLE 2 | Physicochemical characteristics of the diverse treatments at the end of the fermentation process (138 days).

Treatment	Texture (kN/100 g)	Glucose (g/l)	Sucrose (g/l)	Fructose (g/l)	Mannitol (g/l)	Total sugars (g/l)	Acetic acid (g/l)	Lactic acid (g/l)	Citric acid (g/l)	Ethanol (g/l)
CA-C	9.80 (0.24)^{d}	0.11 (0.01)^{a}	0.00 (0.00)^{a}	0.31 (0.00)^{a,b}	0.02 (0.01)^{a}	0.44 (0.03)^{a}	4.23 (0.01)^{b}	7.84 (0.82)^{b}	0.16 (0.00)^{b}	1.91 (0.09)^{b,c}
CA-H	8.31 (0.02)^{c}	0.16 (0.02)^{a}	0.00 (0.00)^{a}	0.26 (0.02)^{a}	0.02 (0.00)^{a}	0.43 (0.02)^{a}	3.99 (0.16)^{b}	10.08 (0.62)^{b}	0.00 (0.00)^{a}	1.27 (0.20)^{b}
FG-C	7.73 (0.17)^{b,c}	13.26 (0.16)^{e}	1.16 (0.13)^{c}	1.81 (0.14)^{d}	1.95 (0.36)^{b}	18.17 (0.20)^{d}	0.07 (0.05)^{a}	0.11 (0.05)^{a}	0.14 (0.01)^{b}	12.06 (0.36)^{a}
FG-H	6.26 (1.17)^{a}	5.54 (0.29)^{d}	0.14 (0.02)^{b}	0.43 (0.03)^{b}	2.45 (0.12)^{c}	8.57 (0.43)^{c}	0.08 (0.02)^{a}	0.18 (0.04)^{a}	0.15 (0.05)^{b}	11.62 (0.31)^{a}
TA-C	6.77 (0.60)^{a,b}	2.53 (0.10)^{b}	0.12 (0.02)^{a,b}	0.37 (0.00)^{a,b}	1.68 (0.01)^{b}	4.71 (0.09)^{b}	0.00 (0.00)^{a}	0.27 (0.05)^{a}	0.19 (0.01)^{b}	11.49 (0.33)^{a}
TA-H	6.54 (0.49)^{a,b}	3.93 (0.09)^{c}	0.01 (0.01)^{a,b}	0.07 (0.03)^{c}	0.29 (0.02)^{a}	4.31 (0.08)^{b}	1.07 (0.38)^{c}	10.19 (2.55)^{b}	0.15 (0.02)^{b}	2.33 (0.67)^{c}

Standard deviation from duplicate measurements in parentheses. Values followed by different superscript letters, within the same column, are statistically different ($p \leq 0.05$) according to LSD post hoc comparison test. CA-C, cured control; CA-H, heat-shocked cured olives; FG-C, refrigerated fresh green control; FG-H, heat-shocked refrigerated fresh green olives; TA-C, control of traditional olives; and TA-H, heat-shocked traditional olives. All treatments were applied to the Aloreña de Malaga cultivar.

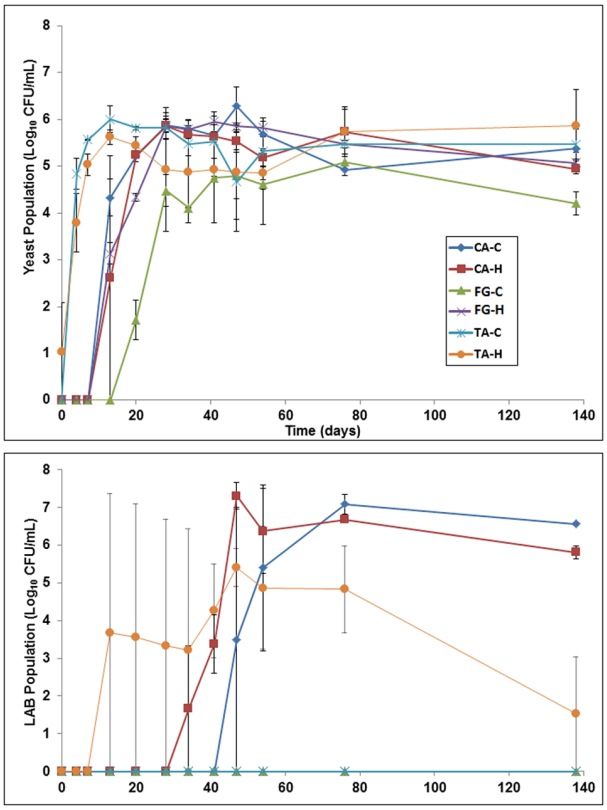

FIGURE 3 | Evolution of the yeast **(upper)** and lactic acid bacteria **(lower)** populations during fermentation in the diverse treatments. Error bars denote standard deviation calculated from duplicate fermentation vessels. CA-C, cured control; CA-H, heat-shocked cured olives; FG-C, refrigerated fresh green control; FG-H, heat-shocked refrigerated fresh green olives; TA-C, control of traditional olives; and TA-H, heat-shocked traditional olives. All treatments were applied to the *Aloreña de Malaga* cultivar.

was 200 mm/min. The firmness, expressed as kN/100 g flesh, was the mean of 10 replicate measurements, each of which was performed on three pitted olives. Individual reducing sugars (glucose, fructose, sucrose and mannitol), organic acids (acetic, lactic, and citric) and ethanol content were determined by HPLC according to the methods developed by Sánchez et al. (2000).

For the counts of the *Enterobacteriaceae,* yeasts and *Lactobacillaceae* populations in brine, samples drawn from the different treatments were spread onto selective media according to the methods described by Rodríguez-Gómez et al. (2015). Counts were expressed as $\log_{10}$ CFU/mL.

Metagenomic Analysis of Bacterial Populations

Microbial genomic DNA from olive and brine samples at the end of the fermentation process (138 days) was extracted as described by Medina et al. (2016) and sent to the Sequencing and Bioinformatic Service of FISABIO (Valencia, Spain) for bacterial metagenomic analysis. 16S rDNA gene amplicons were amplified following the 16S rDNA gene Metagenomic Sequencing

Library Preparation Illumina protocol. The gene-specific sequences used in this protocol target the V3 and V4 region of 16S rDNA gene (Klindworth et al., 2013). Illumina adapter overhang nucleotide sequences were added to the gene-specific sequences. The primer pair were: forward primer (5′-TCGT CGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNG GCWGCAG-3′) and reverse primer (5′-GTCTCGTGGGCTC GGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAA TCC-3′). A multiplexing step was performed using Nextera XT Index Kit (FC-131-1096). 1 μl of the PCR product was run on a Bioanalyzer DNA 1000 chip to verify the size, the expected size on a Bioanalyzer trace should be ∼550 bp. The libraries were sequenced using a 2 × 300 pb paired-end run on a MiSeq Sequencer according to manufacturer's instructions (Illumina). Quality assessment was performed through the use of the prinseq-lite program (Schmieder and Edwards, 2011*)* by applying the following parameters: minimum sequence length of 50 bp, trim_qual_right of 20, trim_qual_type of mean and trim_qual_window of 20 bp.

A metagenomic analysis was performed using the Quantitative Insights into Microbial Ecology (QIIME) pipeline (version1.9.1)[1]. Sequences were sorted by barcode into their respective samples. To calculate alpha diversity indexes, 16S rRNA Operational Taxonomic Units (OTUs) were defined at ≥97% sequence homology. Chimeric sequences were removed using ChimeraSlayer. All reads were classified into the lowest possible taxonomic rank using QIIME and the SILVA108 database. OTUs were assigned by means of uclust (Edgar, 2010) using the script pick_de_novo_otus.py. Alpha diversity was calculated through the alpha_diversity.py by script using different metrics (Chao, Observed Species, Shannon, Simpson, Good's coverage, PD whole tree) after performing a rarefaction analysis. Rarefied OTU tables to 6,500 sequences (lowest number of reads obtained) were used to obtain these alpha diversity metrics. OTU tables to Genus taxonomic level were exported in tab-delimited text format and analyzed using STAMP v2.0.1 (Parks and Beiko, 2010). An ANOVA/Tukey-Kramer (*post hoc*) test was run to elucidate the taxa association in the different grouping variables. The false discovery rate correction (Benjamini and Hochberg, 1995) was finally applied in all cases, and significant differences in taxa were only considered for $p \leq 0.05$ and a q-value below 0.3.

[1]http://qiime.sourceforge.net/

Packaging of Fruits

After 138 days of fermentation, the fruits obtained from the different treatments were washed (12 h) in tap water and then packaged in polyethylene terephthalate (PET) vessels (1.6 L volume). The packages were filled with 0.9 kg of olives, 16 g of seasoning material (a mixture of diced garlic, pepper strips, small pieces of fennel, and thyme) and 0.7 L of cover brine (3.0% NaCl). For each treatment, a total of 6 packages were obtained. Samples for physicochemical, microbiological, and sensory analysis were withdrawn on the 4th and 41st day of packaging.

Sensory Evaluation

The evaluation sheet developed by International Olive Council [IOC] (2010) for the estimation of acidic, salty, bitterness, hardness, and crunchiness attribute scores was used in the present study. Because of the specific sensory characteristics of this table olive speciality, other attributes such as darkening, appreciation of defects, and overall acceptability were also introduced into the evaluation sheet. The panel was composed of 14 expert members. Six of them were from the Instituto de la Grasa (CSIC) staff while the other 6 were from the industry. All of them were chosen because of their usual involvement in previous sensory analyses. Despite this, they were specifically trained (2 h for 2 weeks) for the sensory evaluation of the diverse commercial denominations of *Aloreña de Málaga* table olives. The evaluation sheet consisted of two sections. The first one was devoted to the sample and panelist identification while the second included the attributes to be evaluated, including a final question

TABLE 3 | Number of sequences and OTUs assigned (after removing chloroplast), diversity indexes, and estimated sample coverage for 16S (bacteria) amplicons according to treatments.

Sample	Matrix	Number of reads	Number of OTUs	Coverage	PD whole tree[a]	Chao1[a]	Simpson[a]	Shannon[a]
CA-C-B	Brine	51,667	176	97.90	7.63	615.31	0.34	1.49
CA-H-B	Brine	13,667	197	97.93	2.51	498.86	0.56	2.13
CA-C-E	Fruit	23,335	163	97.86	4.93	748.79	0.21	0.98
CA-H-E	Fruit	13,956	158	98.08	6.29	545.80	0.19	0.92
FG-C-B	Brine	31,582	192	97.95	3.40	557.30	0.56	2.17
FG-H-B	Brine	39,323	302	96.78	1.70	762.35	0.80	3.53
FG-C-E	Fruit	6,583	201	97.83	11.93	589.33	0.72	2.83
FG-H-E	Fruit	27,979	249	97.36	12.11	659.54	0.77	3.29
TA-C-B	Brine	18,888	225	97.43	9.87	765.46	0.47	2.08
TA-H-B	Brine	25,951	180	97.82	7.59	701.43	0.14	0.86
TA-C-E	Fruit	31,416	213	97.63	9.41	686.71	0.52	2.22
TA-H-E	Fruit	23,425	189	97.69	7.67	679.00	0.19	1.07

[a]*Values were estimated after rarefaction to 6,583 sequences. CA-C, cured control; CA-H, heat-shocked cured olives; FG-C, refrigerated fresh green control; FG-H, heat-shocked refrigerated fresh green olives; TA-C, control of traditional olives; and TA-H, heat-shocked traditional olives. All treatments were applied to the Aloreña de Malaga cultivar. B and E stand for samples obtained from brine or epidermis of fruits, respectively.*

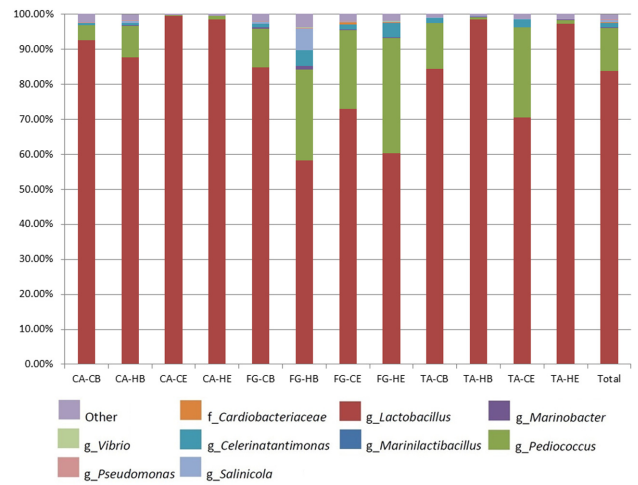

FIGURE 4 | Relative abundance of bacterial species in the different treatments assayed, obtained through metagenomics analysis of 16S RNA gene at the end of the fermentation process in brine (B) and epidermis of fruits (E). CA-C, cured control; CA-H, heat-shocked cured olives; FG-C, refrigerated fresh green control; FG-H, heat-shocked refrigerated fresh green olives; TA-C, control of traditional olives; and TA-H, heat-shocked traditional olives. All treatments were applied to the *Aloreña de Malaga* cultivar.

on overall acceptability. At preselected sampling periods, the samples were offered to panelists, using blue glass according to the recommendations of the standard COI/T.20/Doc.No 5 (Glass for oil tasting) (International Olive Council [IOC], 1987), coded with three digits randomly chosen, and in a balance presentation with respect to PDO. All the attributes were evaluated on an unstructured scale which ranged from 1 to 11, in which 1 was associated with the complete absence of the attribute and 11 to its presence in the highest intensity. The panelists were asked to mark on the scale according to the intensity perceived of each attribute. The sheets were read by the panel leader with 0.1 cm precision.

Statistical Analysis

The data were subjected to an analysis of variance. For this purpose, the one-way ANOVA module of Statistica 7.1 software (Statsoft Inc., Tulsa, OK, United States) was used to check for significant differences among physicochemical, microbiological and sensory attributes as a function of the different treatments assayed. A *post hoc* comparison statistical LSD test was applied using $p \leq 0.05$ as the cut-off level of significance.

RESULTS

Physicochemical Changes during the Fermentation Process

Remarkable differences between heat-shocked and untreated olives were found for pH, titratable acidity, and salt content throughout the 138 days of fermentation (**Figure 1**). In CA treatments, pH increased from the initial 3.0 (first day after brining) up to 3.8 units at the end of the fermentation process. However, in FG treatments and the control following the traditional process (TA-C), a pH value close to 4.3 units was noticed during the entire fermentation time. An entirely different behavior was detected in TA-H, whose pH decreased

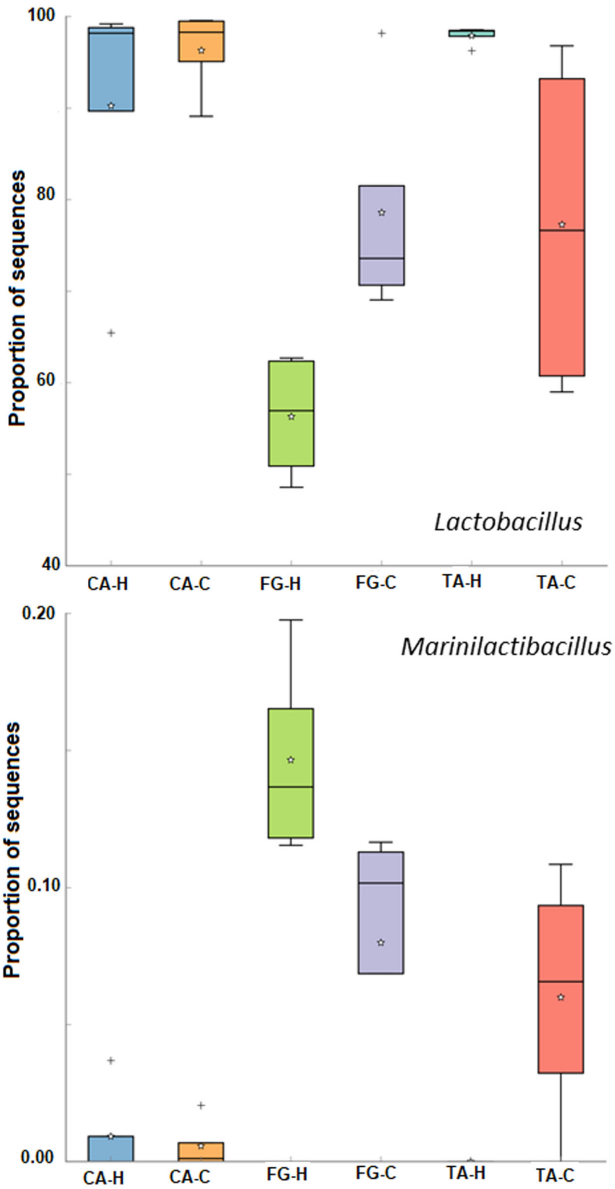

FIGURE 5 | ANOVA Tukey–Kramer *post hoc* comparison test executed with STAMP v2.0.1 to elucidate significant differents for *Lactobacillus* (upper panel) and *Marinilactibacillus* (lower panel) genera in the diverse treatments. Significant differences were only considered for $p \leq 0.05$ and a q-value below 0.3. Meaning of symbols: Cross, star and horizontal line inside box denoted for outlier, mean, and median of the percentage of sequences obtained. CA-C, cured control; CA-H, heat-shocked cured olives; FG-C, refrigerated fresh green control; FG-H, heat-shocked refrigerated fresh green olives; TA-C, control of traditional olives; and TA-H, heat-shocked traditional olives. All treatments were applied to the *Aloreña de Malaga* cultivar.

from an initial 4.3 value to a final 3.9 units at the end of the process. Titratable acidity values were kept constant at approximately 0.4% in FG and TA-C treatments throughout the fermentation period but increased for CA olives and the TA-H treatment. Interestingly, the application of a mild-heat-shock treatment to the fruits favored a higher production of titratable acidity in CA and TA treatments than in their respective controls. The evolution of salt in CA and TA/FG was also completely different, with a lower content in the equilibrium (∼4.5% NaCl) in CA than in TA/FG (∼9.0% NaCl) treatments.

The color data also revealed considerable differences among the diverse *Aloreña de Málaga* denominations (**Figure 2**). The loss in greenness was faster for CA fruits, followed by TA and FG olives. The maximum a^* value, which is associated with the worst green color, was observed in the CA treatments (∼7.5), followed by the control of TA (∼4.5) and FG (∼2.0). Notice the close position of TA-H treatment to FG at the end of the fermentation process (without significant differences between them at $p \leq 0.05$). A similar trend was followed by h_{ab}, although reversed. The lowest value was found in CA treatments (∼78°), followed again by TA-C (∼82°) and FG samples (∼87°). The position of TA-H fruits was again close to FG treatments (∼87°).

At the end of the fermentation process, the texture of CA treatments (which use whole fruits) was higher compared to the cracked olives used for the elaboration of TA and FG olives (**Table 2**). The total sugar content in brine was statistically different ($p \leq 0.05$) in the three *Aloreña de Málaga* commercial denominations. Sugars were practically exhausted in CA treatments but not in TA or, particularly, in FG. The acetic and lactic acid contents were higher ($p \leq 0.05$) in CA and TA-H than in the other treatments. However, the ethanol concentration showed the opposite behavior. The highest values ($p \leq 0.05$) were noticed in TA-C and FG.

Microbiological Changes during the Fermentation Process

Enterobacteriaceae were never found in any treatment. On the contrary, high population levels of yeasts (5.0–6.0 $\log_{10}$ CFU/mL) were always observed. This microbial group first appeared in TA (in both control and heat-treated fruits), then in CA and finally in FG (**Figure 3**, upper panel). Regarding the lactic acid bacteria (LAB) population, this gram-positive bacteria group was only detected in CA and TA-H treatments. The LAB were first noticed in TA-H (from 2 weeks onward) reaching population levels of approximately 5.5 $\log_{10}$ CFU/mL at the 50th day of fermentation. LAB appear later in the CA-H (olives subjected to the heat-shock treatment), with an approximate delay of 3 weeks, and finally in CA-C after 7 weeks of fermentation. In both CA denominations, the LAB population reached levels close to 7.0 $\log_{10}$ CFU/mL (**Figure 3**, lower panel). Except for FG, the heat-shock treatment stimulated the early presence of LAB and their growth. At the end of the fermentation process, the highest count ($p \leq 0.05$) was obtained in the CA treatment, followed by CA-H, and finally the TA-H treatment.

TABLE 4 | Physicochemical and microbiological data obtained for the diverse treatments and packaging storage periods.

		Physicochemical parameters						Microbiological parameters	
Treatment	**Packaging (days)**	**pH**	**Salt (%)**	**Titratable acidity (%)**	**Texture (kN/100 g)**	**Color a^***	**Color h_{ab}**	**LAB ($\log_{10}$ CFU/mL)**	**Yeast ($\log_{10}$ CFU/mL)**
CA-C	4	3.73 (0.01)^{d}	3.70 (0.01)^{b}	0.76 (0.02)^{f}	8.39 (1.52)^{a}	7.28 (0.09)^{e}	75.23 (0.15)^{c,d}	7.03 (0.03)^{a,b,c}	5.18 (0.07)^{c,d}
	41	3.82 (0.01)^{g}	3.81 (0.02)^{d}	1.16 (0.02)^{h}	7.27 (1.41)^{a}	7.43 (0.24)^{e}	74.37 (0.50)^{c}	7.55 (0.05)^{a}	4.34 (0.70)^{b}
CA-H	4	3.65 (0.02)^{a}	3.74 (0.01)^{c}	0.80 (0.03)^{g}	7.14 (0.98)^{a}	6.99 (0.07)^{e}	76.00 (0.68)^{d}	6.58 (0.43)^{b}	4.57 (0.08)^{b,c}
	41	3.59 (0.01)^{c}	3.70 (0.01)^{b}	1.26 (0.03)^{i}	6.48 (1.07)^{a}	6.15 (0.12)^{d}	78.39 (0.07)^{g}	6.73 (0.09)^{b,c}	3.60 (0.65)^{a}
FG-C	4	3.87 (0.08)^{h}	4.19 (0.01)^{e}	0.39 (0.02)^{a}	8.65 (1.53)^{a}	2.22 (0.41)^{a}	86.02 (0.73)^{a,b}	0.00 (0.00)^{f}	4.17 (0.07)^{a,b}
	41	4.04 (0.01)^{b}	4.52 (0.02)^{a}	0.41 (0.01)^{a}	8.92 (1.39)^{a}	3.40 (0.07)^{b}	84.08 (0.24)^{f}	5.03 (0.45)^{d,e}	3.64 (0.25)^{a}
FG-H	4	3.91 (0.01)^{i}	4.56 (0.01)^{a}	0.34 (0.01)^{b}	7.21 (1.54)^{a}	2.56 (0.06)^{a}	85.65 (0.02)^{a}	4.38 (0.46)^{d}	5.20 (0.03)^{c,d}
	41	4.00 (0.02)^{j}	4.43 (0.04)^{f}	0.50 (0.02)^{e}	6.21 (2.02)^{a}	3.28 (0.27)^{b}	84.27 (0.31)^{f}	7.65 (0.75)^{a}	4.17 (0.04)^{a,b}
TA-C	4	3.79 (0.01)^{f}	4.65 (0.01)^{g}	0.31 (0.04)^{c}	7.43 (1.71)^{a}	4.79 (0.08)^{c}	80.94 (0.06)^{e}	4.33 (0.22)^{d}	6.12 (0.02)^{e}
	41	4.03 (0.02)^{b}	4.53 (0.02)^{a}	0.38 (0.01)^{a}	7.19 (1.58)^{a}	4.52 (0.33)^{c}	80.82 (0.37)^{e}	7.17 (0.27)^{a,b,c}	4.05 (0.06)^{a,b}
TA-H	4	3.65 (0.01)^{a}	4.56 (0.01)^{a}	0.35 (0.01)^{b}	6.95 (2.19)^{a}	2.65 (0.31)^{a}	85.93 (0.52)^{a,b}	5.13 (0.03)^{e}	5.51 (0.12)^{d,e}
	41	3.75 (0.02)^{e}	4.83 (0.01)^{h}	0.45 (0.02)^{d}	6.70 (1.61)^{a}	2.11 (0.47)^{a}	86.78 (0.72)^{b}	7.41 (0.21)^{a,c}	4.15 (0.37)^{a,b}

%, expressed as w/v; standard deviation obtained from duplicate measurements in parentheses. Values followed by different superscript letters, within the same column, are statistically different ($p \leq 0.05$) according to the LSD posthoc comparison test. CA-C, cured control; CA-H, heat-shocked cured olives; FG-C, refrigerated fresh green control; FG-H, heat-shocked refrigerated fresh green olives; TA-C, control of traditional olives; and TA-H, heat-shocked traditional olives. All treatments were applied to Aloreña de Malaga cultivar.

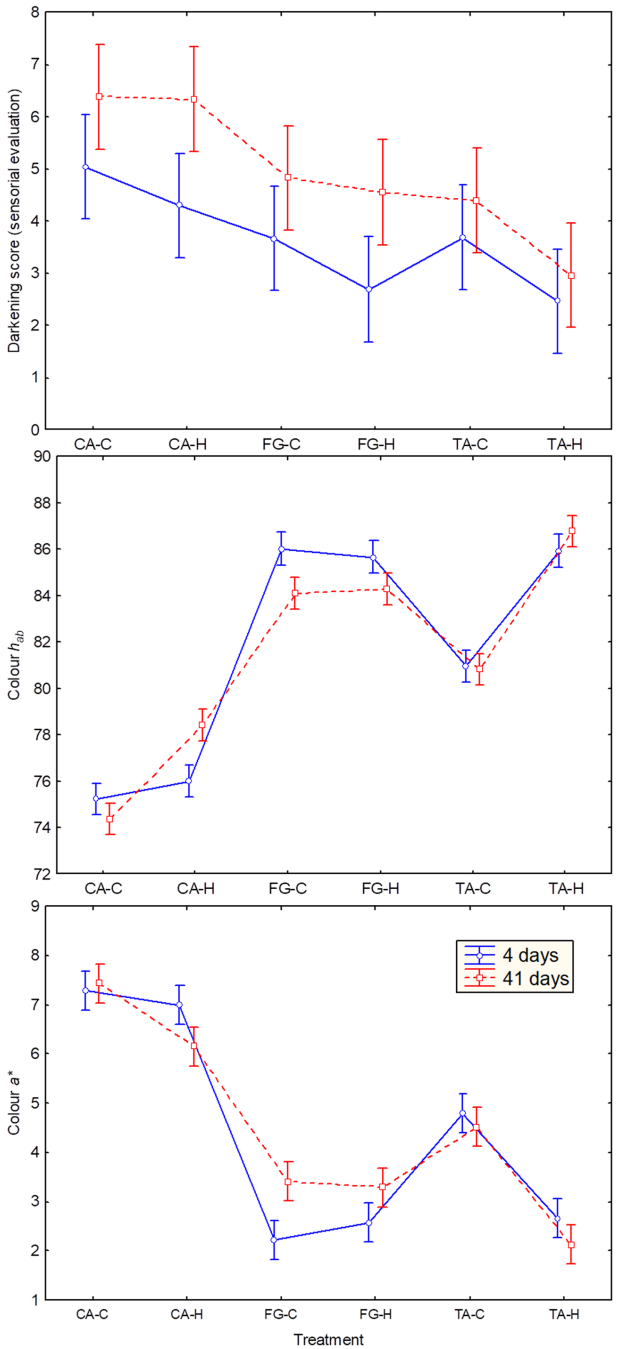

FIGURE 6 | Darkening score assigned by panelist **(upper)**, hue angle (h_{ab}, **middle**), and greenness (a^*, **lower**) concerning the diverse treatments. Error bars for instrumental measurements denote standard deviation calculated from duplicate packaging. CA-C, cured control; CA-H, heat-shocked cured olives; FG-C, refrigerated fresh green control; FG-H, heat-shocked refrigerated fresh green olives; TA-C, control of traditional olives; and TA-H, heat-shocked traditional olives. All treatments were applied to the *Aloreña de Malaga* cultivar

Metagenomic Analysis

A total of 945,386 raw sequences were obtained from the 24 olive samples analyzed in this work. After screening the data for poor quality sequences, the removal of chloroplasts and taxonomically unassigned 16S sequences, 307,772 sequences (an average of 25,647 sequences per sample) were finally used for the metagenomic analysis. Overall, despite the diversity in sequencing depth among samples (**Table 3**), the rarefaction analysis indicated that some reads above 6,583 per sample were satisfactory to obtain good coverage (always above 96%).

Table 3 shows the total of OTUs found in the different samples and their alpha-diversity indexes. In general, a higher biodiversity was noticed for FG *Aloreña* samples, which showed the highest values for Simpson and Shannon indexes. The total number of OTUs assigned ranged from 158 to 302, with an average of 204 observed OTUs per sample. The bacterial phylogenetic assignation of all samples showed that two bacterial phyla (*Proteobacteria* and *Firmicutes*) included the genera with the greatest number of sequences (**Figure 4**). The *Proteobacteria* represented only 2.4% of the total sequences, with genera *Celerinatantimonas* (1.32%), *Salinicola* (0.70%), *Marinobacter* (0.17%), *Pseudomonas* (0.08%), and *Vibrio* (0.06%) as the most representative. They were found in practically all samples. On the contrary, the phyla with the major number of sequences were *Firmicutes* (96.02% of total sequences), with genera *Lactobacillus* (83.67%), *Pediococcus* (12.30%), and *Marinilactibacillus* (0.05%) as the most abundant. **Figure 4** shows the relative abundance of bacterial genera for the different treatments assayed, making a distinction between samples obtained from brine (B) or fruit epidermis (E). The abundance of *Lactobacillus* in all FG samples and the TA-C treatment was the lowest, as confirmed by the application of the Tukey-Kramer *post hoc* test (**Figure 5**, upper panel). The proportion of sequences obtained for *Lactobacillus* genera, regardless of the origin (brine or fruit) was statistically lower ($p \leq 0.05$) in FG-H (56.28%), TA-C (77.27%), and FG-C (78.58%) than in CA-H (90.25%), CA-C (96.30%) and TA-H (97.90%) treatments. On the contrary, the presence of *Marinilactibacillus* genera was statistically higher ($p \leq 0.05$) in FG (0.15 and 0.08% for FG-H and FG-C, respectively) and TA-C samples (0.06%) than in the rest of the samples (which were below 0.01%); that is, this genera showed an opposite behavior compared to *Lactobacillus* (**Figure 5**, lowest panel).

Evaluation of Packaged Fruits

After the fermentation process, the fruits were packaged and subjected to physicochemical and microbiological analyses on the 4th and 41st day of storage (**Table 4**). *Enterobacteriaceae* were never detected in any packaging sample. On the contrary, high populations of LAB and yeasts were found. An increase in LAB population throughout packaging was noticed in practically all treatments while yeast counts had a statistically significant reduction ($p \leq 0.05$), except in the FG-C treatment, during the same period. Concerning physicochemical data, pH ranged from 3.59 (CA-H) to 4.04 (FG-C) at the 41st day of packaging, with a slight trend to increase as the packaging time progressed. After the same period, the salt content ranged from 3.70 (CA-H) to 4.93% (TA-H), with lower values for the CA treatments. Titratable acidity statistically increased ($p \leq 0.05$), from 0.34 (FG-H) on the 4th day to 1.26% (CA-H) on the 41st day of packaging, due to the simultaneous increment in the LAB population.

TABLE 5 | Scores assigned by the panelist to the sensory attributes of the diverse treatments according to packaged storage periods.

Treatment	Packaging storage (*d*)	Hardness	Crunchiness	Acidic	Salty	Bitterness	Browning	Defects	Overall acceptability
CA-C	4	7.60 (2.25)^{a}	7.57 (2.16)^{a}	6.47 (2.65)^{c,d}	4.37 (1.77)^{a,b,c,d}	4.21 (2.10)^{a,c}	5.03 (2.47)^{a,e}	3.88 (2.98)^{a}	6.58 (2.18)^{a,b,c}
	41	7.63 (2.09)^{a}	7.87 (1.50)^{a}	7.07 (2.12)^{c,d}	5.50 (1.47)^{b}	5.75 (2.58)^{b}	6.37 (2.74)^{e}	5.17 (2.87)^{a}	5.42 (1.56)^{b,d}
CA-H	4	7.91 (1.89)^{a}	7.71 (1.67)^{a}	6.01 (2.30)^{c,e}	4.90 (1.67)^{a,b,c,d}	3.60 (1.40)^{a}	4.30 (2.70)^{a,c}	3.13 (1.74)^{a}	7.51 (1.38)^{a}
	41	6.50 (2.42)^{a}	6.48 (2.13)^{a}	7.24 (1.75)^{c,d}	5.15 (1.71)^{b,c,d}	5.22 (2.36)^{b,c}	6.32 (1.45)^{e}	4.54 (2.58)^{a}	5.52 (1.97)^{b,c,d}
FG-C	4	7.86 (1.83)^{a}	8.33 (1.40)^{a}	4.23 (1.88)^{a,b}	3.91 (1.63)^{a,c}	4.92 (2.34)^{a,b,c}	3.66 (2.09)^{a,b,c}	4.10 (2.36)^{a}	6.74 (1.56)^{a,c}
	41	7.77 (1.79)^{a}	7.66 (1.77)^{a}	5.19 (2.12)^{a,b,c,e}	4.34 (1.84)^{a,b,c,d}	4.77 (1.83)^{a,b,c}	4.82 (1.87)^{a}	3.64 (1.90)^{a}	6.55 (1.30)^{a,b,c}
FG-H	4	6.70 (2.63)^{a}	6.91 (2.23)^{a}	3.85 (1.77)^{a}	3.64 (1.22)^{a}	3.57 (1.44)^{a}	2.68 (1.35)^{b}	3.93 (2.03)^{a}	6.88 (1.39)^{a}
	41	6.22 (2.47)^{a}	6.28 (2.38)^{a}	7.54 (2.51)^{d}	5.61 (2.69)^{b}	7.41 (1.98)^{d}	4.54 (1.82)^{a}	5.03 (2.83)^{a}	3.97 (1.86)^{e}
TA-C	4	7.25 (2.21)^{a}	7.42 (2.03)^{a}	4.50 (1.33)^{a,b,e}	3.76 (1.21)^{a}	3.61 (1.55)^{a}	3.68 (0.68)^{a,b,c}	3.85 (2.50)^{a}	6.36 (2.53)^{a,b,c,d}
	41	7.45 (1.46)^{a}	6.93 (1.99)^{a}	6.30 (2.10)^{c,d}	4.82 (2.11)^{a,b,c,d}	5.66 (2.10)^{b,c}	4.39 (2.04)^{a}	4.84 (2.22)^{a}	5.17 (1.49)^{d,e}
TA-H	4	7.09 (1.81)^{a}	6.68 (1.53)^{a}	4.44 (1.42)^{a,b}	4.14 (1.69)^{a,c,d}	3.50 (1.43)^{a}	2.46 (0.83)^{b}	3.79 (2.20)^{a}	6.87 (1.34)^{a}
	41	6.50 (1.79)^{a}	6.68 (1.53)^{a}	5.55 (1.87)^{b,c,e}	5.45 (1.76)^{b,d}	5.73 (2.09)^{b}	2.95 (1.36)^{b,c}	3.24 (1.54)^{a}	6.90 (2.03)^{a}

Standard deviation in parentheses (n = 14). Values followed by different superscript letters within the same column are statistically different ($p \leq 0.05$) according to the LSD post hoc comparison test. CA-C, cured control; CA-H, heat-shocked cured olives; FG-C, refrigerated fresh green control; FG-H, heat-shocked refrigerated fresh green olives; TA-C, control of traditional olives; and TA-H, heat-shocked traditional olives. All treatments were applied to the Aloreña de Malaga cultivar.

The instrumental texture between heat-shocked fruits and their respective controls was not statistically significant ($p \geq 0.05$). The major effects were noticed on the fruits' color expressed as greenness (a^*) and hue angle (h_{ab}). The best color appearance of the fruits was obtained for chilled olives (FG) as well as for the traditional process using heat-shocked fruits (TA-H), which showed significant differences (at $p \leq 0.05$) with respect to the other treatments. On the contrary, the worst instrumental color values were noticed for CA olives. Also, there was a significant ($p \leq 0.05$) loss in color throughout the shelf life in most of the treatments, except in TA-H (**Figure 6**).

With regards to the sensory evaluation (**Table 5**), there were no significant differences ($p \leq 0.05$) among treatments and packaging days for hardness, crunchiness or defects. The first two attributes always obtained good scores (>6.2) while they were lower for the latter (only two treatments exceed 5.0 at the end of packaging). There were significant differences ($p \leq 0.05$) in acidic, salty and bitter among the three *Aloreña de Malaga* denominations and between packaging times but not between heat-shocked olives and their respective controls. Furthermore, acidic, salty and bitterness usually increased in all treatments from the 4th to 41st days. Important browning differences among treatments were detected by panelists, with the highest brown values assigned to CA olives at the end of the storage period (6.3). On the contrary, the lowest values were obtained by TA-H (2.4). In general, browning scores increased as time progressed with statistically significant differences ($p \leq 0.05$) for CA-H and FG-H (**Figure 6**, upper panel). Finally, the overall acceptability score at the beginning of packaging was generally high (>6.5) but decreased considerably in some treatments after 41 days (CA-C, CA-H, FG-H, TA-C), except FG-C and TA-H which kept their high scores throughout the packaging period.

DISCUSSION

Etchells et al. (1964) used hot-water blanching (66–80°C) for a short time (5 min) to rid cucumbers of naturally occurring, interfering, and competitive microbial groups before brining. Inoculation with the desired LAB of the treated material led to the pure culture fermentation of brined cucumbers. The application of a similar treatment to olives (74°C for 3 min) not only inhibited the initial wild microbiota but improved their fermentation (Etchells et al., 1966). The effect was linked to the presence of a LAB inhibitor in the fresh olives that, apparently, was degraded by the heat-shock (Fleming and Etchells, 1967). The use of hot-alkaline solutions improved the fermentation, with a marked enhancement of the acidification rates of *Merhavia* and *Manzanilla* green olives (Juven et al., 1968). Montedoro et al. (2002) was the first to link the lower concentration of HyEDA to a heat treatment of olives. An initial heat-shock treatment (80°C for 10 min) was also applied to reduce the wild microorganisms adhered to the olive epidermis and facilitate the brine and olive surface colonization by *Lactobacillus pentosus* B281 (Argyri et al., 2014). Recently, Ramírez et al. (2017) carried out a mild heat treatment (60°C, 10 min) of olives, followed by a direct brining and inoculation with selected LAB strains. The process caused

oleuropein depletion and reduced the natural bitterness of fruits without the application of any alkali hydrolysis. Apparently, the heat treatment inactivated the β-glucosidase activity of fruits and prevented the formation of antimicrobial compounds like HyEDA while promoting LAB growth.

After these research works, heat-shock should be considered as a promising treatment for LAB growth improvement in brined olives. Obviously, in the case of cultivars with low oleuropein content, such as *Aloreña de Málaga,* the benefits could be even greater. The results obtained in the present study for the cured and traditional denominations have confirmed this hypothesis since a strong LAB growth was observed in CA-H and TA-H denominations (**Figure 3**), which can be linked to the inactivation of the β-glucosidase enzyme and the subsequent absence of HyEDA. However, in not heat treated olives, the formation of inhibitors, although in a limited proportion, was enough to cause a moderate LAB population reduction. This is in agreement with the observations reported by Medina et al. (2007), who found the inhibition of LAB growth even at 0.25 mM concentrations of HyEDA during the storage of natural green olives in brine without alkali treatment. However, the results obtained in this work also indicate an inhibition of the β-glucosidase by temperature (Ramírez et al., 2014) as a consequence of the adequate selection of the heat treatment (60°) which took advantage of the drastic decrease in the activity of this enzyme above 50°C but was good enough to preserve texture, a highly appreciated attribute in *Aloreña de Malaga* olives.

The heat-shock treatment also had a marked effect on the microbiota. In this work, the microbial populations of the olives which received a heat treatment consisted mainly of *Lactobacillus* and *Pediococcus*. In contrast, Medina et al. (2016), using pyrosequencing analysis, reported the presence of undesirable *Celerinatantimonas, Pseudomonas,* and *Propionibacterium* as the most abundant genera detected in traditional industrially fermented fruits while the species of the *Lactobacillaceae* family were in low proportion (3–8%). This work also reveals information about the bacterial biodiversity for CA and FG *Aloreña de Málaga* denominations, whose alpha-biodiversity indexes and number of OTUs obtained in the present work were considerably higher than in previous studies (Medina et al., 2016).

The only disadvantage to exposing olives to a heat treatment could be firmness and color deterioration, with the subsequent impact on consumer acceptance (Brenes et al., 1994). However, no significant effect on olive firmness was found in this work, and the results that are in agreement with those reported by Ramírez et al. (2017). Interestingly, the color of the heat-shocked olives was better than the controls which were browner and had higher a^* values. According to Ramírez et al. (2017), these effects could have been due to the inactivation of another enzyme, the polyphenol-oxidase (PPO), by the heat-shock treatment with the subsequent delay in phenolic compound oxidation, polymerisation, and olive darkening.

In summary, the application of a mild heat-shock to *Aloreña de Málaga* fruits was beneficial, especially for the traditional process, since it favored the growth of the LAB population (especially *Lactobacillus* genera), caused a higher retention of the green appearance, and improved the stability of the packaged olives. Furthermore, all these changes occurred without any adverse effects on the sensory characteristics of the packaged products.

AUTHOR CONTRIBUTIONS

FR-G, MR-B, and VR-G performed the experimental work. AB-C executed the metagenomics analysis. FR-G and FA-L designed the work, while FA-L and AG-F analyzed the results and wrote the paper.

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REFERENCES

Argyri, A. A., Nisiotou, A. A., Mallouchos, A., Panagou, E. Z., and Tassou, C. C. (2014). Performance of two potential probiotic *Lactobacillus* strains from the olive microbiota as starters in the fermentation of heat-shocked green olives. *Int. J. Food Microbiol.* 171, 68–76. doi: 10.1016/j.ijfoodmicro.2013.11.003

Arroyo-López, F. N., Bautista-Gallego, J., Durán-Quintana, M. C., and Garrido-Fernández, A. (2008). Effects of ascorbic acid, sodium metabisulfite and sodium chloride on freshness retention and microbial growth during the storage of Manzanilla-Aloreña cracked table olives. *LWT Food Sci. Technol.* 41, 551–560. doi: 10.1016/j.lwt.2007.05.016

Arroyo-López, F. N., Durán-Quintana, M. C., Romero, C., Rodríguez-Gómez, F., and Garrido-Fernández, A. (2007). Effect of storage process on the sugars, polyphenols, color and microbiological changes in cracked Manzanilla-Aloreña table olives. *J. Agric. Food Chem.* 55, 7434–7444. doi: 10.1021/jf070571b

Balatsouras, G., Tsibri, A., Dallest, T., and Doutsias, G. (1983). Effects of fermentation and its control on the sensory characteristics of Conservolea variety green olives. *Appl. Environ. Microbiol.* 46, 68–74.

Bautista-Gallego, J., Arroyo-López, F. N., Romero-Gil, V., Rodríguez-Gómez, F., and Garrido-Fernández, A. (2011). Evaluating the effects of zinc chloride as a preservative in cracked table olive packing. *J. Food Prot.* 74, 2169–2176. doi: 10.4315/0362-028X.JFP-11-201

Benjamini, Y., and Hochberg, Y. (1995). Controlling the false discovery rate – a practical and powerful approach to multiple testing. *J. R Stat. Soc. Series B Methodol.* 57, 289–300.

Brenes, M., García, P., and Garrido, A. (1994). Influence of salts and pH on the firmness of olives in acid conditions. *J. Food Qual.* 17, 335–346. doi: 10.1111/j.1745-4557.1994.tb00155.x

Chen, Z., Zhu, C., Zhang, Y., Niu, D., and Du, J. (2010). Effects of aqueous chlorine dioxide treatment on enzymatic browning and shelf-life of fresh-cut

asparagus lettuce (*Lactuca sativa* L.). *Post. Biol. Technol.* 58, 232–238. doi: 10.1016/j.postharvbio.2010.06.004

DOUE (2012). Official Journal of the European Union Regulation N°1068/2012. L318/3. 55, 3–4.

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 26, 2460–2461. doi: 10.1093/bioinformatics/btq461

Etchells, J. L., Borg, A. F., Kittel, I. D., Bell, T. A., and Fleming, H. P. (1966). Pure culture fermentation of green olives. *Appl. Environ. Microbiol.* 14, 1027–1041.

Etchells, J. L., Costilow, R. N., Anderson, T. E., and Bell, T. A. (1964). Pure culture fermentation of brined cucumbers. *Appl. Environm. Microbiol.* 12, 523–525.

Fleming, H. P., and Etchells, J. L. (1967). Occurrence of an inhibitor of lactic acid bacteria in green olives. *Appl. Environ. Microbiol.* 15, 1178–1184.

Gallardo-Guerrero, L., Gandul-Rojas, B., Moreno-Baquero, J. M., López-López, A., Bautista-Gallego, J., and Garrido-Fernández, A. (2013). Pigment, physicochemical, and microbiological changes related to the freshness of cracked table olives. *J. Agric. Food Chem.* 61, 3737–3747. doi: 10.1021/jf400240e

Garrido-Fernández, A., Fernández-Díez, M. J., and Adams, R. (1997). *Table Olives. Production and Processing*. London: Chapman & Hall. doi: 10.1007/978-1-4899-4683-6

International Olive Council [IOC] (1987). *Sensory Analysis of Olive Oil Standard Glass For Oil Tasting. COI/T20/Doc n° 5*. Madrid: IOC.

International Olive Council [IOC] (2010). *Sensory Analysis of Table Olives. COI/OT/MO No*1/Rev.1. Madrid: IOC.

International Olive Council [IOC] (2016). *World Table Olive Figures*. Available at: http://www.internationaloliveoil.org/estaticos/view/132-world-table-olive-figures [accessed January 27, 2016].

Juven, B., Samish, Z., Henis, Y., and Jacoby, B. (1968). Mechanism of enhancement of lactic acid fermentation of green olives by alkali and heat treatment. *J. Appl. Microbiol.* 31, 200–207. doi: 10.1111/j.1365-2672.1968.tb00358.x

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., et al. (2013). Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Res.* 41, e1. doi: 10.1093/nar/gks808

López López, A., and Garrido Fernández, A. (2006). *Producción, Elaboración, Composición y Valor Nutricional de la Aceituna Aloreña de Málaga*, ed. S. L. Redagua. Málaga: Pizarra.

Medina, E., Brenes, M., Romero, C., García, A., and de Castro, A. (2007). Main antimicrobial compounds in table olives. *J. Agric. Food Chem.* 55, 9817–9823. doi: 10.1021/jf0719757

Medina, E., Ruiz-Bellido, M. A., Romero-Gil, V., Rodríguez-Gómez, F., Montes-Borrego, M., Landa, B. B., et al. (2016). Assessment of the bacterial community in directly brined *Aloreña de Málaga* table olive fermentations by metagenetic analysis. *Int. J. Food Microbiol.* 236, 47–55. doi: 10.1016/j.ijfoodmicro.2016.07.014

Montedoro, G., Baldioli, M., Selvaggini, R., Begliomini, A. L., Taticchi, A., and Servili, M. (2002). Relationship between phenolic composition of olive fruit and olive oil: the importance of the endogenous enzymes. *Acta Hortic.* 586, 551–556. doi: 10.17660/ActaHortic.2002.586.115

Parks, D. H., and Beiko, R. G. (2010). Identifying biologically relevant differences between metagenomic communities. *Bioinformatics* 26, 715–721. doi: 10.1093/bioinformatics/btq041

Ramírez, E., Brenes, M., de Castro, A., Romero, C., and Medina, E. (2017). Oleuropein hydrolysis by lactic acid bacteria in natural green olives. *LWT Food Sci. Technol.* 78, 165–171. doi: 10.1016/j.lwt.2016.12.040

Ramírez, E., Medina, E., Brenes, M., and Romero, C. (2014). Endogenous enzymes involved in the transformation of oleuropein in Spanish table olive varieties. *J. Agric. Food Chem.* 62, 9569–9575. doi: 10.1021/jf5027982

Rodríguez-Gómez, F., Romero-Gil, V., Arroyo-López, F. N., Bautista-Gallego, J., García-García, P., and Garrido-Fernández, A. (2015). Effect of packaging and storage conditions on microbial survival, physicochemical characteristics and colour of non-thermally preserved Green Spanish-style Manzanilla olives. *LWT Food Sci. Technol.* 63, 367–375. doi: 10.1016/j.lwt.2015.03.095

Sánchez, A. H., De Castro, A., Rejano, L., and Montaño, A. (2000). Comparative study on chemical changes in olive juice and brine during green olive fermentation. *J. Agric. Food Chem.* 48, 5975–5980. doi: 10.1021/jf000563u

Schmieder, R., and Edwards, R. (2011). Quality control and preprocessing of metagenomic datasets. *Bioinformatics* 27, 863–864. doi: 10.1093/bioinformatics/btr026

Segovia-Bravo, K. A., Jarén-Galán, M., García-García, P., and Garrido-Fernández, A. (2009). Browning reactions in olives: mechanism and polyphenols involved. *Food Chem.* 114, 1380–1385. doi: 10.1016/j.foodchem.2008.11.017

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A Probabilistic Decision-Making Scoring System for Quality and Safety Management in *Aloreña de Málaga* Table Olive Processing

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Table olives are one of the most representatives and consumed fermented vegetables in Mediterranean countries. However, there is an evident lack of standardization of production processes and HACCP systems thus implying the need of establishing decision-making tools allowing their commercialization and shelf-life extension. The present work aims at developing a decision-making scoring system by means of a probabilistic assessment to standardize production process of *Aloreña de Málaga* table olives based on the identification of potential hazards or deficiencies in hygienic processes for the subsequent implementation of corrective measures. A total of 658 microbiological and physico-chemical data were collected over three consecutive olive campaigns (2014–2016) to measure the variability and relative importance of each elaboration step on total hygienic quality and product safety. Three representative companies were visited to collect samples from food-contact surfaces, olive fruits, brines, air environment, olive dressings, water tanks, and finished/packaged products. A probabilistic assessment was done based on the establishment of Performance Hygiene and Safety Scores (PHSS 0–100%) through a standardized system for evaluating product acceptability. The mean value of the global PHSS for the *Aloreña de Málaga* table olives processing ($PHHS_{FTOT}$) was 64.82% (90th CI: 52.78–76.39%) indicating the high variability among facilities in the evaluated processing steps on final product quality and safety. Washing and cracking, and selection and addition of olive dressings were detected as the most deficient ones in relation to $PHSS_{Fi}$ values ($p < 0.05$) (mean = 53.02 and 56.62%, respectively). The relative contribution of each processing step was quantified by different experts ($n = 25$) from the *Aloreña de Málaga* table olive sector through a weighted PHSS ($PHSS_W$). The mean value of $PHSS_W$ was 65.53% (90th CI: 53.12–77.52%). The final processing steps obtained higher values for $PHSS_W$ being the finished product the most relevant one (mean = 18.44%; 90% CI: 10.34–25.33%). Sensitivity

analysis concluded that intervention measures focused on reducing the contamination of washing brines could lead to an improvement of $PHSS_{FTOT}$ value to 67.03%. The present work can be potentially applied in the *Aloreña de Málaga* table olive food sector for improving food quality and safety assurance.

Keywords: table olives, HACCP, decision-support system, performance hygiene and safety scores, sensitivity analysis

INTRODUCTION

Table olives are one of the most representative and consumed fermented vegetables in Mediterranean countries (Garrido-Fernandez et al., 1997; Arroyo-López et al., 2012, 2016). According to the recent statistics provided by the International Olive Oil Council (IOOC), European production has raised in 2015/16 to 859.8 mT whereas consumption also showed an increasing trend to 410.7 mT (IOOC, 2017). The global consumption of table olives in recent years has multiplied by 2.7, increasing by 182.0% over the period 1990/91–2016/17. Spain was ranked as the main producer in the world as well as the main consumer with 4.1 kg/person/year.

In the last years, consumers are demanding healthier and more convenient table olive preparations based on traditional processes. In Spain, *Aloreña de Málaga* green table olive has a Protected Designation of Origin (PDO) due to their peculiar characteristics of elaboration and geographical production region (Guadalhorce Valley, Málaga, Spain). Due to its low-to-moderate concentrations of oleuropein, the processing does not include alkaline debittering. Thus, they are produced as directly brined cracked green olives and seasoned with diverse herbs and species before packaging (López-López and Garrido-Fernández, 2006). Their differential characteristics regarding other table olive varieties limits the possibility of applying a heat treatment sufficiently high to destroy or reduce the microbial load in the packaged product. This requires the implementation of alternative preservation processes to allow increasing the shelf-life and further commercialization of finished products.

The microbiological safety of foods is managed by the effective implementation of control measures within a Food Quality Safety Management Systems (FQSMS) including prerequisite programme (PRP) and hazard analysis and critical control points (HACCP) that have been validated, where appropriate, throughout the food chain to minimize contamination and improve food safety (Valero et al., 2017). An integrated approach to food safety covers all sectors of the food chain (Regulation EC 178/2002, Commission Regulation, 2002) in response to requirements demanded by customers, competent authorities and certification bodies. Hygienic requirements for foodstuffs (Regulation EC 852/2004, Commission Regulation, 2004) implemented in the EU have urged the need to develop more sophisticated food quality and safety assurance standards and guidelines (Tzamalis et al., 2016). For the table olives sector, the codex standard (CODEX STAN 66-1981, review 1987 and 2013, Codex Alimentarius Commission, 1981) and the Trade Standard Applying to Table Olives (IOC, 2004) recommend that the product covered by these documents must be prepared and handled in accordance with the appropriate sections of the General Principles of Food Hygiene (CAC/RCP 1-1969; Codex Alimentarius Commission, 1969b), the Code of Hygienic Practice for Low-Acid and Acidified Low-Acid Canned Foods (CAC/RCP 23-1979, Codex Alimentarius Commission, 1969a, 1979), and the Code of Hygienic Practice for Canned Fruit and Vegetable Products (CAC/RCP 2-1969, Codex Alimentarius Commission, 1969a). In addition, the product should comply with any microbiological criteria established in accordance with the Principles for the Establishment and Application of Microbiological Criteria for Foods (CAC/GL 21-1997, Codex Alimentarius Commission, 1997; Regulation (EC) 1441/2007, Commission Regulation, 2007).

The development of a FQSMS requires quantitative tools able to assess the acceptance of final products. However, there is an evident lack of standardization in the table olive sector, thus implying the need of establishing decision-making tools allowing their commercialization and shelf-life extension. Lack of experience, knowledge and human and financial resources make difficult the implementation of standardized FQSMS in industry (Tzamalis et al., 2016). Further, production of table olives as fermented products could not be standardized since several factors such as variations in olive composition according to the season, spontaneous fermentation processes, limited technological capabilities in the company or lack of scientific and technical knowledge by industry's operators. Specifically, the manufacturing process of *Aloreña de Málaga* table olives is carried out by small and medium enterprises placed in, or very close to, the region of production. This fact together with the limited shelf-life of final products due to the presence of high residual sugars, spoilage microorganism, clouding or brines and swelling containers, make the distribution area very limited and do not allow in some cases exportation to other countries (Romero-Gil et al., 2016). There are previous studies dealing with the development of FQSMS in other food commodities demonstrating their usefulness to improve food quality and safety. One of the best examples is the Food Safety Management System- diagnostic instrument (FSMS-DI), which contributes to the measurement of the performance of the FSMS in an organization suggested for edible oil or fresh produce chains (Nanyunja et al., 2015; Ren et al., 2016). Further, development of scoring systems (Stadlmüller et al., 2017) and best practice scores (Tzamalis et al., 2016) for the assessment of FQSMS are also reported. However, these systems are deterministic approaches mainly based on performance of questionnaires or microbial data on targeted hazards, being not potentially applied to the table olive sector. The establishment of risk quality or safety margins by food operators is desirable since quantification of

variability associated to products and processes can be quantified. Furthermore, these measures are in line with the preventive Food Safety Modernization Act approach implemented in US (Grover et al., 2016).

In this study, a probabilistic approach is suggested to assess the performance of quality and safety of *Aloreña de Málaga* table olives production. Based on physico-chemical and microbiological data collected from three representative companies and seasons in Southern Spain, a decision-scoring system was developed establishing Performance Hygiene and Safety Scores (PHSS) to identify potential factors and processing steps to operationalize hygiene and safety of table olive processing.

MATERIALS AND METHODS

Study Design and Facilities

This study was performed in three different small and medium companies dedicated to table olives production located in Southern Spain (Valle del Guadalhorce, Málaga, Spain) which process all *Aloreña de Málaga* table olives. The experimental work was conducted in three consecutive campaigns from 2014 to 2016. Types of samples, processing steps to analyse and sampling planning were previously agreed with the quality inspector of each company as indicated in their different industry's Self-Control Plan (HACCP). The processing steps considered for the present study were based on the traditional elaboration of *Aloreña* table olives and they are shown in **Figure 1**. From each step, different microbiological and physico-chemical analyses were performed as described below to determine their influence on final product quality, hygiene and safety.

FIGURE 1 | Flow diagram of the elaboration process of *Aloreña* table olives shared by the different industries visited in the study.

Microbiological Analyses

Samples Collection

According with the sampling planning (**Tables 1**–**5**), different types of samples were collected in the industry, transferred to sterile containers, transported to the laboratory at refrigeration (2–4°C) conditions and analyzed within 24 h after collection.

Enumeration of Microbial Populations in the Different Types of Samples

To enumerate microbial populations in brines, samples (10 ml), if necessary, were serially diluted in sterile saline solution (0.9% NaCl) and plated (50 μl) in the correspondent culture media described below through using a Spiral Plater model dwScientific (Don Whitley Scientific Limited, UK). After incubation periods at the different temperatures according to the microbial group analyzed in this type of sample [lactic acid bacteria (LAB), yeasts and molds (Y/M), mesophilic bacteria (MB), and *Enterobacteriaceae* (Ent)], colonies were counted by using an Image Analysis System model CounterMat v.3.10 (IUL, Barcelona, Spain). Results were expressed at $\log_{10}$ cfu/ml.

For determination of microorganisms present in olive fruits [MB, Y/M, LAB, Ent, coagulase positive *Staphylococci* (CPS), sulphite reducing clostridia (SRC), *Listeria monocytogenes* (LM), and *Salmonella* sp. (Salm)], two olives (approximately 10 g) were washed with sterile saline solution (0.85% v/v) and deboned at sterile conditions. Then, a decimal dilution of fruit flesh in saline solution (90 ml) was homogenized in a Stomacher 400 Circulator Blender (Seward Laboratory System, UK) for 5 min. Afterwards, 50 μl were plated in the selective culture media. Results were expressed at $\log_{10}$ cfu/g.

To enumerate the number of microorganisms present in olive dressings (MB, Y/M, LAB, Ent, CPS, and SRC), 10 g of each seasoning material (garlic, red pepper and herbal mixture) were singly homogenized for 5 min in Stomacher with 90 ml of buffered peptone water (0.1%). Afterwards, 50 μl of the solutions were plated in the different culture media. Results were expressed at $\log_{10}$ cfu/g.

To determine the presence of microorganisms in water, samples (1,000 ml) were poured into sterile flasks and re-suspended with 5% solution of sodium thiosulfate (Panreac, Barcelona, Spain) to remove the residual effect of free chlorine. Then, samples were filter-sterilized using 0.22 μm diameter filters (Merck Millipore, Massachusetts, US). Then, filters were transferred to different selective media for analysis of the microbial groups specified in the Spanish Royal Decree (RD 140/2003, Royal Decree, 2003) such as MB, coliforms (Col), and SRC. Results were expressed at $\log_{10}$ cfu/ml.

TABLE 1 | Analyses performed, parameters, concentration and scores obtained from samples collected at processing step No. 1 (reception of raw materials and fermentation).

Type of sample (No. analyses)	Units	Parameter*	Mean concentration (95% CI)	Score (mean, 95% CI)
Air environment (16)	cfu/m^3	MB	208.87 (181.44, 236.31)	1.56 (1.95, 2.30)
		Y/M	56.50 (24.98, 88.02)	1.00 (0.74, 1.26)
Olive brine (32)	log_{10} cfu/ml	MB	6.20 (5.91, 6.37)	2.25 (1.77, 2.73)
		Y/M	5.00 (4.61, 5.21)	1.75 (1.35, 2.15)
		LAB	6.30 (5.98, 6.48)	2.00 (1.55, 2.45)
		Ent	<1.30 (–)	0.00 (–)**
Olive fruit (48)	log_{10} cfu/g	MB	5.88 (5.54, 6.06)	1.75 (1.42, 2.08)
		Y/M	4.30 (4.04, 4.47)	1.25 (0.96, 1.54)
		LAB	5.79 (5.48, 5.97)	1.50 (1.16, 1.84)
		Ent	<1.30 (–)	0.00 (–)
		CPS	<1 (–)	0.00 (–)
		SRC	1.07 (1.01, 1.11)	0.50 (0.20, 0.80)
Olive brine (12)	–	pH	4.23 (4.04, 4.42)	1.50 (0.52, 2.48)
	g/100 ml	FA	0.77 (0.62, 0.92)	0.00 (–)
	% (w/v)	NaCl	7.44 (7.10, 7.77)	0.00 (–)
Water (18)	cfu/100 ml	MB	14.30 (<10, 23.96)	0.83 (0.64, 1.02)
		Col	<10 (–)	1.00 (0.28, 1.72)
		SRC	<10 (–)	1.00 (0.28, 1.72)

MB, Mesophilic bacteria; Y/M, yeast/molds; LAB, Lactic-acid bacteria; Ent, Enterobacteriaceae; CPS, coagulase positive Staphylococci; SRC, Sulphite Reducing Clostridia; FA, free acidity; Col, total coliforms.

***CI 95% could not be estimated.*

Microbial air quality was determined by using an Air Sampler (SAS Super 180TM, Scharlab, Barcelona, Spain) searching for MB and Y/M as microbial indicators. The volume of air was fixed at 500 litters. Probable counts (*Pr*, cfu/m^3, statistical probability of multiple particles passing through the same hole) were obtained using a conversion table provided by the manufacturer.

The analysis of food-contact surfaces was carried out using MB and Ent as microbial indicators. Sterile polypropylene swabs (Nuovo Aptaca, Canelli, Italy) with amies medium were used for surface sampling. Each surface was swabbed using a 10 × 10 cm sterile metal template, then the swab head (1–2 cm) was aseptically cut and immersed in 3 ml test tubes of 0.1% buffered peptone water. In the case of handlers' gloves samples, the inner part was swabbed and the area in contact with hands was estimated as 225.07 ± 21.07 cm^2 for men and 188.03 ± 16.08 cm^2 for women (Ren et al., 2010). Results were expressed at cfu/cm^2.

Selective culture media used for enumeration of LAB was to DeMan Rogosa and Sharpe (MRS) agar (Oxoid, Basingstoke, UK) supplemented with 0.02% of sodium azide (Sigma, St. Louis, US) following by an incubation at 37°C for 48 h. Y/M were enumerated with the Yeast Mold agar (YM, Disco, Becton y Dickinson Company, Spark, MD, US) supplemented with 0.005% of gentamycin and oxy-tetracycline sulfate (Oxoid). Samples were incubated at 30°C for 48 h. Ent were counted using Violet Crystal Red Bile Dextrose (VRBD) agar (Merck, Darmstadt, Germany) after an incubation at 37°C for 24 h. MB were enumerated with Plate Count Agar (PCA, Oxoid) after an incubation at 28°C for 24h. CPS were enumerated following the ISO method (ISO: 6888-2: ISO, 1999) in Baird Parker supplemented with fibrinogen and rabbit plasmid (incubation at 37°C for 24 h). SRC were counted using Tryptose Sulphite Cycloserine (TSC) agar (Oxoid) after an incubation at 37°C for 24 h in anaerobic jars. Finally, presence of LM and Salm was confirmed using the ISO methods [ISO 11290-1/-2 (ISO, 2004, 2017) for LM and ISO 6579 (ISO, 2002) for Salm, respectively].

Physico-Chemical Analyses

The analyses of the olive brine for pH, salt, and titratable/free acidity (FA) were carried out using the routine methods described by Garrido-Fernandez et al. (1997). Total sugar content in brine (g/l) was determined by HPLC according to the methods developed by Sánchez et al. (2000) by the summation of values obtained for glucose, fructose, sucrose and mannitol.

Development of a Decision-Making Scoring System to Operationalize Hygiene and Safety of Table Olive Processing

Scoring System

In this study, a quantitative system assessing the food hygiene and safety throughout the elaboration process of table olives was established. This was done through a scoring system assigning different weighted values to microbiological and physico-chemical results obtained at the different steps in the elaboration chain (**Figure 1**). Scores ranged from 0 to 3, indicating the best and worst quality/safety conditions, respectively. Assigned scores and ranges are represented in **Table 6**. which describes how concentrations are translated to scores and the references used. These values were based on previous published studies, Codex standards for table olives, national and European legislations regarding different food criteria applied to samples

TABLE 2 | Analyses performed, parameters, concentration, and scores obtained from samples collected at processing step No. 2 (washing and cracking).

Type of sample (No. analyses)	Units	Parameter*	Mean concentration (95% CI)	Score (mean, 95% CI)
Air environment (16)	cfu/m^3	MB	210 (183.47, 236.77)	2.00 (–)
		Y/M	103 (79.83, 126.91)	1.38 (1.14, 1.61)
Hopper surface (16)	cfu/cm^2	MB	36.60 (5.44, 67.70)	2.63 (2.14, 3.00)
		Ent	73.5 (12.80, 134.17)	1.50 (0.76, 2.24)
Olive fruit (36)	log_{10} cfu/g	MB	5.70 (5.41, 5.87)	1.67 (1.22, 2.11)
		Y/M	4.66 (4.40, 4.82)	0.67 (0.33, 1.00)
		LAB	5.95 (5.76, 6.08)	1.67 (1.22, 2.11)
		Ent	<1.30 (–)**	0.00 (–)
		CPS	1.07 (<1, 1.11)	0.17 (0.03, 0.30)
		SRC	<1.30 (–)	0.00 (–)
Olive brine (24)	log_{10} cfu/ml	MB	5.04 (4.59, 5.25)	1.83 (1.37, 2.30)
		Y/M	4.87 (4.63, 5.03)	1.83 (1.37, 2.30)
		LAB	6.59 (6.03, 6.83)	1.33 (0.73, 1.94)
		Ent	4.38 (3.95, 4.59)	1.33 (0.79, 1.88)
Water (18)	cfu/100 ml	MB	1.34 (1.06, 1.50)	0.83 (0.64, 1.02)
		Col	<1.30 (–)	1.00 (0.28, 1.71)
		SRC	<1.30 (–)	1.00 (0.28, 1.71)

MB, Mesophilic bacteria; Y/M, yeast/molds; LAB, Lactic-acid bacteria; Ent, Enterobacteriaceae; CPS, coagulase positive Staphylococci; SRC, Sulphite Reducing Clostridia; Col, total coliforms.

***CI 95% could not be estimated.*

and parameters evaluated (Al Dagal et al., 1992; Federation des Industries Condimentaires de France, 2000; Royal Decree 1230/2001, Royal Decree, 2001; Royal Decree 140/2003, Royal Decree, 2003; IOC, 2004; Sneed et al., 2004; Regulation EC 1441/2007, Commission Regulation, 2007; Codex 66-1981, 2013, Codex Alimentarius Commission, 1981).

Calculation of Performance Hygiene and Safety Scores

The obtained results were processed and the correspondent scores assigned to each analytical data in accordance to the criteria represented in **Table 6**. Then, a probabilistic model was created in @Risk v7.5 (Palisade Corporation) to quantify variability associated to the elaboration process and to identify potential steps and factors that could influence on the final degree of hygiene and safety.

The variables used for model development were:

- F_i defining the processing step i (**Figure 1**) (i ranges from 1 to 5),
- T_i is the type of sample collected within the ith processing step (i.e., air environment, olive fruits, brines, etc.)
- P_i is the parameter analyzed corresponding to T_i within the processing step F_i (i.e., MB, LAB, pH, etc.) and,
- S_i is the assigned score to the ith parameter, ranging from 0 to 3.

As an example, for the processing step F_1 (reception of raw material and storage), and type of sample T_1 (air environment) the assigned scores as 0, 1, 2, and 3 were summed up for all parameters as follows:

$$(F_1, T_1) = \Sigma S_0(P_i);\ \Sigma S_1(P_i);\ \Sigma S_2(P_i);\ \Sigma S_3(P_i); \quad (1)$$

being P_i the number of parameters evaluated for this type of sample (in this case, MB and Y/M).

Let N_0 to N_3 be the number of times the scores were assigned as 0, 1, 2, and 3, then: $N_0 = \Sigma S_0; N_1 = \Sigma S_1; N_2 = \Sigma S_2; N_3 = \Sigma S_3$. Once N_0 to N_3 values were obtained, within each processing step (F) and type of sample (T), the correspondent probabilities (p) associated to each score were calculated as:

$$p_0 = \frac{N_0}{N_0 + N_1 + N_2 + N_3}; p_1 = \frac{N_1}{N_0 + N_1 + N_2 + N_3};$$
$$p_2 = \frac{N_2}{N_0 + N_1 + N_2 + N_3}; p_3 = \frac{N_3}{N_0 + N_1 + N_2 + N_3} \quad (2)$$

being $p_0 + p_1 + p_2 + p_3 = 1$. A discrete function was implemented in @Risk to assign any possible values from 0 to 3 as a function of the calculated probabilities (p). The resulting values from the discrete distribution (D_1, D_2 … D_i) corresponding to the types of samples and parameters evaluated were summed up to obtain the score for the ith processing step (D_{Fi}), defined as:

$$D_{Fi} = D_1T_1 + D_2T_2 + \ldots + D_iT_i \quad (3)$$

To measure the degree of fulfillment of the ith processing step on product quality and safety, a Performance Hygiene and Safety Score ($PHSS_{Fi}$, %) was obtained. $PHHS_{Fi}$ values ranged from 0 to 100% indicating the percentage of fulfillment of F_i on the overall quality and safety of the process and finished product and was calculated as follows:

$$PHSS_{Fi} = 1 - \left(\frac{D_{Fi}}{D_{maxFi}}\right) x\ 100 \quad (4)$$

TABLE 3 | Analyses performed, parameters, concentration, and scores obtained from samples collected at processing step No. 3 (selection and addition of olive dressings).

Type of sample (No. analyses)	Units	Parameter*	Mean concentration (95% CI)	Score (mean, 95% CI)
Air environment (16)	cfu/m^3	MB	182 (150.76, 213.80)	2.25 (2.02, 2.48)
		Y/M	81.4 (64.62, 98.12)	1.25 (1.02, 1.48)
Conveyor belt (12)	cfu/cm^2	MB	18,000 (0, 23,567)	3.00 (–)**
		Ent	94.2 (22.21, 166.08)	1.33 (0.60, 2.07)
Olive fruit (36)	log_{10} cfu/g	MB	5.19 (5.06, 5.30)	1.50 (1.23, 1.77)
		Y/M	4.07 (3.76, 4.25)	1.00 (0.71, 1.29)
		LAB	4.93 (4.70, 5.08)	1.33 (1.00, 1.67)
		Ent	2.30 (–)	0.50 (0.10, 0.90)
		CPS	<1.30 (–)	0.17 (0.03, 0.30)
		SRC	<1.30 (–)	0.00 (–)
Olive dressing: red pepper (36)	log_{10} cfu/g	MB	3.21 (3.09, 3.30)	0.33 (0.16, 0.50)
		Y/M	3.37 (2.93, 3.59)	0.50 (0.23, 0.77)
		LAB	2.72 (2.02, 2.98)	0.17 (0.03, 0.30)
		Ent	1.70 (1.00, 1.95)	0.50 (0.10, 0.90)
		CPS	2.90 (2.63, 3.07)	1.50 (0.96, 2.04)
		SRC	<1.30 (–)	0.00 (–)
Olive dressing: garlic (36)	log_{10} cfu/g	MB	4.61 (4.26, 4.79)	0.83 (0.40, 1.27)
		Y/M	3.13 (2.80, 3.31)	0.33 (0.16, 0.50)
		LAB	3.80 (3.50, 3.98)	0.83 (0.51, 1.15)
		Ent	3.15 (2.60, 3.38)	1.00 (0.49, 1.50)
		CPS	2.50 (2.16, 2.69)	1.00 (0.49, 1.50)
		SRC	<1.30 (–)	0.00 (–)
Olive dressing: herbs (36)	log_{10} cfu/g	MB	7.34 (6.69, 7.59)	2.50 (2.23, 2.77)
		Y/M	5.72 (5.43, 5.90)	1.67 (1.27, 2.06)
		LAB	5.56 (5.09, 5.78)	1.00 (0.49, 1.50)
		Ent	6.45 (5.75, 6.71)	2.00 (1.50, 2.50)
		CPS	4.42 (3.85, 4.66)	2.50 (2.10, 2.90)
		SRC	2.03 (1.74, 2.21)	1.00 (0.71, 1.29)
Handlers' gloves (12)	cfu/cm^2	MB	2.06 (0.80, 2.35)	1.60 (1.09, 2.11)
		Ent	<1 (–)	0.00 (–)

**MB, Mesophilic bacteria; Y/M, yeast/molds; LAB, Lactic-acid bacteria; Ent, Enterobacteriaceae; CPS, coagulase positive Staphylococci; SRC, Sulphite Reducing Clostridia; FA, free acidity; Col, total coliforms.*

***CI 95% could not be estimated.*

where D_{maxFi} was defined as the maximum score that can be potentially obtained for the processing step F_i (being representative of the worst-case scenario):

$$D_{maxFi} = 3 \times \text{number of parameters evaluated in } F_i.$$

This worst-case scenario was considered as the score 3 was associated to the poorest hygienic conditions. This measure was needed for model development to relativize the PHSS within each processing step.

Finally, the different scores obtained for the five processing steps were then summed up and a global score was obtained (D_{TOT}):

$$D_{TOT} = D_{F1} + D_{F2} + D_{F3} + D_{F4} + D_{F5} \quad (5)$$

With this information, the global Performance Hygiene and Safety Score ($PHSS_{FTOT}$, %) was calculated as:

$$PHSS_{F_{TOT}} = 1 - \left(\frac{D_{TOT}}{D_{\max F_{TOT}}} \right) x\, 100 \quad (6)$$

where $D_{maxF_{TOT}}$ was defined as the maximum score that can be potentially obtained for the five processing steps evaluated.

Calculation of Weighted Performance Hygiene and Safety Scores ($PHSS_w$)

To measure the relative importance of each processing step on the final quality and safety of table olives, an Expert Knowledge Elicitation process (EKE) was performed. Expert elicitation is a process for quantifying expert opinion regarding uncertainties to address research problems in areas where traditional scientific research is infeasible or not yet available. Because uncertainties in the probabilistic model can be described in terms of probability

TABLE 4 | Analyses performed, parameters, concentration, and scores obtained from samples collected at processing step No. 4 (packaging processes).

Type of sample (No. analyses)	Units	Parameter*	Mean concentration (95% CI)	Score (mean, 95% CI)
Air environment (16)	cfu/m^3	MB	184 (150.46, 216.73)	2.17 (1.97, 2.37)
		Y/M	84.2 (63.77, 104.56)	1.33 (1.08, 1.59)
Packaging containers (12)	cfu/cm^2	MB	<1 (–)**	0.50 (0.00, 1.19)
		Ent	<1 (–)	0.00 (–)
Olive fruit (36)	log_{10} cfu/g	MB	4.39 (4.08, 4.58)	1.33 (1.07, 1.60)
		Y/M	3.80 (3.57, 3.95)	1.00 (0.71, 1.29)
		LAB	3.75 (3.11, 3.99)	0.50 (0.23, 0.77)
		Ent	2.68 (2.18, 2.91)	1.50 (0.96, 2.03)
		CPS	<1.30 (–)	0.33 (0.16, 0.50)
		SRC	<1.30 (–)	1.00 (0.49, 1.51)
Olive brine (24)	log_{10} cfu/ml	MB	1.62 (<1.30, 1.84)	0.17 (0.00, 0.33)
		Y/M	<1.30 (–)	0.00 (–)
		LAB	<1.30 (–)	0.00 (–)
		Ent	<1.30 (–)	0.00 (–)
Handlers gloves (12)	cfu/cm^2	MB	2.04 (<1.30, 2.34)	1.40 (0.75, 2.05)
		Ent	<1 (–)	0.60 (0.00, 1.35)
Water (18)	cfu/100 ml	MB	14.30 (<10, 23.96)	0.83 (0.64, 1.02)
		Col	<10 (–)	0.00 (–)
		SRC	<10 (–)	0.00 (–)

MB, Mesophilic bacteria; Y/M, yeast/molds; LAB, Lactic-acid bacteria; Ent, Enterobacteriaceae; CPS, coagulase positive Staphylococci; SRC, Sulphite Reducing Clostridia; Col, total coliforms.

***CI 95% could not be estimated.*

distributions EKE can be considered for the derivation of distribution parameters (Clemen and Winkler, 1999). For the present study, the relative importance of each processing step was quantified by a group of 25 quality inspectors from the table olive sector together with scientists and public health authorities. A percentage from 0 to 100% was individually assigned to each processing step (% F_i) and a triangular distribution with three parameters; most probable number, minimum and maximum. These percentages (% F_i) were included in the model to weight the steps according to the experts' opinion. For the processing step F_i, the weighted PHSS values ($PHSS_w$) were calculated as:

$$PHSS_w = \left(\frac{D_{\max F_{TOT}}}{D_{TOT}}\right) + \left(\frac{\%F_i}{100}\right) x \left(\frac{PHSS_{F_i}}{100}\right) \quad (7)$$

It should be noted that $PHSS_{Fi}$ values provide a measure of the global variability in the elaboration process while $PHSS_w$ values are indicative of the individual contribution of each processing step to the overall quality and safety of the finished product.

Statistical Analyses

Boxplots including the main descriptive statistics (mean, standard deviation, 5th, 95th percentiles) were generated for each model output, i.e., $PHSS_{Fi}$ and $PHSS_w$ values. Descriptive statistics of the final distribution outputs were used to quantify model variability associated to the hygienic-sanitary conditions in each processing step. Uncertainty was considered using the 95% CI for the microbiological results and scores. Further, an ANOVA analysis was also performed to find significant differences between processing steps in relation to the PHSS values calculated ($p < 0.05$).

Further, Spearman correlation coefficients were obtained through a sensitivity analysis to identify the most relevant processing steps, samples and parameters that may exert an influence on the final product quality and safety. To avoid unrealistic results of the model, the Spearman's rank order correlation in the @Risk software was used to assume a previous high-dependence association between microbial loads found in olive fruits and brines ($r = 0.75$). The probabilistic model was run with a MonteCarlo simulation in @Risk v7.5 with 10,000 iterations.

RESULTS AND DISCUSSION

Hygienic-Sanitary Status of *Aloreña* Table Olive Processing

To evaluate the status of the hygienic-sanitary conditions throughout the *Aloreña* table olive processing, a total of 658 microbiological and physico-chemical data were obtained from brines, olive fruits, air environment, food-contact surfaces, food handlers, and water samples in three industries. Besides, finished packed table olives were characterized after processing just before commercialization. The mean concentrations together with the assigned scores to each processing step are represented in **Tables 1–5**.

Processing Step 1: Reception of Raw Materials and Fermentation

In **Table 1**, the status of olive brines and fruits once fermentation was completed indicated a relatively high concentration of MB and Y/M. Olive brines presented a mean value of 6.20 log_{10} cfu/ml of MB while Y/M concentration corresponded to 5.00

TABLE 5 | Analyses performed, parameters, concentration, and scores obtained from samples collected at processing step No. 5 (finished product).

Type of sample (No. analyses)	Units	Parameter*	Mean concentration (95% CI)	Score (mean, 95% CI)
Olive brine (48)	log_{10} cfu/ml	MB	3.08 (3.00, 3.14)	1.67 (1.52, 1.81)
		Y/M	3.20 (3.06, 3.31)	1.33 (1.10, 1.56)
		LAB	3.82 (3.48, 4.01)	1.33 (1.04, 1.62)
		Ent	<1.30 (–)**	0.17 (0.05, 0.28)
		CPS	<1.30 (–)	0.00 (–)
		SRC	<1.30 (–)	0.50 (0.15, 0.85)
Olive fruit (48)	log_{10} cfu/ml	MB	3.89 (3.65, 4.05)	0.83 (0.56, 1.11)
		Y/M	3.85 (3.70, 3.96)	1.33 (1.19, 1.48)
		LAB	4.35 (4.09, 4.52)	1.00 (0.75, 1.25)
		Ent	<1.30 (–)	0.17 (0.05, 0.28)
		CPS	<1.30 (–)	0.50 (0.35, 0.65)
		SRC	<1.30 (–)	0.50 (0.15, 0.85)
		LM	<−1.40 (–)	0.00 (–)
		Salm	<−1.40 (–)	0.00 (–)
Olive brine (24)	–	pH	4.23 (4.14, 4.32)	1.50 (1.11, 1.89)
	mEq/ml	FA	0.31 (0.28, 0.34)	0.50 (0.26, 0.74)
	% (w/v)	NaCl	5.44 (5.36, 5.52)	1.00 (0.75, 1.25)
	g/l	Sugar	2.70 (2.00, 3.40)	0.50 (0.35, 0.65)

MB, Mesophilic bacteria; Y/M, yeast/molds; LAB, Lactic-acid bacteria; Ent, Enterobacteriaceae, CPS, coagulase positive Staphylococci; SRC, Sulphite Reducing Clostridia; LM, L. monocytogenes; Salm, Salmonella sp.; Col, total coliforms.

***CI 95% could not be estimated.*

log_{10} cfu/ml. LAB concentration was also higher than 6 log_{10} cfu/ml. These microbiological values are in agreement with data reported by Arroyo-López (2007) for this type of table olive specialty in this step. In olive fruits, microbial loads were slightly lower though presence of SRC was detected at low levels (around 1 log_{10} cfu/g). Neither Ent nor CPS were detected in brines or fruits samples in this processing step. The absence of Ent was related with the low pH obtained after fermentation of fruits (Garrido-Fernandez et al., 1997). Air contamination was qualified as intermedium for MB (average count of 2.32 log_{10} cfu/m^3) while lower values were obtained for Y/M (1.75 log_{10} cfu/m^3). Regarding physico-chemical data, it should be noted that some deficiencies were denoted regarding pH values of brines, which were slightly higher than 4.3, meaning that these samples would not comply with the requirements stated in the international laws (Codex Alimentarius 1981, rev 2013; IOC, 2004), where maximum allowable pH is 4.3. On the contrary, data obtained for FA and salt can be considered as normal (Garrido-Fernandez et al., 1997; Arroyo-López, 2007). Finally, water samples presented unacceptable values of MB, Col and SRC though these two later groups were detected after samples enrichment.

Processing Step 2: Olive Washing and Cracking

After fermentation of fruits, olives were washed and cracked by industry. Cracking step is considered as a critical control point in the HACCP system since microbial hazards present in contaminated olive fruits can be spread during the cracking process to non-contaminated fruits, brines or food-contact surfaces. The microbiological quality of brines and fruits was very similar to the processing step 1 (**Table 2**). However, presence of Ent was observed at high levels in the hopper surfaces (mean = 1.86 log_{10} cfu/cm^2) and could have been probably transferred to olive brines since more than 4 log_{10} cfu/ml was observed in some of evaluated samples. These loads were also observed by other authors (Arroyo-López, 2007; Alves et al., 2012) ranging from 2.6 to 3.5 log_{10} cfu/ml in brines at the beginning of the fermentation period, but there is no information available on the surface of machinery, containers, operators, etc., in olive industry. The presence of Ent is not desired in table olives because they could jeopardize the stability and safety of finished products (Garrido-Fernandez et al., 1997). Air contamination was classified as "intermediate," according to the microbial concentrations obtained (100–300 cfu/m^3).

Processing Step 3: Selection and Addition of Olive Dressings

In this step, samples collected corresponded to air environment, conveyor belts, handlers' gloves, olive fruits, and olive dressings (red pepper, garlic and herbal mixture). Overall, high microbial counts of MB and Ent were obtained in samples from conveyor belts. Presence of Ent was detected in olive fruits (mean = 2.30 log_{10} cfu/g), and in olive dressings, being higher for the herbal mixture (mean = 6.45 log_{10} cfu/g). Addition of herbs and spices to olive fruits could imply an increase in the microbial load of finished products given the high concentrations of MB, Y/M, LAB, and Ent (Arroyo-López, 2007). Besides, product safety could be compromised since high concentrations of CPS were detected in herb samples (mean = 4.42 log_{10} cfu/g) together with the presence of SRC (**Table 3**). However, the influence

TABLE 6 | Scoring system assigned to the different physico-chemical and microbiological parameters analyzed and samples collected.

Type of sample	Parameters*	Units	Scores				Source**
Air environment	MB and Y/M	cfu/m^3	<10 (0)	10–100 (1)	101–300 (2)	>300 (3)	1
Food-contact surfaces	MB	cfu/cm^2	<1 (0)	1–10 (1)	11–100 (2)	>100 (3)	2
	Ent	cfu/cm^2	<1 (0)	1–5 (1)	5–10 (2)	>10 (3)	2
Olive fruits (semi-elaborated)	MB	cfu/g	$<10^3$ (0)	10^3–10^4 (1)	10^4-10^6 (2)	$>10^6$ (3)	3, 4, 5
	Ent	cfu/g	<20	21–50	51–100	>100	3, 4, 5
	LAB	cfu/g	$<10^3$ (0)	10^3–10^4 (1)	10^4–10^6 (2)	$>10^6$ (3)	3, 4, 5
	Y/M	cfu/g	$<10^3$ (0)	10^3–10^4 (1)	10^4–10^5 (2)	$>10^5$ (3)	3, 4, 5
	CPS	cfu/g	<20 (0)	21–50	51–100	>100	3, 4, 5
	SRC	cfu/g	<20 (0)	–	–	≥20 (3)	3, 4, 5
Olive fruits (finished product) and olive dressings (garlic and red pepper)	MB	cfu/g	$<10^3$ (0)	10^3-10^4 (1)	10^4–10^5 (2)	$>10^5$ (3)	3, 4, 5
	Ent	cfu/g	<20	21–50	51–100	>100	3, 4, 5
	LAB	cfu/g	$<10^2$ (0)	10^2–10^4 (1)	10^4–10^5 (2)	$>10^5$ (3)	3, 4, 5
	Y/M	cfu/g	$<10^2$ (0)	10^2–10^4 (1)	10^4–10^5 (2)	$>10^5$ (3)	3, 4, 5
	CPS	cfu/g	<20 (0)	21–50	51–100	>100	3, 4, 5
	SRC	cfu/g	<20 (0)	–	–	≥20 (3)	3, 4, 5
	LM	cfu/g	<1 /25g (0)	–	–	≥1 /25 g (3)	6
	Salm	cfu/g	<1 /25g (0)	–	–	≥1 /25 g (3)	6
Brines	MB	cfu/ml	$<10^2$ (0)	10^2-10^3 (1)	10^3-10^5 (2)	$>10^5$ (3)	3, 4, 5
	Ent	cfu/ml	<20	21–50	51–100	>100	3, 4, 5
	LAB	cfu/ml	$<10^2$ (0)	10^2–10^3 (1)	10^3–10^5 (2)	$>10^5$ (3)	3, 4, 5
	Y/M	cfu/ml	$<10^2$ (0)	10^2–10^3 (1)	10^3–10^5 (2)	$>10^5$ (3)	3, 4, 5
	pH	–	<4.0 (0)	4.0–4.2 (1)	4.2–4.3 (2)	>4.3 (3)	7
	FA	g/100 ml	>0.3 (0)	0.2–0.3 (1)	0.1–0.2 (2)	<0.1 (3)	7
	NaCl	% (w/v)	>6.0 (0)	5.5–6.0 (1)	5.0–5.5 (2)	<5.5 (3)	7
	Sugar	% (g/l)	<2.0 (0)	2.0–9.0 (1)	9.0–19.0 (2)	>19.0 (3)	7
Olive dressing (herbs)	SRC	cfu/g	<20	21–100	101–10^3	$>10^3$	3, 4, 5
Water	MB	cfu/100 ml	<1 (0)	1–50 (1)	51–100 (2)	>100 (3)	8
	Col	cfu/100 ml	<1 (0)	–	–	≥1 (3)	8
	SRC	cfu/100 ml	<1 (0)	–	–	≥1 (3)	8

**MB, Mesophilic bacteria; Y/M, yeast/molds; LAB, Lactic-acid bacteria; Ent, Enterobacteriaceae; CPS, coagulase positive Staphylococci; SRC, Sulphite Reducing Clostridia; FA, free acidity; Col, total coliforms; LM, L. monocytogenes; Salm, Salmonella sp.*

***1 (Al Dagal et al., 1992); 2 (Sneed et al., 2004); 3 [Codex Standard for Table Olives (Codex 66-1981, 2013, Codex Alimentarius Commission, 1981)]; 4 (Trade Standard Applying to Table Olives, IOC, 2004); 5 [Code des Bonnes Pratiques Loyales pour les Olives de Table (Federation des Industries Condimentaires de France, 2000)]; 6 [Commission Regulation (EC) No 1441/2007 of 5 December 2007 amending Regulation (EC) No 2073/2005 on microbiological criteria for foodstuffs, Commission Regulation, 2007); 7 (Royal Decree 1230/2001, of 8 November, approving the Technical-sanitary Regulation for the elaboration, distribution and sale of table olives); 8 [Royal Decree 140/2003 (Royal Decree, 2003) of 7 February, establishing the sanitary quality criteria of water for human consumption].*

of seasoning material in table olive processing has not been studied in detail in spite of their considerable influence on quality and safety of finished products. Samples from food handlers presented relatively low counts of MB. Ent were not detected in handlers' gloves.

Processing Step 4: Packaging Process

Table 4 represents the microbial counts obtained in the packaging step. A substantial reduction in mean counts were observed in comparison to the previous steps. This could be attributed to the inhibitory effect of salt concentration and pH together with the renovation of brines which imply a reduction in the microbial load of olive fruits. However, low counts of Ent were observed in fruits (mean = 2.68 $\log_{10}$ cfu/g) that could probably be associated to the high concentrations detected in olive dressings and transferred to this step. Olive brines had good microbiological quality as well as water samples. CPS and SRC were not detected in any sample.

Processing Step 5: Finished Product

Finished products just before commercialization presented lower microbial concentration of all groups analyzed which means that contamination during processing can be sporadic and product formulation (especially salt, pH values, and addition of preservatives) does not allow microbial growth during shelf life. In **Table 5**, it can be observed that concentrations were below 4 $\log_{10}$ cfu/g in all samples evaluated. Further, Ent, SRC and CPS were not detected in any sample. Absence or low levels of Ent in finished product is in line with data obtained by other authors which reflect their survival in olive packaging only during the first days (Bautista-Gallego et al., 2010; Romero-Gil et al., 2016). LM and Salm were not detected in any sample of fruits and brines in the finished product. This data is in concordance with the study carried out by Medina et al. (2016), who related the inhibition of diverse food-borne pathogens (among them *Listeria* and *Salmonella*) in *Aloreña de Málaga* brines by the presence of diverse phenolic compounds.

It should be noted that for some samples, pH values and salt concentrations exceeded the recommended limits for table olives (pH > 4.3; NaCl < 6%) which could imply that halotolerant or acidic-resistant microorganisms could proliferate during storage if they are previously present in the intermediate fruits and /or brines. Further, mean content of residual sugar in olive fruits was 2.70 g/l, which could support microbial growth. This is particularly relevant for olive production, since yeasts have the capacity to produce refermentation in presence of residual sugars (Loureiro and Malfeito-Ferreira, 2003). In this context, it is highly important to reduce yeasts concentration during the table olive processing in order to improve product stability and shelf-life (Alves et al., 2012). Bautista-Gallego et al. (2013) related yeasts as the main microbial agent causing instability of *Aloreña de Málaga* packaging at salt concentration above 5.0%, while Romero-Gil et al. (2016) point to LAB as spoilage microorganisms when the salt concentration was below this critical level.

Probabilistic Assessment of Hygiene and Safety of *Aloreña de Málaga* Table Olives

Simulation Results of PHSS and $PHSS_w$ Values

In the present study, a decision-making scoring system was suggested to operationalize hygienic-sanitary conditions in the *Aloreña de Málaga* table olives processing. The degree of fulfillment and the variability in the hygienic and safety conditions was quantified at each processing step ($PHSS_{Fi}$) as well as for the global process through the calculation of $PHHS_{FTOT}$. Besides, the relative importance of processing conditions was quantified by experts' elicitation. This information served to estimate the $PHSS_w$ values.

Figure 2 shows the simulation results of $PHSS_{Fi}$ and $PHSS_{FTOT}$ outputs. The mean value of the global Performance Hygiene and Safety Score for the *Aloreña de Málaga* table olives processing ($PHHS_{FTOT}$) was 64.82% (90th CI: 52.78–76.39%) indicating a variation in the hygienic practices in the evaluated processing steps among different industries. Washing and cracking, and selection and addition of olive dressings were detected as the most deficient steps since the lowest $PHSS_{Fi}$ values were obtained ($p < 0.05$) (mean = 53.02 and 56.62% respectively). Especially for washing and cracking, variability in processing conditions among facilities was the highest (90th CI: 26.67–80.00%) and high contamination of brines and fruits were obtained. Packaging and finished products showed higher $PHSS_{Fi}$ values (mean > 73%) probably attributed to product formulation (combination of low pH and high NaCl levels) together with the addition of new brines and preservatives that contributed to a reduction of microbial contamination at the packaging step.

$PHHS_w$ values were mainly based on the elicitation scores assigned by different experts from the *Aloreña de Málaga* table olive processing sector. Further, triangular distributions with minimum, most probable and maximum scores for each processing step were adjusted. Distributions were used as modeling inputs to estimate the individual contribution of the processing steps to the overall hygienic-sanitary conditions of finished products. In **Table 7**, descriptive statistics and percentiles

TABLE 7 | Elicitation scores (%) assigned by individual experts ($n = 25$) from the *Aloreña de Málaga* table olive sector.

Processing step	Distribution	Mean	S.D.	5th Perc	95th Perc
Reception of raw material and fermentation	RiskTriang(5;10;40)	16.49	6.18	8.02	27.64
Olive washing and cracking	RiskTriang(5;20;30)	17.10	4.61	9.30	24.49
Selection and addition of olive dressings	RiskTriang(10;20;40)	21.46	5.29	13.49	30.84
Packaging process	RiskTriang(10;20;30)	18.86	3.92	12.66	25.61
Finished product	RiskTriang(5;40;40)	26.12	6.74	13.79	36.22

Parameters used for triangular distributions are shown together with simulated statistics.

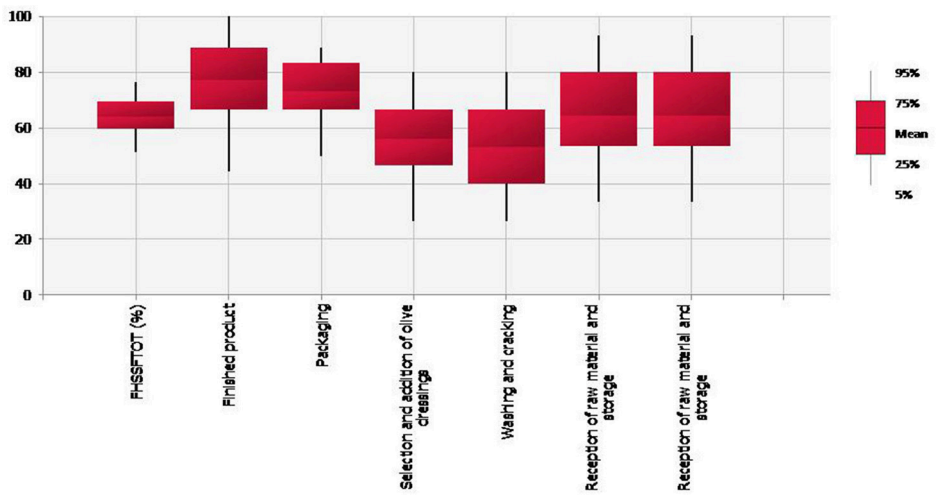

FIGURE 2 | Boxplot representing the mean, 5, 25, 75, and 95th values of the Performance Hygiene and Safety Score at the different processing steps ($PHSS_{Fi}$) together with the global $PHSS_{FTOT}$ (%).

of simulated distributions indicated that the finished product was assigned by experts as the most relevant step from a hygiene and safety point of view, having the highest 95th percentile (36.22%) followed by the selection and addition of olive dressings (30.84%). In contrast, reception of raw materials and fermentation, olive washing and cracking and packaging steps had the lowest 95th percentiles (24.49–27.64%). The main premise behind an expert elicitation method is that the method employed incorporates the knowledge and experience of the experts, and reduces the judgment biases. In the present study, the use of questionnaires allowed to collect information from quality inspectors of the table olive sector. Expert opinions can be used to address important questions and uncertainties in risk analysis. However, one of the limitations of expert elicitation is that sometimes experts may not describe accurately their actual knowledge so that data selection should be taken with caution.

In **Figure 3**, the relative contribution of each processing step on the $PHSS_w$ was represented, according to the values provided by the experts (**Table 7**). Significant differences in $PHSS_w$ values were obtained between packaging process and finished products, and the remaining processing steps ($p < 0.05$). The mean value of $PHSS_w$ was 65.53% (90th CI: 53.12–77.52%), very similar with $PHSS_{FTOT}$ with 64.82%. As above mentioned for $FHSS_{FTOT}$, the final processing steps obtained higher values for $PHSS_w$ being the finished product the most relevant one (mean = 18.44%; 90% CI: 10.34–25.33%). However, it should be noted that $PHSS_w$ values are influenced by the weighting percentage assigned by the experts. In this case, the final steps were considered highly relevant for preserving the stability of the finished product and its shelf-life extension.

Sensitivity Analysis of PHSS and $PHSS_W$ Values on the Type of Sample and Processing Step

In **Figure 4**, Spearman correlation coefficients describing the relative influence of the type of sample on the $PHSS_{FTOT}$ and on the $PHSS_w$ are represented. As $PHSS_{FTOT}$ were calculated without weighting the processing steps (all of them were considered equally relevant for final product quality and safety), correlation coefficients were higher for the primary steps which corresponded to the most contaminated samples. Particularly, the microbiological quality and safety of used brines presented a high correlation (−0.30 for brines used during the reception of raw materials and storage; and −0.28 for brines used during washing and cracking of table olives) with the final $PHSS_{FTOT}$ values, followed by results obtained in processing step 3 (selection and addition of olive dressings) for intermediate fruits and olive dressings.

On the contrary, for $PHSS_w$ values, (**Figure 4B**) the finished product presented the highest correlations (fruits, brines and physico-chemical values) since this step contributed mostly to the increase of $PHSS_w$.

Sensitivity analysis was also performed on the relative variation of each type of sample on the mean $PHSS_{FTOT}$ and $PHSS_w$ values (**Figure 5**). It can be concluded that intervention measures focused on reducing the contamination of washing brines (processing step 2) could lead to an improvement of $PHSS_{FTOT}$ value to 67.03 %. On the contrary, contamination of fruits during washing and cracking could also lead to a reduction of $PHSS_{FTOT}$ values to 60.58%.

Regarding $PHSS_w$, in **Figure 5B**, physico-chemical values and contamination of brines and fruits in the processing step 5 (finished product) produced the widest variation of $PHSS_w$ values. However, as seen in **Table 5**, contamination of brines and fruits were relatively lower than in previous steps, being influenced by the addition of olive dressings as well as by product formulation. It should be remarked that corrective measures implemented during washing and cracking can be equally effective on the $PHSS_w$ (68.11%). It was also identified that cleaning of washing hoppers at processing step 2 could increase the final $PHSS_w$ value up to 68.16%.

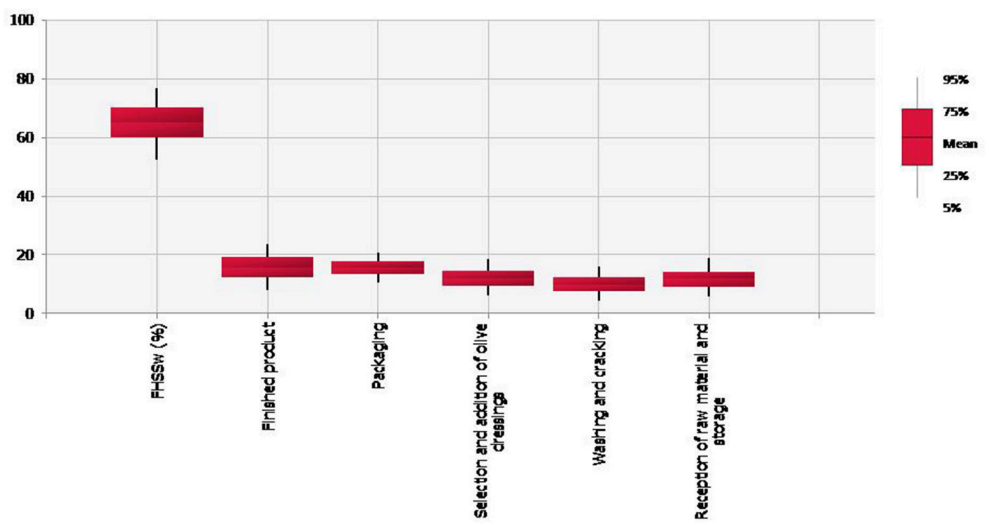

FIGURE 3 | Boxplot representing the mean, 5, 25, 75, and 95th values of the individual contribution of the processing steps on the weighted Performance Hygiene and Safety Score $PHSS_W$ (%).

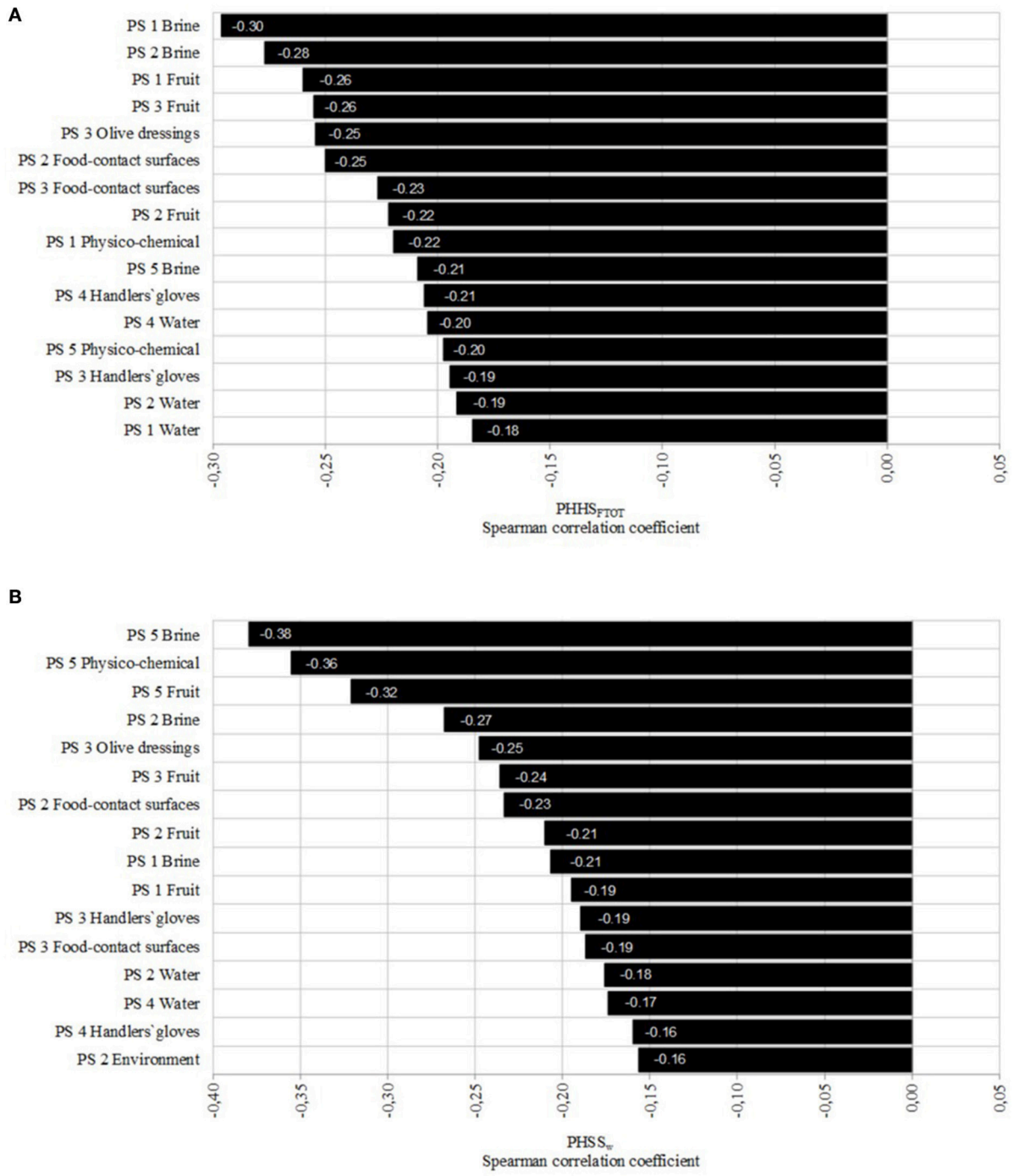

FIGURE 4 | Spearman correlation coefficients describing the relative influence of the type of sample on the final global Performance Hygiene and Safety Score ($PHSS_{FTOT}$) **(A)** and the weighted Performance Hygiene and Safety Score ($PHSS_w$) **(B)**. PS stands for the processing step.

In **Figure 6**, a direct correlation was found between simulated $PHSS_w$ values and relative contributions of each processing step. Simulated results showed that the proportion of directly correlated values was higher for steps 2 (66.4%) and 5 (70.1%). Packaging was identified as the step with lesser proportion of directly correlated values with $PHSS_w$ (57.4%).

To date, there are not probabilistic tools based on the application of FQSMS in the table olive sector. There are other tools in literature in which a systematic analysis of microbial counts was used to assess the degree of performance of a FQSMS (Jacxsens et al., 2009; Lahou et al., 2014). These approaches are based on a selection of critical sampling location, selection

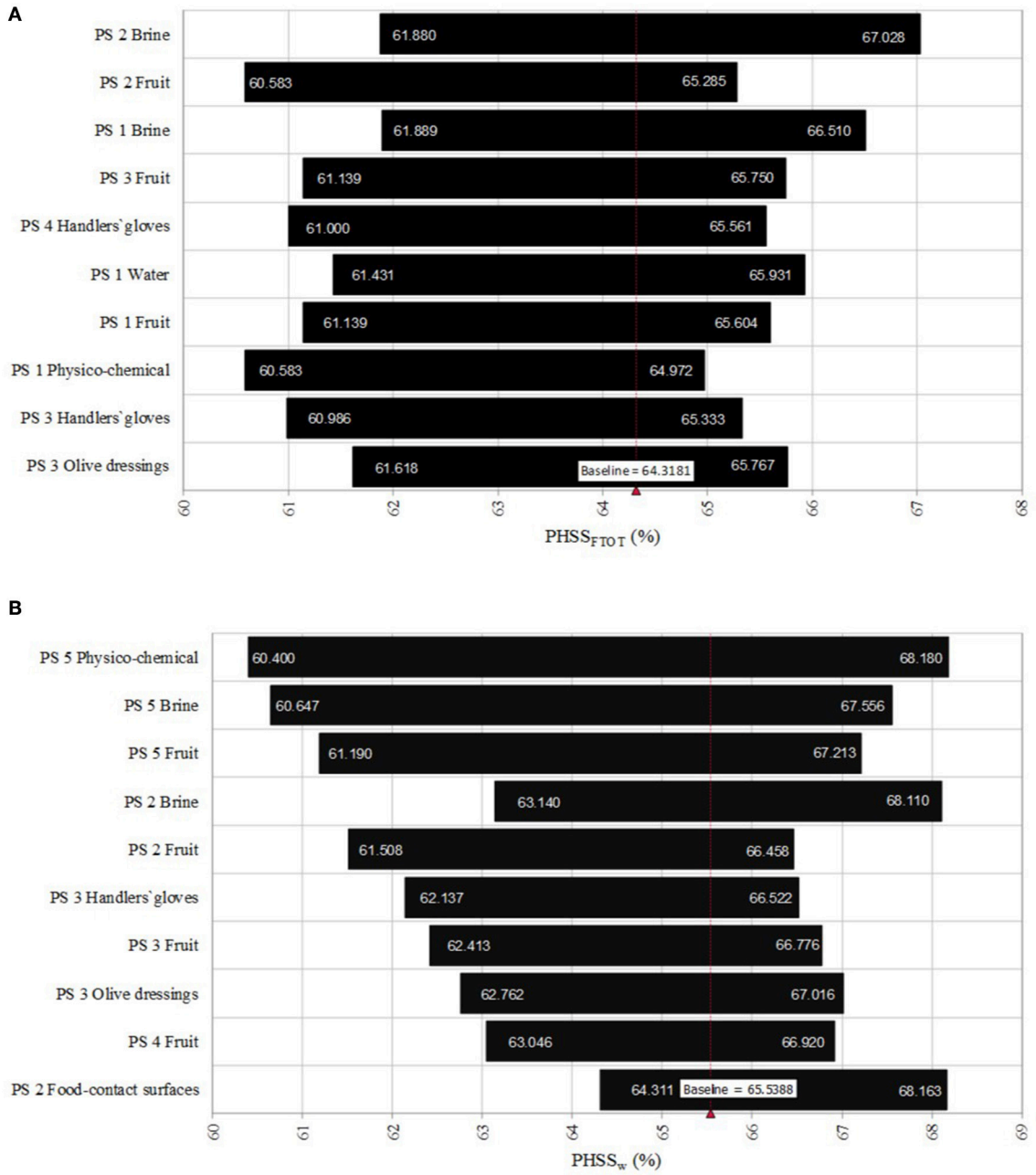

FIGURE 5 | Results of the sensitivity analysis describing the relative influence of the type of sample on the variation of the mean value for the global Performance Hygiene and Safety Score ($PHSS_{FTOT}$) **(A)** and the weighted Performance Hygiene and Safety Score ($PHSS_W$) **(B)**. PS stands for the processing step.

of microbial parameters, assessment of sampling frequency, selection of sampling and analytical methods and data processing and interpretation. Different microbial safety levels are assigned according to the compliance with legal criteria for both microbial hygiene and safety. The approach followed in the present study is in agreement with the principle behind Microbial Assessment Schemes (MAS) in which low concentration of microorganisms and small variability indicate an effective FQSMS (Sampers et al., 2010; Luning et al., 2011).

In conclusion, it is suggested that corrective measures should be focused on reducing the microbial contamination of brines and fruits at primary steps, together with the implementation of novel treatments on olive dressings (irradiation, scalding, ozonization, etc.) to reduce their microbial load since contamination can persist in brines and fruits during table olive processing. According to the suggested approach, these preventive measures can be equally or even more effective than modifying product formulation to

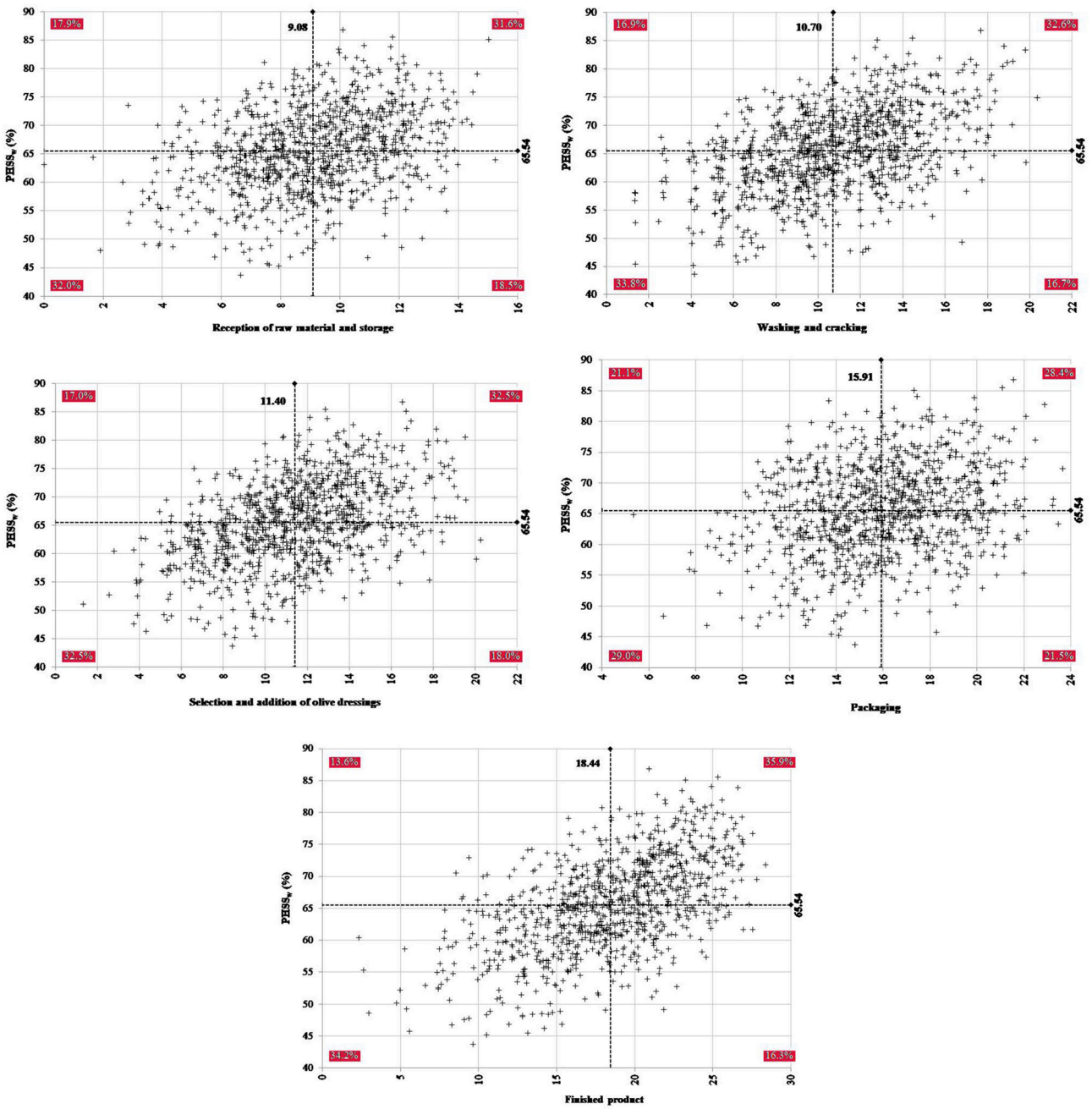

FIGURE 6 | Relationship between simulated PHSS$_W$ values and individual contributions of the evaluated processing steps.

lower pH values and higher salt concentrations. In addition, industry could reduce the levels of salt and preservatives in packaging producing a healthier product. The results presented are currently integrated within a software tool which will provide stakeholders with an easy-to-use, flexible and useful probabilistic decision-making scoring system for the *Aloreña de Málaga* table olive food sector. Furthermore, the approach can be extended to other olive varieties and elaboration methods including alternative treatments and steps as long as the information about scores weighing becomes available.

AUTHOR CONTRIBUTIONS

MR, FR-G, EM, VR, AP, and GP-I executed the experimental work and microbiological and physico-chemical analysis. FA-L, FR, RG-G, and AV planning the experiment and written the manuscript.

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REFERENCES

Al Dagal, M., Mo, O., Fung, D. Y. C., and Kastner, C. (1992). A case study of the influence of microbial quality of air on product shelf life in a meat processing plant. *Dairy Food Environ. Sanita* 12, 69–70.

Alves, M., Gonçalves, T., and Quintas, C. (2012). Microbial quality and yeast population dynamics in cracked green table olives' fermentations. *Food Control* 23, 363–368. doi: 10.1016/j.foodcont.2011.07.033

Arroyo-López, F. N. (2007). *Doctoral Thesis: Conservación y Envasado de Aceitunas de Mesa "Ali-adas" de la Variedad Manzanilla-Alore-a. Dise-o de Modelos Matemáticos Para el Crecimiento e Inhibición de Microorganismos*. Departamento de Cristalografía, Mineralogía y Química Agrícola. Universidad de Sevilla.

Arroyo-López, F. N., Bautista-Gallego, J., Romero-Gil, V., María Baquero, J. M., García-García, P., Jiménez-Díaz, R., et al. (2012). "Fermentation of olive fruit," in *Handbook of Plant-Based Fermented Food and Beverage Technology*, ed Y. H. Hui (Boca Ratón, FL: CRC Press, Taylor and Francis Group), 307–327.

Arroyo-López, F. N., García-García, P., Rodríguez-Gómez, F., and Garrido-Fernández, A. (2016). "Olives: types and consumption," in *The Encyclopaedia of Food and Health*, vol. 4, eds B. Caballero, P. Finglas, and F. Toldrá (Oxford: Academic Press), 167–170.

Bautista-Gallego, J., Arroyo-López, F. N., Durán-Quintana, M. C., and Garrido-Fernández, A. (2010). Fermentation profiles of Manzanilla-Alore-a cracked green table olives in different chloride salt mixtures. *Food Microbiol.* 27, 403–412. doi: 10.1016/j.fm.2009.11.015

Bautista-Gallego, J., Arroyo-López, F. N., Romero-Gil, V., Rodríguez-Gómez, F., García-García, P., and Garrido-Fernández, A. (2013). Microbial stability and quality of seasoned cracked green Alore-a table olives packed in diverse chloride salt mixtures. *J. Food Prot.* 76, 1923–1932. doi: 10.4315/0362-028X.JFP-12-504

Clemen, R. T., and Winkler, R. L. (1999). Combining probability distributions from experts in risk analysis. *Risk Anal.* 19, 187–203. doi: 10.1111/j.1539-6924.1999.tb00399.x

Codex Alimentarius Commission (1969a). *Code of Hygienic Practice for Canned Fruit and Vegetable Products*. CAC/RCP 2-1969.

Codex Alimentarius Commission (1969b). *General Principles of Food Hygiene*. CAC/RCP 1-1969 (Review 1997 and 2003).

Codex Alimentarius Commission (1979). *Code of Hygienic Practice for Low and Acidified Low Acid Canned Foods*. CAC/RCP 23-1979.

Codex Alimentarius Commission (1997). *Principles for the Establishment and Application of Microbiological Criteria for Foods*. CAC/GL 21–1997.

Codex Alimentarius Commission (1981). *Codex Standards for Table Olives*. CODEX STAN 66-1981 (Review 1987 and 2013).

Commission Regulation (2007). Commission Regulation (EC) No 1441/2007 of 5 December 2007 amending Regulation (EC) No 2073/2005 on microbiological criteria for foodstuffs. *Official J. Eur. Union* L322, 12–29. Available online at: http://data.europa.eu/eli/reg/2007/1441/oj

Commission Regulation (2002). Commission Regulation (EC) No 178/2002 of the European parliament and of the council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety. *Official J. Eur. Union* L31, 1–24. Available online at: http://data.europa.eu/eli/reg/2002/178/oj

Commission Regulation (2004). Commission Regulation (EC) No 852/2004 of the European parliament and of the council of 29 April 2004 on the hygiene of foodstuffs. *Official J. Eur. Union*, L226, 3–21. Available online at: http://data.europa.eu/eli/reg/2004/852/2009-04-20

Federation des Industries Condimentaires de France (2000). *Code des pratiques loyales pour les olives de table*.

Garrido-Fernandez, A., Fernandez-Dıez, M. J., and Adams, R. (1997). *Table Olives. Production and Processing*. London: Chapman and Hall.

Grover, A. K., Chopra, S., and Mosher, G. A. (2016). Food safety modernization act: a quality management approach to identify and prioritize factors affecting adoption of preventive controls among small food facilities. *Food Control* 66, 241–249. doi: 10.1016/j.foodcont.2016.02.001

IOC (2004). *Trade Standard Applying to Table Olives. COI/ NC n°1*. Madrid: International Olive Council.

IOOC (2017). *World Table Olive Figures*. International Olive Oil Council. Available online at: http://www.internationaloliveoil.org/estaticos/view/132-world-table-olive-figures (Accessed April 18, 2017).

ISO (2002). *ISO Microbiology of Food and Animal Feeding Stuffs – Horizontal Method for the Detection of Salmonella sp. ISO 6579: 2002*.

ISO (2017). *ISO Microbiology of Food and Animal Feeding Stuffs – Horizontal Method for the Detection and Enumeration of Listeria monocytogenes – Part 1: Detection Method. ISO 11290-1:1996/Amd1:2017*.

ISO (2004). *ISO Microbiology of Food and Animal Feeding Stuffs – Horizontal Method for the Detection and Enumeration of Listeria monocytogenes – Part 2: Enumeration Method. ISO 11290-2:1998/Amd1:2004*

ISO (1999). *ISO Microbiology of Food and Animal Feeding Stuffs – Horizontal Method for the Enumeration of Coagulase-positive Staphylococci (Staphylococcus aureus and Other Species) – Part 2: Technique Using Rabbit Plasma Fibrinogen Agar Medium*. ISO 6888-2:1999.

Jacxsens, L., Kussaga, J., Luning, P. A., Van der Spiegel, M., Devlieghere, F., and Uyttendaele, M. (2009). A microbial assessment scheme to measure microbial performance of Food Safety Management Systems. *Int. J. Food Microbiol.* 134, 113–125. doi: 10.1016/j.ijfoodmicro.2009.02.018

Lahou, E., Jacxsens, L., Van Landeghem, F., and Uyttendaele, M. (2014). Microbiological sampling plan based on risk classification to verify supplier selection and production of served meals in food service operation. *Food Microbiol.* 41, 60–75. doi: 10.1016/j.fm.2014.01.012

López-López, A., and Garrido-Fernández, A. (2006). *Producción, Elaboración, Composición y Valor Nutricional de la Aceituna Alore-a de Málaga*. Málaga: Redagua, S.L., Pizarra.

Loureiro, V., and Malfeito-Ferreira, M. (2003). Spoilage yeasts in the wine industry. *Int. J. Food Microbiol.* 86, 23–50. doi: 10.1016/S0168-1605(03)00246-0

Luning, P. A., Jacxsens, L., Rovira, J., Osés, S. M., Uyttendaele, M., and Marcelis, W. J. (2011). A concurrent diagnosis of microbiological food safety output and food safety management system performance: cases from meat processing industries. *Food Control* 22, 555–565. doi: 10.1016/j.foodcont.2010.10.003

Medina, E., Romero-Gil, V., Garrido-Fernández, A., and Arroyo-López, F. N. (2016). Survival of foodborne pathogens in natural cracked green olives. *Food Microbiol.* 59, 104–111. doi: 10.1016/j.fm.2016.05.017

Nanyunja, J., Jacxsens, L., Kirezieva, K., Kaaya, A. N., Uyttendaele, M., and Luning, P. A. (2015). Assessing the status of food safety management systems for fresh produce production in East Africa: evidence from certified green bean farms in Kenya and noncertified hot pepper farms in Uganda. *J. Food Prot.* 78, 1081–1089. doi: 10.4315/0362-028X.JFP-14-364

Ren, S., Ni, X., Xu, H., Den, J., Wang, Y., Sun, J., et al. (2010). The relationship between hand area and hand contamination. *Am. J. Infect. Control* 39, 66–68. doi: 10.1016/j.ajic.2010.06.014

Ren, Y., He, Z., and Luning, P. A. (2016). A systematic assessment of quality assurance-based food safety management system of Chinese edible oil manufacturer in view of context characteristics. *Total Qual. Man.* 27, 897–911. doi: 10.1080/14783363.2016.1187995

Romero-Gil, V., Rodríguez-Gómez, F., Garrido-Fernández, A., García-García, P., and Arroyo-López, F. N. (2016). *Lactobacillus pentosus* is the dominant species in spoilt packaged Alore-a de Málaga table olives. *LWT Food Sci. Technol.* 70, 252–260. doi: 10.1016/j.lwt.2016.02.058

Royal Decree (2001). *Royal Decree 1230/2001: Technical and Sanitary Regulation Concerning the. Processing, Circulation and Sale of Table Olives*.

Royal Decree (2003). *Royal Decree 140/2003, of 7th February, Approving the Sanitary Quality Criteria of Water for Human Consumption*.

Sampers, I., Jacxsens, L., Luning, P. A., Marcelis, W., Dumoulin, A., and Uyttendaele, M. (2010). Relation between Campylobacter contamination and performance of Food Safety Management Systems in poultry meat industries. *J. Food Protect*. 73, 1447–1457. doi: 10.4315/0362-028X-73.8.1447

Sánchez, A. H., De Castro, A., Rejano, L., and Montaño, A. (2000). Comparative study on chemical changes in olive juice and brine during green olive fermentation. *J. Agr. Food Chem*. 48, 5975–5980 doi: 10.1021/jf000563u

Sneed, J., Strohbehn, C., Gilmore, S. A., and Mendonça, A. (2004). Microbiological evaluation of foodservice contact surfaces in Iowa assisted-living facilities. *J. Am. Diet. Assoc*. 104, 1722–1724. doi: 10.1016/j.jada.2004.08.026

Stadlmüller, L., Matt, M., Peter Stüger, H., Komericki-Strimitzer, T., Jebousek, K., Luttenfeldner, M., et al. (2017). An operational hygiene inspection scoring system for Austrian high-risk companies producing food of animal origin. *Food Control* 77, 121–130. doi: 10.1016/j.foodcont.2017.01.019

Tzamalis, P., Panagiotakos, D., and Drosinos, E. (2016). A 'best practice score' for the assessment of food quality and safety management systems in fresh-cut produce sector. *Food Control* 63, 179–186. doi: 10.1016/j.foodcont.2015.11.011

Valero, A., Medina, E., and Arroyo-López, F. N. (2017). "Microbial hazards and their implications in the production of table olives," in *Foodborne Pathogens and Antibiotic Resistance*, ed O. V. Singh (Hoboken, NJ: Willey and Sons Inc.), 119–138.

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Survival of *Listeria monocytogenes* and *Staphylococcus aureus* in Synthetic Brines. Studying the Effects of Salt, Temperature and Sugar through the Approach of the Design of Experiments

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The fermentation of table olives relies on a complex microbiota of lactic acid bacteria (LAB), yeasts, and enterobacteria. Producers often add sugar to increase the growth rate of LAB, "but this practice could also increase the survival rate of some pathogens. Therefore, the main topic of this paper was to study the effect of sugar, salt and temperature on the survival of *Staphylococcus aureus* and *Listeria monocytogenes* in a synthetic brine through the theory of the Design of Experiments (simplex centroid). The addition of sugar could prolong the survival time of *L. monocytogenes* by 40 days, whereas an increase of the temperature caused a decrease of survival from 18 to 3 days. The survival time of *S. aureus* was prolonged by 50 days by combining sugar (2–4 g/l) and low temperatures (5–15°C). The use of desirability approach and prediction profiles suggests that the prolongation of the survival time of *L. monocytogenes* could be related to a shift in the geometrical shape of the death kinetic. This paper offers a structured statistical approach on the variables acting on the survival of two pathogens in brines and represents the first step to set up and design a predictive approach for olive producers.

Keywords: design of experiment, brine, primary model, predictive microbiology, pathogens

INTRODUCTION

The microbiota of table olives is composed by lactic acid bacteria (LAB), yeasts, enterobacteria, and some other minor groups (clostridia, propionibacteria, Micrococcaceae). Some authors reported that the high amount of salt and the low pH could assure the safety of the product (Medina et al., 2016); however, many researchers reported the occurrence of some pathogenic species, mainly acid-resistant strains (Centers for Disease Control Prevention, 1996, 1999; Medina-Pradas and Arroyo-López, 2015).

The fermentation of table olives is generally a homo-lactic fermentation and if the LAB prevail on the other microorganisms the pH is around 4.5 and the product is stable; however, in traditional fermentations, the process is uncontrolled, and olives might harbor undesirable microorganisms (Argyri et al., 2013).

Several pathogens could be found in olives, like *Listeria monocytogenes*, *Escherichia coli*, *Salmonella* sp., *Staphylococcus aureus*, and to a lesser extent *Clostridium botulinum* (Spyropoulou et al., 2001; Skandamis and Nychas, 2003; Caggia et al., 2004; Pereira et al., 2008; Argyri et al., 2013; Grounta et al., 2013; Medina et al., 2013; Panagou et al., 2013; Medina-Pradas and Arroyo-López, 2015; Tataridou and Kotzekidou, 2015). Panagou et al. (2013) suggested that the contamination of olives by pathogens might be due to poor hygiene, inadequate cleaning and sanitizing of equipment, and failure to washing before brining.

Modeling microorganisms both in food and in real systems is an iterative process, usually starting with a preliminary hypothesis, followed by a step when the initial conjecture needs to be programmed (design of experiments), and then tested (experiments) (van Boekel and Zwietering, 2007). The design of the experiments is a critical step, as it is generally not possible to correct a bad design in a later stage of data processing (van Boekel and Zwietering, 2007).

It is not possible to find a perfect design; however, if the goal of the research is to study the effects of multiple factors on bacterial growth/death curve, the DoE approach (Design of Experiments) could be appropriate.

Different kinds of DoE can be recovered in the literature (full, fractional, or mixture designs); in this paper, three factors were combined through a simple mixture design (simplex-lattice design). In a mixture design, the ratio of the components and their levels are dependent on each other (Flores et al., 2010). The results are generally reported in a simplex coordinate system, where each vertex is the pure blend or the combination where a factor is at the maximum level and the other two at their minimum values, and each of the sides represents a mixture of two components. The interior points in the triangle are mixtures of all ingredients (Myers and Montgomery, 2002). A mixture design offers a mathematical relationship between the factors of the design (input) and the studied parameters (dependent variables) through linear, quadratic or cubic coefficients (Dutcosky et al., 2006). The outputs are polynomial equations, bi-or three-dimensional plots, Pareto charts, desirability and prediction profiles; the main benefit is the reduced number of combinations.

The main goal of this paper was to study the effects of temperature and NaCl on the survival of two pathogens (*Listeria monocytogenes* and *Staphylococcus aureus*) in a synthetic brine. Moreover, sugar was used as an additional variable, as many times the producers of Southern Italy add it to increase the growth rate of LAB. Another additional goal was to assess how these factors could modify the shape of the death kinetic by inducing a shoulder or a tail effect.

MATERIALS AND METHODS

Strains

Listeria monocytogenes and *Staphylococcus aureus* were used in this research. The strains belong to the Culture Collection of the Laboratory of Predictive Microbiology, Dept. SAFE, University of Foggia. They are wild isolates found in foods; some preliminary experiments showed that they could survive on vegetables.

The bacteria were stored at −20°C in Nutrient broth (Oxoid, Milan, Italy), supplemented with 33% of sterile glycerol; before each assay, they were grown in Nutrient broth, incubated at 37°C for 24 h. The microorganisms were centrifuged at 3,000 g for 10 min and the pellet was suspended in a brine prepared with tap water and 4% NaCl. The viable count of these suspensions was 7 log cfu/ml.

Sample Preparation

The brines were prepared with tap water, salt (4.0–10.0%) and sugar (0–4 g/l), as reported in **Table 1**. Then, the brines were sterilized at 121°C for 15 min; after the sterilization, the pH was adjusted to 5.0 through HCl 1.0 N.

The brines were inoculated to 5 log cfu/ml with each strain separately and stored at the temperatures shown in **Table 1**. The concentrations of salt and sugar and the temperature varied according to a simplex centroid.

The viability of the strains was evaluated through the spread plate count three times per week (Nutrient Agar, incubated at 37°C for 24–48 h).

The design was repeated two times and each time the experiments were done on three independent batches ($n = 6$). The volume of each sample was 100 ml.

Primary Models

The results of the viable count were fitted through the equation of Weibull, cast in the form of Mafart et al. (2002):

$$\log N = \log N_0 - \left({}^{t}\!/_{FRT}\right)^{p}.$$

where log N is the count over the time t (log cfu/ml); $\log N_0$ the inoculum (log cfu/ml); FRT, the first reduction time (day), i.e., the time for a 1 log cfu/ml decrease of the bacterial population; p, the shape parameter ($p > 1$ downward curve; $p < 1$, upward curve).

The results were also fitted through the Weibull equation, modified by Bevilacqua et al. (2008a) for the evaluation of the survival time of pathogens:

$$\frac{\log N}{\log N_0} = 1 - \left({}^{t}\!/_{s.t.}\right)^{p}$$

Where *s.t.* is the survival time (days), i.e., the time after which the population is below the detection limit. The fitting was done

TABLE 1 | Simplex centroid.

Sample	Coded values			Values		
	NaCl	Sugar	Temp.	NaCl (%)	Sugar (%)	Temp. (°C)
A	1	0	0	10.0	0.0	5.0
B	0	1	0	4.0	4.0	5.0
C	0	0	1	4.0	0.0	25.0
D	0.5	0.5	0	7.0	2.0	5.0
E	0.5	0	0.5	7.0	0.0	15.0
F	0	0.5	0.5	4.0	2.0	25.0

through the software Statistica for Windows, ver. 12.0 (Statsoft, Tulsa, Okhla.). The goodness of fitting was evaluated through the coefficient R^2.

Secondary Models

FRT, p, and s.t. were used as dependent variables for a multiple regression analysis; salt, sugar, and temperature were the independent variables or categorical factors. The modeling was performed through the option DoE/mixture design of the software Statistica for Windows; salt, sugar, and temperature were used as independent variables and the fitting parameters of Weibull equation as dependent variables. The model was built by using the option "quadratic," for the evaluation of both individual ("salt," "sugar," and "temperature") and interactive effects ("salt*sugar," "salt*temperature," and "temperature*sugar").

The Most Important Output of the Modeling was a Polynomial Equation Reading as follows:

$$y = B_0 + \sum B_i x_i + \sum B_{ij} x_i x_j. \quad (1)$$

where, y, x_i, and x_j are respectively the dependent and the independent variables; B_i and B_{ij} are the coefficients of the model. This model assessed the effects of linear (x_i), and interactive terms ($\Sigma x_i x_j$) of the independent variables on the dependent variable.

The significance of the model was evaluated through the R^2 coefficient adjusted for a multiple regression and the residual mean square error (RMSE), as suggested by Chen and Zhu (2011) for not-linear death kinetics; the significance of each factor was assessed through the Fisher test ($P < 0.05$).

Prediction Profiles and Desirability Approach

The effect of each independent variable (salt, temperature, sugar) on the fitting parameters of the death kinetic of Weibull (p, FRT, and s.t.) was evaluated through the individual desirability functions, estimated as follows:

$$d = \begin{cases} 0, & y \le y_{min} \\ (y - y_{min})/(y_{max} - y_{min}) & y_{min} \le y \le y_{max} \\ 1 & y \le y_{max} \end{cases} \quad (2)$$

Where y_{min} and y_{max} are the minimum and maximum values of the dependent variable, respectively.

The desirability was included in the range 0–1 (0 for the lowest value of p, FRT, and s.t. and 1 for their maximal values). The desirability profiles were built by setting a variable to the coded level 1 (25°C for the temperature, 12% for NaCl, and 4% for sugar) and the other two to their minimum values (5°C for the temperature, 4.0% NaCl, and 0.0% sugar).

RESULTS

Listeria monocytogenes

The results of the viable count of *L. monocytogenes* in the model brines were fitted through the Weibull model (primary model); then, the first reduction time (FRT), the survival time and the shape parameter were used as dependent variables for a DoE approach (secondary model). The first output of a DoE is the table of the standardized effects, which shows the significance of both individual and interactive terms. The FRT of *L. monocytogenes* was significantly affected by the concentrations of salt and sugar and by their interactive term, but the most significant term was NaCl. The survival time was also affected by the interaction temperature/salt; the most significant term was the concentration of sugar (**Table 2**).

The equation could be used to build ternary plots; **Figure 1** shows the effects of the three independent variables on the survival time. The model predicted a survival of *L. monocytogenes* by 40 days or more when sugar was at the coded level 0.50–0.75 (2–3 g/l) and NaCl at 0.25–0.50 (5–7%); the quantitative effect of the temperature was slight.

A ternary plot is an important tool; however, it could not be used to analyze the quantitative effect of each individual term. Thus, the desirability approach was used to counteract this limit.

The desirability is a dimensionless parameter, ranging from 0 to 1 and is the answer to question: how much desired is an output? The reply is: 0 for the worst result and 1 for the best one. Moreover, a desirability profile is often completed by a prediction profile, which shows the predicted values of the dependent variable as a function of the coded values of the factors of the design.

TABLE 2 | Significance of the factors of the simplex centroid on the first reduction time (FRT, days) and on the survival time (s.t., days) of *L. monocytogenes* and *S. aureus* in model brines.

	L. monocytogenes		*S. aureus*	
	Standardized effect	***P*-value**	**Standardized effect**	**Standardized effect**
FRT				
NaCl	10.34	0.000	3.35	0.006
Temperature	–	–	–	–
Sugar	4.85	0.000	6.53	0.000
NaCl*Temp.	–	–	–	–
NaCl*Sugar	2.25	0.044	2.68	0.020
Sugar*Temp.	–	–	2.69	0.020
R^2_{ad}	0.741		0.744	
RMSE	0.427		0.909	
s.t.				
NaCl	6.23	0.000	10.30	0.000
Temperature	–	–	–	–
Sugar	8.55	0.000	4.67	0.001
NaCl*Temp.	2.59	0.024	2.54	0.026
NaCl*Sugar	–	–	4.55	0.001
Sugar*Temp.	–	–	–	–
$R^2_{ad.}$	0.733		0.761	
RMSE	86.715		335.108	

The significance was reported as standardized effects (coefficient of polynomial equation/standard error; absolute value) and P-values. R^2_{ad}, determination coefficient adjusted for a multiple regression. RMSE, residual mean square error.

Figure 2 shows the desirability (**Figures 2C,D**) and the prediction profiles (**Figures 2A,B**) for the effects of the temperature on the first reduction and on the survival time. The model predicted a negative correlation of the temperature with both parameters: an increase of the temperature from 5 to 25°C caused a decrease of the desirability along with decrease of the actual values (from 4 to 0.4 days the first reduction time, and from 18.32 to 2.61 days the survival time) (**Figures 2A,B**).

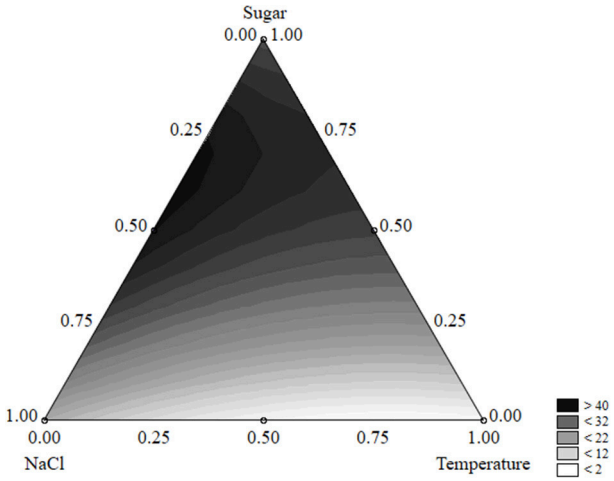

FIGURE 1 | Ternary plot for the effects of sugar, salt, and temperature on the survival time of *L. monocytogenes* (days); each side shows the coded values of the factors (from 0 to 1).

Figure 3 shows the prediction/desirability of the survival time as a function of sugar. The correlation sugar/survival time was not strictly linear. In fact, an increase of the concentration of sugar determined an increase of desirability (**Figure 3B**) and of the actual values up to 40.71 days in presence of 3 g/l of sugar (**Figure 3A**); however, a further increase negatively acted on the survival time.

Finally, **Figure 4** shows the prediction profiles for the effect of salt on the survival time; for this factor, the result was unexpected, as an increase of salt did not negatively act on the survival time. On the other hand, at the highest salt concentration the survival was increased (the predicted value was 18.31 days).

Staphylococcus aureus

The first reduction time of *S. aureus* was affected by the individual terms of sugar and NaCl, and by the interactions sugar/temperature and salt/sugar. The survival time was influenced by the individual terms of salt and sugar and by the interactions salt/temperature and salt/sugar.

Figure 5 shows the ternary plot for the survival time. The survival of the test organism was maximum for the coded level

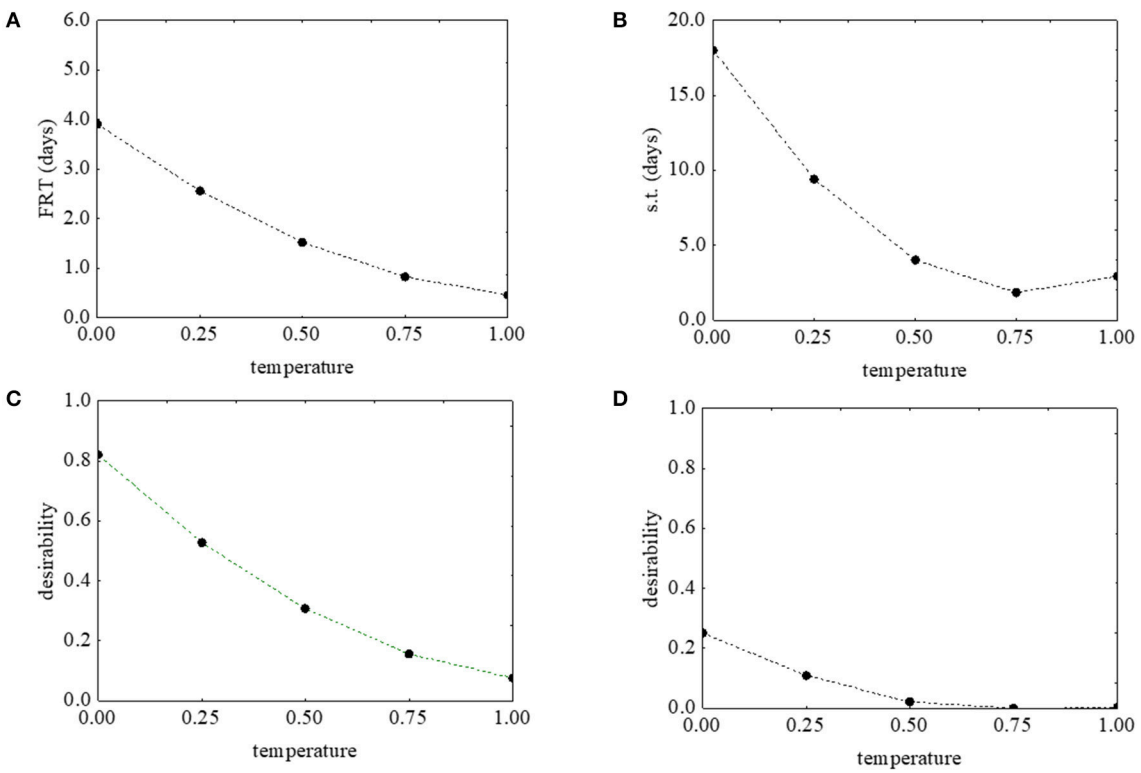

FIGURE 2 | Prediction (**A,B**) and desirability profiles (**C,D**) for the effect of the temperature on the first reduction time (FRT) and on the survival time (s.t.) of *L. monocytogenes*. The x-axis shows the coded values of the factor (0, temperature at 5°C; 1, temperature at 25°C).

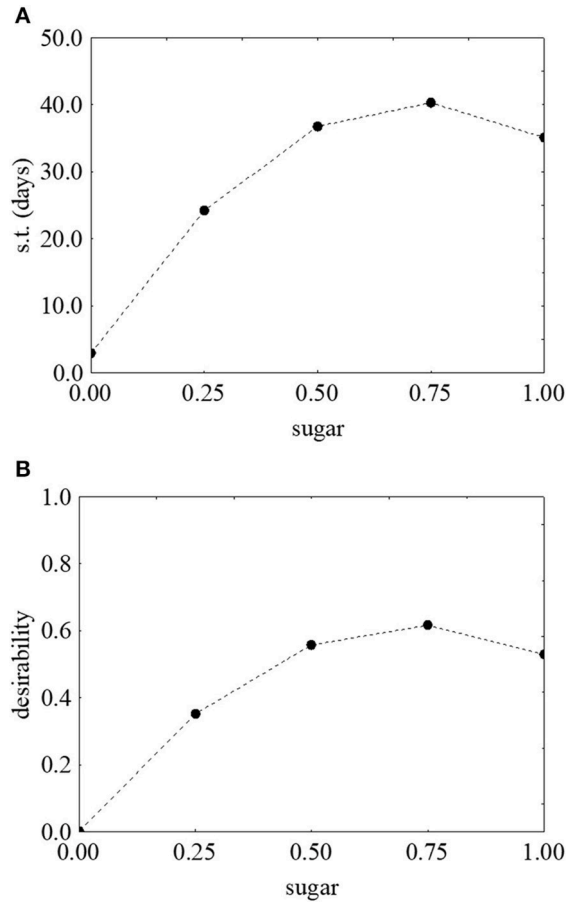

FIGURE 3 | Prediction **(A)** and desirability profiles **(B)** for the effect of sugar on the survival time (s.t.) of *L. monocytogenes*. The x-axis shows the coded values of the factor (0, sugar at 0.0%; 1, sugar at 4.0%).

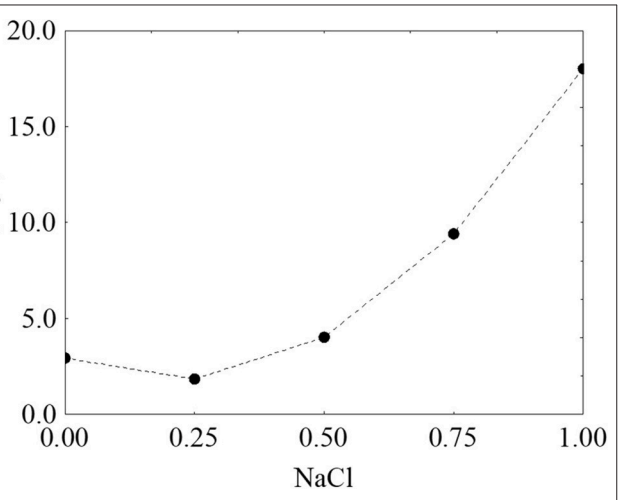

FIGURE 4 | Prediction profile for the effect of NaCl on the survival time (s.t.) of *L. monocytogenes*. The x-axis shows the coded values of the factor (0, NaCl at 4.0%; 1, NaCl at 10%).

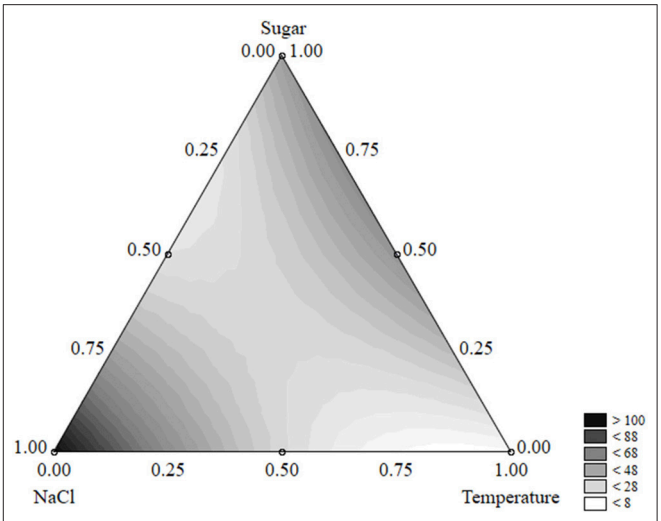

FIGURE 5 | Ternary plot for the effects of sugar, salt, and temperature on the survival time of *S. aureus* (days); each side shows the coded values of the factors (from 0 to 1).

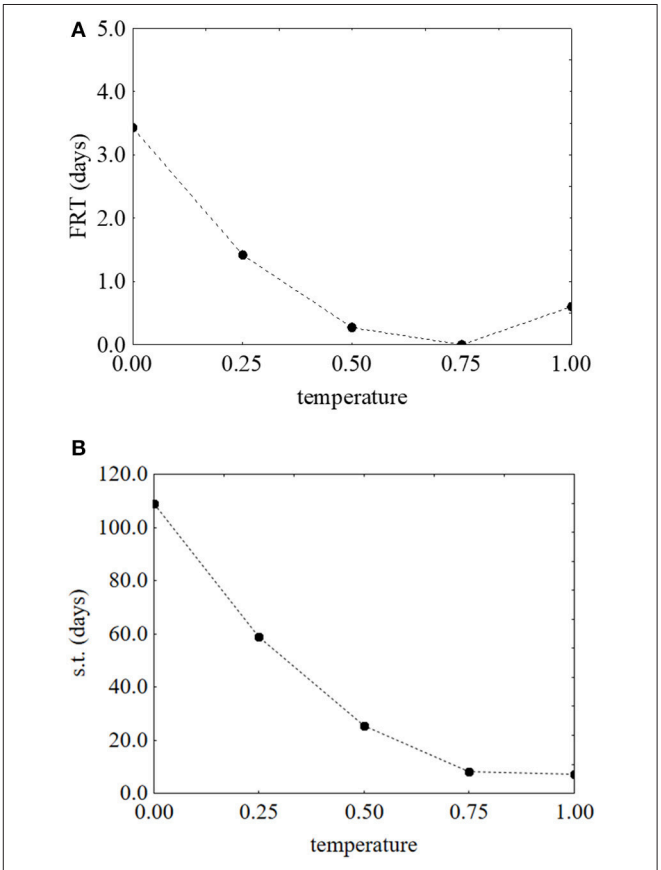

FIGURE 6 | Prediction profiles for the effect of the temperature on the first reduction time (FRT) **(A)** and on the survival time (s.t.) **(B)** of *S. aureus*. The x-axis shows the coded values of the factor (0, temperature at 5°C; 1, temperature at 25°C).

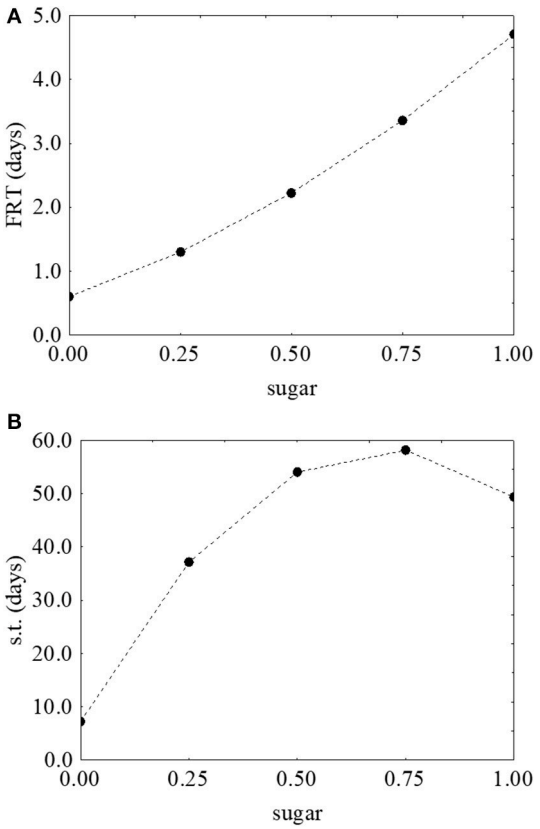

FIGURE 7 | Prediction profiles for the effect of sugar on the first reduction time (FRT) **(A)** and on the survival time (s.t.) **(B)** of *S. aureus*. The x-axis shows the coded values of the factor (0, sugar at 0.0%; 1, sugar at 4.0%).

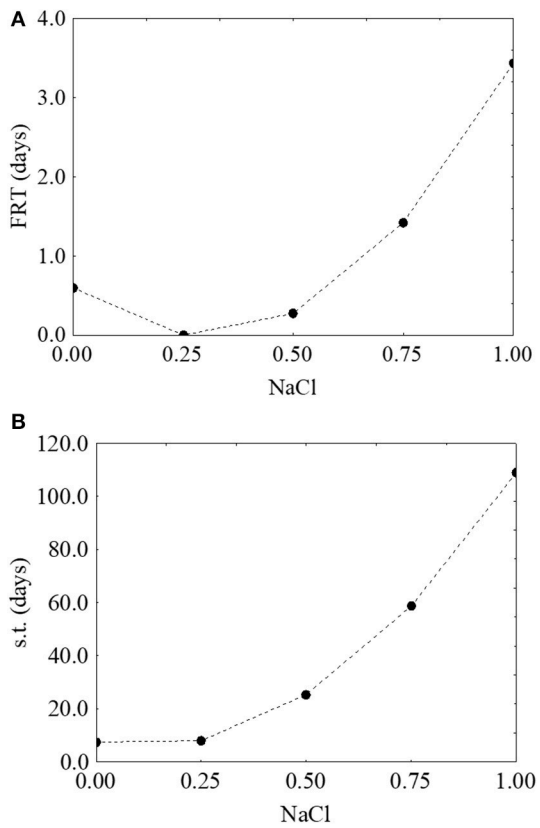

FIGURE 8 | Prediction profiles for the effect of NaCl on the first reduction time (FRT) **(A)** and on the survival time (s.t.) **(B)** of *S. aureus*. The x-axis shows the coded values of the factor (0, NaCl at 4.0%; 1, NaCl at 10%).

"1" of salt (12% NaCl) (>100 days); in addition, the combination of sugar (coded levels 0.5–1, i.e., 2–4 g/l) and low-to-medium temperatures (5–15°C, corresponding to the coded levels 0–0.5) exerted a positive effect on the survival time which was prolonged to 50 days.

As reported for *L. monocytogenes*, the desirability and prediction profiles were also built. The effect of temperature on the first reduction and on the survival time is in **Figure 6**. An increase of the temperature caused a strong decrease of both parameters: the first reduction time from 3.43 to 0.67 days (**Figure 6A**) and the survival time from 109 to 4 days (**Figure 6B**). Sugar increased both the first reduction time (from 0.47 to 4.71 days) (**Figure 7A**) and the survival time (from 5.3 to 59.5 days) (**Figure 7B**).

Finally, the effect of salt was unexpected, as the model suggested a positive rather than a negative effect, with an increase of both the first reduction and survival time as a function of an increase of the concentration of salt (from 0.6 to 3.2 days-**Figure 8A**- and from 7.3 to 112 days-**Figure 8B**, respectively).

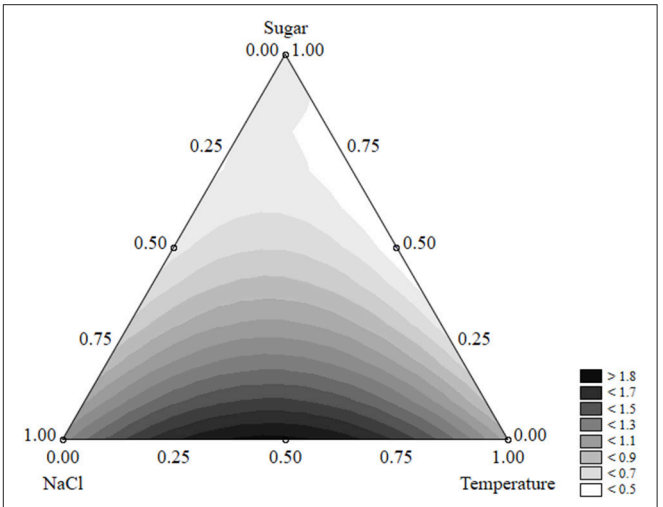

FIGURE 9 | Ternary plot for the effects of sugar, salt, and temperature on the shape parameter of *Listeria monocytogenes*; each side shows the coded values of the factors (from 0 to 1).

Shape of the Death Curves

The effects of the factors of the design on the shape parameter of *L. monocytogenes* are presented in **Figure 9**. p was maximum (>1.8) when salt and temperature were both at the coded value 0.5 (NaCl 7% and temperature 15°C). On the other hand, the concentration of sugar exerted a negative effect and caused

a decrease of p. The approach of desirability and prediction profiles was also used to pinpoint the individual effect of the factors. The shape parameter increased from 1.15 to 1.8 because of a temperature rise from 5 to 15°C; then a further increase of the temperature caused a decrease of p (**Figure 10A**). A similar effect was found for salt, and p was maximum (1.84), when salt was at 7% (coded level 0.5) (**Figure 10B**). Finally, the prediction profiles confirmed the negative effect of sugar (**Figure 10C**).

The effect of the factors of the design on the shape parameter of *S. aureus* was less significant; salt, sugar, and temperature acted on p and caused increases or decreases, but the parameter was always <1 and the shape of the death kinetic was not affected (**Figure 11**).

DISCUSSION

Olives and other fermented vegetables are consumed worldwide (Medina et al., 2013); they do not represent a favorable environment for pathogens (Grounta et al., 2013). However, pathogens were found in olives sold in street markets (Caggia et al., 2004; Franzetti et al., 2011; Romeo et al., 2012). In this paper, *S. aureus* and *L. monoytogenes* were used as model organisms for a preliminary challenge test, aimed at investigating the effect of some factors on their survival in a synthetic brine.

In Southern Italy the fermentation of table olives takes place from the end of September to December, with temperature ranging from 10 to 20–25°C; moreover, salt is in the range 6–12% (Bevilacqua et al., 2010; Perricone et al., 2010). Another practice of Italian producers is the addition of small amounts of sugar to increase the rate of acidification by LAB (Perricone et al., 2010; Bevilacqua, unpublished results). Another technological parameter that can affect the survival of pathogens is pH; in Southern Italy, the fermentation performed by small scale producers results in a final pH around 4.9–5.0. This pH is unusual for fermented foods, as they generally have a pH of 4.6 or lower; however, an incomplete fermentation is a challenge in Southern Italy and a common problem in Apulian Region (Bevilacqua et al., 2008b, 2010). Therefore, this pH was chosen as the pH of the synthetic brine in order to assess the actual risk of pathogen survival for producers which do not use starter cultures.

The effect of temperature on the survival of both pathogens confirms the data of some other authors: the survival in harsh conditions could be enhanced by refrigeration, as suggested by Medina et al. (2013) and Breidt and Caldwell (2011) for table olives, and acidified cucumbers, respectively. The implication of this result could be strong, because it suggests that if a contamination occur throughout or immediately after the fermentation, the pathogen could survive for a long time, because olive containers are generally stored at room temperature in winter before packaging (at least 3–4 months). Then, they are packed and pasteurized at industrial level, but olives for retail markets are not thermally processed.

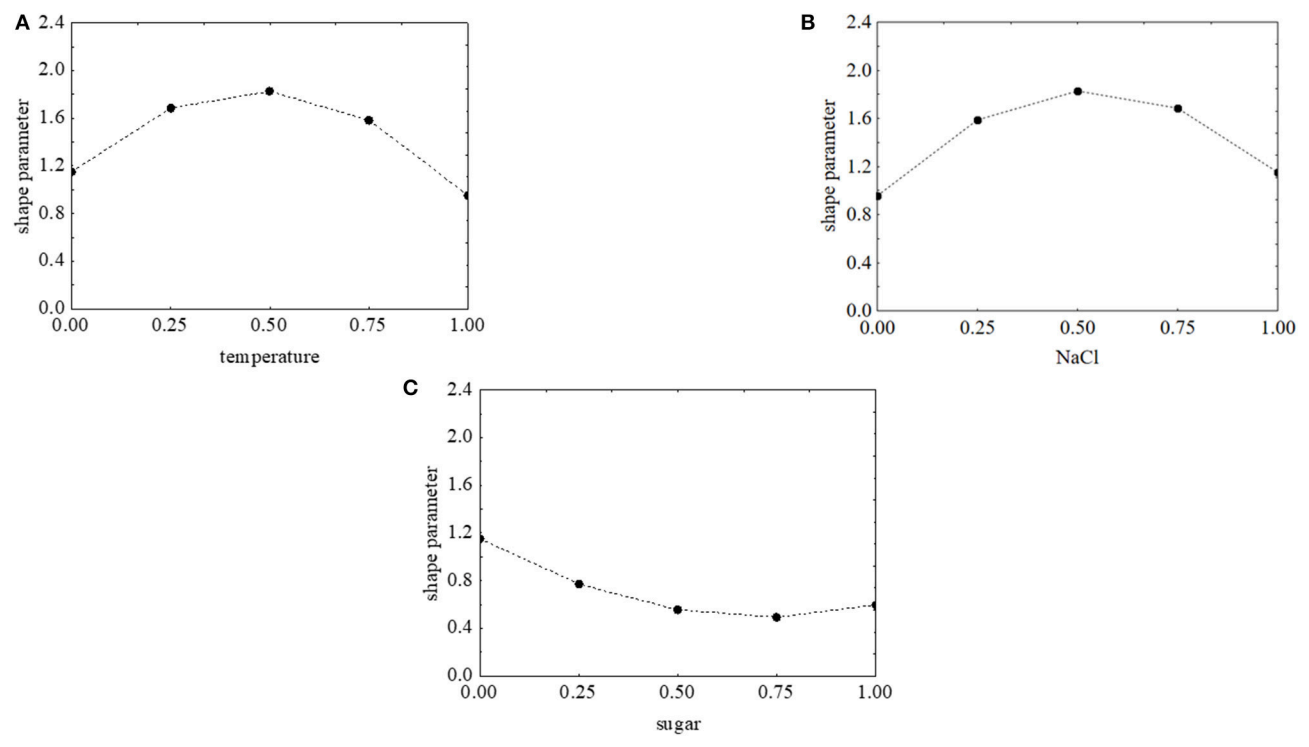

FIGURE 10 | Prediction profiles for the effect of temperature (0, temperature at 5°C; 1, temperature at 25°C) **(A)**, NaCl (0, NaCl at 4.0%; 1, NaCl at 10%) **(B)**, and sugar (0, sugar at 0.0%; 1, sugar at 4.0%) **(C)** on the shape parameter of *L. onocytogenes*. The x-axis shows the coded values of the factor.

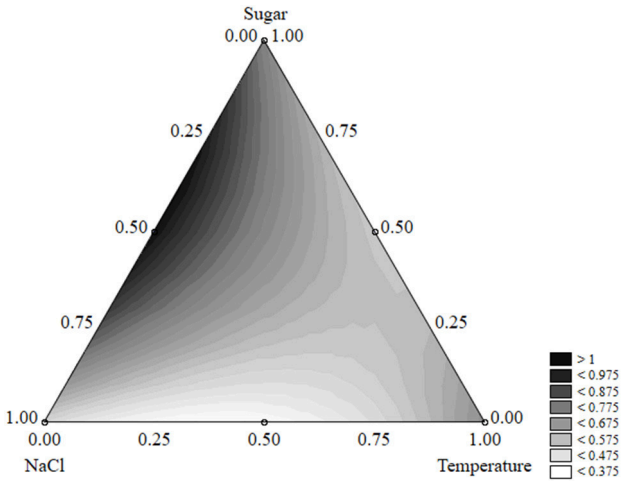

FIGURE 11 | Ternary plot for the effects of sugar, salt, and temperature on the shape parameter of *S. aureus*; each axis shows the coded values of the factors (from 0 to 1).

Sugar enhanced the survival of *L. monoctogenes* and *S. aureus*, although its effect was not strictly linear, as it could interact with the other factors; the same effect was found for salt and this result was unexpected. Few data are available on this topic, but McKellar et al. (2002) studied the survival of *Escherichia coli* STEC in model media, which simulated the aqueous phase of acidic sauces, and reported an apparent increased survival in presence of salt and sugar. Chapman et al. (2006) confirmed this effect on *Escherichia coli* O157 in model acidic sauces. They found a protective effect on the shoulder phase before the inactivation and postulated that this effect could be mediated by the coupling of Na^+ import to H^+ export, thus permitting *E. coli* to maintain the internal pH and allowing its survival (Casey and Condon, 2002).

Concerning the positive effect of sugar, Casey and Condon (2002) suggested that at low water activities, water could be lost from the cytoplasm, resulting in a decrease in cell volume that might effectively concentrate the cytoplasmic constituents and thereby raise the internal pH of the cell.

In the second step of this research, the effects of the factors of centroid on the shape parameter (p) were assessed. When the shape parameter is 1, the death curve is a line and follow the first-order kinetic of Bigelow; on the other hand, when $p < 1$ the death kinetic is an upward curve and for $p > 1$ there is a downward kinetic. Although it does not possess a biological meaning, the shape parameter can be related to the shoulder length (cell count does not undergo significant changes for some days; $p > 1$) and to the tail effect ($p < 1$, residual population at the end of the experiment) (Bevilacqua et al., 2016). The shape of the death kinetics was less affected in *S. aureus*, which always experienced a linear-to-downward trend with a tail effect, probably due to the strong resistance of this strain to the harsh conditions of the brines (salt, low temperatures, etc.,).

In *L. monocytogenes*, the prediction profiles could give some interesting perspectives on the increased survival in presence of salt and sugar, because the lowering of p suggested a possible tail effect. The reasons cannot be elucidated with the data of this paper and further experiments are required to confirm them.

In conclusion, this paper offers a structured statistical approach on the variables acting on the survival of pathogens in brines and represents the first step to set up and design a predictive approach for olive producers. Concerning the addition of sugar in brine, this practice could be a challenge as it could increase the survival of some pathogens and salt could not be able to counteract this effect. Some evidences suggested that the increased survival of *L. monocytogenes* could be the result of a shift of the death curve to an upward kinetic; however, further experiments are required to build a robust mathematical approach on this topic and try to elucidate the molecular mechanisms beyond it.

AUTHOR CONTRIBUTIONS

MC, MS, and AB conceived the study. AB and MC designed the experiments. BS and DC performed the experiments. AB, MS, and MC interpreted the results and modeling. All authors wrote and approved the manuscript. MC funded the research.

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REFERENCES

Argyri, A. A., Lyra, E., Panagou, E. Z., and Tassou, C. C. (2013). Fate of *Escherichia coli* O157:H7, *Salmonella enteritidis* and *Listeria monocytogenes* during storage of fermented green table olives in brine. *Food Microbiol.* 36, 1–6. doi: 10.1016/j.fm.2013.04.001

Bevilacqua, A., Cannarsi, M., Gallo, M., Sinigaglia, M., and Corbo, M. R. (2010). Characterization and implications of *Enterobacter cloacae* strains, isolated from Italian table olives "Bella di Cerignola." *J. Food Sci.* 75, M53–M60. doi: 10.1111/j.1750-3841.2009.01445.x

Bevilacqua, A., Cibelli, F., Cardillo, D., Altieri, C., and Sinigaglia, M. (2008a). Metabolic effects of *Fusarium* spp. on *Escherichia coli* O157:H7 and *Listeria monocytogenes* on raw portioned tomatoes. *J. Food Prot.* 71, 1366–1371. doi: 10.4315/0362-028X-71.7.1366

Bevilacqua, A., Corbo, M. R., Sinigaglia, M., Speranza, B., Campaniello, D., and Altieri, C. (2016). Effects of inulin, fructooligosaccharides/glucose, and pH on the shape of the death kinetic of *Lactobacillus reuteri* DSM 20016. *Int. J. Food Sci. Technol.* 51, 2251–2259. doi: 10.1111/ijfs.13205

Bevilacqua, A., Gallo, M., Cannarsi, M., Corbo, M. R., Sinigaglia, M., and Jaschke-Ferreri, A. (2008b). Preliminary evolution of microbiology quality of "Bella di cerignola" table olives: influence of a not correct storage. *Industrie Alimentari* 47, 612–619.

Breidt, F., and Caldwell, J. M. (2011). Survival of *Escherichia coli* O157:H7 in cucumber fermentation brines. *J. Food Sci.* 76, M198–M203. doi: 10.1111/j.1750-3841.2011.02045.x

Caggia, C., Randazzo, C. L., Salvo, M., Romeo, F., and Giudici, P. (2004). Occurrence of *Listeria monocytogenes* in green table olives. *J. Food Prot.* 10, 2189–2194. doi: 10.4315/0362-028X-67.10.2189

Casey, P. G., and Condon, S. (2002). Sodium chloride decreases the bacteriocidal effect of acid pH on *Escherichia coli* O157:H45. *Int. J. Food Microbiol.* 76, 199–206. doi: 10.1016/S0168-1605(02)00018-1

Centers for Disease Control and Prevention (1996). Outbreak of *Escherichia coli* O157:H7 infections associated with drinking unpasteurized commercial apple juice-British Columbia, California, Colorado, and Washington, October 1996. MMWR Morb. Mortal. *Wkly Rep*. 45:975.

Centers for Disease Control and Prevention (1999). Outbreak of *Salmonella* serotype muenchen infections associated with unpasteurized orange juice-United States and Canada, June 1999. MMWR Morb. *Mortal Wkly Rep*. 48, 582–585.

Chapman, B., Jensen, N., Ross, T., and Cole, M. (2006). Salt, alone or in combination with glucose, can improve the survival of *Escherichia coli* O167 (SRL2) in model acidic sauces. *Appl. Environ. Microbiol*. 72, 5165–5172. doi: 10.1128/AEM.02522-05

Chen, Z., and Zhu, C. (2011). Modelling inactivation by aqueous chlorine dioxide of *Dothiorella gregaria* Sacc. and Fusarium tricinctum (Corda) Sacc. spores inoculated on fresh chestnut kernel. *Lett. Appl. Microbiol*. 52, 676–684. doi: 10.1111/j.1472-765X.2011.03061.x

Dutcosky, S. D., Grossmann, M. V. E., Silva, R. S. S., and Welsch, A. K. (2006). Combined sensory optimization of a prebiotic cereal product using multi-component mixture experiments. *Food Chem*. 98, 630–638. doi: 10.1016/j.foodchem.2005.06.029

Flores, S. K., Costa, D., Yamashita, F., Gerschenson, L. N., and Grossmann, M. V. (2010). Mixture design for evaluation of potassium sorbate and xanthan gum effect on properties of tapioca starch films obtained by extrusion. *Mat. Sci. Eng.* C30, 196–202. doi: 10.1016/j.msec.2009.10.001

Franzetti, L., Scarpellini, M., Vecchio, A., and Planeta, D. (2011). Microbiological and safety evaluation of green table olives marketed in Italy. *Ann. Microbiol*. 61, 843–851. doi: 10.1007/s13213-011-0205-x

Grounta, A., Nychas, G. J. E., and Panagou, E. Z. (2013). Survival of food-borne pathogens on natural black table olives after post-processing contamination. *Int. J. Food Microbiol*. 161, 197–202. doi: 10.1016/j.ijfoodmicro.2012.12.017

Mafart, P., Couvert, O., Gaillard, S., and Leguerinel, I. (2002). On calculating sterility in thermal preservation methods: application of the Weibull frequency distribution model. *Int. J. Food Microbiol*. 72, 107–113. doi: 10.1016/S0168-1605(01)00624-9

McKellar, R. C., Lu, X., and Delaquis, P. J. (2002). A probability model describing the interface between survival and death of *Escherichia coli* O157:H7 in a mayonnaise model system. *Food Microbiol*. 19, 235–247. doi: 10.1006/fmic.2001.0449

Medina, E., Brenes, M., Romero, C., Ramirez, E., and de Castro, A. (2013). Survival of foodborne pathogenic bacteria in table olive brines. *Food Control* 34, 719–724. doi: 10.1016/j.foodcont.2013.06.026

Medina, E., Romero-Gil, V., Garrido-Fernández, A., and Arroyo-López, F. N. (2016). Survival of foodborne pathogens in natural cracked olive brines. *Food Microbiol*. 59, 104–111. doi: 10.1016/j.fm.2016.05.017

Medina-Pradas, E., and Arroyo-López, F. N. (2015). Presence of toxic microbial metabolites in table olives. *Front. Microbiol*. 6:873. doi: 10.3389/fmicb.2015.00873

Myers, R. H., and Montgomery, D. C. (2002). *Response Surface Methodology, 2nd Edn.,* New York, NY: John Wiley & Sons, Inc.

Panagou, E. Z., Nychas, G. J. E., and Sofos, J. N. (2013). Types of traditional Greek foods and their safety. *Food Control*. 29, 32–41. doi: 10.1016/j.foodcont.2012.05.050

Pereira, A. P., Pereira, J. A., Bento, A., and Estevinho, M. L. (2008). Microbiological characterization of table olives commercialized in Portugal in respect to safety aspects. *Food Chem. Toxicol*. 4, 2895–2902. doi: 10.1016/j.fct.2008.05.033

Perricone, M., Bevilacqua, A., Corbo, M. R., and Sinigaglia, M. (2010). Use of *Lactobacillus plantarum* and glucose to control the fermentation of "Bella di Cerignola" table olives, a traditional variety of Apulian region (Southern Italy). *J. Food Sci*. 75, M430–M436. doi: 10.1111/j.1750-3841.2010.01742.x

Romeo, F. V., Piscopo, A., Mincione, A., and Poiana, M. (2012). Quality evaluation of different typical table olive preparations (cv Nocellara del Belice). *Grasas y Aceites* 63, 19–25. doi: 10.3989/gya.058511

Skandamis, P. N., and Nychas, G. J. E. (2003). Modeling the microbial interaction and the death of *Escherichia coli* O157:H7 during the fermentation of Spanish style green table olives. *J. Food Prot*. 66, 1166–1175. doi: 10.4315/0362-028X-66.7.1166

Spyropoulou, K. E., Chorianopoulos, N. G., Skandamis, P. N., and Nychas, G. -J. E. (2001). Survival of *Escherichia coli* O157:H7 during the fermentation of Spanish-style green table olives (conservolea variety) supplemented with different carbon sources. *Int. J. Food Microbiol*. 66, 3–11. doi: 10.1016/S0168-1605(00)00510-9

Tataridou, M., and Kotzekidou, P. (2015). Fermentation of table olives by oleuropeinolytic starter culture in reduced salt brines and inactivation of *Escherichia coli* O157:H7 and *Listeria monocytogenes*. *Int. J. Food Microbiol*. 208, 122–130. doi: 10.1016/j.ijfoodmicro.2015.06.001

van Boekel, M. A. J. S., and Zwietering, M. H. (2007). "Experimental design, data processing and model fitting in predictive microbiology" in *Modelling Microorganisms in Food*, eds, S. Brul, S. Van Gerwen, and M. H. Zwietering (New York, NY: Woodhead Publishing), 22–43.

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Lactobacillus plantarum 5BG Survives During Refrigerated Storage Bio-Preserving Packaged Spanish-Style Table Olives (cv. Bella di Cerignola)

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This paper proposes bio-preservation as a tool to assure quality and safety of Spanish-style table olives cv. Bella di Cerignola. *Lactobacillus plantarum* 5BG was inoculated in ready to sell olives packaged in an industrial plant by using a half-volume brine (4% NaCl; 2% sucrose). The samples were stored at 4°C. The survival of the inoculated strain, the microbiological quality, the sensory scores and the survival of a strain of *Listeria monocytogenes* inoculated in brines were evaluated. The persistence of the *Lb. plantarum* bio-preserving culture was confirmed on olives (≥6.5 Log CFU/g) and in brine (≥7 Log CFU/ml). Bio-preserved olives (SET1) showed a better sensory profile than chemically acidified control olives (SET2) and the texture was the real discriminative parameter among samples. Bio-preserved olives recorded better scores during storage because of their ability to retain good hardness, crunchiness, and fibrousness without cracks. The inoculation of *Lb. plantarum* positively acted on the safety of olives, as the *D*-value of *L. monocytogenes* was reduced from 40 (SET2) to 5 days (SET1). In conclusion, *Lb. plantarum* 5BG and the physico-chemical conditions achieved in the settled procedure are suitable for the industrial packaging of Bella di Cerignola table olives, improving the process by halving brining volumes and avoiding chemical stabilizers, and significantly reducing the salt concentration. The final product is also safely preserved for almost 5 months as suggested by the reduction of the survival rate of *L. monocytogenes*.

Keywords: olives, Bella di Cerignola, bio-preservation, quality, safety

INTRODUCTION

Bio-preservation, defined as the extension of shelf life and food safety by the use of microbial cultures, has been used to ensure high sensory and functional quality of several products i.e., dairy, meat, and vegetables including fermented olives (Ananou et al., 2007; Liu et al., 2008; Trias et al., 2008; De Bellis et al., 2010).

According to the most well-known "Spanish-style" processing method, Spanish-style table olives cv. Bella di Cerignola are debittered and brined in 12–13% NaCl where they undergo spontaneous fermentation by a mixed microbiota mainly composed by lactic acid bacteria (LAB) and yeasts. At the end of the process, fermentation is finished and pH decreases to <4.0 ensuring the preservation of the product. Olives may be stored in bulk in brine with NaCl∼10% and pH <4.0 or, after washing of fruits with water, packaged in small glass containers covered with fresh acidified brine and pasteurized to avoid undesirable fermentation that may affect the quality and safety of the product (Garrido-Fernández et al., 1997). Recently, some packaging solutions as plastic containers or tins are increasingly adopted instead of glass jars to prevent product alteration and to facilitate product managing and logistic organization (Sánchez et al., 2017). In the case of packaging in plastic pouches, the product is stabilized by adding chemical acidifiers and antioxidants with an expiry date no longer than 1 year, while refrigerated storage is recommended after package opening if product is not consumed in a single seating.

Table olives should maintain specific sensory standards as established by the Codex Alimentarius (CODEX/COI, 2013). Particularly, high flesh to stone ratio and a uniform size are the most apparent sensorial attributes. In addition, a firm texture and a color characteristic of the variety (that is green/strawy green for green olives) without white spots or color browning with an equilibrate taste are essential and deeply related to the microbial quality.

To meet consumers and producers demand for alternatives to chemical additives, the ability of a selected *Lactobacillus plantarum* in bio-preserving Spanish-style olives cv. Bella di Cerignola was evaluated in an industrial plant in retail packages. Bioprotective LAB cultures and/or their antimicrobial compounds have already been used to extend shelf life and ensure food safety (Leverentz et al., 2006). The strains used for bio-preservation belong to *Lactobacillus* species and they could generally exert a bacteriostatic or a bactericidal effect, because of the production of bacteriocins and other antimicrobial compounds and/or the competition for nutrients (Gálvez et al., 2010; Bevilacqua et al., 2015).

Particularly, Spanish-style olives fermented with *Lactobacillus pentosus* or *Lb. plantarum* cultures have been stored, using polyethylene (PE) pouches, in modified atmosphere (Argyri et al., 2015) or in brine (Blana et al., 2016). However, in these studies, LAB were applied as starter cultures at the onset of fermentation.

During storage, several pathogens could be found in olives and among them, *Listeria monocytogenes* has been frequently isolated (Caggia et al., 2004; Argyri et al., 2013; Panagou et al., 2013).

In particular, *L. monocytogenes* could survive in brines, as the combinations of pH, salt, and some additives used by producers could enhance its survival for months (Bevilacqua et al., 2018). The exposure of *L. monocytogenes* to a nonlethal pH (4.4÷5.8) for some hours, as may occur in fermented foods, can result in a higher pathogen survival that is of particular concern in products with an extended shelf life as the pathogen well adapts to the conditions used in manufacturing process (exposure to low temperatures, low pH, and elevated salt concentrations) developing defense mechanisms to tolerate these stresses (Farber and Pagotto, 1992; Gandhi and Chikindas, 2007; Uyttendaele et al., 2009; Bevilacqua et al., 2018). To face the risk of pathogen survival, the application of LAB populations as bio-preservatives contribute to warranty quality and safety of vegetable products (Valerio et al., 2013; Iglesias et al., 2017, 2018; Yépez et al., 2017).

The main goal of this research was to use a bio-preserving strain of *Lb. plantarum* during refrigerated storage in order to assure the microbiological and sensorial quality of Spanish-style olives cv. Bella di Cerignola packaged in plastic bags, containing low amount of brine with a minimum salt level and without chemical preservatives. In addition, the ability of the bio-preserving strain to counteract *Listeria monocytogenes* during storage was evaluated.

MATERIALS AND METHODS

Olive Processing and Packaging

Spanish-style table olives used in the present study (cv. Bella di Cerignola), supplied by Cannone Industrie Alimentari SpA, were picked by hand at the green maturation stage during the 2015 crop season and prepared following the Spanish-style method. Olives were placed into tanks and covered with a 1.9% (w/v) NaOH solution for 8 h until the lye penetrated 2/3 of the pit; then fruits were washed with tap water for 10–12 h. Spontaneous fermentation, i.e., fermentation carried out by the indigenous microflora, was performed in plastic tanks keeping fruits (140 kg) submerged in brines (90 kg, 11% NaCl) by the means of perforated caps. After the fermentation, the tanks were stored for 1 year at room temperature increasing the salt concentration in brine up to 10%. *Lb. plantarum* 5BG (belonging to ISPA Collection n°ITEM 17403) was previously isolated from olive brines (Lavermicocca et al., 1998) and used in this study as freeze-dried powder. About 100 kg of olives were withdrawn from tanks, desalted (final NaCl 5%) by washing olives two times with tap water (about 100 L) and two experimental sets were settled up: (1) bio-preserved SET1 was obtained by adding the strain (Log 8 CFU/g of olives) in fresh brine (4% NaCl, 2% sucrose; pH 6.6) for 5 days at 25°C; (2) experimental SET2 was obtained by directly adding desalted olives to a chemically acidified brine (4% NaCl; 0.35% lactic acid; 0.35% citric acid, 0.35% ascorbic acid; pH <4.0).

Olives (400 g) from both sets were packaged in PE bags in the presence of 50 g of brine to obtain olive/brine ratio of 8/1 which was selected in preliminary experiments (unpublished results); bags were heat-sealed using an industrial-scale packaging machine (Tecnovac, Grassobbio, BG, Italy) and kept at 4°C for 1 year. Two independent experiments were performed and, for each analysis and sampling time, two separate bags for each set were prepared. In the case of microbiological and physico-chemical analyses, both brines and olives were analyzed.

Physico-Chemical Analyses

The pH analyses of cover brines and olive fruits were carried out as described in De Bellis et al. (2010); results were the average of 4 and 12 measurements for brines and olives, respectively. The instrumental determination of firmness of olives was determined

using a Kramer shear compression cell fixed to a texture analyzer Machine (Zwickiline Z0.5, Zwick/Roell, Ulm, Germany). Olives from each bag were manually pitted and cut longitudinally, resulting in one piece (about 10 mm × 10 mm × 5 mm) of flesh per olive. The olive pieces (about 1 gr each) were placed in the Kramer cell with the olive surface facing upward. The cross-head speed was set at 400 mm/min and the penetration force was measured in Newton (N). Data were collected and analyzed using the system software (Texture Expert). The maximum force required to penetrate olive section was recorded and firmness was calculated by dividing the maximum shear compression force with the total weight of olive pieces and expressed as N/g of product. Firmness values were the mean of 12 measurements, each of which from 12 different fruits.

The instrumental surface color analyses on the olive fruits was carried out using a Minolta CR-300 (Minolta Ltd., Osaka, Japan) colorimeter. The instrument was calibrated using a reference white tile and color was recorded in the CIE L* (lightness/brightness), a* (redness/greenness; negative values are related to green tones while positive values are associated to red tones) and b* (yellowness/blueness, representing colors on a blue (−) to yellow (+) axis) color scale. Total color difference (ΔE*) was calculated as

$$\sqrt{[(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]} \quad (1)$$

using the color of olives before packaging as a reference

For each sample, 9 different olives from 2 different bags were analyzed to evaluate the skin color. Results were the average of 18 measurements.

Microbiological Analyses

At each sampling time, two bags per set were opened and olive and brine samples were analyzed. Microorganisms adhering to the olive epidermis were determined as follows: at each sampling 50 g of olives each were pitted with a sterile knife under a laminar flow cabinet; 15 g portions from each replicate were homogenized in 135 g of sterile Buffered Peptone Water (BPW, Difco) for 1 min in a Blender (Waring). Decimal dilutions (100 µl or otherwise specified) were plated in duplicate on specific agar media. For LAB enumeration, dilutions were plated onto de Man–Rogosa–Sharpe medium (MRS) and incubated into a 9% CO_2 incubator (Incusafe, Panasonic) at 37°C for 48 h; for total mesophilic count, dilutions were plated on plate count agar (PCA) and incubated at 30°C in aerobiosis; for yeasts and molds, dilutions were plated on Sabouraud Dextrose Agar (SDA) supplemented with chloramphenicol and chlortetracycline (both 0.05 g/l) and incubated at 25°C for 48 h and 5 days, respectively; total counts of Enterobacteriaceae were obtained by pour-plating dilutions (1 ml) in Violet Red Bile Glucose agar (VRBGA), incubated at 37°C for 24 h; Baird-Parker Agar, supplemented with egg yolk tellurite emulsion and incubated at 37°C for 48 h was used for *Staphylococcus* spp. Brines were diluted in a sterile saline solution (9 g/l NaCl) and plated on the same media.

Model Development

To determine the microbial acceptability limit, a modified version of the Gompertz equation was fitted to the experimental data, as reported in previous works (Conte et al., 2009; Del Nobile et al., 2009):

$$\begin{aligned}\log(N(t)) &= \log(N_{\max}) - A \cdot \exp\left[-\exp\left\{\left[\mu_{\max} \cdot 2.71 \frac{\lambda - MAL}{A}\right] + 1\right\}\right] \\ &\quad + A \cdot \exp\left[-\exp\left\{\left[\mu_{\max} \cdot 2.71 \cdot \frac{\lambda - t}{A}\right] + 1\right\}\right]\end{aligned} \quad (2)$$

Where $N(t)$ is the viable cell concentration (CFU/g) at time t, A is related to the difference between the decimal logarithm of maximum bacterial growth attained at the stationary phase and the decimal logarithm of the initial cell load concentration (CFU/g), μ_{max} is the maximum growth rate [Log(CFU/g)/day], λ is the lag time (day), t is the time (day), N_{max} is the break-point (CFU/g), MAL is the microbial acceptability limit or microbiological shelf life (day) (i.e., the storage time at which the population attains the break point).

There are not specific actual microbiological standards for table olives. However, they can be sold in both bulk form in fermentation brine (unpasteurized) and in packages intended for retail sale (pasteurized or possibly unpasteurized); therefore, the limits for ready-to-eat products were used (10^6 CFU/g for yeasts and 10^4 CFU/g for Enterobacteriaceae and *Staphylococcus* spp.) (Food Safety Authority of Ireland, 2001; International Olive Council [IOC], 2004; Commission Regulation [EC], 2007).

The survival of the inoculated strain on olive drupes and in brines during storage was determined as follows: 20% of total presumptive LAB colonies (at least 10 colonies), randomly picked from countable MRS agar plates containing from 50 to 100 colonies, were isolated and checked for purity. Bacterial DNA from each colony was extracted from overnight cultures grown in MRS broth at 37°C as previously described (De Bellis et al., 2010). Genotypic identification of *Lb. plantarum* 5BG was based on the comparison of the REP-PCR profile of each isolate with the specific pattern obtained from the pure culture of *Lb. plantarum* 5BG strain. The amplification products were separated by the Lab-on-a-Chip (LoaC) capillary electrophoresis carried out on a 2100 Bioanalyzer from Agilent Technologies using the DNA 7500 LabChip kit (Agilent Technologies, Waldbronn, Germany). Sample preparation and chip loading was performed according to manufacturer's instructions. DNA fragments were separated electrophoretically and data elaborated by 2100 Expert Software were automatically visualized as peaks in an electropherogram and bands in a gel-like image. Each chip included a sizing ladder containing 10 reference fragments ranging from 50 to 7,000 bp flanked by an upper (10,380 bp) and lower (50 bp) marker. Therefore, the concentration of *Lb. plantarum* 5BG in olives (CFU/g) or in brine (CFU/ml) was calculated on the basis of the number of identified colonies.

Challenge Test

The challenge test was assessed to evaluate the effectiveness of *Lb. plantarum* 5BG to control and/or inhibit pathogens; a wild strain of *L. monocytogenes*, belonging to the culture collection

of the Department of the Science of Agriculture, Food and Environment, University of Foggia, was used as the test organism.

L. monocytogenes was stored at −20°C in Nutrient broth (Oxoid), supplemented with 33% sterile glycerol (J.T. Baker, Milan). Before each assay the strain was grown twice in Nutrient broth incubated at 37°C for 24 h to attain an early stationary phase (ca. 8 Log CFU/ml). Then, the microbial culture was centrifuged twice at 8,000 rpm for 15 min at 4°C; the broth was discarded, and the pathogen suspended in a sterile saline solution (0.9% NaCl)

Olive bag samples of both SET1 and SET2 were inoculated with *L. monocytogenes* through a sterile syringe; the ratio inoculum/brine was ca. 1:10, in order to achieve an initial concentration of *L. monocytogenes* of 5 Log CFU/ml). Olive bags were stored at 4°C for 3 months. Both olives and brines were periodically analyzed to monitor the total mesophilic count, LAB, yeasts, as reported above, and *L. monocytogenes* on Listeria Selective Agar Base (Oxoid), supplemented with Listeria selective supplement (37°C for 24–48 h) (Oxoid). The analyses were performed in duplicate (two bags) over two independent experiments and repeated twice for each bag.

Sensory Analyses

Table olives were subjected to a time intensity descriptive sensory evaluation according to the information provided ISO 13300-1 (2006), modified as follows. The panelists were selected based on their sensory skills (ability to accurately determine and communicate the sensory attributes of a food product) (Meilgaard et al., 1999). Prior to testing, panelists were trained into the sensory vocabulary and into the identification of attributes, by using samples of commercial olives. The panel members were asked to base their judgment evaluating color (spots, uniformity, intense and typical of the fruit), taste (intense and typical of the fruit, salty, acid and bitter), odor (typical of the fruit, acid-off odor, putrid, butyric, metallic and off odors), and texture attributes (hardness, crunchiness, and fibrousness).

The panel members were asked to give a score from 1 to 9 to color, texture, and odor; a score of five was set as the break point. The assignment of the score was based on the following hits:

1. Color: 9 = green color typical of the cultivar, 1 = dark brown
2. Texture: 9 = firm and consistent, 1 = extremely soft
3. Odor: 9 = characteristic olive odor, 1 = extremely off odor.

The panel members were also asked to give a score for the overall quality (where 9 = excellent, 8 = very good, 7 = good, 6 = reasonable, 5 = not good, 4 = disliked, 3 = bad, 2 = very bad, 1 = reject).

Statistical Analysis

All data are presented as mean values ± standard deviation (SD). Microbiological data, expressed as Log CFU/g of pitted olives or Log CFU/ml of brines, and physico-chemical data were compared by using the one-way factor analysis of variance (ANOVA). Significant differences ($p < 0.05$) among groups were determined by using the *post hoc* LSD Fisher test. Paired comparisons were analyzed by Student's *t*-test ($p < 0.05$).

The *L. monocytogenes* count was modeled through the equation of Weibull, as re-parameterized by Mafart et al. (2002):

$$LogN = LogN_0 - (t/\delta)^p \quad (3)$$

where $LogN$ is the count over the time t (Log CFU/ml); $LogN_0$ the inoculum (Log CFU/ml); δ, the first reduction time (day), i.e., the time for a 1 Log CFU/ml decrease of the bacterial population; p, the shape parameter ($p > 1$ downward curve; $p < 1$, upward curve).

The results were also fitted through the Weibull equation, modified by Bevilacqua et al. (2008) for the evaluation of the survival time:

$$\frac{LogN}{LogN_0} = 1 - (t/\text{s.t.})^p \quad (4)$$

where s.t. is the survival time (days), i.e., the time after which the population is below the detection limit.

All statistical analyses were carried out using STATISTICA 6.0 software (StatSoft software package, Tulsa, OK, United States).

RESULTS

Physico-Chemical Quality

Spanish-style fermented Bella di Cerignola desalted olives provided by the industry were used in the present study (pH: 4.67 ± 0.07; firmness: 62.4 ± 11.6 N/g; color parameters L* 55.73 ± 3.40, a* −0.86 ± 0.34 b* 39.34 ± 4.13). The evolution of pH on olive fruits packaged in PE bags in brine during storage at 4°C is presented in **Figure 1**. At the onset of storage (T0), after the 5-days brining in the presence of the preserving strain, the olive pH lowered to 4.50 ± 0.03 ($p < 0.05$). In chemically acidified control SET2, the olive pH remained unvaried ($p > 0.05$). The pH of brines showed values ranging from 4.28 ± 0.02 to 4.27 ± 0.13 in SET1 and from 4.39 ± 0.03 to 4.55 ± 0.06 in SET2, respectively, at T0 and T360. During the entire storage period pH values of inoculated olives and brines were significantly lower ($p < 0.05$) with respect to chemically acidified control.

Instrumental firmness of olives is shown in **Figure 2**. At the onset of storage (T0), olive firmness remained stable ($p > 0.05$) in both sets. During storage, firmness of inoculated olives (SET1) remained stable ($p > 0.05$) until day 180, after that significantly decreased ($p < 0.05$). Whereas, in control SET2, firmness of olives significantly decreased already at day 120 ($p < 0.05$). At each sampling time, bio-preserved olives showed higher ($p < 0.05$) values of firmness compared to control olives.

Variation of color parameters of olives during storage determined comparable ($p > 0.05$) total color differences (ΔE*) between sets with average values of 9.5 ± 2.4 and 10.3 ± 3.0 for SET1 and SET2, respectively. Although color parameters varied ($p < 0.05$) during storage, a similar trend was observed in both sets (**Figure 3**).

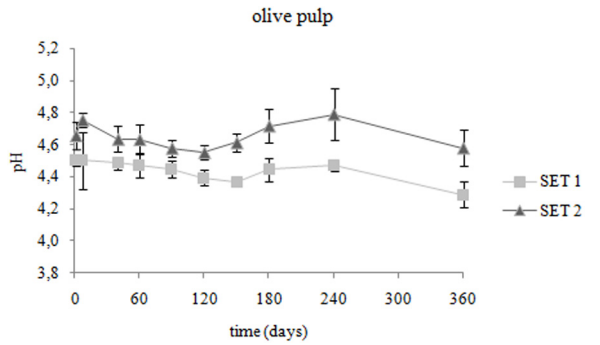

FIGURE 1 | Changes in pH of Bella di Cerignola Spanish-style olives packaged in PTE bags (olive/brine ratio, 8/1): SET1 (bio-preserved), SET2 (control). Means and standard deviations were obtained from measurements made in duplicate packages from 2 different experiments (n = 4).

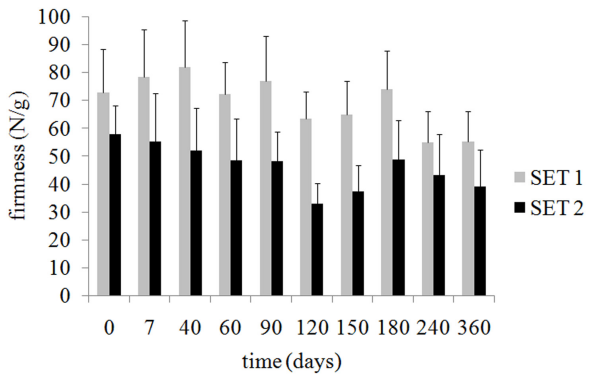

FIGURE 2 | Instrumental firmness of bio-preserved (SET1, gray bars) and control (SET2, black bars) Bella di Cerignola Spanish-style olives packaged in PTE bags (olive/brine ratio, 8/1). Means and standard deviations were obtained from measurements made on three different olives analyzed from duplicate packages per each experiment (n = 12).

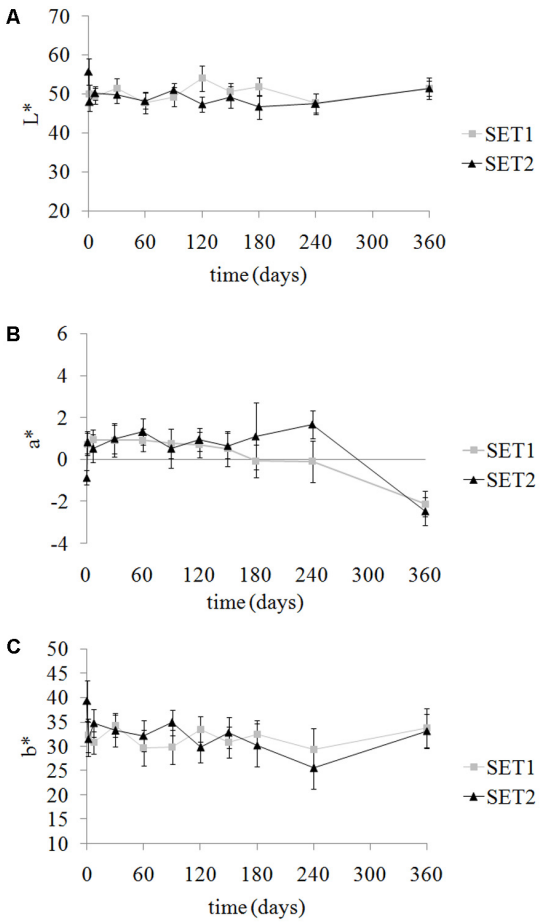

FIGURE 3 | Evolution of color parameters Luminance L* **(A)**, green-red a* **(B)** and blue-yellow b* **(C)** in SET1 (bio-preserved), and SET2 (control) Bella di Cerignola Spanish-style olives packaged in PTE bags (olive/brine ratio, 8/1). Means and standard deviations were obtained from measurements made on nine different olives analyzed in duplicate packages per each experiment (n = 18).

Microbiological Quality

Significant differences in LAB populations ($p < 0.05$) were observed between SET1 and 2 having SET1 higher ($p < 0.05$) microbial loads during the whole experiments. In control SET2, LAB population ranged from 4.63 ± 0.73 to 6.05 ± 0.33 Log CFU/g in olives at T0 and T360, respectively. Values ranged from 5.04 ± 0.28 to 6.51 ± 0.11 Log CFU/ml in brines (data not shown). The presence of the *Lb. plantarum* strain resulted in an increase in total LAB in inoculated SET1 and populations remained stable throughout the experiment (**Figures 4A,B**). The REP-PCR profile of 148 presumptive LAB isolates from olives and brines of SET1 and SET2, were compared to the specific pattern obtained from the pure culture of *Lb. plantarum* 5BG. Bacterial load of the inoculated strain at T0 was 6.56 ± 0.17 Log CFU/g on olives and 7.74 ± 0.06 Log CFU/ml in brines (**Figures 4A,B**). The bio-preserving strain population remained stable on olives until the end of storage while a decrease was observed in brines. In control SET2 none of the REP-PCR profiles of the analyzed colonies was comparable to the *Lb. plantarum* 5BG pattern (data not shown).

Regarding the other microbial populations, the evolution of the total mesophilic count during olive storage is showed in **Figure 5**. A significant ($p < 0.05$) increase of counts was observed in control SET2, while counts remained almost unvaried ($p > 0.05$) in SET1 until 1 year. The corresponding values of the brines (data not shown) appeared to be higher than those found on the pulps during the entire observation period, reaching similar values, about 7.39 and 7.28 Log CFU/ml, respectively, for SET1 and SET2.

Along with the prevailing LAB populations monitored on olive fruits, yeasts were present in both sets during the whole experiment at average values of 5.25 ± 0.88 log CFU/g and 5.30 ± 0.42 log CFU/g, respectively, for SET1 and SET2 and never attained the break-point (6 log CFU/g). In brines a similar trend was observed with comparable ($p > 0.05$) yeast populations found in SET1 (6.34 ± 0.58 log CFU/ml) and SET2 (6.30 ± 0.67

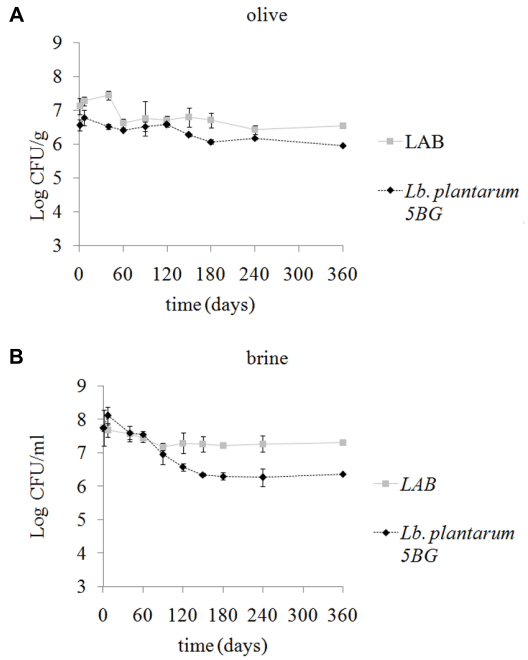

FIGURE 4 | Changes of the LAB population and survival of the bio-preserving strain *Lb. plantarum* 5BG on olives **(A)** and in cover brines **(B)** of SET1 Bella di Cerignola Spanish-style olives packaged in PTE bags (olive/brine ratio, 8/1) and stored at 4°C for 1 year. Means and standard deviations were obtained from measurements made in duplicate packages from two different experiments (n = 4).

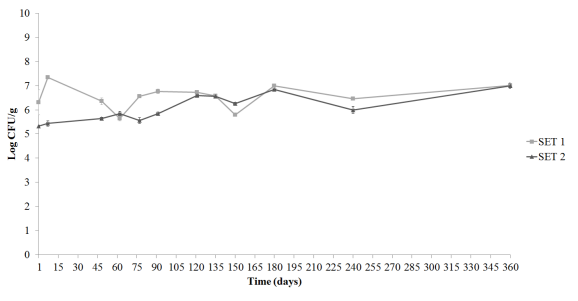

FIGURE 5 | Evolution of mesophilic bacteria in Spanish-style olives pulp packaged in PTE bags and stored at 4°C: SET1 (bio-preserved), and SET2 (control). Means and standard deviations were obtained from measurements made in duplicate packages from two different experiments (n = 4).

log CFU/ml). Molds, staphylococci, and Enterobacteriaceae were always below the detection limit.

Challenge Test

The data of *L. monocytogenes* were fitted through the Weibull model to assess if the presence of *Lb. plantarum* could affect both the survival and the shape of the death kinetics (**Table 1** and **Figure 6**). **Table 1** reports the fitting parameters for SET1 and SET2 inoculated with the pathogen. The Weibull model is characterized by three main parameters, i.e., the initial pathogen count ($LogN_0$), the first reduction time (δ) and

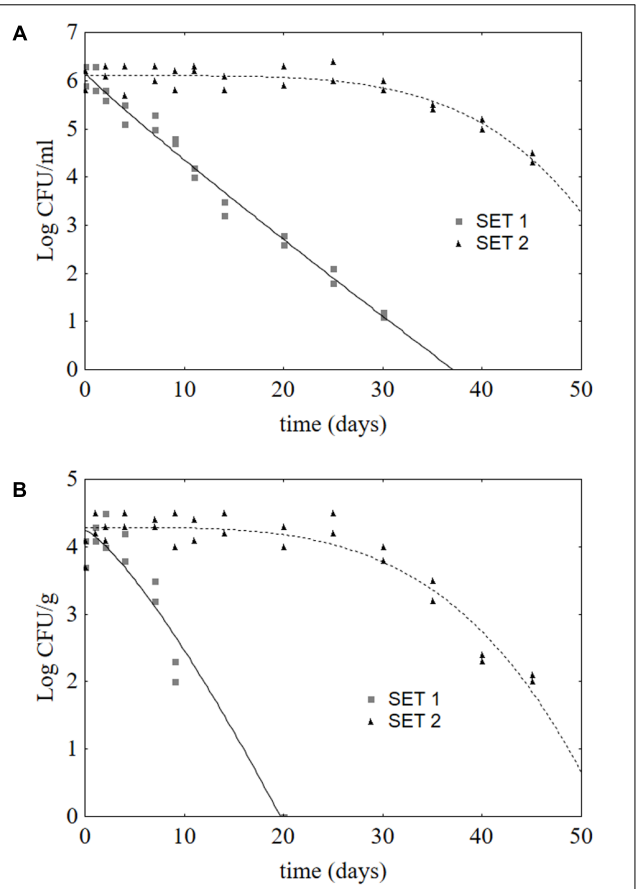

FIGURE 6 | Death kinetics of *L. monocytogenes* in brines **(A)** and on olive surfaces **(B)**. The lines represent the best fitting through the Weibull equation: SET1 (bio-preserved) and SET2 (control).

the shape parameter (p). The first reduction time is similar to the *D*-value for the linear thermal death kinetic and can be used as a simple index of how a treatment affects the survival of the model microorganism. In brines, *L. monocytogenes* experienced a δ of 40.07 ± 0.91 days in the control SET2, thus suggesting a prolonged survival; however, the inoculation of *Lb. plantarum* created conditions that strongly affected *L. monocytogenes* and reduced δ to 5.30 ± 0.86 days. This reduced survival was the result of a significant change in the shape of the death kinetic, as suggested by the shift in the shape parameter (p). In SET2-brine, the shape parameter was 4.72 ± 0.79, whereas in SET1 this parameter was reduced to 0.94 ± 0.08 and the shape of the death kinetic completely changed.

A modified approach of the Weibull equation, proposed by Bevilacqua et al. (2008), contains the survival time of the model organism. *L. monocytogenes* experienced a survival time of 58.77 ± 3.70 days in the control SET2 and 36.63 ± 1.29 days in the brine inoculated with *Lb. plantarum*. The same approach was used to model *L. monocytogenes* on olive surfaces. In the presence of *Lb. plantarum*, a significant reduction of δ (from 35.70 ± 1.10 to 6.37 ± 1.24 days), an effect of the shape parameter

TABLE 1 | Weibull parameters (±standard error) for the death kinetic of *L. monocytogenes* in brines and on olive surface.

	$LogN_0$	δ	*p*	*R*	*s.t.*
Brines					
SET1	6.17 ± 0.14	5.30 ± 0.86	0.94 ± 0.08	0.991	36.63 ± 1.29
SET2	6.10 ± 0.05	40.07 ± 0.91*	4.72 ± 0.79*	0.933	58.77 ± 3.70*
Olives					
SET1	4.25 ± 0.19	6.37 ± 1.24	1.28 ± 0.20	0.972	18.43 ± 1.12
SET2	4.29 ± 0.06	35.70 ± 1.10*	3.84 ± 0.49*	0.950	50.90 ± 1.47*

*SET1, inoculated with Lb. plantarum; SET2, control. $LogN_0$, initial count (Log CFU/ml); δ, first reduction time (time to attain a 1-log decrease, day); p, shape parameter; s.t., survival time (day). *Significant difference between SET1 and SET2 (LSD test, P < 0.05).*

and a decrease of the survival time (from 50.90 ± 1.47 to 18.43 ± 1.12 days) were observed.

Sensory Quality

Both SET1 and SET2 were positively accepted for color, having a uniform appearance and the characteristic color of Spanish-style olives, with slight differences between SET1 and SET2 (**Figure 7**). At the beginning of the storage, the score for odor was lower in SET1 as consequence of the supplementation of *Lb. plantarum*, but for this sample the panelists always recorded an equilibrated odor. On the other hand, they perceived alcoholic odors in SET2 since the 4th month onward and assigned a lower score (**Figure 8**). In SET1, panelists never found smells of butyric, acidic, rancid, and metallic like odors during the entire observation period.

Texture was the real discriminative parameter among samples (**Figure 9**). SET1 recorded, since the beginning and during the whole storage period, a firmer texture, that is a score significantly higher than in SET2, in line with the instrumental data shown in **Figure 2**. Furthermore, bio-preserved olives proved to be better also regarding crunchiness and fibrousness; in fact, panelists underlined a positive mouth texture in relation to the force required to crunch samples with the back molars. On the contrary, SET2 olives revealed a gradually softening with the visual detachment of the pulp. Bio-preserved olives appeared to be positively accepted also in relation to their taste, since they were perceived by panelists as more equilibrated with better scores in relation to acidity and bitterness. SET2 olives had a less intense taste with an excessive bitter and vinegary taste perceived by panelists. In terms of overall quality, reported in **Figure 10**, both samples revealed a gradual decrease; however, the control SET2 was rejected by panelists after 115 days while the bio-preserved olives recorded a longer storage (at least 5 months), thus reflecting the texture trends.

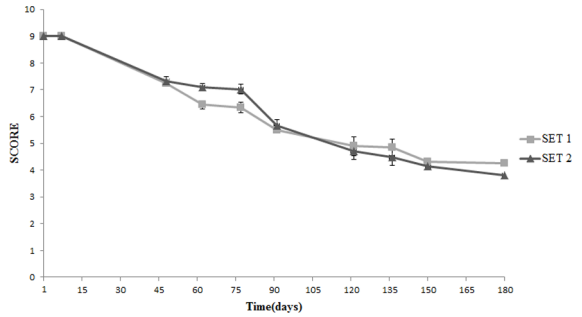

FIGURE 7 | Sensory analysis: evolution of the color in Spanish-style olives packaged in PTE bags and stored at 4°C: SET1 (bio-preserved), and SET2 (control). Means and standard deviations were obtained from measurements made in duplicate packages from two different experiments (*n* = 4).

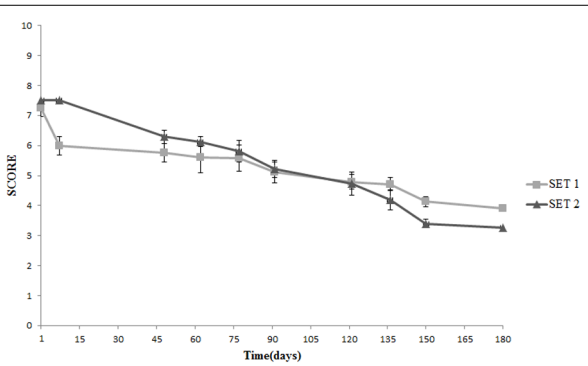

FIGURE 8 | Sensory analysis: evolution of the odor in Spanish-style olives packaged in PTE bags and stored at 4°C: SET1 (bio-preserved), and SET2 (control). Means and standard deviations were obtained from measurements made in duplicate packages from two different experiments (*n* = 4).

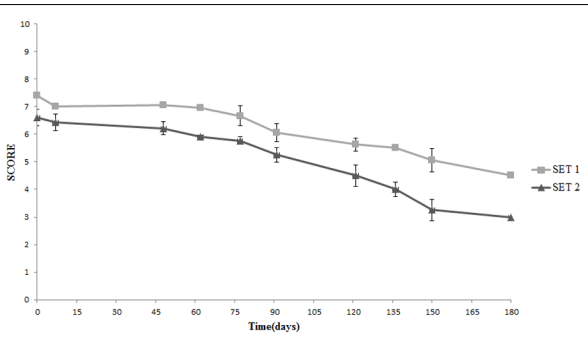

FIGURE 9 | Sensory analysis: evolution of the texture in Spanish-style olives packaged in PTE bags and stored at 4°C: SET1 (bio-preserved), and SET2 (control). Means and standard deviations were obtained from measurements made in duplicate packages from two different experiments (*n* = 4).

DISCUSSION

Packaging in plastic bags is a procedure currently adopted to store Bella di Cerignola Spanish-style olives using 8–10% brine (olive/brine ratio 4/1) in the presence of chemical acidifiers and antioxidants to ensure microbiological stability and sensory quality. However, long-term storage in these conditions cannot be achieved since sensory parameters, particularly texture, experience a strong decrease. In addition, the increasing consumer requirements for low levels of acidity and salt pave the way for the use of bio-protective cultures

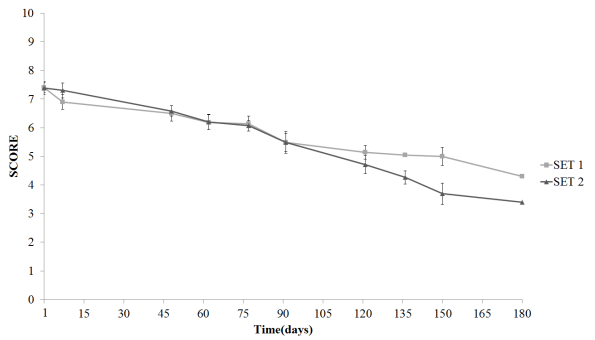

FIGURE 10 | Evolution of the overall sensory quality in Spanish-style olives packaged in PTE bags and stored at 4°C: SET1 (bio-preserved), and SET2 (control). Means and standard deviations were obtained from measurements made in duplicate packages from two different experiments (n = 4).

for a sustainable long-term preservation of table olives. It is noteworthy that the bio-preserving strain used in this study was selected among several strains belonging to *Lb. plantarum*, *Lb. pentosus*, *Lb. casei*, and *Lb. paracasei* spp., isolated from olive environment (Lavermicocca et al., 1998; De Bellis et al., 2010).

In fact, the bio-preserving strain, well adapted in the brine environment, replaced the indigenous LAB population, survived for 1 year and allowed to reach pH values lower than 4.3 in brine as required by the Codex Alimentarius standard (CODEX/COI, 2013).

The combined effects of microbiological and physico-chemical changes occurring during storage (pH, temperature, strain survival) are essential for olive preservation and the application of LAB at the onset of fermentation (Argyri et al., 2015; Blana et al., 2016) or at the moment of packing (Rodríguez-Gómez et al., 2014) represents a valid procedure to preserve Spanish-style table olive quality. Temperature influences the survival rates of LAB in brines and olives as reported in studies on Spanish-style olives, previously fermented with a *Lb. plantarum* strain, thermally treated and packaged in the presence or not of brine (Argyri et al., 2015; Blana et al., 2016). In these studies, during storage at 4°C, a high survival rate of a *Lb. plantarum* strain (96 and 94.1% recovery after 6 months) and no survival or a very low recovery (13.3% after 1 year) has been observed on olives packaged in brine or modified atmosphere, respectively. In our study the bio-preserving strain was able to survive at 4°C under packaging conditions for a long period (1 year) in brines and on olive surface replacing the indigenous LAB population without the need of thermal treatments that can influence the sensory profile. Moreover, the presence of LAB populations can improve the microbiological quality of table olives (Rodríguez-Gómez et al., 2013; Bonatsou et al., 2017). In our study, the total absence of *Enterobacteriaceae*, Staphylococci and molds could be related to the successful long term persistence of the *Lb. plantarum* strain (SET1) that might have hampered the growth of alterative microorganisms and to the chemical acidification in control SET2. The *Lb. plantarum* strain can contribute in preventing spoiling, hence reducing the need for chemical preservatives or thermal treatments in low-salt products. During storage, yeasts were present in brines and on olive surface at high loads in both sets. These microorganisms are known to contribute to the flavor of table olives, however, in some conditions they may be considered spoilage microorganisms (Arroyo-López et al., 2008).

In order to assess if the microbiological and physico-chemical conditions obtained in the applied bio-preserving procedure, could hamper the growth of microbial pathogens, *L. monocytogenes* was chosen as a model since previous researches indicated the prolonged survival of this pathogen on table olives and brine (Bevilacqua et al., 2018). Generally, acid-adapted cells of *L. monocytogenes* can survive during shelf life in acidified or fermented products (Caggia et al., 2004, 2009; Tassou et al., 2009). As a result, in our study the pathogen load remained unvaried on control olives (SET2) until about 30 days. In *Lb. plantarum* bio-preserved olives, a dual effect on *L. monocytogenes* was observed: the decrease of the first reduction time and the change in the shape from a downward to a linear kinetic. These two effects were probably related to an increased death rate resulting from the production of antimicrobial compounds and the antagonistic ability of the strain, as well as to the reduced pH in SET1. Some preliminary experiments for the strain of *L. monocytogenes* used in this paper, in fact, revealed that it could survive a pH 4.0–4.5; however, the combination of acidic pH, low-salt amount and refrigeration could enhance and contribute to the antimicrobial effect of *Lb. plantarum* 5BG. As previously reported, *Lb. plantarum* 5BG, antagonized the human pathogen *Yersinia enterocolitica* in coculture (Lavermicocca et al., 2008) and was able to produce antimicrobial compounds and in particular organic acids responsible for antifungal properties (Valerio et al., 2016). Moreover, the technological suitability of *Lb. plantarum* 5BG was already demonstrated in green and black olive fermentation processes (Lavermicocca et al., 2002).

The evaluation of sensory quality was essential for the final determination of olive shelf life since no microbial alteration was found in any of the experimental samples. A brilliant green color is of great importance for Bella di Cerignola olives. In industrial practice the addition of ascorbic acid is common to prevent oxidation (López et al., 2005; Segovia-Bravo et al., 2011) and it has been demonstrated that it positively affects fruit color regardless the presence of preservatives (Casado et al., 2010).

The correlation between pH values and olive color is well known since low pH values are associated with a light color (Garrido-Fernández et al., 1997). Thus, the lower pH of SET1 could be related to good score for color, as also confirmed by the instrumental measurements. Bio-preserved olives showed changes similar to control olives preserved in chemically acidified brine containing ascorbic acid.

Finally, the taste was the other relevant sensory parameter better preserved in bio-preserved olives. Although the score in the initial part of the storage was lower in SET1 samples, the panelist always recorded an equilibrated taste without any excessive bitter or acidic perception probably due to

the β-Glucosidase activity of *Lb. plantarum* 5BG, which is able to hydrolyze bitter glucosides into no bitter compounds (Lavermicocca et al., 1998). On the contrary, in SET2 samples, above all at the end of storage, the panelists underlined a vinegary and ethanolic perception, responsible for the lower score after 115 days.

CONCLUSION

This paper represents a valuable contribution for a possible scale-up of bio-preservation of table olives, in order to avoid chemical preservatives and/or detrimental thermal treatments. The approach hereby proposed is based on the use of a bio-preserving *Lb. plantarum* strain able to replace wild microbiota and persist at concentration higher than 6 Log CFU/g on packaged Spanish-style olives during refrigerated storage. The environmental stress conditions generated in olives – low pH values, low storage temperature, presence of the bio-preserving strain – influenced the death kinetic of *L. monocytogenes*.

Results obtained in this study are promising even if further efforts are required to validate this approach on other olive varieties.

AUTHOR CONTRIBUTIONS

PL, AC, MDN, and MC conceived of the study. PL, AC, AB, and MC designed the experiments. LA, SL, FV, MP, and AB performed the experiments. PL, LA, FV, AB, MC, and AC interpretated the results. PL, MC, and AC funded the research. All authors wrote and approved the manuscript.

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REFERENCES

Ananou, S., Maqueda, M., Martínze-Bueno, M., and Valdivia, E. (2007). "Biopreservation, an ecological approach to improve the safety and shelf-life of foods," in *Communicating Current Research and Educational Topics and Trends in Applied Microbiology*, ed. A. Méndez-Vilas (Badajoz: Formatex), 475–486.

Argyri, A. A., Lyra, E., Panagou, E. Z., and Tassou, C. C. (2013). Fate of *Escherichia coli* O157:H7, *Salmonella* enteritidis and *Listeria monocytogenes* during storage of fermented green table olives in brine. *Food Microbiol.* 36, 1–6. doi: 10.1016/j.fm.2013.04.001

Argyri, A. A., Nisiotou, A. A., Pramateftaki, P., Doulgeraki, A. I., and Panagou, E. Z. (2015). Preservation of green table olives fermented with lactic acid bacteria with probiotic potential under modified atmosphere packaging. *LWT Food Sci. Technol.* 62, 783–790. doi: 10.1016/j.lwt.2014.11.046

Arroyo-López, F. N., Querol, A., Bautista-Gallego, J., and Garrido-Fernández, A. (2008). Role of yeasts in table olive production. *Int. J. Food Microbiol.* 128, 189–196. doi: 10.1016/j.ijfoodmicro.2008.08.018

Bevilacqua, A., Campaniello, D., Speranza, B., Sinigaglia, M., and Corbo, M. R. (2018). Survival of *Listeria monoctytogenes* and *Staphylococcus aureus* in synthetic brines. Studying the effects of salt, temperature and sugar through the approach of the Design of the Experiments. *Front. Microbiol.* 9:240. doi: 10.3389/fmicb.2018.00240

Bevilacqua, A., Cibelli, F., Cardillo, D., Altieri, C., and Sinigaglia, M. (2008). Metabiotic effects of *Fusarium* spp. on *Escherichia coli* O157:H7 and *Listeria monocytogenes* on raw portioned tomatoes. *J. Food Prot.* 71, 1366–1371. doi: 10.4315/0362-028X.JFP-16-044

Bevilacqua, A., de Stefano, F., Augello, S., Pignatiello, S., Sinigaglia, M., and Corbo, M. R. (2015). Biotechnological innovations for table olives. *Int. J. Food Sci. Nutr.* 66, 127–131. doi: 10.3109/09637486.2014.959901

Blana, V. A., Polymeneas, N., Tassou, C. C., and Panagou, E. Z. (2016). Survival of potential probiotic lactic acid bacteria on fermented green table olives during packaging in polyethylene pouches at 4 and 20°C. *Food Microbiol.* 53, 71–75. doi: 10.1016/j.fm.2015.09.004

Bonatsou, S., Tassou, C. C., Panagou, E. Z., and Nychas, G. E. (2017). Table olive fermentation using starter cultures with multifunctional potential. *Microorganisms* 5:E30. doi: 10.3390/microorganisms5020030

Caggia, C., Randazzo, C. L., Di Salvo, M., Romeo, F., and Giudici, P. (2004). Occurrence of *Listeria monocytogenes* in green table olives. *J. Food Prot.* 67, 2189–2194. doi: 10.4315/0362-028X-67.10.2189

Caggia, C., Scifò, G. O., Restuccia, C., and Randazzo, C. L. (2009). Growth of acid-adapted *Listeria monocytogenes* in orange juice and in minimally processed orange slices. *Food Control* 2, 59–66. doi: 10.1016/j.foodcont.2008.02.003

Casado, F. J., Sánchez, A. H., Rejano, L., de Castro, A., and Montaño, A. (2010). Stability of sorbic and ascorbic acid in packed green table olives during long-term storage as affected by different packing conditions, and its influence on quality parameters. *Food Chem.* 122, 812–818. doi: 10.1016/j.foodchem.2010.03.066

CODEX/COI (2013). *Codex Standard for Table Olives. CODEX STAN 66- 1881. Revision 1987, 2013*. Rome: FAO.

Commission Regulation [EC] (2007). *No 1441/2007 of 5 December 2007 Amending Regulation (EC) No 2073/2005 on Microbiological Criteria for Foodstuffs*. Available at: http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2007:322:0012:0029:EN:PDF

Conte, A., Gammariello, D., Di Giulio, S., Attanasio, M., and Del Nobile, M. A. (2009). Active coating and modified-atmosphere packaging to extend the shelf life of Fiordi Latte cheese. *J. Dairy Sci.* 92, 887–894. doi: 10.3168/jds.2008-1500

De Bellis, P., Valerio, F., Sisto, A., Lonigro, S. L., and Lavermicocca, P. (2010). Probiotic table olives: microbial populations adhering on olive surface in fermentation sets inoculated with the probiotic strain *Lactobacillus paracasei* IMPC2in an industrial plant. *Int. J. Food Microbiol.* 140, 6–13. doi: 10.1016/j.ijfoodmicro.2010.02.024

Del Nobile, M. A., Corbo, M. R., Speranza, B., Sinigaglia, M., Conte, A., and Caroprese, M. (2009). Combined effect of MAP and active compounds on fresh blue fish burger. *Int. J. Food Microbiol.* 135, 281–287. doi: 10.1016/j.ijfoodmicro.2009.07.024

Farber, J. M., and Pagotto, F. (1992). The effect of acid shock on the heat resistance of *Listeria monocytogenes*. *Lett. Appl. Microbiol.* 15, 197–201. doi: 10.1111/j.1472-765X.1992.tb00762.x

Food Safety Authority of Ireland (2001). *Guidance Note No. 3 Guidelines for the Interpretation of Results of Microbiological Analysis of Some Ready to Eat Foods Sampled at the Point-of-Sale*. Dublin: Food Safety Authority of Ireland.

Gálvez, A. M., Grande-Burgos, M. J., Lucas-López, R., and Perez-Pulido, R. (2010). "Natural antimicrobials for food biopreservation", in *Food Biopreservation*, eds A. Galvez, M. J. GrandeBurgos, R. Lucas Lopez, and R. Perez Pulido (New York, NY: Springer), 3–14.

Gandhi, M., and Chikindas, M. L. (2007). Listeria: a foodborne pathogen that knows how to survive. *Int. J. Food Microbiol.* 113, 1–15. doi: 10.1016/j.ijfoodmicro.2006.07.008

Garrido-Fernández, A., Fernández-Diaz, M. J., and Adams, M. R. (1997). *Table Olives: Production and Processing*. London: Chapman & Hall. doi: 10.1007/978-1-4899-4683-6

Iglesias, M. B., Abadias, M., Anguera, M., Sabata, J., and Viñas, I. (2017). Antagonistic effect of probiotic bacteria against foodborne pathogens on fresh-cut pear. *LWT Food Sci. Technol.* 81, 243–249. doi: 10.1016/j.lwt.2017.03.057

Iglesias, M. B., Echeverría, G., Viñas, I., López, M. L., and Abadias, M. (2018). Biopreservation of fresh-cut pear using *Lactobacillus rhamnosus* GG and effect on quality and volatile compounds. *LWT Food Sci. Technol.* 87, 581–588. doi: 10.1016/j.lwt.2017.09.025

International Olive Council [IOC] (2004). *Trade Standard Applied to Table Olives*. Madrid: International Olive Council.

ISO 13300-1 (2006). *Sensory Analysis – General Guidance for the Staff of a Sensory Evaluation Laboratory – Part 1: Staff Responsibilities*. Geneva: ISO.

Lavermicocca, P., Gobbetti, M., Corsetti, A., and Caputo, L. (1998). Characterization of lactic acid bacteria isolated from olive phylloplane and table olive brines. *Ital. J. Food Sci.* 10, 27–39.

Lavermicocca, P., Valerio, F., Lonigro, S. L., Di Leo, A., and Visconti, A. (2008). Antagonistic activity of potential probiotic lactobacilli against the ureolytic pathogen *Yersinia enterocolitica*. *Curr. Microbiol.* 56, 175–181. doi: 10.1007/s00284-007-9069-5

Lavermicocca, P., Valerio, F., Lonigro, S. L., Gobbetti, M., Baruzzi, F., and Morea, M. (2002). Olive fermentations using lactic acid bacteria isolated from olive phylloplane and olive brines. *Acta Hortic.* 586, 621–624. doi: 10.17660/ActaHortic.2002.586.131

Leverentz, B., Conway, W. S., Janisiewicz, W., Abadias, M., Kurtzman, C. P., and Camp, M. J. (2006). Biocontrolof the foodborne pathogens *Listeria monocytogenes* and *Salmonella* enterica serovar Poona on fresh-cut apples with naturally occurring bacterial and yeast antagonists. *Appl. Environ. Microbiol.* 72, 1135–1140. doi: 10.1128/AEM.72.2.1135-1140.2006

Liu, L., O'Conner, P., Cotter, P. D., Hill, C., and Ross, R. P. (2008). Controlling *Listeria monocytogenes* in cottage cheese through heterologous production of enterocin A by *Lactococcuslactis*. *J. Appl. Microbiol.* 104, 1059–1066. doi: 10.1111/j.1365-2672.2007.03640.x

López, A., Montaño, A., García, P., and Garrido, A. (2005). Quantification of ascorbic and dehydroascorbic acid in fresh olive and in commercial presentation of table olives. *Food Sci. Technol. Int.* 11, 199–204. doi: 10.1177/1082013205054421

Mafart, P., Couvert, O., Gaillard, S., and Leguerinel, I. (2002). On calculating sterility in thermal preservation methods: application of the Weibull frequency distribution model. *Int. J. Food Microbiol.* 72, 107–113. doi: 10.1016/S0168-1605(01)00624-9

Meilgaard, M., Civille, G. V., and Carr, B. T. (1999). *Sensory Evaluation Techniques*. Boca Raton, FL: CRC Press. doi: 10.1201/9781439832271

Panagou, E. Z., Nychas, G. J. E., and Sofos, J. N. (2013). Types of traditional Greek foods and their safety. *Food Control* 29, 32–41. doi: 10.1016/j.foodcont.2012.05.050

Rodríguez-Gómez, F., Bautista-Gallego, J., Arroyo-López, F. N., Romero-Gil, V., Jiménez-Díaz, R., Garrido-Fernández, A., et al. (2013). Table olive fermentation with multifunctional Lactobacillus pentosus strains. *Food Control* 34, 96–105. doi: 10.1016/j.fm.2014.03.023

Rodríguez-Gómez, F., Romero-Gil, V., Garrido-Fernandez, A., and Arroyo-Lopez, F. N. (2014). Fortification of table olive packing with the potential probiotic bacteria *Lactobacillus pentosus* TOMC-LAB2. *Front. Microbiol.* 5:467. doi: 10.3389/fmicb.2014.00467

Sánchez, A. H., de Castro, A., López-López, A., Cortés-Delgado, A., Beato, V. M., and Montaño, A. (2017). Retention of color and volatile compounds of Spanish-style green table olives pasteurized and stored in plastic containers under conditions of constant temperature. *LWT Food Sci. Technol.* 75, 685–691. doi: 10.1016/j.lwt.2016.10.027

Segovia-Bravo, K. A., García-García, P., López-López, A., and Garrido-Fernández, A. (2011). Effect of bruising on respiration, superficial color, and phenolic changes in fresh Manzanilla olives (*Olea europaea pomiformis*): development of treatments to mitigate browning. *J. Agric. Food Chem.* 59, 5456–5464. doi: 10.1021/jf200219u

Tassou, C. C., Samaras, F. J., Arkoudelous, J. S., and Mallidis, C. G. (2009). Survival of acid-adapted or not-adapted *Salmonella Enteritidis*, *Listeria monocytogenes* and *Escherichia coli* O157:H7, in traditional greek salads. *Int. J. Food Sci. Technol.* 44, 279–287. doi: 10.1111/j.1365-2621.2007.01684.x

Trias, R., Baeras, L., Montesinos, E., and Badosa, E. (2008). Lactic acid bacteria from fresh fruit and vegetables as biocontrol agents of phytopathogenic bacteria and fungi. *Int. J. Microbiol.* 11, 231–236. doi: 10.2436/20.1501.01.66

Uyttendaele, M., Busschaert, P., Valero, A., Geeraerd, A. H., Vermeulen, A., Jacxsens, L., et al. (2009). Prevalence and challenge tests of *Listeria monocytogenes* in Belgian produced and retailed mayonnaise-based deli-salads, cooked meat products and smoked fish between 2005 and 2007. *Int. J. Food Microbiol.* 33, 94–104. doi: 10.1016/j.ijfoodmicro.2009.05.002

Valerio, F., Di Biase, M., Lattanzio, M. V., and Lavermicocca, P. (2016). Improvement of the antifungal activity of lactic acid bacteria by addition to the growth medium of phenylpyruvic acid, a precursor of phenyllactic acid. *Int. J. Food Microbiol.* 222, 1–7. doi: 10.1016/j.ijfoodmicro.2016.01.011

Valerio, F., Lonigro, S. L., Di Biase, M., de Candia, S., Callegari, M. L., and Lavermicocca, P. (2013). Bioprotection of ready-to-eat probiotic artichokes processed with *Lactobacillus paracasei* LMGP22043 against foodborne pathogens. *J. Food Sci.* 78, M1757–M1763. doi: 10.1111/1750-3841.12282

Yépez, A., Luz, C., Meca, G., Vignolo, G., and Mañes, J. (2017). Biopreservation potential of lactic acid bacteria from Andean fermented food of vegetal origin. *Food Control* 78, 393–400. doi: 10.1016/j.foodcont.2017.03.009

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REVIEW
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Technologies and Trends to Improve Table Olive Quality and Safety

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Table olives are the most widely consumed fermented food in the Mediterranean countries. Peculiar processing technologies are used to process olives, which are aimed at the debittering of the fruits and improvement of their sensory characteristics, ensuring safety of consumption at the same time. Processors demand for novel techniques to improve industrial performances, while consumers' attention for natural and healthy foods has increased in recent years. From field to table, new techniques have been developed to decrease microbial load of potential spoilage microorganisms, improve fermentation kinetics and ensure safety of consumption of the packed products. This review article depicts current technologies and recent advances in the processing technology of table olives. Attention has been paid on pre processing technologies, some of which are still under-researched, expecially physical techniques, such ad ionizing radiations, ultrasounds and electrolyzed water solutions, which are interesting also to ensure pesticide decontamination. The selections and use of starter cultures have been extensively reviewed, particularly the characterization of Lactic Acid Bacteria and Yeasts to fasten and safely drive the fermentation process. The selection and use of probiotic strains to address the request for functional foods has been reported, along with salt reduction strategies to address health concerns, associated with table olives consumption. In this respect, probiotics enriched table olives and strategies to reduce sodium intake are the main topics discussed. New processing technologies and post packaging interventions to extend the shelf life are illustrated, and main findings in modified atmosphere packaging, high pressure processing and biopreservaton applied to table olive, are reported and discussed.

Keywords: table olives, novel processing technologies, starter cultures, probiotic strains, non thermal treatments

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INTRODUCTION

Table olives, which are the most widespread fermented vegetables in the Mediterranean countries, have a great economic significance as a food commodity. Their high nutritional value, the content of bioactive compounds, dietary fibers, fatty acid composition and antioxidants make table olives a valuable functional food. Processes carried out to obtain edible table olives are aimed at the debittering of the fruit, enhancement of the sensory features and to set up chemical-physical and microbiological stability (IOOC, 2004). Agricultural practices carried out in the orchard, including pesticide treatments, soil tillage and harvesting, have an impact on the microbial and chemical quality of the starting material, before processing. From the point of harvesting, a number of pre-processing steps must be carried out to clean the surface of the fruits from soil particles,

reduce microbial load, and pesticide residues. Traditionally, olives are washed in tap or chlorinated water, and then passed to processing. Most of alternative are scarcely used in the table olives sector (washing olives with organic acids, ozonated water, etc.), while researchers are studying techniques to improve quality and safety of the material at the pre-fermentation stage (Degirmencioglu et al., 2014; Degirmencioglu, 2016). Different processing technologies give raise to very distinguishable products. While industry demands new methods to improve the processing of table olives, most of innovations are still at the research stage. The most significant advances in table olive processing are represented by the selection of bacteria and yeasts with positive technological traits (Arroyo-López et al., 2012b; Corsetti et al., 2012). In this respect, starter cultures are being presented to the market to enhance fermentation performances. Autochthonous starters are also studied to drive fermentation processes and address consumers demand for typical food with unique and irreproducible characteristics at the same time (Di Cagno et al., 2013; Campus et al., 2015; Martorana et al., 2015). Moreover, table olives are a suitable matrix to convey probiotics (Lavermicocca et al., 2005; De Bellis et al., 2010; Argyri et al., 2016; Rodríguez-Gómez et al., 2017). Innovative, mild non-thermal technologies have been developed in recent years, and some have been studied in table olives to preserve the natural features of the products to a high extent while reducing spoilage and pathogenic microrganisms (Abriouel et al., 2014; Argyri et al., 2014b). The aim of this review was to provide a consolidated view of current technologies and promising advances in the processing of table olives. Starting with the state of the art of technology, the paper will focus on pre-processing technologies, biotechnological innovations, with particular emphasis in the selection and use of microbial starters and probiotics, new processing technologies and processing plants, while the final part will describe innovative post processing preservation techniques.

TABLE OLIVE PROCESSING TECHNOLOGIES UP TO DATE

Table olives are processed with the aim of reaching an acceptable level of bitterness, to improve sensory characteristics, and to ensure safety of consumption (IOOC, 2004). Up to date, three main processing technologies are used worldwide, namely: the "Spanish style," i.e., debittering of the fruits, soaking them in diluted lye solutions, followed by washing steps to remove the excess of lye, and, finally, a partial fermentation in brine, resulting in "treated olives"; the "Californian style," in which olives are "darkened by oxidation" in lye solutions, alternated with bubbling of air, followed by packaging and retort sterilization; "Natural olives," in which the olives are put into brine and undergo a spontaneous fermentation, in which yeasts and lactic acid bacteria play a major role (Hurtado et al., 2012). The exact and complete definition of all trade and commercial preparations are described in the "Trade Standard Applying to Table Olives" (IOOC, 2004). Oleuropein is the major phenolic compound in olives, and possesses a strong bitter taste. Oleuropein is degraded to simple phenolic compounds by enzymes (β-glucosidases, esterases), both endogenous and of microbial origin (Garrido-Fernández et al., 1997; Tassou et al., 2002). Moreover, it diffuses from the fruit during lye treatments, washing and soaking in brine for fermentation.

Treated Green Olives (Spanish Style)

Spanish style green olives are obtained from fully developed green fruits collected prior to coloring. Such olives must be firm, sound, resistant to a slight pressure between the fingers, and without marks other than their natural pigmentation. The color of the epicarp may vary from green to straw yellow, according to "Reglamentación Técnico Sanitaria para la elaboración, circulación y venta de las aceitunas de mesa" (Boletín Oficial del Estado BOE, 2001). The main varieties processed according to the Spanish style method are "Manzanilla", "Hojiblanca" and "Sevillana" (Gordal). After washing and grading, the olives are soaked in a lye solution (usually between 1.3 e 2.6% w/v. NaOH). The lye treatment permeabilizes the outer tissues of the fruit, increasing the diffusion coefficient of hydrophilic compounds (Rodriguez de la Borbolla y Alcala and Rejano, 1979), from olives to solution. The effect is enhanced by increasing concentration and temperature of lye solutions (Fernández-Diez et al., 1985). Bitter compounds such as oleuropein, while diffusing, undergo an alkaline hydrolysis, resulting in simple, non-bitter, phenolic compounds. The traditional process has been subjected to studies focused on the reduction of lye solutions, the shortening of processing times while reducing damages to olives and, as a result, discards. Pretreatment with diluted lye solutions (0.3% NaOH, temperature below 25°C), prior to the main lye treatment in the processing plant, has beneficial effects on the quality of "Manzanilla" olives, especially when olives are harvested and promptly treated (Navarro Rejano et al., 2008), avoiding peeling (detachment of the outer skin from the fruits) and the occurrence of brown spots on the olives surface. Moreover, the treatment eliminates the resting time of "Manzanilla" olives (24–48 h) prior to lye treatment, usually carried out in the traditional method to reduce the occurrence of peeling. The same authors reported that the use of cold (10–14°C), diluted lye solutions allows the prompt treatment of mechanically harvested olives, without further storage. Calcium salts are used both to prevent peeling during the lye treatment and to improve the final texture. During the processing of olives, processing waste with a high pollutant potential (exhausted lye solutions and washing solutions), is produced. Moreover, the operations carried out in Spanish style processing (and in the Californian style) involve a high consumption of water, which is becoming a more and more scarce natural resource. In order to improve the sustainability of lye based processes, different authors have proposed modifications of traditional operations, with the aim of reducing waste waters, chemical and water consumption as well as energy requirements. The current trend is to reuse lye solutions for 5 to 7 cycles (Sánchez Gómez et al., 2006), before discarding. The washing step that follows the lye treatment refers to soaking the olives with tap water. The objective is to dilute the excess of alkali that penetrated into the olives to a minimum level. Washing implies the loss of soluble compounds, such as sugars, which

are required in sufficient amount to sustain the fermentation step carried out by Lactic Acid Bacteria (LAB) (Rodriguez de la Borbolla y Alcalá and Rejano, 1978). The current practice has reduced this step to one that takes 12–15 h. Fermentation in brine is the final step of the Spanish style processing. Spontaneous fermentations have been progressively substituted with the use of starter cultures. This topic will be discussed comprehensively in another part of the review.

Ripe Olives Processed by Alkaline Oxidation (Californian Style)

Californian style processed olives are produced starting with olives harvested at the green-yellow stage. Olives are stored in acidulated solutions in anaerobic conditions (USA) or aired brines added with calcium salts and organic acids (Spain) (Sánchez Gómez et al., 2006). Olives are then oxidized in horizontal stainless steel or plastic and fiber glass containers in which pressurized air is uniformly bubbled (Fernández-Diez et al., 1985) into the solution. Oxidizing treatments are interspersed with treatments with dilute lye solutions, closing the daily cycle of treatments. The aim of the lye treatments (2–7) is the same as the Spanish style. Throughout the operations the olives darken progressively, due to oxidation of ortodiphenols, hydroxytyrosol and caffeic acid (Brenes et al., 1992; García et al., 1992) to their corresponding quinones. The final lye treatment reaches the stone, then olives are washed several times with water to remove the NaOH and lower the pH in the flesh to around 8 (Fernández-Diez et al., 1985). The number of washes can be lowered by adding HCl to the liquid. It is a common practice to inject CO_2 into the containers to lower the pH to 7.5 before canning (Brenes et al., 1993). To stabilize the dark color, ferrous gluconate is added, at a concentration of 100 ppm of iron in the solution. This phase lasts about 24 h (García et al., 2001). Black olives need a sterilization treatment to ensure microbiological stability. Required lethality at 121°C is $F_0 = 15$, $z = 10$. *Clostridium botulinum* is the reference bacteria for thermal treatment of low acid foods, such as oxidized black olives (IOOC, 2004).

Natural Table Olives (Natural or Greek Style)

Natural table olives are harvested at the green-yellow stage, or fully ripened, previously washed and graded, then submerged into NaCl solutions (6–10% w/v), on which fermentation takes place, mainly due to the metabolism of autochthonous microbiota present in the olives surface and in the processing plant environment (Romero et al., 2004a). Different variables affect the process, which are both intrinsic, such as the olive cultivar used (Medina et al., 2010), the microbial species present over the fruit surface (Nychas et al., 2002), and technological, particularly brines concentration, processing temperature and disinfection practices (Tassou et al., 2002). Debittering is achieved through the diffusion from fruit to brine of the bitter compound oleuropein, and its enzymatic hydrolysis, carried out by microbial and endogenous enzymes (Garrido-Fernández et al., 1997; Tassou et al., 2002). Spoilage or even pathogenic species could develop during the first stages of the fermentation but they usually rapidly succumb to yeasts and LAB since they are more sensitive to salt concentration and acidification of brines which are determined by metabolic activity exerted mainly by LAB. The growth of LAB depends largely on the processing conditions (Abriouel et al., 2011). Yeasts, depending on the species involved, can exert both positive or negative effects (Arroyo-López et al., 2012a). Fermentations are carried out in plastic vats or fiberglass tanks, scarcely controlling salt concentration, pH and microbial evolution. Products are commercialized through packaging in bags or vats using acidified salt solutions.

PRE-PROCESSING OF OLIVES

Chlorine and Its Alternatives in the Surface Disinfection of Table Olive

To ensure microbial safety, table olive processing requires a number of pre-processing steps, such as cleaning, washing, and sanitizing. The objective of these steps are to remove parts of olive tree (stems, leaves, twigs, other debris), soil and pesticide residues, lower the temperature and reduce the undesirable microbial load of the product by thermal, non-thermal, mechanical, or chemical methods. These methods are in common with olives to be processed to obtain olive oil (Ciafardini and Zullo, 2002; Panagou, 2006; Degirmencioglu, 2011a; Degirmencioglu et al., 2011b; Vichi et al., 2015). The surface disinfection methods of olives, prior to processing, can be performed by dipping in or spraying tap water or aqueous antimicrobial solutions (weak organic acids, chlorine and its derivatives, or hydrogen peroxide), by coating with an edible substance, by physical methods, such as ultraviolet light, ultrasounds, electrolyzed water solutions (EWS), ionizing radiations.

Chlorine-Based Agents

Sodium hypochlorite (NaClO), calcium hypochlorite ($Ca(ClO)_2$), and chlorine gas (Cl_2) are extensively used to decrease the initial microbial load of olives surfaces, due to their bactericidal properties (Degirmencioglu et al., 2014; Banach et al., 2015), thus preventing spoilage. In industrial applications, chlorinated water (50–200 ppm as free chlorine, 1–2 min, and pH = 6.0–7.5) are used extensively to wash fruits and vegetables. Studies have shown that chlorine dips can reduce the population of microorganisms below 2 log colony forming unit (cfu), but it has the potential to react with organic materials to form harmful by-products, therefore it should not be used in high doses (Degirmencioglu, 2016).

Gaseous Chlorine Dioxide (ClO_2)

Chlorine dioxide, up to 3 ppm in high-pH solutions, can reach and penetrate microbial cells better than aqueous sanitizers, and its biocidal effectiveness is due to its oxidation power that is 2.5 times greater than chlorine (Allende et al., 2006; Banach et al., 2015; Food and Drug Administration (FDA), 2016a). However, olives should be rinsed with potable water after the washing step (Ciafardini et al., 1994; Degirmencioglu et al., 2014) because it can cause sensory changes (Banach et al., 2015). Treatment with

a ClO_2 wash (10 ppm) followed by dry salting with 10.0% salt, vacuum packaging, and storage at 4°C was more effective than the use of other washing solutions for controlling LAB counts and suppressing yeasts and molds growth.

Weak Organic Acids

Antimicrobial activity exerted by organic acids is attributed to pH reduction. Acidic pH conditions can alter intracellular metabolic activities of microorganisms. In this regard, the type of acid, food composition, the concentration and temperature of acid solution, and the initial microbial load of food, determine the effectiveness of treatments. In dry salted table olives, acetic and lactic acid solutions (2%) are useful as an alternative to ClO_2, when salt concentrations in the washing solutions are 5.0 and 10.0% (Degirmencioglu et al., 2014). To exert antimicrobial effect, organic acids, such as acetic or lactic acid, can be used by dipping or spraying techniques, but taking account of a possible negative impact on sensory properties, such as color, flavor, texture, and on nutritional value (Banach et al., 2015). A combination of aqueous sanitizers, such as organic acids, chlorine, chlorine dioxide, sodium chloride (Degirmencioglu et al., 2014), alone or combined with physical treatments, such as ultrasound (Ramos et al., 2013) is advisable to provide the required level of protection (Hurdle technology).

Ozone (O_3)

Ozone is a natural and strong antimicrobial agent with high reactivity and penetrability (Ramos et al., 2013), acting also on deodorization, decolorization, degradation of mycotoxins, and oxidizing pesticides (Ikeura et al., 2011; Tamaki and Ikeura, 2012; Chen et al., 2013; Bajwa and Sandhu, 2014). It is 1.5 times stronger than chlorine (Kiriş and Velioglu, 2016), and, to exhert an effective antimicrobial effect, it is recommended to use it in water containing 0.03–20.0 ppm of ozone, for up to 5 min at a slightly acidic pH (Ramos et al., 2013; Banach et al., 2015). Ozone application has been used as a post-harvest treatment and to regenerate fermentation brines in table olives, determining some microbial reductions and extending the shelf life (Segovia-Bravo et al., 2008; García-García et al., 2014; Tzortzakis and Chrysargyris, 2017). It has also the potential to reduce phenolics content in fermentation brines, responsible for their pollutant potential, lowering concentrations to 15 and 7 mg/L, in acidic (pH 4.0) and alkaline (pH 10.0) conditions, respectively (Segovia-Bravo et al., 2008).

Ultraviolet (UV) Radiation

UV radiations are classified according to their wavelengths. UV-A ranges from 315 to 400 nm; UV-B, from 280 to 315 nm, and the UV-C, from 100 to 280 nm (Prakash et al., 2000; Artés et al., 2009). UV rays act as an antimicrobial agent against pathogens due to DNA damages, appearing effective in causing cell death (Allende et al., 2006; Birmpa et al., 2013). Nonetheless, at lower doses, microorganisms can remain alive due to their repair mechanisms (Meireles et al., 2016). If the proper precautionary measures are taken, UV is a non-toxic, safe and environment-friendly treatment (Otto et al., 2011), but its use may not be practical due to the presence of organic matter of food or suspended particles in water that could absorb or shield the UV rays (Banach et al., 2015). Therefore, UV-C light (low; 1–4 kJ m^{-2} or high; 10 kJ m^{-2}) which has a synergistic effect with other treatments, should be combined with other preservation techniques such as chilling, ozonated water (10 ppm) activated with UV-light, disinfection solution, mild thermal treatments (90 or 15 min at 45°C) and MAP to preserve quality of foods (Allende et al., 2006). Although UV technology has been explored as a post harvest treatment on a wide variety of fruits and vegetables (Ribeiro et al., 2012), very few contributions can be found of its use on table olives. Cedola et al. (2013) used UV as a post processing and post packaging intervention. The UV treatment reduced the total viable count and lactic acid bacteria on Peranzana, but not on Nocellara. On the contrary, the effect on the yeasts was significant for on both olive varieties. UV was used also on packaging material, thus achieving the inactivation of yeasts and molds. Basing on these results, it could be interesting to acquire more scientific data to evaluate UV as a pre-processing technology.

Electrolyzed Water Solutions (EWS)

EWS has a strong bactericidal effect, both at acidic pH (2.1–4.5, oxidation-reduction potential (ORP) higher than 1,000 mV with presence of hypochlorous acid), or at neutral-basic pH (pH; 5.0–8.5, oxidation-reduction potential; 500–700 mV) (Ramos et al., 2013). Gök and Pazir (2011) tested the combination of sanitizing solutions with subsequent UV treatment (without brine) for disinfecting the surfaces of black Gemlik olives. EO treatment (Electrolized Oxydating water, produced by electrolysis of chlorinated water with 15–80 ppm free chlorine) reduced the microbial load up to −1.6 Log cfu/g, at 80 ppm with the product at a distance of 10 cm from the UV lamp. Although EO have a potential to be used to decontaminate olives as an alternative to the conventional ones, some authors have raised limitations to the employment of the technology due to corrosion of equipment, some detrimental effects on the quality of treated food products, and environmental and human health issues (Rahman et al., 2010). These problems are avoided using EWS produced with low chlorine concentrations, with a nearly neutral pH, by electrolysis of a dilute NaCl solution (0.9%), thus producing low concentration electrolyzed water (LcEW) (Rahman et al., 2010).

Steam Jet-Injection

With or without chemical disinfectants (chlorine), steam jet injection is a heat treatment that destroys microorganisms and inactivates enzymes. Although the heat treatment is usually applied for a short time (≈10 s) a decrease in the sensory attributes of foods (Martín-Diana et al., 2007) and a loss or reduction of the bioavailability of some nutrients (Rico et al., 2008) is observed. Studies reported that the heat treatment leads to the partial destruction of the undesirable microbiota of olives surface (10 min at 80°C), inactivates oxidative enzymes (30 min at 90°C), enhances fermentability, supports the development of probiotic cultures, during fermentation (60°C), and increases permeability of olive tissue cells (3 min at 73–74°C) (Argyri et al., 2014a; Grounta et al., 2015; Ramírez et al., 2015b).

Ionizing Radiations

X-rays, gamma-rays, and electron beams, are capable of producing ions and electrically charged atoms or molecules, acting on water, that forms free radicals, which destroy or inhibit microorganisms (Allende et al., 2006; Ramos et al., 2013). Despite that this method is quite effective in microbial growth control, FDA only approves the use of a maximum level of 1.0 kGy to decontaminate vegetables, to designate them as "fresh" (Ramos et al., 2013). Furthermore, this physical method (0.20–1.00 kGy) has been combined with chemical methods such as chlorinated water (80–200 ppm) and/or by using MAP. These combinations are more efficient than irradiation or chlorination alone, reducing microbial load without adversely affecting sensorial quality of foods (Allende et al., 2006; Ramos et al., 2013; Meireles et al., 2016). Tokuşoğlu (2017) applied gamma irradiation as a post packaging treatment to "Gemlik" olives. The treatment determined a decrease in some nutrients (α-tocopherol, total chlorophyll, and total carotene) during 8 months of shelf life observations.

Ultrasounds (US)

Ultrasounds are sonic waves at high amplitude (Otto et al., 2011; Misra, 2015; Meireles et al., 2016) that form cavitation bubbles (Seymour et al., 2002). These bubbles collapse generating the mechanical energy responsible for the disinfecting action (detachment), and the free radicals formation (destruction) (Seymour et al., 2002; Sagong et al., 2011). On the other hand, this treatment is not significantly effective on reducing high level microbial contamination, despite being safe and environmentally friendly (Meireles et al., 2016). Therefore, it should be used in combination with aqueous disinfectants, such as organic acid (Ramos et al., 2013), chlorinated water (Seymour et al., 2002), hot water (55°C) (Allende et al., 2006), which improve the effectiveness of these methods (Seymour et al., 2002; São José et al., 2014). Current literature on olive fruit surface decontamination with US and ionizing irradiations requires more data before their application in table olive technology can be adopted by producers. Remarkably, ultrasound treatment is able to speed up and promote NaOH-free olive debittering, without causing any undesirable changes (Habibi et al., 2015, 2016). Ultrasound is an applicable novel technique minimizing debittering time of olive fruits and decreasing NaOH concentration, thus reducing the number of wash-cycles required for the completion of debittering, and at the same time, resulting in substantial reduction in water usage.

Removal/Reduction of Pesticide in Table Olive

Pesticides are widely used in commercial agriculture to fight weeds, molds, and pests, and increase farm productivity (Kiriş and Velioglu, 2016). Most pesticide residues are retained on the surface of fruits and vegetables peels (Bajwa and Sandhu, 2014; Kiriş and Velioglu, 2016), and as part of Good Agricultural Practice (GAP), pesticides should be implemented at the applicable doses. Attention should be paid to the time between the application of pesticide and harvesting (González-Rodríguez et al., 2011; Bajwa and Sandhu, 2014). Pesticides can be classified as hydrophilic or lipophilic. The latter exhibit higher residual levels in food due to their non-solubility in water (Ikeura et al., 2011; Iizuka and Shimizu, 2014), and most of pesticides residues are retained on fruit and vegetable surface, and may be also absorbed into the flesh (Bajwa and Sandhu, 2014). The various food processing methods such as washing (added with strong oxidizing agents, such as ozone, chlorine dioxide), peeling, brushing, blanching, cooking, boiling, etc., can be used to reduce pesticide contamination (González-Rodríguez et al., 2011; Bajwa and Sandhu, 2014; Misra, 2015; Degirmencioglu, 2016; Qi et al., 2018).

Agrochemicals are used extensively to decrease losses (García-Reyes et al., 2007; Amvrazi and Albanis, 2009; Gómez-Almenar and García-Mesa, 2015). Nevertheless, the misuse of pesticides by farmers can lead to a possible contamination, entailing a risk for the health of consumers and the environment, because olives spontaneously fallen to the ground have higher pesticide levels residues than olives collected directly from the tree (Guardia-Rubio et al., 2006a,b; García-Reyes et al., 2007; Kaushik et al., 2009; Ikeura et al., 2011; Bajwa and Sandhu, 2014; Gómez-Almenar and García-Mesa, 2015).

Results of researches on removal of pesticide residues from olive products documented that pesticides, adsorbed on the dust and adhering to the fruit, may be removed, from no reduction to a 45% decrease, depending on the efficiency of the washing step applied (Cabras et al., 1997; Guardia-Rubio et al., 2006a, 2007a,b,c; García-Reyes et al., 2007). At this stage, the effectiveness of the washing process can be improved by cleaning washing machines every day and changing the washing water with fresh water at the starting of the day (Ruiz-Medina and Llorent-Martínez, 2012), and it is enhanced by using chemical agents (Degirmencioglu, 2016). For that purpose, chlorine based products (10–500 ppm), ozone (1–3 ppm), electrolyzed oxidizing water (EO) alone or with combined chlorine solutions (120 mg/L chlorine, for 15 min), hydrogen peroxide (10–100 ppm), weak and strong acids, potassium permanganate ($KMnO_4$, 0.001%), NaCl solution (5–10%), baking soda ($NaHCO_3$, 5–10%), vinegar (0.1%) for several minutes, and ultrasonic cleaners (García-Reyes et al., 2007; Kaushik et al., 2009; Bajwa and Sandhu, 2014; Gómez-Almenar and García-Mesa, 2015; Qi et al., 2018) can be effectively used.

Experiences of cleaning technologies applied in the olive oil sector can be translated to the table olive processing. Agrochemicals concentrates in washing water during cleaning, decreasing the washing efficiency over time. The use of $FeCl_3$ as coagulant, and activated charcoal (200 mg/L) as adsorbent, is sufficient to eliminate pesticide residues in washing water (Guardia-Rubio et al., 2008).

Chlorine dioxide (ClO_2) is the most powerful oxidizing agent, and several reports have shown that it can remove or reduce pesticides from fruit and vegetable surfaces. The effectiveness in aqueous solutions depends on its concentration, pH, treatment time and temperature, thickness of olives peel, the initial concentration of pesticide on olives peel, and the types of pesticide (Hwang et al., 2002; García-Reyes et al., 2007; Chen et al., 2014; Kiriş and Velioglu, 2016).

Ozone (O_3) is a natural substance in the atmosphere and it is generated through a high voltage electrical discharge or by UV light irradiation (Tamaki and Ikeura, 2012). There are several reports on the use of ozone to remove pesticides in foods without causing flavor changes, by immersion in ozonized solutions. It is reported that high pH values, high ozone dosages, combined with ultrasound or UV light are favorable for pesticide degradation (Xiong et al., 2011; Tamaki and Ikeura, 2012; Misra, 2015). However for better removal efficiency, the ozone dosage, treatment time and temperature, bubble size of ozone, and product geometry should be standardized (Wu et al., 2007; Misra, 2015). Kiriş and Velioglu (2016) studied key olive oil processing factors that influence the removal efficiency of pesticide residues with ozone, and they found that ozonated water wash cycles (2 and 5 min) efficienctly removed pesticides, depending on the structural properties of the pesticide itself, olive fruit variety, oil and water content, and oil extraction technology.

The application of intense UV light also can promote the photodegradation of some pesticides by direct photolysis due to their potential to absorb light (Nieto et al., 2009). The authors attempted to develop a simple UV immersion system (200–280 nm, 150 W) to reduce the amount of pesticides in virgin olive oil depending on the treatment time and temperature (15, 20, 25, and 30°C). While these results indicated the possibility of using UV light as an effective, low-cost process for the destruction of pesticides in olive oil and in table olives, no further progress has been reported in this regard.

Ultrasound (US) degradation of pesticides has received much attention in the last years, but the use of US technology for pesticide degradation still remains under-researched. All the studies have indicated the US power (frequency, intensity, amplitude), ambient conditions (temperature, pressure, treatment time), product properties (viscosity and surface tension) and chemical structure of the pesticide, to be the most significant factors influencing the degradation of pesticides. However, long treatment times such as 1-2 h should be performed to achieve significant reduction in pesticide concentration, resulting in significant loss of bioactive compounds and worsening of chemical quality (Misra, 2015).

BIOTECHNOLOGICAL INNOVATIONS

Starter Cultures

Fermentation can occur spontaneously, as a result of the development of the microflora colonizing the environment, the raw material, and the tools with which food is obtained and manipulated, or it can be performed using part of a previous successful fermentation batch to inoculate a new batch (back-slopping), or by addition of starter cultures (Leroy and De Vuyst, 2004). Microorganisms used to obtain fermented foods belong to several taxonomic groups, mostly to LAB, both homo- and heterophermentative, and others such as micrococci, propionibacteria, yeasts, and molds (Bavaro et al., 2017; Bonatsou et al., 2017). It is possible to distinguish selected and natural starter cultures. Selected starter cultures (SSC), which are simpler than natural ones, made up of a low, defined number of species and strains (sometimes they are monocultures), chosen for their strong aptitude for fulfilling the biochemical processes required by each production technology and for their suitability to be grown at a laboratory. SSC are widely applied to different fermented food productions that no longer posses geographic niches and typicity. Since selected strains have strong technological and growth abilities, they become easily dominant over natural microflora when added to raw material in high concentration. The consequent dramatic decrease of microbial biodiversity results in loss of manifold peculiar characteristics and flattening of fermented food sensory profile (Corsetti et al., 2012; Martorana et al., 2015). Industrial level productions are often driven by SSC (Hurtado et al., 2012).

Natural starter cultures (NSC) are made up of microorganisms that colonize production environments, tools and raw materials.

NSC are complex and undefined cultures, consisting of an indefinite number of species and strains, starter and non-starter, coexisting in equilibrium. NCS strains composition is not reproducible in a place other than that of their origin, thus they are thought to be capable of enriching products with peculiar sensory features that bind them to the territory (Leroy and De Vuyst, 2004; Bassi et al., 2015). Usually, NSC characterize the most typical and high quality agri-food products (Martorana et al., 2015).

The main advantages of their use stem primarily from their high biodiversity. Indeed, a large number of strains are capable of following different and complementary metabolic pathways, so that the development of one becomes functional to the development of another, mutually enhancing their abilities (Gatti et al., 2004).

Moreover, biodiversity grants resistance to phages infections on natural cultures, which are usually strain-specific. Indeed, even if one out of the many strains present in the microbial community succumbs to viral attacks, other phage-insensitive individuals will survive and be able to fill the functions that have been carried out by the infected strain until then. On the contrary, fermentations relying on SSC, often made up of one or two strains (Heperkan, 2013), can be impaired by a phage attack, with fatal consequences on the final product (Lanza, 2013).

The main pros and cons of each kind of culture, which should be borne in mind in order to choose the most suitable starter, are summarized in **Table 1**.

Among vegetables, table olives are the most widespread fermented food in Western countries, particularly in the Mediterranean area (Panagou et al., 2008; Wacher et al., 2010; Aponte et al., 2012; Bonatsou et al., 2015, 2017; Tufariello et al., 2015). Although the use of starters is widely diffused in the production of many fermented foods (dairy, meat, sourdough), it is still relatively limited in table olives and in vegetable fermentation, in general (Di Cagno et al., 2008; Aponte et al., 2012; Heperkan, 2013; Iorizzo et al., 2016; Bonatsou et al., 2017). Important table olive varieties, such as Kalamáta, Conservolea, Manzanilla, Sevillana, Hojiblanca, Bella di Cerignola and Ascolana Tenera, are mainly still processed without starter cultures addition (Panagou et al., 2008; Aponte et al., 2010, 2012; Bevilacqua et al., 2010, 2015; Corsetti et al., 2012; De Angelis et al., 2015; Rodríguez-Gómez et al., 2017). However, interest in SSC is increasing in industrial table

TABLE 1 | Pros and cons of selected and natural starter cultures.

Type of culture	Pros	Cons
Selected	Easy to prepare and manage	Poorly flexible system
	Easy check for purity and activity	High sensitivity to phage attack, cultures rotations are needed
	Standardized activity	Not suitable for many PDO, traditional and typical products
Natural	Low sensitivity to phage attack	Problems in the case of predominance of low performances strains
	Better adaptability to the raw material to be processed	Difficult to standardize natural starter culture reproduction, concentrations and performances
	Symbiotic effects	Original balance among species and strains difficult to preserve and check
	Unique sensory quality of the product (the only allowed for many PDO, traditional and typical products)	Difficulties in products sensory quality standardization

olives production in Spain, other Mediterranean countries and Argentina (Heperkan, 2013).

Vegetable fermentations are difficult to control, mainly because this kind of raw material cannot be thermal treated without resulting in damage of the product texture, and abnormal phenomena are usually kept under control merely adequately modulating process parameters, such as salt and pH. Starter application, mainly aimed to quicken fruit debittering, contributes to prevent the development of spoilage and pathogenic bacteria, prolonging olive preservation by brine acidification, and improving their sensory properties (Romeo, 2012).

When selecting microorganisms for table olive fermentation, there are many factors to be taken into account. First, the preferability of the species changes depending on the cultivar, type of processing, and geographic area the raw material come from Argyri et al. (2016). Furthermore, microorganism growth rate, ability to survive brine adverse conditions (low temperature, high pH values and NaCl concentration), adhere to olive cuticle, and survive freeze-drying process are criteria to be followed when selecting strains to be used as starter cultures (Heperkan, 2013). Moreover, of no lesser importance, the resistance to organic acids and polyphenols, ability of rapidly metabolizing fermentable substrates and oleuropein, aptitude to produce aromas, synthesize bacteriocins, and colonize the brine by countering the development of undesired microorganisms (i.e., *Enterobacteriaceae*, *Clostridium*, *Pseudomonas*, *Staphylococcus,* and *Listeria*), must be considered (Bevilacqua et al., 2010; Corsetti et al., 2012; Bleve et al., 2015).

Besides the technological efficency of microorganisms, as for all the substances intentionally added to foods, their safety must be checked and proven, unless they are recognized among qualified experts, as having been adequately shown to be safe under the conditions of their intended use (Generally Recognized As Safe, GRAS) (Food and Drug Administration (FDA), 2016b), or have the QPS (Qualified Presumption of Safety) status (European Food Safety Authority [EFSA], 2017).

In literature dealing with biodiversity of table olive microflora, it is possible to find exhaustive lists of bacteria, yeasts and molds species, identified both with classical and molecular, culture-dependent and –independent, techniques (Bautista-Gallego et al., 2011b, 2013a; Botta and Cocolin, 2012; Corsetti et al., 2012; Heperkan, 2013; Campus et al., 2015; De Angelis et al., 2015; Bavaro et al., 2017; Bonatsou et al., 2017; Comunian et al., 2017).

Table olive starter cultures, so far mainly developed for Spanish-style processing, are made up of one or two strains belonging to facultative heterofermentative mesophilic lactobacilli species, above all *Lactobacillus plantarum, Lactobacillus pentosus*, less frequently, *Lactobacillus paraplantarum*, *Lactobacillus casei*, *Lactobacillus brevis*, *Leuconostoc mesenteroides*, *Pediococcus acidilactici* and, more rarely, Enterococci. In some cases, bacteria have been associated with yeasts, such as *Debaryomyces spp.*, *Saccharomyces spp.*, *Candida spp., Pichia spp.*, *Rhodotorula spp.*, in order to favor and enhance their development (De Castro et al., 2002; Corsetti et al., 2012; Heperkan, 2013; Argyri et al., 2016; Bonatsou et al., 2017.

Autochthonous LAB Starter Cultures

As stated above, the use of starters in table olive fermentation is quite recent and not common yet. Back-slopping, a technological expedient borrowed from other fermented food technologies (such as dairy products, sourdough and wine), relying on autochthonous microflora, has been the first attempt to improve and speed up table olive fermentation process, and can be considered a precursor of the starter culture method (Aponte et al., 2012; Corsetti et al., 2012). However, this practice is not risk-free. Indeed, together with useful autochthonous microflora, even pathogen or spoilage bacteria could be potentially inoculated, and allowed to reach high and dangerous concentrations in the new food batch if the fermentation is unsuccessful. This is why back-slopping is being progressively abandoned in various fermentation processes and, in some cases, it has become even illegal (New Zealand Food Safety Authority [NZFSA], 2008).

In recent years, the consumer demand for more traditional and homemade products, with unique characteristics, different and distinguishable from the others present on the market, is increasing (Corsetti et al., 2012; Medina et al., 2016). To satisfy this demand, overcome the back-slopping drawbacks and avoid the standardization of the product as a result of using commercial SSC, it has been thought to isolate autochthonous strains from successful natural fermentations, test them for their technological abilities, and use the most promising strains as starters. Indeed, autochthonous strains are generally considered to be more adapted to the microflora naturally present in the raw material to be processed than allochthonous ones, and therefore they are able to dominate the microbiota, counteract spoilage microflora, and drive the fermentation from the very early phases of the process (Di Cagno et al., 2008;

Aponte et al., 2012; Bevilacqua et al., 2015). Furthermore, autochthonous starters cultures (ASC) are the most suitable for PDO, IGP productions (often the only starters allowed by PDO specifications), as they are considered to be able to bind the product to the region where it comes from. Notwithstanding this, ASC are still less widespread than commercial SSC, particularly in table olive production, and there are only a few studies dealing with autochthonous starter selection (Leal-Sánchez et al., 2003; Di Cagno et al., 2008, 2013; Aponte et al., 2012; Campus et al., 2015; Tataridou and Kotzekidou, 2015; Tufariello et al., 2015). However, it should be kept in mind that the use of 1–3 selected strains, though autochthonous, is not a guarantee of good fermentation performances and counteraction of spoilage/pathogenic microflora, nor results in peculiar qualities that make the product clearly distinguishable from other analogous ones produced in different geographical areas (Aponte et al., 2012; Durante et al., 2017). As reported above, NSC, because of the complexity of strains that make them up, with different technological abilities and metabolic pathways, are considered as the most suitable starter to grant a specific identity to a fermented food (Leroy and De Vuyst, 2004; Bassi et al., 2015). Martorana et al. (2015) recently reported that ASC can be effectively used to drive the fermentation process of Nocellara del Belice olives, when used as a "Pied de cuve," both strengthened or not with the inoculum of authoctonous *L. pentosus* strain.

Therefore, the back-slopping strategy can be a good starting point to be enhanced and implemented following a new technological approach, in order to keep its advantages and avoid the drawbacks. A compromise between back-slopping and a SSC (both allochtonous and autochthonous) might be a "semi-natural" ASC consisting of an undefined number of strains. The Selected Inoculum Enrichment (SIE) method, recently proposed and applied to *Tonda di Cagliari* table olives (a cultivar of Sardinia island, Italy) represents a new concept in the use of LAB starters in table olive processing (Campus et al., 2015, 2017; Comunian et al., 2017). Indeed, with the SIE method the number of desirable microorganisms initially present in the brined olives was increased, ensuring a more reliable and faster process than spontaneous fermentation, inoculating only useful bacteria. This complex culture was added to the natural microflora of a new fermentation process without operating any previous selection of particular strains.

Such a mix of autochthonous strains have undergone, as a whole, a natural selection that have made them more adapted to the specific brine conditions. It was observed to be able to counteract spoilage microflora and preserve the product better than allochthonous SSC and natural microbiota, colonizing the environment from the very early stage of fermentation until its end (Campus et al., 2015; Comunian et al., 2017). Therefore, SIE could be a successful technology to be applied at an industrial scale to obtain fermented products, not only table olives, as good as the spontaneously fermented ones, while still guaranteeing safety, quality constancy and reproducibility.

Yeasts With Positive Technological Traits

Yeasts are part of table olive natural microbiota, coexisting with LAB during the whole fermentation process, but with a wider species biodiversity.

The yeast species detected in table olive fermentation, their prevalence and dynamics change depending on the geographic area, cultivar, interaction with other microorganisms (LAB) and processing technology (salt concentration, temperature, pH, nutrients, oxygen) (Bleve et al., 2014; Leventdurur et al., 2016). In general, higher salt concentrations and phenolic compounds or low pH levels favor yeast development to the detriment of LAB (Bautista-Gallego et al., 2015; Benítez-Cabello et al., 2015; Porru et al., 2018). Therefore, their involvement is particularly important in natural olives, when fruits are not lye treated and phenolic compounds partly inhibit LAB development (Arroyo-López et al., 2012b). Moreover, the presence of the various yeasts species was observed to vary even during the whole fermentation time (their number increased during the fermentation), because of and depending on their abilities to adapt to the evolution of the physico-chemical conditions of olives and brines (Pereira et al., 2015; Arroyo-López et al., 2016).

A list of the yeast species most frequently reported in recent literature (from 2006 up today), as part of the microbiota colonizing 20 table olives cultivars, processed in 6 countries (Spain, Portugal, Greece, Italy, Tunisia, and Turkey), is reported in **Table 2**.

Candida boidinii, Debaryomyces hansenii, and *Pichia membranifaciens* resulted the most geographically diffused species, since they were detected in all the countries, but one (*D. hansenii* was not detected in Turkey, while *C. boidinii* and *P. membranifaciens* were not in Tunisia). Among the other species reported in **Table 2**, *Candida*, *Pichia* (included *Wickerhamomyces anomalus,* current name of *Pichia anomala)*, and *Saccharomyces* were the most frequent genera detected. The other genera (apart from *Rhodotorula*, which was detected in 4 cultivars from Greece, Portugal and Spain), were found only in 1–2 cultivars.

The presence of yeasts can have positive or negative consequences on table olive sensory characteristics and shelf-life; (Nisiotou et al., 2010; Arroyo-López et al., 2012b,c). Indeed, though they can be responsible of clouding of brines, off-flavors, off-odors, and gas-pocket defects. For instance, among frequently isolated species strains of *S. cerevisiae* and *W. anomalus* have been thought to be responsible for producing high amounts of CO_2, causing gas-pocket defects, while *D. hansenii* and some species of *Rhodotorula* genus showed the ability to degrade fruits cell wall polysaccharides or to produce polygalacturonases causing olives softening (Arroyo-López et al., 2008).

However, strains belonging to the same yeast species can significantly contribute to the improvement of table olive quality and safety. Indeed, they have a role in: (a) favoring useful bacteria growth, thus enhancing lactic acid production needed to preserve the product from spoilage microorganisms; (b) acting as biocontrol agents against fungi and other undesirable yeast and bacteria species; (c) producing compounds that positively affect flavor and texture (i.e., ethanol, glycerol, esters,

TABLE 2 | List of the most frequently cited yeasts species in recent literature.

Species	Country and cultivar						References
	Greece	Italy	Portugal	Spain	Tunisia	Turkey	
Aureobasidum pullulans	Conservolea		Manzanilla				Nisiotou et al., 2010; Alves et al., 2012
Bullera variabilis					Greek style black olives		Fendri et al., 2013
Candida cf apicola				Aloreña			Abriouel et al., 2011
Candida atlantica						Gemlik	Leventdurur et al., 2016
Candida boidinii	Conservolea	Bosana, Nocellara del Belice, Leccino, Istrana nera, Peranzana, Cellina di Nardò	Negrinha de Freixo	Arbequina, Aloreña		Gemlik	Arroyo-López et al., 2006; Hurtado et al., 2008; Nisiotou et al., 2010; Tofalo et al., 2013; Bleve et al., 2014; Martorana et al., 2015; Pereira et al., 2015; Leventdurur et al., 2016; Porru et al., 2018
Candida butyri/aaseri	Conservolea		Manzanilla	Spanish style green olives, Arbequina		Gemlik	Hurtado et al., 2008; Nisiotou et al., 2010; Alves et al., 2012; Lucena-Padrós et al., 2014; Leventdurur et al., 2016
Candida diddensiae		Bosana, Nocellara del Bellice	Manzanilla	Arbequina, Aloreña			(Arroyo-López et al., 2006; Hurtado et al., 2008; Alves et al., 2012; Martorana et al., 2015; Porru et al., 2018)
Candida glabrata				Manzanilla			Hernández et al., 2007
Candida holmii				Aloreña			Arroyo-López et al., 2006
Candida humicola				Manzanilla			Hernández et al., 2007
Candida inconspicua				Manzanilla			Hernández et al., 2007
Candida ishiwadae		Cellina di Nardò					Tofalo et al., 2013
Candida maris				Manzanilla			Hernández et al., 2007
Candida membranifaciens		Nocellara del Belice		Arbequina			Hurtado et al., 2008; Martorana et al., 2015
Candida norvegica			Negrinha de Freixo				Pereira et al., 2015
Candida oleophila			Manzanilla				Alves et al., 2012
Candida parapsilosis		Brandofino, Manzanilla, Nocellara del Belice, Passanulara		Arbequina; Manzanilla			Hernández et al., 2007; Hurtado et al., 2008; Aponte et al., 2010
Candida rugosa				Manzanilla			Hernández et al., 2007
Candida silvae	Conservolea						Nisiotou et al., 2010
Candida sorbosa				Manzanilla			Arroyo-López et al., 2012a
Candida sp.	Halkidiki						Grounta et al., 2015
Candida tartarivorans		Cellina di Nardò					Bleve et al., 2014
Candida thaimueangensis				Spanish style green olives			Lucena-Padrós et al., 2014
Candida tropicalis			Negrinha de Freixo				Pereira et al., 2015
Candida zeylanoides				Manzanilla			Hernández et al., 2007
Cryptococcus laurentii				Manzanilla	Greek style black olives		Hernández et al., 2007; Fendri et al., 2013

(Continued)

TABLE 2 | Continued

Species	Country and cultivar						References
	Greece	Italy	Portugal	Spain	Tunisia	Turkey	
Cryptococcus albidus					Greek style black olives		Fendri et al., 2013
Cystofilobasidium capitatum	Conservolea						Nisiotou et al., 2010
Cyteromyces matritensis			Manzanilla				Alves et al., 2012
Debaryomyces carsonii		Cellina di Nardò					Bleve et al., 2014
Debaryomyces etchellsii		Leccino					Bleve et al., 2014
Debaryomyces hansenii	Conservolea, Halkidiki, Kalamàta	Cellina di Nardò	Negrinha de Freixo	Manzanilla	Greek style black olives		Hernández et al., 2007; Nisiotou et al., 2010; Fendri et al., 2013; Bleve et al., 2014, 2015; Grounta et al., 2015; Pereira et al., 2015
Dekkera bruxellensis				Aloreña	Greek style black olives		Hernández et al., 2007; Fendri et al., 2013
Galactomyces reessii			Negrinha de Freixo				Pereira et al., 2015
Geotrichum candidum				Manzanilla; Aloreña			Arroyo-López et al., 2006, 2012a
Hanseniaspora guilliermondii				Aloreña			Arroyo-López et al., 2006
Issatchenkia occidentalis				Aloreña			Arroyo-López et al., 2006
Kluyveromyces lactis				Arbequina			Hurtado et al., 2008
Kluyveromyces marxianus				Manzanilla			Hernández et al., 2007
Metschnikowia pulcherrima	Conservolea						Nisiotou et al., 2010
Meyerozyma sp.						Gemlik	Leventdurur et al., 2016
Nakazawaea molendini-olei		Bosana					Porru et al., 2018
Pichia anomala (current name *Wickerhamomyces anomalus*)	Conservolea, Kalamàta	Bosana, Nocellara del Belice, Cellina di Nardò, Bella di Cerignola		Arbequina, Manzanilla	Gemlik		Hernández et al., 2007; Hurtado et al., 2008; Nisiotou et al., 2010; Tofalo et al., 2013; Bleve et al., 2014, 2015; Grounta et al., 2015; Martorana et al., 2015; Leventdurur et al., 2016; Porru et al., 2018
Pichia galeiformis		Peranzana, Nocellara del Belice, Cellina di Nardò		Gordal, Manzanilla			Arroyo-López et al., 2012a; Tofalo et al., 2013; Benítez-Cabello et al., 2015
Pichia guilliermondii	Halkidiki, Conservolea	Castriciana, Manzanilla, Passanulara	Negrinha de Freixo	Manzanilla			Hernández et al., 2007; Aponte et al., 2010; Nisiotou et al., 2010; Grounta et al., 2015; Pereira et al., 2015
Pichia kluyveri	Conservolea	Brandofino, Castriciana, Manzanilla, Nocellara del Belice, Passanulara		Arbequina			Hurtado et al., 2008; Aponte et al., 2010; Nisiotou et al., 2010
Pichia kudriavzevii					Greek style black olives	Gemlik	Leventdurur et al., 2016
Pichia farinosa							Fendri et al., 2013
Pichia manshurica	Conservolea	Peranzana, Nocellara del Belice, Cellina di Nardò	Negrinha de Freixo				Nisiotou et al., 2010; Tofalo et al., 2013; Pereira et al., 2015

(Continued)

TABLE 2 | Continued

Species	Country and cultivar						References
	Greece	Italy	Portugal	Spain	Tunisia	Turkey	
Pichia membranifaciens	Conservolea, Kalamàta	Cellina di Nardò e Leccino	Negrinha de Freixo	Gordal, Arbequina	Greek style black olives		Hurtado et al., 2008; Nisiotou et al., 2010; Fendri et al., 2013; Bleve et al., 2014, 2015; Benítez-Cabello et al., 2015; Pereira et al., 2015
Pichia minuta				Arbequina			Hurtado et al., 2008
Pichia rhodanensis				Arbequina			Hurtado et al., 2008
Rhodotorula diobovatum	Conservolea						Nisiotou et al., 2010
Rhodotorula glutinis			Negrinha de Freixo	Arbequina, Manzanilla, Aloreña			Arroyo-López et al., 2006; Hernández et al., 2007; Hurtado et al., 2008; Pereira et al., 2015
Rhodotorula graminis			Negrinha de Freixo	Aloreña			Arroyo-López et al., 2006; Pereira et al., 2015
Rhodotorula minuta				Manzanilla			Hernández et al., 2007
Rhodotorula mucilaginosa	Conservolea		Manzanilla	Spanish style green olives			Nisiotou et al., 2010; Alves et al., 2012; Lucena-Padrós et al., 2014
Saccharomyces cerevisiae	Kalamàta, Conservolea	Bosana, Cellina di Nardò, Leccino, Istrana nera/bianca, Peranzana, Nocellara del Belice, Bella di Cerignola	Negrinha de Freixo, Manzanilla	Spanish style green olives, Aloreña, Manzanilla			Arroyo-López et al., 2006; Hernández et al., 2007; Nisiotou et al., 2010; Abriouel et al., 2011; Alves et al., 2012; Tofalo et al., 2013; Bleve et al., 2014, 2015; Lucena-Padrós et al., 2014; Pereira et al., 2015; Porru et al., 2018
Saccharomyces sp.						Gemlik	Leventdurur et al., 2016
Saturnispora mendoncae				Spanish style green olives			Lucena-Padrós et al., 2014
Schwanniomyces etchellsii						Gemlik	Leventdurur et al., 2016
Sporobolomyces roseus					Greek style black olives		Fendri et al., 2013
Torulaspora delbruekii				Manzanilla	Greek style black olives		Hernández et al., 2007; Fendri et al., 2013
Trichosporum cutaneum				Manzanilla			Hernández et al., 2007
Zygoascus hellenicus						Gemlik	Leventdurur et al., 2016
Zygosaccharomyces bailii				Aloreña	Greek style black olives		Arroyo-López et al., 2006; Fendri et al., 2013
Zygosaccharomyces mrakii		Leccino	Manzanilla				Alves et al., 2012; Bleve et al., 2014
Zygotorulaspora mrakii		Bosana					Porru et al., 2018
Zygowilliopsis californica	Conservolea						Nisiotou et al., 2010

other volatile compounds, free fatty acids); (d) biodegrading polyphenols (such as oleuropein which gives olives a bitter taste); (e) preventing unsaturated fatty acid oxidation and peroxide formation (catalase positive yeast strains) (Arroyo-López et al., 2008, 2012b; Bevilacqua et al., 2013; Bleve et al., 2014).

In recent years, the interest in studying and developing yeast starter cultures for table olive processing, both alone or in combination with LAB is growing (Psani and Kotzekidou, 2006; Arroyo-López et al., 2008, 2012b; Nisiotou et al., 2010; Bevilacqua et al., 2013, 2015). Even a sequential inoculation strategy (firsly yeasts, then bacteria) was developed to take advantage of the beneficial effect exerted by yeast on LAB growth (Tufariello et al., 2015). Furthermore, yeasts ability to favor mixed species (bacteria and yeast) biofilm formation, both on biotic (drupes) and abiotic (fermenter vats) surfaces, was extensively studied in table olives processing. *C. boidinii*, *C. sorbosa*, *D. hansenii, Geotrichum candidum*, *Pichia galeiformis*, *P. guilliermondii* and *W. anomalus* were the yeast species proved to be the most effective ones in favoring mixed species biofilm formation (particularly on olive skins) especially in combination with *Lb. pentosus*. Indeed, they displayed capability to develop a propitious environment assuring *Lb. pentosus* survival, enhancing its development and starter activity (Arroyo-López et al., 2012a; Domínguez-Manzano et al., 2012; Grounta and Panagou, 2014; Benítez-Cabello et al., 2015; Grounta et al., 2015; León-Romero et al., 2016; Porru et al., 2018). LAB and yeast populations of the biofilms adhering to the olives skin were estimated in about 6–7 and 3–5 log cfu/g, respectively (Grounta and Panagou, 2014; Benítez-Cabello et al., 2015), while those colonizing olive fermentation vessels were quantified in 3.0–4.5 and 4.0–4.6 log cfu/cm^2 (LAB and yeast counts, respectively). Biofilm populations, particularly yeasts, were able to survive cleaning treatments of vessels and were still alive even when vessels were left to dry for 60 days (between 2.76 and 3.94 log cfu/cm^2, while LAB were not detectable; Grounta et al., 2015).

Furthermore, it is noteworthy that the genera/species most frequently isolated from not inoculated brines and drupes are also the ones that showed many of the positive technological traits that make yeast strains good candidates as starter culture for table olives. Indeed, *C. boidinii* which belongs to the most widespread genuswas recognized to have a positive effect on olive flavor, by means of the formation of esters from drupes free fatty acids thanks to its strong lipase and esterase activity (Hernández et al., 2007; Rodríguez-Gómez et al., 2010; Bautista-Gallego et al., 2011b; Arroyo-López et al., 2012c; Pereira et al., 2015). While, *S. cerevisiae* and several species of the *Pichia* genus (catalase positive yeasts) showed antioxidant activity, useful to protect fruits from unsaturated fatty acid oxidation and peroxide formation (Hernández et al., 2007). Strains of *W. anomalus*, a species with a strong tendency to grow in harsh environmental conditions, such as low pH and high NaCl concentrations, in addition to high lipase and esterase activity, showed strong β-glucosidase activity (Bautista-Gallego et al., 2011b; Arroyo-López et al., 2012c; Romero-Gil et al., 2013; Bonatsou et al., 2015). Therefore, it contributes both to fruit debittering by phenolic compounds biodegradation, and reduction of the large quantities of olive wastewater produced when a lye treatment is applied (Arroyo-López et al., 2012c).

Among the other frequently isolated species, strains of *D. hansenii* and *S. cerevisiae* were reported, like many other less common yeasts species, to favor LAB development, both synthesizing vitamins, aminoacids and purins, or metabolizing complex carbon sources (Arroyo-López et al., 2012c). Moreover, *D. hansenii*, as *P. membranifaciens*, *C. boidinii*, and *W. anomalus*, showed strain-specific killer activity (toxic proteins or glycoproteins production) against spoilage yeasts (Psani and Kotzekidou, 2006; Arroyo-López et al., 2008, 2012c; Hernández et al., 2008). When selecting starter strains, this last one is an important feature to be investigated and searched foras well as the technological traits, because it allows to reduce salt content or chemical preservatives use, thus obtaining a more natural product with an improved shelf-life (Arroyo-López et al., 2012c).

Table Olives as Carrier and Source of Probiotic LAB

Probiotics are defined as "Live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (FAO/WHO, 2001). Their claimed benefits are numerous. In fact, they are believed to be capable of: equilibrating the intestinal microbiota, thus reducing the risk of gastro-intestinal diseases; improving nutrients bioavailability; prevent or reduce allergies. Furthermore, they are presumed to have antimutagenic, anticarcinogenic (reduction of the risk of colon, liver, and breast cancers), hypocholesterolemic, antihypertensive, anti-osteoporosis, immunomodulatory effects (Behnsen et al., 2013; Kechagia et al., 2013; Papadimitriou et al., 2015). However, despite the increase of studies documenting these effects for strains isolated from different sources, benefits of foods containing probiotics are not taken for granted, but must be scientifically proven and certificated by the authorities in charge, before they can be claimed on the food labels. The European Parliament and Council of The European Union (2006), issued the EC Regulation N. 1924/2006 in order to protect consumers and facilitate their choice. At present, the only probiotic health claim approved for foods in EU, by the European Food Safety Authority (EFSA), is that related to live yogurt cultures, recognized to be able to "improve lactose digestion of the product in individuals who have difficulty digesting lactose" [EFSA, 2010]. In regard to USA, probiotics are not considered "health claims," but "structure/function claims" (for instance "promote digestion" or "help maintain normal cholesterol levels"). Thus, while health claims need a pre-approval by US Food and Drug Administration (FDA), before the product is put on the market, the structure/function claims do not. However, manufacturers are responsible for their veracity and their label must show the following disclaimer: "This statement has not been evaluated by the Food and Drug Administration. This product is not intended to diagnose, treat, cure, or prevent any disease" (Hoffmann, 2013). At present, even in USA, no qualified health claim has been

approved for any probiotic microorganism (Szajewska et al., 2016).

Nevertheless, consumers are more and more interested in functional products in general, including those that contain probiotic microorganisms.

In the last decades, various studies on probiotics conveyed by products of vegetal origin (i.e., fruit, fermented vegetables, cereals) were carried out (Granato et al., 2010; Peres et al., 2012; Abriouel et al., 2017; Huerta-Vera et al., 2017). Among vegetable products, table olives are considered a very interesting matrix to support the survival of both added selected commercial probiotic strains, and wild-type autochthonous ones that naturally colonize drupes and brines even during refrigerated shelf-life (Lavermicocca et al., 2005; Rodríguez-Gómez et al., 2014b, 2017; Argyri et al., 2015). Indeed, olives proved to support probiotics survival as or better than dairy products, and better than other vegetal substrates, maybe because of the nutrients release from the fruits, the presence of prebiotic substances, and their microstructure. In particular, the rough olive surface seems to protect bacteria from the acid environment, and favor the formation of mixed LAB and yeast communities biofilms, able to vehicle probiotics to human gastrointestinal (GI) tract (De Bellis et al., 2010; Ranadheera et al., 2010; Arroyo-López et al., 2012a; Blana et al., 2014; Rodríguez-Gómez et al., 2014a,b, 2017; Grounta et al., 2015).

Strains belonging to L. plantarum and L. pentosus, the most represented LAB starter species, among other less frequently isolated species (i.e., L. paracasei subsp. paracasei, L. casei, L. paraplantarum, Leuconostoc mesenteroides, and Ln. pseudomesenteroides), colonizing different cultivars of table olives (Conservolea, Halkidiki, Aloreña, Gordal, Manzanilla, Nocellara Etnea, and Bella di Cerignola), have been extensively tested for their *in vitro* probiotic characteristics (Bevilacqua et al., 2010; Argyri et al., 2013; Bautista-Gallego et al., 2013a; Botta et al., 2014; Pérez Montoro et al., 2016). Furthermore, potential probiotic strains have been studied also for their technological abilities, in order to develop multifunctional starters able to both control fermentation and grant probiotic characteristics to the product (Bevilacqua et al., 2010; De Bellis et al., 2010; Rodríguez-Gómez et al., 2013). In any case, when looking for probiotic strains, both functional characterization and safety assessment must be performed, following the FAO/WHO (2002) guidelines. First, strains intended to be added to food must have the QPS (in EU), or be GRAS (in USA), and, if intended to be administered to at-risk subjects, accomplish even more strict quality standards (Fijan, 2014; Sanders et al., 2016; Abriouel et al., 2017). The successive step should be checking their capacity to colonize the host. In fact, this is an indispensable prerequisite, which makes probiotics able to exert their beneficial effects (Kechagia et al., 2013; Papadimitriou et al., 2015). Therefore, isolates from naturally fermented table olives were tested for their ability to survive low pH values, resist bile acid, hydrolyse bile salts, adhere to mucus and/or human epithelial cells, and autoaggregate(Bevilacqua et al., 2010; Argyri et al., 2013; Bautista-Gallego et al., 2013a; Botta et al., 2014; Pérez Montoro et al., 2016). Moreover, haemolytic activity, antibiotic resistance and bacteriocin production as well as other important safety features were assessed. The latter characteristic is important to counteract pathogen development not only in human GIT, but also in food matrices (Pérez Montoro et al., 2016).

L. plantarum, *L. paracasei subsp. paracasei*, and, particularly, *L. pentosus* strains turned out to be the most promising probiotic species, sometimes showing even better performances than *L. rhamnosus* GG (LGG) and *L. casei* Shirota probiotic strains, used as control (Bevilacqua et al., 2010; Argyri et al., 2013; Bautista-Gallego et al., 2013a; Botta et al., 2014; Pérez Montoro et al., 2016).

However, *in vitro* tests positive results are not sufficient to demonstrate the actual *in vivo* efficacy of putative probiotic strains. Reduced risk of disease, improvement in illness symptoms, or well-being promotion, as a result of their use, must be proved through animal and clinical studies, in order to define a strain as probiotic (FAO/WHO, 2002). Unfortunately, regulations of probiotics is still quite vague. Often the same schemes/protocols used in drugs development are applied to probiotic foods, and this limiting approach, even though could be positive for consumers protection, may be responsible to restrain innovation and research on foods health benefits, preventing consumers to access to potential helpful information (van Loveren et al., 2012; Hill et al., 2014).

INNOVATIONS IN TABLE OLIVE PROCESSES AND PROCESSING PLANTS

Salt Reduction and Replacement

As reported earlier in this article, current practices in Sevillan style processing are quite stable and widespread among producers, with the main technological applied innovation being the selection and use of starter cultures, as described above. On the other hand, in recent literature, a large amount of contributions on optimization of processing parameters of table olive dealt with NaCl reduction and partial substitution with other salts. Indeed, salt intake is a topic of concern, because its excessive consumption has been related to hypertension and cardiovascular diseases (World Health Organization (WHO), 2012), expecially in those countries where the daily intake of NaCl is substantially higher than the recommendations (U.S. Dept. of Agriculture U.S. Dept. of Health Human Services, 2010). Therefore, the formulation of low salt foods, including fermented vegetables, to improve consumers' health, is a challenge to be addressed by food scientists. Fadda et al. (2014) compared the impact of two different salt concentrations on the texture and antioxidant activity of natural table olives. The texture profile did not reveal any difference between treatments, while the antioxidant activity was preserved more in 7% salt brined table olives than in the 4%. Among NaCl replacers, calcium chloride, potassium chloride, and magnesium chloride are the most investigated, mainly because of the positive health effects of Ca, K, and Mg intake, especially associated with a low Na diet (Leiba et al., 2005), and their inclusion in the list of nutrients of food regulations (Bautista-Gallego et al., 2013b). The reduction of NaCl in fermentation brines of table olives is advisable, since it has positive effects on LAB growth, as reported

in naturally black olives, (Tassou et al., 2002, 2007), depending also on NaCl concentration. Its partial substitution with $CaCl_2$ has been reported to affect the olive texture (Tassou et al., 2007), and usually has a detrimental effect on *Enterobacteriaceae* (Bautista-Gallego et al., 2010) and Yeasts growth (Bautista-Gallego et al., 2015). A partial substitution of NaCl with other chlorine salts ($CaCl_2$ and KCl) did not alter the fermentation profile of black olives, while substantially reducing the sodium content, improving the final product. On the other hand, other authors reported the effect of calcium addition on reducing the diffusion of soluble sugars from olive to brine, with a detrimental effect on acidity development and pH drop (Bautista-Gallego et al., 2011a; Rodríguez-Gómez et al., 2012). In Manzanilla olives, moderate (20–30 g/L) addition of $CaCl_2$ can be advisably used in substitution of NaCl, in the presence of the same amount of KCl, being stimulating for LAB growth, positively affecting acidity development, while not stimulating the growth of *Enterobacteriaceae* (Bautista-Gallego et al., 2015). From a sensory point of view, the addition of other salts other than NaCl in the processing of natural green olives and black olives resulted in a reduced salt concentration product with acceptable sensory characteristics (Di Silva, 2000; Mulé et al., 2000), and no impact on saltiness perception when KCl, NaCl and $CaCl_2$ are added in the same proportion to the brine (Marsilio et al., 2002). High levels of $CaCl_2$, however, could lead to excessive hardness of tissues resulting in a negative impact on the overall sensory characteristics. In a study conducted on Conservolea natural black olives, only 4% KCl plus 4% NaCl, among the mixture tested, could finally produce olives with lower sodium content without affecting the "traditional" taste (Panagou et al., 2011). The use of $ZnCl_2$ has also been proposed to fortify fermented vegetables and as an alternative preservative to potassium sorbate. Treatments have been shown to have a positive impact on texture, physico-chemical features, and sensory properties. Olive packaging with 0.75 g/L of $ZnCl_2$ led to olives with higher final sugars concentration, due to selective inhibition of microflora, lower pH and higher titratable acidity of the brines, having a positive effect on sensory properties (Bautista-Gallego et al., 2011c). Bautista-Gallego et al. (2013c) obtained better scores for instrumental firmness and the lowest scores for the kinesthetic sensations, bitterness and saltiness, for olives treated with $ZnCl_2$ (0.075% w/v) rather than those containing potassium sorbate (1.2% w/v).

Optimization of Processing Factors and Innovative Processing Plants

Natural green olives are processed through several months in order to reduce the bitterness at a palatable level and ensure chemical and microbiological stability. The main mechanism involved is the slow diffusion of bitter phenols from pulp to brine (Romero et al., 2004a,b; Arroyo-López et al., 2005). Many chemical-physical variables influence the natural process, namely the phenolic content in pulp, linked to the variety and degree of ripeness (Campus et al., 2015), salt concentration in brine (Fadda et al., 2014), acidification of brines, which can favor acid hydrolysis of oleuropein (Gikas et al., 2006; Servili et al., 2006), endogenous (Ramírez et al., 2016), and microbial hydrolases (Ciafardini et al., 1994; Servili et al., 2006), with β-glucosidase and esterase activity. Recently, alternative transformation protocols have been proposed in order to enhance the fermentation profile and accelerate the debittering of natural table olives. Ramírez et al. (2017) studied the effect of sodium chloride, acid concentration, and storage temperature on the enzymatic hydrolysis of oleuropein. Temperature optimum for endogenous β-glucosidases is cultivar dependent (Ramírez et al., 2014), with the pH-optimum around 5 (Romero-Segura et al., 2009; Kara et al., 2011; Ramírez et al., 2014), whileesterase activity decreases drastically at lower pH values. The cited authors proposed the following protocol: in the first stage (1 month of brining), a low concentration of NaCl (60 g/L) and acetic acid (2 g/L) together with a low storage temperature (10°C), which facilitates the action of endogenous enzymes (β-glucosidase and esterase), to favor the hydrolysis of the bitter phenol. In the second stage, concentrations and temperature of storage are raised (140 g/L NaCl, 16 g/L acetic acid and 40°C) to favor chemical hydrolysis of oleuropein, for a few months (Ramírez et al., 2017).

During processing, phenolic compounds present in the olive give rise to antimicrobial chemical species that influence the fermentation, affecting the growth of LAB and thus the chemical and microbiological stability (Medina et al., 2007). This argument is of concern only if olives are not treated with NaOH, since the treatments eliminate the phenolic compounds largely. In this regard, olives processed without the addiction of alkali exhibit different behaviors, depending on the variety. The addition of nutrients (MRS medium or Yeasts extract) during the fermentation of "difficult" varieties, such as Manzanilla, has been proposed to overcome the inhibitory activity of oleuropein-derived antimicrobial compounds (Medina et al., 2010).

In special conditions, such as processing plants located in cold zones, the use of starter cultures has been studied and physico-chemical variables optimized to drive the fermentation in such environments. Durán Quintana et al. (1999), using response surface methodology and *L. plantarum* starter cultures, reported that the most effective combination of factors were: initial pH of 5.0; 3%,w/v, NaCl; incubation at 12°C. Thus, normal fermentation processes can be obtained in cold regions when appropriate initial conditions and starter cultures are used.

In face of innovations proposed to drive the fermentation processes (Bevilacqua et al., 2015), scarce are the research contributions to the development of novel table olive processing plants. Usually, table olives are processed in closed static reactors with no strict control of processing parameters (temperature, salt concentration, acidity development), without any kind of automation in the control and monitoring of such parameters. An innovative processing plant, which allowed the real time control of main process parameters (pH, temperature, O_2 and CO_2 dissolved, salinity of brine) has been described recently (Campus et al., 2017). A starter driven process in the innovative plant, with continuous recirculation of brine, at controlled temperature, has been compared to static natural fermentation in plastic vats. The authors reported the evolution of relevant process parameters, chemico-physical determinations, instrumental texture, and

microbiological analyses. In the innovative process, a more rapid acidification onset was observed, pH reaching lower values than the control. Inoculated autochthonous *L. pentosus* strains have multiplied rapidly, supplanting *Enterobacteriaceae* in short time. No differences were found between samples in the instrumental texture profile analysis parameters (TPA). Notably, olives processed in the innovative plant were debittered in 3 months, as depicted by HPLC determination of phenolic compounds and Descriptive Sensory Analyses results, while naturally fermented olives took over 180 days to reach an acceptable level of bitter taste. Slight differences were found in few other sensory descriptors.

INNOVATIVE POST PROCESSING TECHNIQUES

After the fermentation, the table olives are packed in glass or plastic containers, tins, and plastic (single- or multi-laminated) or aluminum pouches, to improve their economic value and expand markets. These packaging materials are filled with brine, which may contain additives for improving the microbial quality, and protecting sensorial features (Panagou, 2004; Degirmencioglu, 2011a; Degirmencioglu et al., 2014). In some cases, modified atmosphere packaging (MAP), vacuum packaging (VP), active packaging (AP), and packaging by edible coating and film (ECP) (Ščetar et al., 2010), can alternatively be used.

Modified Atmosphere Packaging (MAP) and Vacuum Packaging (VP)

MAP is one of the most important food preservation methods. It consists in creating an atmosphere composed by CO_2, O_2, and /or N_2 in a pack, regulating the ripening process, extending shelf life, controlling or reducing microbial spoilage during storage and distribution (Valdés et al., 2014). Vacuum packaging is an efficient method in which the food is packed with low oxygen permeability plastic film, then air is evacuated, and the package sealed. However, the gaseous atmosphere of the vacuum package can change due to microbial metabolism and chemical reactions within the packed food; the oxygen from the external environment can permeate into the packaging; therefore, over time, the atmosphere in the package may be different from the original atmosphere (Degirmencioglu et al., 2014). The use of MAP and VP for extending shelf life of table olives, at ambient or low temperature, has been well noted and proven in several studies (Panagou et al., 2002; Panagou, 2006; Degirmencioglu, 2011a; Degirmencioglu et al., 2011b, 2014; Mantzouridou and Tsimidou, 2011; Doulgeraki et al., 2012; Argyri et al., 2015). As reported by these researchers, dipping the olives in anti-microbial solutions before packaging and storing them under pressure in a CO_2 atmosphere, at 4°C, controlled microbial activity effectively and minimized the production of mycotoxins, retaining the quality characteristics of the packed products.

Edible Coating Packaging (ECP)

ECP have been used for preserving and extending shelf life of foods. Semi-permeable films or coatings are formed on the surface of foods, to control moisture loss, suppress respiration, and carry out other functions. These films or coating are made by dipping in or spraying on foods a proper solution which, after time, forms a continuous layer in contact with the food surface. In producing edible coatings, emulsifiers and plasticizers are often used for improving coating performances. Edible coatings and films may act as carriers of antioxidants, antimicrobials, flavoring and coloring compounds, which are added to enhance food stability, safety and quality (Ščetar et al., 2010; Moutsatsou et al., 2011; Khalifa et al., 2016). Also, several antioxidant or antimicrobial agents can be coated, incorporated, immobilized, or surface modified into edible coatings, such as organic acids, essential oils, fatty acid esters, polypeptides, phenolic compounds, nitrites, and sulfites (Ščetar et al., 2010; Carpiné et al., 2015; Martillanes et al., 2017).

Nowadays, the modern trend is to use natural polymers, such as chitosan, incorporated with natural additives, such as vanillin, olive leaf extract, cinnamdehyde, eugenol, carvacrol or cellulose derivates, such as carboxymethyl cellulose and methyl cellulose, as food coating material. Chitosan and hydroxy-propyl-methyl-cellulose (HPMC) were tested in combination with MAP (100% N_2, 80% CO_2 - 20% Air) by Moutsatsou et al. (2011), with the aim to propose a new type of preservation and packaging of table olives. The coating was successful in reducing the weight loss, maintenaning color and firmness and extending shelf life of the olives, while chitosan proved to be more efficient than HPMC as olives coating, when they were packaged in 100% air. Ramírez et al. (2015a) studied the combined effect of an edible coating (commercial coatings employed to prevent citrus post harvest deseases) and a nitrogen atmosphere to avoid the occurrence of brown spotting of Manzanilla olives mechanically harvested and processed as Spanish-style green olives. The percentage of olives free of any brown spots ranged between 35–50, 10–25, and 50–65% for fruit directly processed, stored under nitrogen, or coated and stored under nitrogen, respectively. Therefore, the postharvest storage of coated olives under nitrogen resulted to be a good method to prevent bruise damage in mechanically harvested fruit.

Non-thermal Processes

Decontamination and shelf life extension of foods are closely related to microbial quality and biochemical and enzymatic reactions. The non-thermal technologies, such as high hydrostatic pressure (HHP), ultraviolet (UV), ultrasound, osmotic dehydration, or irradiation, are used for controlling the growth of microorganisms and the sensory, physical and chemical parameters, during food processing and storage (Tokuşoglu et al., 2010; Delgado-Adamez et al., 2013; Manzocco and Nicoli, 2015).

Contamination of olives may be due to inadequate hygienic conditions (raw material, water, additives, fermentation vehicle, personel etc.) during harvest, pre-treatment, fermentation/processing and storage stages (Argyri et al., 2014b). Most fermented or processed olives are distributed to market "in bulk" and they could be exposed to high risk of contamination from the environment (Panagou et al., 2013; Argyri et al., 2014b). The final product may be marketed or exported safely in packaging materials (Degirmencioglu, 2011a; Panagou et al., 2013) by using a thermal pasteurization or

physical packaging methods, for extending the shelf life (Kadam et al., 2012).

Among the non-thermal technologies, high hydrostatic pressure (HHP) is applied to liquid and solid foods, in the range of 50–1,000 MPa (Delgado-Adamez et al., 2013; Argyri et al., 2014b). Generally, moderate pressure (up to 200–300 MPa) decreases the rate of reproduction and growth of microorganisms, whereas higher pressure (300–700 MPa) inactivates microbial activity (Tokuşoglu et al., 2010), largely in dependence of the target microorganism. More recently, HHP has been used in a wide variety of applications; however, there are only a limited number of studies on the application of HHP to fermented vegetable foods, such as table olives (Tokuşoglu et al., 2010; Pradas et al., 2012; Abriouel et al., 2014; Argyri et al., 2014b).

Olives have a high content of functional micronutrients, fatty acids, bioactive phytochemicals (polyphenols, tocopherols, and phytosterols), having antioxidant, anti-inflammatory, antimicrobial properties, which have well-known positive effects on human health (Tokuşoglu et al., 2010; Delgado-Adamez et al., 2013). On the other side, table olives act as a suitable substrate for the production of mycotoxins (especially citrinin) produced by filamentous fungi *Penicillium, Aspergillus*, and *Monascus* (Tokuşoglu et al., 2010). Nevertheless, thermal processing of table olives induces some quality deterioration, resulting in softening of olive tissue, loss of green color or change to brown, and development of cooking taste (Dimou et al., 2013; Abriouel et al., 2014). For this purpose, optimization of the HHP conditions is important for bioactive compounds stability and quality of table olives, as an alternative to thermal processing (Argyri et al., 2014b).

Different levels of HHP (250–600 MPa for 5–30 min) showed to be more effective than thermal pasteurization (80°C for 20 min) in reducing the yeast and mold populations, the mycotoxin level (citrinin), and extending the shelf life of table olives and derived products (Tokuşoglu et al., 2010; Sánchez et al., 2012; Delgado-Adamez et al., 2013; Abriouel et al., 2014; Argyri et al., 2014b). Notably, the total phenolic and hydroxytyrosol levels were increased by factors of 2.1–2.5 and 0.8–2.0, respectively, while oleuropein decreased after HHP (Tokuşoglu et al., 2010). Similar results were also reported for Cornezuelo olives treated by HHP (400 MPa for 5 and 10 min and 600 MPa for 5 and 10 min). Olives treated by HHP had higher stability in terms of pH and free acidity values, and the HHP treatment (400 MPa for 5 min) can be used to prevent the formation of gas in the packed olives and to improve the sensory characteristics of Cornezuelo dressed olives (Pradas et al., 2012).

The color makes a key contribution to the marketability of table olives, and the vivid green color is an essential characteristic of the product, especially in Spanish-style processing (Sánchez-Gómez et al., 2013; Argyri et al., 2014b); it must be noted that HHP treatments caused a moderate degradation of the color of the processed olives (Pradas et al., 2012; Delgado-Adamez et al., 2013; Abriouel et al., 2014; Argyri et al., 2014b). For preserving and improving the color of HHP-treated olives, the addition of ascorbic acid (15 g/L) or purging with gaseous nitrogen has been suggested (Arroyo-López et al., 2008).

Biopreservation

Biopreservation is the use of microorganisms and their metabolites (organic acids, ethanol, carbon dioxide, antifungal compunds, bacteriocins, etc.) with the aim of extending the shelf life and improving the safety of foods (Settanni and Corsetti, 2008; Di Cagno et al., 2013). In particular, biopreservation by use of LAB cultures and their metabolites, including bacteriocins, has gained interest beacause of their antibacterial (Chen and Hoover, 2003; Bhattacharyya and Bhattacharjee, 2007) and antifungal activity (Schnürer and Magnusson, 2005). LAB with biopreservation activity should conveniently originate from the same type of food material as they are intended for use in (Vescovo et al., 1995, 1996). LAB strains involved in the fermentation of table olives were found to produce bacteriocins, and to being active *in vitro* against other LAB strains and against various genera of bacteria, including spoilage and pathogenic ones, such as Propionibacterium, Clostridium (Jiménez-Díaz et al., 1993; Delgado et al., 2005; Ruiz-Barba et al., 2010), and Helicobacter pylori (Brito et al., 2012). Studies has proven that some strains isolated from brined table olives can adhere to porcine jejune epithelial cells (Bevilacqua et al., 2010), and survive low pH and bile concentrations, as previously stated in section Table olives as carrier and source of Probiotic LAB, exherting probiotic activity (see section Table olives as carrier and source of Probiotic LAB). The ideal biopreserving bacteria should successfully lead successfully the fermentation, supplant spoilage and pathogenic microrganisms, and be active (or its antimicrobial metabolites) also during shelf life. Among Yeasts, the species Wickerhamomyces anomalus and Pichia membranifaciens have inhibitory activity against a considerable number of microorganisms (Santos et al., 2000; Passoth et al., 2011), as extensively reviewed in section Yeasts with positive technological traits.

CONCLUSIONS

The improvement of processing technologies is an essential step toward better, safer and more profitable table olive productions. In this review, conventional and advanced pre-processing operations, transformation and post packaging interventions have been reviewed and discussed. For sanification purposes, alternative to chlorine agents, alone or combined with other treatments, have been experimented and, in suitable conditions, proved to be effective in reducing the starting contamination of microbes and filth, although more data are needed to evaluate the suitability of innovative techniques such as ionizing radiations, unltrasounds and electrolyzed water solutions. Chlorine oxyde and ozone have been also employed to effectively reduce pesticide in table olive under law level. Starter cultures are the main applied biotechnological innovation in table olive processing. LAB and yeasts are the main microbial groups studied, and several strains have been selected and characterized for their technological traits. LAB strains with positive traits are capable to drive the fermentation process, supplanting spoilage microflora and favoring the onset of physical-chemical conditions that ensure microbial stability. Yeasts can exhert

positive or negative effects, depending on the species and strains involved. Authoctonous starters have advantages over single strain starters, in therms of adaptability and characterization of typical table olive preparations. Researchers have paid attention in the enhancement of healthiness of table olive, considered as a potential media to vehicle probiotics. Moreover, in recent literature, many contributions on processing parameters optimization dealt with NaCl reduction. Due to its implication in human health, partial sodium salt replacement with other salts have been showed to be possible and advisable at the same time. Alternative processing protocols have been described, in order to shorten time or obtain olives in disfavourable climate conditions. The contributions dealing with innovative processing plants are scarce, although incouraging results have been published recently. Among packaging interventions, MAP is the most promising and industrializable technique to prolong shelf life. Thermal post packaging treatment, such as pasteurization, are effective in reducing microbial contamination, but have detrimental effects on canned products. HPP has been proposed as an effective non thermal stabilization technique, which ensures microbial and enzymatic stability, minimizing the impact over sensory and nutritional features. As a concluding remark, bioremediation gives interesting perpsectives of a multifintional use of starter cultures, not only to drive the fermentation process but also to guarantee table olive preservability over time.

AUTHOR CONTRIBUTIONS

MC conceived the idea for the article, prepared and edited sections Table Olive Processing Technologies Up to Date and Innovations in Table Olive Processes and Processing Plants. ND prepared and edited sections Pre-processing of Olives and Innovative Post-processing Techniques. RC prepared and edited section Biotechnological Innovations and the tables. All authors reviewed the manuscript and approved it for publication.

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REFERENCES

Abriouel, H., Benomar, N., Gálvez, A., and Pulido, R. P. (2014). Preservation of Manzanilla Alorena cracked green table olives by high hydrostatic pressure treatments singly or in combination with natural antimicrobials. *LWT Food Sci. Technol.* 56, 427–431. doi: 10.1016/j.lwt.2013.09.012

Abriouel, H., Benomar, N., Lucas, R., and Gálvez, A. (2011). Culture-independent study of the diversity of microbial populations in brines during fermentation of naturally-fermented Aloreña green table olives. *Int. J. Food Microbiol.* 144, 487–496. doi: 10.1016/j.ijfoodmicro.2010.11.006.

Abriouel, H., Montoro, B. P., Casado Muñoz, M. D. C., Knapp, C. W., Gálvez, A., and Benomar, N. (2017). In silico genomic insights into aspects of food safety and defense mechanisms of a potentially probiotic *Lactobacillus pentosus* MP-10 isolated from brines of naturally fermented Aloreña green table olives. *PLoS ONE* 12:e0176801. doi: 10.1371/journal.pone.0176801

Allende, A., Tomas-Barberan, F. A., and Gil, I. M. (2006). Minimal processing for healthy traditional foods. *Trends Food Sci. Technol.* 17, 513–519. doi: 10.1016/j.tifs.2006.04.005

Alves, M., Gonçalves, T., and Quintas, C. (2012). Microbial quality and yeast population dynamics in cracked green table olives' fermentations. *Food Control.* 23, 363–368. doi: 10.1016/j.foodcont.2011.07.033

Amvrazi, E. G., and Albanis, T. A. (2009). Pesticide residue assessment in different types of olive oil and preliminary exposure assessment of Greek consumers to the pesticidresidues detected. *Food Chem.* 113, 253–261. doi: 10.1016/j.foodchem.2008.06.073

Aponte, M., Blaiotta, G., La Croce, F., Mazzaglia, A., Farina, V., and Settanni, L. (2012). Use of selected autochthonous lactic acid bacteria for Spanish style table olive fermentation. *Food Microbiol.* 12, 8–16. doi: 10.1016/j.fm.2011.10.005

Aponte, M., Ventorino, V., Blaiotta, G., Volpe, G., and Farina, V., Avellone, et al. (2010). Study of green Sicilian table olive fermentations through microbiological, chemical and sensory analyses. *Food Microbiol.* 27, 162–170. doi: 10.1016/j.fm.2009.09.010

Argyri, A. A., Nisiotou, A. A., Mallouchos, A., Panagou, E. Z., and Tassou, C. C. (2014a). Performance of two potential probiotic *Lactobacillus* strains from the olive microbiota as starters in the fermentation of heat shocked green olives. *Int. J. Food Microbiol.* 171, 68–76. doi: 10.1016/j.ijfoodmicro.2013.11.003

Argyri, A. A., Nisiotou, A. A., Pramateftaki, P., Doulgeraki, A. I., Panagou, E. Z., and Tassou, C. C. (2015). Preservation of green table olives fermented with lactic acid bacteria with probiotic potential under modified atmosphere packaging. *LWT-Food Sci. Technol.* 62. 783–790. doi: 10.1016/j.lwt.2014.11.046

Argyri, A. A., Panagou, E. Z., and Tassou, C. C. (2016). "Probiotics from the olive microbiota," in *Probiotics, Prebiotics, and Synbiotics. Bioactive Foods in Health Promotion*, eds R. R. Watson and V. R. Preedy (Cambridge: Academic Press), 371–389.

Argyri, A. A., Panagou, E. Z., Nychas, G. J. E., and Tassou, C. C. (2014b). Nonthermal pasteurization of fermented green table olives by means of high hydrostatic pressure processing. *Bio.Med. Res. Int.* 2014:515623. doi: 10.1155/2014/515623

Argyri, A. A., Zoumpopoulou, G., Karatzas, K. A. G., Tsakalidou, E., Nychas, G. J. E., Panagou, E. Z., et al. (2013). Selection of potential probiotic lactic acid bacteria from fermented olives by *in vitro* tests. *Food Microbiol.* 33, 282–291. doi: 10.1016/j.fm.2012.10.005

Arroyo-López, F. N., Bautista-Gallego, J., Domínguez-Manzano, J., Romero-Gil, V., Rodriguez-Gómez, F., García-García, P., et al. (2012a). Formation of lactic acid bacteria-yeasts communities on the olive surface during Spanish-style Manzanilla fermentations. *Food Microbiol.* 32: 295–301. doi: 10.1016/j.fm.2012.07.003

Arroyo-López, F. N., Bautista-Gallego, J., Duran-Quintana, M. C., Rodríguez-Gómez, F., Romero-Barranco, C., and Garrido-Fernández, A. (2008). Improvement of the storage process for cracked table olives. *J. Food Eng.* 89, 479–487. doi: 10.1016/j.jfoodeng.2008.06.001

Arroyo-López, F. N., Durán-Quintana, M. C., Ruiz-Barba, J. L., Querol, A., and Garrido-Fernández, A. (2006). Use of molecular methods for the identification of yeast associated with table olives. *Food Microbiol.* 23, 791–796. doi: 10.1016/j.fm.2006.02.008

Arroyo-López, F. N., Medina, E., Ruiz-Bellido, M. Á., Romero-Gil, V., Montes-Borrego, M., and Landa, B. B. (2016). Enhancement of the knowledge on fungal communities in directly brined aloreña de málaga green olive fermentations by metabarcoding analysis. *PLoS ONE* 11:e0163135. doi: 10.1371/journal.pone.0163135

Arroyo-López, F. N., Romero, C., Duran-Quintana, M. C., Lopez-Lopez, A., García- García, P., and Garrido-Fernández, A. (2005). Kinetic study of the physicochemical and microbiological changes in "Seasoned" olives during the shelf-life period. *J. Agric. Food Chem*. 53, 5285–5292. doi: 10.1021/jf050501

Arroyo-López, F. N., Romero-Gil, V., Bautista-Gallego, J., Rodríguez-Gómez, F., Jiménez-Díaz, R., García-García, P., et al. (2012b). Potential benefits of the application of yeast starters in table olive processing. *Front. Microbiol.* 3:161. doi: 10.3389/fmicb.2012.00161

Arroyo-López, F. N., Romero-Gil, V., Bautista-Gallego, J., Rodríguez-Gómez, F., Jiménez-Díaz, R., García-García, P., et al. (2012c). Yeasts in table olive processing: desirable or spoilage microorganisms? *Int. J. Food Microbiol.* 160, 42–49. doi: 10.1016/j.ijfoodmicro.2012.08.003

Artés, F., Gomez,., P., Aguayo, E., Escalona, V., and Artes-Hernandez, F. (2009). Sustainable sanitation techniques for keeping quality and safety of fresh-cut plant commodities. *Postharv. Biol. Technol.* 51, 287–296. doi: 10.1016/j.postharvbio.2008.10.003

Bajwa, U., and Sandhu, K. S. (2014). Effect of handling and processing on pesticide residues in food-a review. *J. Food Sci. Tech*. 51, 201–220. doi: 10.1007/s13197-011-0499-5

Banach, J. L., Sampers, I., Van Haute, S., and van der Fels-Klex, H. J. (2015). Effect of disinfectants on preventing the cross-contamination of pathogens in fresh produce washing water. *Int. J. Environ. Res. Public Health.* 12, 8658–8677. doi: 10.3390/ijerph120808658

Bassi, D., Puglisi, E., and Cocconcelli, P. S. (2015). Comparing natural and selected starter cultures in meat and cheese fermentations. *Curr. Opin. Food Sci.* 2, 118–122. doi: 10.1016/j.cofs.2015.03.002

Bautista-Gallego, J., Arroyo-Lopez, F. N., Duran-Quintana, M. C., and Garrido-Fernandez, A. (2010). Fermentation profiles of Manzanilla-Alorena cracked green table olives in different chloride salt mixtures. *Food Microbiol.* 27:403–412. doi: 10.1016/j.fm.2009.11.015

Bautista-Gallego, J., Arroyo-López, F. N., Rantsiou, K., Jiménez-Díaz, R., Garrido-Fernández, A., and Cocolin, L. (2013a). Screening of lactic acid bacteria isolated from fermented table olives with probiotic potential. *Food Res. Int*. 50, 135–142. doi: 10.1016/j.foodres.2012.10.004

Bautista-Gallego, J., Arroyo-Lopez, F. N., Romero-Gil, V., Rodriguez-Gomez, F., and Garrido-Fernandez, A. (2011c). Evaluating the effects of zinc chloride as a preservative in cracked table olive packing. *J. Food Protect.* 74, 2169–2176. doi: 10.4315/0362-028X.JFP-11-201

Bautista-Gallego, J., Arroyo-López, F. N., Romero-Gil, V., Rodríguez-Gómez, F., Garcia-Garcia, P., and Garrido-Fernández, A. (2011a). Chloride salt mixtures affect Gordal cv. green Spanish-style table olive fermentation. *Food Microbiol.* 28, 1316–1325. doi: 10.1016/j.fm.2011.06.002

Bautista-Gallego, J., Arroyo-López, F. N., Romero-Gil, V., Rodríguez-Gómez, F., García-García, P., and Garrido-Fernández, A. (2015). Fermentation profile of green Spanish-style Manzanilla olives according to NaCl content in brine. *Food Microbiol.* 49, 56–64. doi: 10.1016/j.fm.2015.01.012

Bautista-Gallego, J., Moreno-Baquero, J. M., Garrido-Fernandez, A., and Lopez-Lopez, A. (2013c). Development of a novel Zn fortified table olive product. *LWT-Food Sci. Technol.* 50, 264–271. doi: 10.1016/j.lwt.2012.05.017

Bautista-Gallego, J., Rantsiou, K., Garrido-Fernández, A., Cocolin, L., and Arroyo-López, F. N. (2013b). Salt reduction in vegetable fermentation: reality or desire? *J. Food. Sci.* 78, 1095–1100. doi: 10.1111/1750-3841.12170

Bautista-Gallego, J., Rodríguez-Gómez, F., Barrio, E., Querol, A., Garrido-Fernández, A., and Arroyo-López, F. N. (2011b). Exploring the yeast biodiversity of green table olive industrial fermentations for technological applications. *Int. J. Food Microbiol.* 147, 89–96. doi: 10.1016/j.ijfoodmicro.2011.03.013

Bavaro, S., Susca, A., Frisvad, J. C., Tufariello, M., Chytiri, A., Perrone, G., et al. (2017). Isolation, characterization, and selection of molds associated to fermented black table olives. *Front. Microbiol.* 8, 1356. doi: 10.3389/fmicb.2017.01356.

Behnsen, J., Deriu, E., Sassone-Corsi, M., and Raffatellu, M. (2013). Probiotics: properties, examples, and specific applications. *Cold Spring Harb. Perspect. Med*. 3:a010074. doi: 10.1101/cshperspect.a010074

Benítez-Cabello, A., Romero-Gil, V., Rodríguez-Gómez, F., Garrido-Fernández, A., Jiménez-Díaz, R., and Arroyo-López, F. N. (2015). Evaluation and identification of poly-microbial biofilms on natural green Gordal table olives. *Antonie Van Leeuwenhoek*. 108, 597–610. doi: 10.1007/s10482-015-0515-2

Bevilacqua, A., Altieri, C., Corbo, M. R., Sinigaglia, M., and Ouoba, L. I. I. (2010). Characterization of lactic acid bacteria isolated from Italian Bella di Cerignola table olives: selection of potential multifunctional starter cultures. *J. Food Sci.* 75, 536–544. doi: 10.1111/j.17503841.2010.01793.x

Bevilacqua, A., Beneduce, L., Sinigaglia, M., and Corbo, M. R. (2013). Selection of yeasts as starter cultures for table olives. *J. Food Sci*. 78, 742–751. doi: 10.1111/1750-3841.12117

Bevilacqua, A., De Stefano, F., Augello, S., Pignatiello, S., Sinigaglia, M., and Corbo, M. R. (2015). Biotechnological innovations for table olives. *Int. J. Food Sci. Nutr*. 66, 127–131. doi: 10.3109/09637486.2014.959901

Bhattacharyya, B. K., and Bhattacharjee, D. (2007). Bacteriocin: a biological food preservative. *J. Food Sci. Technol*. 44, 459–464.

Birmpa, A., Sfika, V., and Vantarakis, A. (2013). Ultraviolet light and ultrasound as nonthermal treatments for the inactivation of microorganisms in fresh ready-to-eat foods. *Int. J. Food Microbiol.* 167, 96–102. doi: 10.1016/j.ijfoodmicro.2013.06.005

Blana, V. A., Grounta, A., Tassou, C. C., Nychas, G. -J. E., and Panagou E, Z. (2014). Inoculated fermentation of green olives with potential probiotic *Lactobacillus pentosus* and *Lactobacillus plantarum* starter cultures isolated from industrially fermented olives. *Food Microbiol.* 38, 208–218. doi: 10.1016/j.fm.2013.09.007

Bleve, G., Tufariello, M., Durante, M., Grieco, F., Ramires, F. A., Mita, G., et al. (2015). Physico-chemical characterization of natural fermentation of Conservolea and Kalamáta table olives and development of a protocol for the pre-selection of fermentation starters. *Food Microbiol*. 46, 368–382. doi: 10.1016/j.fm.2014.08.021

Bleve, G., Tufariello, M., Durante, M., Perbellini, E., Ramires, F. A., and Grieco, F., et., al (2014). Physico-chemical and microbiological characterization of spontaneous fermentation of Cellina di Nardò and Leccino table olives. *Front. Microbiol*. 5:570. doi: 10.3389/fmicb.2014.00570

Boletín Oficial del Estado BOE (2001). *Real Decreto 1230/2001*. Reglamentación Técnico-sanitaria Para la Elaboración, Circulación y Venta de las Aceitunas de Mesa. BOE núm 279, 21/11/2001, Madrid.

Bonatsou, S., Benítez, A., Rodríguez-Gómez, F., Panagou, E. Z., and Arroyo-López, F. N. (2015). Selection of yeasts with multifunctional features for application as starters in natural black table olive processing. *Food Microbiol.* 46, 66–73. doi: 10.1016/j.fm.2014.07.011

Bonatsou, S., Tassou, C. C., Panagou, E. Z., and Nychas, G. J. E. (2017). Table olive fermentation using starter cultures with multifunctional potential. *Microorganisms* 5:30. doi: 10.3390/microorganisms5020030

Botta, C., and Cocolin, L. (2012). Microbial dynamics and biodiversity in table olive fermentation: culture-dependent and independent approaches. *Front. Microbiol*. 3:245. doi: 10.3389/fmicb.2012.00245

Botta, C., Langerholc, T., Cencič, A., and Cocolin, L. (2014). *In vitro* selection and characterization of new probiotic candidates from table olive microbiota. *PLoS ONE* 9:e94457. doi: 10.1371/journal.pone.0094457

Brenes, M., García, P., and Garrido, A. (1992). Phenolic compounds related to the black colour formed during the elaboration of ripe olives. *J. Agric. Food Chem*. 40, 1192–1196. doi: 10.1021/jf00019a023

Brenes, M., García, P., Romero, C., and Garrido, A. (1993). Estudio de los factores que afectan a la velocidad de neutralización de la pulpa durante la elaboración de aceitunas tipo negras. *Grasas y Aceites*. 44, 190–194.

Brito, D., Serrano, C., Peres, C., Delgado, A., Pereira, A., Oleastro, M., et al. (2012). Lactobacilli from fermented portuguese table-olives are able to inhibit the human pathogen helicobacter pylori. *Acta Hortic.* 949, 463–468. doi: 10.17660/ActaHortic.2012.949.68

Cabras, P., Angioni, A., Garau, V. L., Melis, M., Pirisi, F. M., Karim, M., et al. (1997). Persistence of insecticide residues in olives and olive oil. *J. Agric. Food Chem*. 45, 2244–2247. doi: 10.1021/jf960922e

Campus, M., Cauli, E., Scano, E., Piras, F., Comunian, R., Paba, A., et al. (2017). Towards controlled fermentation of table olives: lab starter driven process in an automatic pilot processing plant. *Food Bioprocess Tech*. 10, 1063–1073. doi: 10.1007/s11947-017-1882-7.

Campus, M., Sedda, P., Cauli, E., Piras, F., Comunian, R., Paba, A., et al. (2015). Evaluation of a single strain starter culture, a selected inoculum enrichment, and natural microflora in the processing of Tonda di Cagliari natural table olives: impact on chemical, microbiological, sensory and texture quality. *LWT Food Sci. Technol.* 64, 671–677. doi: 10.1016/j.lwt.2015.06.019

Carpiné, D., Dagostin, J. L. A., Bertan, L. C., and Mafra, M. R. (2015). Development and characterization of soy protein isolate emulsion-based edible films with added coconut oil for olive oil packaging: barrier, mechanical, and thermal properties. *Food Bioprocess Tech*. 8, 1811–1823. doi: 10.1007/s11947-015-1538-4

Cedola, A., Sciretta, N., Corbo, M. R., Sinigaglia, M., and Bevilacqua, A. (2013). Using an UV-treatment for microbial stabilization of table olives. *Ind. Aliment*. 57, 14–19.

Chen, H., and Hoover, D. G. (2003). Bacteriocins and their food applications. *Compreh. Rev. Food Sci. Food Safety* 2, 82–100. doi: 10.1111/j.1541-4337.2003.tb00016.x

Chen, J. Y., Lin, Y. J., and Kuo, W. C. (2013). Pesticide residue removal from vegetables by ozonation. *J. Food Eng.* 114, 404–411. doi: 10.1016/j.jfoodeng.2012.08.033

Chen, Q., Wang, Y., Chen, F., Zhang, Y., and Liao, X. (2014). Chlorine dioxide treatment for the removal of pesticide residues on fresh lettuce and in aqueous solution. *Food Control*. 40, 106–112. doi: 10.1016/j.foodcont.2013.11.035

Ciafardini, G., and Zullo, B. A. (2002). Survival of micro-organisms in extra virgin olive oil during storage. *Food Microbiol*. 19, 105–109. doi: 10.1006/fmic.2001.0458

Ciafardini, G., Marsillio, V., Lanza, B., and Pozzi, N. (1994). Hydrolysis of oleuropein by *Lactobacillus plantarum* strains associated with olive fermentation. *Appl. Env. Microbiol.* 60, 4142–4147.

Comunian, R., Ferrocino, I., Paba, A., Daga, E., Campus, M., Di, S.alvo, et al. (2017). Evolution of microbiota during spontaneous and inoculated Tonda di Cagliari table olives fermentation and impact on sensory characteristics. *LWT Food Sci. Technol.* 84, 64–72. doi: 10.1016/j.lwt.2017.05.039

Corsetti, A., Perpetuini, G., Schirone, M., Tofalo, R., and Suzzi, G. (2012). Application of starter cultures to table olive fermentation: an overview on the experimental studies. *Front. Microbiol*. 3:248. doi: 10.3389/fmicb.2012.00248

De Angelis, M., Campanella, D., Cosmai, L., Summo, C., Rizzello, C. G., and Caponio, F. (2015). Microbiota and metabolome of un-started and started Greek-type fermentation of Bella di Cerignola table olives. *Food Microbiol*. 52, 18–30. doi: 10.1016/j.fm.2015.06.002

De Bellis, P., Valerio, F., Sisto, A., Lonigro, S. L., and Lavermicocca, P. (2010). Probiotic table olives: microbial populations adhering on olive surface in fermentation sets inoculated with the probiotic strain *Lactobacillus paracasei* IMPC2.1 in an industrial plant. *Int.J. Food Microbiol.* 140, 6–13. doi: 10.1016/j.ijfoodmicro.2010.02.024

De Castro, A., Montaño, A., Casado, F., Sánchez, A., and Rejano, L. (2002). Utilization of *Enterococcus casseliflavus* and *Lactobacillus pentosus* as starter cultures for Spanish-style green olive fermentation. *Food Microbiol.* 19:637644. doi: 10.1006/fmic.2002.0466

Degirmencioglu, N. (2011a). Influence of temperature and modified atmosphere on the microbial profile of packed Gemlik dry-Salted olives. *J. Food Saf.* 31, 115–124. doi: 10.1111/j.1745-4565.2010.00274.x

Degirmencioglu, N. (2016). "Modern techniques in the production of table olives," in *Products from Olive Tree*, eds D. Boskou and M. L. Clodoveo (Zagreb: InTech), 215–234.

Degirmencioglu, N., Gürbüz, O., Degirmencioglu, A., Sahan, Y., and Özbey, H. (2011b). Effect of MAP and vacuum sealing on sensory qualities of dry salted olive. *Food Sci. Biotechnol*. 20, 1307–1313. doi: 10.1007/s10068-011-0180-9

Degirmencioglu, N., Gürbüz, O., Degirmencioglu, A., and Yildiz, S. (2014). Effect of pre-treatments on microbial growth and sensory properties of dry-salted olives. *J. Food Prot*. 77, 1527–1537. doi: 10.4315/0362-028X.JFP-14-036

Delgado, A., Brito, D., Peres, C., Noe-Arroyo, F., and Garrido-Fernández, A. (2005). Bacteriocin production by *Lactobacillus* pentosus B96 can be expressed as a function of temperature and NaCl concentration. *Food Microbiol*. 22, 521–528. doi: 10.3989/gya.2008.v59.i3.513

Delgado-Adamez, J., Franco, M. N., Sánchez, J., De Miguel, C., Ramírez, M. R., and Martín-Vertedor, D. (2013). Comparative effect of high pressure processing and traditional thermal treatment on the physicochemical, microbiology, and sensory analysis of olive jam. *Grasas y Aceites* 64, 432–441. doi: 10.3989/gya.023613

Di Cagno, R., Coda, R., De Angelis, M., and Gobbetti, M. (2013). Exploitation of vegetables and fruits through lactic acid fermentation. *Food Microbiol*. 33, 1–10. doi: 10.1016/j.fm.2012.09.003

Di Cagno, R., Surico, R. F., Siragusa, S., De Angelis, M., Paradiso, A., Minervini, F., et al. (2008). Selection and use of autochthonous mixed starter for lactic acid fermentation of carrots, French beans or marrows. *Int. J. Food Microbiol*. 127, 220–228. doi: 10.1016/j.ijfoodmicro.2008.07.010

Di Silva, A. (2000). Preliminary results of a new processing in order to obtain green table olives with low sodium content. *Ind. Aliment*. 39, 844–847.

Dimou, A., Panagou, E., Stoforos, N. G., and Yanniotis, S. (2013). Analysis of thermal processing of table olives using computational fluid dynamics. *J. Food Sci*. 78, 1695–1703. doi: 10.1111/1750-3841.12277.

Domínguez-Manzano, J., Olmo-Ruiz, C., Bautista-Gallego, J., Arroyo-López, F. N., Garrido-Fernández, A., and Jiménez-Díaz, R. (2012). Biofilm formation on abiotic and biotic surfaces during Spanish style green table olive fermentation. *Int. J. Food Microbiol*. 157, 230–238. doi: 10.1016/j.ijfoodmicro.2012.05.011

Doulgeraki, A. I., Hondrodimou, O., Iliopoulos, V., and Panagou, E. Z. (2012). Lactic acid bacteria and yeast heterogeneity during aerobic and modified atmosphere packaging storage of natural black Conservolea olives in polyethylene pouches. *Food Control*. 26, 49–57. doi: 10.1016/j.foodcont.2012.01.006

Durán Quintana, M. C., García García, P., and Garrido Fernández, A. (1999). Establishment of conditions for green table olive fermentation at low temperature *Int. J. Food Microbiol*. 51, 133–143. doi: 10.1016/S01168-1605(99)00123-3

Durante, M., Tufariello, M., Tommasi, L., Lenucci, M. S., Bleve, G., and Mita, G. (2017). Evaluation of bioactive compounds in black table olives fermented with selected microbial starters. *J. Sci. Food Agric*. 98, 96–103. doi: 10.1002/jsfa.8443

European Food Safety Authority (EFSA) (2010). Scientific Opinion on the substantiation of health claims related to live yoghurt cultures and improved lactose digestion (ID 1143, 2976) pursuant to Article 13(1) of Regulation (EC) No 1924/2006. *EFSA J.* 8:1763. doi: 10.2903/j.efsa.2010.1763

European Food Safety Authority [EFSA] (2017). Scientific Opinion on the update of the list of QPS-recommended biological agents intentionally added to food or feed as notified to EFSA. *EFSA J*. 15, 4664–4841. doi: 10.2903/j.efsa.2017.4664

European Parliament and Council of The European Union (2006). Regulation (EC) No 1924/2006 of the European Parliament and of the Council of 20 December 2006 on nutrition and health claims made on foods. *Offic. J. Euro. Union* L404, 9–25.

Fadda, C., Del Caro, A., Sanguinetti, A. M., and Piga, A. (2014). Texture and antioxidant evolution of naturally green table olives as affected by different sodium chloride brine concentrations. *Grasas y Aceites*. 65:e002. doi: 10.3989/gya.037213

FAO/WHO (2001). *Expert Consultation. Health and Nutritional Properties of Probiotics in Food including Powder Milk with Live Lactic Acid Bacteria.* Available online at: http://www.fao.org/3/a-a0512e.pdf (Accessed Nov 17, 2017).

FAO/WHO (2002). *Joint FAO/WHO Working Group Report on Drafting Guidelines for the Evaluation of Probiotics in Food.* London, ON: FAO/WHO.

Fendri, I., Chamkha, M., Bouaziz, M., Labat, M., Sayadi, S., and Abdelkafi, S. (2013). Olive fermentation brine: biotechnological potentialities and valorization. *Environ. Technol*. 34, 183–193. doi: 10.1080/09593330.2012.689364

Fernández-Diez, M. J., Castro, R., Garrido, A., González, F., González, F., Nosti, M., et al. (1985). *Biotecnología de la Aceituna de Mesa*. Madrid;Sevilla: Servicio de Publicaciones del CSIC.

Fijan, S. (2014). Microorganisms with claimed probiotic properties: an overview of recent literature. *Int. J. Environ. Res. Public Health.* 11, 4745–4767. doi: 10.3390/ijerph110504745

Food and Drug Administration (FDA) (2016a). *Chlorine dioxide.Code of Federal Regulations. Sec. 173.300.* Available online at: http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/ CFRSearch.cfm?fr=173.300 (Accessed June 21, 2016).

Food and Drug Administration (FDA) (2016b). *Substances Generally Recognized as Safe; Final Rule. Federal Register Rules and Regulations, Vol. 81, No. 159: 54959-55055.* Available online at: https://www.gpo.gov/fdsys/pkg/FR-2016-08-17/pdf/2016-19164.pdf. *Errata corrige, Vol. 81, No. 174: 62004.* Available online at: https://www.gpo.gov/fdsys/pkg/FR-2016-09-08/pdf/C1-2016-19164.pdf (Accessed Aug 25, 2017)

García, P., Brenes, M., Romero, C., and Garrido, A. (2001). Colour fixation in ripe olives. Effect of the type of iron salt and other processing factors. *J. Sci. Food Agric*. 81, 1364–1370. doi: 10.1002/jsfa.954

García, P., Brenes, M., Vattan, T., and Garrido, A. (1992). Kinetic study at different pH of the oxidation process to produce ripe olives. *J. Sci. Food Agric*. 60, 327–331. doi: 10.1002/jsfa.2740600309

García-García, P., Arroyo-López, N. F., and Rodríguez-Gómez, F. (2014). Partial purification of iron solutions from ripe table olive processing using ozone and electro-coagulation. *Sep. Purif. Technol.* 133, 227–235. doi: 10.1016/j.seppur.2014.06.011

García-Reyes, J. F., Ferrer, C., Gómez-Ramos, M. J., Fernández-Alba, A. R., and Molina-Díaz, A. (2007). Determination of pesticide residues in olive oil and olives. *Trends Analyt. Chem*. 26, 239–251. doi: 10.1016/j.trac.2007.01.004

Garrido-Fernández, A., Fernández-Díez, M. J., and Adams, R. (1997). *Table Olives. Production and Processing.* Chapman & Hall: London.

Gatti, M., Trivisano, C., Fabrizi, E., Neviani, E., and Gardini, F. (2004). Biodiversity among *Lactobacillus helveticus* strains isolated from different natural whey starter cultures as revealed by classification trees. *Appl. Environ. Microbiol*. 70, 182–190. doi: 10.1128/AEM.70.1.182-190.2004

Gikas, E., Papadoloulos, N., and Tsarbopoulos, A. (2006). Kinetic study of the acidic hydrolysis of oleuropein, the major bioactive metabolite of olive oil. *J. Liq. Chromatogr. Relat. Technol.* 29, 497–508. doi: 10.1080/10826070500474113

Gök, S., and Pazir, F. (2011). "Efficacy of some non thermal operations on shelf life of packaged black olive in the absence of brine," in *11th International Congress on Engineering and Food* (Athens).

Gómez-Almenar, M. C., and García-Mesa, J. A. (2015). Determination of pesticide residues in olives by liquid extraction surface analysis followed by liquid chromatography/tandem mass spectrometry. *Grasas y Aceites* 66:e078. doi: 10.3989/gya.0828142

González-Rodríguez, R. M., Rial-Otero, R., Cancho-Grande, B., Gonzalez-Barreiro, C., and Simal-Gándara, J. (2011). A review on the fate of pesticides during the processes within the food-production chain. *Crit. Rev. Food Sci. Nutr.* 51, 99–114. doi: 10.1080/10408390903432625

Granato, D., Branco, G. F., Nazzaro, F., Cruz, A. G., and Faria, J. A. F. (2010). Functional foods and non dairy probiotic food development: trends, concepts, and products. *Compr. Rev. Food Sci. Food Saf.* 9, 292–302. doi: 10.1111/j.1541-4337.2010.00110.x

Grounta, A., and Panagou, E. Z. (2014). Mono and dual species biofilm formation between *Lactobacillus pentosus* and *Pichia membranifaciens* on the surface of black olives under different sterile brine conditions. *Ann. Microbiol*. 64, 1757–1767. doi: 10.1007/s13213-014-0820-4

Grounta, A., Doulgeraki, A. I., and Panagou, E. Z. (2015). Quantification and characterization of microbial biofilm community attached on the surface of fermentation vessels used in green table olive processing. *Int. J. Food Microbiol.* 203, 41–48. doi: 10.1016/j.ijfoodmicro.2015.03.001

Guardia-Rubio, M., Ayora-Cañada, M. J., and Ruiz-Medina, A. (2007b). Effect of washing on pesticide residues in olives. *J. Food Sci.* 72, 139–143. doi: 10.1111/j.1750-3841.2006.00252.x

Guardia-Rubio, M., Fernández-de Córdova, M. L., Ayora-Cañada, M. J., and Ruiz-Medina, A. (2006a). Simplified pesticide multiresidue analysis in virgin olive oil by gas chromatography with thermoionic specific, electron-capture and mass spectrometric detection. *J. Chromatogr. A*. 1108, 231–239. doi: 10.1016/j.chroma.2006.01.006

Guardia-Rubio, M., Marchal-López, R. M., Ayora-Cañada, M. J., and Ruiz-Medina, A. (2007c). Determination of pesticides in olives by gas chromatography using different detection systems. *J. Chromatogr. A*. 1145, 195–203. doi: 10.1016/j.chroma.2007.01.068

Guardia-Rubio, M., Ruiz-Medina, A., Molina-Díaz, A., and Ayora-Cañada, M. J. (2006b). Influence of harvesting method and washing on the presence of pesticide residues in olives and olive oil. *J. Agric. Food Chem.* 54, 8538–8544. doi: 10.1021/jf0615911

Guardia-Rubio, M., Ruiz-Medina, A., Molina-Díaz, A., and Ayora-Cañada, M. J. (2008). Pesticide residues in washing water of olive oil mills: effect on olive washing efficiency and decontamination proposal. *J. Sci. Food Agric.* 88, 2467–2473. doi: 10.1002/jsfa.3364

Guardia-Rubio, M., Ruiz-Medina, A., Pascual Reguera, M. I., and Fernández de Córdova, M. L. (2007a). Multiresidue analysis of three groups of pesticides in washing waters from olive processing by solid-phase extraction-gas chromatography with electron capture and thermionic specific detection. *Microchem. J.* 85, 257–264. doi: 10.1016/j.microc.2006.06.005

Habibi, M., Golmakani., M. T., Farahnaky, A., Mesbahi, G., and Majzoobi, M. (2016). NaOH-free debittering of table olives using power ultrasound. *Food Chem.* 192, 775–781. doi: 10.1016/j.foodchem.2015.07.086

Habibi, M., Golmakani, M. T., Mesbahi, G., Majzoobi, M., and Farahnaky, A. (2015). Ultrasound accelerated debittering of olive fruits. *Innov. Food Sci. Emerg. Technol.* 31, 105–115. doi: 10.1016/j.ifset.2015.06.014

Heperkan, D. (2013). Microbiota of table olive fermentations and criteria of selection for their use as starters. *Front. Microbiol.* 4:143. doi: 10.3389/fmicb.2013.00143

Hernández, A., Martín, A., Aranda, E., Pérez-Nevado, F., and Córdoba, M. G. (2007). Identification and characterization of yeast isolated from the elaboration of seasoned green table olives. *Food Microbiol.* 24, 346–351. doi: 10.1016/j.fm.2006.07.02

Hernández, A., Martín, A., Córdoba, M. G., Benito, M. J., Aranda, E., and Pérez-Nevado, F. (2008). Determination of killer activity in yeasts isolated from the elaboration of seasoned green table olives. *Int. J. Food Microbiol.* 121, 178–188. doi: 10.1016/j.ijfoodmicro.2007.11.044

Hill, C., Guarner, F., Reid, G., Gibson, G. R., Merestein, D. J., Pot, B., et al. (2014). Expert consensus document. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nat. Rev. Gastroenterol. Hepatol.* 11, 506–514. doi: 10.1038/nrgastro.2014.66

Hoffmann, D. E. (2013). Health claim regulation of probiotics in the USA and the EU: is there a middle way? *Benef. Microb.* 4, 109–115. doi: 10.3920/BM2012.0033

Huerta-Vera, K., Flores-Andrade, E., Pérez-Sato, J. A., Morales-Ramos, V., Pascual-Pineda, L. A., and Contreras-Oliva, A. (2017). Enrichment of banana with *Lactobacillus rhamnosus* using double emulsion and osmotic dehydration. *Food Bioprocess Tech*. 10, 1053–1062. doi: 10.1007/s11947-017-1879-2

Hurtado, A., Reguant, C., Bordons, N., and Rozés, N. (2012). Lactic acid bacteria from fermented table olives. *Food Microbiol.* 31, 1–8. doi: 10.1016/j.fm.2012.01.006

Hurtado, A., Reguant, C., Esteve-Zarzoso, B., Bordons, A., and Rozès, N. (2008). Microbial population dynamics during the processing of Arbequina table olives. *Food Res. Int.* 41, 738–744. doi: 10.1016/j.foodres.2008.05.007

Hwang, E., Cash, J. N., and Zabik, M. J. (2002). Chlorine and chlorine dioxide treatment to reduce or remove EBDCs and ETU residues in a solution. *J. Agric. Food Chem*. 50, 4734–4742. doi: 10.1021/jf020307c

Iizuka, T., and Shimizu, A. (2014). Removal of pesticide residue from Brussels sprouts by hydrostatic pressure. *Innov. Food Sci. Emerg. Technol.* 22, 70–75. doi: 10.1016/j.ifset.2014.01.009

Ikeura, H., Kobayashi, F., and Tamaki, M. (2011). Removal of residual pesticide, fenitrothion, in vegetables by using ozone microbubbles generated by different methods. *J. Food Eng.* 103, 345–349. doi: 10.1016/j.jfoodeng.2010.11.002

IOOC (2004). *Trade Standard Applying To Table Olives. International Olive Oil Council COI/T20/Doc No 1.* Madrid: IOOC.

Iorizzo, M., Lombardi, S. J., Macciola, V., Testa, B., Lustrato, G., Lopez, F., et al. (2016). Technological potential of *Lactobacillus* strains isolated from fermented green olives: *In vitro* studies with emphasis on oleuropein-degrading capability. *Sci. World J.* 2016:1917592. doi: 10.1155/2016/1917592

Jiménez-Díaz, R., Ríos-Sánchez, R. M., Desmazeaud, M., Ruiz-Barba, J. L., and Piard, J.-C., (1993). Plantaricins S and T, two new bacteriocins produced by Lactobacillus plantarum LPCO10 isolated from a green olive fermentation. *Appl. Environ. Microbiol.* 59, 1416–1424. doi: 10.3989/gya.2006.v57.i3.51

Kadam, P. S., Jadhav, B. A., Salve, R. V., and Machewad, G. M. (2012). Review on the high pressure technology (HPT) for food preservation. *J. Food Process. Technol.* 3, 1–5. doi: 10.4172/2157-7110.1000135

Kara, H. E., Sinan, S., and Turan, Y. (2011). Purification of b-glucosidase from olive (*Olea europaea* L.) fruit tissue with specifically designed hydrophobic interaction chromatography and characterization of the purified enzymatic. *J. Chromatogr. B Analyt. Technol. Biomed Life Sci.* 879, 1507–1512. doi: 10.1016/j.jchromb.2011.03.036

Kaushik, G., Satya, S., and Naik, S. N. (2009). Food processing a tool to pesticide residue dissipation- A review. *Food Res. Int.* 42, 26–40. doi; 10.1016/j.foodres.2008.09.009

Kechagia, M., Basoulis, D., Konstantopoulou, S., Dimitriadi, D., Gyftopoulou, K., Skarmoutsou, N., et al. (2013). Health benefits of probiotics: a review. *ISRN Nutrition* 481651. doi: 10.5402/2013/481651

Khalifa, I., Barakat, H., El-Mansy, H., and Soliman, S. A. (2016). Effect of chitosan-olive oil processing residues coatings on keeping quality of cold-storage strawberry (*Fragaria ananassa*.var.Festival). *Food Qual.* 39, 504–515. doi: 10.1111/jfq.12213

Kiriş, S., and Velioglu, Y. S. (2016). Reduction in pesticide residue leels in olives by ozonated and tap water treatments and their transfer into olive oil. *Food Addit. Contam. Part A*. 33, 128–136. doi: 10.1080/19440049.2015.1114683

Lanza, B. (2013). Abnormal fermentations in table-olive processing: microbial origin and sensory evaluation. *Front. Microbiol.* 4:91. doi: 10.3389/fmicb.2013.00091

Lavermicocca, P., Valerio, F., Lonigro, S. L., de Angelis, M., Morelli, L., and Callegari, M. L. (2005). Study of adhesion and survival of lactobacilli and bifidobacteria on table olives with the aim of formulating a new probiotic food. *Appl. Environ. Microbiol.* 71, 4233–4240. doi: 10.1128/AEM.71.8.4233-4240.2005

Leal-Sánchez, M. V., Ruiz-Barba, J. L., Sánchez, A. H., Rejano, L., Jiménez-Díaz, R., and Garrido, A. (2003). Fermentation profile and optimization of green olive fermentation using *Lactobacillus plantarum* LPCO10 as a starter culture. *Food Microbiol.* 20, 421–430. doi: 10.1016/S0740-0020(02)00147-8

Leiba, A., Vald, A., Peleg, E., Shamiss, A., and Grossman, E. (2005). Does dietary recall adequately assess sodium, potassium and calcium intake in hypertensive patients?. *Nutrition* 21, 462–466. doi: 10.1016/j.nut.2004.08.021

León-Romero, Á., Domínguez-Manzano, J., Garrido-Fernández, A., Arroyo-López, F. N., and Jiménez-Díaz, R. (2016). Formation of *in vitro* mixed-species biofilms by *Lactobacillus pentosus* and yeasts isolated from Spanish-style green table olive fermentations. *Appl. Environ. Microbiol.* 82, 689–695. doi: 10.1128/AEM.02727-15

Leroy, F., and De Vuyst, L. (2004). Lactic acid bacteria as functional starter cultures for the food fermentation industry. *Trends Food Sci. Technol.* 15, 67–78. doi: 10.1016/j.tifs.2003.09.004

Leventdurur, S., Sert-Aydin, S., Boyaci-Gunduz, C. P., Agirman, B., Ben Ghorbal, A., Francesca, N., et al. (2016). Yeast biota of naturally fermented black olives in different brines made from cv. Gemlik grown in various districts of the Cukurova region of Turkey. *Yeast* 33, 289–301. doi: 10.1002/yea.3170

Lucena-Padrós, H., Caballero-Guerrero, B., Maldonado-Barragán, A., and Ruiz-Barba, J. L. (2014). Genetic diversity and dynamics of bacterial and yeast strains associated to Spanish-style green table-olive fermentations in large manufacturing companies. *Int. J. Food Microbiol.* 190, 72–78. doi: 10.1016/j.ijfoodmicro.2014.07.035

Mantzouridou, F., and Tsimidou, M. Z. (2011). Microbiological quality and biophenol content of hot air-dried Thassos cv. table olives upon storage. *Eur. J. Lipid Sci. Technol.* 113, 786–795. doi: 10.1002/ejlt.201000453

Manzocco, L., and Nicoli, M. C. (2015). Surface processing: existing and potential applications of ultraviolet light. *Crit. Rev. Food Sci. Nutr.* 55, 469–484. doi: 10.1080/10408398.2012.658460

Marsilio, V., Campestre, C., Lanza, B., De Angelis, and, M., and Russi, F. (2002). Sensory analysis of green table olives fermented in different saline solutions. *Acta Hortic.* 586, 617–620. doi: 10.17660/ActaHortic.2002.586.130

Martillanes, S., Rocha-Pimienta, J., Cabrera-Bañegil, M., Martín-Vertedor, D., and Delgado-Adámez, J. (2017). "Application of phenolic compounds for food preservation: Food additive and active packaging," in *Phenolic Compounds-Biological Activity,* eds M. Soto-Hernandez, M. Palma-Tenango and M. del Rosario Garcia-Mateos (Zagrep: InTech), 39–58.

Martín-Diana, A., Rico, D., Barry-Ryan, C., Frías, J., Henehan, G., and Barat, J. (2007). Efficacy of steamer jet-injection as alternative to chlorine in fresh-cut lettuce. *Postharv. Biol. Technol.* 45, 97–107. doi: 10.1016/j.postharvbio.2007.01.013

Martorana, A., Alfonzo, A., Settanni, L., Corona, O., La Croce, F., Caruso, T., et al. (2015). An innovative method to produce green table olives based on "pied de cuve" technology. *Food Microbiol.* 50, 126–140. doi: 10.1016/j.fm.2015.03.008

Medina, E., Brenes, M., Romero, C., García, A., and de Castro, A. (2007). Main antimicrobial compounds in table olives. *J. Agric. Food Chem.* 55, 9817–9823. doi: 10.1021/jf0719757

Medina, E., Gori, C., Servili, M., de Castro, A., Romero, C., and Brenes, M. (2010). Main variables affecting the lactic acid fermentation of table olives. *Int. J. Food Sci. Technol.* 45, 1291–1296. doi: 10.1111/j.1365-2621.2010.02274.x

Medina, E., Ruiz-Bellido, M. A., Romero-Gil, V., Rodríguez-Gómez, F., Montes-Borrego, M., Landa, B. B., et al. (2016). Assessment of the bacterial community in directly brined *Aloreña de Málaga* table olive fermentations by metagenetic analysis. *Int. J. Food Microbiol.* 236, 47–55. doi: 10.1016/j.ijfoodmicro.2016.07.014

Meireles, A., Giaouris, E., and Simões, M. (2016). Alternative disinfection methods to chlorine for use in the fresh-cut industry. *Food Res. Int.* 82, 71–85. doi: 10.1016/j.foodres.2016.01.021

Misra, N. N. (2015). The contribution of non-thermal and advanced oxidation Technologies towards dissipation of pesticide residues. *Trends Food Sci. Technol.* 45, 229–244. doi: 10.1016/j.tifs.2015.06.005

Moutsatsou, P., Tzia, C., Kerasiotis, T., and Skondras, D. (2011). *Prolongation of Table Olive Shelf-life by Combining Edible Coating Application and Modified Atmosphere Packaging (MAP).* Available online at: http://www.icef11.org/content/papers/aft/AFT1063.pdf

Mulé, R., Fodale, A. S., Bati, C. B., Tucci, A., and di Pisa, A. (2000). Preliminary results of a new processing in order to obtain green table olives with a low sodium content. *Ind. Aliment.* XXXIX, 844–847.

Navarro Rejano, L., Sánchez-Gómez, A. H., and Vega Macías, V. (2008). Nuevas tendencias en el tratamiento alcalino "cocido" de las aceitunas verdes aderezadas al estilo español o sevillano. *Grasas y Aceites* 59, 197–204. doi: 10.3989/gya.2008.v59.i3.509

New Zealand Food Safety Authority [NZFSA] (2008). *Food (Uncooked Comminuted Fermented Meat) Standard 2008.* Available online at: http://www.mpi.govt.nz/document-vault/11398 (Accessed Sept 25, 2017).

Nieto, L. M., Hodaifa, G., and Casanova, M. S. (2009). Elimination of pesticide residues from virgin olive oil by ultraviolet light: preliminary results. *J. Hazard Mater.* 168, 555–559. doi: 10.1016/j.jhazmat.2009.02.030

Nisiotou, A. A., Chorianopoulos, N., Nychas, G. J. E., and Panagou, E. Z. (2010). Yeast heterogeneity during spontaneous fermentation of black Conservolea olives in different brine solutions. *J. Appl. Microbiol.* 108, 396–405. doi: 10.1111/j.1365-2672.2009.04424.x

Nychas, G. J., Panagou, E. Z., Parker, M. L., Waldron, K. W., and Tassou, C. C. (2002). Microbial colonization of naturally black olives during fermentation and associated biochemical activities in the cover brine. *Lett. Appl. Microbiol.* 34, 173–177. doi: 10.1046/j.1472-765x.2002.01077.x

Otto, C., Zahn, S., Rost, F., Zahn, P., Jaros, D., and Rohm, H. (2011). Physical methods for cleaning and disinfection of surfaces. *Food Eng. Rev.* 3, 171–188. doi: 10.1007/s12393-011-9038-4

Panagou, E. Z. (2004). Effect of different packing treatments on the microbiological and physicochemical characteristics of untreated green olives of the Conservolea cultivar. *J. Sci. Food Agric.* 84, 757–764. doi: 10.1002/jsfa.1732

Panagou, E. Z. (2006). Greek dry-salted olives: monitoring the dry-salting process and subsequent physicochemical and microbiological profile during storage under different packing conditions at 4 and 20 degrees C. *LWT Food Sci. Technol.* 39, 323–330. doi: 10.1016/j.lwt.2005.02.017

Panagou, E. Z., Hondrodimou, O., Mallouchos, A., and Nychas, G. J. E. (2011). A study on the implications of NaCl reduction in the fermentation profile of Conservolea natural black olives. *Food Microbiol.* 28, 1301–1307. doi: 10.1016/j.fm.2011.05.008

Panagou, E. Z., Nychas, G. J. E., and Sofos, J. N. (2013). Types of traditional Greek foods and their safety. *Food Control.* 29, 32–41. doi: 10.1016/j.foodcont.2012.05.050

Panagou, E. Z., Schillinger, U., Franz, C. M. A. P., and Nychas, G.-J. E. (2008). Microbiological and biochemical profile of cv. Conservolea naturally black olives during controlled fermentation with selected strains of lactic acid bacteria. *Food Microbiol.* 25, 348–358. doi: 10.1016/j.fm.2007.10.005

Panagou, E. Z., Tassou, C. C., and Katsaboxakis, K. Z. (2002). Microbiological, physicochemical and organoleptic changes in dry-salted olives of Thasos variety stored under different modified atmospheres at 4°C and 20°C. *Int. J. Food Sci. Technol.* 37, 635–641. doi: 10.1046/j.1365-2621.2002.00590.x

Papadimitriou, K., Zoumpopoulou, G., Folign,é, B., Alexandraki, V., Kazou, M., Pot, B., et al. (2015). Discovering probiotic microorganisms: in vitro, in vivo, genetic and omics approaches. *Front. Microbiol.* 6:58. doi: 10.3389/fmicb.2015.00058

Passoth, V., Olstorpe, M., and Schnürer, J. (2011). Past, present and future research directions with *Pichia anomala*. A. *Van Leeuw.* 99, 121–125. doi: 10.1007/s10482-010-9508-3

Pereira, E. L., Ramalhosa, E., Borges, A., Pereira, J. A., and Baptista, P. (2015). Yeast dynamics during the natural fermentation process of table olives (*Negrinha de Freixo* cv.). *Food Microbiol.* 46, 582–586. doi: 10.1016/j.fm.2014.10.003

Peres, C. M., Hernandez-Mendoza, A., Peres, C., and Malcata, F. X. (2012). Review on fermented plant materials as carriers and sources of potentially probiotic lactic acid bacteria with an emphasis on table olives. *Trends Food Sci. Technol.* 26, 31–42. doi: 10.1016/j.tifs.2012.01.006

Pérez Montoro, B., Benomar, N., Lavilla Lerma, L., Castillo Gutiérrez, S., Gálvez, A., and Abriouel, H. (2016). Fermented Aloreña table olives as a source of potential probiotic *Lactobacillus pentosus* strains. *Front. Microbiol.* 7:1583. doi: 10.3389/fmicb.2016.01583

Porru, C., Rodríguez-Gómez, F., Benítez-Cabello, A., Jiménez-Díaz, R., Zara, G., Budroni, M., et al. (2018). Genotyping, identification and multifunctional features of yeasts associated to Bosana naturally black table olive fermentations. *Food Microbiol.* 69, 33–42. doi: 10.1016/j.fm.2017.07.010

Pradas, I., Del Pino, B., Peña, F., Ortiz, V., Moreno-Rojas, J. M., Fernández-Hernández, A., et al. (2012). The use of high hydrostatic pressure (HHP) treatments for table olives preservation. *Innov. Food Sci. Emerg. Technol.* 13, 64–68. doi: 10.1016/j.ifset.2011.10.011

Prakash, A., Inthajak, P., Huibregtse, H., Caporaso, F., and Foley, D. M. (2000). Effects of low-dose gamma irradiation and conventional treatments on shelf life and quality characteristics of diced celery. *J. Food Sci.* 65, 1070–1075. doi: 10.1111/j.1365-2621.2000.tb09420.x

Psani, M., and Kotzekidou, P. (2006). Technological characteristics of yeast strains and their potential as starter adjuncts in Greek-style black olive fermentation. *World J. Microbiol. Biotechnol,* 22:1329–1336. doi: 10.1007/s11274-006-9180-y

Qi, H., Huang, Q., and Hung, Y. C. (2018). Effectiveness of electrolyzed oxidizing water treatment in removing pesticide residues and its effect on produce quality. *Food Chem.* 239, 561–568. doi: 10.1016/j.foodchem.2017.06.144

Rahman, S. M. E., Ding, T., and Oh, D.-H. (2010). Inactivation effect of newly developed low concentration electrolyzed water and other sanitizers against microorganisms on spinach. *Food Contr.* 21, 1383–1387. doi: 10.1016/j.foodcont.2010.03.011

Ramírez, E., Brenes, M., García, P., Medina, E., and Romero, C. (2016). Oleuropein hydrolysis in natural green olives: importance of the endogenous enzymes. *Food Chem.* 206, 204–209. doi: 10.1016/j.foodchem.2016.03.061

Ramírez, E., Gandul-Rojas, B., Romero, C., Brenes, M., and Gallardo-Guerrero, L. (2015b). Composition of pigments and colour changes in green table olives related to processing type. *Food Chem.* 166, 115–124. doi: 10.1016/j.foodchem.2014.05.154

Ramírez, E., Medina, E., Brenes, M., and Romero, C. (2014). Endogenous enzymes involved in the transformation of oleuropein in Spanish table olive varieties. *J. Agric. Food Chem.* 62, 9569–9575. doi: 10.1021/jf5027982

Ramírez, E., Medina, E., García, P., Brenes, M., and Romero, C. (2017). Optimization of the natural debittering of table olives. *LWT Food Sci. Technol.* 77, 308–313. doi: 10.1016/j.lwt.2016.11.071

Ramírez, E., Sanchez, A. H., Romero, C., and Brenes, M. (2015a). Combined use of nitrogen and coatings to improve the quality of mechanically harvested Manzanilla olives. *Food Chem.* 171, 50–55. doi: 10.1016/j.foodchem.2014.08.103

Ramos, B., Miller, F. A., Brandão, T. R. S., Teixeira, P., and Silva, C. L. M. (2013). Fresh fruits and vegetables—An overview on applied methodologies to improve its quality and safety. *Innov. Food Sci. Emerg. Technol.* 20, 1–15. doi: 10.1016/j.ifset.2013.07.002

Ranadheera, R. D. C. S., Baines, S. K., and Adams, M. C. (2010). Importance of food in probiotic efficacy. *Food Res. Int.* 43, 1–7. doi: 10.1016/j.foodres.2009.09.009

Ribeiro, C., Canada, J., and Alvarenga, B. (2012). Prospects of UV radiation for application in postharvest technology. *J. Food Agric.* 24, 586–597. doi: 10.9755/ejfa.v24i6.1467

Rico, D., Martin-Diana, A. B., Barry-Ryan, C., Frias, J. M., Henehan, G. T. M., and Barat, J. M. (2008). Use of neutral electrolysed water (EW) for quality maintenance and shelf-life extension of minimally processed lettuce. *Innov. Food Sci. Emerg. Technol.* 9, 37–48. doi: 10.1016/j.ifset.2007.05.002

Rodriguez de la Borbolla y Alcalá, J. M., and Rejano, N. L. (1978). Sobre la preparación de la aceituna estilo sevillano. El lavado de los frutos tratados con lejía. *Grasas y Aceites* 29, 281–291.

Rodriguez de la Borbolla y Alcala¸, J. M., and Rejano, N. L. (1979). Sobre la preparacio'n de aceitunas de estilo sevillano. La fermentacion. *Grasas y Aceites* 30, 175–185.

Rodríguez-Gómez, F., Arroyo-López, F. N., López-López, A., Bautista-Gallego, J., and Garrido-Fernández, A. (2010). Lipolytic activity of the yeast species associated with the fermentation/storage phase of ripe olive processing. *Food Microbiol.* 27, 604–612. doi: 10.1016/j.fm.2010.02.003

Rodríguez-Gómez, F., Bautista-Gallego, J., Arroyo-López, F. N., Romero-Gil, V., Jiménez-Díaz, R., Garrido-Fernández, A., et al. (2013). Table olive fermentation with multifunctional *Lactobacillus pentosus* strains. *Food Control.* 34, 96–105. doi: 10.1016/j.foodcont.2013.04.010

Rodríguez-Gómez, F., Bautista-Gallego, J., Romero-Gil, V., Arroyo-López, F. N., Garrido-Fernández, A., and García-García, P. (2012). Effects of salt mixtures on Spanish green table olive fermentation performance. *LWT Food Sci. Technol.* 46, 56–63. doi: 10.1016/j.lwt.2011.11.002

Rodríguez-Gómez, F., Romero-Gil, V., Arroyo-López, F. N., Roldán-Reyes, J. C., Torres-Gallardo, R., Bautista-Gallego, J., et al. (2017). Assessing the challenges in the application of potential probiotic lactic acid bacteria in the large-scale fermentation of Spanish-style table olives. *Front. Microbiol.* 8:915. doi: 10.3389/fmicb.2017.00915

Rodríguez-Gómez, F., Romero-Gil, V., Bautista-Gallego, J., García-García, P., Garrido-Fernández, A., and Arroyo-López, F. N. (2014a). Production of potential probiotic Spanish-style green table olives at pilot plant scale using multifunctional starters. *Food Microbiol.* 44, 278–287. doi: 10.1016/j.fm.2014.03.023

Rodríguez-Gómez, F., Romero-Gil, V., García-García, P., Garrido-Fernández, A., and Arroyo-López, F. N. (2014b). Fortification of table olive packing with the potential probiotic bacteria *Lactobacillus pentosus* TOMC-LAB2. *Front. Microbiol.* 5:467. doi: 10.3389/fmicb.2014.00467

Romeo, F. V. (2012). "Microbiological aspects of table olives, olive germplasm," in *The Olive Cultivation, Table Olive and Olive Oil Industry in Italy,* ed I. Muzzalupo (Zagreb: InTech), 321–342.

Romero, C., Brenes, M., García, P., García, A., and Garrido, A. (2004a). Polyphenol changes during fermentation of naturally black olives. *J. Agric. Food Chem.* 52, 1973–1979. doi: 10.1021/jf030726p

Romero, C., Brenes, M., Yousfi, K., Garcia, P., Garcia, A., and Garrido, A. (2004b). Effect of cultivar and processing method on the contents of polyphenols in table olives. *J Agric Food Chem* 52, 479–484. doi: 10.1021/jf030525l

Romero-Gil, V., Bautista-Gallego, J., Rodríguez-Gómez, F., García-García, P., Jiménez-Díaz, R., Garrido-Fernández, A., et al. (2013). Evaluating the individual effects of temperature and salt on table olive related microorganisms. *Food Microbiol.* 33, 178–184. doi: 10.1016/j.fm.2012.09.015

Romero-Segura, C., Sanz, C., and Perez, A. G. (2009). Purification and characterization of an olive fruit b-glucosidase involved in the biosynthesis of virgin olive oil phenolics. *J. Agric. Food Chem.* 57, 7983–7988. doi: 10.1021/jf901293c

Ruiz-Barba, J. L., Caballero-Guerrero, B., Maldonado-Barragán, A., and Jiménez- Díaz, R. (2010). Coculture with specific bacteria enhances survival of Lactobacillus plantarum NC8, an autoinducer-regulated bacteriocin producer, in olive fermentations. *Food Microbiol.* 27, 413–417. doi: 10.1016/j.fm.2009.10.002

Ruiz-Medina, A., and Llorent-Martínez, E. J. (2012). "Evaluation of the contamination by herbicides in olive groves,"in *Herbicides-Properties, Synthesis and Control of Weeds*, ed M. N. Hasaneen (Zagrep: InTech), 207–226.

Sagong, H. G., Lee, S. Y., Chang, P. S., Heu, S., Ryu, S., Choi, Y. J., et al. (2011). Combined effect of ultrasound and organic acids to reduce *Escherichia coli* O157:H7, *Salmonella typhimurium*, and *Listeria monocytogenes* on organic fresh lettuce. *Int. J. Food Microbiol.* 145, 287–292. doi: 10.1016/j.ijfoodmicro.2011.01.010

Sánchez Gómez, A. H., García García, P., and Rejano Navarro, L. (2006). Trends in table olive production. *Grasas y Aceites* 57, 86–94. doi: 10.3989/gya.2006.v57.i1.24

Sánchez, J., De Miguel, C., Ramirez, M. R., Delgado, J., Franco, M. N., and Martín, D. (2012). Effect of high hydrostatic pressure versus thermal pasteurization on the microbiological, sensory aspects and oxidative stability of olive pate. *Grasas y Aceites* 63, 100–108. doi: 10.3989/gya.071211

Sánchez-Gómez, A. H., García-García, P., and Garrido Fernández, A. (2013). Spanish-style green table olive shelf-life. *Int. J. Food Sci. Technol.* 48, 1559–1568. doi: 10.1111/ijfs.12124

Sanders, M. E., Merenstein, D. J., Ouwehand, A. C., Reid, G., Salminem, S., Cabana, M. D., et al. (2016). Probiotic use in at-risk populations. *J. Am. Pharm. Assoc.* 56, 680–686. doi: 10.1016/j.japh.2016.07.001

Santos, A., Marquina D., Leal J. A., and Peinado J. M. (2000). (1→6)-β-d-Glucan as cell wall receptor for *Pichia membranifaciens* Killer toxin. *Appl. Environ. Microbiol.* 66, 1809–1813. doi: 10.1046/j.1472-765x.2002.01053.x

São José, J. F. B. D., Andrade, N. J., d., Ramos, A. M., Vanetti, M. C. D., Stringheta, P. C., and Chaves, J. B. P. (2014). Decontamination by ultrasound application in fresh fruits and vegetables. *Food Control.* 45, 36–50. doi: 10.1016/j.foodcont.2014.04.015

Ščetar, M., Kurek, M., and Galić, K. (2010).Trends in fruit and vegetable packaging. *Croatian J. Food Technol. Biotechnol. Nutr.*5, 69–86.

Schnürer, J., and Magnusson, J. (2005). Antifungal lactic acid bacteria as biopreservatives. *Trends Food Sci. Technol.* 16, 70–78. doi:10.1016/j.tifs.2004.02.014

Segovia-Bravo, K. A., García-García, P., Arroyo-López, F. N., Lopez-Lopez, A., and Garrido-Fernández, A. (2008). Ozonation process for the regeneration and recycling of Spanish green table olive fermentation brines. *Eur. Food Res. Technol.* 227, 463–472. doi: 10.1007/s00217-007-0742-5

Servili, M., Settanni, L., Veneziani, G., Esposto, S., Massitti, O., Taticchi, A., et al. (2006). The use of *Lactobacillus pentosus* 1MO to shorten the debittering process time of black table olives (cv. Itrana and Leccino): a pilotscale application. *J. Agric. Food Chem.* 54, 3869–3875. doi: 10.1021/jf053206y

Settanni, L., and Corsetti, A. (2008). Application of bacteriocins in vegetable food biopreservation. *Int. J. Food Microbiol.* 121, 123–138. doi: 10.1016/j.ijfoodmicro.2007.09.001

Seymour, I. J., Burfoot, D., Smith, R. L., Cox, L. A., and Lockwood, A. (2002). Ultrasound decontamination of minimally processed fruits and vegetables. *Int. J. Food Sci. Technol.* 37, 547–557. doi: 10.1046/j.1365-2621.2002.00613.x

Szajewska, H., Konarska, Z., and Kołodziej, M. (2016). Probiotic bacterial and fungal strains: claims with evidence. *Dig. Dis.* 34, 251–259. doi: 10.1159/000443359

Tamaki, M., and Ikeura, H. (2012). "Removal of residual pesticides in vegetables using ozone microbubbles," in *Pesticides-Recent Trends in Pesticide Residue Assay,* ed R. P. Soundararajan (Zagrep: InTech), 103–118.

Tassou, C. C., Katsabouxakis, C. Z., Georget, D. M. R., Parker, M. L., Waldrom, K. W., Smith, A. C., et al. (2007). Effect of calcium chloride on mechanical properties and microbiological characteristics of cv. Conservolea naturally black olives fermented at different sodium chloride levels. *J. Sci. Food. Agr.* 87, 1323–1331. doi: 10.1002/jsfa.2823

Tassou, C. C., Panagou, E. Z., and Katsaboxakis, K. Z. (2002). Microbiological and physicochemical changes of naturally black olives fermented at different temperatures and NaCl levels in the brines. *Food Microbiol.* 19, 605–615. doi: 10.1006/fmic.2002.0480

Tataridou, M., and Kotzekidou, P. (2015). Fermentation of table olives by oleuropeinolytic starter culture in reduced salt brines and inactivation of *Escherichia coli* O157:H7 and *Listeria monocytogenes*. *Int. J. Food Microbiol.* 208, 122–130. doi: 10.1016/j.ijfoodmicro.2015.06.001

Tofalo, R., Perpetuini, G., Schirone, M., Suzzi, G., and Corsetti, A. (2013). Yeast biota associated to naturally fermented table olives from different Italian cultivars. *Int. J. Food Microbiol.* 161, 203–208. doi: 10.1016/j.ijfoodmicro.2012.12.011

Tokuşoğlu, Ö. (2017). "Alfa-tocopherol, chlorophyll and carotenoid contents of oils obtained from less salty black table olives preserved by vacuum, MAP and gamma irradiation technologies," in *10th World Congress on Nutrition & Food Sciences* (Osaka).

Tokuşoglu, Ö., Alpas, H., and Bozoglu, F. (2010). High hydrostatic pressure effects on mold flora, citrinin mycotoxin, hydroxytyrosol, oleuropein phenolics and antioxidant activity of black table olives. *Innov. Food Sci. Emerg. Technol.* 11, 250–258. doi: 10.1016/j.ifset.2009.11.005

Tufariello, M., Durante, M., Ramires, F. A., Grieco, F., Tommasi, L., Perbellini, E., et al. (2015). New process for production of fermented black table olives using selected autochthonous microbial resources. *Front. Microbiol.* 6:1007. doi: 10.3389/fmicb.2015.01007

Tzortzakis, N., and Chrysargyris, A. (2017). Postharvest ozone application for the preservation of fruits and vegetables. *Food Rev. Int.* 33, 270–315. doi: 10.1080/87559129.2016.1175015

U.S. Dept. of Agriculture and U.S. Dept. of Health and Human Services (2010). *Dietary Guidelines for Americans. 7th Edn.* Washington, DC: U.S. Government Printing Office.

Valdés, A., Mellinas, A. C., Ramos, M., Garrigós, M. C., and Jiménez, A. (2014). Natural additives and agricultural wstes in biopolymer formulations for food packaging. *Front. Chem.* 2, 1–10. doi: 10.3389/fchem.2014.00006

van Loveren, H., Sanz, Y., and Salminen, S. (2012). Health claims in Europe: probiotics and prebiotics as case examples. *Annu. Rev. Food Sci. Technol.* 3, 247–261. doi: 10.1146/annurev-food-022811-101206

Vescovo, M., Orsi, C., Scolari, G., and Torriani, S., (1995). Inhibitory effect of selected lactic acid bacteria on microflora associated with ready-to-eat vegetables. *Lett. Appl. Microbiol.* 21, 121–125. doi: 10.1111/j.1472-765X.1995.tb01022.x

Vescovo, M., Torriani, S., Orsi, C., Macchiarolo, F., and Scolari, G. (1996). Application of antimicrobial-producing lactic acid bacteria to control pathogens in ready-to-use vegetables. *J. Appl. Bacteriol.* 81, 113–119. doi: 10.1111/j.1365-2672.1996.tb04487.x

Vichi, S., Boynuegri, P., Caixach, J., and Romero, A. (2015). Quality losses in virgin olive oil due to washing and short term storage before olive milling. *Eur. J. Lipid Sci. Technol.* 117, 2015–2022. doi: 10.1002/ejlt.201500066

Wacher, C., Díaz-Ruiz, G., and Tamang, J. P. (2010). "Fermented vegetable products," in *Fermented Foods and Beverages of the World,* eds J. P. Tamang and K. Kailasapathy (Boca Raton, FL: CRC Press; Taylor & Francis Group), 149–190.

World Health Organization (WHO) (2012). *Sodium Intake for Adults and Children.* Available online at: http://www.who.int/nutrition/publications/guidelines/sodium_intake/en/

Wu, J., Luan, T., Lan, C., Lo, T. W. H., and Chan, G. Y. S. (2007). Removal of residual pesticides on vegetable using ozonated water. *Food Control* 18, 466–472. doi: 10.1016/j.foodcont.2005.12.011

Xiong, Z., Cheng, X., and Sun, D. (2011). Pretreatment of heterocyclic pesticide wastewater using ultrasonic/ozone combined process. *J. Environ. Sci.* 23, 725–730. doi: 10.1016/S1001-0742(10)60465-2

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In Vitro and *in Vivo* Selection of Potentially Probiotic Lactobacilli From Nocellara del Belice Table Olives

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Table olives are increasingly recognized as a vehicle as well as a source of probiotic bacteria, especially those fermented with traditional procedures based on the activity of indigenous microbial consortia, originating from local environments. In the present study, we report characterization at the species level of 49 Lactic Acid Bacteria (LAB) strains deriving from Nocellara del Belice table olives fermented with the Spanish or Castelvetrano methods, recently isolated in our previous work. Ribosomal 16S DNA analysis allowed identification of 4 *Enterococcus gallinarum*, 3 *E. casseliflavus*, 14 *Leuconostoc mesenteroides*, 19 *Lactobacillus pentosus*, 7 *L. coryniformis,* and 2 *L. oligofermentans*. The *L. pentosus* and *L. coryniformis* strains were subjected to further screening to evaluate their probiotic potential, using a combination of *in vitro* and *in vivo* approaches. The majority of them showed high survival rates under *in vitro* simulated gastro-intestinal conditions, and positive antimicrobial activity against *Salmonella enterica* serovar Typhimurium, *Listeria monocytogenes* and enterotoxigenic *Escherichia coli* (ETEC) pathogens. Evaluation of antibiotic resistance to ampicillin, tetracycline, chloramphenicol, or erythromycin was also performed for all selected strains. Three *L. coryniformis* strains were selected as very good performers in the initial *in vitro* testing screens, they were antibiotic susceptible, as well as capable of inhibiting pathogen growth *in vitro.* Parallel screening employing the simplified model organism *Caenorhabditis elegans*, fed the *Lactobacillus* strains as a food source, revealed that one *L. pentosus* and one *L. coryniformis* strains significantly induced prolongevity effects and protection from pathogen-mediated infection. Moreover, both strains displayed adhesion to human intestinal epithelial Caco-2 cells and were able to outcompete foodborne pathogens for cell adhesion. Overall, these results are suggestive of beneficial features for novel LAB strains, which renders them promising candidates as starters for the manufacturing of fermented table olives with probiotic added value.

Keywords: foodborne bacteria, fermented foods, nematode, autochtonous bacteria, plant food matrix

INTRODUCTION

The most recent definition of "probiotic" was formulated by an expert consultation of international scientists working on behalf of FAO/WHO (Food and Agriculture Organization/World Health Organization), and refers to viable, non-pathogenic microorganisms (bacteria and/or yeast) that when ingested in adequate amounts, are able to reach and colonize the Gastro-Intestinal (GI) tract and to confer health benefits to the host (FAO/WHO, 2006). The main benefits associated with probiotic intake include gut health and immune modulation (Ritchie and Romanuk, 2012; Hill et al., 2014). In particular, probiotic consumption can influence the microbial composition and balance within the intestinal microbiota. Production of antimicrobial substances such as organic acids, hydrogen peroxide, antifungal peptides, and bacteriocins, contributes to decrease harmful microorganisms and promotes growth and stability of beneficial bacteria, such as lactobacilli and bifidobacteria (Magnusson and Schnürer, 2001; Baker et al., 2009; Abriouel et al., 2012; Amund, 2016; Hegarty et al., 2016). Probiotic capacity greatly varies among strains belonging to different genera and species. The most common bacterial strains employed as probiotics are found within LAB (Lactic Acid Bacteria) species belonging to the *Bifidobacterium*, *Lactobacillus,* and *Streptococcus* genera (Saulnier et al., 2009). Members of the *Lactobacillus* genus are especially relevant as foodborne probiotics because they can be exploited also from the technological viewpoint, as their metabolic properties lead to production of a wide spectrum of molecules conferring specific organoleptic quality to fermented products. Moreover, several lactobacilli are considered Generally Recognized as Safe (GRAS) and are largely used as starter and/or protective cultures in fermented vegetables, sausages, fish and dairy products (Giraffa et al., 2010; Garrigues et al., 2013; Montoro et al., 2016).

The growing demand for plant-based foods is presently driving selection of bacteria which are able to grow on fermentable vegetable sources (Granato et al., 2010). Vegetable fermented foods such as table olives, pickles, sauerkraut, and kimchi, are slowly overtaking the role of fermented products of animal origin (dairies and sausages) as leading source of live bacteria in human diets, and they are also increasingly considered as novel reservoirs of yet uncharacterized probiotic strains (Ranadheera et al., 2010). Table olives could therefore represent a natural source for the isolation of novel probiotic bacterial strains. This is especially true for those olives fermented with traditional procedures relying on the activity of indigenous microbial consortia of environmental origin. The microbiota of processed olives and brines includes, among others, several LAB species such as *L. plantarum*, *L. pentosus*, *L. paracasei*, *L. rhamnosus*, *Leuconostoc mesenteroides* (Arroyo-López et al., 2008; Hurtado et al., 2012; Zinno et al., 2017).

Fermented vegetable matrices are presently recognized not only as a source, but also as a vehicle of probiotic bacteria. Recent studies demonstrated that LAB species isolated from different table olive *cultivars* exhibit probiotic features, such as resistance to acid and bile salts, antimicrobial activity and interaction with intestinal epithelial cells. This suggests their potential application as novel probiotic candidates for *in vivo* studies in animals and humans (Bevilacqua et al., 2010; Abriouel et al., 2012; Argyri et al., 2013; Botta et al., 2014; Montoro et al., 2016). However, simplified *in vitro/in vivo* models represent useful and less expensive screening tools to identify probiotic strains from a large number of microbial candidates. Human intestinal epithelial Caco-2 cells are a well characterized enterocyte-like cell line, capable of expressing the morphological and functional differentiation features which are typical of mature enterocytes, including cell polarity and a functional brush border (Sambuy et al., 2005). The Caco-2 cell line has been extensively used as a reliable *in vitro* system to study the adhesion capacity of lactobacilli as well as their probiotic effects, such as protection against intestinal injury induced by pathogens (Liévin-Le Moal et al., 2002; Resta-Lenert and Barrett, 2003; Roselli et al., 2006; Montoro et al., 2016).

The nematode *Caenorhabditis elegans* is becoming an increasingly valuable *in vivo* model to study host-probiotic interactions. Its success lies in the transparency of the body, in the small size and in the absence of ethical issues. Moreover, it is inexpensive to maintain and suitable for screening studies (Clark and Hodgkin, 2014). *C. elegans* is a powerful tool to test the effects of ingested bacteria on host physiology and it can also be useful in providing mechanistic insights on the beneficial effects of probiotics. A growing number of studies employing the *C. elegans* model system demonstrated that ingestion of lactobacilli and bifidobacteria can prolong the lifespan of nematodes and modify host defense (Kim and Mylonakis, 2012; Komura et al., 2013; Park et al., 2015). The *L. gasseri* strain SBT2055, which was reported to exert beneficial effects in mice and humans, showed a positive impact on longevity and/or aging in this nematode (Nakagawa et al., 2016). The health-promoting *L. delbrueckii* subspecies *bulgaricus* was also found to increase the lifespan of nematodes (Zanni et al., 2017), further highlighting the power of this *in vivo* model.

The aim of the present study was the identification and characterization of novel potentially probiotic strains of *L. pentosus* and *L. coryniformis*, deriving from a LAB collection of isolates from Nocellara del Belice table olives (Zinno et al., 2017). We used a combination of *in vitro* and *in vivo* approaches, including Caco-2 cell cultures and the *C. elegans* nematode model, to select specific strains displaying beneficial host-microbe interactions. The autochtonous nature of the food fermenting microbiota of origin, as well as the GRAS status of LAB species, allows to employ these strains as starter cultures in food fermentations, with the added value of providing health promoting traits.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

The LAB strains described in this work, as well as reference strains *L. plantarum* ATCC® 14917™, *L. pentosus* ATCC® 8041™ and *L. rhamnosus* GG ATCC® 53103™ (LGG), were grown in De Man Rogosa Sharpe (MRS) medium for 24–48 h at 30 or 37°C under anaerobic conditions. The enterotoxigenic *Escherichia coli* strain K88 (ETEC, O149:K88ac,

provided by The Lombardy and Emilia Romagna Experimental Zootechnic Institute, Reggio Emilia, Italy) and *E. coli* strain OP50 were grown in Luria-Bertani (LB) broth at 37°C overnight. *Salmonella enterica* serovar Typhimurium LT2 and *Listeria monocytogenes* OH were routinely grown in Tryptone Soya Broth (TSB) at 37 and 30°C, respectively. All media and supplements were provided by Oxoid (Milan, Italy).

Species Identification

For taxonomical identification, 16S rDNA gene fragments were amplified from LAB isolates using the P0-P6 primer pair (P0: 5′-GAGAGTTTGATCCTGGCT-3′; P6: 5′-CTACGGCTACCTTGTTAC-3′; Di Cello and Fani, 1996). Two μl of DNA extracted by microlysis were used for PCR amplification with the following program: 95°C for 10 min, 30 cycles at: 94°C for 1 min, 55°C for 90 s, 72°C for 150 s; one step at 55°C for 10 min followed by a final step at 72°C for 10 min. Amplified PCR fragments were analyzed by gel electrophoresis in 0.8% agarose in 1X TAE and then purified with NucleoSpin Gel and PCR clean-up purification kit (Macherey-Nagel, Germany). Sequencing of purified 16S rDNA fragments was performed at Bio-Fab Research (Italy) laboratories. For taxonomical identification, DNA sequences were compared with those reported in the BLAST NCBI (National Center for Biotechnology Information, Bethesda, USA) database. Nucleotide sequences of the amplified 16S rDNA from each LAB isolate were submitted to GenBank, and the corresponding accession numbers are reported in **Table 1**.

Multiplex PCR Assay

L. plantarum/*L. pentosus* strains were subjected to a multiplex PCR assay using the *recA* gene-based primers paraF (5′-GTC ACA GGC ATT ACG AAA AC-3′), pentF (5′-CAG TGG CGC GGT TGA TAT C-3′), planF (5′-CCG TTT ATG CGG AAC ACC TA-3′), and pREV (5′-TCG GGA TTA CCA AAC ATC AC-3′; Torriani et al., 2001). The PCR mixture included 1.5 mM $MgCl_2$, the primers paraF, pentF, and pREV (0.25 μM each), 0.12 μM primer planF, 0.2 mM dNTPs, 3 U *Taq* DNA Polymerase (Invitrogen, Carlsbad, USA). Two μl of DNA extracted by microlysis were used for the reaction. PCR programs consisted of an initial denaturation step at 94°C for 3 min, followed by 30 cycles of amplification (denaturation at 94°C for 30 s, annealing at 56°C for 10 s, and elongation at 72°C for 30 s), and a final extension step at 72°C for 5 min. The PCR products were visualized on a 2% agarose gel in 1X TAE buffer and digitally captured by using ImageQuant LAS 4000 (GE Healthcare Life Sciences, Little Chalfont, UK).

Rep-PCR Fingerprinting

Two μl of DNA extracted by microlysis from selected *L. coryniformis* and *L. pentosus* strains (Microzone, Haywards Heath, UK) were used for PCR amplification with primer GTG_5 (5′-GTGGTGGTGGTGGTG-3′), as previously described (Zinno et al., 2017), or with the ERIC primer (ERIC1R: 5′-ATGTAAGCTCCTGGGGATTCAC-3′; ERIC2: 5′-AAGTAAGTGACTGGGGTGAGCG-3′; de La Puente-Redondo et al., 2000).

For ERIC Rep-PCR, DNA amplification was carried out in 25 μl PCR mixture containing 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 3 U of *Taq* DNA Polymerase (Invitrogen, Carlsbad, USA) and 0.6 μM primers. Each cycle consisted of an initial denaturation step at 95°C for 3 min followed by 30 cycles of amplification (94°C for 1 min, 40°C for 1 min, 72°C for 1 min), and a final extension step at 72°C for 8 min. Amplified products were analyzed by gel electrophoresis (80 V for 4 h) in 1.8% agarose in 1X TAE. The gels were visualized under UV and digitally captured by using ImageQuant LAS 4000 (GE Healthcare Life Sciences, Little Chalfont, UK).

Acid and Bile Salt Tolerance Assay

Tolerance to gastrointestinal conditions of selected *L. coryniformis* and *L. pentosus* strains was evaluated according to (Vizoso Pinto et al., 2006). Three ml of overnight bacterial suspensions were centrifuged at 5,000 × g for 15 min at 4°C and the corresponding pellet was diluted 1:1 in a sterile electrolyte solution simulating salivary juice (Simulated Salivary Juice, SSJ), composed of 6.2 g/l NaCl, 2.2 g/l KCl, 0.22 g/l $CaCl_2$, 1.2 g/l $NaHCO_3$ pH 6.9 in which lysozyme was added to a final concentration of 100 mg/l. The mixed suspension was incubated for 5 min at 37°C. Subsequently, the sample was diluted 3:5 with Simulated Gastric Juice (SGJ) containing 6.2 g/l NaCl, 2.2 g/l KCl, 0.22 g/l $CaCl_2$, 1.2 g/l $NaHCO_3$ pH 2.5 and 3 g/l pepsin, and incubated for 1 h at 37°C. After incubation, 1 ml aliquot of the sample was serially diluted and plated, in triplicate, onto MRS agar. The remaining sample was diluted 1:4 in Simulated Pancreatic Juice (SPJ) consisting of 6.4 g/l $NaHCO_3$, 0.239 g/l KCl, 1.28 g/l NaCl, 0.5% bile extract, 0.1% pancreatin at pH 7.2, and incubated for 3 h at 37°C. At 2 and 3 h incubation times, 1 ml aliquots were withdrawn, serially diluted and plated on MRS agar. Aliquots of overnight inocula were also tested to determine the CFU/ml at the initial time point (t_0) for each strain. In parallel, control samples were treated with Phosphate Buffered Saline (PBS) and subjected to the same procedure. Survival capacity was calculated as the percentage of 1– [(log CFU/ml_{t0}-log CFU/ml_{SPJ3h})/log CFU/ml_{t0}], where CFU/ml_{SPJ3h} represented the total viable counts (CFU/ml) for each strain at the final time point of incubation in SPJ, and CFU/ml_{t0} represented the total viable counts at the initial time point. All enzymes and salts used in the assay were provided by Sigma Aldrich (Milan, Italy).

Antibiotic Susceptibility Tests

Antibiotic susceptibility was performed for a selected panel of antibiotics, namely ampicillin, tetracycline, chloramphenicol, and erythromycin, chosen as representatives of the most commonly used pharmacological classes of antimicrobials. For *L. pentosus*, each antibiotic was used at the breakpoint concentration defined by the FEEDAP Panel (EFSA, 2012). For *L. coryniformis*, which was not listed in the EFSA guidance document, we referred to the antibiotic concentrations reported by (Lara-Villoslada et al., 2007). Two μl of overnight bacterial cultures (OD_{600} = 1.3) were spotted onto MRS agar plates containing ampicillin, tetracycline, chloramphenicol or erythromycin, which were used at the following breakpoint

TABLE 1 | List of LAB isolates deriving from Nocellara del Belice table olives fermented with Sivigliano or Castelvetrano methods and related species identified by 16S rDNA sequencing.

Strain ID	Bacterial species	% Identity with reference species in BLAST database	Source of isolation	GenBank accession number
C303.8	*Enterococcus gallinarum*	99	Castelvetrano	MG585222
C301.1	*Enterococcus gallinarum*	99	Castelvetrano	MG585223
C302.1	*Enterococcus casseliflavus*	99	Castelvetrano	MG585224
C302.4	*Enterococcus casseliflavus*	99	Castelvetrano	MG585225
C303.6	*Enterococcus casseliflavus*	99	Castelvetrano	MG585226
C304.2	*Leuconostoc mesenteroides*	99	Castelvetrano	MG585227
I307.27	*Leuconostoc mesenteroides*	99	Sivigliano	MG953414
G307.7	*Leuconostoc mesenteroides*	99	Sivigliano	MG585228
G3010.28	*Leuconostoc mesenteroides*	99	Sivigliano	MG585229
G3010.29	*Leuconostoc mesenteroides*	99	Sivigliano	MG585230
H306.1	*Leuconostoc mesenteroides*	99	Sivigliano	MG585231
I307.20	*Leuconostoc mesenteroides*	99	Sivigliano	MG585232
I307.22	*Leuconostoc mesenteroides*	99	Sivigliano	MG585233
I307.29	*Leuconostoc mesenteroides*	99	Sivigliano	MG585234
I306.9	*Leuconostoc mesenteroides*	99	Sivigliano	MG585235
I3010.34	*Leuconostoc mesenteroides*	99	Sivigliano	MG585236
L309.4	*Leuconostoc mesenteroides*	98	Sivigliano	MG585237
C305.2	*Leuconostoc mesenteroides*	99	Castelvetrano	MG585238
C305.16	*Leuconostoc mesenteroides*	99	Castelvetrano	MG585257
C305.5	*Lactobacillus pentosus*	99	Castelvetrano	MG585239
D301.4	*Lactobacillus pentosus*	99	Castelvetrano	MG585240
D302.23	*Lactobacillus pentosus*	98	Castelvetrano	MG585241
D302.29	*Lactobacillus pentosus*	99	Castelvetrano	MG585242
G306.1	*Lactobacillus pentosus*	99	Sivigliano	MG585243
G306.2	*Lactobacillus pentosus*	99	Sivigliano	MG585244
G308.65	*Lactobacillus pentosus*	99	Sivigliano	MG953413
H3010.5	*Lactobacillus pentosus*	99	Sivigliano	MG585245
I306.2	*Lactobacillus pentosus*	99	Sivigliano	MG585246
H308.2	*Lactobacillus pentosus*	99	Sivigliano	MG585247
I308.32	*Lactobacillus pentosus*	100	Sivigliano	MG585248
D303.36	*Lactobacillus pentosus*	99	Castelvetrano	MG585249
H3010.1	*Lactobacillus pentosus*	99	Sivigliano	MG585250
I306.12	*Lactobacillus coryniformis*	99	Sivigliano	MG585251
H307.1	*Lactobacillus coryniformis*	99	Sivigliano	MG585252
C305.1	*Lactobacillus coryniformis*	99	Castelvetrano	MG585253
H307.6	*Lactobacillus coryniformis*	99	Sivigliano	MG585254
C303.1	*Lactobacillus oligofermentans*	99	Castelvetrano	MG585255
G3010.31	*Lactobacillus oligofermentans*	99	Sivigliano	MG585256
C371.10	*Enterococcus gallinarum*	97	Castelvetrano	MG585258
C373.1	*Enterococcus gallinarum*	98	Castelvetrano	MG585259
D371.5	*Lactobacillus pentosus*	99	Castelvetrano	MG585260
D372.20	*Lactobacillus pentosus*	99	Castelvetrano	MG585261
D373.37	*Lactobacillus pentosus*	99	Castelvetrano	MG585262
I379.8	*Lactobacillus pentosus*	99	Sivigliano	MG585263
G377.8	*Lactobacillus pentosus*	99	Sivigliano	MG585264
G378.30	*Lactobacillus pentosus*	99	Sivigliano	MG585265
H376.2	*Lactobacillus coryniformis*	98	Sivigliano	MG585266
H376.5	*Lactobacillus coryniformis*	99	Sivigliano	MG585267
H377.3	*Lactobacillus coryniformis*	99	Sivigliano	MG585268

concentrations: 2 mg/l, 32 mg/l, 8 mg/l, 1 mg/l, respectively, for *L. pentosus*, and 10 mg/l, 30 mg/l, 30 mg/l, 15 mg/l, respectively, for *L. coryniformis*. Plates were incubated for 24 h at 37°C in anaerobic conditions. Strains able to grow were considered resistant (R). The Minimum Inhibitory Concentration (MIC) for ampicillin and erythromycin of selected resistant strains was determined by broth microdilution assay, as described in (Devirgiliis et al., 2008). The antibiotic concentrations tested ranged from 1 to 20 mg/l and from 0.5 to 10 mg/l for ampicillin and erythromycin, respectively. Antibiotics were provided by Sigma Aldrich (Milan, Italy).

Antimicrobial Activity

To evaluate the antagonistic activity of *L. coryniformis* and *L. pentosus* strains against pathogens the agar double-layer diffusion method was performed (Damaceno et al., 2017). The indicator strains used were: *S. enterica* serovar Typhimurium LT2, *L. monocytogenes* OH and ETEC K88. Two μl/each of *L. coryniformis* and *L. pentosus* overnight cultures ($OD_{600} = 1.3$) were spotted onto MRS agar and incubated at 37°C for 24 h in anaerobic conditions. After incubation, cells were killed by chloroform exposure for 30 min. Plates were then overlaid with 7 ml TSA soft agar, which had been previously inoculated with 1% (v/v) of each pathogen indicator strain, and incubated at the corresponding optimal growth temperature for 24 h. The antagonist activity was recorded as the diameter (mm) of growth inhibition halo around each spot.

Caco-2 Cell Culture and Growth Conditions

The human intestinal Caco-2/TC7 cell line was provided by Monique Rousset (Institute National de la Santé et de la Recherche Médicale, INSERM, France). These cells derive from parental Caco-2 cells, exhibit a more homogeneous expression of differentiation traits, and have been reported to express higher metabolic and transport activities than the original cell line, more closely resembling small intestinal enterocytes (Caro et al., 1995). The Caco-2/TC7 cells were routinely maintained at 37°C in an atmosphere of 10% CO_2/95% air at 90% relative humidity and grown on plastic tissue culture flasks (Becton Dickinson, Milan, Italy) in Dulbecco's modified Minimum Essential Medium (complete DMEM: 3.7 g/L $NaHCO_3$, 4 mM glutamine, 10% heat inactivated fetal calf serum, 1% nonessential amino acids, 10^5 U/l penicillin and 100 mg/l streptomycin). All cell culture reagents were from Euroclone (Milan, Italy).

Competition Assay for Pathogen Adhesion to Caco-2 Cells

Caco-2 cells were seeded in 12-well plates (Becton Dickinson) and, after confluency, were left for 14-17 days to allow differentiation (Sambuy et al., 2005). Medium was changed three times a week. Complete DMEM was replaced with antibiotic- and serum-free DMEM 16 h before the assay. To test the capacity of the selected *L. pentosus* and *L. coryniformis* strains to compete with pathogens for adhesion to Caco-2 cells, *S. enterica* serovar Typhimurium LT2 and *L. monocytogenes* OH pathogens were used as test strains. Preliminary experiments were performed to set up the optimal growth conditions for pathogens as well as for lactobacilli, to ensure that each strain could be used at the exponential growth phase. The viability of pathogens and lactobacilli in DMEM was also previously verified. On the day of the assay, overnight bacterial cultures were diluted 1:10 in LB (pathogens) or MRS media (lactobacilli) and grown for 4, 5, or 6 h to the exponential growth phase, according to the respective optimal conditions previously identified for each strain. After monitoring the OD_{600}, appropriate amounts of bacterial cells were harvested by centrifugation at 5,000 × g for 10 min, resuspended in antibiotic- and serum-free DMEM and added to cell monolayers at a concentration of 1×10^7 CFU/well (pathogens) or 1×10^8 CFU/well (lactobacilli). Cells were incubated at 37°C for 1.5 h with either one of the two pathogens, alone or in combination with one of the two *Lactobacillus* strains. After incubation at 37°C for 1.5 h, non-adhering bacteria were removed by 5 washes with Hanks' Balanced Salt solution (HBSS: 137 mM NaCl, 5.36 mM KCl, 1.67 mM $CaCl_2$, 1 mM $MgCl_2$, 1.03 mM $MgSO_4$, 0.44 mM KH_2PO_4, 0.34 mM Na_2HPO_4, 5.6 mM glucose) and cell monolayers were lysed with 1% Triton-X-100, according to (Roselli et al., 2006). Adhering, viable pathogen cells were quantified by plating appropriate serial dilutions of Caco-2 lysates on Violet Red Bile Glucose Agar (VRBGA, for *Salmonella*) or Listeria Selective Agar Base (Oxford, for *Listeria*). These two media were selective for pathogens, preventing the growth of lactobacilli.

C. elegans Strain and Growth Conditions

The wild-type *C. elegans* strain, Bristol N2, was grown at 16°C on Nematode Growth Medium NGM plates covered by a layer of *E. coli* OP50, LGG, *L. coryniformis,* or *L. pentosus* strains, which were prepared as described in (Zanni et al., 2015).

C. elegans Lifespan Assay

Synchronized N2 adults were placed to lay embryos for 2 h on NGM plates, lawned with different bacteria, and then sacrificed. All lifespan assays started when the progeny became fertile (t0). Animals were transferred to new plates seeded with fresh lawns and monitored daily. They were scored as dead when they no longer responded to gentle touch with a platinum wire. At least 60 nematodes per condition were used in each experiment.

Estimation of Bacterial Titer Within the Nematode Gut

For each experiment, 10 days old animals were washed and lysed according to (Uccelletti et al., 2010). Worm lysates were then plated onto MRS-agar plates. The number of CFU was counted after 48 h of incubation at 37°C, anaerobically.

Nematode Brood Size Measurements

Progeny production was evaluated according to (Zanni et al., 2015) with some modifications. Briefly, synchronized worms obtained as above were grown on NGM plates seeded with bacteria and then allowed to lay embryos at 16°C. Next, animals were transferred onto a fresh bacteria plate every day, and the number of progeny was counted with a Zeiss Axiovert 25 microscope. The procedure was repeated until the mother worms stopped laying eggs. Each day the progeny production

was recorded and was compared with the OP50- or LGG-fed nematodes.

Pharyngeal Pumping Assay

Pharyngeal pumping was analyzed as described in (Uccelletti et al., 2008) under Zeiss Axiovert 25 microscope by counting the number of grinder contractions of 10 animals for each treatment, during a period of 30 s. The analysis was performed in 13-days-adult worms, grown on different bacteria starting from embryo stage.

Body Bending Evaluation

The locomotion behavior of nematodes, fed with different bacteria from embryos hatching, was analyzed by body bending counting after 30 s. After several washes in M9 buffer to remove bacteria, nematodes were placed in 10 µl of M9 buffer allowing them to swim freely. About 10 worms for each experimental condition were monitored.

Lipofuscin Analysis

The autofluorescence of intestinal lipofuscin was measured as an index of senescence at day 13 of adulthood. Randomly selected worms from the plate lawned with bacteria were washed three times with M9 buffer. Worms were then placed onto a 3% agar pad containing 20 mM sodium azide. Lipofuscin autofluorescence was detected by fluorescence microscopy (Zeiss Axiovert 25).

C. elegans Killing Assay

For killing assay 35 mm-NGM plates were spread with 60 µl of *L. pentosus* D303.36 or *L. coryniformis* H307.6 mixed with *S. enterica* serovar thyphimurium LT2 or *L. monocytogenes* OH, in 1:1 ratio; the strains were grown as indicated above. *C. elegans* synchronous L4 larvae were transferred onto the bacterial lawn and incubated at 25°C. Worms were monitored every day. Nematodes fed with pathogen alone were taken as control. A worm was considered dead when it failed to respond to touch.

Statistical Analysis

All experiments were performed at least in triplicate. Data are presented as mean ± SD. Prior to the analysis, normal distribution and homogeneity of variance of all variables were assumed with Shapiro-Wilk and Levene's tests, respectively. For *in vivo* experiments in *C. elegans* the statistical significance was determined by Student's *t*-test or one-way ANOVA analysis coupled with a Bonferroni post test (GraphPad Prism 5.0 software, GraphPad Software Inc., La Jolla, CA, USA). For *in vitro* experiments, statistical significance was evaluated by one-way ANOVA, followed by *post-hoc* Tukey honestly significant difference (HSD) test. Statistical univariate analysis was performed with the "Statistica" software package (version 5.0; Stat Soft Inc., Tulsa, OK). Differences with p-values < 0.05 were considered significant and were indicated as follows: $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

RESULTS

Species Identification of LAB Isolates

A total of 49 Lactic Acid Bacteria strains deriving from Nocellara del Belice table olives fermented with Spanish or Castelvetrano methods, recently collected in our previous work (Zinno et al., 2017), were characterized at the species level. Ribosomal 16S DNA sequencing allowed the identification of 4 *Enterococcus gallinarum*, 3 *E. casseliflavus*, 14 *L. mesenteroides*, 7 *L. coryniformis* and 2 *L. oligofermentans* (**Table 1**). For 19 isolates, however, similarity searches using sequenced ribosomal DNA fragments retrieved ambiguous species assignments, as the genomic sequences matched both *L. pentosus* and *L. plantarum* with similar scores. These isolates were therefore subjected to a multiplex PCR assay, using *recA* gene-based primers, which are able to discriminate *L. plantarum*, *L. paraplantarum,* and *L. pentosus* species (Torriani et al., 2001). The PCR results clarified that all 19 strains belonged to the *L. pentosus* species (Figure S1). *L. pentosus* and *L. coryniformis* isolates were chosen as potential probiotic candidates for further screening, as these two species are widely employed as starter and/or protective cultures in table olive manufacturing. To assess the presence of unique strains, these isolates were subjected to strain typing using a combination of GTG_5 rep-PCR (**Figure 1**) and of Enterobacterial Repetitive Intergenic Consensus sequence PCR (ERIC-PCR, data not shown). The results shown in **Figure 1** revealed the presence of distinct fingerprinting profiles characterizing each strain within both species, indicating that each of the 19 *L. pentosus* and 7 *L. coryniformis* isolates represented a unique strain. Therefore, they were all subjected in parallel to the subsequent assays aimed at characterizing probiotic capacity.

In Vitro Tolerance to Simulated Gastro-Intestinal Conditions

Tolerance to gastrointestinal conditions was assayed using a series of sequential treatments that simulate bacterial transit along the mammalian GI tract. As shown in **Table 2**, each treatment differentially affected survival of the tested strains. In particular, 1 h incubation in SGJ, characterized by acidic pH (2.5), exerted a mild but significant reduction of bacterial counts for all the *L. coryniformis* and *L. pentosus* strains. Percent survival was close to 90% in all cases, with the exception of *L. coryniformis* isolate I306.12 which was almost unaffected by this treatment (98% survival), while two other *L. coryniformis* strains (C305.1 and H376.2) were unable to survive this condition (**Table 2**). Subsequent treatment of the surviving strains with SPJ containing bile salts and pancreatin, displayed a more severe impact on overall bacterial survival, as revealed by the inability of 5 *L. pentosus* strains to tolerate pancreatic conditions after 2 h of incubation (**Table 2**). Both 2 and 3 h treatments in SPJ are usually employed to mimick transit time in the GI tract. In our case however, 3 h incubation in SPJ did not affect overall bacterial survival any further than the 2 h incubation timepoint, with the exception of the *L. pentosus* strain G378.30 which did not survive, and of 5 *L. pentosus* strains that showed a decrease of about 1.5 log in CFU/ml. In order to compare the results

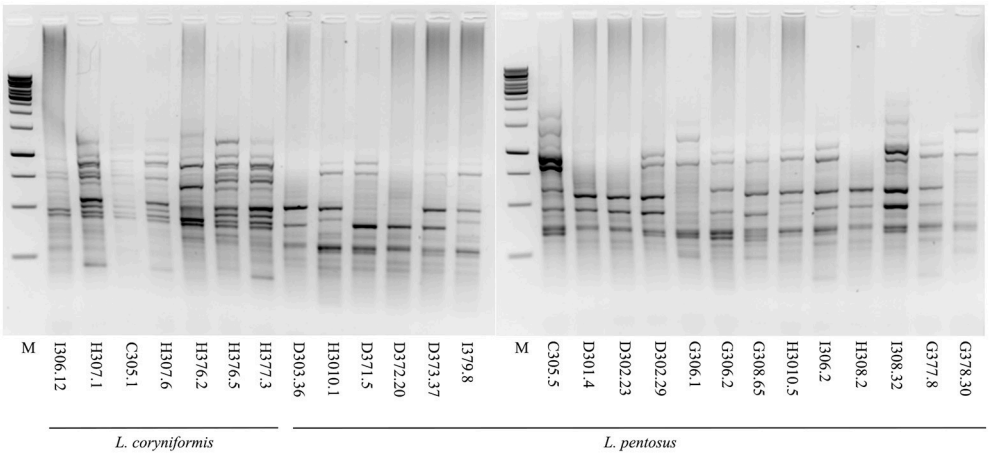

FIGURE 1 | Strain typing of the selected *L. pentosus* and *L. coryniformis* isolates by rep-PCR. Agarose gel electrophoresis of GTG_5 rep-PCR fingerprinting profiles of *L. coryniformis* and *L. pentosus* strains. M: 1 kb DNA ladder (Microzone, UK).

obtained, survival capacity was calculated for each strain as well as for the reference probiotic strain LGG (see Materials and Methods). Overall, the capacity of the tested strains to tolerate gastrointestinal conditions ranged between 50 and 80%. Notably, 7 *L. pentosus* and 4 *L. coryniformis* strains showed survival capacities similar or higher than those of the reference probiotic strain LGG, namely about 70% at the end of both treatments (**Table 2**).

Antibiotic Resistance

The *L. coryniformis* and *L. pentosus* strains were analyzed for resistance to ampicillin, tetracycline, chloramphenicol, and erythromycin, as representatives of different pharmacological classes of antimicrobials widely employed in human and veterinary medicine. The selected antibiotics were used at the breakpoint concentrations proposed by the European Food Safety Agency (EFSA) for *L. pentosus* (EFSA, 2012), while for *L. coryniformis* they were chosen according to (Lara-Villoslada et al., 2007). **Table 3** shows the antibiotic resistance panel for each tested bacterial strain. Overall, the majority of the tested strains displayed susceptibility to the selected antibiotics. However, all *L. pentosus* strains displayed resistance to ampicillin, with the exception of isolate C305.5. As for the other antibiotics, all 19 *L. pentosus* strains were sensitive to tetracycline and chloramphenicol, while 3 strains, namely D303.36, H3010.1, and I379.8, displayed phenotypic resistance to erythromycin. All *L. coryniformis* strains resulted susceptible to all tested antibiotics (**Table 3**). The ampicillin and erythromycin resistant *L. pentosus* strains were further investigated by quantifying their MIC values for the two antibiotics. MIC values above the breakpoints, corresponding to 2 mg/l for ampicillin and 1 mg/l for erythromycin, were considered indicative of phenotypic antibiotic resistance. The results show that ampicillin MIC values ranged between 2 and 20 mg/l, with variable distribution within the 18 analyzed strains. Three of them, namely *L. pentosus* D303.36, H3010.1, and I379.8, which were resistant to both antibiotics, also displayed erythromycin MIC values of 1.125 or 2.5 mg/l (**Table 3**).

Antimicrobial Activity Against Pathogens

The great majority of the *L. pentosus* and *L. coryniformis* strains displayed antagonistic activity in the agar double-layer diffusion test against all three pathogens chosen as indicator strains (*S. enterica* serovar Typhimurium LT2, *L. monocytogenes* OH, and ETEC K88). The strength of such inhibition was variable among the different lactobacilli, as shown by broad variability of the inhibition halo diameters on all 3 pathogen test strains (Table S1). The only exception was represented by *L. pentosus* strain C305.5, which was unable to inhibit growth of *S. enterica* serovar Typhimurium LT2 (**Table 4**). In particular, the majority of the 19 *L. pentosus* strains produced inhibition halo diameters above median value when tested against all three pathogens. The *L. coryniformis* strains, on the other hand, showed overall inhibition halo diameters between 1 mm and the corresponding median value against all three pathogens, with the exception of two strains, namely H307.1 and H377.3, both displaying inhibition halo diameters above the median against *Salmonella* and ETEC (**Table 4**). Notably, both of these latter strains had shown survival capacities comparable to that of the well characterized probiotic strain LGG following simulated gastro-intestinal condition, and were both susceptible to all tested antibiotics.

In Vivo Screening in *C. elegans*

In vivo screening of the *L. pentosus* and *L. coryniformis* strains for health-promoting traits was performed in the *C. elegans* model system, which was shown to display beneficial effects in response to administration of probiotic bacteria (Nakagawa et al., 2016). Lifespan analysis was initially used as a pre-screening assay to select those bacterial strains able to prolong worm longevity. To this aim, the lifespan of animals separately fed each of the isolated *Lactobacillus* strains starting from embryo hatching, was compared with that of control worms grown on LGG as the

TABLE 2 | Survival of *L. pentosus* and *L. coryniformis* strains under *in vitro* simulated gastro-intestinal conditions.

Bacterial species	Strain ID	t0	SGJ	SPJ $_{2h}$	SPJ $_{3h}$	Survival capacity (%)
L. pentosus	C305.5	8.20 ± 0.02^{a}	7.53 ± 0.03^{b}	6.18 ± 0.02^{c}	6.14 ± 0.03^{c}	74.92
	D301.4	9.12 ± 0.08^{a}	7.75 ± 0.15^{b}	0	0	0
	D302.23	8.93 ± 0.03^{a}	8.40 ± 0.07^{b}	0	0	0
	D302.29	9.23 ± 0.003^{a}	8.70 ± 0.05^{b}	0	0	0
	G306.1	8.31 ± 0.18^{a}	7.77 ± 0.05^{b}	5.97 ± 0.07^{c}	5.94 ± 0.07^{c}	71.44
	G306.2	8.91 ± 0.12^{a}	8.43 ± 0.02^{b}	6.99 ± 0.02^{c}	6.91 ± 0.05^{c}	77.60
	G308.65	9.09 ± 0.06^{a}	8.30 ± 0.02^{b}	0	0	0
	H3010.5	9.49 ± 0.09^{a}	8.48 ± 0.05^{b}	6.20 ± 0.12^{c}	4.72 ± 0.15^{d}	49.76
	I306.2	8.61 ± 0.13^{a}	8.07 ± 0.09^{b}	6.69 ± 0.03^{c}	6.50 ± 0.10^{c}	75.47
	H308.2	8.63 ± 0.24^{a}	8.10 ± 0.05^{b}	6.66 ± 0.04^{c}	5.99 ± 0.10^{d}	69.43
	I308.32	9.19 ± 0.01^{a}	8.06 ± 0.02^{b}	0	0	0
	G377.8	9.38 ± 0.32^{a}	8.23 ± 0.01^{b}	6.77 ± 0.02^{c}	6.44 ± 0.07^{c}	68.64
	G378.30	9.17 ± 0.19^{a}	8.08 ± 0.07^{b}	5.52 ± 0.30^{c}	0	0
	D303.36	9.05 ± 0.14^{a}	8.54 ± 0.03^{b}	5.21 ± 0.07^{c}	4.68 ± 0.04^{d}	51.65
	H3010.1	8.92 ± 0.21^{a}	6.81 ± 0.35^{b}	5.01 ± 0.06^{c}	4.74 ± 0.15^{c}	53.17
	D371.5	8.66 ± 0.04^{a}	7.84 ± 0.04^{b}	5.62 ± 0.51^{c}	5.32 ± 0.05^{c}	61.43
	D372.20	9.10 ± 0.06^{a}	7.85 ± 0.03^{b}	5.99 ± 0.03^{c}	4.56 ± 0.33^{d}	50.09
	D373.37	8.53 ± 0.05^{a}	7.57 ± 0.09^{b}	6.27 ± 0.2^{c}	6.23 ± 0.02^{c}	72.99
	I379.8	8.95 ± 0.04^{a}	8.25 ± 0.03^{b}	7.02 ± 0.04^{c}	6.88 ± 0.02^{d}	76.88
L. coryniformis	I306.12	8.71 ± 0.093^{a}	8.56 ± 0.12^{a}	6.80 ± 0.25^{b}	6.71 ± 0.03^{b}	77.04
	H307.1	8.80 ± 0.09^{a}	8.09 ± 0.13^{b}	6.13 ± 0.06^{c}	6.18 ± 0.04^{c}	70.20
	C305.1	8.25 ± 0.01	0	0	0	0
	H307.6	9.66 ± 0.24^{a}	8.54 ± 0.04^{b}	7.80 ± 0.05^{c}	7.09 ± 0.10^{d}	73.44
	H376.2	8.55 ± 0.05	0	0	0	0
	H376.5	8.35 ± 0.01^{a}	7.54 ± 0.14^{b}	5.55 ± 0.41^{c}	5.49 ± 0.15^{c}	65.82
	H377.3	8.76 ± 0.06^{a}	8.21 ± 0.04^{b}	6.98 ± 0.04^{c}	6.96 ± 0.01^{c}	79.49
L. rhamnosus	GG	8.7 ± 0.021^{a}	8.1 ± 0.39^{b}	6.9 ± 0.04^{c}	6.1 ± 0.04^{d}	69.91

Values represent mean log (CFU/ml) ± standard deviation. Distinct letters indicate significance at p value < 0.05 among log (CFU/ml) values for the different tested conditions, within each strain.

Survival capacity is expressed as the percentage of 1– [(log CFU/ml$_{t0}$ – log CFU/ml$_{SPJ3h}$)/log CFU/ml$_{t0}$].

only bacterial source, or on the standard *E. coli* OP50 diet. The results are reported in Figure S2. Among the tested strains, the *L. pentosus* D303.36 diet induced a relevant increase in *C. elegans* longevity (Figure S2A), and animals fed *L. coryniformis* H307.6 showed similar viability with respect to those fed the probiotic strain LGG (Figure S2B). On the other hand, when compared to the control OP50 diet, none of the other tested strains determined significant increase in worm lifespan, with the exception of *L. pentosus* D371.5 (Figure S2A) and *L. coryniformis* H376.2 (Figure S2B), which led to significant lifespan reduction when used as the sole dietary source of bacteria. The *L. coryniformis* strain I306.12 induced an embryonic lethal phenotype, since embryos failed to develop into larvae (data not shown).

Therefore, the two *L. pentosus* D303.36 and *L. coryniformis* H307.6 strains were considered as the most promising candidates in terms of health-promoting features, and were selected for further analysis. **Figure 2A** shows that nematode median survival was recorded at days 18 and 15 when worms were fed *L. pentosus* D303.36 and *L. coryniformis* H307.6, respectively, as compared to 9.5 days in the case of OP50-fed worms. The above described effects on the longevity phenotype were not observed when worms were fed heat-killed bacteria, as shown in **Figure 2B**.

Microscopic observation allowed to evaluate other physiological effects promoted by the *Lactobacillus* strains in *C. elegans*: animals fed *L. pentosus* D303.36 or *L. coryniformis* H307.6 displayed reduced size with respect to OP50-fed animals along all developmental stages, similarly to the effect of feeding the probiotic strain LGG (**Figure 2C**). Moreover, *C. elegans* progeny production was significantly reduced when nematodes were fed *L. pentosus* D303.36 or *L. coryniformis* H307.6, with about 60% reduction of progeny number in both cases as compared to OP50-fed animals. A similar reduction was also observed in the case of LGG-fed animals, although to a lesser extent (**Figure 2D**).

Subsequently, aging biomarkers were analyzed in order to evaluate the prolonged lifespan of *C. elegans* supplemented with the different isolates at 13 days of adulthood. The neuromuscular functionality of nematodes was investigated by measuring contractions of the pharynx to assess whether *L. pentosus* D303.36 and *L. coryniformis* H307.6 strains could

TABLE 3 | Antibiotic resistance of *L. pentosus* and *L. coryniformis* strains.

		Antibiotic[a]			
Bacterial species	**Strain ID**	**Ampicillin (2 mg/l)**	**Tetracycline (32 mg/l)**	**Chloramphenicol (8 mg/l)**	**Erythromycin (1 mg/l)**
L. pentosus	C305.5	S	S	S	S
	D301.4	R (20)	S	S	S
	D302.23	R (20)	S	S	S
	D302.29	R (2)	S	S	S
	G306.1	R (8)	S	S	S
	G306.2	R (4)	S	S	S
	G308.65	R (16)	S	S	S
	H3010.5	R (4)	S	S	S
	I306.2	R (8)	S	S	S
	H308.2	R (12)	S	S	S
	I308.32	R (8)	S	S	S
	G377.8	R (4)	S	S	S
	G378.30	R (8)	S	S	S
	D303.36	R (16)	S	S	R (2.5)
	H3010.1	R (4)	S	S	R (1.25)
	D371.5	R (4)	S	S	S
	D372.20	R (8)	S	S	S
	D373.37	R (8)	S	S	S
	I379.8	R (4)	S	S	R (2.5)
		Ampicillin (10 mg/l)	**Tetracycline (30 mg/l)**	**Chloramphenicol (30 mg/l)**	**Erythromycin (15 mg/l)**
L. coryniformis	I306.12	S	S	S	S
	H307.1	S	S	S	S
	C305.1	S	S	S	S
	H307.6	S	S	S	S
	H376.2	S	S	S	S
	H376.5	S	S	S	S
	H377.3	S	S	S	S

[a]*Each antibiotic was used at the microbiological breakpoint indicated in parenthesis, according to the bacterial species.*
LAB strains resulting sensitive or resistant to antibiotic are referred as S or R, respectively. MIC values (expressed as mg/l) for resistant strains are indicated in parenthesis.

TABLE 4 | Antimicrobial activity of *L. pentosus* and *L. coryniformis* strains against indicator pathogens.

		Pathogen strain		
Bacterial species	**Strain ID**	***S. enterica* serovar Typhimurium LT2**	***L. monocytogenes* OH**	**ETEC K88**
L. pentosus	C305.5	–	+	+
	D301.4	++	+	++
	D302.23	++	++	++
	D302.29	++	+	+
	G306.1	++	+	+
	G306.2	++	++	++
	G308.65	++	+	+
	H3010.5	+	++	++
	I306.2	++	++	++
	H308.2	++	+	++
	I308.32	++	++	+
	G377.8	+	++	++
	G378.30	+	++	++
	D303.36	++	+	+
	H3010.1	++	++	++
	D371.5	+	++	++
	D372.20	+	++	++
	D373.37	+	+	++
	I379.8	+	++	++
L. coryniformis	I306.12	+	+	+
	H307.1	++	+	++
	C305.1	+	+	+
	H307.6	+	+	+
	H376.2	+	+	+
	H376.5	+	+	+
	H377.3	++	+	++

Inhibitory activities refer to the measured inhibition halo diameter and are indicated as: – (diameter <1 mm);
+ (1 mm < diameter < median value); ++ (diameter > median value).

impact on *C. elegans* swallowing capacity. Pharyngeal pumping rates increased when each of the two strains were administered to nematodes, with respect to the OP50 control (**Figure 3A**). In parallel, analysis of the locomotion behavior was performed to determine possible modifications of *C. elegans* mobility. Similarly to LGG-fed worms, body bending was increased when feeding *L. coryniformis* H307.6 as compared to OP50. On the other hand, the *L. pentosus* D303.36-based diet did not show any effect (**Figure 3B**). Evaluation of intestinal lipofuscin accumulation was performed as an additional aging biomarker. Fluorescence microscope analysis revealed a reduced fluorescent signal, diffused throughout the body of nematodes fed *L. pentosus* D303.36 or *L. coryniformis* H307.6. Similar results were obtained when feeding the probiotic control strain LGG. By contrast, nematodes fed OP50 showed intense fluorescence accumulating in large granules along the intestine, typical of aged animals (**Figure 3C**).

To evaluate their colonization capacity, bacteria were recovered from *C. elegans* gut and quantified by measuring CFUs at 10-days adulthood stage. Both *L. pentosus* D303.36 and *L. coryniformis* H307.6 strains showed a colonization capacity almost identical to that of the probiotic control strain LGG (data not shown).

Probiotic strains were reported to protect *C. elegans* against infection mediated by several pathogens (Park et al., 2014; Neuhaus et al., 2017). Since both *L. pentosus* D303.36 and *L. coryniformis* H307.6 strains displayed antimicrobial activity against the three tested pathogens (**Table 4**), these two LAB strains were evaluated also for their protective potential in *C. elegans* against pathogen infection mediated death. *S. enterica* serovar Thyphimurium LT2 or *L. monocytogenes* OH were chosen for the assay as they represent important foodborne pathogens. The results in **Figure 4** demonstrate that *C. elegans* displayed reduced survival on NGM medium when fed *S. enterica* Thyphimurium LT2 alone, as compared to nematodes supplemented with co-cultures of the same pathogen with *L. pentosus* D303.36 or *L. coryniformis* H307.6. In the case of *L. monocytogenes* OH, on the other hand, neither one of the

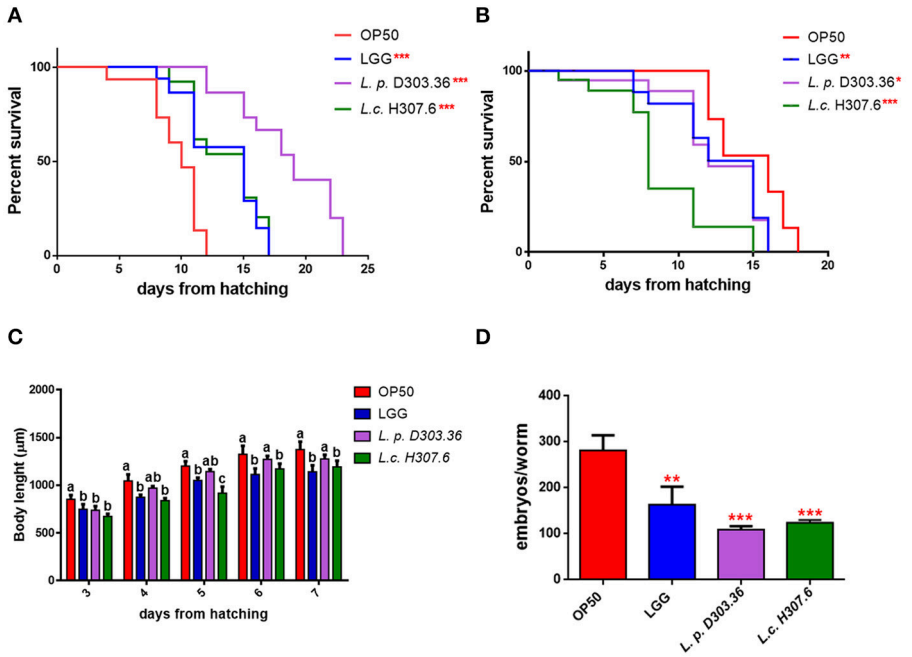

FIGURE 2 | Effect of the *L. pentosus* D303.36 and *L. coryniformis* H307.6 strains on *C. elegans* lifespan, body length, and fertility. **(A)** Kaplan–Mèier survival plots of N2 fed *L. p.* D303.36 and *L. c.* H307.6 strains, starting from embryo hatching; $n = 60$ for each single experiment. Lifespans of OP50- and LGG-fed animals are reported as controls. **(B)** Survival of *C. elegans* fed heat killed bacterial strains. Statistical analysis was evaluated by one-way ANOVA with the Bonferroni *post-test*; asterisks indicate significant differences ($^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$). **(C)** Effect of bacteria on larval development. Worm length was measured from head to tail at the indicated time points. Statistical analysis was performed by one-way ANOVA with the Bonferroni *post-test*; different letters indicate significant differences ($p < 0.05$). **(D)** Embryo production per worm in animals fed different bacterial strains. Bars represent the mean of three independent experiments ($^{**}p < 0.01$; $^{***}p < 0.001$).

co-cultures was able to abrogate premature death of the animals (data not shown).

Reduction of Pathogen Adhesion to Human Intestinal Epithelial Cells by *L. pentosus* D303.36 and *L. coryniformis* H307.6 Strains

Several pathogens, including *S. enterica* serovar Typhimurium, *L. monocytogenes* and ETEC, are able to adhere to the brush border of intestinal cells, damaging the structure of tight or adherens junctions (Boyle and Finlay, 2003; Köhler et al., 2007). Increasing evidence highlights the capacity of lactobacilli to inhibit pathogen adhesion to the intestinal mucosa and counteract the associated inflammatory processes, thus preventing intestinal disease in both humans and animals (Zhou et al., 2010; Asahara et al., 2011). The two *L. pentosus* D303.36 and *L. coryniformis* H307.6 strains were therefore analyzed for their capacity to reduce pathogen adhesion to Caco-2 cells, which represent a valuable *in vitro* model of human intestinal epithelium. Both strains, which were previously tested for their adhesion ability to Caco-2 cells (data not shown), were co-cultured with intestinal cells in combination with *S. enterica* serovar Typhimurium LT2 or *L. monocytogenes* OH.

Treatment of intestinal cells with *L. pentosus* D303.36 or *L. coryniformis* H307.6 reduced adhesion of *S. enterica* serovar Typhimurium LT2 by about 0.5 log CFU/ml (**Figure 5**). Statistical analysis performed by ANOVA revealed p-values < 0.05, indicating that, although to a mild extent, both strains were able to significantly counteract pathogen attachment to the cells. On the other hand, no protective effect of *L. pentosus* D303.36 or *L. coryniformis* H307.6 against *L. monocytogenes* OH could be observed (data not shown).

DISCUSSION

Table olives are increasingly recognized as a potential natural source of probiotic bacteria that could be exploited to obtain a health-promoting functional product (Bonatsou et al., 2017). Different olive *cultivars* are characterized by specific autochtonous fermenting microbiota (Heperkan, 2013), representing a valuable reservoir of novel strains of environmental origin. In particular, Nocellara del Belice is an important Italian olive *cultivar*, awarded the official PDO designation (Protected Designation of Origin, EC Regulation No 134/1998), whose microbial composition is dominated by several LAB species of technological and health-related interest (Aponte et al., 2010, 2012; Zinno et al., 2017). In the present work, a combination of *in vitro* and *in vivo* approaches was used to select novel potentially probiotic *Lactobacillus* strains deriving from a LAB collection of isolates from Nocellara del Belice table olives fermented with Spanish or Castelvetrano

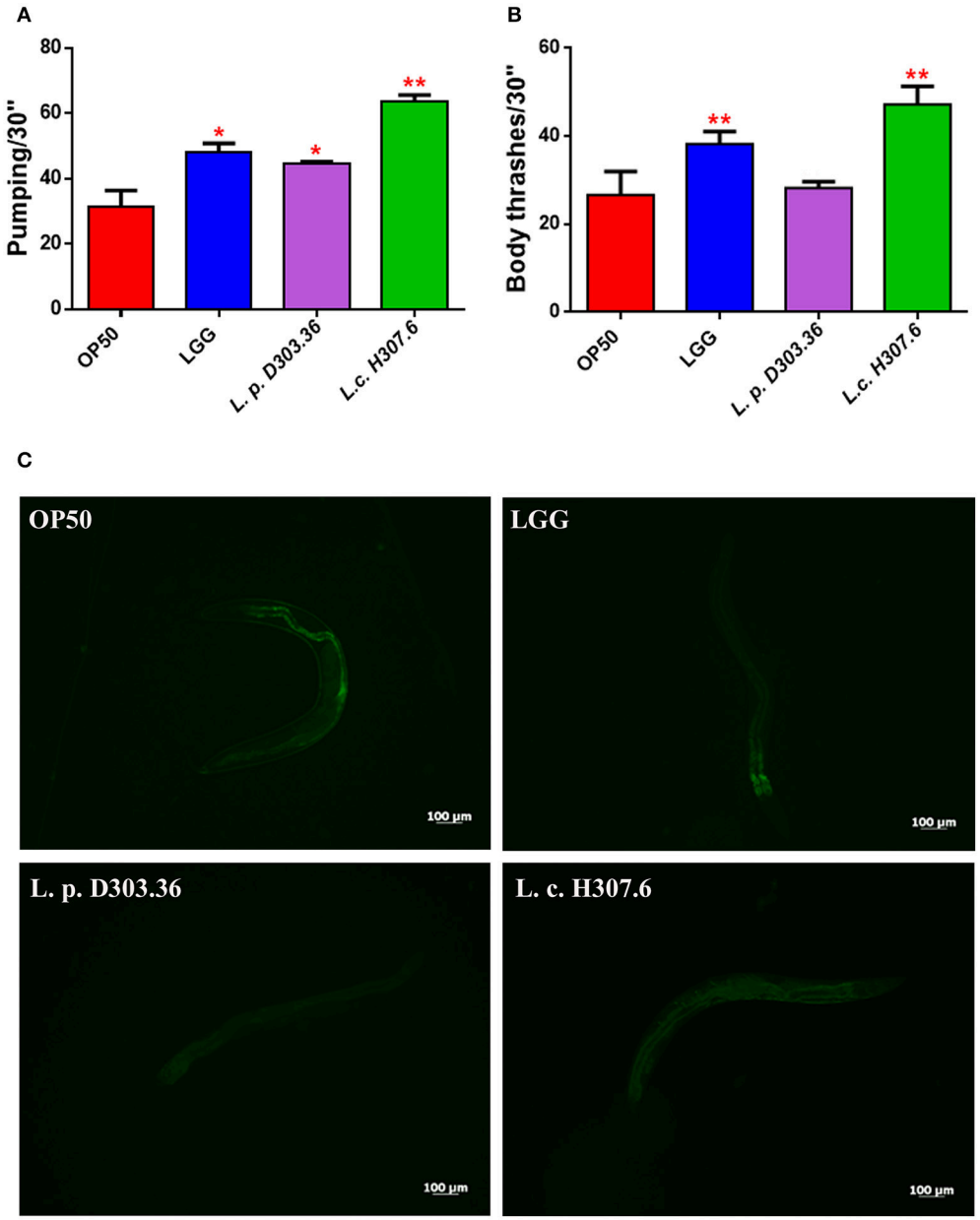

FIGURE 3 | Analysis of aging markers in *C. elegans* fed the *L. pentosus* D303.36 and *L. coryniformis* H307.6 strains. **(A)** Pumping rate of 13-days-old worms, measured for 30 s and determined from the mean of 10 worms for each bacterial strain. Worms fed OP50 or LGG were used as controls. **(B)** Body bend frequency, measured for 30 s, of *C. elegans* fed different *Lactobacillus* strains or OP50. Statistical analysis was performed by one-way ANOVA with the Bonferroni *post-test*; asterisks indicate significant differences (*$p < 0.05$, **$p < 0.01$). **(C)** Autofluorescence of lipofuscin granules in *C. elegans* fed different bacterial strains on day 13. Ten worms were used for each measurement. Scale bar = 100µm.

methods, that was previously established in our laboratory (Zinno et al., 2017). Characterization of LAB isolates at the species level identified *Leuconostoc mesenteroides*, *L. pentosus,* and *L. coryniformis* as the predominant species, along with *L. oligofermentans*, *E. gallinarum,* and *E. casseliflavus* as minor components. Among these isolates, *L. pentosus* and *L. coryniformis* were chosen as potential probiotic candidates for further analysis since increasing experimental evidence reports on health-promoting features displayed by several strains belonging to these species (Olivares et al., 2006; Abriouel et al., 2017; Bendali et al., 2017). Probiotic traits are known to be strain-specific (Saulnier et al., 2009; Amund, 2016), it is therefore important to consider that all selected *L. pentosus* and *L. coryniformis* isolates analyzed in this work displayed distinct fingerprinting profiles, therefore representing unique and novel strains.

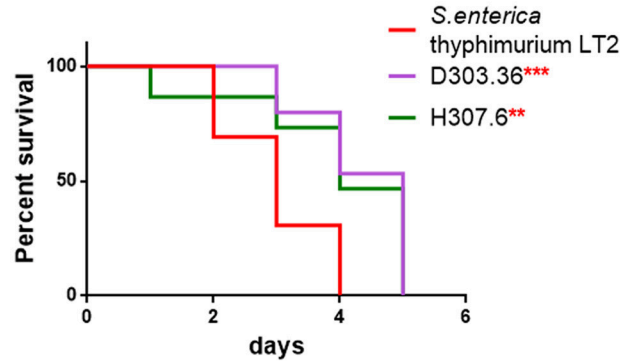

FIGURE 4 | Rescuing potential of *L. pentosus* D303.36 and *L. coryniformis* H307.6 against *Salmonella enterica* infection. Kaplan–Mèier Survival plot of *C. elegans* fed *L. pentosus* D303.36 or *L. coryniformis* H307.6 in a 1:1 co-colture with *S. enterica* serovar Thyphimurium LT2. Worms fed *Salmonella* alone were taken as control (**$p < 0.01$; ***$p < 0.001$).

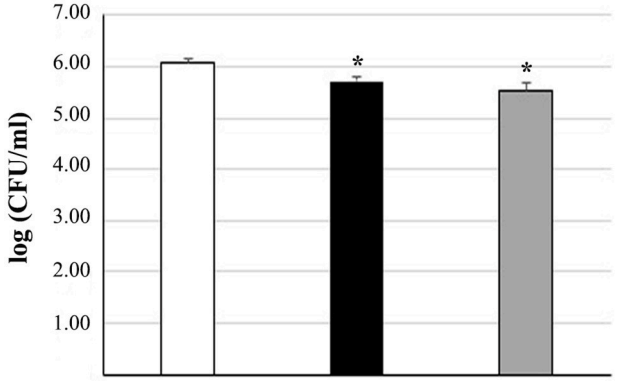

FIGURE 5 | Reduction of *Salmonella enterica* adhesion to Caco-2 cells by *L. pentosus* D303.36 and *L. coryniformis* H307.6. Cell counts of viable *S. enterica* serovar Thyphimurium LT2 adhering on differentiated Caco-2 cells treated with: *S. enterica* alone (control, white column); *S. enterica* in combination with *L. pentosus* D303.36 (black column) or *L. coryniformis* H307.6 (gray column). Columns represent the mean ± SD of four independent experiments. Data are reported as log of bacterial CFU recovered after plating. Statistical analysis was performed by one-way ANOVA, followed by *post-hoc* Tukey honestly significant difference (HSD) test. Asterisks indicate significant differences (*$p < 0.05$ vs. control).

Resistance to the harsh conditions of the upper GI tract is a key pre-requisite for efficient colonization by a probiotic strain (Dicks and Botes, 2010). As a first predictive phenotypic trait, tolerance to gastrointestinal conditions was assessed by evaluating the survival capacity of each strain in comparison with the well-characterized, commercial probiotic strain *L. rhamnosus* GG. Overall, the majority of the investigated strains showed 50–80% survival capacity to gastric and pancreatic juice treatments, with strain-dependent variability. Notably, 11 of the 26 *Lactobacillus* strains assayed in this study displayed survival rates equal or higher than that of the well characterized probiotic control strain LGG (70%) at the end of simulated digestion treatments, thus pointing at the diet, and to fermented foods in particular, as a relevant source of live microorganisms that can reach the host gut microbiota in a metabolically active state, where they can transiently colonize and interact with resident gut bacteria. An important trait to be verified for safety purposes concerns antibiotic resistance profiling (Imperial and Ibana, 2016). To this aim, all the *L. coryniformis* and *L. pentosus* strains were also analyzed for resistance to ampicillin, tetracycline, chloramphenicol or erythromycin, as representatives of distinct pharmacological classes of antimicrobials commonly used in human and veterinary medicine (Aminov, 2017). While the majority of the tested strains were susceptible to these antibiotics, all but one of the *L. pentosus* isolates showed phenotypic resistance to ampicillin, with a few of them diplaying erythromycin resistance as well. These strains will be subjected to further analysis to identify the corresponding antibiotic-resistance determinants and their genomic contexts, so that possible horizontal transmission can be ascertained.

Probiotics are known to be effective in preventing or counteracting foodborne infections by reducing the growth of enteric pathogens through different mechanisms, involving competitive exclusion or the production of inhibitory molecules (Karami et al., 2017; Mathipa and Thantsha, 2017). We therefore tested the antimicrobial activity exerted by the *L. pentosus* and *L. coryniformis* strains against three common pathogens, namely *S. enterica* serovar Typhimurium, *L. monocytogenes* and ETEC. The great majority of the *Lactobacillus* isolates screened in this work resulted to be active against all three pathogens *in vitro*, although with variable, strain-specific efficacy.

At the end of this *in vitro* screening, 3 *L. coryniformis* strains appeared to be good candidate probiotics on the basis of their positive performance under simulated gastro-intestinal digestion, antibiotic susceptibility, and antimicrobial activity. However, only one of these strains was also selected by parallel screening for health-promoting traits in the *in vivo* nematode model *C. elegans*. This simplified model organism lives on bacteria as the only food source, but a substantial number of bacterial cells escape the grinding capacity of the worm larynx and can proceed to colonize the nematode gut (Nakagawa et al., 2016). The *L. coryniformis* H307.6 strain was able to significantly increase *C. elegans* lifespan as compared to the OP50 control strain, overlapping the effect exerted by the well characterized probiotic strain LGG, while positively impacting also on other well-established aging biomarkers such as pharyngeal pumping rate, body size, brood size, and lipofuscin (Lee et al., 2015). These results further confirm the ability of specific LAB strains to extend nematode lifespan as reported in previous studies (Ikeda et al., 2007; Komura et al., 2013; Nakagawa et al., 2016). Moreover, the *L. coryniformis* H307.6 displayed health-promoting activities also in host defense against *S. enterica* serovar Typhimurium, both *in vitro* (inhibiting pathogen growth as well as competing with pathogen for intestinal cell adhesion), and *in vivo* (increasing survival of infected worms). It is worth mentioning in this respect that host-pathogen interactions have been investigated in *C. elegans* for a number of pathogens of human and animal origin (Clark and Hodgkin, 2014), including *S. enterica* and *L. monocytogenes* which are able to colonize

TABLE 5 | Summary table listing the main features displayed by the candidate probiotic strains identified in this study.

Species/strain ID	*C. elegans*		GI tract survival (%)	Antimicrobial activity			Antibiotic resistance	
	Longevity	Colonization		*In vitro*	Caco-2	*C. elegans*	Growth on antibiotic*	MIC (mg/ml)
L. coryniformis H307.1	–	ND	70.20	+++	ND	ND	S	NA
L. coryniformis H377.3	–	ND	79.49	+++	ND	ND	S	NA
L. coryniformis H307.6	+	+	73.44	+	+(*Salmonella*)	+(*Salmonella*)	S	NA
L. pentosus D303.36	++	+	51.65	++	+(*Salmonella*)	+(*Salmonella*)	R(Amp, Ery)	Amp = 16Ery = 2.5

**growth in the presence of breakpoint concentration of antibiotics; R, Resistant to the specified antibiotics; S, Susceptible to all tested antibiotics.*
MIC, Minimum Inhibitory Concentration, determined only for strains which survived breakpoint concentrations for the specified antibiotics.
ND, Not Determined; NA, Not Applicable.

the worm gut and infect the nematode (Aballay et al., 2000; Thomsen et al., 2006). A second isolate, namely *L. pentosus* D303.36, was positively selected in *C. elegans* as a lifespan extending, health-promoting strain. This strain, however, did not display good performance with respect to tolerance to GI tract conditions, and was also resistant to ampicillin and erythromycin. Therefore, further molecular characterization is necessary to exclude potential horizontal transmission of antibiotic resistance before it can be considered a promising probiotic candidate.

On the other hand, *L. coryniformis* strains H307.1 and H377.3, that were selected as very good performers in the initial *in vitro* testing screens, were both antibiotic susceptible, as well as capable of inhibiting pathogen growth in the agar double-layer diffusion assay. However, neither one of these two strains could positively impact on *C. elegans* longevity. **Table 5** summarizes the main features displayed by the candidate probiotic strains identified in this study. In light of the recent findings indicating that probiotic capacity of mixed foodborne microbial consortia might be more effective than single strain supplementation (Foligné et al., 2016; Roselli et al., 2017), testing these strains as members of a multistrain probiotic complex could open new avenues for their applications in vegetable food fermentations.

CONCLUSIONS

Extensive *in vitro* and *in vivo* characterization of 26 *Lactobacillus* strains previously isolated from fermented Nocellara del Belice table olives, led to the identification of various potential candidate probiotics. Of these, three *L. coryniformis* strains displayed good probiotic features *in vitro*, although only one of them could also exert prolongevity and protective effects in the simplified model organism *C. elegans*. The GRAS status of Lactobacilli allows to consider their application as starters of fermentation with probiotic added value. However, further validation in *in vivo* trials with more complex animal or human systems should be performed to gain deeper understanding of their potential health promoting features for human health.

AUTHOR CONTRIBUTIONS

CD and DU conceived and designed the experiments. CD and DU wrote the paper. GP and CP critical revision of manuscript. ES performed animal experiments/treatments. PZ and BG performed microbiological analyses. MR and BG performed cell culture experiments.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.00595/full#supplementary-material

REFERENCES

Aballay, A., Yorgey, P., and Ausubel, F. M. (2000). *Salmonella typhimurium* proliferates and establishes a persistent infection in the intestine of *Caenorhabditis elegans*. *Curr. Biol.* 10, 1539–1542. doi: 10.1016/S0960-9822(00)00830-7

Abriouel, H., Benomar, N., Cobo, A., Caballero, N., Fernández Fuentes, M. Á., Pérez-Pulido, R., et al. (2012). Characterization of lactic acid bacteria from naturally-fermented Manzanilla Alorena green table olives. *Food Microbiol.* 32, 308–316. doi: 10.1016/j.fm.2012.07.006

Abriouel, H., Perez Montoro, B., Casimiro-Soriguer, C. S., Pérez Montoro, A. J., Knapp, C. W., Caballero Gómez, N., et al. (2017). Insight into potential probiotic markers predicted in Lactobacillus pentosus MP-10 genome sequence. *Front. Microbiol.* 8:891. doi: 10.3389/fmicb.2017.00891

Aminov, R. (2017). History of antimicrobial drug discovery: major classes and health impact. *Biochem. Pharmacol.* 133, 4–19. doi: 10.1016/j.bcp.2016.10.001

Amund, O. D. (2016). Exploring the relationship between exposure to technological and gastrointestinal stress and probiotic functional properties of lactobacilli and bifidobacteria. *Can. J. Microbiol.* 62, 715–725. doi: 10.1139/cjm-2016-0186

Aponte, M., Blaiotta, G., Croce, F. L., Mazzaglia, A., Farina, V., Settanni, L., et al. (2012). Use of selected autochthonous lactic acid bacteria for Spanish-style table olive fermentation. *Food Microbiol.* 30, 8–16. doi: 10.1016/j.fm.2011.10.005

Aponte, M., Ventorino, V., Blaiotta, G., Volpe, G., Farina, V., Avellone, G., et al. (2010). Study of green Sicilian table olive fermentations through microbiological, chemical and sensory analyses. *Food Microbiol.* 27, 162–170. doi: 10.1016/j.fm.2009.09.010

Argyri, A. A., Lyra, E., Panagou, E. Z., and Tassou, C. C. (2013). Fate of *Escherichia coli* O157:H7, Salmonella Enteritidis and *Listeria monocytogenes* during storage of fermented green table olives in brine. *Food Microbiol.* 36, 1–6. doi: 10.1016/j.fm.2013.04.001

Arroyo-López, F. N., Querol, A., Bautista-Gallego, J., and Garrido-Fernandez, A. (2008). Role of yeasts in table olive production. *Int. J. Food Microbiol.* 128, 189–196. doi: 10.1016/j.ijfoodmicro.2008.08.018

Asahara, T., Shimizu, K., Takada, T., Kado, S., Yuki, N., Morotomi, M., et al. (2011). Protective effect of *Lactobacillus casei* strain Shirota against lethal infection with multi-drug resistant *Salmonella enterica* serovar Typhimurium DT104 in mice. *J. Appl. Microbiol.* 110, 163–173. doi: 10.1111/j.1365-2672.2010.04884.x

Baker, H. C., Tran, D. N., and Thomas, L. V. (2009). Health benefits of probiotics for the elderly: a review. *J. Foodserves* 20, 250–252. doi: 10.1111/j.1748-0159.2009.00147.x

Bendali, F., Kerdouche, K., Hamma-Faradji, S., and Drider, D. (2017). *In vitro* and *in vivo* cholesterol lowering ability of Lactobacillus pentosus KF923750. *Benef. Microbes* 8, 271–280. doi: 10.3920/BM2016.0121

Bevilacqua, A., Cannarsi, M., Gallo, M., Sinigaglia, M., and Corbo, M. R. (2010). Characterization and implications of *Enterobacter cloacae* strains, isolated from Italian table olives "Bella di Cerignola". *J. Food Sci.* 75, M53–M60. doi: 10.1111/j.1750-3841.2009.01445.x

Bonatsou, S., Tassou, C. C., Panagou, E. Z., and Nychas, G. E. (2017). Table olive fermentation using starter cultures with multifunctional potential. *Microorganisms* 5:30. doi: 10.3390/microorganisms5020030

Botta, C., Langerholc, T., Cencic, A., and Cocolin, L. (2014). *In vitro* selection and characterization of new probiotic candidates from table olive microbiota. *PLoS ONE* 9:e94457. doi: 10.1371/journal.pone.0094457

Boyle, E. C., and Finlay, B. B. (2003). Bacterial pathogenesis: exploiting cellular adherence. *Curr. Opin. Cell Biol.* 15, 633–639. doi: 10.1016/S0955-0674(03)00099-1

Caro, I., Boulenca, X., Rousset, M., Meunier, V., Bourrié, M., Julian, B., et al. (1995). Characterisation of a newly isolated Caco-2 clone (TC-7), as a model of transport processes and biotransformation of drugs. *Int. J. Pharm.* 116, 147–158. doi: 10.1016/0378-5173(94)00280-I

Clark, L. C., and Hodgkin, J. (2014). Commensals, probiotics and pathogens in the Caenorhabditis elegans model. *Cell. Microbiol.* 16, 27–38. doi: 10.1111/cmi.12234

Damaceno, Q. S., Souza, J. P., Nicoli, J. R., Paula, R. L., Assis, G. B., Figueiredo, H. C., et al. (2017). Evaluation of potential probiotics isolated from human milk and colostrum. *Probiotics Antimicrob. Proteins* 9, 371–379. doi: 10.1007/s12602-017-9270-1

de La Puente-Redondo, V. A., del Blanco, N. G., Gutiérrez-Martín, C. B., García-Peña, F. J., and Rodríguez Ferri, E. F. (2000). Comparison of different PCR approaches for typing of *Francisella tularensis* strains. *J. Clin. Microbiol.* 38, 1016–1022.

Devirgiliis, C., Caravelli, A., Coppola, D., Barile, S., and Perozzi, G. (2008). Antibiotic resistance and microbial composition along the manufacturing process of Mozzarella di Bufala Campana. *Int. J. Food Microbiol.* 128, 378–384. doi: 10.1016/j.ijfoodmicro.2008.09.021

Di Cello, F., and Fani, R. (1996). A molecular strategy for the study of natural bacterial communities by PCR-based techniques. *Minerva Biotecnol.* 8, 126–134.

Dicks, L. M., and Botes, M. (2010). Probiotic lactic acid bacteria in the gastro-intestinal tract: health benefits, safety and mode of action. *Benef. Microbes* 1, 11–29. doi: 10.3920/BM2009.0012

EFSA (2012). Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance. *EFSA J.* 10:2740. 10.2903/j.efsa.2012.2740

FAO/WHO (2006). *Probiotics in Food. Health and nutritional properties and guidelines for evaluation*. Available online at: http://www.fao.org/food/food-safety-quality/a-z-index/probiotics/en/

Foligné, B., Parayre, S., Cheddani, R., Famelart, M. H., Madec, M. N., Plé, C., et al. (2016). Immunomodulation properties of multi-species fermented milks. *Food Microbiol.* 53, 60–69. doi: 10.1016/j.fm.2015.04.002

Garrigues, C., Johansen, E., and Crittenden, R. (2013). Pangenomics–an avenue to improved industrial starter cultures and probiotics. *Curr. Opin. Biotechnol.* 24, 187–191. doi: 10.1016/j.copbio.2012.08.009

Giraffa, G., Chanishvili, N., and Widyastuti, Y. (2010). Importance of lactobacilli in food and feed biotechnology. *Res. Microbiol.* 161, 480–487. doi: 10.1016/j.resmic.2010.03.001

Granato, D., Branco, G. F., Nazzaro, F., Cruz, A. G., and Faria, J. A. F. (2010). Functional foods and non-dairy probiotic food development: trend, concepts, and products. *Compr. Rev. Food Sci. Saf.* 9, 292–302. doi: 10.1111/j.1541-4337.2010.00110.x

Hegarty, J. W., Guinane, C. M., Ross, R. P., Hill, C., and Cotter, P. D. (2016). Bacteriocin production: a relatively unharnessed probiotic trait? *F1000Res* 5, 2587. doi: 10.12688/f1000research.9615.1

Heperkan, D. (2013). Microbiota of table olive fermentations and criteria of selection for their use as starters. *Front. Microbiol.* 4:143. doi: 10.3389/fmicb.2013.00143

Hill, C., Guarner, F., Reid, G., Gibson, G. R., Merenstein, D. J., Pot, B., et al. (2014). Expert consensus document. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nat. Rev. Gastroenterol. Hepatol.* 11, 506–514. doi: 10.1038/nrgastro.2014.66

Hurtado, A., Reguant, C., Bordons, A., and Rozès, N. (2012). Lactic acid bacteria from fermented table olives. *Food Microbiol.* 31, 1–8. doi: 10.1016/j.fm.2012.01.006

Ikeda, T., Yasui, C., Hoshino, K., Arikawa, K., and Nishikawa, Y. (2007). Influence of lactic acid bacteria on longevity of *Caenorhabditis elegans* and host defense against salmonella enterica serovar enteritidis. *Appl. Environ. Microbiol.* 73, 6404–6409. doi: 10.1128/AEM.00704-07

Imperial, I. C., and Ibana, J. A. (2016). Addressing the antibiotic resistance problem with probiotics: reducing the risk of its double-edged sword effect. *Front. Microbiol.* 7:1983. doi: 10.3389/fmicb.2016.01983

Karami, S., Roayaei, M., Hamzavi, H., Bahmani, M., Hassanzad-Azar, H., Leila, M., et al. (2017). Isolation and identification of probiotic Lactobacillus from local dairy and evaluating their antagonistic effect on pathogens. *Int. J. Pharm. Investig.* 7, 137–141. doi: 10.4103/jphi.JPHI_8_17

Kim, Y., and Mylonakis, E. (2012). Caenorhabditis elegans immune conditioning with the probiotic bacterium Lactobacillus acidophilus strain NCFM enhances gram-positive immune responses. *Infect. Immun.* 80, 2500–2508. doi: 10.1128/IAI.06350-11

Köhler, H., Sakaguchi, T., Hurley, B. P., Kase, B. A., Reinecker, H. C., and McCormick, B. A. (2007). *Salmonella enterica* serovar Typhimurium regulates intercellular junction proteins and facilitates transepithelial neutrophil and bacterial passage. *Am. J. Physiol. Gastrointest. Liver Physiol.* 293, G178–G187. doi: 10.1152/ajpgi.00535.2006

Komura, T., Ikeda, T., Yasui, C., Saeki, S., and Nishikawa, Y. (2013). Mechanism underlying prolongevity induced by bifidobacteria in *Caenorhabditis elegans*. *Biogerontology* 14, 73–87. doi: 10.1007/s10522-012-9411-6

Lara-Villoslada, F., Sierra, S., Martín, R., Delgado, S., Rodríguez, J. M., Olivares, M., et al. (2007). Safety assessment of two probiotic strains, *Lactobacillus coryniformis* CECT5711 and *Lactobacillus gasseri* CECT5714. *J. Appl. Microbiol.* 103, 175–184. doi: 10.1111/j.1365-2672.2006.03225.x

Lee, J., Kwon, G., and Lim, Y. H. (2015). Elucidating the mechanism of weissella-dependent lifespan extension in *Caenorhabditis elegans*. *Sci. Rep.* 5:17128. doi: 10.1038/srep17128

Liévin-Le Moal, V., Amsellem, R., Servin, A. L., and Coconnier, M. H. (2002). *Lactobacillus acidophilus* (strain LB) from the resident adult human gastrointestinal microflora exerts activity against brush border damage promoted by a diarrhoeagenic *Escherichia coli* in human enterocyte-like cells. *Gut* 50, 803–811. doi: 10.1136/gut.50.6.803

Magnusson, J., and Schnürer, J. (2001). *Lactobacillus coryniformis* subsp. coryniformis strain Si3 produces a broad-spectrum proteinaceous antifungal compound. *Appl. Environ. Microbiol.* 67, 1–5. doi: 10.1128/AEM.67.1.1-5.2001

Mathipa, M. G., and Thantsha, M. S. (2017). Probiotic engineering: towards development of robust probiotic strains with enhanced functional properties and for targeted control of enteric pathogens. *Gut Pathog.* 9, 28. doi: 10.1186/s13099-017-0178-9

Montoro, B. P., Benomar, N., Lavilla Lerma, L., Castillo Gutiérrez, S., Gálvez, A., and Abriouel, H. (2016). Fermented alorena table olives as a source of potential probiotic Lactobacillus pentosus strains. *Front. Microbiol.* 7:1583. doi: 10.3389/fmicb.2016.01583

Nakagawa, H., Shiozaki, T., Kobatake, E., Hosoya, T., Moriya, T., Sakai, F., et al. (2016). Effects and mechanisms of prolongevity induced by *Lactobacillus gasseri* SBT2055 in *Caenorhabditis elegans*. *Aging Cell* 15, 227–236. doi: 10.1111/acel.12431

Neuhaus, K., Lamparter, M. C., Zölch, B., Landstorfer, R., Simon, S., Spanier, B., et al. (2017). Probiotic *Enterococcus faecalis* symbioflor® down regulates virulence genes of EHEC *in vitro* and decrease pathogenicity in a *Caenorhabditis elegans* model. *Arch. Microbiol.* 199, 203–213. doi: 10.1007/s00203-016-1291-8

Olivares, M., Díaz-Ropero, M. A., Gómez, N., Lara-Villoslada, F., Sierra, S., Maldonado, J. A., et al. (2006). Oral administration of two probiotic strains, *Lactobacillus gasseri* CECT5714 and Lactobacillus coryniformis CECT5711, enhances the intestinal function of healthy adults. *Int. J. Food Microbiol.* 107, 104–111. doi: 10.1016/j.ijfoodmicro.2005.08.019

Park, M. R., Oh, S., Son, S. J., Park, D. J., Kim, S. H., et al. (2015). *Bacillus licheniformis* isolated from Traditional Korean Food resources enhances the longevity of *Caenorhabditis elegans* through serotonin signaling. *J. Agric. Food Chem.* 63, 10227–10233. doi: 10.1021/acs.jafc.5b03730

Park, M. R., Yun, H. S., Son, S. J., Oh, S., and Kim, Y. (2014). Short communication: development of a direct *in vivo* screening model to identify potential probiotic bacteria using *Caenorhabditis elegans*. *J. Dairy Sci.* 97, 6828–6834. doi: 10.3168/jds.2014-8561

Ranadheera, R. D. C. S., Baines, S. K., and Adams, M. C. (2010). Importance of food in probiotic efficacy. *Food Res. Int.* 43, 1–7. doi: 10.1016/j.foodres.2009.09.009

Resta-Lenert, S., and Barrett, K. E. (2003). Live probiotics protect intestinal epithelial cells from the effects of infection with enteroinvasive *Escherichia coli* (EIEC). *Gut* 52, 988–997. doi: 10.1136/gut.52.7.988

Ritchie, M. L., and Romanuk, T. N. (2012). A meta-analysis of probiotic efficacy for gastrointestinal diseases. *PLoS ONE* 7:e34938. doi: 10.1371/journal.pone.0034938

Roselli, M., Devirgiliis, C., Zinno, P., Guantario, B., Finamore, A., Rami, R., et al. (2017). Impact of supplementation with a food-derived microbial community on obesity-associated inflammation and gut microbiota composition. *Genes Nutr.* 12:25. doi: 10.1186/s12263-017-0583-1

Roselli, M., Finamore, A., Britti, M. S., and Mengheri, E. (2006). Probiotic bacteria *Bifidobacterium animalis* MB5 and *Lactobacillus rhamnosus* GG protect intestinal Caco-2 cells from the inflammation-associated response induced by enterotoxigenic *Escherichia coli* K88. *Br. J. Nutr.* 95, 1177–1184. doi: 10.1079/BJN20051681

Sambuy, Y., De Angelis, I., Ranaldi, G., Scarino, M. L., Stammati, A., and Zucco, F. (2005). The Caco-2 cell line as a model of the intestinal barrier: influence of cell and culture-related factors on Caco-2 cell functional characteristics. *Cell Biol. Toxicol.* 21, 1–26. doi: 10.1007/s10565-005-0085-6

Saulnier, D. M., Spinler, J. K., Gibson, G. R., and Versalovic, J. (2009). Mechanisms of probiosis and prebiosis: considerations for enhanced functional foods. *Curr. Opin. Biotechnol.* 20, 135–141. doi: 10.1016/j.copbio.2009.01.002

Thomsen, L. E., Slutz, S. S., Tan, M. W., and Ingmer, H. (2006). *Caenorhabditis elegans* is a model host for *Listeria monocytogenes*. *Appl. Environ. Microbiol.* 72, 1700–1701. doi: 10.1128/AEM.72.2.1700-1701.2006

Torriani, S., Felis, G. E., and Dellaglio, F. (2001). Differentiation of *Lactobacillus plantarum*, *L.* pentosus, and *L. paraplantarum* by recA gene sequence analysis and multiplex PCR assay with recA gene-derived primers. *Appl. Environ. Microbiol.* 67, 3450–3454. doi: 10.1128/AEM.67.8.3450-3454.2001

Uccelletti, D., Pascoli, A., Farina, F., Alberti, A., Mancini, P., Hirschberg, C. B., et al. (2008). APY-1, a novel *Caenorhabditis elegans* apyrase involved in unfolded protein response signalling and stress responses. *Mol. Biol. Cell* 19, 1337–1345. doi: 10.1091/mbc.E07-06-0547

Uccelletti, D., Zanni, E., Marcellini, L., Palleschi, C., Barra, D., and Mangoni, M. L. (2010). Anti-pseudomonas activity of frog skin antimicrobial peptides in a *Caenorhabditis elegans* infection model: a plausible mode of action *in vitro* and *in vivo*. *Antimicrob. Agents Chemother.* 54, 3853–3860. doi: 10.1128/AAC.00154-10

Vizoso Pinto, M. G., Franz, C. M., Schillinger, U., and Holzapfel, W. H. (2006). *Lactobacillus* spp. with *in vitro* probiotic properties from human faeces and traditional fermented products. *Int. J. Food Microbiol.* 109, 205–214. doi: 10.1016/j.ijfoodmicro.2006.01.029

Zanni, E., Laudenzi, C., Schifano, E., Palleschi, C., Perozzi, G., Uccelletti, D., et al. (2015). Impact of a complex food microbiota on energy metabolism in the model organism *Caenorhabditis elegans*. *Biomed Res. Int.* 2015:621709. doi: 10.1155/2015/621709

Zanni, E., Schifano, E., Motta, S., Sciubba, F., Palleschi, C., Mauri, P., et al. (2017). Combination of metabolomic and proteomic analysis revealed different features among *Lactobacillus delbrueckii* subspecies bulgaricus and lactis strains while *in vivo* testing in the model organism *Caenorhabditis elegans* highlighted probiotic properties. *Front. Microbiol.* 8:1206. doi: 10.3389/fmicb.2017.01206

Zhou, Y., Qin, H., Zhang, M., Shen, T., Chen, H., Ma, Y., et al. (2010). *Lactobacillus plantarum* inhibits intestinal epithelial barrier dysfunction induced by unconjugated bilirubin. *Br. J. Nutr.* 104, 390–401. doi: 10.1017/S0007114510000474

Zinno, P., Guantario, B., Perozzi, G., Pastore, G., and Devirgiliis, C. (2017). Impact of NaCl reduction on lactic acid bacteria during fermentation of Nocellara del Belice table olives. *Food Microbiol.* 63, 239–247. doi: 10.1016/j.fm.2016.12.001

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frontiers in Microbiology

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Fermentation of Nocellara Etnea Table Olives by Functional Starter Cultures at Different Low Salt Concentrations

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Nocellara Etnea is one of the main Sicilian cultivars traditionally used to produce both olive oil and naturally fermented table olives. In the present study, the effect of different salt concentrations on physico-chemical, microbiological, sensorial, and volatile organic compounds (VOCs) formation was evaluated in order to obtain functional Nocellara Etnea table olives. The experimental design consisted of 8 treatments as follow: fermentations at 4, 5, 6, and 8% of salt with (E1-E4 samples) and without (C1-C4 samples) the addition of starters. All the trials were carried out at room temperature (18 ± 2°C) and monitored for an overall period of 120 d. In addition, the persistence of the potential probiotic *Lactobacillus paracasei* N24 at the end of the process was investigated. Microbiological data revealed the dominance of lactic acid bacteria (LAB), starting from the 7th d of fermentation, and the reduction of yeasts and enterobacteria in the final product inoculated with starters. VOCs profile highlighted a high amount of aldehydes at the beginning of fermentation, which significantly decreased through the process and a concomitant increase of alcohols, acids, esters, and phenols. In particular, esters showed an occurrence percentage higher in experimental samples rather than in control ones, contributing to more pleasant flavors. Moreover, acetic acid, ethanol, and phenols, which often generate off-flavors, were negatively correlated with mesophilic bacteria and LAB. It is interesting to note that salt content did not affect the performances of starter cultures and slightly influenced the metabolome of table olives. Sensory data demonstrated significant differences among samples registering the highest overall acceptability in the experimental sample at 5% of NaCl. The persistence of the *L. paracasei* N24 strain in experimental samples, at the end of the process, revealed its promising perspectives as starter culture for the production of functional table olives with reduced salt content.

Keywords: NaCl content, probiotic strain, metabolomics, microbiota, REP-PCR analysis

INTRODUCTION

The greater consumer's attention for healthy food is confirmed by the growing trend in fermented vegetables consumption, such as table olives (International Olive Council (IOC), 2016). Table olives are mainly produced in several Mediterranean countries, such as Spain, Italy, and Greece and in Sicily, two main cultivars (Nocellara del Belice and Nocellara Etnea) are growth. In particular, Nocellara Etnea cv is mainly cultivated in the Central and Eastern area of Sicily, among the provinces of Enna, Catania, Messina, Syracuse, and Ragusa. The drupes, elliptical in shape and slightly asymmetric, are characterized by a uniform and a large size and by late harvesting. The relationship between core and pulp is very high and this character makes this cultivar one of the best for the production of green table olives. The latter are mostly obtained by a spontaneous process in which the hydrolysis of oleuropein is relied on enzymatic activities of indigenous microorganisms, and on the plasmolytic effect of salt. This process is mainly dominated by lactic acid bacteria (LAB) and yeasts, which form a natural consortium (Randazzo et al., 2010). However, during the spontaneous fermentation spoilage microorganisms, such as *Enterobacteriaceae* and *Propionibacteriaceae* may occur. It is well established that *Lactobacillus plantarum* and *Lactobacillus pentosus* are the main detected species, due to their versatile adaptation to the brine environment (Ruiz-Barba et al., 1994; G-Alegria et al., 2004; Bautista-Gallego et al., 2010; Randazzo et al., 2011, 2012; Hurtado et al., 2012; Cocolin et al., 2013; Tofalo et al., 2014), and different strains are widely used as starter cultures in several table olive fermentations (Arroyo-López et al., 2012; Hurtado et al., 2012; Randazzo et al., 2014).

During olive fermentation, coarse salt is added in order to reduce the water activity, preventing the growth of spoilage microorganisms, and to improve taste and textures of the final product (Bautista-Gallego et al., 2013a). The EU Member States try to implement national nutritional policies with the aim to decrease salt intake according to the European Commission suggestion (European Council, 2010). The strategy to set up table olives with a reduced daily Na intake, which has been established at 5 g salt, by WHO/FAO [World Health Organisation (WHO)/Food 2003] is one of the main goals of food industry. Different chloride salts, such as KCl, $CaCl_2$, and $ZnCl_2$, have been evaluated as replacers for NaCl (Bautista-Gallego et al., 2013b), especially in Spanish style green olives (Bautista-Gallego et al., 2010, 2011). The reduction in Na and the increase in other salts may lead to a more equilibrated mineral composition in table olives, ameliorating the consumers' diet, and enhancing the perception of the nutritional value of the olives. Nevertheless, the effects of NaCl replacement with other salts could affect the microbiota evolving in the fermentation process of table olives (Bautista-Gallego et al., 2015; Mateus et al., 2016), as well as impact the sensorial quality of the final product (Zinno et al., 2017). Potential NaCl reduction depends on characteristics linked with the cultivar, its composition, other ingredients, processing, and technological parameters (Bautista-Gallego et al., 2013a), which should be well addressed before their implementation at the industrial scale. Furthermore, the final product must be safe from the microbiological point of view. It is already established that a reduction in NaCl might be responsible for an increase of pathogens such as *Clostridium botulinum* (Simpson et al., 1995).

Nowadays, based on the increasing consumers' demand, the production of healthier table olives is of great industry importance, taking into account the potential market of table olives as a functional food. In fact, functional table olives can provide a concrete opportunity to convey the benefits that are already appreciated by consumers in dairy sectors. It has already been demonstrated that table olives represent a good vehicle to transport probiotics to humans for both their microarchitecture and the presence of nutrients (Lavermicocca et al., 2005; Valerio et al., 2006; Randazzo et al., 2017).

The aim of the present study was (i) to set up a fermentation, at laboratory scale, of Nocellara Etnea table olives with reduced level of NaCl; (ii) to evaluate the effect of the NaCl reduction on the physico-chemical, microbiological, and sensorial parameters compared to fermentation carried out without starter cultures.

MATERIALS AND METHODS

Bacterial Strains and Olive Processing

In the present study two lyophilized LAB strains, *L. plantarum* UT2.1 and the potential probiotic *Lactobacillus paracasei* N24, belonging to the Di3A microbial collection, previously screened for their technological and functional features, and already applied as starter cultures at industrial scale (Randazzo et al., 2017) were used. Each strain was directly inoculated into fresh brine (1:1 ratio) to reach a final cell density of 7 log colony forming units per ml (cfu/ml). Olives of Nocellara Etnea cultivar, kindly provided by a local company (Consoli srl, Adrano, Sicily) were processed, at laboratory scale, following the Sicilian style method. After harvesting, about 3 kg of olives were subjected to quality control, to remove damaged fruits, washed with tap water, directly placed in sterile glass vessels, and covered with ∼3 l of sterile brine. The experimental design consisted of 8 treatments as follows: fermentations at 4, 5, 6, and 8% of salt with the addition of starters (E1-E4 samples); fermentations at 4, 5, 6, and 8% of salt without the addition of starters (C1-C4 samples). All fermentation trials were carried out at room temperature (18 ± 2°C), and monitored for an overall period of 120 d. The brine salt concentration was maintained at each initial level by adding marine salt. Fresh brine was periodically supplied to maintain olives totally dipped in order to inhibit growth of molds on the brine surfaces. The experimental trials were carried out in triplicate. The progression of the fermentation was followed by monitoring pH, titratable acidity and the shift in microbial populations in brine throughout the process.

Physico-Chemical and Total Polyphenol Determination of Brine Samples

Fifty ml of each brine sample were taken at 60 and 120 days of fermentation. The pH values of brines were monitored by a pH meter (H19017, Microprocessor, Hanna Instruments). Total free acidity was measured by titration and expressed as the percentage of lactic acid (g/100 ml brine). Total polyphenol content was

colorimetrically determined in brine samples at 60 and 120 d, using Folin-Ciocalteu reagents, according to Singleton (1974). Polyphenols were measured in triplicate and expressed as mg/l of gallic acid.

Microbiological Analyses of Brine Samples

For the microbiological characterization, brine samples were analyzed at 1, 7, 15, 30, 60, 90, and 120 days of fermentation. At each sampling time, brines were serially diluted, using sterile quarter-strength Ringer's solution (QRS), and plated in triplicate on the following agar media (all provided from Oxoid Italy), and conditions: Plate Count Agar, incubated at 32 ± 2°C for 48 h, for total mesophilic bacteria; de Man-Rogosa-Sharp agar, supplemented with cycloheximide (5 ml/l), anaerobically incubated at 32°C for 24–48 h, for LAB count; Sabouraud Dextrose Agar, supplemented with chloramphenicol (0.05 g/l), incubated at 25°C for 4 days, for yeast count; Violet Red Bile Glucose Agar, aerobically incubated at 37°C for 24 h, for Enterobacteriaceae count; Mannitol Salt Agar, incubated at 32°C for 48 h, for staphylococci enumeration; Mac Conkey incubated at 32°C for 24–48 h for *Escherichia coli* determination. Results were expressed as $\log_{10}$ cfu/ml.

Volatile Organic Compound (VOC) Analysis by Gas Chromatography-Mass-Spectrometry (GC-MS)

VOCs, detected in brine samples at 1, 60, and 120 d of fermentation, were sampled using a solid-phase microextraction (SPME). The SUPELCO SPME (Bellefonte, PA) fiber holder and fiber used were coated with divinylbenzene/polydimethylsiloxane (DV/PDMS), 65 mm. Before the first extraction, the fiber was conditioned in the GC injector port at 300°C for 1 h, according to the manufacturer's recommendation. Ten ml of brine sample were added to a 35 ml vial. Extraction temperature of head-space and time were 40°C and 20 min, respectively. One g of NaCl was added to increase extraction rate of VOCs. The samples were gently vortexed during extraction using a magnetic stirrer. Fiber exposition was prolonged for 20 min at 40°C. Thermal desorption was performed in the injector at 230°C for 1 min (Sabatini et al., 2008; Malheiro et al., 2011). The identification of the extracted VOCs was carried out using a GC instrument (HP GC6890, Hewlett Packard, Palo Alto, CA), coupled to a MS detector (HP MS5973) (Panagou and Tassou, 2006). The gas chromatograph was equipped with a 30 m 0.25 mm i.d. 0.25 mm film thickness fused-silica capillary column (DB-WAX J&W Scientific) and the injector temperature was 230°C. The conditions applied were as those previously reported (Randazzo et al., 2017). The quantification of VOCs was determined with the internal standard method spiking propionic acid, ethanol, ethyl acetate, benzaldehyde and guaiacol to all analyzed samples. All analyses were performed in duplicate and the results were expressed in μg/l of brine.

Isolation and Genetic Identification of Lactic Acid Bacteria

From each MRS agar plate, at the highest dilution, of both E (E1-E4) and C (C1-C4) brine samples at 1, 60, and 120 d of fermentation, the 20% of total number of colonies was randomly selected, purified, checked for catalase activity and Gram reaction, and microscopically examined before storing in liquid culture using 20% glycerol at −80°C. The random colony selection from the highest dilution plates allowed us to collect 400 LAB isolates. Total genomic DNA of isolates was extracted from overnight cultures according to the method described by Pino et al. (2018). DNA concentration and DNA quality were assessed by measuring optical density using Fluorometer Qubit (Invitrogen, Carlsbad, 278 CA, USA).

All LAB isolates were subjected to *Rec*A and *Tuf* gene species-specific PCR following the protocol previously described by (Torriani et al., 2001; Ventura et al., 2003).

REP-PCR Analysis

In order to evaluate the viability of the potential probiotic *L. paracasei* N24 throughout the fermentation, lactobacilli isolated from E samples at 60 and 120 d (60 isolates), ascribed to *L. paracasei* species through the aforementioned species-specific multiplex PCR, were subjected to REP-PCR analysis, using the $(GTG)_5$-primer, as described by Gevers et al. (2001). PCR amplicons were separated on a 1.5% agarose gel (w/v) in 1X TAE buffer (40 mM Tris, 20 mM acetic acid and 1 mM EDTA) under highly standardized conditions (55 V, 400 mA, 16 h at 4°C). At regular intervals a reference marker (6 μl each composed of 1.10 μl Molecular Ruler 500 bp (Bio-Rad), 1.40 μl Molecular Ruler 100 bp (Bio-Rad), 2 μl TE buffer (1 mM EDTA, 10 mM Tris–HCl, pH 8.0) and 1.50 μl loading dye), was loaded for normalization. Profiles were visualized under ultraviolet light, after staining with ethidium bromide. Digitized images of gels were normalized and analyzed by the BioNumerics 7.6.2 software (Applied Maths, Belgium). Similarity matrices of densitometric curves of the gel tracks were calculated with Pearson's product-moment correlation coefficient. Subsequent cluster analyses of similarity matrices were performed by unweighted pair group method with arithmetic averages (UPGMA).

Table Olives Sensory Evaluation

The sensory assessment of table olives was performed by a trained sensory panel consisting of 10 panelists (6 females and 4 males, aged from 22 to 40 years), according to the method reported by the International Olive Council (International Olive Council (IOC), 2011). Olives were tasted for negative sensations (abnormal fermentation such as musty, rancid, cooking effect, soapy, metallic, earthy, and winey-vinegary), based on the classification reported by International Olive Council (IOC) (2011, 2016), while a descriptive analysis was carried out for descriptors corresponding to gustatory sensations (acidity, saltiness, and bitterness) and kinaesthetic sensations (hardness, fibrousness, and crunchiness). In addition, an overall acceptability descriptor was considered as an indication of the overall quality. Sensory data were acquired by a direct computerized registration system (FIZZ Biosystemes. Couternon, France).

Statistical Analysis

Statistical analysis of chemical data was performed using a one-way analysis of variance with repeated measures of the GLM procedure by SAS (2001), considering the different treatments as variable. Means were separated by a Least Significant Difference (LSD) test when a significant treatment ($P < 0.05$) was observed. Microbiological data and VOCs were analyzed by ANOVA (One-way Analysis of Variance) using Tukey's *post-hoc* test, in order to assess the overall differences among samples. Statistical analysis was performed using XLSTAT PRO 5.7 (Addinsoft, New York, USA) and the reference level of significance was 0.05 in all the assays. Sensory data were submitted to one-way ANOVA using the software package Statgraphics® Centurion XVI (Statpoint Technologies, INC.) using samples as treatments. The significance was tested by means of the F-test. To differentiate the samples, the mean values were submitted to the multiple comparison test using the LSD procedure. In order to correlate the

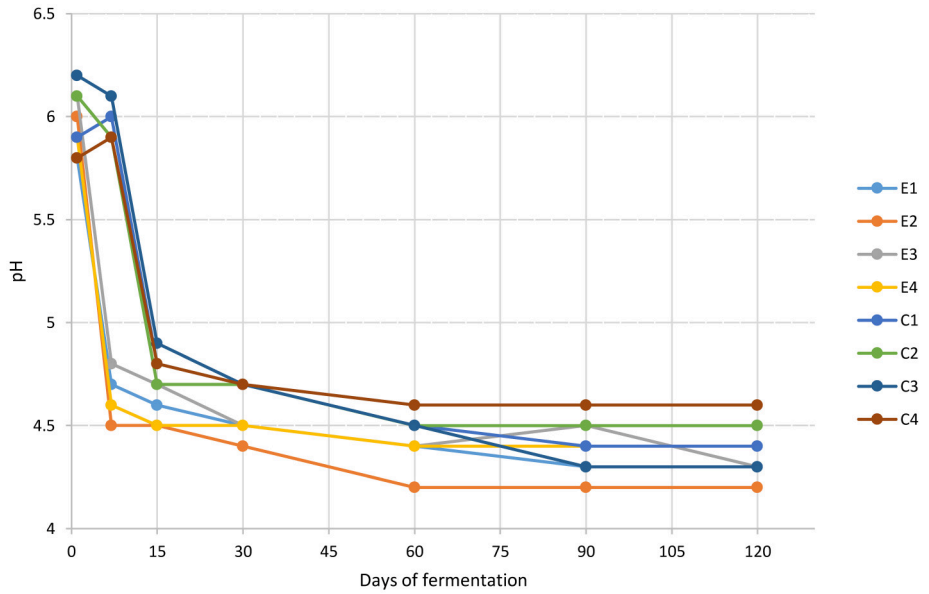

FIGURE 1 | Changes in pH throughout the fermentation of Nocellara Etnea experimental and control samples.

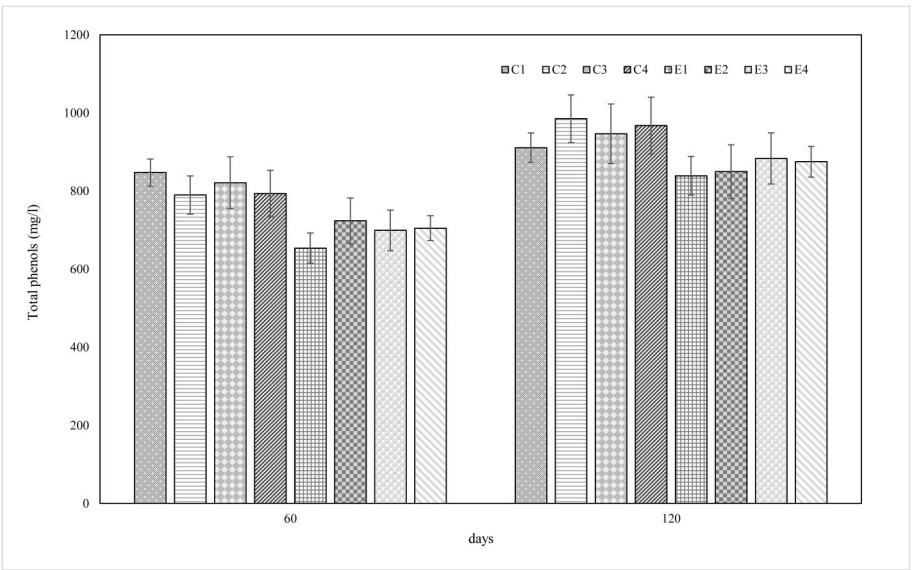

FIGURE 2 | Total phenolic content (mg/l) of control (C) experimental (E) brine samples at 60 and 120 days of fermentation.

experimental and control brine samples to volatile compounds, data obtained at 1, 60, and 120 d of fermentation were subjected to principal component analysis (PCA) using MATLAB, achieving high data compression efficiency of the original data.

Similarities between the microbiota and metabolome profiles of experimental and control brine samples were carried out by PermutMatrix software. Data correlations between microbiota (mesophilic bacteria, LAB, staphylococci, yeasts, enterobacteria) and VOCs were computed using Statistica v. 7.0 and elaborated through PermutMatrix software.

RESULTS

Physico-Chemical Data

The pH values, showed in **Figure 1**, dropped faster in experimental samples rather than in control ones reaching a value between 4.5 and 4.8 at 7 days of fermentation. The lowest pH value (4.2) was revealed by E2 sample at 60 days. At the end of the process, pH values ranged from 4.6 to 4.2. The titratable acidity, expressed as the percentage of lactic acid (g/100 ml brine), was determined at 60 and 120 d of fermentation. Overall, no differences were detected among experimental and control samples and results showed an increase during the fermentation process, reaching an average value of 0.37 g/100 ml brine in the final products (data not shown). Results of total phenols (TP) content are presented in **Figure 2**. TP detected in brines showed quite differences among E and C samples while within each group similarities on TP content was achieved. In addition, statistically significant differences were noticed between samples at 60 and 120 d of fermentation with a range of 911.2–985.2 mg/l for C samples and of 839.2–967.8 mg/l for E samples.

Microbial Counts of Brine Samples Through the Fermentation

Microbial counts, expressed as $\log_{10}$ cfu/ml, of both experimental (E) and control (C) brine samples, at different salt concentrations (4, 5, 6, and 8%), are reported in **Table 1**. Viable mesophilic bacteria exhibited different behavior among samples. In detail, a steady trend in samples inoculated with starter cultures (E1-E4), was recorded, with the exception of E4 sample (at 8% of NaCl), in which, from a lower initial value (6.65 log unit), a slight decrease throughout the fermentation was observed. Control samples (C1-C4) exhibited a mean initial value of 4.6 $\pm$ 0.07 log unit, which significantly increased from the 15th day of fermentation, reaching a final mean value of 5.43 $\pm$ 0.09 log unit (**Table 1**). Similar behavior was observed for LAB population, which reached the highest concentration value in all inoculated samples (E1-E4). The highest value (8.58 log unit) was detected after 60 days of fermentation in sample E2, obtained adding 5% of salt. Among the control samples (C1-C4), a similar initial LAB count was found (average value of 4.4 $\pm$ 0.20 log cfu/ml) with the exception of C4 sample, which presented the lowest LAB cell density (**Table 1**). At the end of the fermentation (120 days) no statistically significant differences ($P > 0.05$) were achieved among samples, that exhibited an average value of 5.88 $\pm$ 0.15 log cfu/ml. Yeasts were present at an initial average level of 3.93 $\pm$ 0.08 log cfu/ml and 4.18 $\pm$ 0.17 log cfu/ml, in E and C samples, respectively. These densities slightly increased through the fermentation process, achieving, at 120 days, an average value of 5.51 $\pm$ 0.13 log unit, with the exception of samples E1, E2, and E3, which exhibited the lowest yeast count (**Table 1**). At the beginning of fermentation, the staphylococci level was quite similar among inoculate and un-inoculated samples, with a slight increase at 30 and 60 days, and followed by a decrease to a final average value of 3.53 $\pm$ 0.19 cfu/ml. Enterobacteria counts significantly decreased from the 30th day of fermentation, reaching a final value below 2 log. In addition, *E. coli* was never detected in any brine samples analyzed.

Volatile Organic Compound (VOC) Detection by Gas Chromatography-Mass-Spectrometry (GC-MS)

Volatile organic compounds (VOCs) of E and C brine samples at 1, 60, and 120 d of fermentation, are reported in **Table 2**. The assessment allowed the identification of 47 compounds as acids, alcohols, esters, aldehydes, and phenols. Overall, total VOCs exhibited a growing trend during the fermentation reaching an average value of 2349.70 μg/l after 120 days. In particular, the highest values were registered in all control samples, with a mean value of 3215.23 μg/l. In detail, in all samples, at beginning of fermentation, aldehydes represented the main VOCs, and after 60 days, they significantly decreased, whereas alcohols, acids, esters and phenols increased. At the end of fermentation (120 d), differences were observed among brine samples. Zooming on each chemical class, it is possible to assert that overall, the detected amounts of each compounds were sample-dependent. Among acids, the acetic acid was the most abundant compound, with the highest values in control samples, whereas hexanoic and propionic acids were more abundant in experimental samples (**Table 2**). Among alcohols, ethanol dominated the fermentation process, especially in control samples, followed by isoamyl- and phenyl-ethyl alcohol. Among esters, the highest amount was achieved by ethyl-acetate, followed by ethyl lactate. A different trend was revealed for butanoic-acid-2-methylester, which showed the highest value in the E2 sample, followed by control samples. The most abundant aldehyde and phenols were nonanal and cresol, respectively. Evaluating the VOCs occurrence percentage on E and C samples at 120 days of fermentation (**Figure S1**), it is interesting to note that esters and acids were mainly present in all E samples with the highest occurrence percentage in E2 (31.7 and 11.3%, respectively). Alcohols, phenols, and aldehydes were also detected at high occurrence percentage in E2 sample (**Figure S1**). **Figures 3A,B** showed the PCA plot of distribution of C (C1-C4) and E (E1-E4) samples, at different days of fermentation, in the PC1–PC2 plane. Based on the loadings (data not shown), component 1, that represent the 80.88% of the variability, can be viewed as an esters factor, while the second principal component (variance 11.64%),

TABLE 1 | Microbial counts expressed as $\log_{10}$ CFU/ml of 3 replicates ± standard deviation of the main microbial groups detected in Nocellara Etnea table olives at different salt concentrations (4, 5, 6, 8%) trough experimental (E) and spontaneous (C) fermentation (1 to 120 days).

DAYS OF FERMENTATION							
	1	7	15	30	60	90	120
MESOPHILIC BACTERIA							
	7.36 ± 0.05^{d}	7.75 ± 0.06^{g}	7.53 ± 0.20^{e}	7.74 ± 0.05^{e}	7.44 ± 0.18^{d}	7.15 ± 0.12^{d}	7.35 ± 0.08^{f}
E1							
E2	7.31 ± 0.06^{d}	7.25 ± 0.30^{f}	7.51 ± 0.06^{e}	7.01 ± 0.12^{d}	7.40 ± 0.10^{d}	7.11 ± 0.14^{d}	7.01 ± 0.08^{ef}
E3	7.23 ± 0.09^{d}	6.95 ± 0.43^{e}	7.49 ± 0.06^{e}	6.99 ± 0.02^{d}	6.65 ± 0.09^{c}	6.93 ± 0.10^{d}	7.05 ± 0.14^{e}
E4	6.56 ± 0.11^{c}	6.75 ± 0.18^{d}	6.53 ± 0.24^{d}	5.94 ± 0.41^{c}	5.12 ± 0.37^{a}	5.82 ± 0.24^{b}	5.73 ± 0.13^{c}
C1	4.21 ± 0.06^{a}	4.40 ± 0.21^{a}	4.83 ± 0.33^{a}	5.20 ± 0.09^{a}	5.11 ± 0.11^{a}	5.36 ± 0.08^{a}	5.07 ± 0.05^{a}
C2	4.38 ± 0.10^{a}	4.53 ± 0.15^{ab}	5.02 ± 0.21^{b}	5.92 ± 0.09^{c}	5.36 ± 0.07^{ab}	5.60 ± 0.06^{bc}	5.39 ± 0.06^{b}
C3	4.89 ± 0.09^{b}	4.81 ± 0.05^{ac}	5.51 ± 0.18^{c}	5.97 ± 0.11^{c}	5.45 ± 0.09^{ab}	5.40 ± 0.12^{ac}	5.53 ± 0.13^{bc}
C4	4.82 ± 0.03^{b}	4.68 ± 0.18^{a}	5.21 ± 0.21^{b}	5.68 ± 0.28^{b}	5.63 ± 0.12^{b}	5.58 ± 0.23^{ac}	5.74 ± 0.12^{cd}
LACTIC ACID BACTERIA							
	7.09 ± 0.10^{d}	7.52 ± 0.12^{g}	8.08 ± 0.25^{g}	8.72 ± 0.15^{f}	8.13 ± 0.12^{e}	7.85 ± 0.13^{d}	7.83 ± 0.07^{de}
E1							
E2	7.12 ± 0.10^{de}	7.59 ± 0.13^{g}	7.38 ± 0.30^{d}	8.27 ± 0.07^{e}	8.49 ± 0.05^{d}	8.58 ± 0.08^{e}	8.23 ± 0.11^{d}
E3	7.02 ± 0.21^{de}	7.14 ± 0.05^{f}	7.15 ± 0.35^{de}	7.03 ± 0.10^{b}	7.64 ± 0.07^{c}	7.86 ± 0.11^{d}	7.79 ± 0.15^{de}
E4	7.03 ± 0.11^{e}	7.37 ± 0.27^{e}	7.34 ± 0.09^{df}	7.02 ± 0.21^{abc}	7.30 ± 0.40^{b}	7.05 ± 0.53^{c}	7.04 ± 0.30^{c}
C1	4.51 ± 0.20^{abc}	4.69 ± 0.13^{b}	5.81 ± 0.05^{c}	7.36 ± 0.11^{cd}	7.83 ± 0.05^{c}	6.88 ± 0.13^{b}	6.00 ± 0.08^{a}
C2	4.48 ± 0.23^{c}	4.79 ± 0.18^{bc}	5.96 ± 0.07^{c}	7.21 ± 0.07^{ad}	7.32 ± 0.06^{b}	6.04 ± 0.09^{a}	5.96 ± 0.11^{a}
C3	4.21 ± 0.16^{b}	4.57 ± 0.09^{bd}	5.53 ± 0.13^{b}	7.10 ± 0.13^{ad}	6.74 ± 0.12^{a}	6.78 ± 0.10^{b}	5.67 ± 0.06^{a}
C4	3.93 ± 0.21^{a}	3.85 ± 0.09^{a}	5.12 ± 0.21^{a}	6.95 ± 0.35^{a}	6.58 ± 0.14^{a}	5.96 ± 0.42^{a}	5.94 ± 0.36^{a}
YEASTS							
	4.00 ± 0.01^{d}	4.63 ± 0.13^{c}	5.27 ± 0.28^{a}	5.13 ± 0.06^{a}	5.47 ± 0.18^{a}	5.20 ± 0.12^{ab}	4.61 ± 0.05^{b}
E1							
E2	3.97 ± 0.10^{c}	4.18 ± 0.06^{b}	5.35 ± 0.31^{a}	5.61 ± 0.09^{a}	5.56 ± 0.11^{a}	5.60 ± 0.15^{b}	4.11 ± 0.06^{a}
E3	3.84 ± 0.01^{a}	5.05 ± 0.14^{d}	5.62 ± 0.31^{ab}	5.28 ± 0.07^{a}	5.51 ± 0.10^{a}	5.14 ± 0.11^{a}	4.30 ± 0.05^{ab}
E4	3.93 ± 0.21^{b}	3.46 ± 0.11^{a}	5.81 ± 0.18^{b}	5.23 ± 0.21^{a}	5.68 ± 0.30^{a}	5.12 ± 0.24^{a}	5.50 ± 0.23^{c}
C1	4.21 ± 0.12^{g}	5.34 ± 0.30^{d}	6.73 ± 0.18^{c}	6.25 ± 0.12^{b}	6.28 ± 0.10^{b}	5.32 ± 0.09^{ab}	5.58 ± 0.09^{c}
C2	4.28 ± 0.23^{bcd}	6.12 ± 0.32^{e}	6.61 ± 0.21^{c}	6.85 ± 0.11^{c}	6.48 ± 0.11^{bc}	5.28 ± 0.14^{ab}	5.72 ± 0.08^{c}
C3	4.09 ± 0.09^{e}	5.03 ± 0.11^{d}	5.61 ± 0.11^{ab}	6.16 ± 0.08^{b}	5.47 ± 0.05^{ab}	5.00 ± 0.09^{a}	5.36 ± 0.14^{c}
C4	4.14 ± 0.23^{f}	4.96 ± 0.16^{cd}	5.78 ± 0.23^{ab}	5.63 ± 0.31^{a}	5.91 ± 0.26^{ab}	5.32 ± 0.27^{ab}	5.40 ± 0.11^{c}
STAPHYLOCOCCI							
	4.76 ± 0.08^{b}	4.23 ± 0.14^{a}	4.72 ± 0.21^{bc}	4.83 ± 0.26^{b}	4.79 ± 0.15^{ab}	4.19 ± 0.18^{a}	3.30 ± 0.23^{ac}
E1							
E2	4.18 ± 0.32^{a}	4.42 ± 0.33^{a}	4.97 ± 0.35^{cd}	4.12 ± 0.15^{a}	4.65 ± 0.07^{a}	4.44 ± 0.23^{ac}	3.72 ± 0.32^{bcd}
E3	4.10 ± 0.16^{a}	4.33 ± 0.18^{a}	4.54 ± 0.15^{ab}	4.21 ± 0.31^{a}	4.64 ± 0.09^{a}	4.93 ± 0.21^{bd}	3.14 ± 0.20^{a}
E4	4.03 ± 0.21^{a}	4.45 ± 0.23^{a}	4.26 ± 0.32^{a}	4.83 ± 0.21^{bc}	4.62 ± 0.26^{ab}	4.30 ± 0.21^{ac}	3.04 ± 0.18^{a}
C1	4.81 ± 0.14^{b}	5.18 ± 0.11^{b}	5.44 ± 0.11^{ef}	5.18 ± 0.08^{bc}	5.18 ± 0.12^{ac}	4.62 ± 0.05^{acd}	3.58 ± 0.11^{c}
C2	4.76 ± 0.18^{ab}	5.05 ± 0.19^{b}	5.73 ± 0.21^{f}	5.24 ± 0.18^{c}	5.11 ± 0.15^{a}	4.74 ± 0.10^{bcd}	3.74 ± 0.14^{cd}
C3	4.27 ± 0.11^{a}	5.04 ± 0.09^{b}	5.53 ± 0.09^{ef}	4.72 ± 0.06^{b}	4.98 ± 0.10^{a}	4.63 ± 0.06^{bcd}	3.96 ± 0.07^{d}
C4	4.34 ± 0.12^{a}	5.08 ± 0.30^{b}	5.11 ± 0.12^{de}	4.77 ± 0.26^{b}	4.73 ± 0.34^{a}	4.23 ± 0.31^{ac}	3.82 ± 0.31^{cd}
ENTEROBACTERIA							
	2.99 ± 0.24^{b}	2.33 ± 0.28^{b}	2.40 ± 0.18^{cd}	1.03 ± 0.11^{a}		< 1	< 1
E1					< 1		
E2	2.24 ± 0.06^{a}	2.13 ± 0.14^{bc}	1.09 ± 0.10^{a}	< 1	< 1	< 1	< 1
E3	2.00 ± 0.13^{a}	2.26 ± 0.09^{bc}	1.84 ± 0.16^{c}	1.32 ± 0.13^{a}	1.05 ± 0.21^{a}	< 1	< 1
E4	2.19 ± 0.07^{a}	1.74 ± 0.21^{ac}	1.45 ± 0.23^{b}	1.19 ± 0.21^{a}	1.02 ± 0.07^{a}	< 1	< 1
C1	3.91 ± 0.09^{c}	4.11 ± 0.15^{d}	3.53 ± 0.15^{e}	2.37 ± 0.24^{b}	2.32 ± 0.11^{b}	1.63 ± 0.13^{b}	1.14 ± 0.05^{a}
C2	3.82 ± 0.13^{c}	4.02 ± 0.21^{dg}	3.13 ± 0.09^{de}	2.50 ± 0.18^{b}	2.67 ± 0.15^{bc}	1.84 ± 0.21^{b}	1.45 ± 0.12^{ab}
C3	3.89 ± 0.18^{c}	3.97 ± 0.12^{df}	3.23 ± 0.18^{e}	2.15 ± 0.09^{b}	2.31 ± 0.13^{b}	1.54 ± 0.18^{b}	1.55 ± 0.08^{b}
C4	3.98 ± 0.12^{c}	3.31 ± 0.23^{de}	3.64 ± 0.14^{e}	2.06 ± 0.09^{b}	2.09 ± 0.21^{bd}	1.14 ± 0.13^{a}	1.39 ± 0.03^{ab}

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

TABLE 2 | Volatile organic compounds (VOCs) expressed as μg/l of Nocellara Etnea brine samples at different salt concentrations (4, 5, 6, 8%) under controlled (E) and spontaneous fermentation (C) at 1, 60, and 120 days.

		E1			E2			E3			E4			C1			C2			C3			C4		
	RT	1	60	120	1	60	120	1	60	120	1	60	120	1	60	120	1	60	120	1	60	120	1	60	120
Acids		**0.00**	**142.29**	**113.26**	**0.00**	**69.69**	**154.59**	**0.00**	**69.55**	**108.06**	**0.00**	**75.84**	**107.60**	**0.00**	**69.52**	**139.04**	**0.00**	**71.35**	**136.14**	**0.00**	**60.25**	**134.61**	**0.00**	**59.23**	**131.78**
Acetic acid	24.78	0.00	66.75^{bc}	63.24^{b}	0.00	45.06^{a}	81.84^{d}	0.00	42.76^{a}	58.34^{b}	0.00	48.01^{a}	60.21^{b}	0.00	69.52^{c}	108.83^{e}	0.00	70.23^{c}	105.24^{e}	0.00	60.25^{b}	103.91^{e}	0.00	59.23^{b}	102.85^{e}
Propionic acid	29.74	0.00	4.02^{c}	9.08	0.00	5.38^{d}	31.23^{e}	0.00	1.03^{b}	0.00	0.00	1.02^{b}	0.09^{a}	0.00	0.00	3.00^{c}	0.00	0.00	2.85^{c}	0.00	0.00	3.15^{c}	0.00	0.00	3.13^{c}
Isobutyric acid	31.43	0.00	8.96^{f}	7.01^{e}	0.00	1.26^{a}	3.04^{c}	0.00	5.39^{d}	1.67^{a}	0.00	5.65^{d}	1.88^{ab}	0.00	0.00	2.12^{ab}	0.00	1.12^{a}	2.64^{c}	0.00	0.00	2.36^{c}	0.00	0.00	2.29^{b}
Butanoic acid	35.01	0.00	2.31^{e}	1.78^{d}	0.00	0.94^{c}	4.51^{f}	0.00	0.59^{b}	1.52^{d}	0.00	0.89^{c}	1.01^{c}	0.00	0.00	0.25^{a}	0.00	0.00	0.23^{a}	0.00	0.00	0.95^{c}	0.00	0.00	0.86^{c}
Hexanoic acid	36.19	0.00	0.00	30.12^{e}	0.00	7.93^{a}	32.98^{e}	0.00	16.54^{cd}	45.34^{f}	0.00	17.23^{d}	43.21^{f}	0.00	0.00	15.21^{c}	0.00	0.00	14.21^{c}	0.00	0.00	12.56^{b}	0.00	0.00	11.99^{b}
2-Ethylheptanoic acid	57.20	0.00	60.25^{d}	2.03^{a}	0.00	9.12^{c}	0.99^{a}	0.00	3.24^{a}	1.19^{a}	0.00	3.04^{a}	1.20^{a}	0.00	0.00	9.63^{b}	0.00	0.00	10.97^{b}	0.00	0.00	11.68^{b}	0.00	0.00	10.66^{b}
Alcohol		**19.20**	**632.36**	**434.45**	**48.32**	**548.94**	**449.10**	**29.40**	**1041.55**	**1082.59**	**30.01**	**1047.93**	**1084.68**	**67.71**	**1056.92**	**2165.14**	**61.46**	**1048.11**	**2019.43**	**69.49**	**1028.98**	**1966.09**	**64.05**	**937.38**	**1961.51**
Ethanol	3.33	0.00	250.14^{ab}	236.95^{ab}	0.00	350.21^{b}	165.24^{a}	0.00	736.45^{c}	768.00^{c}	0.00	735.84^{c}	769.12^{c}	0.00	705.91^{c}	1754.23^{d}	0.00	694.81^{c}	1613.28^{d}	0.00	681.21^{c}	1570.21^{d}	0.00	601.25^{c}	1568.91^{d}
Isoamylalcohol	11.58	5.95^{a}	81.25^{c}	60.01^{b}	0.00	59.11^{b}	71.98^{c}	2.81^{a}	65.34^{b}	88.28^{c}	2.89^{a}	65.21^{b}	87.56^{c}	0.00	84.87^{c}	172.13^{d}	0.00	83.14^{c}	169.87^{d}	0.00	79.56^{c}	160.85^{d}	0.00	79.11^{c}	159.27^{d}
1-Hexanol	19.23	1.03^{a}	9.08^{b}	11.56^{c}	0.00	13.03^{cd}	14.74^{d}	0.00	12.11^{c}	11.37^{c}	0.00	13.84^{d}	11.59^{c}	0.00	15.21^{d}	18.25^{e}	0.00	12.24^{c}	18.12^{e}	0.00	16.02^{d}	16.59^{d}	0.00	14.96^{d}	18.07^{e}
cis Hexen 1 ol	20.84	0.00	81.65^{c}	41.21^{a}	0.00	35.83^{a}	30.98^{a}	0.00	67.32^{b}	55.41^{b}	0.00	67.89^{b}	54.98^{b}	0.00	61.23^{b}	88.46^{c}	0.00	62.29^{b}	88.23^{c}	0.00	61.21^{b}	87.69^{c}	0.00	58.41^{b}	86.30^{c}
3-Octenol	25.13	0.00	1.96^{c}	2.58^{de}	0.00	0.99^{a}	2.01^{c}	0.00	2.45^{d}	1.69^{b}	0.00	2.39^{cd}	1.72^{b}	0.00	1.20^{a}	3.02^{e}	0.00	2.03^{c}	2.91^{e}	0.00	1.45^{ab}	3.01^{e}	0.00	1.11^{a}	2.47^{d}
1-Heptanol	25.79	2.36^{a}	9.02^{f}	3.99^{c}	3.01^{b}	4.96^{d}	5.12^{d}	2.07^{a}	5.34^{d}	2.79^{ab}	2.05^{a}	5.58^{d}	2.81^{ab}	3.45^{bc}	9.52^{f}	6.13^{e}	3.25^{b}	13.21^{g}	6.09^{e}	3.56^{bc}	11.23^{g}	5.99^{de}	3.23^{b}	9.98^{f}	5.59^{d}
1-Octanol	31.12	0.00	21.96^{d}	2.46^{a}	14.89^{c}	2.78^{a}	12.56^{c}	13.12^{c}	11.03^{c}	8.42^{b}	12.97^{c}	12.01^{c}	9.01^{b}	20.31^{d}	21.23^{d}	4.01^{a}	20.28^{d}	21.43^{d}	3.95^{a}	21.59^{d}	24.47^{d}	3.54^{a}	20.85^{d}	22.83^{d}	3.48^{a}
1-Nonanol	35.91	8.74^{a}	68.32^{c}	0.00	5.87^{a}	0.00	0.00	4.02^{a}	0.00	0.00	4.65^{a}	0.00	0.00	4.99^{a}	27.14^{b}	0.00	4.85^{a}	26.93^{b}	0.00	5.80^{a}	24.61^{b}	0.00	5.01^{a}	23.74^{b}	0.00
Benzyl Alcohol	47.77	0.00	43.21^{e}	0.00	0.00	9.18^{a}	16.35^{b}	0.00	29.06^{d}	0.00	0.00	30.15^{d}	0.00	0.00	21.93^{c}	0.00	0.00	18.36^{c}	0.00	0.00	20.25^{c}	0.00	0.00	19.26^{c}	0.00
Phenylethyl alcohol	50.96	0.00	62.35^{a}	75.69^{s}	0.00	72.85^{a}	130.12^{c}	0.00	112.45^{b}	146.62^{c}	0.00	115.02^{b}	147.89^{c}	0.00	105.47^{b}	118.91^{b}	0.00	110.29^{b}	116.98^{b}	0.00	105.26^{b}	118.21^{b}	0.00	103.61^{b}	117.42^{b}
1-Undecanol	53.54	1.12^{a}	1.59^{a}	0.00	21.92^{d}	0.00	0.00	7.38^{c}	0.00	0.00	7.45^{c}	0.00	0.00	36.27^{e}	3.21^{b}	0.00	31.22^{e}	3.38^{b}	0.00	36.44^{e}	3.71^{b}	0.00	32.98^{e}	3.12^{b}	0.00
1-Dodecanol	63.35	0.00	1.83^{a}	0.00	2.63^{b}	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	2.69^{b}	0.00	0.00	1.86^{a}	0.00	0.00	2.10^{a}	0.00	0.00	1.98^{a}	0.00	0.00
Esters		**0.00**	**392.42**	**287.06**	**0.00**	**287.25**	**434.10**	**0.00**	**346.49**	**490.94**	**0.00**	**356.58**	**480.07**	**0.00**	**379.66**	**626.99**	**0.00**	**247.02**	**458.30**	**0.00**	**375.88**	**641.68**	**0.00**	**378.62**	**640.34**
Ethyl acetate	2.75	0.00	126.38^{a}	127.54^{a}	0.00	127.91^{a}	150.52^{a}	0.00	134.65^{a}	197.56^{ab}	0.00	140.83^{a}	189.12^{ab}	0.00	201.30^{b}	347.21^{c}	0.00	154.28^{a}	256.81^{bc}	0.00	202.70^{b}	352.42^{c}	0.00	205.61^{b}	350.14^{c}
Ethyl propanoate	3.56	0.00	0.00	0.00	0.00	0.00	7.12	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Ethyl butanoate	4.07	0.00	0.00	0.00	0.00	1.87^{a}	8.96^{c}	0.00	2.30^{a}	10.45^{d}	0.00	2.19^{a}	11.02^{d}	0.00	8.86^{c}	0.00	0.00	4.56^{b}	0.00	0.00	9.86^{cd}	0.00	0.00	8.88^{c}	0.00
Butanoic acid 2 methylester	5.44	0.00	6.53^{a}	11.04^{b}	0.00	14.76^{b}	93.11^{f}	0.00	5.67^{a}	11.98^{b}	0.00	6.01^{a}	12.86^{b}	0.00	24.39^{c}	69.23^{e}	0.00	13.32^{b}	62.13^{d}	0.00	23.62^{c}	74.37^{e}	0.00	25.84^{c}	72.86^{e}
Butanoic acid 3 methylester	5.85	0.00	2.86^{a}	5.42^{b}	0.00	12.90^{d}	65.01^{f}	0.00	4.34^{b}	17.63^{e}	0.00	4.16^{b}	17.84^{e}	0.00	2.51^{a}	9.89^{c}	0.00	1.23^{a}	9.61^{c}	0.00	2.84^{a}	9.56^{c}	0.00	2.51^{a}	9.99^{c}
Isoamylacetate	7.47	0.00	2.69^{a}	16.31^{b}	0.00	9.31^{b}	11.82^{b}	0.00	4.53^{a}	14.11^{b}	0.00	4.86^{a}	14.82^{b}	0.00	3.22^{a}	45.23^{c}	0.00	1.75^{a}	42.18^{c}	0.00	3.65^{a}	48.51^{c}	0.00	3.46^{a}	47.02^{c}
Hexanoic acid methylester	9.90	0.00	0.00	2.18	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Ethyl hexanoate	12.29	0.00	16.89^{d}	13.02^{cd}	0.00	8.75^{b}	3.99^{a}	0.00	11.09^{c}	18.34^{d}	0.00	11.21^{c}	19.05^{d}	0.00	9.06^{b}	3.98^{a}	0.00	4.56^{a}	3.21^{a}	0.00	9.17^{b}	4.57^{a}	0.00	9.01^{b}	4.32^{a}
Ethyl lactate	18.49	0.00	61.53^{e}	56.25^{e}	0.00	34.39^{c}	42.98^{d}	0.00	42.34^{d}	80.67^{f}	0.00	42.76^{d}	81.12^{f}	0.00	23.95^{b}	51.74^{e}	0.00	11.98^{a}	32.12^{c}	0.00	22.86^{b}	45.79^{d}	0.00	23.12^{b}	49.56^{de}
Butanoic acid 2-hydroxy-3-methylester	23.10	0.00	4.02^{a}	4.41^{a}	0.00	0.00	4.01^{a}	0.00	0.00	3.40^{a}	0.00	0.00	3.98^{a}	0.00	0.00	7.98^{c}	0.00	0.00	6.36^{b}	0.00	0.00	8.82^{c}	0.00	0.00	8.17^{c}
Ethyl octanoate	23.44	0.00	9.63^{d}	1.84^{b}	0.00	1.78^{b}	0.34^{a}	0.00	2.66^{bc}	0.32^{a}	0.00	2.97^{c}	0.56^{a}	0.00	8.99^{d}	1.82^{b}	0.00	1.50^{b}	0.96^{ab}	0.00	9.61^{d}	1.38^{b}	0.00	9.09^{d}	1.79^{b}
Ethyl-3-hydroxybutyrate	28.92	0.00	10.27^{e}	7.08^{d}	0.00	11.15^{e}	7.21^{d}	0.00	2.98^{c}	0.00	0.00	2.81^{c}	0.00	0.00	8.60^{de}	1.97^{b}	0.00	0.00	0.89^{a}	0.00	0.00	1.49^{ab}	0.00	0.00	1.89^{b}

(Continued)

TABLE 2 | Continued

		E1			E2			E3			E4			C1			C2			C3			C4	
	RT	1	60	120	1	60	120	1	60	120	1	60	120	1	60	120	1	60	120	1	60	120	1	60	120
Pentanoic acid 2-hydroxy-4-methyl-ethylester	30.00	0.00	16.83^{f}	7.21^{c}	0.00	5.16^{b}	7.85^{c}	0.00	10.45^{d}	12.49^{e}	0.00	9.99^{d}	13.15^{e}	0.00	6.92^{c}	15.19^{e}	0.00	3.69^{a}	7.21^{c}	0.00	6.78^{c}	15.53^{e}	0.00	6.77^{c}	15.15^{e}
Decanoic acid methylester	32.95	0.00	4.10^{c}	2.00^{b}	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.52^{a}	0.00	0.00	0.00	0.00	0.00	0.68^{a}	0.00	0.00	0.49^{a}
Ethyl decanoate	35.77	0.00	5.18^{c}	1.74^{b}	0.00	1.42^{b}	0.36^{a}	0.00	1.45^{b}	1.46^{b}	0.00	1.24^{b}	1.51^{b}	0.00	1.74^{b}	5.01^{c}	0.00	1.79^{b}	2.38^{b}	0.00	1.96^{b}	4.78^{c}	0.00	1.86^{b}	5.02^{c}
Ethyl benzoate	36.74	0.00	5.30^{bc}	4.81^{b}	0.00	12.38^{d}	3.86^{b}	0.00	3.89^{b}	5.87^{c}	0.00	3.92^{b}	5.71^{bc}	0.00	2.45^{a}	5.63^{c}	0.00	1.54^{a}	1.99^{a}	0.00	2.71^{a}	5.29^{bc}	0.00	2.41^{a}	5.86^{c}
eAcetic acid, 2 phenylethyl	43.01	0.00	3.04^{c}	1.83^{b}	0.00	8.72^{d}	2.31^{b}	0.00	0.99^{a}	2.54^{bc}	0.00	1.05^{a}	2.45^{bc}	0.00	1.65^{b}	3.98^{c}	0.00	0.96^{a}	1.75^{b}	0.00	1.85^{b}	3.99^{c}	0.00	1.72^{b}	4.14^{c}
Methyl Hydrocinnamate	45.91	0.00	19.98^{d}	4.89^{b}	0.00	0.00	7.16^{bc}	0.00	10.34^{c}	13.69^{d}	0.00	10.58^{c}	13.63^{d}	0.00	10.69^{c}	4.82^{b}	0.00	5.65^{b}	2.13^{a}	0.00	10.74^{c}	4.87^{b}	0.00	10.36^{c}	4.39^{b}
Ethyl dodecanoate	46.20	0.00	15.03^{bc}	0.30^{a}	0.00	9.63^{b}	0.51^{a}	0.00	10.45^{b}	1.32^{a}	0.00	10.75^{b}	1.41^{a}	0.00	1.06^{a}	20.23^{c}	0.00	0.80^{a}	8.96^{b}	0.00	1.32^{a}	20.64^{c}	0.00	1.12^{a}	21.03^{c}
Ethyl Hydrocinnamate	48.97	0.00	82.16^{d}	19.19*a*	0.00	27.12^{ab}	16.98^{a}	0.00	98.36^{d}	99.11^{d}	0.00	101.25^{e}	91.84^{de}	0.00	64.31^{c}	32.59^{b}	0.00	39.41^{b}	19.61^{a}	0.00	66.21^{c}	38.99^{b}	0.00	66.86^{c}	38.52^{b}
Aldehides		**213.48**	**113.96**	**28.62**	**104.29**	**32.79**	**75.99**	**82.71**	**55.02**	**31.62**	**82.85**	**55.29**	**32.38**	**166.44**	**46.50**	**76.45**	**165.87**	**48.18**	**52.66**	**168.02**	**74.49**	**75.31**	**165.79**	**44.82**	**75.50**
Octanal	14.81	19.20^{e}	19.85^{e}	2.85^{a}	11.85^{d}	4.17^{b}	5.89^{bc}	8.34^{c}	13.99^{d}	4.63^{b}	8.17^{c}	14.02^{de}	4.83^{b}	17.12^{e}	6.09^{bc}	10.23^{d}	17.45^{e}	7.52^{c}	5.21^{b}	16.95^{e}	12.28^{d}	9.96^{d}	16.98^{e}	5.48^{b}	10.32^{d}
Nonanal	20.78	86.98^{g}	27.01^{d}	11.98^{a}	43.92^{e}	11.01^{a}	32.18^{d}	37.23^{de}	20.25^{c}	10.43^{a}	37.41^{de}	20.28^{c}	10.79^{a}	71.23^{f}	15.21^{b}	25.96^{cd}	70.84^{f}	15.48^{b}	19.85^{c}	69.82^{f}	32.11^{d}	25.63^{cd}	70.94^{f}	15.02^{b}	26.41^{cd}
3-Octanal	21.64	0.00	2.04^{a}	5.21^{b}	0.00	1.74^{a}	1.89^{a}	0.00	2.05^{a}	0.00	0.00	2.10^{a}	0.00	0.00	2.21^{a}	2.49^{a}	0.00	2.15^{a}	2.56^{a}	0.00	2.01^{a}	2.06^{a}	0.00	2.07^{a}	2.30^{a}
Decanal	26.85	107.30^{h}	41.21^{ef}	4.56^{a}	47.52^{f}	6.87^{b}	21.02^{d}	35.74^{e}	6.45^{b}	4.91^{a}	35.76^{e}	6.58^{b}	5.01^{a}	73.24^{g}	10.85^{c}	21.56^{d}	72.61^{g}	10.78^{c}	10.11^{c}	76.23^{g}	16.57^{cd}	21.54^{d}	73.01^{g}	10.20^{c}	19.99^{d}
Benzaldehyde	28.08	0.00	23.85^{e}	4.02^{b}	1.00^{a}	9.00^{c}	15.01^{d}	1.40^{a}	12.27^{c}	11.65^{c}	1.51^{a}	12.31^{c}	11.75^{c}	4.85^{b}	12.14^{c}	16.21^{d}	4.96^{b}	12.25^{c}	14.93^{d}	5.02^{b}	11.52^{c}	16.12^{d}	4.86^{b}	12.05^{c}	16.48^{d}
Phenols		**0.00**	**202.76**	**181.60**	**0.00**	**129.26**	**255.00**	**0.00**	**9.85**	**52.39**	**0.00**	**9.99**	**52.58**	**0.00**	**148.47**	**412.65**	**0.00**	**116.02**	**395.65**	**0.00**	**123.91**	**376.73**	**0.00**	**126.67**	**374.95**
Guaiacol	47.25	0.00	16.93^{a}	0.00	0.00	0.00	31.08^{b}	0.00	0.00	11.84^{a}	0.00	0.00	12.01^{a}	0.00	61.02^{c}	70.25^{d}	0.00	57.16^{c}	63.55^{c}	0.00	64.13^{c}	54.23^{c}	0.00	66.84^{cd}	55.01^{c}
Creosol (Homoguaiacol)	52.57	0.00	185.23^{f}	175.28^{f}	0.00	116.01^{e}	205.83^{f}	0.00	3.65^{a}	32.57^{c}	0.00	3.89^{a}	32.41^{c}	0.00	58.62^{d}	301.25^{g}	0.00	17.89^{b}	292.14^{g}	0.00	18.30^{b}	284.60^{g}	0.00	18.76^{b}	281.52^{g}
Phenol	55.36	0.00	0.60^{a}	1.96^{a}	0.00	1.41^{c}	4.02^{b}	0.00	0.59^{a}	1.01^{a}	0.00	0.63^{a}	1.04^{a}	0.00	6.98^{bc}	10.21^{c}	0.00	5.69^{b}	9.81^{c}	0.00	5.12^{b}	9.78^{c}	0.00	5.05^{b}	9.46^{c}
4-Ethyl phenol	63.20	0.00	0.00	4.36^{a}	0.00	11.84^{b}	14.07^{b}	0.00	5.61^{a}	6.97^{a}	0.00	5.47^{a}	7.12^{a}	0.00	21.85^{c}	30.94^{d}	0.00	35.28^{e}	30.15^{d}	0.00	36.36^{e}	28.12^{d}	0.00	36.02^{e}	28.96^{d}
Total		**232.68**	**1483.79**	**1044.99**	**152.61**	**1067.93**	**1368.78**	**112.11**	**1522.46**	**1765.60**	**112.86**	**1545.63**	**1757.31**	**234.15**	**1701.07**	**3420.27**	**227.33**	**1530.68**	**3062.18**	**237.51**	**1663.51**	**3194.42**	**229.84**	**1546.72**	**3184.08**

a–g for each compound different superscript letters in the same raw are significantly different ($P < 0.05$).

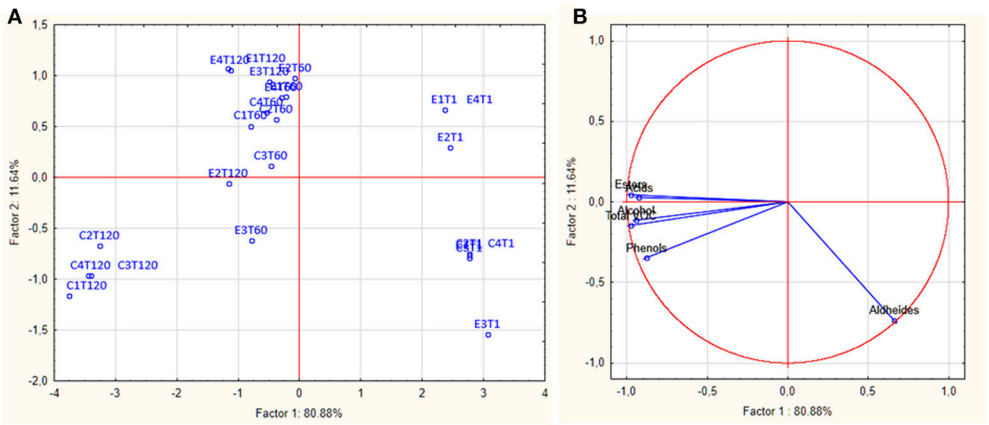

FIGURE 3 | PCA plot **(A)** and score plot **(B)** showing the distribution of experimental (E) and control (C) samples through the fermentation process.

was mainly represented by aldehydes. Score plots are effective in showing the difference among samples and in separating them in the graphs. In detail, all E and C samples at 1 day of fermentation highlighted a positive contribution by component 2 planes. After 120 d, C samples were characterized by a positive contribution of alcohols whereas E samples principally by esters.

Permutation Analysis and Correlations Between Microbiota and Metabolome of Nocellara Etnea Table Olives

Similarities in the observed microbial counts and metabolomics profiles between samples at 60 and 120 days of fermentation were estimated using the PermutMatrixEN software (**Figure 4**). In detail, two clusters were revealed, showing that overall the samples grouped based on the addition of starters and on time of the fermentation. It is interesting to note that salt content did not discriminate samples, with the exception of the experimental samples E1 and E2 at 60 and 120 d, respectively, which exhibited unique profiles. In fact, these samples showed the most divergent microbial and metabolic profiles, with a strong presence of ethyl-3-hydroxybutyrate, LAB, mesophilic bacteria, isobutyric acid, 2-ethyleptanoic acid, 1-dodecanol, decanoic acid methyl ester, methyl idrocynnamate, 1-octanol, 1-nonanol, ethyl octanoate, benzyl alcohol, staphylococci, octanal, decanal, benzaldehyde, nonanal in the E1 sample at 60 days, and of butanoic acid 2 methylester, propionic acid, ethyl propanoate, butanoic acid 3 methylester, hexanoic acid, phenylethyl alcohol, ethyl butanoate in the E2 sample at 120 days. Compared to 60 days of fermentation, control samples at 120 days showed different microbial and metabolomics profiles with a strong increase in acetic acid, phenol, ethanol, isoamyl alcohol, ethyl acetate, 1-hexanol, isoamylacetate, butanoic acid 2-hydroxy-3-methylester, creosol (homoguaiacol), 3-octenol, cis hexen 1 ol, ethyl decanoate, ethyl dodecanoate, and nonanal. Evaluating E samples, both, time of fermentation and NaCl content, affected the microbial and metabolomics profiles. High similarity was found between samples treated with 6 and 8% of NaCl after 60 and 120 d of fermentation, characterized by a lower amount of guaiacol, 4-ethyl phenol and enterobacteria.

Correlations between microbial and metabolomics data are shown in **Figure 5**. Among organic acids, propionic, isobutyric acid, butanoic, and exanoic compounds were negatively correlated with yeasts and staphylococci, and positively correlated ($r > 0.30$, $p < 0.05$) with mesophilic bacteria. One exception was the acetic acid, which, was positively correlated ($r = 0.238$, $p = 0.045$) with enterobacteria and negatively with LAB ($r = -0.735$; $p = 0.048$). Zooming on the metabolomics, the acetic acid was positively correlated ($r > 0.70$, $p < 0.05$) with alcohols (ethanol, isoamylalcohol,1-hexanol, cis hexen 1 ol and 3-octenol) and esters (ethyl acetate, butanoic acid 2 methylester, isoamylacetate, butanoic acid, and 2-hydroxy-3-methylester). Alcohols, with the exception of 3-octenol, phenylethyl alcohol and 1-dodecanol, were positively correlated with enterobacteria and yeasts. In addition, ethanol, isoamylalcohol, 1-hexanol, cis hexen 1 ol, and 3-octenol were negatively correlated with mesophilic bacteria and LAB.

Molecular Identification of *Lactobacillus* spp. Isolates

Four hundred isolates from MRS plates were considered lactobacilli based on their positive Gram reaction, non-motility, absence of catalase activity, and spore formation, and rod or coccal shape. Presumptive lactobacilli were identified by using multiplex PCR and were ascribed to *L. plantarum*, *L. pentosus*, *L. paracasei*, and *Lactobacillus casei* species, and their occurrence percentage in E and C samples, at 1, 60, and 120 days of fermentation is illustrated in **Figure 6**. In detail, C samples, as expected, exhibited at both day 1 and after 60 days, a high occurrence (70%) of *L. plantarum* accompanied by *L. pentosus* (30%). After 120 d, a slight occurrence (20%) of *L. casei* was detected. In E samples, 40% of isolates were ascribed to both *L. paracasei* and *L. plantarum*. The remaining 20% was identified as *L. pentosus*. Similar occurrence was revealed after 60 d of fermentation. Different species occurrence was highlighted at

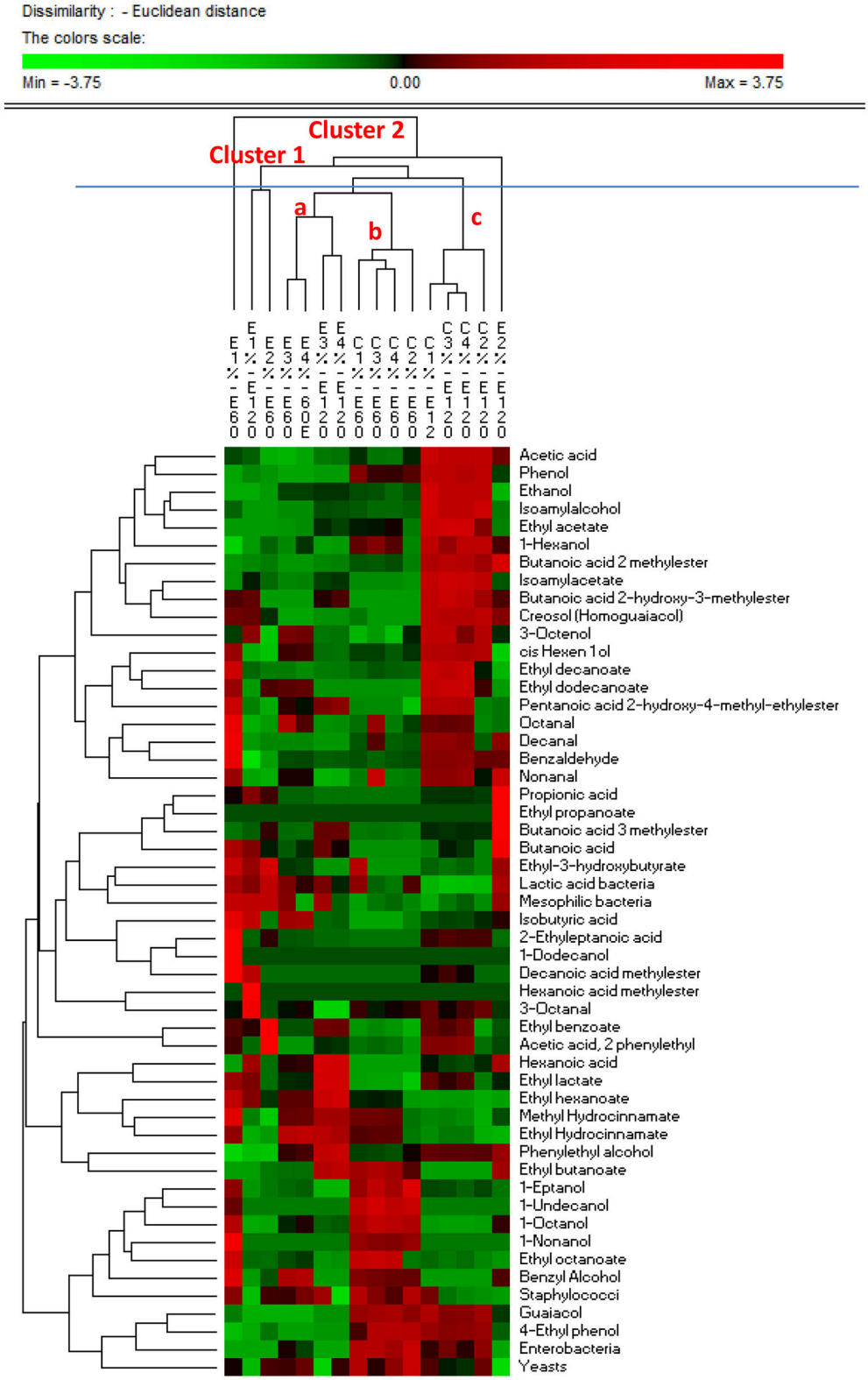

FIGURE 4 | PermutMatrixEN analysis of the microbial and metabolomics profiles of experimental (E) and control (C) Nocellara Etnea under different salt concentrations (4, 5, 6, 8%) at 60 and 120 days.

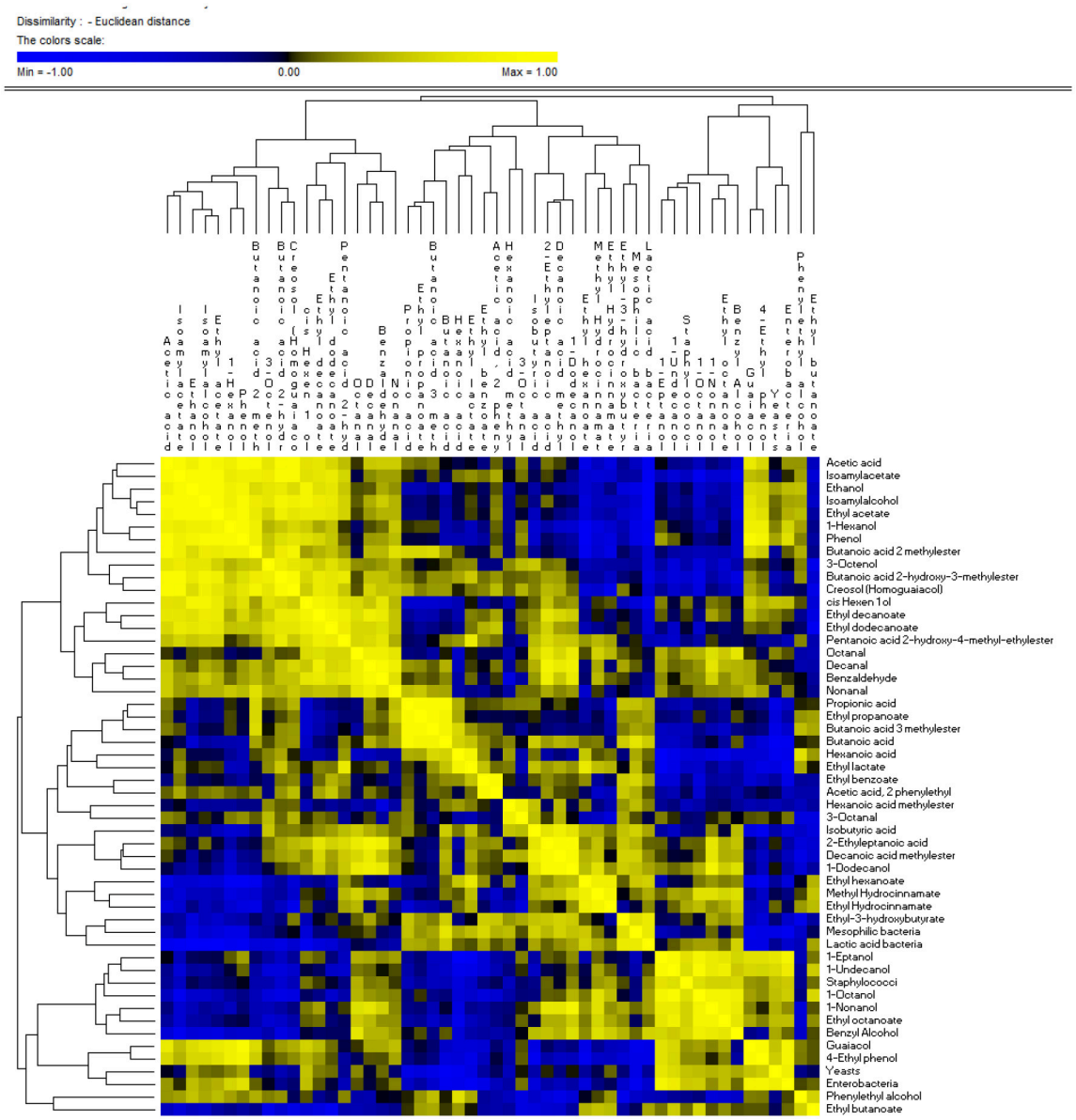

FIGURE 5 | Significant correlations between microbial and metabolomics data found after 60 and 120 days of fermentation of experimental (E) and control (C) Nocellara Etnea under different salt concentrations (4, 5, 6, 8%).

120 days, since the majority of the isolates were identified as *L. paracasei* (60%), followed by *L. pentosus* (30%) and *L. plantarum* (10%) (**Figure 6**). It is interesting to note that among the 60 isolates identified as *L. paracasei*, the majority of the occurrence was revealed in E sample at 5% of salt (E2).

Viability of *L. paracasei* N24 Strain Through Table Olives Fermentation

In order to evaluate the viability of *L. paracasei* strain N24 in the experimental table olives at 60 and 120 d, DNA of 60 strains was submitted to REP-PCR. Results are reported in **Figures 7A,B**. All strains clustered together with the *L. paracasei* N24 strain (with a percentage of similarity higher than 85%), with the exception of 17 strains, which exhibited different profiles.

Sensory Data

Results of sensory analysis are reported in **Table 3**. No off-odors were detected in any samples as inferred by the low scores of the taste panel for this organoleptic perception. Overall, regarding the gustatory sensations (acidity, saltiness, and bitterness), differences among E and C samples at different salt content were detected, with the exception of bitterness descriptor. In detail, E samples received similar and moderate values in

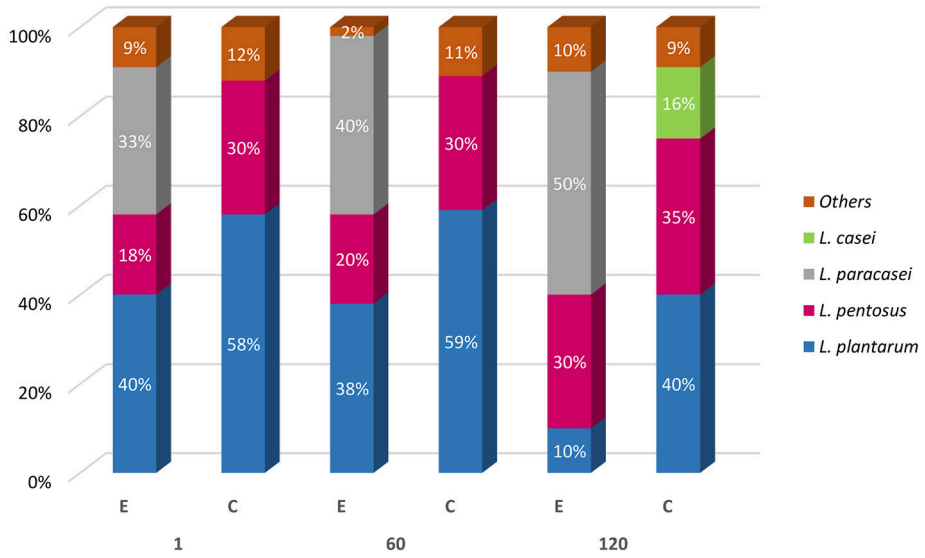

FIGURE 6 | Molecular identification of *Lactobacillus* spp. isolates from experimental (E) and control (C) samples at 1, 60, and 120 days of fermentation.

acidity, while C ones exhibited a higher score, with the highest value for the sample with 8% of salt (C4). A similar trend was observed for the saltiness score, with lower value in E samples. Only samples made adding 8% of salt (E4 and C4) showed significant differences, registering a saltiness score of about 8.1. Regarding kinaesthetic sensations (hardness, fibrousness, and crunchiness) no statistical differences were achieved among samples, registering a mean score of 7.2. Finally, E olives received higher scores for the overall acceptability descriptor, with the exception of E1 sample, which showed an average score similar to those obtained by C samples. Experimental E2 sample exhibited the highest overall acceptability score (8.8 ± 0.82).

DISCUSSION

Consumer's acceptance and attitude toward functional foods determine the market success, which is growing steadily, mainly toward vegetables, fruits, and cereal products due to vegetarianism emergence, lactose intolerance, cholesterolemia, and food allergies (Granato et al., 2010; Ranadheera et al., 2010; Peres et al., 2012; Martins et al., 2013). Among the non-dairy functional foods, table olives represent a good food matrix to carry active viable bacteria into the gastrointestinal tract (Lavermicocca et al., 2005; Valerio et al., 2006; Abriouel et al., 2012; Randazzo et al., 2017). Table olives are considered functional food because of their nutritional value related to the presence of phenolic compounds and monounsaturated fatty acids (Buckland and Gonzalez, 2015). Nevertheless, their preparation relies on the use of NaCl as the main ingredient of the brine especially for reducing undesirable spoilage and pathogenic microorganisms ensuring, thus, the microbiological safety and quality of the final product (Taormina, 2010; Albarracín et al., 2011). In recent years, public health and regulatory authorities have recommended the reduction of dietary intake of sodium because of its association to hypertension [World Health Organisation (WHO), 2003, 2007], and to cardiovascular diseases (Ortega et al., 2011). In Mediterranean regions, populations eat considerably high amounts of table olives and, subsequently, ingest greater amounts of salt; hence, NaCl reduction in table olives is strongly recommended. Recently, several studies have been focused on the replacement of NaCl with other salts, such as KCl and $CaCl_2$ (Bautista-Gallego et al., 2010, 2013a,b; Mateus et al., 2016; Zinno et al., 2017), and results are not fully in areement. In particular, while Bautista-Gallego et al. (2010) showed that *Enterobacteriaceae* growth was slightly stimulated by high $CaCl_2$ contents, Mateus et al. (2016) revealed that the presence of potassium and calcium chlorides in the brines caused an increase of the enterobacteria death rate. Up to now only De Bellis et al. (2010) have proposed to study table olive processing at reduced NaCl concentration, without any salt replacement. Based on our previous reported data (Randazzo et al., 2017), with the aim to set up functional table olives from Nocellara Etnea cultivar, in the present study the fermentation was carried out at different salt contents (4, 5, 6, and 8%), by using starter cultures constituting of the promising probiotic strain *L. paracasei* N24 and by the strain *L. plantarum* UT2.1. The fermentation without the use of starter cultures was used as control. It is well established that both salt content and pH value are the main parameters controlling the pathogens growth in fermented products, such as *C. botulinum*. Taormina (2010) has already reported that the probability of growth and toxin production of *C. botulinum* at 5% NaCl decreased as the pH and storage temperature was decreased. In this context, our data reveled that all experimental brines have had a pH value below 4.5, with the exception of control sample C4, and a constant temperature of ca 18°C, which guarantee the pathogens growth inhibition. Overall, in contrast to De Bellis et al. (2010), who observed

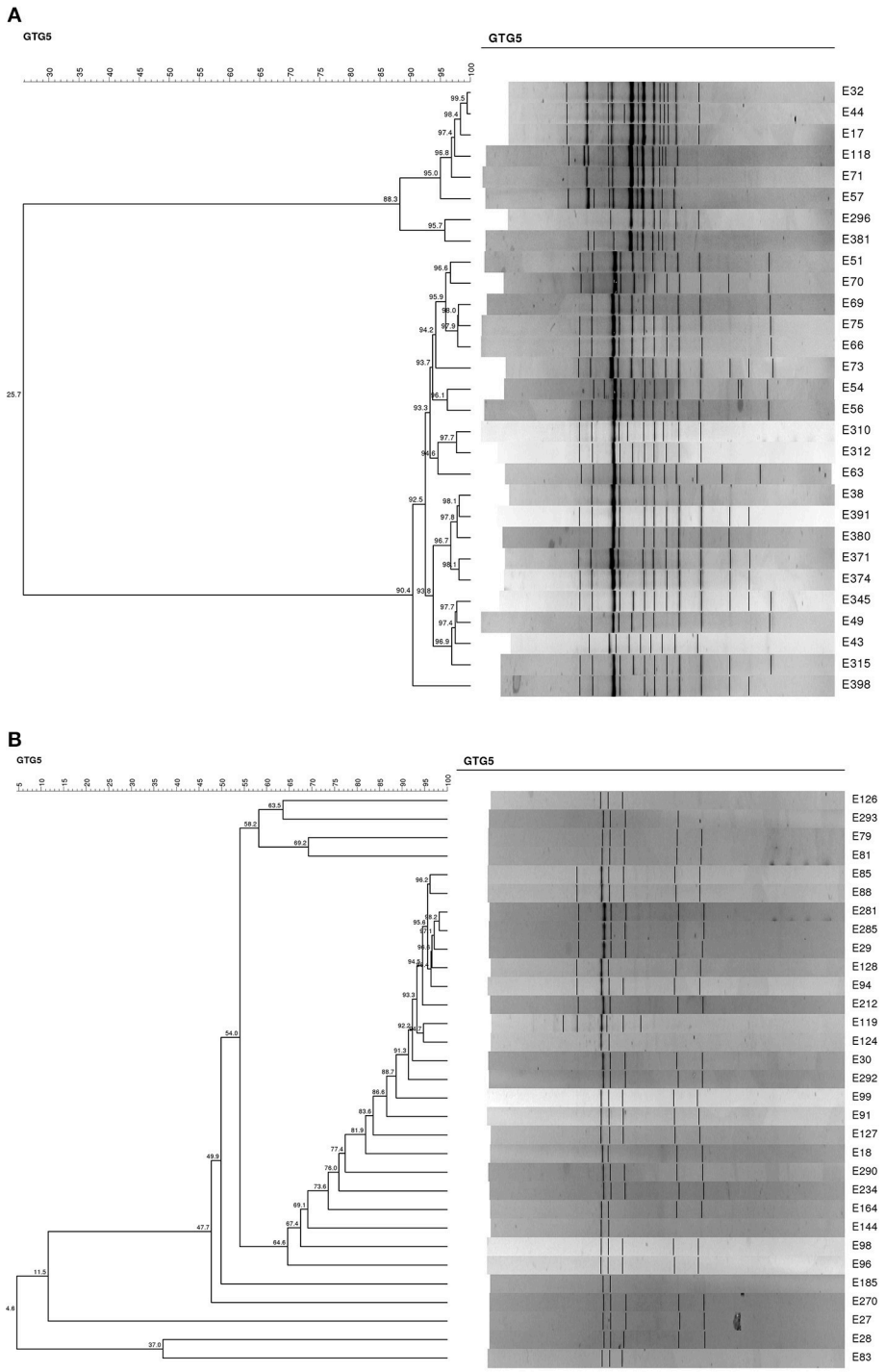

FIGURE 7 | (A) Dendrogram generated after cluster analysis of the digitized $(GTG)_5$-PCR fingerprints of the lactobacilli isolated from experimental table olives at 60 and 120 days. **(B)**. Dendrogram generated after cluster analysis of the digitized $(GTG)_5$-PCR fingerprints of the lactobacilli isolated from experimental table olives at 60 and 120 days.

alterative processes in spontaneous fermentation at 4% of NaCl, in the present study, samples treated with different salt content obtained similar scores in terms of "abnormal fermentation" sensory attribute. Slight sensory differences were detected only for bitterness and acidity descriptors among experimental and control samples, with a higher score in control ones. These

TABLE 3 | Sensory data of the experimental (E) and control (C) table olives.

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	
E1	1.1 ± 0.29^{a}	0.4 ± 0.28^{a}	0.7 ± 0.16^{a}	0.2 ± 0.12^{ab}	0.5 ± 0.12^{a}	1.3 ± 0.12^{a}	0.9 ± 0.31^{a}	3.7 ± 0.36^{a}	6.1 ± 0.29^{a}	2.6 ± 0.12^{a}	5.3 ± 0.61^{ab}	3.2 ± 0.12^{a}	6.4 ± 0.23^{a}	8.5 ± 0.12^{a}
E2	1.3 ± 0.49^{a}	0.3 ± 0.25^{a}	0.5 ± 0.17^{a}	0.0 ± 0.05^{ab}	0.6 ± 0.12^{a}	1.1 ± 0.29^{a}	1.1 ± 0.23^{a}	3.6 ± 0.3^{a}	6.3 ± 0.41^{a}	2.7 ± 0.09^{a}	6.9 ± 0.05^{bc}	3.3 ± 0.14^{a}	6.7 ± 0.16^{a}	8.8 ± 0.82^{a}
E3	1.4 ± 0.24^{a}	0.4 ± 0.23^{a}	0.7 ± 0.21^{a}	0.3 ± 0.16^{a}	0.5 ± 0.05^{a}	1.3 ± 0.26^{a}	1.1 ± 0.28^{a}	3.7 ± 0.12^{a}	6.5 ± 0.29^{ab}	2.8 ± 0.37^{a}	5.2 ± 0.78^{a}	3.5 ± 0.34^{a}	6.3 ± 0.12^{a}	8.5 ± 0.21^{a}
E4	1.3 ± 0.53^{a}	0.5 ± 0.28^{a}	0.8 ± 0.12^{a}	0.2 ± 0.05^{ab}	0.5 ± 0.29^{a}	1.1 ± 0.61^{a}	0.8 ± 0.41^{a}	4.5 ± 0.21^{ab}	7.3 ± 0.41^{ab}	2.6 ± 0.12^{a}	5.3 ± 0.16^{a}	3.3 ± 0.21^{a}	6.3 ± 0.31^{a}	8.2 ± 0.29^{a}
C1	1.3 ± 0.12^{a}	0.2 ± 0.21^{a}	0.7 ± 0.16^{a}	0.3 ± 0.05^{ab}	0.7 ± 0.21^{a}	1.1 ± 0.58^{a}	0.9 ± 0.37^{a}	5.3 ± 0.61^{bc}	6.4 ± 0.05^{ab}	5.3 ± 0.08^{b}	7.2 ± 0.08^{c}	3.4 ± 0.35^{a}	6.6 ± 0.43^{a}	7.9 ± 0.33^{a}
C2	1.4 ± 0.21^{a}	0.3 ± 0.34^{a}	0.6 ± 0.16^{a}	0.4 ± 0.16^{b}	0.7 ± 0.12^{a}	1.3 ± 0.29^{a}	1.1 ± 0.25^{a}	5.4 ± 0.86^{bc}	6.4 ± 0.90^{ab}	5.5 ± 0.86^{b}	7.3 ± 0.37^{c}	3.5 ± 0.11^{a}	6.7 ± 0.38^{a}	7.7 ± 0.61^{a}
C3	1.4 ± 0.37^{a}	0.4 ± 0.24^{a}	0.7 ± 0.21^{a}	0.2 ± 0.05^{ab}	0.8 ± 0.33^{a}	1.3 ± 0.54^{a}	1.2 ± 0.53^{a}	5.4 ± 0.37^{bc}	6.3 ± 0.37^{a}	5.3 ± 0.78^{b}	7.2 ± 0.57^{c}	3.8 ± 0.23^{a}	6.5 ± 0.29^{a}	7.6 ± 0.57^{a}
C4	1.3 ± 0.12^{a}	0.4 ± 0.40^{a}	0.7 ± 0.21^{a}	0.2 ± 0.12^{ab}	0.5 ± 0.37^{a}	1.2 ± 0.61^{a}	1.2 ± 0.61^{a}	6.2 ± 0.53^{c}	7.7 ± 0.12^{b}	5.2 ± 0.53^{b}	7.2 ± 0.53^{c}	3.2 ± 0.40^{a}	6.5 ± 0.26^{a}	7.6 ± 0.57^{a}

a–c for each descriptor, in the same column, with different superscript letters are significantly different ($P < 0.05$).

differences could be therefore attributed to the added starter cultures. In particular, the higher contents of acetic acid, ethanol, and phenols, associated to vinegary, fatty smell, and bitter aroma, respectively, in control samples, could justify the highest acidity and bitterness scores registered by panelists. On the contrary, it is interesting to note that the experimental sample at 5% of NaCl (E2) showed the highest overall acceptability score and, based on the similarity tree, this sample exhibited a unique profile. Even in the experimental samples a high amount of propionic acid was detected, panelists did not reveal any off-flavors, indicating that the defect generated by propionic acid was probably hidden by other compounds such as esters, as indicated by Blana et al. (2014). In fact, among total VOCs, esters showed an occurrence percentage higher in experimental samples rather than in control samples, contributing to more pleasant flavors (Sabatini et al., 2008). Evaluating both PCA data on VOCs and similarity tree, brine samples were mainly grouped based on fermentation time, in discordance to Blana et al. (2014), that demonstrated the importance of salt content on the fermentation profiles. The present study revealed that salt contents slightly influences the metabolome of table olives; however, the overall characteristics of the final products were strongly time-dependent. In addition, the salt content did not affect the performances of *L. paracasei* N24 and *L. plantarum* UT2.1 starter cultures used. They were effective in accelerating the fermentation process, quickly reducing the pH from the 7th day of fermentation, inhibiting spoilage bacteria in all experimental samples. In fact, at the end of the fermentation, *Enterobacteriaceae* were countable only in control samples. This microbial group, as suggested by Medina-Pradas et al. (2017), can negatively influence the quality and safety of table olives, causing gas pockets spoilage, or producing metabolites that affect the final aroma. Moreover, the present work allowed to asses that starters, rather than NaCl replacement with $CaCl_2$ and/or KCl, as discussed by Mateus et al. (2016), has an effect on the reduction of the yeast population. In fact, in the present study yeast decreasing could be attributed to the intense competition between LAB and yeasts for nutrients. It is noteworthy that yeasts are involved in the VOCs formation; nevertheless, a high occurrence of this microbial group could be responsible of undesirable fermentation. In the present study, yeasts were positively correlated with the main alcohols and phenols detected, which could generate off-flavors. Hence, the use of starter cultures is strongly recommended in table olives fermentation also in order to inhibit spoilage bacteria and control the autochthonous yeast growth. Evaluating the lactobacilli behavior, *L. plantarum* and *L. pentosus* were the main species detected at the end of the fermentation in all samples, confirming their key role in table olive fermentation. In addition, a high survival rate of the promising probiotic N24 strain was depicted in all experimental samples. This evidence confirms its technological suitability to be used as starter in olive fermentations as well as its ability to survive during the process regardless of brine salt concentration. In addition, the promising probiotic *L. paracasei* N24 strain exhibited the highest occurrence in experimental sample at 5% of salt (E2). The latter was clearly separated from the remaining treatments, exhibiting a unique metabolomics profile, which

generate sensorial traits appreciate by panelists. Hence, data of the present investigation revealed promising perspectives for the application of *L. paracasei* N24 strain as starter cultures for the production of table olives with increased added value.

CONCLUSION

Results of the present study demonstrated that both brine microbial population and VOCs were slightly affected by salt content while a strong influence was determined by time of fermentation. The reduction of NaCl content, without any replacement with other salts resulted in a successful fermentation of Nocellara Etnea table olives. The final products fulfilled microbiological criteria and exhibited more appreciate sensorial characteristics. In addition, the formulation of probiotic table olives with low salt content is healthier and more suitable for consumers at risk of hypertension, opening new perspectives for their production at industrial scale.

AUTHOR CONTRIBUTIONS

AP, AT, and KV performed the experiments, and analyzed data. AP and CR wrote the manuscript. MD, CR, and CC designed the study and contributed to data interpretation. AT and KV co-wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.01125/full#supplementary-material

Figure S1 | Total phenolic content (mg/l) of control (C) experimental (E) brine samples at 60 and 120 days of fermentation.

REFERENCES

Abriouel, H., Benomar, N., Cobo, A., Caballero, N., Fernández Fuentes, M. Á., Pérez-Pulido, R., et al. (2012). Characterization of lactic acid bacteria from naturally-fermented Manzanilla Aloreña green table olives. *Food Microbiol.* 32, 308–316. doi: 10.1016/j.fm.2012.07.006

Albarracín, W., Sánchez, I. C., Grau, R., and Barat, J. M. (2011). Salt in food processing; usage and reduction: a review. *Int. J. Food Sci. Technol.* 46, 1329–1336. doi: 10.1111/j.1365-2621.2010.02492.x

Arroyo-López, F. N., Bautista-Gallego, J., Domínguez-Manzano, J., Romero-Gil, V., Rodriguez-Gómez, F., García-García, P., et al. (2012). Formation of lactic acid bacteria-yeasts communities on the olive surface during Spanish-style Manzanilla fermentations. *Food Microbiol.* 32, 295–301. doi: 10.1016/j.fm.2012.07.003

Bautista-Gallego, J., Arroyo-López, F. N., Durán-Quintana, M. C., and Garrido-Fernández, A. (2010). Fermentation profiles of Manzanilla-Aloreña cracked green table olives in different chloride salt mixture. *Food Microbiol.* 27, 403–412. doi: 10.1016/j.fm.2009.11.015

Bautista-Gallego, J., Arroyo- López, F. N., López – López, A., and Garrido-Fernández, A. (2011). Effect of chloride salt mixtures on selected attributes and mineral content of fermented cracked Aloreña olives. *Food Sci. Technol.* 44, 120–129. doi: 10.1016/j.lwt.2010.06.027

Bautista-Gallego, J., Arroyo-López, F. N., Rantsiou, K., Jiménez-Díaz, R., Garrido-Fernández, A., and Cocolin, L. (2013a). Screening of lactic acid bacteria isolated from fermented table olives with probiotic potential. *Food Res. Int.* 50, 135–142. doi: 10.1016/j.foodres.2012.10.004

Bautista-Gallego, J., Arroyo-López, F. N., Romero-Gil, V., Rodríguez-Gómez, F., García-García, P., and Garrido-Fernández, A. (2015). Fermentation profile of green Spanish-style Manzanilla olives according to NaCl content in brine. *Food Microbiol.* 49, 56–64. doi: 10.1016/j.fm.2015.01.012

Bautista-Gallego, J., Rantsiou, K., Garrido-Fernández, A., Cocolin, L., and Arroyo-López, F. N. (2013b). Salt reduction in vegetable fermentation: reality or desire? *J. Food. Sci.* 78, 1095–1100. doi: 10.1111/1750-3841.12170

Blana, V. A., Grounta, A., Tassou, C. C., Nychas, G. J., and Panagou, E. Z. (2014). Inoculated fermentation of green olives with potential probiotic *Lactobacillus pentosus* and *Lactobacillus plantarum* starter cultures isolated from industrially fermented olives. *Food Microbiol.* 38, 208–218. doi: 10.1016/j.fm.2013.09.007

Buckland, G., and Gonzalez, C. A. (2015). The role of olive oil in disease prevention: a focus on the recent epidemiological evidence from cohort studies and dietary intervention trials. *Br. J. Nutr.* 113, S94–S101. doi: 10.1017/S0007114514003936

Cocolin, L., Alessandria, V., Botta, C., Gorra, R., De Filippis, F., Ercolini, D., et al. (2013). NaOH-debittering induces changes in bacterial ecology during table olives fermentation. *PLoS ONE* 8:e69074. doi: 10.1371/journal.pone.0069074

De Bellis, P., Valerio, F., Sisto, A., Lonigro, S. L., and Lavermicocca, P. (2010). Probiotic table olives: microbial populations adhering on olive surface in fermentation sets inoculated with the probiotic strain *Lactobacillus paracasei* IMPC2.1 in an industrial plant. *Int. J. Food Microbiol.* 140, 6–13. doi: 10.1016/j.ijfoodmicro

European Council (2010). *Council Conclusions of 8 June on Action to Reduce Population Salt Intake for Better Health*. European Council. Union. 11.11.2010 C305/3-C305/5.

G-Alegria, E., Lopez, I., Ruiz, J. I., Sáenz, J., and Fernández, E., Zarazaga, M., et al. (2004). High tolerance of wild *Lactobacillus plantarum* and *Oenococcus oeni* strains to lyophilisation and stress environmental condition of acid pH and ethanol. *FEMS Microbiol. Lett.* 230, 53–61. doi: 10.1016/S0378-1097(03)00854-1

Gevers, D., Huys, G., and Swings, J. (2001). Applicability of rep-PCR fingerprinting for identification of *Lactobacillus* species. *FEMS Microbiol. Lett.* 205, 31–36. doi: 10.1111/j.1574-6968.2001.tb10921.x

Granato, D., Branco, G. F., Nazzaro, F., Cruz, A. G., and Faria, J. A. F. (2010). Functional foods and nondairy probiotic food development: trends, concepts, and products. *Comp. Rev. Food Sci. Food Safety* 9, 292–302. doi: 10.1111/j.1541-4337.2010.00110.x

Hurtado, A., Reguant, C., Bordons, A., and Rozès, N. (2012). Lactic acid bacteria from fermented table olives. *Food Microbiol.* 31, 1–8. doi: 10.1016/j.fm.2012.01.006

International Olive Council (IOC) (2011). *Sensory Analysis of Table Olives*. Document COI/OT/MO No 1/Rev. 2, Madrid.

International Olive Council (IOC) (2016). Market Newsletter No 108 – September 2016. Available Online at: http://www.internationaloliveoil.org/news/view/686-year2016-news/762-marketnewsletter-september-2016

Lavermicocca, P., Valerio, F., Lonigro, S. L., De Angelis, M., Morelli, L., Callegari, M. L., et al. (2005). Adhesion and survival of lactobacilli and bifidobacteria on table olives with the aim of formulating a new probiotic food. *Appl. Environ. Microbiol.* 71, 4233–4240. doi: 10.1128/AEM.71.8.4233-4240.2005

Malheiro, R., de Pinho, P. G., Casal, S., Bento, A., and Pereira, J. A. (2011). Determination of the volatile profile of stoned table olives from different varieties by using HS-SPME and GC/IT-MS. *J. Sci. Food Agric.* 91, 1693–1701. doi: 10.1002/jsfa.4372

Martins, E. M. F., Mota Ramos, A., Silva Lago Vanzela, E., César Stringheta, P., de Oliveira Pinto, C. L., and Martins, J. M. (2013). Products of vegetable origin:

a new alternative for the consumption of probiotic bacteria. *Food Res. Int*. 51, 764–770. doi: 10.1016/j.foodres.2013.01.047

Mateus, T., Santo, D., Saúde, C., Pires-Cabral, P., and Quintas, C. (2016). The effect of NaCl reduction in the microbiological quality of cracked green table olives of the Maçanilha Algarvia cultivar. *Int. J. Food Microbiol.* 218, 57–65. doi: 10.1016/j.ijfoodmicro.2015.11.008

Medina-Pradas, E., Pérez-Díaz, I. M., Garrido-Fernández, A., and Arroyo-López, F. (2017). "Review of vegetable fermentations with particular emphasis on processing modifications, microbial ecology, and spoilage," in *The Microbiological Quality of Food Foodborne Spoilers*, eds A. Bevilacqua, M. R. Corbo, and M. Sinigaglia (New York, NY: Elsevier), 211–236.

Ortega, R. M., López Sobaler, A. M., Ballesteros, J. M., Pérez Farinós, N., Rodríguez Rodríguez, E., Aparicio, A., et al. (2011). Estimation of salt intake by 24h urinary sodium excretion in a representative sample of Spanish adults. *Br. J. Nutr*. 105, 787–794. doi: 10.1017/S000711451000423X

Panagou, E. Z., and Tassou, C. C. (2006). Changes in volatile compounds and related biochemical profile during controlled fermentation of cv. Conservolea green olives. *Food Microbiol*. 23, 738–746. doi: 10.1016/j.fm.2006.02.005

Peres, C. M., Peres, C., Hernández-Mendoza, A., and Xavier Malcata, F. (2012). Review on fermented plant materials as carriers and sources of potentially probiotic lactic acid bacteria e with an emphasis on table olives. *Trends Food Sci. Technol*. 26, 31–42. doi: 10.1016/j.tifs.2012.01.006

Pino, A., Liotta, L., Randazzo, C. L., Todaro, A., Mazzaglia, A., De Nardo, F., et al. (2018). Polyphasic approach to study physico-chemical, microbiological and sensorial characteristics of artisanal Nicastrese goat's cheese. *Food Microbiol*. 70, 143–154. doi: 10.1016/j.fm.2017.09.005

Ranadheera, R. D. C. S., Baines, S. K., and Adams, M. C. (2010). Importance of food in probiotic efficacy. *Food Res. Int.* 43, 1–7. doi: 10.1016/j.foodres.2009.09.009

Randazzo, C. L., Fava, G., Tomaselli, F., Romeo, F. V., Pennino, G., Vitello, E., et al. (2011). Effect of kaolin and copper based products and of starter cultures on green table olive fermentation. *Food Microbiol.* 28, 910–919. doi: 10.1016/j.fm.2010.12.004

Randazzo, C. L., Rajendram, R., and Caggia, C. (2010). "Lactic acid bacteria in table olive fermentation," in *Olives and Olive Oil in Health and Disease Prevention,* eds R. Victor, Preedy, and Ronald Ross Watson (Oxford: Academic Press), 369–376. doi: 10.1016/B978-0-12-374420-3.00041-3

Randazzo, C. L., Ribbera, A., Pitino, I., Romeo, F. V., and Caggia, C. (2012). Diversity of bacterial population of table olives assessed by PCR-DGGE analysis. *Food Microbiol.* 32, 87–96. doi: 10.1016/j.fm.2012.04.013

Randazzo, C. L., Todaro, A., Pino, A., Corona, O., and Caggia, C. (2017). Microbiota and metabolome during controlled and spontaneous fermentation of Nocellara Etnea table olives. *Food Microbiol.* 65, 136–148. doi: 10.1016/j.fm.2017.01.022

Randazzo, C. L., Todaro, A., Pino, A., Pitino, I., Corona, O., Mazzaglia, A., et al. (2014). Giarraffa and Grossa di Spagna naturally fermented table olives: effect of starter and probiotic cultures on chemical, microbiological and sensory traits. *Food Res. Int*. 62, 1154–1164. doi: 10.1016/j.foodres.2014.05.056

Ruiz-Barba, J. L., Cathcart, D. P., Warner, P. J., Jiménez-Diáz, R. (1994). Use of *Lactobacillus plantarum* LPC010, a bacteriocin producer, as a starter culture in Spanish style green olive fermentation. *Appl. Environ. Microbiol*. 60, 2059–2064.

Sabatini, N., Mucciarella, M. R., and Marsilio, V. (2008). Volatile compounds in uninoculated and inoculated table olives with *Lactobacillus plantarum* (*Olea europaea* L., cv. *Moresca Kalamata). LWT Food Sci. Technol*. 41, 2017–2022. doi: 10.1016/j.lwt.2007.12.002

SAS (2001). *User's Guide: Statistics, ver. 8.1. 2001*. Cary, NC: SAS INST. INC.

Simpson, M. V., Smith, J. P., Dodds, K., Ramaswamy, H. S., Blanchfield, B., and Simpson, B. K. (1995). Challenge studies with *Clostridium botulinum* in a sous-vide spaghetti and meat sauce product. *J. Food Prot.* 58, 229–234. doi: 10.4315/0362-028X-58.3.229

Singleton, V. L. (1974). "Analytical fractionation of the phenolic substances of grapes and wines and some practical uses of such analyses," in *Chemistry of Winemaking. Advances in Chemistry Series, Vol. 137*, ed A. D. Webb (Washington, DC: American Chemical Society), 184–211.

Taormina, P. J. (2010). Implications of salt and sodium reduction on microbial food safety. *Crit. Rev. Food Sci. Nutr*. 50, 209–227. doi: 10.1080/10408391003626207

Tofalo, R., Perpetuini, G., Schirone, M., Fasoli, G., Suzzi, G., and Corsetti, A. (2014). *Lactobacillus pentosus* dominates spontaneous fermentation of Italian table olives. *LWT Food Sci. Technol*. 57, 710–717. doi: 10.1016/j.lwt.2014.01.035

Torriani, S., Felis, G. E., and Dellaglio, F. (2001). Differentiation of *Lactobacillus plantarum*, *L.* pentosus, and *L. paraplantarum* by recA gene sequence analysis and multiplex PCR assay with rec gene-derived primers. *Appl. Environ. Microbiol*. 67, 3450–3454. doi: 10.1128/AEM.67.8.3450-3454.2001

Valerio, F., De Bellis, P., Lonigro, S. L., Morelli, L., Visconti, A., and Lavermicocca, P. (2006). *In vitro* and *in vivo* survival and transit tolerance of potentially probiotic strains carried by artichokes in the gastrointestinal tract. *Appl. Environ. Microbiol.* 72, 3042–3045. doi: 10.1128/AEM.72.4.3042-3045.2006

Ventura, M., Canchaya, C., Meylan, V., Klaenhammer, T. R., and Zink, R. (2003). Analysis, characterization, and loci of the *tuf* genes in *Lactobacillus* and *Bifidobacterium* species and their direct application for species identification. *Appl. Environ. Microbiol*. 69, 6908–6922. doi: 10.1128/AEM.69.11.6908-6922.2003

World Health Organisation (WHO) (2003). *Diet, Nutrition and the Prevention of Chronic Disease Report of a Joint WHO/FAO Expert Consultation.* Technical Report Series No, Geneva. 916.

World Health Organisation (WHO) (2007). *Reducing Salt Intake in Populations*. Report of a WHO Forum and Technical Meeting. WHO Document Publication Services, Geneva.

Zinno, P., Guantario, B., Perozzi, G., Pastore, G., and Devirgiliis, C. (2017). Impact of NaCl reduction on lactic acid bacteria during fermentation of Nocellara del Belice table olives. *Food Microbiol.* 63, 239–247. doi: 10.1016/j.fm.2016.12.001

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Assessing the Challenges in the Application of Potential Probiotic Lactic Acid Bacteria in the Large-Scale Fermentation of Spanish-Style Table Olives

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This work studies the inoculation conditions for allowing the survival/predominance of a potential probiotic strain (*Lactobacillus pentosus* TOMC-LAB2) when used as a starter culture in large-scale fermentations of green Spanish-style olives. The study was performed in two successive seasons (2011/2012 and 2012/2013), using about 150 tons of olives. Inoculation immediately after brining (to prevent wild initial microbiota growth) followed by re-inoculation 24 h later (to improve competitiveness) was essential for inoculum predominance. Processing early in the season (September) showed a favorable effect on fermentation and strain predominance on olives (particularly when using acidified brines containing 25 L HCl/vessel) but caused the disappearance of the target strain from both brines and olives during the storage phase. On the contrary, processing in October slightly reduced the target strain predominance on olives (70–90%) but allowed longer survival. The type of inoculum used (laboratory vs. industry pre-adapted) never had significant effects. Thus, this investigation discloses key issues for the survival and predominance of starter cultures in large-scale industrial fermentations of green Spanish-style olives. Results can be of interest for producing probiotic table olives and open new research challenges on the causes of inoculum vanishing during the storage phase.

Keywords: Green Spanish-style table olives, probiotic starter culture, large-scale fermentation, inoculum survival

INTRODUCTION

Table olives are a fermented vegetable of the Mediterranean basin with many centuries of history. Among the different types of olive elaborations, green Spanish-style is the most well-known industrial process (Garrido-Fernández et al., 1997). Borbolla y Alcalá et al. (1952) were among the first researchers to apply pure starter cultures in this elaboration. Since then, many investigations

were conducted with the objective of ensuring the microbiological control of the fermentation process to produce adequate and safe products (Martorana et al., 2015; Tataridou and Kotzekidou, 2015). The adhesion of lactobacilli and bifidobacteria on the epidermis of the processed olives (Lavermicocca et al., 2005) and the formation of true biofilms on green Spanish-style table olives (Arroyo-López et al., 2012a; Domínguez-Manzano et al., 2012) have been reported recently. Such discoveries have encouraged researchers to investigate the use of multifunctional starter cultures for producing potential probiotic olives at laboratory and pilot plant scales. In fact, this step is an essential requirement to turn table olives into a carrier of beneficial microorganisms to the human body. Rodríguez-Gómez et al. (2013) assessed the technological characteristics and the dominance of several strains of *Lactobacillus pentosus* in 100 L fermentation vessels. Blana et al. (2014) used single and combined cultures of *L. pentosus* and *Lactobacillus plantarum*, isolated from industrially fermented olives, for processing Halkidiki olives in 14 L capacity plastic vessels. Both potential probiotic strains successfully colonized the olive surface, although *L. pentosus* B281 presented the most desirable characteristics for predominance. In heat shocked green olives, the results were similar, and higher recoveries of the *L. pentosus* B281 strain were observed (Argyri et al., 2014). The commercial probiotic *Lactobacillus rhamnosus* GG was also recovered from inoculated samples of natural green Italian cultivars, demonstrating its survival in the olive brine matrix (Randazzo et al., 2014). Sisto and Lavermicocca (2012) also reported the suitability of *Lactobacillus paracasei* IMPC2.1 to make olives with potential probiotic properties. Their results showed that the strain formed a biofilm on the fruits and persisted in high numbers during fermentation. The biofilm formation was also observed on Conservolea natural black olives processed with the functional starter culture *L. pentosus* B281 (alone or in co-inoculation with *Pichia membranifaciens*). Molecular analysis revealed that the bacteria successfully colonized the black olive surface and presented a high recovery rate. On the contrary, recovery of the yeast was limited (Grounta et al., 2016). Recently, Bevilacqua et al. (2015) made a review of the most relevant biotechnological innovations in table olives, based on both traditional and innovative starter cultures.

Despite the substantial effort devoted to the use of starter culture in green Spanish-style table olives, the fermentation process at industrial scale remains virtually spontaneous. Furthermore, the complete predominance of a starter culture at large-scale for producing fermented probiotic olives still constitutes an important technological challenge. The objective of this work was the investigation of the factors that may affect the survival/predominance of a multifunctional starter culture at a large-scale level (16 cubic meter fermentation vessels), under the current processing technology (open tops of containers and environmental conditions prevailing in the fermentation yards). Aiming at the selection of the most favorable conditions for the assurance of the appropriate strain implantation, the present study assessed the effects of time of inoculation, time of the processing season, inoculum history, and initial pH correction on the fermentation profile and inoculum predominance.

MATERIALS AND METHODS

Experimental Design and Olives

This work is related to experiments carried out in two successive seasons: 2011/2012 (inoculation, 26 September 2011) and 2012/2013 (inoculations: 27 September 2012 and 25 October 2012). The study was carried out at JOLCA, S.A. facilities in Huevar del Aljarafe (Seville, Spain), using Manzanilla olives picked by hand at the green maturation stage.

The experiments were always carried out in 16,000 L industrial fermentation vessels, containing 9,856 kg olives and 6,374 L brine. The debittering process and washing were similar in both seasons, and the operations were always performed at environment temperature. The lye treatment was conducted in above-ground vessels of the olive debittering section of the industry, with a 2.6% NaOH solution, which was let to penetrate approximately 2/3 of the flesh (∼6 h). The lye was then removed, and tap water was added to cover the fruits. After 8 h washing, the exhausted water was substituted with an 11% (w/v) NaCl solution for initial salt absorption (∼10 h). Then, the olives were transferred into underground vessels situated in the fermentation yard. During this operation, the HCl proportions specified for each treatment (see below) were progressively incorporated to the circulating brine, to facilitate their homogeneous distribution.

In 2011/2012 season, the design consisted of three duplicate independent treatments (in total six fermentation vessels) which were identified as IF1 (control, spontaneous), IF2 (inoculated with TOMC-LAB2, LAB2 in short) and IF3 (inoculated with TOMC-LAB4, LAB4 in short) (**Figure 1**). The LAB strains were selected because of their promising potential probiotic characteristics (Bautista-Gallego et al., 2013; Arroyo-López et al., 2014). In 2011/2012 season, all fermentation vessels were filled with the same acidified brine [25 L food grade HCl (35% w/w)/vessel]. After allowing 3 days for partial equilibrium, the inoculation was performed by adding 5 L of laboratory-adapted *L. pentosus* LAB2 and LAB4 cultures. The pre-adaptation was achieved by growing the two strains in MRS broth (Oxoid LTD, Basingstoke, England), added with 4% NaCl, till the stationary phase was reached (24 h).

In 2012/2013 season, the design included the following variables: processing time (September vs. October), inoculum type (laboratory vs. industry pre-adaptation), and the proportion of acid added to the initial brine [25 vs. 10 L food grade HCl (35%, w/w)/vessel]. Therefore, for each season time, the experiment consisted of a duplicate complete 2^2 factorial design on inoculum type and proportion of acid added plus the corresponding controls without inoculation. The design required a total of 10 industrial fermentation vessels. The temperature averages during processing were 27 and 22°C in September and October, respectively. The acronyms of the treatments were obtained by combining the variable (history/type of inoculation, HCl volume added, and processing time) levels. E.g., A25S, refers to a treatment performed in September (S), using a brine added with 25 L HCl (35% w/w), and inoculated with a pre-adapted in the industry starter (A). A flow chart with a detailed explanation of the experimental designs and the acronyms of treatments is shown in **Figure 1**.

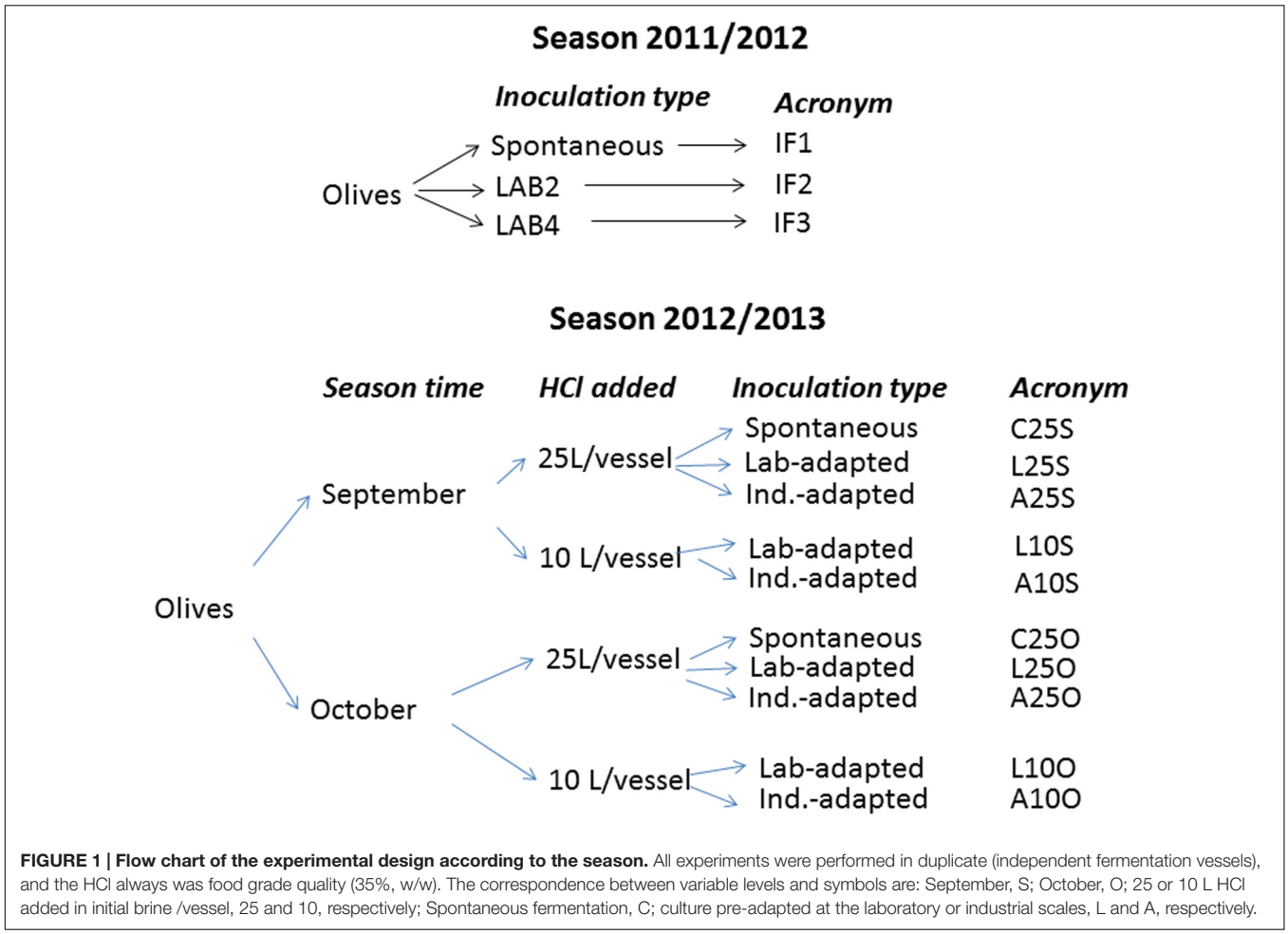

FIGURE 1 | Flow chart of the experimental design according to the season. All experiments were performed in duplicate (independent fermentation vessels), and the HCl always was food grade quality (35%, w/w). The correspondence between variable levels and symbols are: September, S; October, O; 25 or 10 L HCl added in initial brine /vessel, 25 and 10, respectively; Spontaneous fermentation, C; culture pre-adapted at the laboratory or industrial scales, L and A, respectively.

In 2012/2013, based on the results of the previous season, only the *L. pentosus* LAB2 strain was used, and the inocula were added twice (when the olives were transferred into the underground fermentation vessel and at the following day).

Bacterial Strains and Brine Inoculation

In season 2011/2012, cultures (LAB2 and LAB4) were prepared and pre-adapted in the laboratory using 5 L flasks with MRS broth supplemented with 4% NaCl and 2.5% glucose. When the populations reached the stationary phase, approximately 9 $\log_{10}$ CFU/mL according to plate counting in MRS agar medium, the contents of the flasks were introduced directly into the fermentation vessels. The inoculum was distributed into the large fermentation vessels by using a pump provided with an appropriate sterile stainless steel tube, which was introduced at diverse deeps and directions. Later, the fermentation brines were fully homogenized, without aeration, by recycling the solution with a pump.

In 2012/2013 season, the laboratory pre-adapted inoculum (L) was prepared similarly to those of the 2011/2012 season but using LAB2 only. For the preparation of the industry pre-adapted to brine inoculum (A), 1 L of a previously grown in MRS LAB2 culture was added to a plastic drum, containing 200 L of fresh brine, which had the following composition: 4% NaCl, 0.1% MRS broth and 2.5% glucose. The drums were let at environment temperature (20–30°C) for 1 day, after which the LAB2 population reached ∼8 $\log_{10}$ CFU/mL. Then, 100 L of the pre-adapted inoculum were added to 10, and 25 L HCL treated fermentation vessels, using the same procedure of 2011/2012 season. The operation (re-inoculation) was repeated at the following day (24 h) to counteract the usual partial initial population decay after the first inoculum addition. Therefore, each fermentation vessel was inoculated twice.

Physicochemical Analysis

The analyses of pH, titratable and combined acidity in the cover brines were performed in samples withdrawn from the bottom, half and top of the fermentation vessels, using standard methods (Garrido-Fernández et al., 1997). Their averages were recorded and used for further studies.

Microbiological Analysis

The brine samples (100 μL), withdrawn at selected fermentation periods, were plated [directly or after appropriate dilutions with a sterile peptone water (0.9% peptone, wt/vol)], using

a Spiral System model dwScientific (Dow Whitley Scientific Limited, England) on specific selective media. The use of an automatized spiral system reduces considerably the error associated with the plating methodology. *Enterobacteriaceae* were counted on Crystal-violet Neutral-Red bile glucose (VRBD) agar (Merck, Darmstadt, Germany), lactic acid bacteria (LAB) were spread onto de Man, Rogosa and Sharpe (MRS) agar (Oxoid) supplemented with 0.02% (wt/vol) sodium azide (Sigma, St. Louis, MO, USA), and yeasts were grown on yeast–malt–peptone–glucose medium (YM) agar (Difco, Becton and Dickinson Company, Sparks, MD, USA) supplemented with oxytetracycline and gentamicin sulfate (0.005%, wt/vol) as selective agents. The plates were incubated at 30°C for 48 h (bacteria) and 72 h (yeasts) and counted using a Flash&Go (IUL, Barcelona, Spain) image analysis system. Brine counts were expressed as $\log_{10}$ CFU/mL.

To determine the microorganisms adhered to the olive epidermis, the enzymatic protocol developed by Böckelmann et al. (2003) for the detachment of biofilms was followed. Briefly, fruits (25 g) from each fermentation vessel were randomly taken and washed for 1 h with 250 mL of a sterile phosphate buffered saline (PBS) solution. Then, the olives were transferred into 50 mL of a PBS solution with the following enzymes: 14.8 mg/L lipase (L3126), 12.8 mg/L β-galactosidase (G-5160), and 21 μL/L α-glucosidase (G-0660) (Sigma-Aldrich, St. Louis, MO, USA). To achieve biofilm disintegration and removal of the adhered cells, the fruits were incubated at 30°C in this enzyme cocktail with slight shaking (150 rpm). After 12 h, the olives were removed, and the resulting suspension centrifuged at 9,000 × *g* for 10 min at 4°C. Finally, the pellet was re-suspended in 2 mL of PBS and spread onto the different culture media described above. Olive counts were expressed as $\log_{10}$ CFU/g olive (average weight and surface: 4.08 ± 0.46 g and 10.99 ± 1.01 cm^2, respectively; $n = 50$).

Characterization of LAB Populations Adhered on Olives Epidermis

For characterization of the lactobacilli population, repetitive bacterial DNA element fingerprinting analysis (rep-PCR) with primer GTG_5 was followed using the protocol described in Versalovic et al. (1994). The PCR reaction in a final volume of 25 μL contained: 5 μL of 5x MyTaq reaction buffer (5 mM dNTPs and 15 mM $MgCl_2$), 0.1 μL of My Taq DNA polymerase (BiolineReactives, United Kingdom), 1 μL GTG_5 primer (25 μM), 13.9 μL deionized H20 and 5 μL DNA (∼20 ng/μL). PCR amplification was carried out in a thermal cycler (Master Cycler Pro, Eppendorf) with the following program: 95°C for 5 min as initial step plus 30 cycles at: (1) denaturation at 95°C for 30 s, (2) annealing at 40°C for 1 min, and (3) extension at 65°C for 8 min, with a final step of 65°C for 16 min to conclude the amplification. This methodology was used to determine the recovery frequency of the inoculated strain at the time of the maximal population of LAB. Ten isolates were randomly picked from each fermentation vessel, making a total of 160 isolates for the two seasons. Their pattern profiles of bands (from 100 up to 3,000 bp) were compared with the strains used to inoculate the fermentation vessels (LAB2 and LAB4). For this purpose, PCR products were electrophoresed on a 2% agarose gel and visualized under ultraviolet light by staining with ethidium bromide. The resulting fingerprints were digitally captured and analyzed with the BioNumerics 6.6 software package (Applied Maths, Kortrijk, Belgium). The similarity among digitalized profiles was calculated using the Pearson product-moment correlation coefficient. Dendrograms were obtained by means of the Unweighted Pair Group Method using Arithmetic Average (UPGMA) clustering algorithm and the automatic calibration tool for the determination of the optimization and curve smoothing parameters.

Scanning Electron Microscopy

For "*in situ*" observations of the microbiota adhered to the olive epidermis, the method developed by Kubota et al. (2008) was followed. Olives were taken from each fermentation vessel at the moment of maximum population of LAB and washed twice for 1 h with a 100 mM phosphate buffer (pH 7.0). Then, the fruits were placed for 2 h in the same phosphate buffer with 5% glutaraldehyde and then washed several times. Slices (0.5 cm^2) of the olive epidermis were dehydrated in increasing concentrations of ethanol (50, 70, 80, 90, 95, and 100%) and fixed onto glass slides. Finally, samples were sputtered with gold, using a Scancoat Six scanning electron microscopy (SEM) sputter coater equipment (Edwards, Gat, Israel), for 180 s and observed with a scanning electron microscopy model JSM-6460LV (Jeol, Ltd, Tokyo, Japan).

Data Analysis

The physicochemical and microbiological characteristics of each treatment were followed, and their averages recorded. The effect of the treatments was studied by following throughout processing the averages ($n = 4$) of each variable level (l = 2) over the two other variables. Simultaneously, their confidence intervals ($p = 0.05$) at each sample point were also estimated. As an approximation, the observation of non-overlapping confidence intervals of the two levels, at several successive sampling points, was considered as a significant effect of the variable in that interval; that is, during that time, the analyzed variable exerted a significant effect (difference) on such parameter at $p < 0.05$. The typical estimations of the effects over time were not intended because not all the fermentation vessels were sampled at the same time and the measures over time were not considered as independent. When necessary, the scales were re-adjusted to facilitate comparisons among treatments. The data analyses and graphs were performed using SigmaPlot release 13.0 (Systat Software, Inc., 2008).

RESULTS AND DISCUSSION

The survival/predominance of starter cultures at large-scale fermentations, in the current state of the art of the industrial technology, is not a trivial issue. Ruiz-Barba and Jiménez-Díaz (2012) have presented a novel *L. pentosus* paired starter culture that was tested in several industrial 10-ton vessels. Apparently, the starter rapidly colonized the brines, dominated the native

microbiota, and persisted throughout fermentation. However, this is not usual. De Bellis et al. (2010) inoculated industrial plastic vessels (190 kg olives) with the probiotic strain *L. paracasei* IMPC2.1, using 4 and 8% NaCl at room temperature and 4°C. Results indicated that the *L. paracasei* strain successfully colonized the olive surface and dominated the LAB population. Besides this, there was a considerable genetic diversity of LAB species colonizing the olive surface, mainly at 8% NaCl. Lucena-Padrós et al. (2014) have confirmed this microbial diversity at industrial scale in a recent survey carried out at several locations; the spontaneous fermentation was dominated by *L. pentosus,* but there was always a diverse secondary microbiota, including some new bacterial species. In fact, the actual processing conditions prevailing in the current fermentation yards together with the industrial high volume vessels with considerable brine surface exposed to air makes difficult the survival of any starter culture over the environmental microbiota by just controlling the physicochemical conditions. If the objective is the control of the microbiota adhered to the olive surface, the challenge may be even harder. Therefore, establishing conditions for starter culture survival/predominance not only in the cover brine but also on the olive biofilms is a priority for the production of potentially probiotic olives. The present work was designed with this objective.

Among other environmental factors prevailing during green Spanish-style fermentation, several studies consider salt concentration as playing an important role for LAB inoculum survival (De Bellis et al., 2010; Blana et al., 2014). However, in this work, it was let constant (∼6%, w/v) at the equilibrium due to the vast acceptance of this level by industry (Garrido-Fernández et al., 1997). Furthermore, recent studies have demonstrated that LAB growth is usually inhibited at higher than 8% salt concentrations (Romero-Gil et al., 2013). Also, some starter cultures can be applied in alkaline conditions (Sánchez et al., 2001) or without pH correction (Ruiz-Barba and Jiménez-Díaz, 2012), although the addition of diverse proportions of HCl in the initial brine is rather common. In fact, its incorporation reduces the exposure of fermenting olives to high pH levels and limits the riks of undesirable microbial populations such as *Enterobacteriaceae* (Garrido-Fernández et al., 1997). Hence, acidification of initial brines was adopted, although with different levels according to treatments. The use of these conditions was forced by industry to prevent any risk that might compromise the quality and safety of the product involved in the experiment (16 fermentation tanks of 10-ton olives, for a total of about 157.696 kg of fruits).

Tentative Inoculation (2011/2012 Season)

The results obtained in 2011/2012 season constituted the basis for the selection of the treatments applied in the following trial of experiments (2012/2013 season). Thus, they merit some concise comments. The main interest of this tentative essay was focussed on the performance/predominance of the LAB strains, using inoculation conditions based on the literature. In general, the information on this aspect is limited. De Bellis et al. (2010), Ruiz-Barba and Jiménez-Díaz (2012), and Blana et al. (2014) give detailed information on the experiments but not on when the inocula were added. Therefore, the inoculation of microorganisms was performed on the third post-brining day when, supposedly, the solution was already transformed into a sufficiently nutritious fermentation medium. In further experiments on the same fermentation type, Rodríguez-Gómez et al. (2013) applied a similar post-brining delay before inoculation.

In general, the changes in the physicochemical characteristics during fermentation followed the typical trend for green Spanish-style table olives (Garrido-Fernández et al., 1997). It was characterized by a fast pH decrease, a simultaneous (although of opposed direction) and rapid production of acid (which took place in a reduced period, ∼20 days), and a fast combined acidity and salt concentration equilibrium. In brine, the microbial population was composed of *Enterobacteriaceae* (for a short period), LAB and yeasts. On olives, the images showed the typical biofilm formation (Arroyo-López et al., 2012a; Rodríguez-Gómez et al., 2013), even in the spontaneous fermentation (control). The biofilms were mainly composed of LAB and yeast, whose counts indicated a high population at the ∼20th day which, as usual, slowly declined after the fermentation phase (Rodríguez-Gómez et al., 2013). However, unexpectedly, the biofilm also included *Enterobacteriaceae*, which was still detected after 3 months of fermentation (a longer period than in brine), but not at the end of the process (population below the detection limit, 1.2 $\log_{10}$ CFU/mL). Apparently, their embedding in the biofilm might have supported a longer presence on the olives than in the brine. In any case, the LAB control on the biofilms showed that from the 15th day (possible the moment of the maximum population) onward, the profiles of the isolates from the spontaneous fermentation (control) had low similarities with those from the inocula. Please, consult the Supplementary Figures S1–S3 to obtain more information of these processes.

Therefore, if only the standard brine control had been followed, the fermentation with LAB2 and LAB4 would have been assessed as successful; but, in fact, it failed since the objective of the inoculum predominance was not achieved. However, the experiment was useful for showing that the industrial probiotic fermentations require strengthening the hygienic conditions (for preventing the occurrence of *Enterobacteriaceae* in the biofilms) and re-considering the conditions of inoculation. A way of improving starter survival/predominance could be the correction of the initial pH by HCl addition and the incorporation of the LAB inoculum as soon as possible, which could serve as a mechanism of biocontrol and promotion of the proper fermentation. The experiments of the following season were performed considering these aspects.

Effects of the Variables Studied in the Second Season (2012/2013) on the Fermentation Profile

Based on the previous results, in this season the starter was always added just after transferring the olives into the underground fermentation vessels (to reduce as much as possible the initial wild population, including *Enterobacteriaceae* and spontaneous LAB). The variables studied in this season, were: (i) initial pH of

the brine (controlled at two levels by 25 L vs. 10 L HCl addition), (ii) processing time [at the beginning of the season (September), olives are richer in polyphenols and sugars, and the temperature is more favorable than in October], and (iii) inoculum history (laboratory vs. industry pre-adaptation, which might facilitate its rapid growth in the industrial vessels).

It is noteworthy that the average initial pH in the just filled fermentation vessels were 5.1 ($\pm$ 0.2), in September (beginning of the season), and 5.5 ($\pm$ 0.1), in October (final of the season). The effects of the different variables on this parameter had been stressing. Only the season time showed a significant effect during the first 40 days (**Figure 2**). The pH level was initially higher in the olives processed in October than in those prepared in September, although the differences were progressively decreasing and disappeared at about the 50th day. After this time, the evolution was similar. Notice that the slight increase in pH in October, due to the leaching of the NaOH excess before reaching equilibrium, could, eventually, have been stressing for LAB growth (Sánchez et al., 2001). In the treatments processed in September, the pH decreased quite rapidly during fermentation and reached the equilibrium at around the 18th day after brining. However, in those prepared in October, after the first increase, the pH decrease was slower, and the equilibrium was reached later, at around the 50th day due to the lowest LAB counts during the initial fermentation phase (see below). On the contrary, the changes in pH over the other variables showed that the type of inoculum and the proportion of HCl added to the initial brine had not significant effects on this parameter (**Figures 2B,C**). In general, the changes in pH were similar to those observed in other experiments with Greek cultivars (Blana et al., 2014) or to those observed inoculating *L. paracasei* IMPC2.1, a strain of human origin, in Bella di Cerignola debittered green olives (Sisto and Lavermicocca, 2012).

The titratable acidity over time was influenced again by the season time (**Figures 3A**), being the acid formation faster in September when most of the acid was produced before the 20th fermentation day. The onward production was rather slow, with concentrations showing a progressive increase. The average titratable acidity in the spontaneous fermentation corresponding to this time (data not shown) was slightly above those of the other fermentation vessels during storage, possibly due to the LAB disappearance in the inoculated vessels after fermentation. In the treatments applied in October, the titratable acidity was formed more progressively (lower production rates than in September) but following a similar trend in all fermentation systems (even control) (**Figure 3A**). Hence, the performance of *L. pentosus* LAB2 at industrial scale behaved a little bit worse than the wild LAB populations in September but similarly to them in October. Hence, the effect of processing time on titratable acidity (**Figure 3A**) showed that, in September, the acid production rates (and their levels) were clearly above those treated in October not only during the 50 first days but also in the storage phase. Furthermore, the titratable acidity in the olives processed in September did not decline during the study while tended to decrease along the storage in the vessels processed in October, probably due to a certain progressive consumption of the lactic acid by yeasts (Garrido-Fernández et al., 1997). However, the changes in the titratable acidity were not affected by the type/history of inoculation used or by the proportion of HCl added to the initial brine (**Figures 3B,C**). During large-scale green Spanish-style table olive fermentation devoted to probiotic production using Halkidiki cultivar, a faster acidification at 8% NaCl than at 10% was observed (Blana et al., 2014). In the present study, the salt levels in the equilibrium were initially in the range 6–8%; then, the slowest acid production in October vessels may have been due to the effect of a low sugar concentration at this time (ripening of fruits) or to the level of temperature, about 5°C lower in October.

With respect to other physicochemical characteristics, the initial salt concentration was 6.7–7.6°Bé in September, and 6.4–6.7°Bé in October. However, such difference hardly affected LAB growth since it would eventually had favored them in in the olives processed in October. In any case, since no specific control was exerted on this parameter, their evolutions should be considered as the habitual for this processing at the season times essayed (Garrido-Fernández et al., 1997). The combined acidity plays an important role during fermentation, due to its buffering capacity (Garrido-Fernández et al., 1997). The combined acidity levels in brine rapidly rose due to the organic matter leakage into the brine. No effect of season time, type of inoculation, and HCl added to the initial brines were observed on salt or combined acidity, although fermentation vessels with 25 L HCl added led, as expected, to the lowest non-significant levels of the last parameter. More information on the evolution of these parameters during fermentation are included in the Supplementary Figures S4, S5.

Regarding microbial populations, the *Enterobacteriaceae* were detected only in brine during the first fermentation week and always below 3 $\log_{10}$ CFU/mL (data not shown), confirming that the initial pH correction had a favorable effect on the control of these microorganisms (Garrido-Fernández et al., 1997). This behavior contrast with the *Enterobacteriaceae* presence during about the 30 days period reported by De Bellis et al. (2010) in experiments inoculated with *L. paracasei* IMPC2.1. As expected, the LAB population was higher in the inoculated fermentation brines than in the control fermentation vessels ($\sim$6.0 vs.$\sim$ 2.0–3.2 $\log_{10}$ CFU/mL), regardless of treatments. In September, such difference was maintained during all the phase of active fermentation. On the contrary, in October, the initial LAB populations in several treatments decreased, but the decay was followed by later recoveries (data not shown).

The trends followed by LAB were quite dependent on the processing time (**Figure 4**). In general, the populations in the vessels inoculated with starter cultures followed entirely different trends along the first fermentation period, which was characterized by an immediate growth in September vs. slight decrease followed by a recovering in October (**Figure 4A**). This different behavior resulted in September in a faster rapid growth of the LAB, that reached a maximum average population slightly above those treatments processed in October. The differences between the LAB in brine behaviors according to processing times were also observed along the fermentation. In September, the LAB populations decreased sharply and disappeared around the 40th day. In the treatments processed

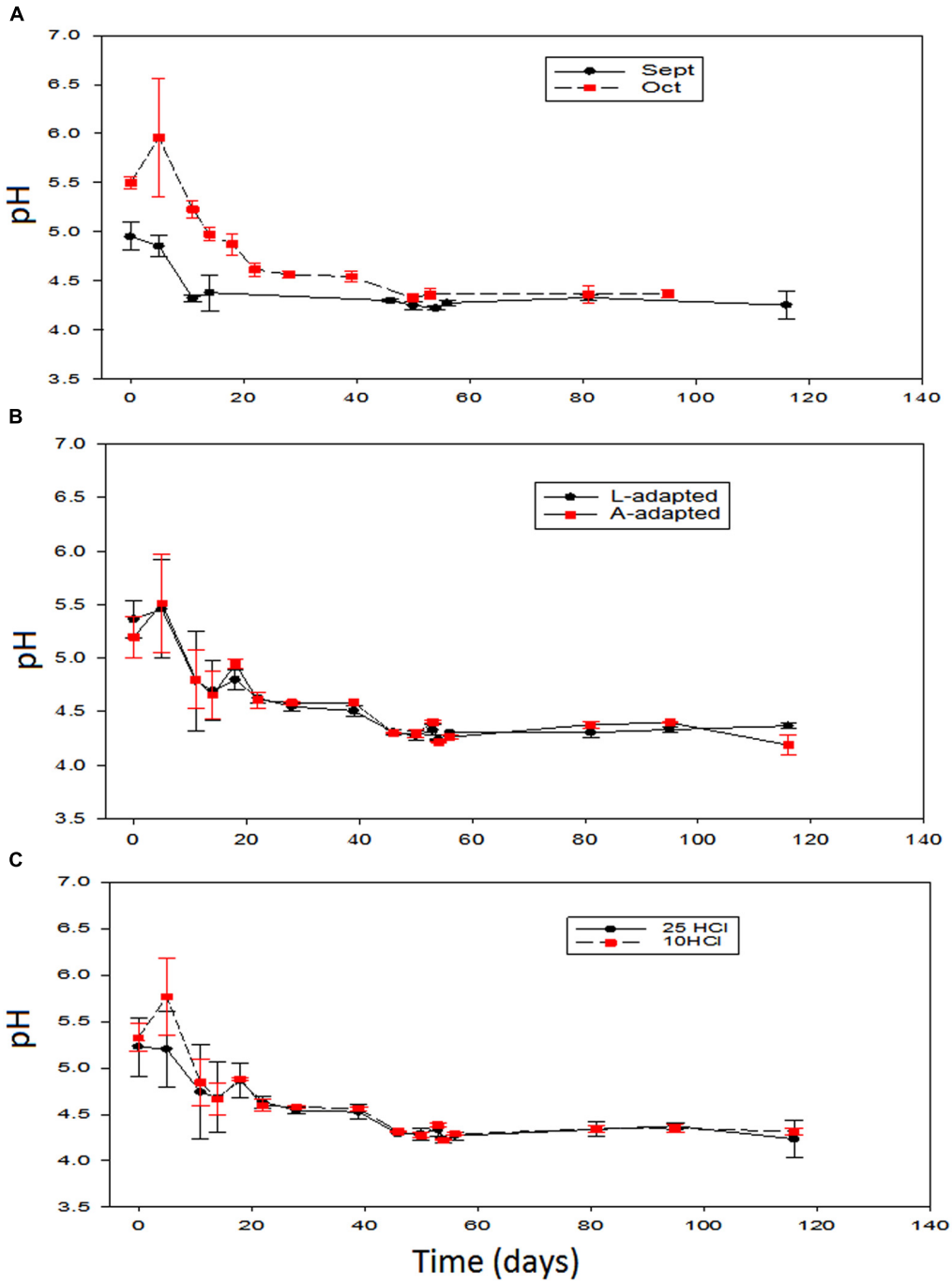

FIGURE 2 | Application of potential probiotic starter (LAB2) at industrial scale (season 2012/2013). Changes in pH (± CL, *n* = 4 for each level) according to time season **(A)**, type of inoculum **(B)**, and proportion of HCl added to fermentation vessels **(C)**. The values are always averages over the other two non-included in the graph variables. See **Figure 1** for the the meanings of symbols.

in October, on the contrary, the LAB population only suffered a slight progressive decrease after the maximum (**Figure 4A**). Apparently, there were some environmental characteristics in September inoculated experiments that strongly, and preferably, affected LAB2 and caused its disappearance after fermentation. Among them, olive polyphenols (with higher concentrations in September than in October, Medina et al., 2010) or the own LAB metabolites (lactic acid or hydrogen peroxide) produced during fermentation. However, in September, such characteristics affected less markedly the wild LAB population (control) which survived at a moderate level (4 $\log_{10}$ CFU/mL) (data not shown). Conversely, the strong inhibitory effect above-commented was

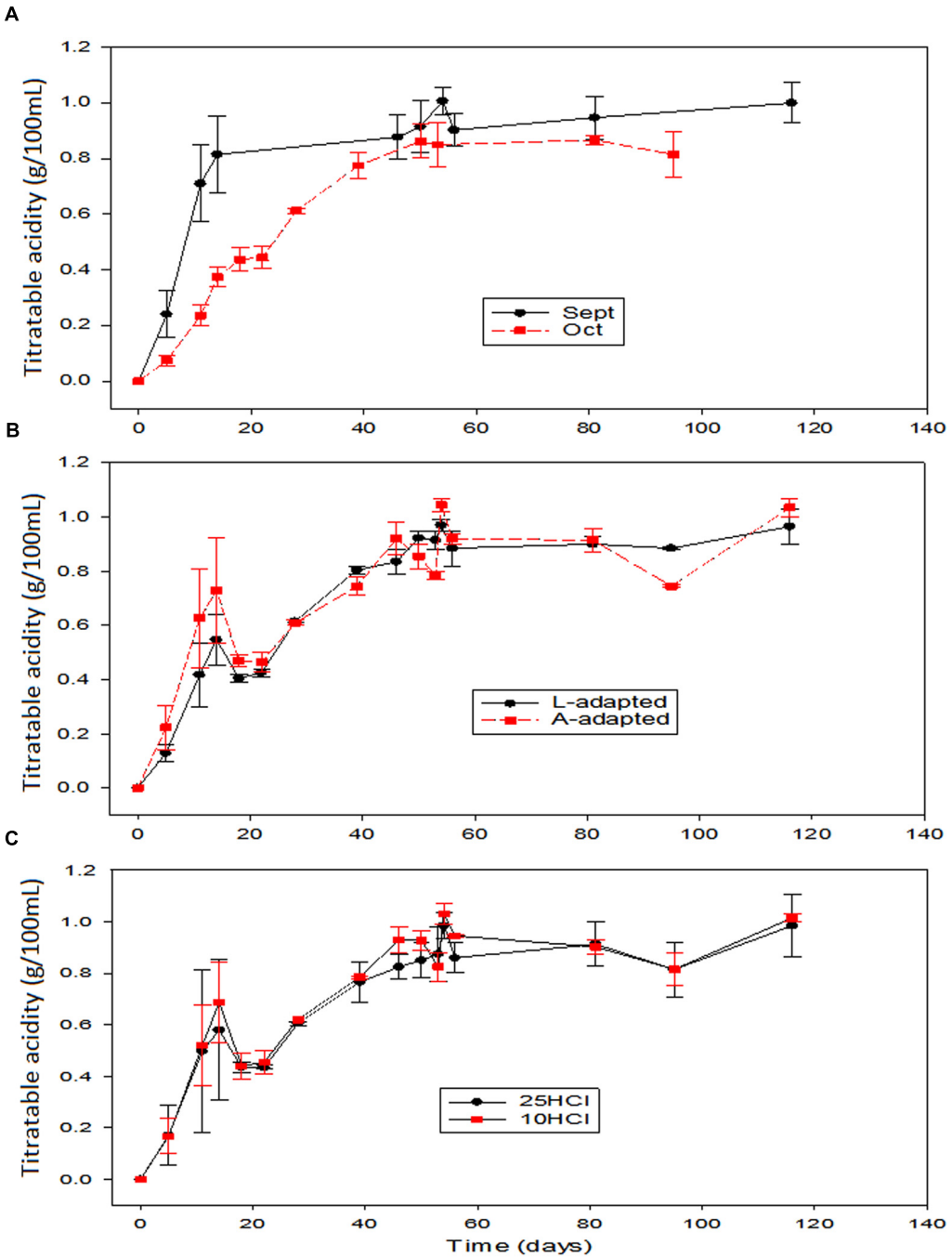

FIGURE 3 | Application of potential probiotic starter (LAB2) at industrial scale (season 2012/2013). Changes in titratable acidity (± CL, $n = 4$ for each level) according to time season **(A)**, type of inoculum **(B)**, and proportion of HCl added to fermentation vessels **(C)**. The values are always averages over the other two non-included in the graph variables. See **Figure 1** for the the meanings of symbols.

not observed in October, when the LAB population after fermentation always followed a trend similar to that found in normal processes (Panagou et al., 2003). The effects of the type of inoculation or the amount of HCl in the initial brines on the LAB populations in brine over the other two variables were never significant (**Figures 4B,C**). Argyri et al. (2014), on the contrary, did not find any sharp LAB decrease during fermentation or storage, indicating that their potential probiotic *Lactobacillus* were more resistant to inhibitors, the heat shock could have transformed them, or the strain did not produce toxic substances. Furthermore, in the case of the inoculation with *L. paracasei* IMPC2.1 Italian green olives, the LAB population also had a

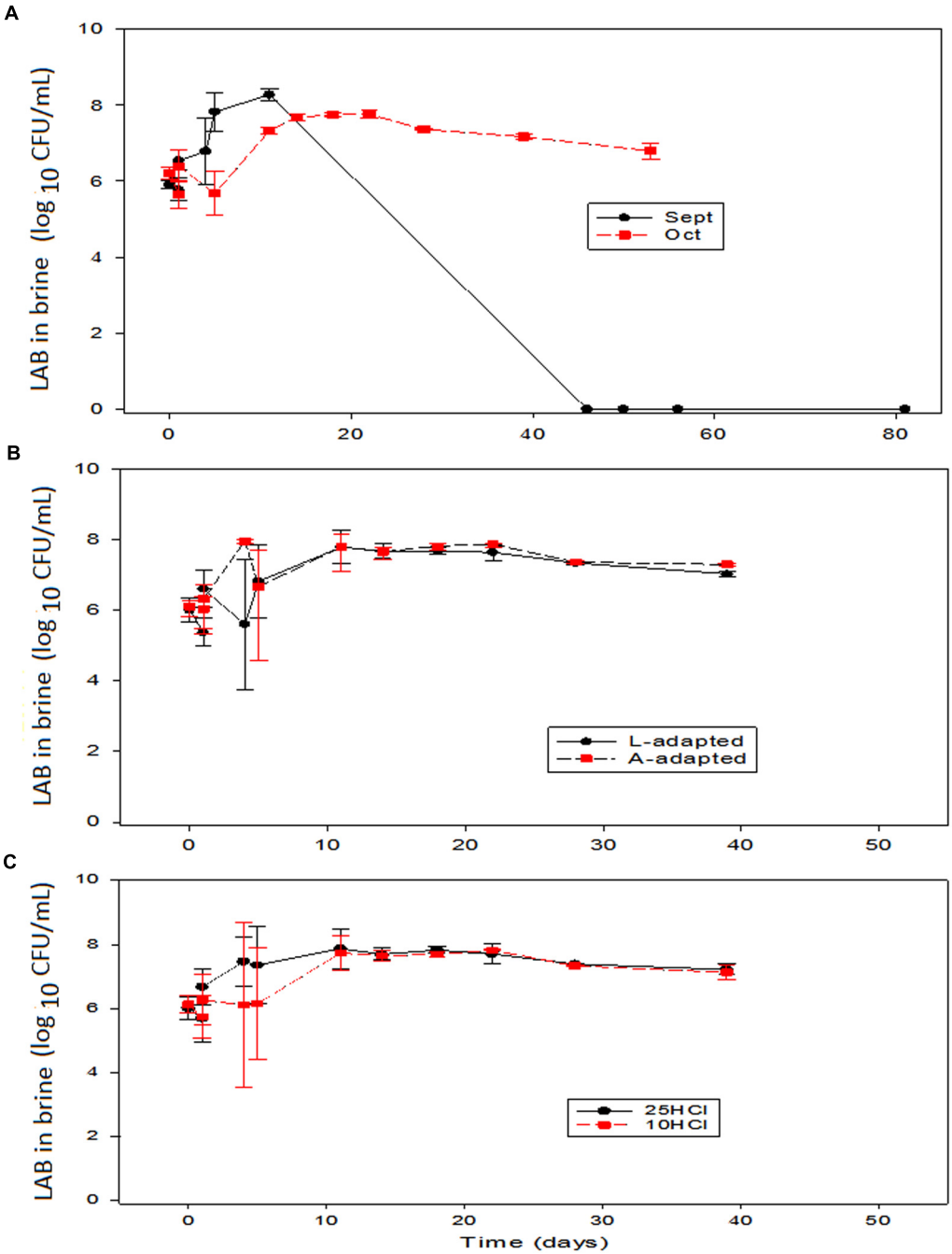

FIGURE 4 | Application of potential probiotic starter (LAB2) at industrial scale (season 2012/2013). Changes in LAB in brine (± CL, $n = 4$ for each level) according to time season **(A)**, type of inoculum **(B)**, and proportion of HCl added to fermentation vessels **(C)**. The values are always averages over the other two non-included in the graph variables. See **Figure 1** for the meanings of symbols.

noticeable important decrease (De Bellis et al., 2010). In the case of the olive inoculation with *L. paracasei* IMPC2.1, the inoculum reduced the population of wild *L. pentosus* during the fermentation process but did not prevent its predominance from the 20th day up to the end of the process (Sisto and Lavermicocca, 2012).

The presence of biofilm on the olives was detected shortly after brining and only included LAB and yeasts. Then, the composition was similar to that already found at pilot plant scale (Rodríguez-Gómez et al., 2014a). LAB population changes were rather similar to those observed in brine, and the main effect on olives was that of processing time (**Figure 5A**). Inoculation caused a more rapid

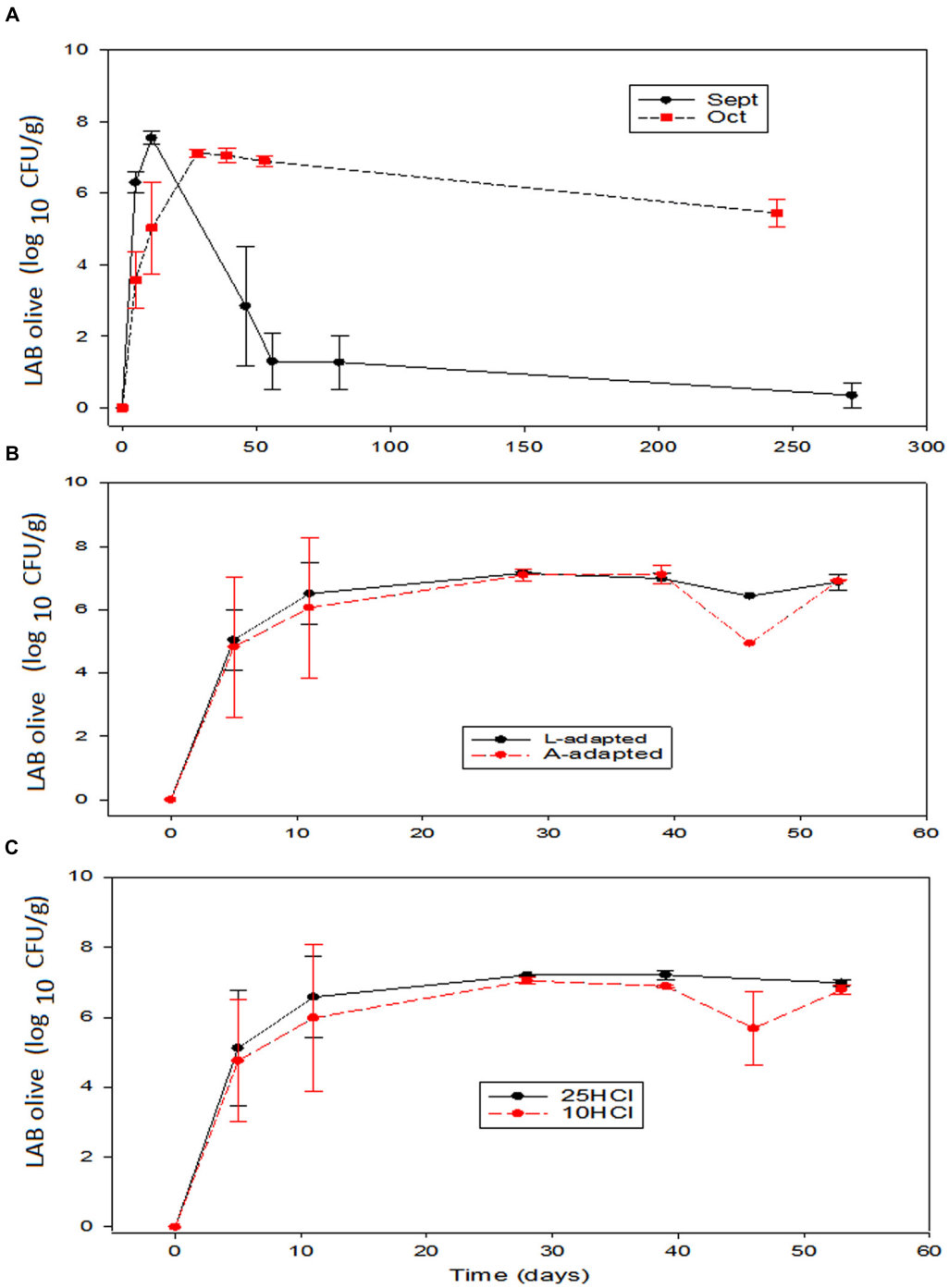

FIGURE 5 | Application of potential probiotic starter (LAB2) at industrial scale (season 2012/2013). Changes in LAB on olives (± CL, n = 4 for each level) according to time season **(A)**, type of inoculum **(B)**, and proportion of HCl added to fermentation vessels **(C)**. The values are always averages over the other two non-included in the graph variables. See **Figure 1** for the meanings of symbols.

biofilm formation and a slightly higher maximum population in September. However, after reaching the maximum, the trends followed depended on the processing time. The LAB counts on olives processed in September had a similar rapid decline than in brine and this population rapidly reduced below the detection limits ($\leq$1.2 $\log_{10}$ CFU/mL). Therefore, the same inhibitory effect on LAB observed in September processed brines was also noticed on the LAB adhered to the olive surface. The causes of such inhibitions are probably the same above-mentioned for brines (Medina et al., 2010). On the contrary, the LAB population on

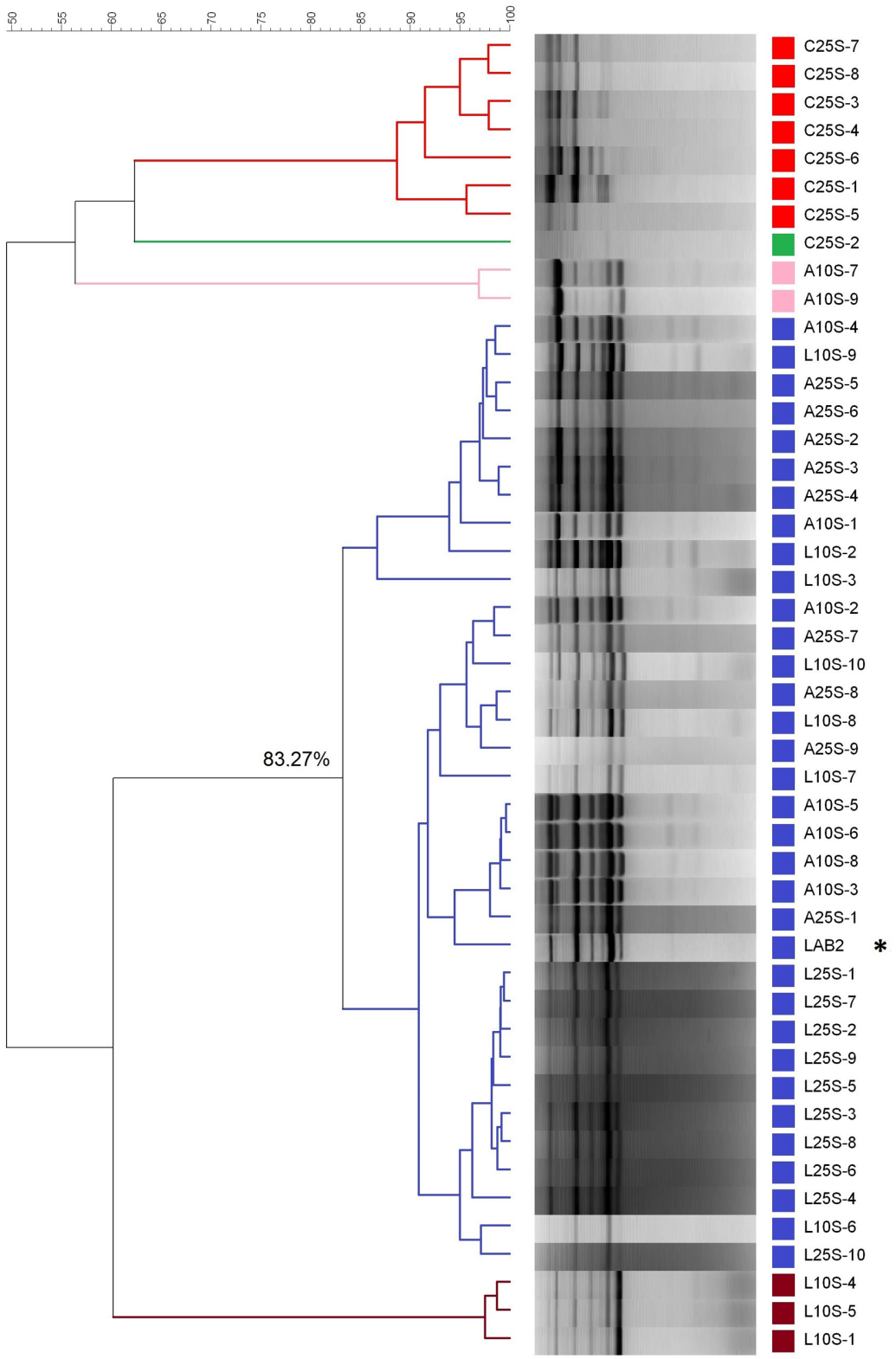

FIGURE 6 | Clustering analysis of the diverse LAB isolates obtained from the fermentation experiments carried out in September during 2012/2013 industrial fermentations. L and A stand for laboratory and industry pre-adapted inocula, respectively; 25 and 10 stand for the proportions of HCl/vessel added to the initial brine; C stands for the spontaneous fermentation. The last number refers to the order of isolation. *Stands for the profile of inoculum LAB2.

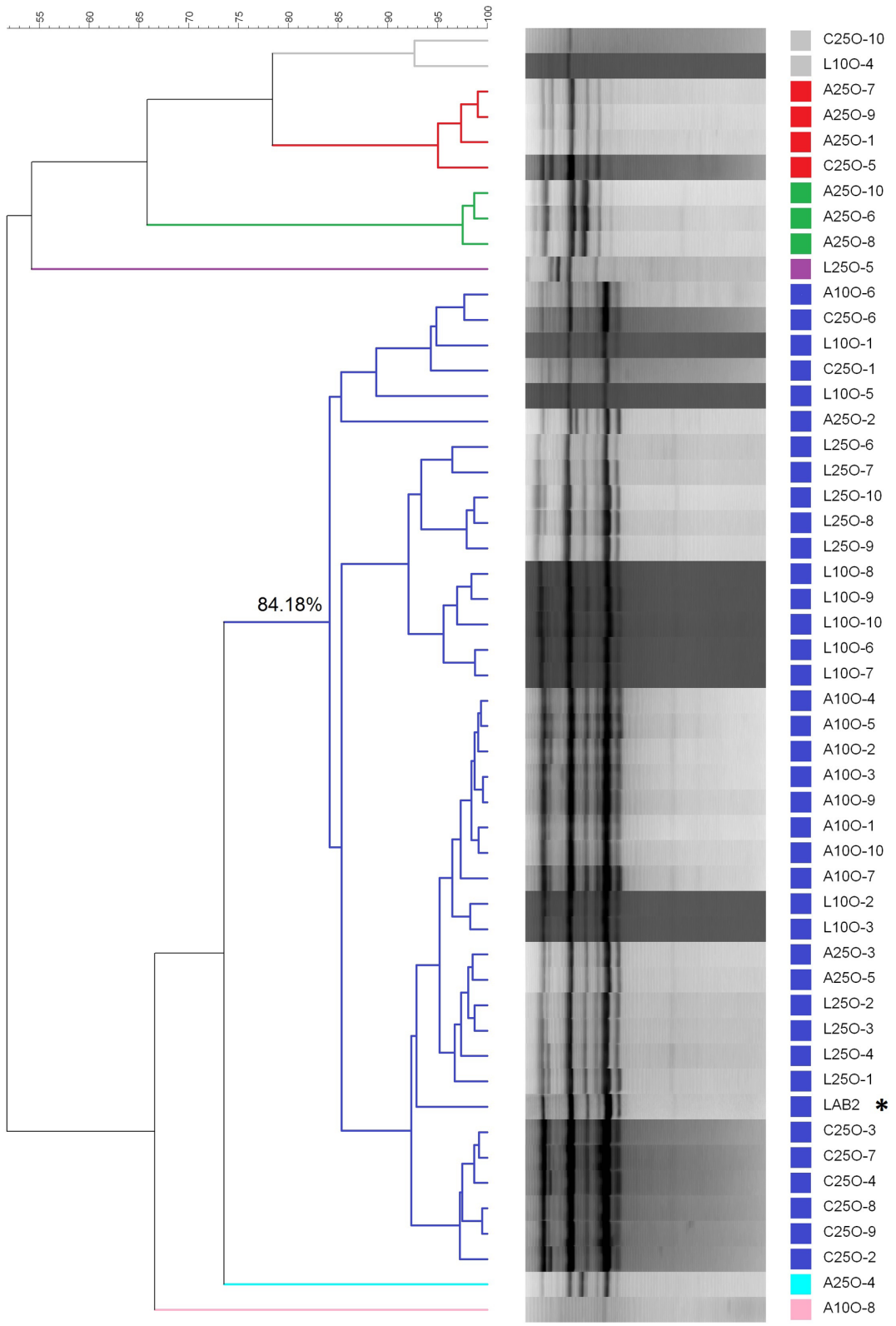

FIGURE 7 | Clustering analysis of the diverse LAB isolates obtained from the industrial fermentation experiments carried out in October during 2012/2013 season. L and A stand for laboratory and industry scale pre-adapted inocula, respectively; 25 and 10 stand for the proportions of HCl/vessel added to the initial brine; C stands for the spontaneous fermentation. The last number refers to the order of isolation. *Stands for the profile of inoculum LAB2.

the olives processed in October had only a slight decline in the storage period, and the survival was extended for above 250 days (**Figure 5A**). The survival on the fruits processed in October was then comparable to that found on the olives preserved in bulk, where a negative effect of the salt is always observed (Rodríguez-Gómez et al., 2014b). Conversely, the same organisms were not inhibited in similar experiments carried out at pilot plant scale (Rodríguez-Gómez et al., 2014a). Finally, inoculation type and the amount of HCl added caused negligible effects on LAB growth and survival (**Figures 5B,C**).

Regarding the evolution of yeast populations, it was observed an initial decreasing trend in brine, followed by an increase during the first period of fermentation to reach population levels around 4 $\log_{10}$ CFU/mL. Then, their counts did not show significant changes. For a period (between the 18th to the 40th day), the use of industry- pre-adapted starter culture, apparently, led to a lower yeast population than the laboratory-adapted one. The effect of the proportion of HCl added to the initial brine on the yeast populations was never significant. In general, yeasts counts were always comparable to those habitually found in green Spanish-style table olives during fermentation (Garrido-Fernández et al., 1997; Arroyo-López et al., 2012b). Furthermore, their levels were also comparable with those reported in other probiotic fermentation experiments in which the yeast growth was also allowed spontaneous (De Bellis et al., 2010; Argyri et al., 2014). In the case of the growth of yeasts on the olive surface, the yeast populations on the olives processed in September were generally above those found on fruits processed in October. On the contrary, no appreciable effects of inoculation and proportion of HCl added to the initial brine on yeast population on olives were observed. However, yeasts were found on the olive biofilm during the entire fermentation and storage period, in agreement with the role attributed to yeast on the initiation of the biofilm formation (León-Romero et al., 2016). Their changes followed similar trends to those found in other investigations (Argyri et al., 2014; Rodríguez-Gómez et al., 2014a). Supplementary Figures S6, S7 give more information of the evolution of this microbial group.

Predominance of the Inoculum on the Olive Surface in the Second Season (2012/2013)

A molecular genotyping was performed to all the LAB isolates obtained from the biofilms at the moment of the maximum population to determine the frequency of recovery of the inoculum in the diverse treatments assayed.

The dendrogram, based on the rep-PCR profiles using the GTG_5 primer, revealed the presence of five clusters (below 83% similarity) in the isolates from olives processed in September (**Figure 6**). This cut-point was chosen according to previous studies carried out by Rodríguez-Gómez et al. (2014c), who found a reproducibility of 86.9 ± 3.4% in assays with LAB2 inoculum. On the one side, the isolates from the control treatment (C25S), spontaneous fermentation, were clearly different from the rest of the strains, with which, in the best case, only shared a 60% similarity. The biggest cluster was composed of 33 isolates, which included the LAB2 strain making the 71.73% of the total of isolates obtained. The percentage similarity shared within them was above 83%. Therefore, all these isolates could be assimilated to the inoculum profile. The other three groups consisted of one, two and three isolates respectively and shared a low similarity with the LAB2 inoculum. With respect to the treatments processed in October, the molecular analysis was carried out on 50 isolates (10 for each treatment). The dendrogram showed the presence of seven clusters below 83% similarity, some of them with only one isolate (**Figure 7**). The most numerous consisted of 38 isolates and had a minimum 84% similarity with the LAB2 profile, making 76% of total isolates obtained. Also most of the isolates from the spontaneous control (C25O) were included in this cluster, even if apart from those isolates from the olives of the inoculated fermentation vessels, possibly due to a process of cross contamination of this fermentation vessel. The other groups included the rest of the isolates, which shared a relatively low proportion of similarly. These isolates could hardly be related to the profile of the inoculum because the maximum similarity observed with it was 75% and most of them were even below 55%. Similarities of LAB2 in the case of this work were slightly higher than those observed at pilot scale (79%) (Rodríguez-Gómez et al., 2014a).

Based on the dendrograms from the LAB on olives in treatments processed in September and in October, the predominance (%) on the particular treatments were inferred (**Table 1**). The results showed that the predominance was not dependent on the type of inoculum used (laboratory or industry-adapted) but was always higher in September than in October. Also, the use of 25 L HCl/fermentation vessels for the pH correction led to fairly good predominance in September (100% in L25S and A25S) but slightly lower in October (90.3%). The results showed that, despite the current fermentation yard drawbacks, a 100% predominance of the inoculated strains could

TABLE 1 | Predominance (expressed as percentage) of the inoculated *Lactobacillus pentosus* LAB2 strain at the moment of maximum LAB population in the diverse treatments assayed.

	Treatments applied in September (S)					Treatments applied in October (O)				
	A25S	**A10S**	**L25S**	**L10S**	**C25S**	**A25O**	**A10O**	**L25O**	**L10O**	**C25O**
Inoculum	100	78	100	70	0	30	90	90	90	80
*Other LAB	0	22	0	30	100	70	10	10	10	20

**Percentage (%) of the predominance of other spontaneous LAB profiles. A stands for pre-adapted inoculum in the industry, while L stands for inoculum adapted in the laboratory. 25 stands for the addition of 25 L of HCl to fermentation tanks, while 10 stands for the addition of 10 L. C stands for the control treatments.*

be possible, using a strong strain starter culture like *L. pentosus* LAB2 with proved ability for predominance at laboratory and pilot plant scales (Rodríguez-Gómez et al., 2014a).

Therefore, *L. pentosus,* in general, is a very well-adapted species to the green Spanish-style fermentation processes. In fact, Tofalo et al. (2014) found that *L. pentosus* was the dominant species (53–69%) in diverse Italian table olive fermentations. Blana et al. (2014) reported predominance frequencies ranging from 80 to 100% of their *L. pentosus* starter in Halkidiki fermentations. Argyri et al. (2014) also indicated in heat shocked olives high recoveries (100 or 95%, in 10 and 8% NaCl brines, respectively) for *L. pentosus* B281 but markedly lower proportions (59 or 55, in 10 and 8% NaCl brines, respectively) for *L. plantarum* B282. Furthermore, *L. plantarum* B282 failed to colonize the olive surface at 10% NaCl (Blana et al., 2014). Bella di Cerignola, inoculated with *L paracaseie* IMC2.1, showed a considerable genetic diversity (mainly at 8% NaCl) but *L. pentosus* was the most frequently isolated species. Hence, the results suggest that *L. pentosus* from autochthonous microflora might eventually have a favorable competence against strains from other species or environments.

CONCLUSION

The predominance of potential probiotic strains during large-scale fermentations is not a risk-free challenging task due to the current conditions prevailing in the industrial fermentation yards, the competitiveness of the wild environmental microflora, and to the possible integration of *Enterobacteriaceae* into the polymicrobial biofilm formed on the olive surface. A detailed study of the physicochemical and microbiological changes during fermentation showed that: (i) an immediate post-brining inoculation (to reduce the presence of initial wild microorganisms), (ii) the use of a re-inoculation, to replace the LAB that could have eventually died after the first one, and (iii) an early processing in the season led to a general improvement of the inoculum survival. On the contrary, inoculum history (laboratory vs. industry pre-adaptation) or HCL addition (25 L vs. 10 HCl L/vessel) had not or limited influences. The complete inhibition of LAB2 (and almost any LAB) during the storage phase in the inoculation experiments conducted in September was unexpected and is currently under investigation.

AUTHOR CONTRIBUTIONS

FR-G, VR-G, JR-R, RT-G, and JB-G performed the experimental work. FR-G, AG-F, PG-G, and FA-L designed the work, analyzed the results and wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.00915/full#supplementary-material

REFERENCES

Argyri, A. A., Nisiotou, A. A., Mallouchos, A., Panagou, E. Z., and Tassou, C. C. (2014). Performance of two potential probiotic *Lactobacillus* strains from the olive microbiotas starters in the fermentation of heat shocked green olives. *Int. J. Food Microbiol.* 171, 68–76. doi: 10.1016/j.ijfoodmicro.2013.11.003

Arroyo-López, F. N., Bautista-Gallego, J., Domínguez Manzano, J., Romero-Gil, V., Rodríguez Gómez, F., García García, P., et al. (2012a). Formation of lactic acid bacteria-yeasts communities on the olive surface during the Spanish-style Manzanilla fermentations. *Food Microbiol.* 32, 295–301. doi: 10.1016/j.fm.2012.07.003

Arroyo-López, F. N., Romero-Gil, V., Bautista-Gallego, J., Rodríguez Gomez, F., Jimenez-Diaz, R., García García, P., et al. (2012b). Potential benefits of the application of yeast starters in table olive processing. *Front. Microbiol.* 3:161. doi: 10.3389/fmicb.2012.00161

Arroyo-López, F. N., Blanquet Diot, S., Denis, S., Thévenot, J., Chalancon, S., Alric, M., et al. (2014). Survival of pathogenic and lactobacilli species of fermented olives during simulated human digestion. *Front. Microbiol.* 5:540. doi: 10.3389/fmicb.2014.00540

Bautista-Gallego, J., Arroyo López, J., Rantsiou, K., Jiménez Díaz, R., Garrido Fernández, A., and Cocolin, L. (2013). Screening of lactic acid bacteria isolated from fermented table olives with probiotic potential. *Food Res. Int.* 50, 135–142. doi: 10.1016/j.foodres.2012.10.004

Bevilacqua, A., De Stefano, F., Augello, S., Pignatiello, S., Sinigaglia, M., and Corbo, M. R. (2015). Biotechnological innovations for table olives. *Int. J. Food Sci. Nutr.* 186, 271–276. doi: 10.3109/09637486.2014.959901

Blana, V. A., Grounta, A., Tassou, C.-C., Nychas, G.-J. E., and Panagou, E. P. (2014). Inoculated fermentation of green olives with potential probiotic *Lactobacillus pentosus* and *Lactobacillus plantarum* starter cultures isolated from industrially fermented olives. *Food Microbiol.* 38, 208–218. doi: 10.1016/j.fm.2013.09.007

Böckelmann, U., Szewzyk, U., and Grohmann, E. (2003). A new enzymatic method for the detachment of particle associated soil bacteria. *J. Microbiol. Methods* 55, 201–211. doi: 10.1016/S0167-7012(03)00144-1

Borbolla y Alcalá, J. M. R., de, Gómez Herrrera, C., and Izquierdo Tamayo, A. (1952). El uso de cultivos puros de lactobacilos en el aderezo de aceitunas verdes. *Grasas Aceites* 3, 91–94.

De Bellis, P., Valerio, F., Sisto, A., Lonigro, S. L., and Lavermicoca, P. (2010). Probiotic table olives: microbial populations adhering on olive surface in fermentations sets inoculated with the probiotic strain *Lactobacillus paracasei* IMPC2.1 in an industrial plant. *Int. J. Food Microbiol.* 140, 6–13. doi: 10.1016/j.ijfoodmicro.2010.02.024

Domínguez-Manzano, J., León-Romero, A., Olmo-Ruiz, C., Bautista-Gallego, J., Arroyo-López, F. N., Garrido-Fernández, A., et al. (2012). Biofilm formation on

abiotic and biotic surfaces during Spanish-style green table olive fermentation. *Int. J. Food Microbiol.* 157, 230–238. doi: 10.1016/j.ijfoodmicro.2012.05.011

Garrido-Fernández, A., Fernández-Díez, M. J., and Adams, R. M. (1997). *Table Olives. Production and Processing*. London: Chapman and Hall. doi: 10.1007/978-1-4899-4683-6

Grounta, A., Doulgeraki, A. I., Nychas, G. E. N., and Panagou, E. Z. (2016). Biofilm formation on Conservolea natural black olives during single and combined inoculation with functional *Lactobacillus pentosus* starter culture. *Food Microbiol.* 56, 35–44. doi: 10.1016/j.fm.2015.12.002

Kubota, H., Senda, S., Nomura, N., Tokuda, H., and Uchiyama, H. (2008). Biofilm formation by lactic acid bacteria and resistance to environmental stress. *J. Biosci. Bioeng.* 106, 381–386. doi: 10.1263/jbb.106.381

Lavermicocca, P., Valerio, F., Lonigro, S. L., De Angelis, M., Morelli, L., Callegari, M. L., et al. (2005). Study of the adhesion and survival of lactobacilli and bifidobacteria on table olives with the aim of formulating a new probiotic food. *Appl. Environ. Microbiol.* 71, 4233–4240. doi: 10.1128/AEM.71.8.4233-4240.2005

León-Romero, A., Domínguez-Manzano, J., Garrido-Fernández, A., Arroyo-López, F. N., and Jiménez-Díaz, R. (2016). Formation of in vitro mixed-species biofilms by *Lactobacillus pentosus* and yeasts isolated from Spanish-style green table olive fermentations. *Appl. Environ. Microbiol.* 82, 689–695. doi: 10.1128/AEM.02727-15

Lucena-Padrós, H., Caballero-Guerrero, B., Maldonado-Barragán, A., and Ruiz Barba, J. L. (2014). Microbial diversity and dynamics of Spanish-style green table olive fermentations in large manufacturing companies through culture-dependent techniques. *Food Microbiol.* 42, 154–165. doi: 10.1016/j.fm.2014.03.020

Martorana, A., Alfonso, A., Settanni, L., Corona, O., La Croce, F., Caruso, T., et al. (2015). Effect of mechanical harvest of drupes on the quality characteristics of green fermented table olives. *J. Sci. Food Agric.* 96, 2004–2017. doi: 10.1002/jsfa.7311

Medina, E., Gori, C., Servili, M., de Castro, A., Romero, C., and Brenes, M. (2010). Main variables affecting the lactic acid fermentation of table olives. *Int. J. Food Sci. Technol.* 45, 1291–1298. doi: 10.1111/j.1365-2621.2010.02274.x

Panagou, E. Z., Tassou, C. C., and Katsaboxakis, C. Z. (2003). Induced lactic acid fermentation of untreated green olives of the Conservolea cultivar by *Lactobacillus pentosus*. *J. Sci. Food Agric.* 83, 667–674. doi: 10.1002/jsfa.1336

Randazzo, C. I., Todaro, A., Pino, A., Pitino, I., Corona, O., Mazzaglia, A., et al. (2014). Giarrafa and Grossa di Spagna naturally fermented table olives: effect of starter and probiotic cultures on chemical, microbiological and sensory traits. *Food Res. Int.* 62, 1154–1164. doi: 10.1016/j.foodres.2014.05.056

Rodríguez-Gómez, F., Bautista-Gallego, J., Arroyo-López, F. N., Romero-Gil, V., Jiménez-Díaz, R., García-García, P., et al. (2013). Table olive fermentation with multifunctional *Lactobacillus pentosus* strains. *Food Control* 34, 96–105. doi: 10.1016/j.fm.2014.03.023

Rodríguez-Gómez, F., López-López, A., Romero-Gil, V., Arroyo-López, F. N., Moreno-Baquero, J. M., Garrido-Fernández, A., et al. (2014a). Production of potential probiotic green Spanish-style table olives at pilot plant scale using multifunctional starters. *Food Microbiol.* 44, 278–287. doi: 10.1016/j.fm.2014.03.023

Rodríguez-Gómez, F., López-López, A., Romero-Gil, V., Arroyo-López, F. N., Moreno-Baquero, J. M., Garrido-Fernández, A., et al. (2014b). Effect of post-fermentation storage on Spanish-style green Manzanilla olives. *LWT Food Sci. Technol.* 57, 789–793. doi: 10.1016/j.lwt.2014.01.041

Rodríguez-Gómez, F., Romero-Gil, V., García-García, P., Garrido-Fernández, A., and Arroyo-López, F. N. (2014c). Fortification of table olive packing with the potential probiotic bacteria *Lactobacillus pentosus* TOMC-LAB2. *Front. Microbiol.* 5:467. doi: 10.3389/fmicb.2014.00467

Romero-Gil, V., Bautista-Gallego, J., Rodríguez-Gómez, F., García-García, P., Jiménez-Días, R., Garrido-Fernández, A., et al. (2013). Evaluating the individual effects of temperature and salt on table olive related microorganisms. *Food Microbiol.* 33, 178–184. doi: 10.1016/j.fm.2012.09.015

Ruiz-Barba, J. L., and Jiménez-Díaz, R. (2012). A novel *Lactobacillus pentosus*-paired starter culture for Spanish-style green olive fermentation. *Food Microbiol.* 30, 253–259. doi: 10.1016/j.fm.2011.11.004

Sánchez, A. H., Rejano, L., Montaño, A., and de Castro, A. (2001). Utilization at high pH of starter cultures of lactobacilli for Spanish-style Green olive fermentation. *Int. J. Food Microbiol.* 67, 115–122. doi: 10.1016/S0168-1605(01)00434-2

Sisto, A., and Lavermicocca, P. (2012). Suitability of a probiotic *Lactobacillus paracasei* strain as a starter culture in olive fermentation and development of the innovative patented product "probiotic table olives". *Front. Microbiol.* 3:174. doi: 10.3389/fmicb.2012.00174

Tataridou, M., and Kotzekidou, P. (2015). Fermentation of table olives by oleuropeinolytic starter culture in salt brines and inactivation of *Escherichia coli* 0157:H7 and *Listeria monocytogenes*. *Int. J. Food Microbiol.* 208, 122–130. doi: 10.1016/j.ijfoodmicro.2015.06.001

Tofalo, R., Perpetuini, R., Schirone, M., Ciarrocchi, A., Fasoli, G., Suzzi, G., et al. (2014). *Lactobacillus pentosus* dominates spontaneous fermentation of Italian table olives. *LWT Food Sci. Technol.* 57, 710–717. doi: 10.1016/j.lwt.2014.01.035

Versalovic, J., Schneider, M., Bruijn, F. J., and Lupski, J. R. (1994). Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. *Methods Mol. Cell. Biol.* 5, 25–40.

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Insight into Potential Probiotic Markers Predicted in *Lactobacillus pentosus* MP-10 Genome Sequence

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Lactobacillus pentosus MP-10 is a potential probiotic lactic acid bacterium originally isolated from naturally fermented Aloreña green table olives. The entire genome sequence was annotated to *in silico* analyze the molecular mechanisms involved in the adaptation of *L. pentosus* MP-10 to the human gastrointestinal tract (GIT), such as carbohydrate metabolism (related with prebiotic utilization) and the proteins involved in bacteria–host interactions. We predicted an arsenal of genes coding for carbohydrate-modifying enzymes to modify oligo- and polysaccharides, such as glycoside hydrolases, glycoside transferases, and isomerases, and other enzymes involved in complex carbohydrate metabolism especially starch, raffinose, and levan. These enzymes represent key indicators of the bacteria's adaptation to the GIT environment, since they involve the metabolism and assimilation of complex carbohydrates not digested by human enzymes. We also detected key probiotic ligands (surface proteins, excreted or secreted proteins) involved in the adhesion to host cells such as adhesion to mucus, epithelial cells or extracellular matrix, and plasma components; also, moonlighting proteins or multifunctional proteins were found that could be involved in adhesion to epithelial cells and/or extracellular matrix proteins and also affect host immunomodulation. *In silico* analysis of the genome sequence of *L. pentosus* MP-10 is an important initial step to screen for genes encoding for proteins that may provide probiotic features, and thus provides one new routes for screening and studying this potentially probiotic bacterium.

Keywords: Aloreña table olives, *Lactobacillus pentosus*, probiotics, *in silico* analysis, carbohydrate metabolism, host interaction

INTRODUCTION

The *Lactobacillus* genus belongs to the LAB group, which currently comprises of 222 species described in List of Prokaryotic Names with Standing in Nomenclature "LPSN"[1] (February 2017). In this context, *Lactobacillus* represents a highly heterogeneous taxonomic group encompassing species with various physiological, biochemical and genetic characteristics that reflect their capacity to colonize many ecological niches and to respond to several environmental stresses (De Angelis and Gobbetti, 2004; Pot et al., 2014). Lactobacilli have been isolated from different sources [e.g., plants, foods, and the mucosal surfaces (i.e., from oral, gastrointestinal, and reproductive tracts) of mammalian hosts], and they have widely been used as starter cultures in food fermentations, due to their safe-history of use, and also as protective cultures because of their production of antimicrobial substances (e.g., bacteriocins, peroxide, diacetyl, among others) (Leroy and de Vuyst, 1999; Heller, 2001; Hansen, 2002; Holzapfel, 2002; Giraffa et al., 2010; Franz et al., 2011; Garrigues et al., 2013). Thus, the Food and Drug Administration and European Food Safety Authority certify some *Lactobacillus* species as Generally Recognized As Safe (GRAS) or having a Qualified Presumption of Safety (QPS), respectively (Bernardeau et al., 2008). Furthermore, many *Lactobacillus* species represent main components of the global probiotic market: *L. acidophilus*, *L. bulgaricus*, *L. plantarum*, *L. brevis*, *L. reuteri*, *L. johnsonii*, *L. casei*, *L. rhamnosus*, and *L. salivarius*. Specifically, some *L. pentosus* strains have exerted probiotic effects such as the acceleration of IgA secretion in saliva and the enhancement of IgA production in the small intestine (Kotani et al., 2010; Izumo et al., 2011), which have aroused great interest due to vegetal origin (Pérez Montoro et al., 2016). Generic mechanisms for underlying probiotic effects can be linked to taxonomic groups (genus or species); however, specific mechanisms tend to be strain-specific (Hill et al., 2014). As such, whole genome sequencing (WGS) remains the best way to better understand the genetic and metabolic potential of each species/strain, to demonstrate the plasticity of their phylogenetic relationships, metabolic pathways, adaptation, fitness and safety (Jolley and Maiden, 2010; Maiden et al., 2013).

Lactobacillus pentosus MP-10 is a potential probiotic LAB isolated from naturally fermented Aloreña green table olives (Abriouel et al., 2011) and has exhibited several probiotic capacities when tested *in vitro* such as good growth and survival capacities under simulated gastro-intestinal conditions, ability to auto-aggregate, and co-aggregate with pathogenic bacteria, adherence to intestinal and vaginal cell lines, antagonistic activity against pathogens and fermentation of several prebiotics and lactose (Pérez Montoro et al., 2016). However, the putative health-promoting capacities of this strain may depend on genetic characteristics and the interactions within its ecological niche (O'Sullivan et al., 2009); for this reason, the whole-genome sequence obtained by Abriouel et al. (2016) and the subsequent annotation will improve our knowledge about the functionality of this strain, its adaptation to the human gastrointestinal tract (GIT) and its interaction within the host. As such, we carried out *in silico* analysis of *L. pentosus* MP-10's carbohydrate metabolism and the factors that affect their interaction with the host with the aim to identify genes as potential probiotic markers.

[1]http://www.bacterio.net

RESULTS AND DISCUSSION

General Metabolic Features of a Probiotic *Lactobacillus pentosus* MP-10

Figure 1 shows the frequency of KEGG functional annotations obtained by BlastKOALA (KEGG tool; last updated March 4, 2016), which assigned approximately half (45.7%) of the genes to KEGG annotations corresponding to environmental information processing (443 genes), genetic information processing (413 genes), carbohydrate metabolism (279), amino acid metabolism (173), cellular processes (164 genes), nucleotide metabolism (90 genes), energy metabolism (87 genes), metabolism of cofactors and vitamins (87 genes), human disease factors (70 genes), lipid metabolism (62 genes), among others.

To highlight the molecular mechanisms involved in the adaptation of *L. pentosus* MP-10 to the human GIT, we focused the *in silico* analysis on carbohydrate metabolism related to prebiotic utilization and the proteins involved in host interactions, since the adaptation of probiotics is mainly represented by the enrichment of mucus-binding proteins and enzymes involved in breakdown of complex carbohydrates (Ventura et al., 2012).

In silico analysis has some limitations related with the prediction accuracy which in turn depends on the algorithm used and the phenotype data from experiments (Ng and Henikoff, 2006); however, to avoid incorrect predictions all the annotations made in the present study were curated manually.

Carbohydrate Metabolism Related with Prebiotic Utilization

Over 8% of the identified genes in *L. pentosus* MP-10 genome are involved in carbohydrate metabolism (279 of 3558 genes), which is similar to the most-studied bifidobacterial genomes and 30% higher than other gastrointestinal (GIT)-resident bacteria (Ventura et al., 2009). The abundance of carbohydrate metabolism genes in *L. pentosus* MP-10 is important with respect to its possible adaptation to the microhabitats of gastrointestinal environment and its interaction with human host, and thus may enhance its survival, competitiveness and persistence.

Lactobacillus pentosus MP-10 is a facultatively hetero-fermentative LAB, and its genome possesses genes for both the phosphoketolase and Embden-Meyerhof pathways (EMP). Thus, it can potentially ferment carbohydrates mainly via the EMP, utilizing glucose, and converting it to pyruvate and then to lactate (glycolysis). However, in the absence of six-carbon sugars (e.g., glucose, et al.), *L. pentosus* MP-10 would possibly ferment five-carbon carbohydrates such as xylose, xylulose, arabinose, or ribose via the phosphoketolase pathway (PK), as reported for other *L. pentosus* strains (Bustos et al., 2005). Analysis by BlastKOALA indicated that

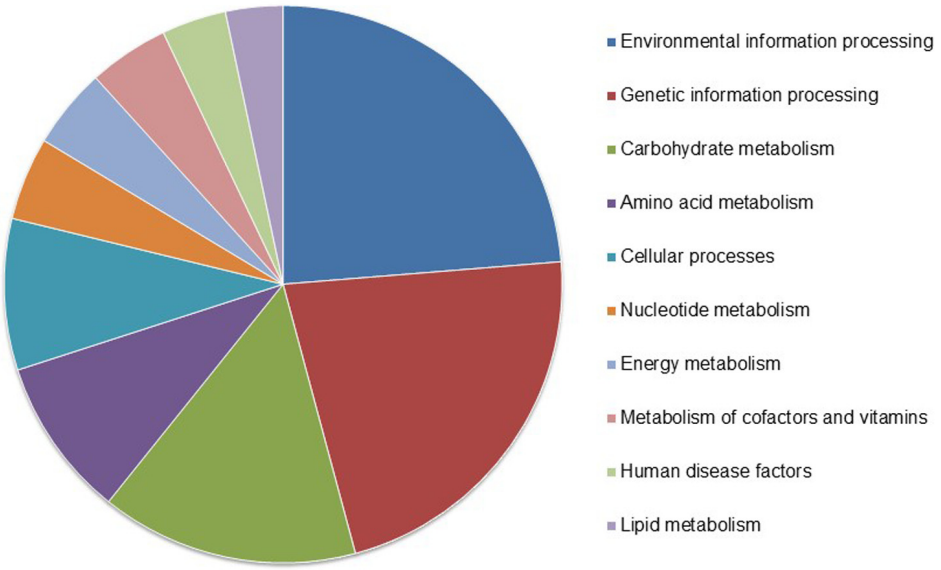

FIGURE 1 | BlastKOALA results of functional categories predicted in *Lactobacillus pentosus* MP-10 genome and their frequencies.

EMP (complete pathway), pentose phosphate pathway (PP) (both oxidative and non-oxidative complete pathways), and galactose degradation pathway (complete Leloir pathway) form the central core of carbohydrate metabolism in *L. pentosus* MP-10; however, the Entner-Doudoroff pathway (ED) appears incomplete.

Lactobacillus pentosus MP-10 has been shown to be able to ferment *in vitro* a variety of carbohydrates such as glucose, galactose, fructose, lactose, saccharose, and lactulose (Pérez Montoro et al., 2016). *In silico* analysis of the annotated genome sequence of *L. pentosus* MP-10 also predicted its capacity to ferment several simple carbohydrates of both five-carbon and six-carbon sugars such as mannose, inositol, ribose, arabinose, rhamnose, maltose, xylose, xylulose, and trehalose; furthermore, we also predicted its ability to use complex carbohydrates such as cellulose, xylan (hemicellulose), starch, raffinose, chitin, and levan (**Figure 2**). These carbohydrates can either be dietary compounds or carbon sources derived from the metabolism of the gastrointestinal microbiota (Korakli et al., 2002). Ultimately, 15 carbohydrate utilization pathways were predicted in *L. pentosus* MP-10 genome sequence: glycolysis/gluconeogenesis, citrate cycle, PP pathway, pentose, and glucuronate interconversions, fructose and mannose metabolism, galactose metabolism, ascorbate, and aldarate metabolism, starch and sucrose metabolism, amino sugar and nucleotide sugar metabolism, pyruvate metabolism, glyoxylate and dicarboxylate metabolism, propanoate metabolism, butanoate metabolism, C5-branched dibasic acid metabolism and inositol phosphate metabolism. As such, the wide repertoire of enzymes involved in the fermentation of various carbohydrate substrates is reflected in its relatively large genome size, which is also corroborated by the significantly abundant number of genes for the phosphoenolpyruvate- (PEP) dependent sugar phosphotransferase system (PTS) (77 genes) and the presence of specific genes or gene clusters involved in carbohydrate utilization by *L. pentosus* MP-10.

The possible adaptation and enrichment of *L. pentosus* MP-10 in GIT could be predicted by the presence of genes encoding various carbohydrate-modifying enzymes able to modify oligo- and polysaccharides. These enzymes are produced by intestinal microbial communities and are required for the metabolism of plant- and host-derived carbohydrates (e.g., cellulose, xylan, and pectin), since mammals have limited evolved abilities to hydrolyze complex polysaccharides for digestion (Cantarel et al., 2012). Among these enzymes, many were predicted in *L. pentosus* MP-10 genome and belong to several CAZY "Carbohydrate-Active Enzymes" families (**Table 1**): glycoside hydrolases or glycosylases (15 genes); hexosyl- (15 genes), pentosyl- (13 genes) and phospho-transferases (13 genes); and also isomerases (24 genes).

Furthermore, the presence of sugar ABC transporters, carbohydrate esterases, glycosyl transferases, polysaccharide lyases, permeases, and PEP-PTS (PEP; PTS) components required for the uptake and metabolism of plant and host-derived carbohydrates were predicted in the *L. pentosus* MP-10 genome, as similarly reported for the probiotic *Bifidobacterium* (Kim et al., 2009). This arsenal of genes coding for carbohydrate-modifying enzymes predicted in *L. pentosus* MP-10 genome could represent a key indicator of this bacterium's adaptation to the GIT environment, as these genes are involved in the metabolism and transport of carbohydrates non-digestible by human enzymes. Glycosyl (hexosyl-, pentosyl-, and phospho-) transferases are involved in the biosynthesis of disaccharides, oligosaccharides and polysaccharides by transferring sugar moieties from an activated donor to a specific substrate (Lairson et al., 2008); the resulting

glycoconjugates (as part of the glycome) play an important role in the establishment of environment- and host-specific interactions (Kay et al., 2010). Glycoside hydrolases are able to hydrolyze the glycosidic bond between two or more carbohydrates, and also between carbohydrate and non-carbohydrate moieties. The most common predicted genes found in *L. pentosus* MP-10 were coding for oligo-1,6-glucosidase, beta-galactosidase, alpha-L-rhamnosidase, and 6-phospho-beta-glucosidase among others (with several GH families), playing a key role not only in carbohydrate hydrolysis but also their action as retaining enzymes involved in the synthesis of oligosaccharides that may be selectively used as prebiotics by *L. pentosus* MP-10 and other gastrointestinal probiotic bacteria (**Table 1**).

Regarding isomerases, we observed several carbohydrate isomerases involved in the glycolytic pathway; however, the presence of different copies of phosphoglycerate mutase may indicate that gene-products may accomplish other functions as a moonlighting protein (Candela et al., 2007).

Complex Carbohydrate Metabolism

Lactobacillus pentosus MP-10 has the capacity to metabolize complex carbohydrates (e.g., starch, cellulose, galactan, xylan, pullulan, pectins, and gums). For example, glycogen metabolism plays an important role in survival and fitness of LAB in their ecological niche by contributing to cellular processes such as carbohydrate metabolism, energy production, stress response, and cell–cell communication (Eydallin et al., 2007, 2010). The glycogen metabolism operon (*glg*) predicted in *L. pentosus* MP-10 is encoded by a 9608-base chromosomal region and consists of *glgBCDAP-apu* genes (XX999_00114 to XX999_00119), which are co-transcribed as polycistronic mRNA (**Table 2**). The organization of the core genes (*glgBCDAP*) is identical to many bacteria, with the exception of two additional glycogen synthase genes exclusive to *L. pentosus* MP-10 (XX999_01233 and XX999_02081) which are homologous with *Bacillus subtilis* 168 and *Mycobacterium tuberculosis* CDC 1551, respectively (**Table 2**). Furthermore, genes *amyB* and *pgcA* coding for alpha-amylase 2 and phosphoglucomutase, respectively, are distantly located from the *glg* operon (**Table 2** and **Figure 2B**). According to Goh and Klaenhammer (2014), the glycogen gene cluster organization might differ depending on the bacterial species and origin; in this study, the glycogen gene cluster is composed of *glgBCDAP-apu-amyB-pgcA* genes and the other two glycogen synthase genes (XX999_01233 and XX999_02081). Glycogen metabolism is predicted as an additional trait in *L. pentosus* MP-10, as it will contribute to probiotic activities and the retention of this bacterium in highly competitive and dynamic niches, such as the gastrointestinal

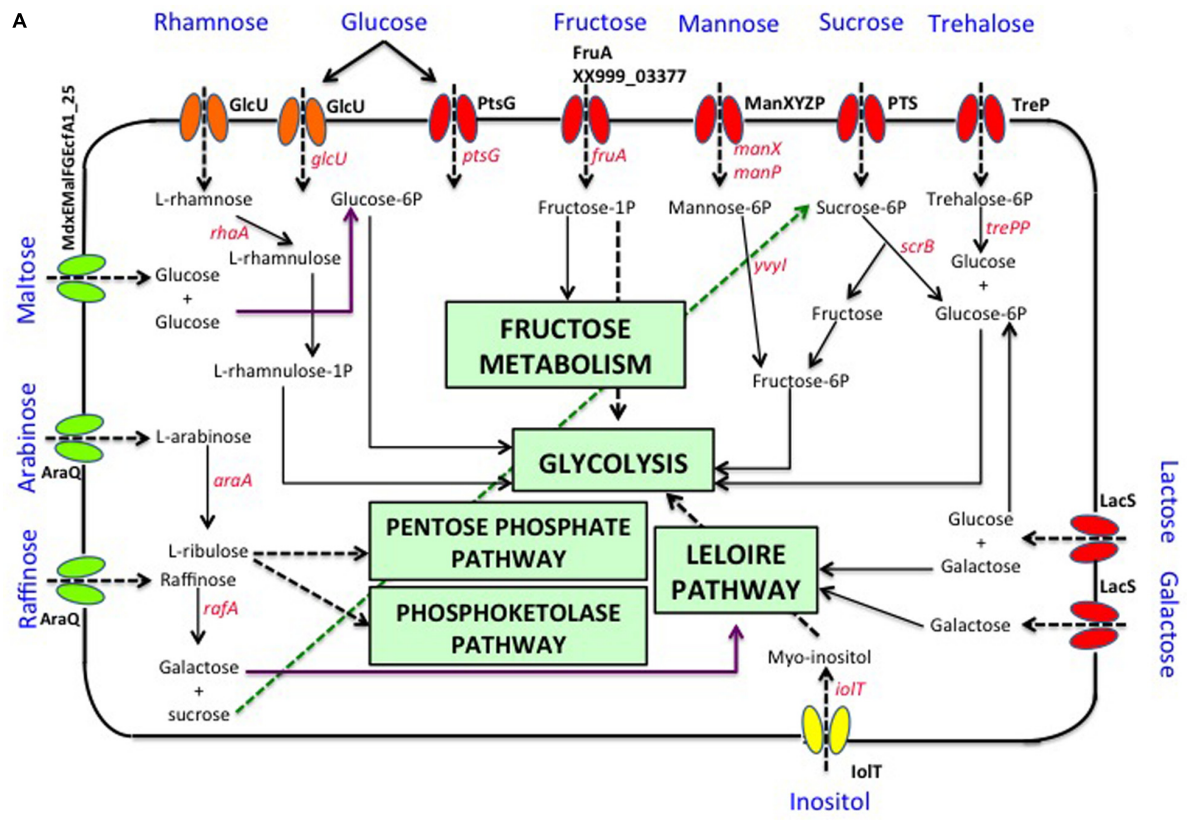

FIGURE 2 | Continued

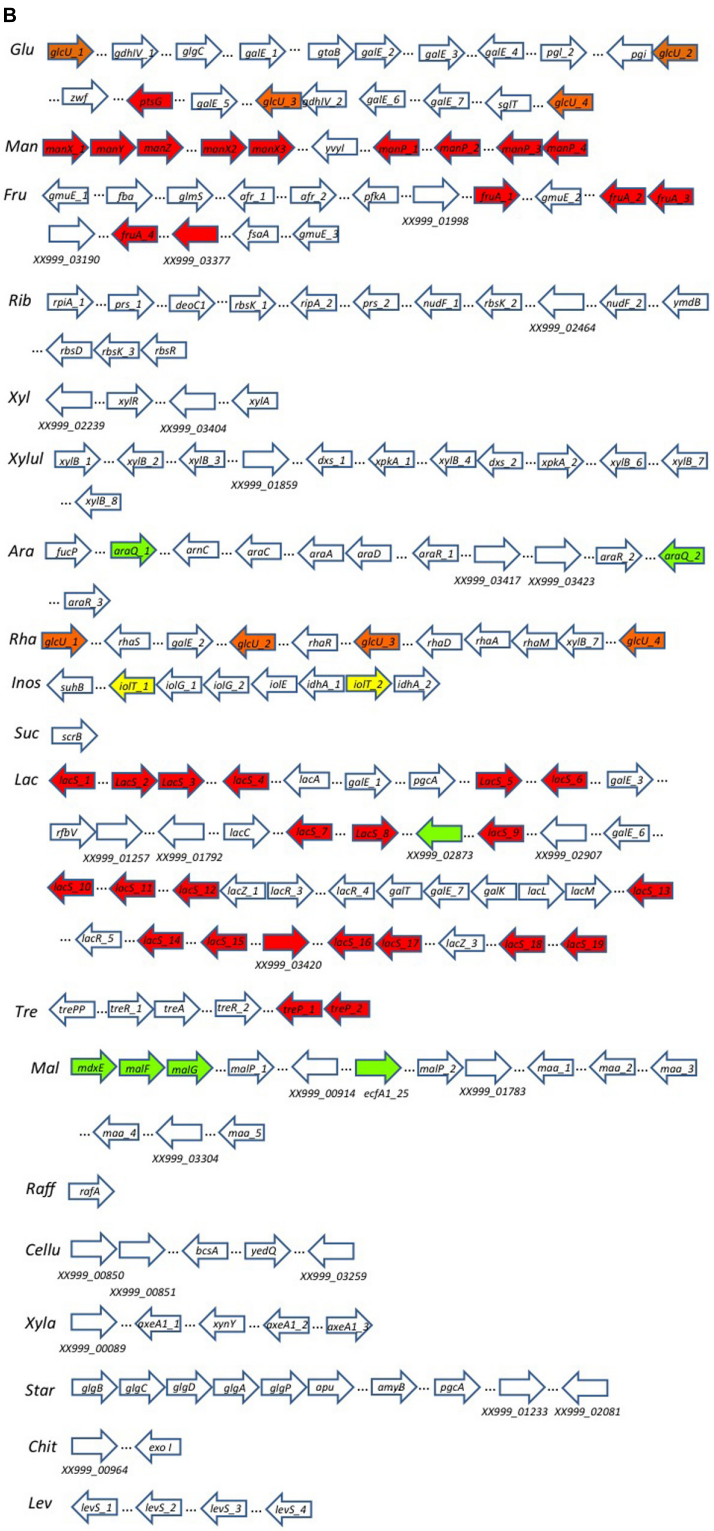

FIGURE 2 | Organization of gene clusters encoding proteins predicted to be involved in carbohydrate utilization as prebiotics by *L. pentosus* MP-10. **(A)** Pathway reconstruction as predicted by genome annotation: PTS (phosphotransferase system), red; MFS (Major Facilitator Superfamily), yellow; ABC Transporter, green; GRP (Glucose/Ribose Porter Family), orange. **(B)** Genetic loci of interest: *Ara*, arabinose; *Cellu*, cellulose; *Chit*, chitin; *Fru*, fructose; *Glu*, glucose; *Inos*, inositol; *Lac*, lactose–galactose loci; *Lev*, levan; *Mal*, maltose; *Man*, mannose; *Raff*, raffinose; *Rha*, rhamnose; *Rib*, ribose; *Star*, starch; *Suc*, sucrose; *Tre*, trehalose; *Xyl*, xylose; *Xyla*, xylan; *Xylul*, xylulose.

TABLE 1 | Putative carbohydrate-modifying enzymes identified in the genome sequence of *Lactobacillus pentosus* MP-10.

	Enzyme	Gene	Gene ID	EC number	CAZy Family*
Hexosyltransferases	Glycogen phosphorylase	*glgP*	XX999_00118	EC:2.4.1.1	GT35
	Maltose phosphorylase	*mapA*	XX999_00299	EC:2.4.1.8	GH65
	Cellulose synthase (UDP-forming)	*bcsA*	XX999_01782	EC:2.4.1.12	GT6
	1,4-alpha-glucan branching enzyme**	*glgB*	XX999_01507	EC:2.4.1.18	GH13, GH57
	Starch synthase**	*glgA*	XX999_00114	EC:2.4.1.21	GT5
	Poly(glycerol-phosphate) alpha-glucosyltransferase	*tagE*	XX999_00117	EC:2.4.1.52	GT4
	Alpha,alpha-trehalose phosphorylase	E2.4.1.64	XX999_01349	EC:2.4.1.64	GH65
	Peptidoglycan glycosyltransferase	*pbp2A*	XX999_01350	EC:2.4.1.129	GT51
	N-acetylglucosaminyldiphosphoundecaprenol *N*-acetyl-beta-D-mannosaminyltransferase	*tagA*	XX999_02448	EC:2.4.1.187	–
			XX999_02762		
			XX999_02763		
		murG		EC:2.4.1.227	GT28
				EC:2.4.1.337	–
	Undecaprenyldiphospho-muramoylpentapeptide beta-*N*-acetylglucosaminyltransferase	*bgsB*	XX999_03361	EC:2.4.1.–	GH1, GH3, GH5, GH13, GH16, GH17, GH20, GH27, GH31, GH32, GH33, GH35, GH39, GH65, GH70, GH72, GH94, GH112, GH130
		rfaB	XX999_01483	EC:2.4.1.– 3.4.–.–	
	1,2-diacylglycerol 3-alpha-glucosyltransferase	*mrcA*	XX999_00670	EC:2.4.1.–	
	UDP-D-galactose:(glucosyl)LPS alpha-1,6-D-galactosyltransferase**	*icaA*	XX999_02161	EC:2.4.1.–	GH1, GH3, GH5, GH13, GH16, GH17, GH20, GH27, GH31, GH32, GH33, GH35, GH39, GH65, GH70, GH72, GH94, GH112, GH130
		cpoA	XX999_01307		
			XX999_01219		
					GH1, GH3, GH5, GH13, GH16, GH17, GH20, GH27, GH31, GH32, GH33, GH35, GH39, GH65, GH70, GH72, GH94, GH112, GH130
			XX999_01806		
	Penicillin-binding protein 1A**				
					GH1, GH3, GH5, GH13, GH16, GH17, GH20, GH27, GH31, GH32, GH33, GH35, GH39, GH65, GH70, GH72, GH94, GH112, GH130
	Poly-beta-1,6-*N*-acetyl-D-glucosamine synthase**		XX999_01594		
	1,2-diacylglycerol-3-alpha-glucose alpha-1,2-galactosyltransferase**		XX999_01308		

(Continued)

TABLE 1 | Continued

	Enzyme	Gene	Gene ID	EC number	CAZy Family*
Pentosyltransferases	Adenine phosphoribosyltransferase	*apt*	XX999_01330	EC:2.4.2.7	GH10
	Hypoxanthine phosphoribosyltransferase	*hpt*	XX999_02067	EC:2.4.2.8	GH10
	Uracil phosphoribosyltransferase	*upp*	XX999_00627	EC:2.4.2.9	GH10
	Pyrimidine operon attenuation protein/uracil phosphoribosyltransferase	*pyrR*	XX999_02348	EC:2.4.2.9	GH10
	Orotate phosphoribosyltransferase	*pyrE*	XX999_01829	EC:2.4.2.10	GH10
	Amidophosphoribosyltransferase	*purF*	XX999_02638	EC:2.4.2.14	GH10
	ATP phosphoribosyltransferase	*hisG*	XX999_02631	EC:2.4.2.17	GH10
	Anthranilate phosphoribosyltransferase	*trpD*	XX999_02648	EC:2.4.2.18	GH10
	Xanthine phosphoribosyltransferase	*xpt*	XX999_02513	EC:2.4.2.22	GH10
	tRNA-guanosine34 transglycosylase	*tgt*	XX999_01714	EC:2.4.2.29	GH10
	triphosphoribosyl-dephospho-CoA synthase	*citG*	XX999_01169	EC:2.4.2.52	–
	Glutamine amidotransferase**	*hisH*	XX999_02268	EC:2.4.2.-	GH10
	S-adenosylmethionine:tRNA ribosyltransferase-isomerase	*queA*	XX999_01135	EC:2.4.99.17	–
			XX999_02510		
			XX999_02269		
Phosphotransferases	Glucokinase	*glk*	XX999_01642	EC:2.7.1.2	–
	Fructokinase	*scrK*	XX999_00302	EC:2.7.1.4	–
	Rhamnulokinase	*rhaB*	XX999_03099	EC:2.7.1.5	–
	Galactokinase	*galK*	XX999_03468	EC:2.7.1.6	–
	6-phosphofructokinase	pfkA	XX999_03415	EC:2.7.1.11	–
	Gluconokinase	*gntK*	XX999_03299	EC:2.7.1.12	–
	Ribokinase	*rbsK*	XX999_01922	EC:2.7.1.15	–
	Xylulokinase	*xylB*	XX999_01285	EC:2.7.1.17	–
	1-phosphofructokinase	*fruK*	XX999_00576	EC:2.7.1.56	–
	Glycerate 2-kinase	*glxK*	XX999_02236	EC:2.7.1.165	–
	Phosphoglycerate kinase	*pgk*	XX999_03490	EC:2.7.2.3	–
	Ribose-phosphate diphosphokinase	prsA	XX999_03492	EC:2.7.6.1	–
	Glucose-1-phosphate adenylyltransferase	glgC	XX999_02075	EC:2.7.7.27	–
			XX999_03125		
			XX999_03346		
			XX999_00881		
			XX999_00563		
			XX999_02133		
			XX999_00115		
			XX999_00116		

(Continued)

TABLE 1 | Continued

	Enzyme	Gene	Gene ID	EC number	CAZy Family*
Glycosylases (glycosyl hydrolases)	Oligo-1,6-glucosidase	*malL*	XX999_00306	EC:3.2.1.10	GH13, GH31
	Alpha-glucosidase**	*malZ*	XX999_00309	EC:3.2.1.20	GH4, GH13, GH31, GH63, GH97, GH122
	Alpha-galactosidase**	*galA*	XX999_03453	EC:3.2.1.22	GH4, GH27, GH31, GH36, GH57, GH97, GH110
	Beta-galactosidase**	*lacZ*	XX999_03369	EC:3.2.1.23	
	Alpha-mannosidase	E3.2.1.24	XX999_03302	EC:3.2.1.24	GH1, GH2, GH3, GH35, GH39, GH42, GH50, GH59, NC
	Beta-fructofuranosidase**	*sacA*	XX999_03300	EC:3.2.1.26	
	Xylan 1,4-beta-xylosidase**	*xynB*	XX999_03301	EC:3.2.1.37	GH31, GH38, GH92
	Alpha-L-rhamnosidase	*ramA*	XX999_03309	EC:3.2.1.40	GH32, GH68, GH100
	Beta-*N*-acetylhexosaminidase**	*nagZ*	XX999_03287	EC:3.2.1.52	GH1, GH3, GH5, GH30, GH39, GH43, GH51, GH52, GH54, GH116, GH120
	Cyclomaltodextrinase**	*ma*	XX999_03438	EC:3.2.1.54	
	Non-reducing end alpha-L-arabinofuranosidase**	*abfA*	XX999_03461	EC:3.2.1.55	GH78, GH106, CE15
	6-phospho-beta-glucosidase	*bglA*	XX999_00304	EC:3.2.1.86	GH3, GH5, GH18, GH20, GH84, GH116, NC
	Alpha,alpha-phosphotrehalase	*treC*	XX999_03314	EC:3.2.1.93	GH13, GH57
	Mannosylglycerate hydrolase				
	Alpha-D-xyloside xylohydrolase	*mngB*	XX999_02624	EC:3.2.1.170	
		xylS	XX999_03313	EC:3.2.1.177	GH2, GH3, GH10, GH43, GH51, GH54, GH62
			XX999_03312		GH1, GH4
			XX999_02682		GH13
			XX999_03314		GH38, GH63
			XX999_00538		
			XX999_02708		
			XX999_02709		
			XX999_02906		
			XX999_03006		
			XX999_03053		
			XX999_03350		
			XX999_03357		
			XX999_03358		
			XX999_03459		
			XX999_00377		
			XX999_03347		
			XX999_03495		
					GH31

(Continued)

TABLE 1 | Continued

	Enzyme	Gene	Gene ID	EC number	CAZy Family*
Isomerases	Ribulose-phosphate 3-epimerase	*rpe*	XX999_01689	EC:5.1.3.1	–
	UDP-glucose 4-epimerase	*galE*	XX999_00804	EC:5.1.3.2	GT1
	Aldose 1-epimerase	*galM*	XX999_01230	EC:5.1.3.3	–
	L-ribulose-5-phosphate 4-epimerase	*araD*	XX999_02084	EC:5.1.3.4	–
	N-acylglucosamine-6-phosphate 2-epimerase	*nanE*	XX999_03032	EC:5.1.3.9	–
	UDP-*N*-acetylglucosamine 2-epimerase (non-hydrolyzing)	*wecB*	XX999_03298	EC:5.1.3.14	GT4
	L-rhamnose mutarotase	*rhaM*	XX999_00914	EC:5.1.3.32	–
	2-epi-5-epi-valiolone epimerase	*cetB*	XX999_01783	EC:5.1.3.33	–
	D-allulose-6-phosphate 3-epimerase	*alsE*	XX999_03304	EC:5.1.3.-	–
	Triose-phosphate isomerase	*tpiA*	XX999_03394	EC:5.3.1.1	–
	L-arabinose isomerase	*araA*	XX999_03407	EC:5.3.1.4	–
	Xylose isomerase	*xylA*	XX999_01209	EC:5.3.1.5	–
	Ribose-5-phosphate isomerase	*rpiA*	XX999_03414	EC:5.3.1.6	–
	Mannose-6-phosphate isomerase	*manA*	XX999_00348	EC:5.3.1.8	–
	Glucose-6-phosphate isomerase	*pgi*	XX999_03373	EC:5.3.1.9	–
	L-rhamnose isomerase	*rhaA*	XX999_00882	EC:5.3.1.14	–
	1-(5-phosphoribosyl)-5-[(5-phosphoribosylamino)methylideneamino]imidazole-4-carboxamide isomerase	*hisA*	XX999_03393	EC:5.3.1.16	–
		trpF	XX999_03493	EC:5.3.1.24	–
		hxlB	XX999_00477	EC:5.3.1.27	–
	Phosphoribosylanthranilate isomerase	*pgm*	XX999_00762	EC:5.4.2.2	–
	6-phospho-3-hexuloisomerase	*pgmB*	XX999_02356	EC:5.4.2.6	–
	Phosphotransferases (phosphomutases)	*glmM*	XX999_02452	EC:5.4.2.10	–
	Beta-phosphoglucomutase	*gpmA*	XX999_03413	EC:5.4.2.11	–
	Phosphoglucosamine mutase	*gpmB*	XX999_02509	EC:5.4.2.12	–
	Phosphoglycerate mutase (2,3-diphosphoglycerate-dependent)		XX999_01716		
	Phosphoglycerate mutase (2,3-diphosphoglycerate-independent)		XX999_03454		
			XX999_00856		
			XX999_00121		
			XX999_00179		
			XX999_00910		
			XX999_00758		
			XX999_03037		
			XX999_00318		
			XX999_00974		
			XX999_00975		
			XX999_01026		
			XX999_01833		
			XX999_02136		
			XX999_02714		
			XX999_02790		

**Last update 17/02/2017. **Carbohydrate-binding module (CBM) proteins. NC, non-classified; GH, Glycoside Hydrolase; GT, Glycosyl Transferase; CE, Carbohydrate Esterase.*

TABLE 2 | Genes necessary for the glycogen metabolism in *Lactobacillus pentosus* MP-10 isolated from naturally fermented Aloreña table olives.

Gene ID	Gene	Gene length (bp)	Protein (Uniref_protein)	GO terms
XX999_00114	*glgB*	1623	1,4-alpha-glucan branching enzyme GlgB (UniRef100:P30538)	1,4-alpha-glucan branching enzyme activity (MF); hydrolase activity, hydrolyzing O-glycosyl compounds (MF); glycogen biosynthetic process (BP); cation binding (MF)
XX999_00115	*glgC*	1140	Glucose-1-phosphate adenylyltransferase (UniRef100:P39122)	ATP binding (MF); glycogen biosynthetic process (BP); glucose-1-phosphate adenylyltransferase activity (MF)
XX999_00116	*glgD*	1173	Glycogen biosynthesis protein GlgD (UniRef100:P39124)	Glycogen biosynthetic process (BP); nucleotidyltransferase activity (MF)
XX999_00117	*glgA*	1440	Glycogen synthase (UniRef100:P39125)	Glycogen biosynthetic process (BP); starch synthase activity XX999_00297
XX999_00118	*glgP*	2403	Glycogen phosphorylase (UniRef100:P39123)	Glycogen metabolic process (BP); glycogen phosphorylase activity (MF); pyridoxal phosphate binding (MF)
XX999_00119	*apu*	1818	Amylopullulanase precursor (UniRef100:P16950)	Starch binding (MF); alpha-amylase activity (MF); carbohydrate metabolic process (BP); metal ion binding (MF); pullulanase activity (MF)
XX999_00297	*amyB*	1323	Alpha-amylase 2 (UniRef100:P14898)	Alpha-amylase activity (MF); cytoplasm (CC); carbohydrate metabolic process (BP); metal ion binding (MF)
XX999_00856	*pgcA*	1728	Phosphoglucomutase (UniRef100:P18159)	Magnesium ion binding (MF); phosphoglucomutase activity (MF); cytosol (CC); glycogen biosynthetic process (BP); glucose metabolic process (BP); enterobacterial common antigen biosynthetic process (BP); galactose catabolic process (BP)
XX999_01233	*XX999_01233*	1032	Glycogen synthase (UniRef100:P9WMY8)	Glycogen (starch) synthase activity (MF); glycogen biosynthetic process (BP)
XX999_02081	*XX999_02081*	1041	Glycogen synthase (UniRef100:P9WMY8)	Glycogen (starch) synthase activity (MF); glycogen biosynthetic process (BP)

BP, Biological process; CC, Cellular component; MF, Molecular function.

environment, similarly as the probiotic *L. acidophilus* (Goh and Klaenhammer, 2013). The presence of more than one glycogen synthase gene in *L. pentosus* MP-10 indicates the capacity of these bacteria to store carbohydrates in the form of glycogen.

Lactobacillus pentosus MP-10 possesses genes predicted as levansucrase (*levS_1, levS_2, levS_3*, and *levS_4*) with identities ranging from 44.07 to 62.4% with *levS* gene from *L. sanfranciscensis* (**Table 3**; Rhee et al., 2002; Tieking et al., 2005), which are responsible for levan polymers [fructan polymers composed of β(2,6)-linked fructose units] and the fructo-oligosaccharide (FOS) 1-kestose production with prebiotic effects. This bacterium is capable to produce levan [with β-2,6 glycosidic bonds, produced by levansucrases (E.C. 2.4.1.10)] but not inulin-fructan types as no inulosucrase genes were detected in *L. pentosus* MP-10 genome. This is the first report of levansucrase in *L. pentosus*; this enzyme has only been reported in other LAB (*L. sanfranciscensis L. reuteri*, *L. johnsonii*, *L. gasseri*, *L. crispatus*, *L. plantarum*, *L delbrueckii*, and *L. vaginalis* among others). Alignments of the amino acid sequence of LevS proteins of *L. pentosus* MP-10 (LevS1, LvS2, LevS3, and LevS4) with levansucrase proteins of other lactic acid bacteria revealed less similarity and formed a separate cluster in the phylogenetic tree (**Figure 3**).

Regarding other enzymes involved in complex carbohydrate degradation, we found genes coding for a protein similar to chitooligosaccharide deacetylase of *E. coli* K12 and beta-hexosaminidase involved in chitin degradation pathway as part of glycan degradation. Further, several genes coding for enzymes involved in the degradation of plant structural polysaccharides such as cellulose, ß-glucan, and xylan were predicted in *L. pentosus* MP-10 genome (**Table 3**). In this context, a gene coding for a protein similar to cellulase/esterase CelE from *Clostridium thermocellum* ATCC 27405, which is a multifunctional enzyme involved in the degradation of plant cell wall polysaccharides, was identified in *L. pentosus* MP-10 genome necessary for cellulose and xylan digestion by both human and animals (**Table 3**). Moreover, endo-1,4-beta-xylanase, acetylxylan esterase (three genes) and polysaccharide deacetylase were predicted in *L. pentosus* MP-10 genome sequence being involved in xylan catabolic pathway. Alpha-galactosidase coding gene was also detected in *L. pentosus* MP-10 genome sequence and is involved in raffinose degradation (**Table 3**), which was previously shown *in vitro* by Pérez Montoro et al. (2016). Furthermore, *L. pentosus* MP-10 also had genes coding for cellulose synthase (two genes exclusive to *L. pentosus* MP-10 and two other genes) involved in cellulose synthesis (**Table 3**), which could accumulate cellulose on the cell wall surface as an extracellular matrix for cell adhesion and biofilm formation to protect the bacteria. Cellulose production has been reported in lactic acid bacteria (Adetunji and Adegoke, 2007); however, no reports were found of cellulase production, although some *Lactobacillus* sp. genomes exhibit cellulase genes such as *L. delbrueckii* subsp. *bulgaricus* CNCM I-1519 (UniProtKB-G6F519) and

TABLE 3 | Genes necessary for complex carbohydrate metabolism in *Lactobacillus pentosus* MP-10 isolated from naturally fermented Aloreña table olives.

Carbohydrate	Gene ID	Gene	Gene length (bp)	Protein (Uniref_protein)	Identity (%)	E-value	GO terms
Levan	XX999_02538	*levS_1*	2448	Levansucrase (UniRef100:Q70XJ9)	44.07	2e-07	Extracellular region (CC); cell wall (CC); carbohydrate metabolic process (BP); carbohydrate utilization (BP); metal ion binding (MF); levansucrase activity (MF)
	XX999_02724	*levS_2*	3078	Levansucrase (RefSeq:Q70XJ9)	46.67	3e-24	
	XX999_02966	*levS_3*	2688	Levansucrase (UniRef100:Q70XJ9)	50.4	2e-06	Extracellular region (CC); cell wall (CC); membrane (CC)
	XX999_02983	*levS_4*	6552	Levansucrase (UniRef100:Q70XJ9)	62.4	1e-09	Extracellular region (CC); cell wall (CC); carbohydrate metabolic process (BP); carbohydrate utilization (BP); metal ion binding (MF); levansucrase activity (MF)
Chitin	XX999_00964	*XX999_00964*	759	Hypotheticalprotein	26.87	8e-23	Polysaccharide catabolic process (BP); cytoplasm (CC); chitin catabolic process (BP); chitin disaccharide deacetylase activity (MF); metal ion binding (MF); diacetylchitobiose catabolic process (BP)
	XX999_03477	*exo I*	1851	Beta-hexosaminidase (UniRef100:P96155)	25.73	8e-12	Polysaccharide catabolic process (BP); beta-N-acetylhexosaminidase activity (MF); chitin catabolic process (BP); periplasmic space (CC)
Raffinose	XX999_03302	*rafA*	2217	Alpha-galactosidase (UniRef100:P16551)	33.16	4e-96	Carbohydrate metabolic process (BP); raffinose alpha-galactosidase activity (MF)
Cellulose	XX999_00850	*XX999_00850*	1446	Cellulose synthase regulator protein (CLUSTERS:PRK11114)	–	–	–
	XX999_00851	*XX999_00851*	702	Cellulose synthase regulator protein (CLUSTERS:PRK11114)	–	–	–
	XX999_01507	*bcsA*	1986	Cellulose synthase catalytic subunit [UDP-forming] (UniRef100:P37653)	27.89	3e-65	Plasma membrane (CC); UDP-glucose metabolic process (BP); integral component of membrane (CC); cellulose synthase (UDP-forming) activity (MF); cyclic-di-GMP binding (MF); bacterial cellulose biosynthetic process (BP)
	XX999_02472	*yedQ*	1194	Putative diguanylate cyclase YedQ (UniRef100:P76330)	28.91	7e-20	Negative regulation of bacterial-type flagellum-dependent cell motility (BP); GTP
	XX999_03259	*XX999_03259*	984	Hypothetical protein (UniRef100:P10477)	24.64	3e-06	lipid metabolic process (BP); cellulase activity (MF); hydrolase activity, acting on ester bonds (MF); cellulose catabolic process (BP)

(Continued)

TABLE 3 | Continued

Carbohydrate	Gene ID	Gene	Gene length (bp)	Protein (Uniref_protein)	Identity (%)	E-value	GO terms
Xylan	XX999_00089	*XX999_00089*	588	Polysaccharide deacetylase (UniRef100:P54865)	30.77	7e-05	Hydrolaseactivity, actingoncarbon-nitrogen (butnotpeptide) bonds (MF); polysaccharidebinding (MF); endo-1,4-beta-xylanase activity (MF); xylancatabolicprocess (BP)
	XX999_01054	*axeA1_1*	798	Acetylxylan esterase precursor (UniRef100:D5EV35)	26.82	2e-11	Xylancatabolicprocess (BP); acetylxylan esterase activity (MF)
	XX999_02525	*xynY*	918	Endo-1,4-beta-xylanase Y precursor (UniRef100:P51584)	29.51	3e-29	Endo-1,4-beta-xylanase activity (MF); cellulosome (CC); xylancatabolicprocess (BP)
	XX999_03401	*axeA1_2*	837	Acetylxylan esterase precursor (UniRef100:D5EV35)	27.63	4e-12	Xylancatabolicprocess (BP); acetylxylan esterase activity (MF)
	XX999_03577	*axeA1_3*	714	Acetylxylan esterase precursor (UniRef100:D5EV35)	27.59	3e-12	Xylancatabolicprocess (BP); acetylxylan esterase activity (MF)

BP, Biological process; CC, Cellular component; MF, Molecular function.

L. plantarum (UniProtKB – A0A1C9HK74). For probiotic bacteria, such as *E. coli* Nissle 1917, cellulose production is required for adhesion of bacteria to the gastrointestinal epithelial cell line HT-29, to the mouse epithelium *in vivo*, and for enhanced cytokine production (Monteiro et al., 2009). Thus, the role of cellulose production in *L. pentosus* MP-10 must be investigated in depth.

Overall, the repertoire of enzymes coding genes identified in *L. pentosus* MP-10 genome highlight the attractiveness of this bacterium as potential probiotic for human and animal.

Molecular Mechanisms Involved in the Interaction with the Host

Probiotic lactobacilli can mimic the same mechanisms used by the pathogens in the colonization process, thus they can express cell surface proteins such as key probiotic ligands that interact with host receptors resulting in several probiotic effects—thus inducing signaling pathways in the host (Voltan et al., 2008). The identification and characterization of these proteins, often strain-specific, involved in the molecular communication or interaction with the host are necessary to evaluate *a priori* the probiotic potential of *Lactobacillus* sp. candidates. Here, the possible interaction between *L. pentosus* MP-10 and the intestinal host cells, the target of most interactions with probiotics (Lebeer et al., 2010), may be bioinformatically predicted from the genome sequence. For example, several extracellular proteins (reviewed by Sánchez et al., 2008) were predicted in *L. pentosus* MP-10 to be involved in mucus adhesion: MucBP domain protein (codified by two genes determined in this study), lipoprotein signal peptidase (*lspA* gene) and moonlighting proteins such as glutamine-binding periplasmic protein (*glnH* genes) and elongation factor Tu (*tuf* gene) (**Table 4**). The high genetic heterogeneity of MucBP proteins among *Lactobacillus* species (and strains) was reported by Mackenzie et al. (2010) for MUB and MUB-like proteins in *L. reuteri*. MucBP domain proteins are bacterial peptidoglycan-bound proteins, which are ligands or effector molecules contributing to specific properties such as adherence to the host, auto-aggregation and/or co-aggregation with pathogenic bacteria (Pérez Montoro et al., 2016)—as reported by Mackenzie et al. (2010) for MUB in *L. reuteri*. However, this should be further investigated for *L. pentosus* MP-10 under different conditions. Adhesion to mucus has been attributed to other molecules such as the *Lactobacillus* surface protein A (LspA), reported as mucus binding protein in *L. salivarius* UCC118 (van Pijkeren et al., 2006), which was also found in *L. pentosus* MP-10 (**Table 4**). Mucus binding proteins in *L. pentosus* MP-10 may have a dual role: (1) being involved in the adhesion of this bacterium to the host cells and thus reinforcing the protection of the mucosal barrier and the competitive exclusion of pathogens, and (2) these proteins could also be implicated in the induction of mucin secretion by the host as reported for other lactobacilli (Mack et al., 2003). These finding are corroborated by the fact that *L. pentosus* MP-10 was able to adhere to Caco-2 and HeLa 229 cell lines and also co-aggregate with different

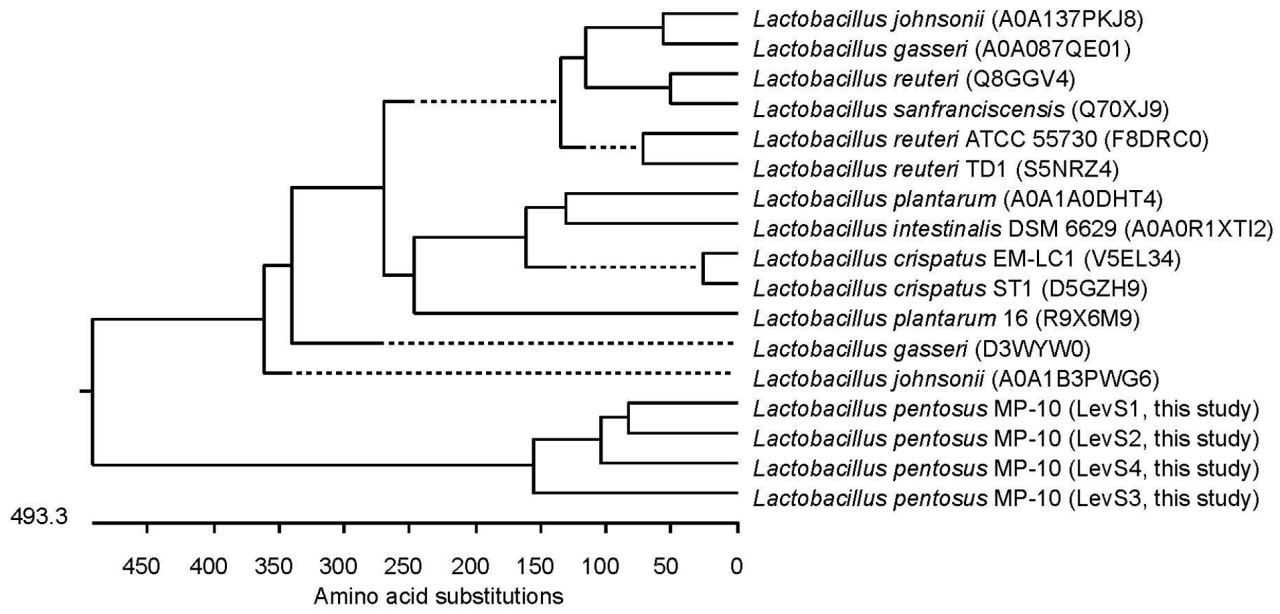

FIGURE 3 | Phylogenetic relationships of *L. pentosus* MP-10 and other *Lactobacillus* sp. inferred from the alignment of levansucrase proteins. The sequences were aligned and the most parsimonious phylogenetic trees were constructed using the CLUSTAL W of Lasergene program, version 14 (MegAlign 14, Inc., Madison, WI, USA). The scale below indicates the number of amino acid substitutions. Accession numbers are indicated in parentheses.

pathogens (*Escherichia coli*, *Staphylococcus aureus*, *Listeria innocua*, and *Salmonella* Enteritidis) (Pérez Montoro et al., 2016) by means of cell-wall surface molecules. However, further studies are required to demonstrate the target cell-wall surface molecules involved in such adhesion to intestinal cells.

Other proteins predicted to be involved in adhesion to epithelial cells or extracellular matrix include: poly-beta-1,6-*N*-acetyl-D-glucosamine synthase, collagen binding protein, manganese ABC transporter substrate-binding lipoprotein precursor and moonlighting proteins such as elongation factor Tu, glyceraldehyde-3-phosphate dehydrogenase, 10 and 60 kDa chaperonins, enolase, 2 glutamine synthetase, and glucose-6-phosphate isomerase (**Table 4**). The poly-beta-1,6-*N*-acetyl-D-glucosamine synthase encoded by *L. pentosus* MP-10 was similar to *E. coli* K12 (33.89% identity), and it has been reported to be a surface polysaccharide involved in biofilm formation by this strain (Matthysse et al., 2008). However, the role of this protein in lactobacilli has not been determined. Furthermore, we predicted the presence of collagen-binding protein specific to *L. pentosus* MP-10, which could be involved in their adhesion to epithelial cells/extracellular matrix proteins similarly as shown other lactobacilli such as *L. reuteri* NCIB 11951 (Roos et al., 1996) and *L. fermentum* RC-14 (Heinemann et al., 2000). Thus, this could be of vital importance for effective colonization and also competitive displacement of gut pathogens (Yadav et al., 2013).

On the other hand, the manganese ABC transporter substrate-binding lipoprotein precursor predicted in *L. pentosus* MP-10, similar to *Streptococcus pneumoniae* ATCC BAA-334 (51.96% identity), has been described as an important factor in pathogenesis and infection, since it acts as an adhesin involved on adherence to extracellular matrix (Dintilhac et al., 1997). Furthermore, the manganese ABC transporter substrate-binding lipoprotein precursor has also been detected in different *Lactobacillus* sp. such as *L. plantarum*, *L.* rhamnosus, and *L. delbrueckii* among others being involved in cell adhesion (UniprotKB).

The moonlighting proteins, or multifunctional proteins such as elongation factor Tu and chaperonin GroEL, have been involved in the adhesion to epithelial cells and/or extracellular matrix proteins and also in host immunomodulation in *L. johnsonii* NCC 533 (Granato et al., 2004; Bergonzelli et al., 2006; Sánchez et al., 2008), while α-enolase has been involved in adhesion to epithelial cells and/or extracellular matrix proteins and also plasma components in *L. crispatus* ST1 (Antikainen et al., 2007). Glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate mutase have been involved in the adhesion to plasma components in *L. crispatus* ST2 (Antikainen et al., 2007; Candela et al., 2007). Furthermore, Kainulainen et al. (2012) showed that glutamine synthetase and glucose-6-phosphate isomerase have also been involved in adhesion to epithelial cells. However, the role of these moonlighting proteins in *L. pentosus* MP-10 has not yet been determined, requiring for this purpose further mutation or proteomic studies.

CONCLUSION

Lactobacillus pentosus MP-10 has harbored in its genome several genes putatively involved in their adaptation to the human GIT—particularly those involved in carbohydrate metabolism related

TABLE 4 | Genes coding for extracellular proteins with roles in adhesion or interaction with the host as predicted from genome annotation of *Lactobacillus pentosus* MP-10 isolated from naturally fermented Aloreña table olives.

Gene ID	Gene	Gene length (bp)	Protein (Uniref_protein/Pfam)*	Identity (%)	E-value	Organism	GO terms
XX999_01369	*XX999_01369*	11817	MucBP domain protein (Pfam:PF06458.6)	–	–	–	Mucin-Binding Protein
XX999_01708	*XX999_01708*	6885					
XX999_00892	*glnH_1*	1437	Glutamine-binding periplasmic protein	40.98	5e-43	*Escherichia coli* O157:H7	Transporter activity (MF); amino acid transport (BP); periplasmic space (CC)
XX999_02287	*glnH_3*	840	precursor (UniRef100:P0AEQ5)	31	1e-29		
XX999_01827	*lspA*	450	Lipoprotein signal peptidase (UniRef100:C4ZPV3)	55.5	1e-10	*Escherichia coli* K12	Aspartic-type endopeptidase activity (MF); plasma membrane (CC); integral component of membrane (CC)
XX999_02097	*tuf*	1188	Elongation factor Tu (UniRef100:P0DA82)	77.08	0.0	*Streptococcus pyogenes* ATCC BAA-595	Translation elongation factor activity (MF); GTPase activity (MF); GTP binding (MF); cytoplasm (CC)
XX999_01594	*pgaC_1*	1314	Poly-beta-1,6-N-acetyl-D-glucosamine	33.89	3e-66	*Escherichia coli* K12	Plasma membrane (CC); metabolic process (BP); acetylglucosaminyltransferase activity (MF); integral component of membrane (CC); cell adhesion involved in biofilm formation (BP)
XX999_02115	*pgaC_2*	1356	synthase (UniRef100:P75905)	25.97	1e-19		
X999_01138	*psaA_1*	942	Manganese ABC transporter substrate-binding	51.96	6e-113	*Streptococcus pneumoniae* ATCC BAA-334	Plasma membrane (CC); cell adhesion (BP); metal ion transport (BP); metal ion binding (MF)
XX999_02913	*psaA_2*	894	binding lipoprotein precursor	27.21	7e-23		
XX999_03164	*psaA_3*	909	(UniRef100:P0A4G2)	25.09	4e-13		
XX999_00883	*eno2*	1329	Enolase 2 (UniRef100:Q042F4)	78.65	0.0	*Lactobacillus gasseri* ATCC 33323	Phosphopyruvate hydratase complex (CC); magnesium ion binding (MF); phosphopyruvate hydratase activity (MF); extracellular region (CC); glycolytic process (BP); cell surface (CC)
XX999_00880	*gap*	1023	Glyceraldehyde-3-phosphate dehydrogenase (UniRef100:Q59309)	57.86	2e-137	*Clostridium pasteurianum*	Glyceraldehyde-3-phosphate dehydrogenase (NAD++) (phosphorylating) activity (MF); cytoplasm (CC); glucose metabolic process (BP); glycolytic process (BP); NADP binding (MF); NAD binding (MF)
XX999_02862	*XX999_02862*	1884	Collagen binding domain protein	–	–	–	–

(Continued)

TABLE 4 | Continued

Gene ID	Gene	Gene length (bp)	Protein (Uniref_protein/Pfam)*	Identity (%)	E-value	Organism	GO terms
XX999_00818	*groS*	285	10 kDa chaperonin (UniRef100:Q07200)	61.96	6e-37	*Geobacillus stearothermophilus*	ATP binding (MF); cytoplasm (CC); protein folding (BP)
XX999_00819	*groL*	1626	60 kDa chaperonin (UniRef100:Q04IQ3)	75.79	0.0	*Staphylococcus aureus* Mu50	ATP binding (MF); cytoplasm (CC); protein refolding (BP)
XX999_01649	*pgiglnA*	1347	Glutamine synthetase (UniRef100:P60890)	67.86	0.0	*Streptococcus pneumoniae* D39	Glutamate-ammonia ligase activity (MF); ATP binding (MF); cytoplasm (CC); glutamine biosynthetic process (BP); nitrogen fixation (BP)
XX999_02452	*pgi*	1353	Glucose-6-phosphate isomerase (UniRef100:P81181)	64.96	0.0	*Lactococcus lactis* subsp. *lactis* IL1403	Glucose-6-phosphate isomerase activity (MF); cytoplasm (CC); gluconeogenesis (BP); glycolytic process (BP)

BP, Biological process; CC, Cellular component; MF, Molecular function.

to prebiotic utilization, and also the proteins involved in the interaction with host tissues. Enzymes involved in carbohydrate modification and complex-carbohydrate metabolism are highly represented in *L. pentosus* MP-10 genome, which may enhance their survival, competitiveness, and persistence in a competitive GIT niche. Furthermore, we found genes encoding mucus-binding proteins—involved in the adhesion to mucus, epithelial cells or extracellular matrix, to plasma components—and also moonlighting proteins, or multifunctional proteins, predicted to be involved in their adhesion to epithelial cells and/or extracellular matrix proteins and also involved in host immunomodulation. In conclusion, *in silico* analysis of the *L. pentosus* MP-10 genome sequence highlights the attractiveness of this bacterium as a potential probiotic for human and animal hosts, and offers opportunities for further investigation of novel routes for screening and studying these bacteria.

MATERIALS AND METHODS

Genomic DNA Sequence of *L. pentosus* MP-10

The complete genome sequence of *L. pentosus* MP-10, obtained by using PacBio RS II technology (Abriouel et al., 2016) and deposited at the EMBL Nucleotide Sequence Database under accession numbers FLYG01000001 to FLYG01000006, was annotated as described by Abriouel et al. (in press). Briefly, the assembled genome sequences were annotated using the Prokka annotation pipeline, version 1.11 (Seemann, 2014), which predicted tRNA, rRNA, and mRNA genes and signal peptides in the sequences using Aragorn, RNAmmer, Prodigal, and SignalP, respectively (Laslett and Canback, 2004; Lagesen et al., 2007; Hyatt et al., 2010).

In Silico Analysis of Carbohydrate Metabolism in *L. pentosus* MP-10

The annotated genome sequence was used to detect the putative genes involved in carbohydrate metabolism, their products, and the associated GO terms. Furthermore, the carbohydrate metabolic pathways were reconstructed by using BlastKOALA (last update March 4, 2016) as part of the KEGG (Kyoto Encyclopedia of Genes and Genome) tool in the pathway database[2] for annotating genomes; here, we used the annotated genes predicted in *L. pentosus* MP-10 genome as the input query.

In Silico Analysis of Proteins Involved in Interaction with Host

The annotated genome sequence was screened for mucus-binding proteins, proteins involved in adhesion to epithelial/extracellular matrix proteins, plasma components, and host immunomodulation as described in the literature (Roos et al., 1996; Heinemann et al., 2000; Granato et al., 2004; Bergonzelli et al., 2006; van Pijkeren et al., 2006; Antikainen et al., 2007; Candela et al., 2007; Sánchez et al., 2008; Mackenzie et al., 2010; Kainulainen et al., 2012).

[2]http://www.genome.jp/kegg/pathway.html

AUTHOR CONTRIBUTIONS

HA, NB, CK, and AG drafted the manuscript. HA, NB, BPM, CC-S, APP, NCG, SC-G, and ME-M analyzed the data; All authors discussed the results, commented on the manuscript, and approved the final version.

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REFERENCES

Abriouel, H., Benomar, N., Lucas, R., and Gálvez, A. (2011). Culture-independent study of the diversity of microbial populations in brines during fermentation of naturally fermented Aloreña green table olives. *Int. J. Food Microbiol.* 144, 487–496. doi: 10.1016/j.ijfoodmicro.2010.11.006

Abriouel, H., Pérez Montoro, B., Casado Muñoz, M. C., Knapp, C. W., Gálvez, A., and Benomar, N. (in press). Genomic insights into safety aspects and defense mechanisms of a potentially probiotic *Lactobacillus pentosus* MP-10 isolated from brines of naturally fermented Aloreña green table olives. *PLoS ONE*.

Abriouel, H., Peìrez Montoro, B., Casado Munþoz, M. D. C., Lavilla Lerma, L., Hidalgo Pestanþa, M., Caballero Goìmez, N., et al. (2016). Complete genome sequence of a potential probiotic, *Lactobacillus pentosus* MP-10, isolated from fermented Alorenþa table olives. *Genome Announc.* 4:e854-16. doi: 10.1128/genomeA.00854-16

Adetunji, V. O., and Adegoke, G. O. (2007). Bacteriocin and cellulose production by lactic acid bacteria isolated from West African soft cheese. *Afr. J. Biotechnol.* 6, 2616–2619.

Antikainen, J., Kuparinen, V., Lähteenmaki, K., and Korhonen, T. K. (2007). pH-dependent association of enolase and glyceraldehyde-3-phosphate dehydrogenase of *Lactobacillus crispatus* with the cell wall and lipoteichoic acids. *J. Bacteriol.* 189, 4539–4543.

Bergonzelli, G. E., Granato, D., Pridmore, R. D., Marvin-Guy, L. F., Donnicola, D., and Corthésy-Theulaz, I. E. (2006). GroEL of *Lactobacillus johnsonii* La1 (NCC 533) is cell surface associated: potential role in interactions with the host and the gastric pathogen *Helicobacter pylori*. *Infect. Immun.* 74, 425–434.

Bernardeau, M., Vernoux, J. P., Henri-Dubernet, S., and Gueguen, M. (2008). Safety assessment of dairy microorganisms: the *Lactobacillus* genus. *Int. J. Food Microbiol.* 126, 278–285.

Bustos, G., Moldes, A. B., Cruz, J. M., and Domiìnguez, J. M. (2005). Influence of the metabolism pathway on lactic acid production from hemicellulosic trimming vine shoots hydrolyzates using *Lactobacillus pentosus*. *Biotechnol. Prog.* 21, 793–798.

Candela, M., Bergmann, S., Vici, M., Vitali, B., Turroni, S., Eikmanns, B. J., et al. (2007). Binding of human plasminogen to *Bifidobacterium*. *J. Bacteriol.* 189, 5929–5936.

Cantarel, B. L., Lombard, V., and Henrissat, B. (2012). Complex carbohydrate utilization by the healthy human microbiome. *PLoS ONE* 7:e28742. doi: 10.1371/journal.pone.0028742

De Angelis, M., and Gobbetti, M. (2004). Environmental stress responses in *Lactobacillus*: a review. *Proteomics* 4, 106–122.

Dintilhac, A., Alloing, G., Granadel, C., and Claverys, J.-P. (1997). Competence and virulence of *Streptococcus pneumoniae*: Adc and PsaA mutants exhibit a requirement for Zn and Mn resulting from inactivation of putative ABC metal permeases. *Mol. Microbiol.* 25, 727–739.

Eydallin, G., Montero, M., Almagro, G., Sesma, M. T., Viale, A. M., Munoz, F. J., et al. (2010). Genome-wide screening of genes whose enhanced expression affects glycogen accumulation in *Escherichia coli*. *DNA Res.* 17, 61–71. doi: 10.1093/dnares/dsp028

Eydallin, G., Viale, A. M., Moran-Zorzano, M. T., Munoz, F. J., Montero, M., Baroja-Fernandez, E., et al. (2007). Genome-wide screening of genes affecting glycogen metabolism in *Escherichia coli* K-12. *FEBS Lett.* 581, 2947–2953.

Franz, C. M. A. P., Cho, G. Y., and Holzapfel, W. H. (2011). "Probiotics: taxonomy and technological features," in *Probiotic and Prebiotic Foods: Technology, Stability and Benefits to Human Health*, eds N. P. Shah, A. Gomes da Cruz, and J. de Assis Fonseca Faria (Hauppauge, NY: Nova Science Publishers), 1–23.

Garrigues, C., Johansen, E., and Crittenden, R. (2013). Pangenomics—an avenue to improved industrial starter cultures and probiotics. *Curr. Opin. Biotechnol.* 24, 187–191. doi: 10.1016/j.copbio.2012.08.009

Giraffa, G., Chanishvili, N., and Widyastuti, Y. (2010). Importance of lactobacilli in food and feed biotechnology. *Res. Microbiol.* 161, 480–487. doi: 10.1016/j.resmic.2010.03.001

Goh, Y. J., and Klaenhammer, T. R. (2013). A functional glycogen biosynthesis pathway in *Lactobacillus acidophilus*: expression and analysis of the *glg* operon. *Mol. Microbiol.* 89, 1187–1200. doi: 10.1111/mmi.12338

Goh, Y. J., and Klaenhammer, T. R. (2014). Insights into glycogen metabolism in *Lactobacillus acidophilus*: impact on carbohydrate metabolism, stress tolerance and gut retention. *Microb. Cell Fact.* 13:94. doi: 10.1186/s12934-014-0094-3

Granato, D., Bergonzelli, G. E., Pridmore, R. D., Marvin, L., Rouvet, M., and Corthésy-Theulaz, I. E. (2004). Cell surface-associated elongation factor Tu mediates the attachment of *Lactobacillus johnsonii* NCC533 (La1) to human intestinal cells and mucins. *Infect. Immun.* 72, 2160–2169.

Hansen, E. B. (2002). Commercial bacterial starter cultures for fermented foods of the future. *Int. J. Food Microbiol.* 78, 119–131.

Heinemann, C., van Hylckama Vlieg, J. E. T., Janssen, D. B., Busscher, H. J., van der Mei, H. C., and Reid, G. (2000). Purification and characterization of a surface-binding protein from *Lactobacillus fermentum* RC-14 that inhibits adhesion of *Enterococcus faecalis* 1131. *FEMS Microbiol. Lett.* 190, 177–180.

Heller, K. J. (2001). Probiotic bacteria in fermented foods: product characteristics and starter organisms. *Am. J. Clin. Nutr.* 73, 374s–379s.

Hill, C., Guarner, F., Reid, G., Gibson, G. R., Merenstein, D. J., Pot, B., et al. (2014). The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nat. Rev. Gastroenterol. Hepatol.* 11, 506–514. doi: 10.1038/nrgastro.2014.66

Holzapfel, W. H. (2002). Appropriate starter culture technologies for small-scale fermentation in developing countries. *Int. J. Food Microbiol.* 75, 197–212.

Hyatt, D., Chen, G.-L., LoCascio, P. F., Land, M. L., Larimer, F. W., and Hauser, L. J. (2010). Prodigal: prokaryotic gene recognition and translation initiation site identification. *BMC Bioinformatics* 11:119. doi: 10.1186/1471-2105-11-119

Izumo, T., Izumi, F., Nakagawa, I., Kitagawa, Y., Shibata, H., and Kiso, Y. (2011). Influence of *Lactobacillus pentosus* S-PT84 ingestion on the mucosal immunity of healthy and *Salmonella* Typhimurium-infected mice. *Biosci. Microflora* 30, 27–35. doi: 10.12938/bifidus.30.27

Jolley, K. A., and Maiden, M. C. (2010). BIGSdb: scalable analysis of bacterial genome variation at the population level. *BMC Bioinformatics* 11:595. doi: 10.1186/1471-2105-11-595

Kainulainen, V., Loimaranta, V., Pekkala, A., Edelman, S., Antikainen, J., Kylväjä, R., et al. (2012). Glutamine synthetase and glucose-6-phosphate isomerase are adhesive moonlighting proteins of *Lactobacillus crispatus* released by epithelial cathelicidin LL-37. *J. Bacteriol.* 194, 2509–2519. doi: 10.1128/JB.06704-11

Kay, E., Lesk, V. I., Tamaddoni-Nezhad, A., Hitchen, P. G., Dell, A., Sternberg, M. J., et al. (2010). Systems analysis of bacterial glycomes. *Biochem. Soc. Trans.* 38, 1290–1293. doi: 10.1042/BST0381290

Kim, J. F., Jeong, H., Yu, D. S., Choi, S.-H., Hur, C.-G., Park, M.-S., et al. (2009). Genome sequence of the probiotic bacterium *Bifidobacterium animalis* subsp. *lactis* AD011. *J. Bacteriol.* 191, 678–679. doi: 10.1128/JB.01515-08

Korakli, M., Gänzle, M. G., and Vogel, R. F. (2002). Metabolism by bifidobacteria and lactic acid bacteria of polysaccharides from wheat and rye, and exopolysaccharides produced by *Lactobacillus sanfranciscensis*. *J. Appl. Microbiol.* 92, 958–965.

Kotani, Y., Shinkai, S., Okamatsu, H., Toba, M., Ogawa, K., Yoshida, H., et al. (2010). Oral intake of *Lactobacillus pentosus* strain b240 accelerates salivary immunoglobulin A secretion in the elderly: a randomized, placebo-controlled, double-blind trial. *Immun. Ageing* 7:11. doi: 10.1186/1742-4933-7-11

Lagesen, K., Hallin, P., Rødland, E. A., Stærfeldt, H.-H., Rognes, T., and Ussery, D. W. (2007). RNAmmer: consistent and rapid annotation of ribosomal RNA genes. *Nucleic Acids Res.* 35, 3100–3108.

Lairson, L. L., Henrissat, B., Davies, G. J., and Withers, S. G. (2008). Glycosyltransferases: structures, functions, and mechanisms. *Annu. Rev. Biochem.* 77, 521–555.

Laslett, D., and Canback, B. (2004). ARAGORN, a program to detect tRNA genes and tmRNA genes in nucleotide sequences. *Nucleic Acids Res.* 32, 11–16.

Lebeer, S., Vanderleyden, J., and De Keersmaecker, S. C. J. (2010). Host interactions of probiotic bacterial surface molecules: comparison with commensals and pathogens. *Nat. Rev. Microbiol.* 8, 171–184. doi: 10.1038/nrmicro2297

Leroy, F., and de Vuyst, L. (1999). Temperature and pH conditions that prevail during fermentation of sausages are optimal for production of the antilisterial bacteriocin sakacin K. *Appl. Environ. Microbiol.* 65, 974–981.

Mack, D. R., Ahrne, S., Hyde, L., Wei, S., and Hollingsworth, M. A. (2003). Extracellular MUC3 mucin secretion follows adherence of *Lactobacillus* strains to intestinal epithelial cells *in vitro*. *Gut* 52, 827–833.

Mackenzie, D. A., Jeffers, F., Parker, M. L., Vibert-Vallet, A., Bongaerts, R. J., Roos, S., et al. (2010). Strain-specific diversity of mucus-binding proteins in the adhesion and aggregation properties of *Lactobacillus reuteri*. *Microbiol.* 156, 3368–3378. doi: 10.1099/mic.0.043265-0

Maiden, M. C., Jansen van Rensburg, M. J., Bray, J. E., Earle, S. G., Ford, S. A., Jolley, K. A., et al. (2013). MLST revisited: the gene-by-gene approach to bacterial genomics. *Nat. Rev. Microbiol.* 11, 728–736. doi: 10.1038/nrmicro3093

Matthysse, A. G., Deora, R., Mishra, M., and Torres, A. G. (2008). Polysaccharides cellulose, poly-β-1,6-*N*-acetyl-D-glucosamine, and colanic acid are required for optimal binding of *Escherichia coli* O157:H7 strains to alfalfa sprouts and K-12 strains to plastic but not for binding to epithelial cells. *Appl. Environ. Microbiol.* 74, 2384–2390. doi: 10.1128/AEM.01854-07

Monteiro, C., Saxena, I., Wang, X., Kader, A., Bokranz, W., Simm, R., et al. (2009). Characterization of cellulose production in *Escherichia coli* Nissle 1917 and its biological consequences. *Environ. Microbiol.* 11, 1105–1116. doi: 10.1111/j.1462-2920.2008.01840.x

Ng, P. C., and Henikoff, S. (2006). Predicting the effects of amino acid substitutions on protein function. *Annu. Rev. Genomics Hum. Genet.* 7, 61–80.

O'Sullivan, O., O'Callaghan, J., Sangrador-Vegas, A., McAuliffe, O., Slattery, L., Kaleta, P., et al. (2009). Comparative genomics of lactic acid bacteria reveals a niche-specific gene set. *BMC Microbiol.* 9:50. doi: 10.1186/1471-2180-9-50

Pérez Montoro, B., Benomar, N., Lavilla Lerma, L., Castillo Gutiérrez, S., Gálvez, A., and Abriouel, H. (2016). Fermented Aloreña table olives as a source of potential probiotic *Lactobacillus pentosus* strains. *Front. Microbiol.* 7:1583.

Pot, B., Felis, G. E., De Bruyne, K., Tsakalidou, E., Papadimitriou, K., Leisner, J., et al. (2014). "The genus Lactobacillus," in *Lactic Acid Bacteria: Biodiversity and Taxonomy*, eds W. P. Holzapfel and B. J. B. Wood (Hoboken, NJ: John Wiley & Sons, Inc), 249–353.

Rhee, S.-K., Song, K. B., Kim, C. H., Park, B. S., Jang, E. K., and Jang, K. H. (2002). "Levan," in *Biopolymers. Polysaccharides I: Polysaccharides from Prokaryotes*, eds A. Steinbüchel, S. D. Baets, and E. H. Vandamme (Weinheim: Wiley VCH), 351–378.

Roos, S., Aleljung, P., Robert, N., Lee, B., Wadstrom, T., Lindberg, M., et al. (1996). A collagen binding protein from *Lactobacillus reuteri* is part of an ABC transporter system? *FEMS Microbiol. Lett.* 144, 33–38.

Sánchez, B., Bressollier, P., and Urdaci, M. C. (2008). Exported proteins in probiotic bacteria: adhesion to intestinal surfaces, host immunomodulation and molecular cross-talking with the host. *FEMS Immunol. Med. Microbiol.* 54, 1–17. doi: 10.1111/j.1574-695X.2008.00454.x

Seemann, T. (2014). Prokka: rapid prokaryotic genome annotation. *Bioinformatics* 30, 2068–2069. doi: 10.1093/bioinformatics/btu153

Tieking, M., Ehrmann, M. A., Vogel, R. F., and Ganzle, M. G. (2005). Molecular and functional characterization of a levansucrase from the sourdough isolate *Lactobacillus sanfranciscensis* TMW 1.392. *Appl. Microbiol. Biotechnol.* 66, 655–663.

van Pijkeren, J.-P., Canchaya, C., Ryan, K. A., Li, Y., Claesson, M. J., Sheil, B., et al. (2006). Comparative and functional analysis of sortase-dependent proteins in the predicted secretome of *Lactobacillus salivarius* UCC118. *Appl. Environ. Microbiol.* 72, 4143–4153.

Ventura, M., Turroni, F., Canchaya, C., Vaughan, E. E., O'toole, P. W., and Sinderen, D. V. (2009). Microbial diversity in the human intestine and novel insights from metagenomics. *Front. Biosci.* 14:3214–3221.

Ventura, M., Turroni, F., Motherway, M. O. C., Macsharry, J., and Van Sinderen, D. (2012). Host-microbe interactions that facilitate gut colonization by commensal bifidobacteria. *Trends Microbiol.* 20, 467–476. doi: 10.1016/j.tim.2012.07.002

Voltan, S., Martines, D., Elli, M., Brun, P., Longo, S., Porzionato, A., et al. (2008). *Lactobacillus crispatus* M247-derived H2O2 acts as a signal transducing molecule activating peroxisome proliferator activated receptor-γ in the intestinal mucosa. *Gastroenterol.* 135, 1216–1227. doi: 10.1053/j.gastro.2008.07.007

Yadav, A. K., Tyagi, A., Kaushik, J. K., Saklani, A. C., Grover, S., and Batish, V. K. (2013). Role of surface layer collagen binding protein from indigenous *Lactobacillus plantarum* 91 in adhesion and its anti-adhesion potential against gut pathogen. *Microbiol. Res.* 168, 639–645. doi: 10.1016/j.micres.2013.05.003

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Sensory Assessment by Consumers of Traditional and Potentially Probiotic Green Spanish-Style Table Olives

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This work presents the sensory characterization by consumers of traditionally and potentially probiotic green Spanish-style table olives. To this aim, green Manzanilla olives from the same lot were debittered, washed, and brined in the same way; then, one sub-lot was allowed fermenting spontaneously while another one was inoculated with a putative probiotic bacterium (*Lactobacillus pentosus* TOMC-LAB2). After fermentation, the olives from both sub-lots were packed with fresh brine to reach 5.5 g/100 mL NaCl and 0.6 g lactic acid/100 mL in the equilibrium. The stabilized olives were then subjected to sensory evaluation by 200 consumers, and the results were analyzed by ANOVA and multivariate statistical techniques. In a first approach, consumers perceived the spontaneously fermented olives as similar to the potentially probiotic product. However, a biplot based on Canonical Variate Analysis (CVA) showed differences between them in the Salty and *Overall score*. When data from the consumer test were assessed by PLS analysis, regardless of the fermentation system, *Overall score,* and *Buying predisposition* were significantly driven by Appearance, Odor, Salty (negatively), Hardness, and Crispness.

Keywords: green Spanish-style table olives, potentially probiotic olives, consumer test, principal component analysis, predictive biplot, canonical variate analysis

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INTRODUCTION

Diverse foods are used as carriers for delivering probiotics to humans. Until recently, the term probiotic was almost exclusively associated with dairy products. However, Chiu et al. (1) showed that *Pediococcus pentosaceus* and *Lactobacillus plantarum* isolated from pickled vegetables were able to inhibit the *Salmonella* invasion in mice. Atrend toward the use of other food systems like vegetables as carriers (2–4) is nowadays evident. A first approach can be the addition of the potentially probiotic organism. *L. plantarum* and *Lactobacillus paracasei* survived in artichokes for at least 90 days, and the anchoring to the product improved their survival through the gastrointestinal tract; particularly *L. paracasei* IMPC2.1 was recovered from stools (5). *L. paracasei*-enriched artichokes had a favorable effect on constipated patients (6) and *L. paracasei LMGP22043* incorporated into artichokes transiently colonized the gut, antagonized with *Escherichia coli* and *Clostridium* spp. and increased the genetic diversity of lactic acid bacteria (LAB) (7). The adhesion of Lactobacilli and bifidobacteria onto ripe table olives with the aim of formulating a new probiotic food has also been reported (8).

But the lactic acid fermentation of vegetables is an ancient tradition from all around the world and would be an attractive mechanism for incorporating the appropriate bacterium into their final products. The use of LAB with specific characteristics as starter cultures is a common practice in diverse plant materials. Its application may lead to faster acidification and other technological, nutritional, health, or safety advantages as well as to produce a favorable influence on the organoleptic properties (9). The use of the probiotic *L. plantarum* L4 and *Leuconostoc mesenteroides* LMG 7954 as starter cultures for cabbage head fermentations led to products which could be considered as probiotic [alive cell counts >6 $\log_{10}$ CFU/g product; Verganovim (10)]. A starter culture of *L. plantarum* optimized the lactic acid fermentation of York cabbage and produced a ∼5 $\log_{10}$ CFU/mL increment in bacterial growth. The population level persisted for longer than 15 days' storage in a cold room (4°C); therefore, inoculation can be used for developing potentially probiotic products (4). However, the matrix may play an important role in the probiotic development and efficacy (2). Due to the peculiar characteristics of table olives (11), the use of an adapted starter culture could result essential for its implantation. Mourad and Nour-Eddine (12) were the first to make an *in vitro* a *L. plantarum* preselection from olive microbiota based on probiotic criteria. Bevilacqua et al. (13) chosen potential multifunctional starter cultures from Bella di Cerignola table olives. Hurtado et al. (14) reviewed the use of LAB starters and outlined the possibility of using table olives as probiotics. Bautista-Gallego et al. (15) made a screening of LAB, mainly *Lactobacillus pentosus*, from wild Spanish-cultivar fermented table olives to be used later as probiotic starters. Argyri et al. (16) have selected from Greek cultivars several strains of *L. plantarum* and *L. paracasei* with similar properties than *Lactobacillus casei S*hirota and *Lactobacillus rhamnosus.* Botta et al. (17) investigated the microbiota of the Italian cultivar Nocellara Etnea to identify new probiotics LAB with the same objective, suggesting *L. plantarum* S11T3E as an interesting candidate. Peres et al. (18) also characterized the potential probiotic features of strains of LAB from Galega fermentations, finding 10 strains belonging to *L. plantarum* and *Lactobacillus paraplantarum* who had probiotic value. Recently, Guantario et al. (19) found one strain of *L. pentosus* and *Lactobacillus coryniformis* who were able to out-compete foodborne pathogens for cell adhesion and were promising stater candidates for manufacturing table olives with probiotic added value.

In any case, the presence of the strain in the brine does not assure its intake. It is essential to study their incorporation onto the olives since only these are ingested. De Bellis et al. (20) used *L. paracasei* IMPC2.1 to control the green Spanish-style fermentation and colonize the olive surface. A biofilm formation on glass and fruits during green Gordal (21) and Manzanilla (22) table olive fermentation has been reported. The imposition of the probiotic strain on olives has been demonstrated to be highly dependent on its characteristics, circumstance that confirms the importance of the matrix and a proper starter. In green olives cv. Halkidiki, inoculated with *L. pentosus* B281 and *L. plantarum* 282, the first strain was best adapted to the fermentation environment and survived in high number in both low (8% NaCl) and high (10% NaCl) brines while the second did not survive at 10% NaCl; besides, when inoculated on co-culture, only *L. pentosus* B281 was recovered at a high number (>90%) from the olive fruits (23). Similar results were also observed using heat shocked green olives where both strains dominated over the indigenous LAB, but *L. pentosus* B281showed also higher proportions of recovery in the 10% NaCl brine and dominate in the case of co-culture (24). As observed, an adequate starter can provide a considerable LAB load on fermented olives. An exhaustive report on the diverse strains of LAB and yeasts proposed as starter cultures can be found elsewhere (25). As a result, several reports have emphasized the role of table olives as adequate carriers for delivering probiotic bacteria to humans (26, 27), particularly when the LAB strains were previously isolated and characterized from olive microbiota (25). Among the challenges mentioned by Champagne and Gardner (28) for the probiotic production are the determination, in the product, of the appropriate cell population, and the effect that the potentially probiotic starters had on the sensory properties of the fermented products. In inoculated fermented green Spanish-style olives from the diverse cultivars and countries, the sensory characteristics of the fermented product were comparable to those of the traditional lactic acid fermentation (23, 29), but when using heat socked green olives, the sensory assessment of products showed higher preference for the olives from probiotic inoculated fermentations (24). The analysis of consumer preferences for table olives, in the case of Albanian urban consumers, is an interesting approach to disclose the motivation regarding driving olive consumption, mainly with the interest of improving the local offer (30). However, the comparative response of consumers against the traditional and probiotic origin packaged table olives has been scarcely studied and still represents an attractive research issue.

The goal of this work was the sensory characterization of traditional spontaneously fermented and potentially probiotic green Spanish-style Manzanilla table olives. The study is based on the data obtained from the evaluation of olives from both fermentation systems by 200 consumers. The results are analyzed by ANOVA and multivariate techniques. The study may be useful for predicting the possible response of consumers against the eventual commercialization of potentially probiotic table olives.

MATERIALS AND METHODS

Processing

The experiment was carried out with Manzanilla fruits (*Olea Europaea pomiformis*) from the same lot debittered, washed, and brined similarly. In short, it consisted on treating 60 kg olives with 40 L lye (2.2 g NaOH/100 mL) until this reached 2/3 of the flesh (∼5 h), followed by an overnight washing to remove the excess of alkali. The debittered fruits were then brined with 40 L 11% NaCl solution, acidified with 0.5 L 10 N HCl. After 2 days, the pH was down corrected to 7.5 units at equilibrium by bubbling CO_2. Finally, the olives were subjected to two different fermentation systems. One sub-lot was allowed fermenting spontaneously while another one was inoculated with the potentially probiotic bacteria *L. pentosus*

TOMC-LAB2 (LAB2), which population size was chosen to reach ≈6 $\log_{10}$CFU/mL after addition to the fermenters. The strain had been isolated from wild spontaneous green Spanish-style fermentation processes and was selected because its promising probiotic results according to *in vitro* phenotypic tests (high resistance to gastric and pancreatic digestion, hydrophobicity, auto-aggregation, or capacity of deconjugating bile salts) (15). Their fermentative processes are described elsewhere (31).

Fermented Olive Packaging

The fruits from two replicates of the spontaneous and LAB2 inoculated processes were thoroughly washed and packaged in plastic containers (5 kg olives and 2.7 L brine). The characteristics of the packing brine were fixed to reach, after equilibrium in a cold room (7°C), 5.5 g NaCl/100 mL, and 0.6 g lactic acid/100 mL, similar to the usual concentrations of these parameters in the commercial presentations (11). Before being tested by consumers, samples from each replicate of both fermentation methods were withdrawn and analyzed. The study included brine parameters, fruit characteristics, and commercial classification (after tempered at room temperature).

Physicochemical Analyses

The physicochemical characteristics of the cover brines were analyzed according to the methods used routinely in table olive control (11). The olive color was measured using a BYK-Gadner Model 9000 Color View Spectrophotometer (Silver Spring, MD, USA), covering the samples with a box which had a matt black interior. Olive color was expressed as CIE L^*, a^*, b^* parameters. Color Index (*Ci*) was estimated by the formula: $\mathrm{Ci} = \frac{-2^*R_{560} + R_{590} + 4^*R_{635}}{3}$, where *R*s are for the reflectance at 560, 590, and 635 nm, respectively (32). Recorded data were the average of 10 olive measurements.

The firmness of the olives was measured on three pitted olives using a Kramer shear compression cell coupled to an Instron Universal Machine (Canton, MA, USA) with a crosshead speed of 200 mm/min. Recorded data were the mean of three olive replicates, expressed as N/100 g flesh.

Microbiological Analyses

The microbiological analyses of brines and olives were performed according to previously described procedures (29, 31, 33). Briefly, appropriate dilutions of the brine samples were plated using a Spiral System model DwScientific (Don Whitley Sci. Ltd., Shipley, UK). The following media were used for the examination of the usual microbiota: VRBD (Crystal-violet Neutral-red bile glucose)-agar (Merck, Darmstadt, Germany) for *Enterobacteriaceae*; MRS (de Man, Rogosa and Sharpe)-agar (oxoid) supplemented with 0.02% (w/v) sodium azide (Sigma, St., Luis, USA) for LAB; and YM (yeast-malt-peptone-glucose)-agar (Difco™, Becton, and Dickison Company, Sparks, MD, USA) supplemented with oxytetracycline and gentamicin sulfate as selective agents, for yeasts. Plates were incubated for 24 h at 30°C for *Enterobacteriaceae* and 48 h at 30°C for yeasts and LAB; Counting was achieved by a CounterMat v.3.10 (IUL, Barcelona, Spain) image analysis system. Results were expressed as $\log_{10}$CFU/mL.

Characterization of the LAB Population on Olives

Before the sensory evaluation, the biofilms from the packaged olives (of both fermentation methods) were detached, and 20 LAB isolates from the suspension were randomly obtained and coded S, for spontaneous, and P, for probiotic. The lactobacilli were subjected to RAPD-PCR analysis with primer OPL_5 according to the protocol described by Rossi et al. (34). Their banding profiles (from 100 up to 4,000 bp) were then compared with those of the inoculated strain. For this purpose, PCR products were electrophoresed on a 2% agarose gel and visualized under ultraviolet light by staining with ethidium bromide. The resulting fingerprints were digitally captured and analyzed with the BioNumerics 6.6 software package (Applied Maths, Kortrijk, Belgium). The similarity among the digitalized profiles was calculated using the Pearson product-moment correlation coefficient. The dendrogram was generated using the Unweighted Pair Group Method using the Arithmetic Average (UPGMA) clustering algorithm.

Olive Classification

Before the consumer test, the packaged olives were subjected to classification analysis to assure they had proper commercial quality. The evaluation was conducted in the standardized testing room of the Food Biotechnology Department (IG-CSIC, Sevilla, Spain) under controlled environmental conditions (temperature, humidity and light), using individual booths. The panelists were 12 experienced judges of the Department's staff, habitual consumers of olives with a high level of training due to their participation for decades in the development of the method for the sensory analysis of table olives, issued by the International Olive Council (35, 36). For the test, the evaluation sheet suggested by IOC (36), with an unstructured 10 cm scale, anchored to 1 (no perception) and 11 (extremely intense) was used. In this test, only those attributes used for olive classification according to the IOC methodology (36) were evaluated. Therefore, the assessment in this phase was limited to abnormal fermentation and other defects (e.g., musty, rancid, cooking taste, soapy, metallic, earthy, and winery-vinegary). The marks on the sheet were measured, from the left anchor, with the precision of one decimal place. The analyses were performed in duplicate.

Consumer Test

The consumer test was based on the gustatory (Acid, Salty, and Bitter) and kinesthetic sensations (Hardness, Fibrousness, and Crispness) of the evaluation sheet from the Methods of Sensory Analysis of Table Olives (36). However, the final sheet also included two additional queries: *Overall score* (liking) and *Buying predisposition*. The same 10 cm unstructured scale (anchor rating 1–11) and measurement procedure as in olive classification were applied. The test was performed in one of the most popular Markets in Seville (Mercado de la Encarnación), in a place close to a table olive shop. Only self-reported table olive consumers, at least 2–3 times a month olive consumer, non-diet restrictions, and persons who agree to perform the test were chosen. As the LAB tested were selected from the natural wild microbiota of spontaneous fermentation, no special

issues related to health risks were required according to our Ethical Committee. The packaged samples from spontaneously or LAB2 fermented olives, previously classified as Extra/Fancy, were offered to people, under white light, in 150 mL plastic jars labeled with a randomly chosen three-digit code. The fruits were presented as a completely randomized block design. Water was provided to consumers for rinsing their palates between samples, and a 2 min rest was enforced to minimize the carry-over effect. There was no pre-established period for performing each session. The total number of panelists recruited was 200 (105 women and 95 men, with age ranging 20–65 years old).

Statistical Data Analysis

Panel results for the olive classification were analyzed according to the procedure established in the Method of Sensory Analysis of Table Olives (35). Data from the consumer assessment were subjected to ANOVA, and *post-hoc* tests with the objective of finding possible differences between fermentation systems within attributes, *Overall score*, and *Buying predisposition*. Also, the data were subjected to Discriminant Analysis (DA). For mapping the samples and visualizing their relationships with the sensory variables, Principal Component Analysis (PCA), Predictive Biplots (PB), and Canonical Variate Analysis (CVA), which determine the position of the points by the two first canonical variates were used. Also, PLS regression was applied to associate sensory attributes with the *Overall score* and *Buying predisposition* and study the relationship between them. The analyses were performed with XLSTAT 2014 (Addinsoft, Paris, France) and the Biplot GUI package which provides a graphical user interface for the construction of, interaction with, and manipulation of biplots in R (37).

RESULTS AND DISCUSSION

Characteristics of the Spontaneously Fermented and LAB2 Inoculated Packaged Olives

The fermentation process was considered finished after 4 months (the sugars had been exhausted). Then, the physicochemical conditions, quite similar in both fermentation systems, were approximately: pH, 4.0, lactic acid, 15 g/L; NaCl, 55 g/L. Then, the inoculated with LAB-2 (or just probiotic) and the spontaneously processed olives were conditioned and packaged as specified in the Material and Methods section. After equilibrium in the fresh brine used for packaging, the physicochemical and microbiological features of the containers from both fermentations showed similar levels (**Table 1**). Furthermore, NaCl and titratable acidity concentrations at equilibrium were also close to the presumed levels.

The lack of significance of the differences between the two methods in pH and LAB populations in brine, which were lower in the probiotic than in the spontaneously fermented olives, was due to the high standard errors found for these parameters in the olive packages which olives followed the spontaneous process. Such variability can be associated with the usual diverse evolution of non-controlled replicates which is the biggest obstacle for achieving homogeneous products with the traditional process (11).

The surface color of the packaged olives (**Table 2**) was also similar, regardless of the fermentation system, with very close levels for CIE L^*, a^*, b^* parameters and color index. Only the luminance (L^*) of the probiotic fruits was slightly (non-significantly) higher than the values found in the spontaneously fermented olives, due to their lower pH. L^* *and* b^* values of the color parameters were very similar to those found in the

TABLE 1 | Average values of the physicochemical characteristics and microbial populations in the packaging brine from spontaneously and LAB2 fermented olives.

Fermentation method	NaCl (g/100mL)	pH	Titratable acidity (g/100mL)*	Combined acidity (Eq/L)	Microbial population in brine (log_{10}CFU/mL)	
					LAB	Yeast
Probiotic	5.44 (0.02)	3.63 (0.02)	0.69 (0.05)	0.059 (<0.001)	3.62 (<0.01)	4.32 (<0.01)
Spontaneous	5.46 (0.07)	4.00 (0.32)	0.61 (0.04)	0.060 (0.001)	4.23 (1.04)	4.24 (0.16)

*Standard error in parenthesis; No statistical difference between processing methods was found within any parameter; *expressed as lactic acid; LAB, lactic acid bacteria; Enterobacteriaceae were never found.*

TABLE 2 | Average values of surface color, firmness, and microbial load on packaged fruits from spontaneously and LAB2 fermented olives.

Fermentation method	Surface color				Firmness (N/100 g pitted olives)	Microbial load on the olive surface (log_{10}CFU/olive)	
	L^*	a^*	b^*	Color index		LAB	Yeast
Probiotic	54.3 (0.3)	2.6 (0.3)	35.9 (0.2)	26.9 (0.41)	1128 (105)	7.59 (0.04)	4.25 (0.76)
Spontaneous	52.9 (0.3)	2.9 (0.3)	35.3 (0.4)	26.2 (0.18)	1237 (132)	6.71 (0.81)	4.67 (<0.01)

*Standard error in parenthesis; No statistical difference between processing methods was found within any parameter; *CIE, parameters; LAB, lactic acid bacteria; Enterobacteriaceae were never found.*

fermented product, but those of *a** had increased slightly (31). The values of the three parameters were initially lower (about 48, 27, and 1.83, respectively) in green cv. Halkidiki table olives, fermented with potentially probiotic strains, and packaged under modified atmosphere, regardless of the storage temperature (4 or 20°C). But at the end of the product storage, the values of *L** *and b** were similar to those found in this work (38).

Firmness was also statistically the same in spontaneously fermented and probiotic olives (**Table 2**). However, packaging had sensibly reduced firmness with respect to the fermented product which was about 1,600 and 1,400 N/100 pitted olives for spontaneous and LAB2 fermented olives, respectively (31). Comparison with other similar Greek products is difficult because the different measurements technologies used, although no marked changes with storage time were observed (38).

Finally, LAB and yeast populations in the olive biofilms from both fermentation methods were similar, but the average in probiotic fruits was higher in the LAB and lower in yeast counts. However, the high variability of the LAB in the spontaneous process and yeast in the probiotic olives prevented the differences from being significant. The opposed relationship between LAB and yeast populations in the biofilm on these olives, regardless of the processing method, are comparable to those found in other works (21, 22) or in packaged olives (39). Also, the microbial populations on olives in this work are comparable with those found in Halkidiki cultivar fermented with probiotic *L. pentosus* and *L. plantarum*, packaged under controlled atmosphere and stored at 20°C; however, at 4°C the LAB population increased, but the yeast counts were less affected. To notice that, in this case, there were no differences in behavior among inoculated and spontaneous treated olives and at the end of the storage period, the influence of temperature was reduced but retaining the prevalence of LAB (38).

In any case, according to Verganovi (10), the LAB population in the olive biofilm of the probiotic olives could provide a considerable intake of the putative probiotic LAB2 strain (**Table 2**). This high value of LAB on the olives supports the suitability of table olives as carriers of potentially probiotic organisms (25–27). When added this characterisitic to the natural composition of olives, which includes mono and polyunsaturated fat (11 and 1.2 g/100 g olive flesh, respectively), dietary fiber (2.6 g/100 g olive flesh), vitamin E (4.6 mg α-tocopherol/100 g olive flesh), phystosterols (2,700 mg/kg olive fat), and the practical absence of cholesterol and sugars (40–43) as well as the numerous properties attributed to the olive polyphenols (anticancer, hemoprotective, anti-inflammatory, antimicrobial, antihypertensive, among others) (44), the table olives may be considered as a potential symbiotic food.

Therefore, according to the previous comments on this work, the physicochemical characteristics (NaCl, pH, titratable acidity, and combined acidity), olive color (*L**, *a**, *b**, and *CI*), firmness, and microbial populations in brines and on olives (LAB and yeasts) of the packaged putative probiotic olives were similar to those from spontaneous fermentation and the differences observed by consumers could only come from the applied fermentations systems (potential probiotic over spontaneous).

Characterization of the LAB Population in the Olive Biofilm

The molecular analysis of the 20 LAB strains isolated from the biofilms, both the spontaneous and probiotic fermented packaged olives, showed that the profiles of the LAB population on the fruits from the probiotic olives was 90% similar to that of the LAB2 strain used as starter (**Figure 1**). On the contrary, the LAB isolates from the spontaneously fermented packaged olives only had 4.5% similarity with LAB2 and, therefore, their strains were completely different. Furthermore, the isolates from the spontaneous fermentation formed six different clusters. As a result, the packaged fruits from the inoculated fermentation were, effectively, carriers of the putative probiotic LAB2 strain used as starter while the olives from the spontaneous process carried only wild strains (with six different genotypes). The consumer test was then performed between real potentially probiotic carriers of LAB2 (inoculated olives) and wild LAB (spontaneous fermentation).

In the already mentioned packaging of cv. Halkidiki under controlled atmosphere, the survival rate of *L. pentosus* B281 was always higher than that of and *L. plantarum* B282, and with time the percentage of both decreased sensibly, and only *L. pentosus* B281 was found after 168 days (38). In Spanish-style Manzanilla olives fermented with putative probiotic LAB, the highest counts (6.2–6.5 $\log_{10}$CFU/mL) on olive epidermis were found in fruits stored in glass jars and plastic pouches at 22°C. In general, the correlation between the LAB and yeasts populations were always negative (39). In fortified Manzanilla packaging, the inoculated treatments always had higher populations on the olive surface (4.41–6.77 $\log_{10}$CFU/cm^2) than their respective controls (0.00–4.33 $\log_{10}$CFU/cm^2) and the added strain (*L. pentosus* TOMC-LAB2) was recovered at the end of the shelf life (200 days) at frequencies ranging 53–100% (45).

Packaged Olive Classification

The objective of this test was checking that the olives from the two fermentation methods (spontaneous and probiotic) had appropriate commercial quality. Then, it was considered as a first step in the evaluation of the packaged fruits. After analyzing the scores assigned by the panel to abnormal fermentation and other defects, the average values (data not shown) were very close to 1.0. Then, the levels obtained were far below the set upper limit (3.0) for considering any sign of spoilage according to the Sensory Analysis of Table Olives (36) and, subsequently, the olives from both fermentation methods were classified as Extra (or Fancy). Therefore, both the spontaneously fermented and potentially probiotic packaged olives had the highest commercial quality and were appropriate for consumer evaluation.

Results of the Consumer Test

Sensory Scores of Spontaneously Fermented and Potentially Probiotic Packaged Olives

The average scores for the gustatory and kinesthetic sensations as well as for *Overall score* and *Buying predisposition* were

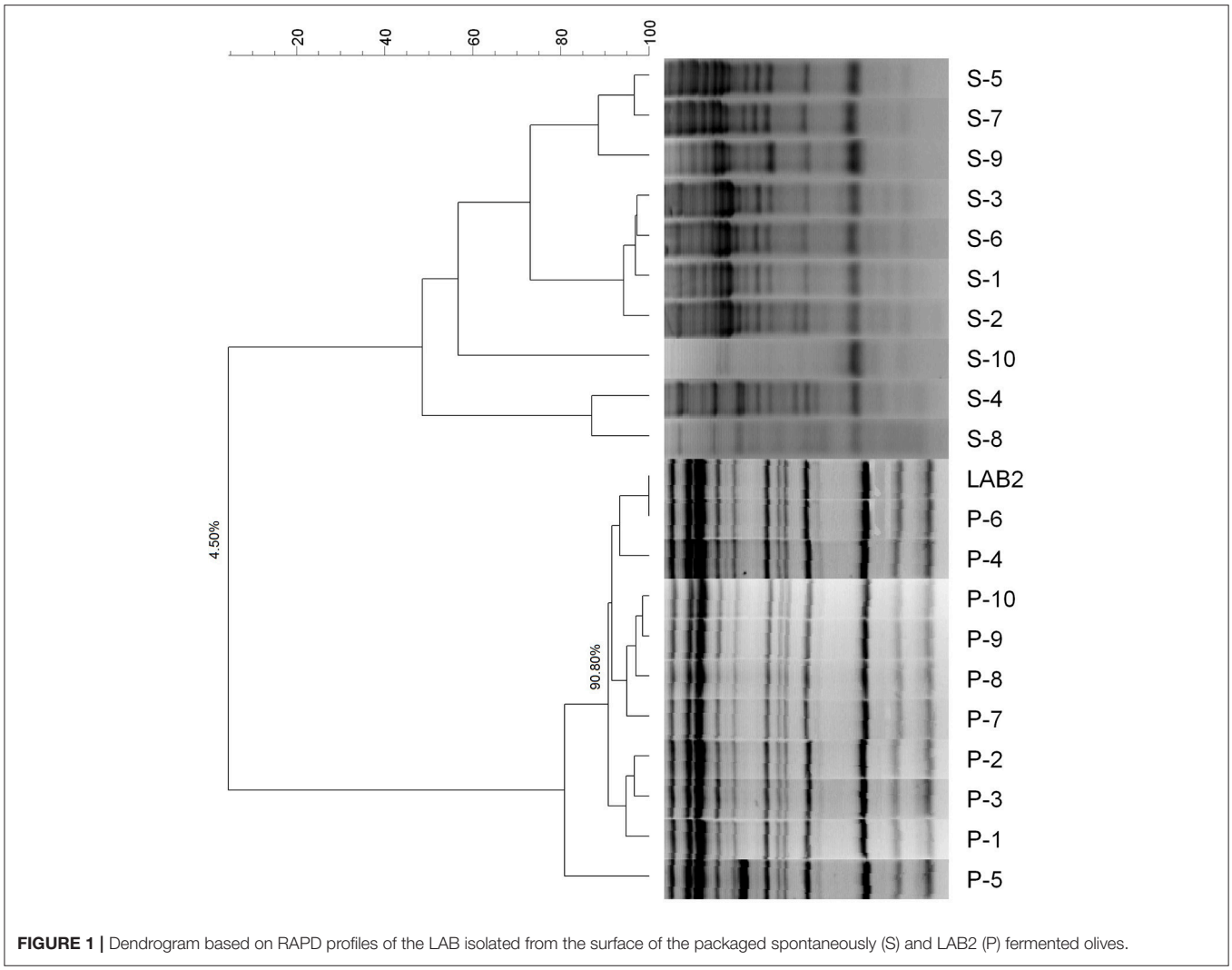

FIGURE 1 | Dendrogram based on RAPD profiles of the LAB isolated from the surface of the packaged spontaneously (S) and LAB2 (P) fermented olives.

similar and did not lead to any significant difference ($p < 0.05$) between the spontaneously fermented and potential probiotic packaged olives (**Table 3**). Also, the proportions of consumers who assigned a higher *Overall score* to the potentially probiotic and to the spontaneous olives (or vice versa) were statistically the same according to the χ^2-test.

The cv. Halkidiki fermented as Spanish-style table olives with *L. pentosus* B281, *L. plantarum* B282 or subjected to the spontaneous process were also sensory analyzed after packaging in modified atmosphere. At the beginning of the storage, the inoculated samples were preferred (higher acceptability index scores) over those from the traditional process; furthermore, those treated with *L. pentosus* B281 were chosen preferently. However, storage reduced acceptability, although retaining the preferences for the inoculate olives. After 12 months storage period, those olives which followed the spontaneous process were almost unacceptable while *L. plantarum* B282 retained acceptability index similar to those stored for 6 months and was preferred to those fermented with *L. pentosus* B281 (38). After packaging, the cv. Manzanilla fermented with diverse strains of *L. pentosus* showed, for all the attributes evaluated (36), levels around the score center (5.5–6.4) while the fruits from the spontaneous processed developed a clear abnormal fermentation, circumstance that reinforces the convenience of inoculation as a way of the safety initiation of green Spanish-style fermentation (29). Randazzo et al. (46), studied the sensory effect of using probiotic cultures as starters in non-lye treated Giarrafa and Grossa di Spagna and results were significantly cultivar-dependent. In heat-shocked olives, the salty scores were strongly associated with brine concentration but received similar acidity punctuation, except control (lowest) at 8% NaCl. Regarding bitterness, the inoculated process had high levels at 8% NaCl but the control and inoculated with *L. pentosus* were perceived as more bitter when processed at 10% NaCl. The inoculated olives (*L. pentosus and L. plantarum)* at 8% NaCl were the preferred olives, followed by those fermented with *L. plantarum* at 10% NaCl (24).

However, the tendencies observed in this work regarding several attributes like Acid (possibly due to the slightly lower pH of the probiotic packaged olives), Bitter, Salty (also

TABLE 3 | Average values of sensory attributes from the consumer test and *Overall score* and *Buying predisposition*, according to olive processing method.

Sensory variable	Average score		Statistical comparison		Preference		Preference test	
	Probiotic	Spontaneous	*F*-value*	*p*-value	Probiotic	Spontaneous	X^2	*p*-value
Appearance	9.05 (1.55)	8.99 (1.54)	0.1661	0.68	94	106	0.0036	0.0000
Odor	7.87 (2.08)	8.06 (1.88)	0.9691	0.32	92	102	0.0064	0.0000
Acid	5.27 (2.51)	5.06 (2.50)	0.7179	0.40	110	90	0.0100	0.0000
Bitter	3.21 (2.29)	3.18 (2.24)	0.0127	0.91	111	89	0.0100	0.0000
Salty	7.31 (1.91)	7.13 (1.93)	0.6968	0.40	105	95	0.0025	0.0000
Hardness	4.70 (1.92)	4.56 (1.89)	0.5445	0.46	102	98	0.0004	0.0000
Fibrousness	3.97 (2.24)	3.94 (2.21)	0.0356	0.85	99	101	0.0004	0.0000
Crispness	3.77 (2.12)	3.78 (2.23)	0.0033	0.95	94	109	0.0036	0.0000
Overall score	7.96 (1.73)	8.20 (1.56)	2.2855	0.13	92	108	0.0064	0.0004
Buying predisposition	7.10 (2.61)	7.33 (2.52)	0.7540	0.39	77**	101**	0.0196	0.0004

*Preference, highest values in the Overall score, is also included. Standard deviation in parenthesis; *F values for 1 and 398 fd; **same score, 22.*

possibly related to the pH level), and Crispness should not be underestimated. They may indicate the presence of some subtle trends in consumer preferences whose in-depth study could be of interest.

Multivariate Analysis of the Scores Assigned by Consumers

There were many significant ($p < 0.05$) correlations among the attribute scores. For example, Appearance vs. Odor (0.462), Bitter (−0.270), Fibrousness (−0.221), *Overall score* (0.265), and *Buying predisposition* (0.269); Acid vs. Bitter (0.369) and Hardness (0.355); Bitter vs. Fibrousness (0.360), and Crispness (0.455); or *Overall score* vs. *Buying predisposition* (0.688). Therefore, the data were appropriate for multivariate exploration.

The PCA of the sensory variables extracted four eigenvalues higher than 1 which accounted for ∼64.39% of the total variance. Their projection onto the plane of the first two Dimensions (D1 and D2) (∼51% of variance), after varimax rotation, showed that Appearance and Odor were closely related but negatively linked to D1 (**Figure 2**). Crispness, Fibrousness, and Bitter were also closely related and positively correlated to D1 (**Figure 2**). D1 could then be associated with maturation degree. In fact, the opposed relationship between the previous two groups of attributes is reasonable since the less mature (higher Bitterness, Fibrousness, and Crispness) the more difficult is obtaining the typical Spanish-style characteristics (Odor and Appearance) after fermentation. D2 was linked to Hardness which, in turn, was hardly associated to Odor and Appearance or Fibrousness, Crispness and Bitter because of the close to 90° angle (cosine = 0) between the first and the last two groups (**Figure 2**). Finally, the vectors of Acid and Salty (mainly), although following similar directions (linked to some correlation), were relatively short, indicating that they might be not well represented by the first two Dimensions; in fact, Acid was closely related to D3 (correlation, 0.6379) and Salty to D4 (0.796). The graph was then useful to show the association among variables in an intuitive way and

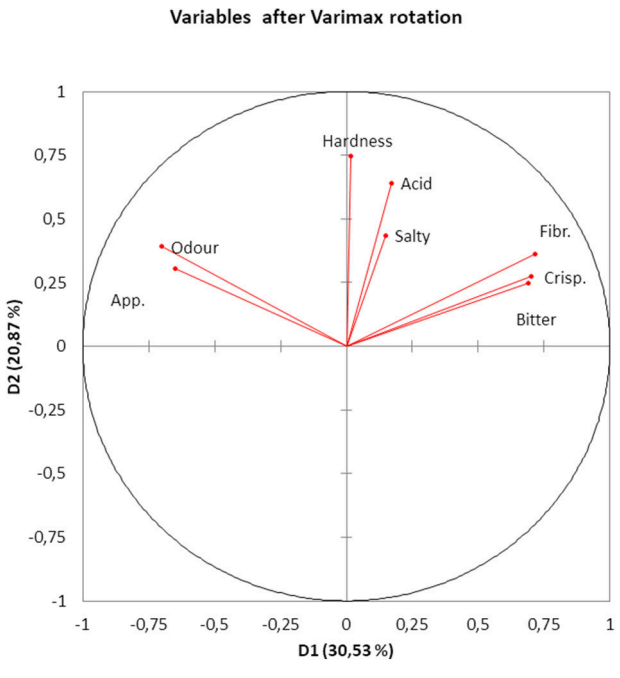

FIGURE 2 | PCA analysis of the result from the consumer test, based on all responses. Projection of variables onto the first two Principal Components. App., Appearance; Fibr., Fibrousness; Crisp., Crispness.

revealed that the number of variables evaluated in the Sensory Evaluation of Table Olives (36) could be reduced and the analysis simplified.

In case of Halkidiki cultivar Spanish-style table olives, clustering was mainly achieved regarding storage time, with the greatest distance between olives stored for 168 days and the rest with slight differences between 0 and 370 days storage. By using PCA, three segregated groups were noticed, with time being

also the most influential parameter. Also, olives samples after 6 months storage were strongly correlated with L^* while those with 12 months storage were linked to b^* values. Besides, the just packaged olives were associated with acidity, a_W, and a^* values and the olives at the end of storage with pH (38).

Due to the high number of consumers and their random distribution close to and around the origin (without any clear segregation between potential spontaneously fermented and probiotic olives), their relationships in the plane of the first two Dimensions were difficult to interpret. Furthermore, the DA led to a correct overall classification of around 50%, similar to that expected just by random assignation to groups. However, a more in-depth study of the tendencies mentioned above could still be of interest.

Mapping the Sensory Tendencies by Biplot

One approach to this study was accomplished by condensing the answers of consumers into a reduced number of data. In this way, the number of cases would be manageable without losing information. To this aim, the initial 200 consumers were randomly assigned to 8 groups of 25 consumers each, and their sensory variable averages estimated. The values obtained (eight averages per fermentation method), representing the population of answers, were now a manageable number of cases, with an easy visualization on any graph and, particularly, on the biplots.

Usually, a biplot simultaneously provides information on both the samples and the variables of a data matrix in two and, if necessary, three dimensions. A recent improvement consists of incorporating information on the original variables by linear or non-linear axes. Implementation of the goodness of fit measurements, convex hulls, and classification regions markedly assist in the interpretation of results (37). In this work, the PCA biplot with predictive axes was applied to the data matrix formed with the eight averages of each sensory attribute and their respective *Overall score* and *Buying predisposition*. Apart from the relationships among variables, in the biplot obtained (**Figure 3**) because of the use of calibrated axes, it is possible to read the predicted values (and their errors) of each point (average of 25 answers) by projecting perpendicular lines onto the respective axes. For example, the predicted (average) values for the left upper point in the second quadrant would be: Appearance, 8.7 (with a relative error of 8.3%); Odor, 7.90 (14.6%); Acid 5.73 (1.1%); Bitter, 3.69 (3.9%);

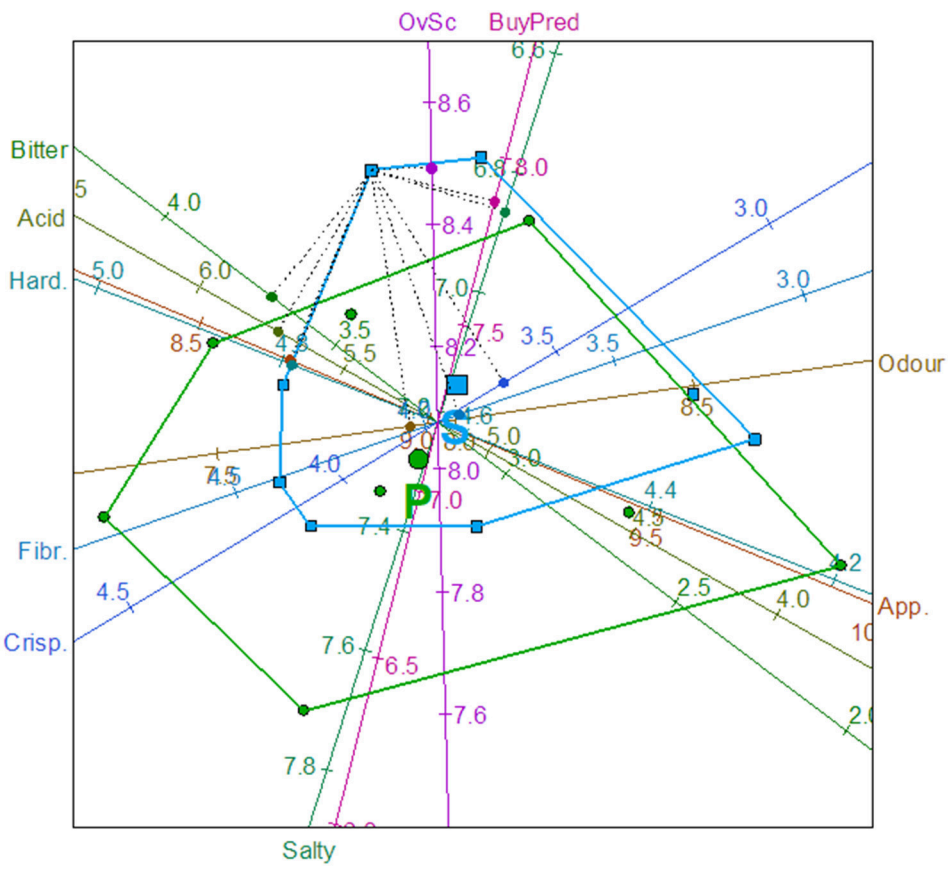

FIGURE 3 | PCA Biplot of the average over 25 consumers according to sensory variables (App., Appearance; Hard., Hardness; Fibr., Fibrousness; Crisp., Crispness), *Overall score* (OvSc), and *Buying predisposition* (Buypre). Convex hulls for spontaneously (S) and potentially probiotic (P) fermented olives. Predictions for one average, are also shown.

Salty, 6.87 (11.0%); Hardness, 4.79 (5.9%); Fibrousness, 3.91 (15%); Crispness, 3.62 (17.6%); *Overall score*, 8.49 (12.7%), and *Buying predisposition*, 7.87 (11.6%). The predictions of the biplot were then fairly good for some variables (e.g., Acid and Bitter) while were relatively less precise for others (e.g., Fibrousness and Crispness). However, the most relevant information from this graph (**Figure 3**) is the disposition of the averages. The centroids of the convex hulls (with the corresponding symbols in a bigger size) were near and the regions associated with spontaneous (S) and probiotic (P) olive fermentations overlapped. Nevertheless, the displacement observed between their convex hulls indicates that there was a tendency for scoring the probiotic olives to the bottom of the graph, although the biplot was not efficient enough for disclosing the possible small differences between the two fermentation methods.

However, a biplot based on the CVA, whose axes are estimated as linear combinations of the original variables that maximally separate the group means, could be even more convenient. With the CVA predictive biplot, the potentially probiotic (on the left) and spontaneously fermented (on the right) olives were segregated (**Figure 4**). Most of the calibrated variable axes are close to the vertical canonical axis and, as a result, had a limited segregating capacity; however, Salty and *Overall score*, which form a close to 90° axes, can be considered responsible for the segregation. The biplot also associated the region on the left with the probiotic olives and that on the right with the spontaneous method. Probiotic fermented olives are then characterized by 7.2–7.4 Salty scores and 7.8–8.1 *Overall scores* while spontaneously fermented olives have lower than 7.2 Salty score and higher than 8.1 *Overall score*.

Relationship Between Sensory Attributes With the Overall Score and Buying Predisposition

Using the sensory attributes as independent variables and the *Overall score* and *Buying predisposition* as a dependent (responses), the relationships between them in the whole matrix of data were quantified by PLS. The application of this statistical tool is rather appropriate since it is particularly useful in case of correlations among predictor variables. Quality indices of the regression for the model with two components (Q^2 accumulated, 0.155; R^2Y accum, 0.206; and R^2X, 0.440) showed a low proportion of the total, Y and X variance explanation.

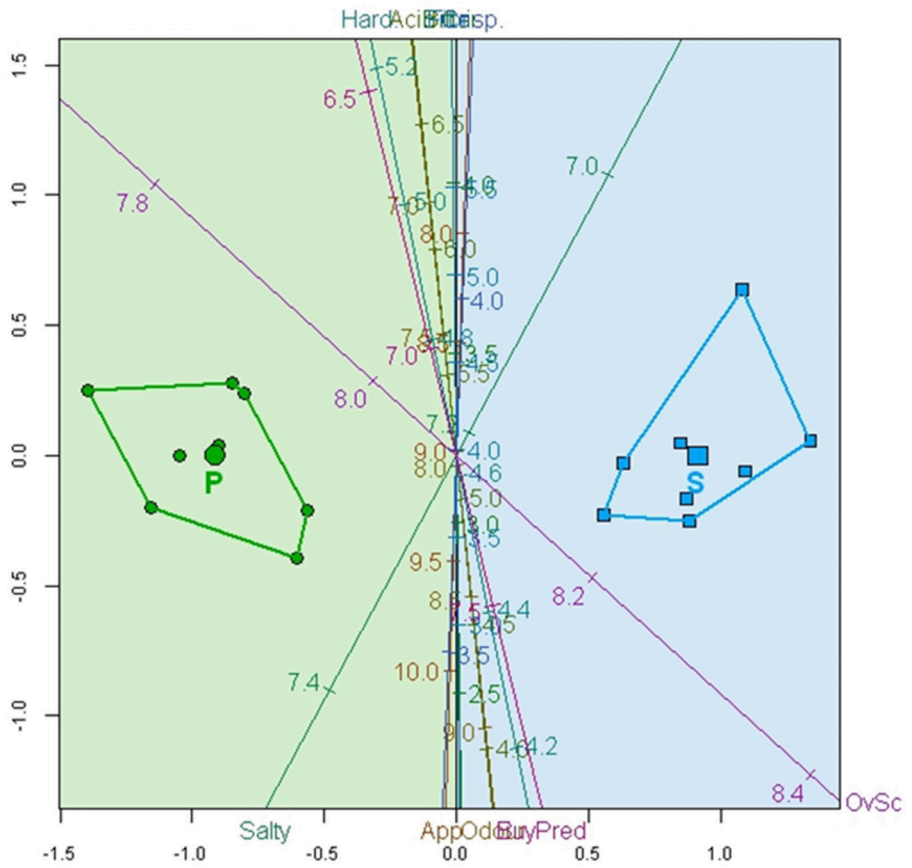

FIGURE 4 | Biplot based on CVA analysis of the average over 25 consumers according to sensory variables (App., Appearance; Hard., Hardness; Fibr., Fibrousness; Crisp., Crispness), *Overall score* (OvSc), and *Buying predisposition* (Buypre). Convex hulls for spontaneously (S) and potentially probiotic (P) fermented olives are also included.

Apparently, some of the motivations of consumers for scoring or buying table olives might not be included in the official evaluation sheet for table olives (36). Subsequently, the analyzed sensory variables had a limited prediction power. The most important variables in the two-component model were Odor and Appearance, in agreement with their proximity to the component t1 and t2 plane (**Figure 5**). Apart from t1, *Overall score and Buying predisposition* were also clearly associated with Hardness, Appearance, and Odor while were negatively related to Salty (**Figure 5**). Mathematically, the equation of *Overall score* and *Buying predisposition* as a function of the sensory attributes were deduced and their standardized coefficients, standard errors, and confidence limits estimated (**Table 4**), with the standardized coefficients being a measurement of their contributions to the responses, regardless of their physical levels. Most of the coefficients were significant and, therefore, predictions can still represent an attractive tool to disclose consumers' attitude. The equations for *Overall score* and *Buying predisposition* had similar structures (coefficients), in agreement with their high correlation (**Figures 4**, **5**); that is, the same attributes have significant contributions (positively and negatively) to both *Overall score* and *Buying predisposition* (**Table 4**). Appearance, Odor, Hardness, and Crispness (positive contributions) improve consumer perception while Salty has a substantial adverse impact.

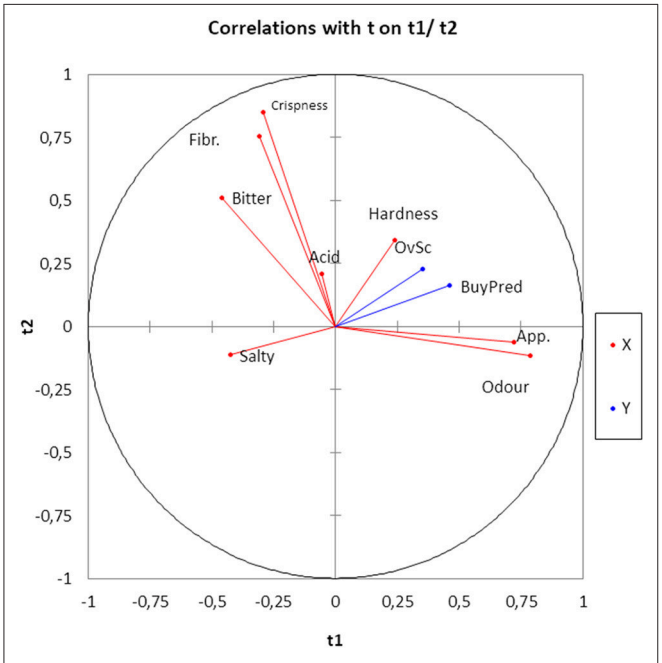

FIGURE 5 | Sensory variables, *Overall score* and *Buying predisposition* projection onto the t1 and t2 plane as the deduced by PLS analysis. Fibr, Fibrousness; OvSC, *Overall score,* BuyPred, *Buying predisposition;* App, Appearance.

The equations (in physical values) were the following:

Overall score = 5.06 + 0.22*App. + 0,17*Odor − 1.73E − 03*Acid − 5.26E − 05*Bitter − 0.20*Salty + 0.10*Hard + 6.17E − 02*Fibr. + 0.12*Crisp.

Buying predisposition = 2.01 + 0.41*App. + 0.32*Odor − 4.97E − 03*Acid − 3.66E − 02*Bitter − 0.33*Salty + 0.17*Hard + 6.25E − 02*Fibr. + 0.15*Crisp.

The performance of these equations was checked by representing the scores assigned by the consumer to these variables vs. the predicted values (obtained from the equations). To facilitate the observation, only 25 cases of those used for the model development and validation, randomly selected, are included in the graph (**Figure 6**). Only a reduced number of cases are outside the Confidence Limits (CL) ($p \leq 0.05$), regardless of the response.

According to the equations, for increasing table olive valorization by consumers, Appearance, Odor (appropriate fermentation), Hardness, and Crispness should be improved (mainly the first two attributes). On the contrary, the Salty perception should be reduced because of its sensible negative influence on the *Overall score* and *Buying predisposition*. The significant negative contribution of Salty (the highest) to *Overall score* and *Buying predisposition* is in agreement with the consumer concern for the association of salt with cardiovascular diseases (47) and the firm determination of consumers (and authorities) to reduce its intake (48–50). Since the consumers' scores regarding salt reflected was not influenced by any previous questing on this issue, they reflected their

TABLE 4 | Standardized coefficients for the *Overall score* and *Buying predisposition* estimated by PLS-R as a function of the sensory attributes.

Sensory variable	*Overall score*				*Buying predisposition*			
	Coeff.	SE	LCL	UCL	Coeff.	SE	LCL	UCL
Appearance	0.200*	0.047	0.108	0.293	0.242*	0.040	0.164	0.319
Odor	0.194*	0.052	0.092	0.295	0.241*	0.036	0.171	0.312
Acid	−0.003	0.065	−0.131	0.126	−0.005	0.077	−0.156	0.146
Bitter	0.000	0.037	−0.073	0.073	−0.032	0.042	−0.115	0.050
Salty	−0.228*	0.058	−0.343	−0.114	−0.250*	0.069	−0.385	−0.114
Hardness	0.116*	0.045	0.028	0.204	0.128*	0.053	0.024	0.232
Fibrousness	0.082	0.064	−0.044	0.208	0.054	0.056	−0.056	0.164
Crispness	0.159*	0.048	0.065	0.252	0.128*	0.053	0.025	0.232

*SE, standard error; LCL and UCL, Low and upper confidence interval (for $p \leq 0.05$); Coeff., coefficients for independent variables; *significant at $p \leq 0.05$.*

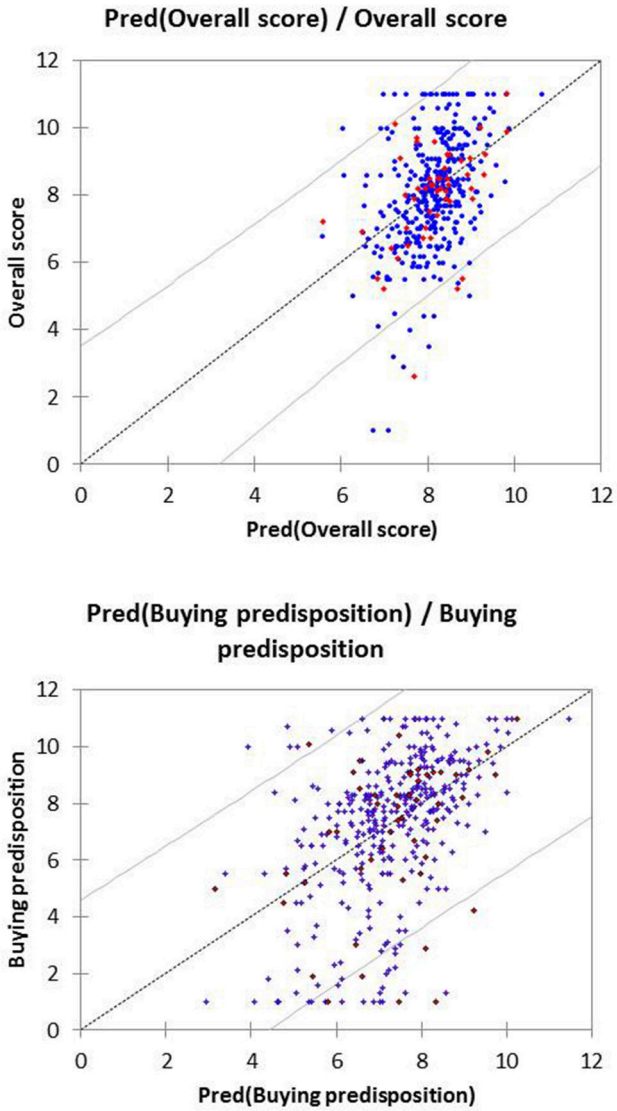

FIGURE 6 | Prediction and their confidence limits ($p \leq 0.05$) from the PLS analysis of the *Overall score* and *Buying predisposition* as a function of the sensory variables. Only 25 cases (active and validation) randomly selected are represented, as examples.

current spontaneous attitude. Therefore, reducing the Salty perception, and the clarification of the relationship among this attribute with pH and Acid (frequently not well differentiated), should then be a priority for a better understanding of the consumers' attitude not only toward probiotic but to table olives in general. Furthermore, due to the favorable influence of low Salty scores on table olive appreciation by consumers, a possible reduction in the salt level of the potentially probiotic olives could be a very straightforward and efficient strategy to facilitate their commercialization. However, the motivation of consumers is by no means simple. In a survey with the objective of studying the motivation of the Albanian population for the consumption of olives, including their origin, Zhllima et al. (30) chosen a limited number of questions namely origin (imported/local), color (green/dark brown), type (plain, pitted, and stuffed) as well as the price they were willing to pay. Using Cojoint Choice Experiment and Latent Class Analysis, the authors were able to segment the demand into four main classes, associating Class 1, mainly with origin; Class 2, with color; Class 3, with price; and Class 4, with price and type. Therefore, many another aspects should also be borne in mind when commercializing table olives. In any case, due to the favorable influence of low Salty scores on table olive appreciation by consumers, a possible reduction in the salt level of the potentially probiotic olives could be a very straightforward and convenient strategy to facilitate their introduction in the market.

CONCLUSION

Overall, green Spanish-style potentially probiotic olives are perceived by consumers as similar to the traditional product. In fact, ANOVA and DA were unable to disclose any differences among them. However, the application of Predictive Biplot based on CVA revealed consumer trends based mainly on the Salty perception and *Overall score*. The sensory attributes with favorable influence on the *Overall score* and the *Buying predisposition* were Appearance, Odor, and Crispness (in a lower proportion); on the contrary, Salty had a marked adverse effect. Therefore, an association of probiotic olives with low Salty perception could facilitate its commercialization.

AUTHOR CONTRIBUTIONS

AL-L, JM-B, PG-G, and AG-F performed the consumers' test. FR-G characterized the physicochemical and microbiological characteristics of the products. AL-L and AG-G designed and supervised the experiment, analyzed and interpreted the data, and drafted the manuscript. All authors approved the final version of the paper.

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REFERENCES

1. Chiu HH, Tsai CC, Hsich HY, Tsen HY. Screening from pickled vegetables the potential probiotic strains of lactic acid bacteria able to inhibit the Salmonella invasion in mice. *J Appl Microbiol*. (2008) 104:605–12. doi: 10.1111/j.1365-2672.2007.03573.x
2. Ranadheera RDCS, Baines SK, Adams MC. Importance of food in probiotic efficacy. *Food Res Int.* (2010) 43:1–7. doi: 10.1016/j.foodres.2009.09.009
3. Rivera-Espinoza Y, Gallardo-Navarro Y. Non-dairy probiotic products. *Food Microbiol.* (2010) 27:1–11. doi: 10.1016/j.fm.2008.06.008
4. Jaiswal AK, Gupta S, Abu-Ghannam N. Optimisation of lactic acid fermentation of York cabbage for the development of potential probiotic products. *Int J Food Sci Tech*. (2012) 47:1605–12. doi: 10.1111/j.1365-2621.2012.03010.x
5. Valerio F, De Bellis P, Lonigro SL, Morelli L, Visconti A, Lavermicocca P. *In vitro* and *in vivo* survival and transit tolerance of potentially probiotic strains carried by artichokes in the gastroinstestinal tract. *Appl Environ Microb*. (2006) 72:3042–5. doi: 10.1128/AEM.72.4.3042-3045.2006
6. Valerio F, Russo F, de Candia S, Riezzo G, Orlando A, Lonigro SL, et al. Effects of probiotic *Lactobacillus paracasei*-enriched artichokes on constipated patients. *J Clin Gastroenterol.* (2010) 44 (Suppl. 1), S49–53. doi: 10.1097/MCG.0b013e3181d2dca4
7. Valerio F, de Candia S, Lonigro SL, Russo F, Riezzo G, Orlando A, et al. Role of the probiotic strains *Lactobacillus paracasei* LMGP22043 carried by artichokes in influencing faecal bacteria and biochemical parameters in human subjects. *J Appl Microbiol.* (2011) 111:155–64. doi: 10.1111/j.1365-2672.2011.05019.x
8. Lavermicocca P, Valerio F, Lonigro SL, De Angelis M, Morelli L, Callegari ML, et al. Study of the adhesion and survival of Lactobacilli and Bifidobacteria on table olives with the aim of formulating a new probiotic food. *Appl Environ Microb.* (2005) 71:4233–40. doi: 10.1128/AEM.71.8.4233-4240.2005
9. Leroy F, De Vuyst L. Lactic acid bacteria as functional starter cultures for the food industry. *Trends Food Sci Technol.* (2004) 15:67–78. doi: 10.1016/j.tifs.2003.09.004
10. Verganović J, Pavunc AL, Gjuračić K, Špoljarec M, ŠuŠković J, Kos B. Improved sauerkraut production with probiotic strain *Lactobacillus plantarum* L4 and *Leuconostoc mesenteroides* LMG 7954. *J Food Sci.* (2011) 76:M124–9. doi: 10.1111/j.1750-3841.2010.02030.x
11. Garrido-Fernández A, Fernández-Díez MJ, Adams RM. (1997). *Table Olives. Production and Processing.* London: Chapman and Hall.
12. Mourad K, Nour-Eddine K. *In vitro* preselection criteria for the probiotic *Lactobacillus plantarum* strains of fermented olives. *Int J Probiotics Prebiotics* (2006) 1:27–32.
13. Bevilacqua A, Altieri C, Corbo MR, Sinigaglia M, Ouoba LII. Characterization of lactic acid bacteria isolated from Italian Bella di Cerignola table olives: selection of potential multifunctional starter cultures. *J Food Sci.* (2010) 75:M536–44. doi: 10.1111/j.1750-3841.2010.01793.x
14. Hurtado A, Reguant C, Bordons A, Rozès N. Lactic acid bacteria from fermented table olives. *Food Microbiol.* (2012) 31:1–8. doi: 10.1016/j.fm.2012.01.006
15. Bautista-Gallego J, Arroyo-López FN, Kantsiou K, Jiménez-Díaz R, Garrido-Fernández A, Cocolin L. Screening of lactic acid bacteria isolated from fermented table olives with probiotic potential. *Food Res Int*. (2013) 50:135–42. doi: 10.1016/j.foodres.2012.10.004
16. Argyri AA, Zoumpopulou G, Karatzas KA, TsaKalidou E, Nychas GJ, Panagou EZ, et al. Selection of potential probiotic lactic acid bacteria from fermented olives by *in vitro* tests. *Food Microbiol.* (2013) 33:282–91. doi: 10.1016/j.fm.2012.10.005
17. Botta C, Langerhold T, Cencic A, Cocolin L. *In vitro* selection and characterization of new probiotic candidates from table olive microbiota. *PLoS ONE* (2014) 9:e95457. doi: 10.1371/journal.pone.0094457
18. Peres CM, Alves M, Hernández-Mendoza A, Moreira L, Silva S, Bronze MR, et al. Novel isolates of lactobacilli from fermented Portuguese olive as potential probiotics. *LWT Food Sci Technol.* (2014) 59:234–46. doi: 10.1016/j.lwt.2014.03.003
19. Guantairo B, Zinno P, Schifano E, Roselli M, Perozzid G, Palleschi C, et al. *In vitro* and *in vivo* selection of potentially probiotic lactobacilli from Nocellara del Belice table olives. *Front Microbiol.* (2018) 9:595. doi: 10.3389/fmicb.2018.00595
20. De Bellis P, Valerio F, Sisto A, Lonigro SL, Lavermicoca P. Probiotic table olives: microbial populations adhering on olive surface in fermentations sets inoculated with the probiotic strain *Lactobacillus paracasei* IMPC2.1 in an industrial plant. *Int J Food Microbiol*. (2010) 140:6–13. doi: 10.1016/j.ijfoodmicro.2010.02.024
21. Domínguez-Manzano J, León-Romero A, Olmo-Ruiz C, Bautista-Gallego J, Arroyo-López FN, Garrido-Fernández A, et al. Biofilm formation on abiotic and biotic surfaces during Spanish-style green table olive fermentation. *Int J Food Microbiol*. (2012) 157:230–8. doi: 10.1016/j.ijfoodmicro.2012.05.011
22. Arroyo-López FN, Bautista-Gallego J, Domínguez-Manzano J, Romero-Gil V, Rodríguez-Gómez F, García-García P, et al. Formation of lactic acid bacteria-yeasts communities on the olive surface during the Spanish-style Manzanilla fermentations. *Food Microbiol*. (2012) 32:295–301. doi: 10.1016/j.fm.2012.07.003
23. Blana VA, Grounta A, Tassou CC, Nychas, GJ. E., Panagou EZ. Inoculated fermentation of green olives with potential probiotic *Lactobacillus pentosus* and *Lactobacillus plantarum* starter cultures isolated from industrially fermented olives. *Food Microbiol*. (2014) 38:208–18. doi: 10.1016/j.fm.2013.09.007
24. Argyri AA, Nicosiotou AA, Malloucos A, Panagou EZ, Tassou CC. Performance of two potential probiotic Lactobacillus strains from the olive microbiota as starters in the fermentation of heat shocked green olives. *Int J Food Microbiol*. (2014) 171:68–76. doi: 10.1016/j.ijfoodmicro.2013.11.003
25. Argyri AA, Panagou EZ, Tassou CC. Probiotics from olive microbiota. In: R. R. Watson, V. Preedy editors. *Probiotics, Prebiotics, and Synbiotics. Bioactive Foods in Health Promotion*. Oxford: Academic Press (2016). p. 371–89.
26. Peres CM, Peres C, Hernández-Mendoza A, Malcata FX. Review on fermented plant materials as carriers and sources of potentially probiotic lactic acid bacteria-with an emphasis in table olives. *Trends Food Sci Tech.* (2012) 26:31–42. doi: 10.1016/j.tifs.2012.01.006
27. Lavermicocca P, Rossi M, Russo F, Srirajaskanthan R. (2016). Table olives: A carrier for delivering probiotic bacteria to humans. In: V. R. Preddy, R. R. Watson editors *Olives and Olive Oil in Health and Disease prevention*. Oxford: Academic Press. p. 735–743.
28. Champagne CP, Gardner NJ. Challenges in the addition of probiotic cultures to foods. *Crit Rev Food Sci Nutr*. (2005) 45:61–84. doi: 10.1080/10408690590900144
29. Rodríguez-Gómez F, Bautista-Gallego J, Arroyo-López FN, Romero-Gil V, Jiménez-Díaz R, Garrido-Fernández A. Table olive fermentation with multifunctional *Lactobacillus pentosus* strains. *Food Control* (2013) 34:96–105. doi: 10.1016/j.foodcont.2013.04.010
30. Zhllima E, Chan-Halbrendt C, Merkaj E, Imami D, Vercuni A, Quinami I. Analysis of consumer preferences for table olives-The case of Albanian urban consumers. *J Food Products Market.* (2015) 21:521–32. doi: 10.1080/10454446.2013.807407
31. Rodríguez-Gómez F, Romero-Gil V, Bautista-Gallego J, García-García P, Garrido-Fernández A, Arroyo-López FN Production of potential probiotic Spanish-style green table olives at pilot plant using multifunctional starters. *Food Microbiol.* (2014) 44:278–87. doi: 10.1016/j.fm.2014.03.023
32. Sánchez-Gómez AH, Rejano-Navarro L, Montaño-Asquerino A. Colour determination on green Spanish-style Manzanilla olives. *Grasas Aceites* (1985) 36:258–261.
33. Böckelmann U, Szewzyk U, Grohmann E. A new enzymatic method for the detachment of particle-associated soil bacteria. *J Microbiol Meth*. (2003) 55:201–11. doi: 10.1016/S0167-7012(03)00144-1
34. Rossi F, Torriani S, Dellaglio F. Identification and clustering of dairy propionibacteria by RAPD-PCR and CGE-REA methods. *J Appl Microbiol.* (1998) 85:956–64. doi: 10.1111/j.1365-2672.1998.tb05259.x
35. IOC (2010). *Guidelines for Tasters and Panel Leader Training in the Sensory Assessment of Table Olives and Panel Management According to Standard COI/OT/MO Doc. N° 1 2010. T.OT/GFMO November 2010.* Madrid: IOOC.
36. IOC (2010). *Sensory Analysis of Table Olives. COI/OT/N° 1/Rev 1 February 2010.* Madrid: IOOC.
37. La Grage A, le Roux N, Gardner-Lubbe S. BiplotGUI: interactive biplots in R. *J Stat Softw.* (2009) 30:1–37. doi: 10.18637/jss.v030.i12
38. Argyri AA, Nisiotou AA, Pramateftaki P, Doulgeraki AI, Panagou EZ, Tassou CC. Preservation of green table olives fermented with lactic acid bacteria with

probiotic potential under modified atmosphere packaging. *LWT Food Sci. Technol.* (2015) 62:783–90. doi: 10.1016/j.lwt.2014.11.046
39. Rodríguez-Gómez F, Romero-Gil V, Arroyo-López FN, García-García P, Garrido Fernández A. Effect of packaging and storage conditions on microbial survival, physicochemical characteristics and colour of non-thermally preserved green Spanish-style Manzanilla olives. *LWT Food Sci Technol.* (2015) 63:367–75. doi: 10.1016/j.lwt.2015.03.095
40. López A, Montaño A, Garrido A. Evaluation of vitamin E by HPLC in a variety of olive-based foodstuffs. *J Am Oil Chem Soc.* (2005) 82:129–33. doi: 10.1007/s11746-005-1054-9
41. López A, Montaño A, García P, Garrido A. Fatty acid profile of table olives and its multivariate characterization using unsupervised (PCA) and supervised (DA) chemometrics. *J Agric Food Chem.* (2006) 54:6747–53. doi: 10.1021/jf0612474
42. López-López A, Jiménez-Araujo A, García-García P, Garrido-Fernández A. Multivariate analysis for the evaluation of fiber, sugars, and organic acids in commercial presentations of table olives. *J Agric Food Chem.* (2007) 55:10803–11. doi: 10.1021/jf0720638
43. López-López A, Montaño A, Ruíz-Méndez MV, Garrido-Fernández A. Sterols, fatty alcohols, and triterpenic alcohols in commercial table olives. *J Am Oil Chem Soc.* (2008) 85:253–62. doi: 10.1007/s11746-007-1186-6
44. Kiritsakis A, Shahidi F. Table olives, processing, nutritional and health implications. In: S. G. Kailis, A. Kiritsakis editors. *Olives and Olive Oil as Functional Foods: Bioactivity, Chemistry and Processing.* Oxford: John Wiley & Sons(2017). p. 295–324.
45. Rodríguez-Gómez F, Romero-Gil V, García-García P, Garrido-Fernández A, Arroyo-López FN. Fortification of table olive packaging with the potential probiotic bacteria *Lactobacillus pentosus* TOMC-LAB2. *Front Microbiol.* (2014) 4:467. doi: 10.3389/fmicb.2014.00467
46. Randazzo AL, Todaro A, Pino A, Pitino I, Corona O, Mazzaglia A, et al. Giarraffa and Grossa di Spagna naturally fermented olives: Effect of starter and probiotic cultures on chemical, microbiological and sensory traits. *Food Res Int.* (2014) 62:1154–64. doi: 10.1016/j.foodres.2014.05.056
47. O'Donnell M, Mente A, Yusuf S. Sodium intake and cardiovascular health. *Circ Res.* (2015) 116:1046–57. doi: 10.1161/CIRCRESAHA.116.303771
48. WHO (2012) (World Health Organization). *Guideline: Sodium Intake for Adults and Children.* Available online at: http://www.who.int
49. Institute of Medicine. *A Population-Based Policy and Systems Change Approach to Prevent and Control Hypertension.* Washington, DC: The National Academic Press (2010).
50. Kloss L, Meyer JD, Graeve L, Vetter W. Sodium intake and its reduction by food reformulation in the European Union-a review. *NFS J.* (2015) 1:9–19. doi: 10.1016/j.nfs.2015.03.001

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Reduction of the Bitter Taste in Packaged Natural Black Manzanilla Olives by Zinc Chloride

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The work assays the use of various concentrations of $ZnCl_2$ (0.0–0.1%, w/v) in packaged natural black Manzanilla table olives. The transformations were followed for 4 months. The presence of Zn modified the leaching of total sugars (sucrose, glucose, fructose, and mannitol) into the brine, which decreased as the $ZnCl_2$ content increased. Over the study, sucrose and glucose were exhausted while fructose, although consumed, left some final residues and the use of mannitol was limited. Titratable acidity was always gradually formed causing the subsequent pH decrease, which stabilized at ≈3.5. Acetic and mainly lactic acid were also formed during the assay, reaching the highest level of lactic acid in the 0.050% $ZnCl_2$ treatment, followed by the Control. The acids were formed by lactic acid bacteria (LAB) (*Lactobacillus pentosus*, 39%, and *Lactobacillus plantarum,* 61%). However, the most outstanding Zn effect was found on the olive sensory characteristics: its presence markedly reduced the bitter notes, increased the overall appreciation, and the treatment containing 0.075% $ZnCl_2$ had the highest scores in hardness, crunchiness, and overall appreciation. Therefore, the addition of $ZnCl_2$ into packaged natural table olives may lead to healthy products with desirable sensory characteristics which, in turn, could promote consumption.

Keywords: preservation, natural black table olives, packaging, zinc fortified olives, sensory analysis

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INTRODUCTION

The world consolidated table olive production for 2014/15 season was 2.6×10^6 tons with the main producers being the EU (0.9×10^6), followed by Egypt (0.5×10^6), Turkey (0.4×10^6), and Algeria (0.2×10^6) (1). Unfortunately, the International Olive Council does not provide information on the distribution among types; however, it is well known that countries like Egypt, Turkey, Algeria, or Greece are great producers of natural black table olives. The most worldwide famous natural black olive presentations are based on the Greek Kalamata variety (2). In general, table olives are a good source of polyphenols, but hydroxytyrosol, tyrosol, tyrosol acetate or 1-acetoxy pinoresinol (3) and triterpenic acids (mainly maslinic and oleanolic acids, with ≈ 2000 mg/kg flesh), among others, are in higher concentrations in natural olives than in Spanish-style in which the lye treatment and washing cause partial leaching (4, 5). The high concentrations of triterpenes give natural olives antitumoral, cardioprotective, anti-inflammatory, and antioxidant activities (6). Also, natural black olives are an excellent source of anthocyanins, responsible for their pink-purple color (3, 7). The particular nutritional characteristics of natural olives are favored by the soft

processing, which only implies a direct brining followed by spontaneous fermentation. The process produces then, a markedly reduced volume of wastewaters concerning other table olive methods. Natural black olives are usually packaged in brine, frequently added with various seasoning materials. Among this presentation, the Greek Kalamata olives, which typically include vinegar and good quality olive oil in the initial cover brine, are particularly appreciated by consumers. However, the packaged natural black table olives require for its stabilization pasteurization or preservatives, which may deteriorate the sensory characteristics (8).

Zinc chloride ($ZnCl_2$) has shown in the synthetic medium good inhibitory effect against yeasts strains of the genera *Saccharomyces*, *Wickerhamomyces*, *Debaryomyces*, *Issatchenkia*, *Candida*, *Pichia*, *Kluyveromyces*, and *Torulaspora* isolated from table olives (9). This effect could open new alternatives to the use of this salt in fermented vegetables, which are usually prone to spoilage by yeasts, not only as a fortifying agent but also as a possible preservative (9). Its application at 0.050 and 0.075% $ZnCl_2$ levels in cracked Manzanilla-Aloreña table olive packaging led to a significant reduction in the *Enterobacteriaceae* and yeast populations and increased the lactic acid bacteria (LAB) population (10). Also, the *Aloreña de Málaga* prepared with 0.075% $ZnCl_2$ in the cover brine represented a novel Zn fortified table olive presentation (11). On the contrary, $ZnCl_2$ added to green Spanish-style table olive packaging showed a lower inhibitory effect than potassium sorbate against yeasts (12). The loss of inhibitory efficiency could not be attributed to hydroxytyrosol or NaCl since, in their presence, the fungicidal activity of $ZnCl_2$ and $ZnSO_4$ against table olive yeasts, using synthetic medium, remained inalterable (13). Therefore, the role of zinc salts in table olives still requires further investigation even more in natural black table olives where there are no studies about zinc use. Also, the zinc is an essential trace element for humans due to its role in many physiological functions in the living systems (14). Diverse zinc salts can be used in the manufacture of food supplements (15). Therefore, the investigation of Zn effects on table olive products and its possible use as a fortifying agent or preservative is of scientific and practical interest.

The objective of this work was to study the transformations on the physicochemical, microbiological, and sensory characteristics of natural black Manzanilla table olives caused by the presence of $ZnCl_2$ in the packaging brine. The addition may also enhance the healthy properties of the natural olive compounds with those provided by the zinc. Then, the product could represent an attractive, functional table olive trade preparation.

MATERIALS AND METHODS

Experimental Design and Samples

The natural black stored in brine Manzanilla olives used in the present study were provided by a local producer of the region of Seville (Spain). The pitted stored fruits were placed in plastic bags (420 g olives + 350 ml brine) and seasoned, as usual, with small pieces of thyme. Then, four different brines were used for packaging in bags. The first (Control) consisted of the brine habitually used by the industry, which contained (w/v): 0.4% potassium sorbate, 0.6% benzoic acid, 2.0% NaCl, 0.2% citric acid, 0.6% of ascorbic acid, and 0.25% of lactic acid. The other three brines were similar to that used by the producer, except for the absence of preservatives, but added with 0.050, 0.075, and 0.100% (w/v) $ZnCl_2$, respectively. After filling, a total of 22 containers per treatment were closed and kept at room temperature (20 ± 3°C) for a period similar to that of real shelf life (4 months). Periodically, two replicate containers per treatment were removed and analyzed in duplicate. The first samples were collected 4 h after packaging.

Physicochemical Analysis

The analysis of brines for pH, chloride concentration, titratable acidity, and combined acidity were carried out using the standard methods developed for table olives (8). The concentrations of sugars and organic acids in brines were determined by HPLC according to the methods developed by Rodríguez-Gómez et al. (16).

CIE coordinates L^* (lightness), a^* (negative values indicate green while positive values indicate magenta), and b^* (negative values indicate blue and positive values indicate yellow) of the fruits were obtained using a BYK-Gardner Model 9000 Color-view spectrophotometer. Samples were covered with a box with a matt black interior to prevent interference by stray light. Each determination was the average of 20 olive measurements. The color index (Ci) was estimated using the formula:

$$C_i = \frac{-2R_{560}+R_{590}+2R_{630}+2R_{640}}{3} \quad (1)$$

where the Rs stand for the reflectance values at 560, 590, 630, and 640 nm, respectively (17).

Instrumental firmness was measured using the protocol described by Bautista-Gallego et al. (18). Briefly, a Kramer shear compression cell coupled to an Instron Universal Testing Machine (Canton, MA) was used. The crosshead speed was 200 mm/min. The firmness (shear compression force) was the mean of 20 measurements, each of which was performed on one cracked pitted fruit from which the stone had been previously removed. The result was expressed as kN/100 g pitted olive.

Microbiological Analysis

Brine samples and their decimal dilutions were plated using a Spiral System model dwScientific (Dow Whitley Scientific Limited, England) on the appropriate media. Subsequently, plates were counted using a CounterMat v.3.10 (IUL, Barcelona, Spain) image analysis system, and the results expressed as $\log_{10}$ CFU/mL. *Enterobacteriaceae* were counted on VRBD (Crystal-violet Neutral-Red bile glucose)-agar (Merck, Darmstadt, Germany), total viable counts were determined on plate count agar (tryptone-glucose-yeast) (Oxoid), LAB on MRS (de Man, Rogosa and Sharpe)-agar (Oxoid) with 0.02% (w/v) sodium azide (Sigma, St. Luis, USA), and yeasts on YM (yeast-malt-peptone-glucose medium)-agar (DifcoTM, Becton and Dickinson Company, Sparks, MD, USA) supplemented with oxytetracycline and gentamicin sulfate as selective agents for yeasts. Plates

were incubated in aerobiosis at 30°C for 48–72 h. Changes in the microbial populations were assessed by counts at selected sampling times.

Brine samples (100 mL) were collected in sterile conditions at the end of the study (∼4 months) and plated on the yeast and LAB selective media described above. A total of 160 isolates, 80 LAB, and 80 yeasts (10 for each treatment and replicate) were randomly selected and purified by subsequent re-streaking on YM or MRS agar, respectively. The different LAB isolates were identified at species level using multiplex PCR analysis of the *rec*A with species-specific primers for *Lactobacillus pentosus*, *Lactobacillus plantarum,* and *Lactobacillus paraplantarum*, following the protocol described by Torriani et al. (19). Yeasts were stored at −80°C and subjected to further identification, probiotic, and technological tests which will be considered separately.

Sensory Analysis

The sensory analysis was conducted by a panel of 13 experienced judges (6 men and 7 women) from the staff of the Food Biotechnology Department. All panelists had participated in previous table olive classification tests (20, 21), had between 4 and 15 years' experience, and were familiar with sensory studies on table olive presentations. The tests were performed in individual booths under controlled conditions of light, temperature, and humidity. For the analysis, the descriptors included in the Sensory Analysis for Table Olives (22) sheet, added with an overall appreciation, were used, due to its familiarity to panelists. Despite this background, the panelists were trained for the proper assessment of the descriptors and the objectives of this experiment (23). The samples were served in the cups described in the Method for Sensory Analysis of Table Olives (24), coded with a 3-digit random number, and presented in a randomized balanced order to the panelists (25). Only four runs per session were performed. Samples were analyzed in triplicate.

The olives were scored according to a 10-cm unstructured scale. Anchor ratings were 1 (no perception) and 11 (extremely strong) for gustatory perceptions and low and high levels for kinesthetic sensations (26). The marks on the evaluation sheet were transformed into data by taking measurements (in 0.1 cm precision) from the left anchor.

Olives from the various treatments were also subjected to comparisons among them, using the sheets developed for the R-index. In these tests, panelists were asked to look for differences between a previously known sample and those supplied during the test (including the reference sample). The answers to be selected were S-sure (S), S-unsure (S?), N-unsure (N?), and N-Sure (N) and their preferences. Data collected were processed according to Lee and Van Hout (27).

Statistical Analysis

Data were processed using diverse statistical programmes according to the objective of the analysis, using ANOVA to study significant differences among treatments at particular sample points when necessary. Sigma Plot 13 (Systat Software, Inc.) was also used for most graphic presentations. Regarding the sensory analysis, the study of the panel behavior and characterization of products were performed using XLSTAT for Excel v 19.4 (AddisonSoft, France) and Panel Check v1.4.2 (a joint project by Nofima Mat., Technical University of Denmark, and the Department of Food Science of the University of Copenhagen). The data were subjected to ANOVA, considering the effects of treatments as fixed and those of assessors, sessions and their interactions as random, and other statistical tools provided by the packages. When appropriate, data were also auto-scaled and centered (28), to prevent bias due to differences in scales, or centered by assessors, a transformation that is particularly recommended when the analysis is focussed on the products and not on panelists, as in this study (29).

All treatments and attributes were also subjected to PCA, which is a procedure for condensing the maximum amount of variance in the minimum number of uncorrelated variables (usually called principal components or Factors) to prevent multicollinearity (30).

RESULTS AND DISCUSSION

Changes in the Physicochemical Characteristics of Brines Along the Storage

Sugars

After 4 h packaging, there were remarkable initial concentrations of total sugar in brines which ranged from 0.95(±0.05) g/L, in the Control, to 0.61 (±0.01) g/L, in the 0.100% $ZnCl_2$ treatments. The presence of sugars in the initial packaging brines are a consequence of their high levels in the raw materia used for the presentations which, in turn, is due to their slow diffusion from the plain fruits into the brines during the previous storage, caused by the limited permeability of the skin when olives are not lye treated (8). However, after pitting, the solubilisation was faster and the concentrations only a few hours after packaging reached considerable levels (**Table 1**). The differences between the Control (without Zn) and the treatments with the two highest concentrations of zinc salt used were significant at $p < 0.05$ but not with respect to those containing only 0.050% $ZnCl_2$. Besides, there was no significant total sugar content between treatments with the two intermediate level of the salt (**Table 1**). In any case, the averages of the initial total sugar concentration as the Zn content increased was progressively lower. This delay in the sugars' release in the presence of Zn can be related to a similar phenomenon-observed in Gordal fermentation when Ca was added to the brine and may be due to the formation of complexes with flesh components and incrase of consistence, caused by the divalent character of both cations, with the subsequent decrease in difusion (16).

The most abundant contributors to initial total sugars were fructose (0.43 ± 0.02–0.35 ± 0.01 g/L), followed by sucrose (0.18 ± 0.02–0.14 ± 0.00 g/L), glucose (0.11 ± 0.04–not detected g/L), and mannitol (0.16 ± 0.02–0.13 ± 0.01 g/L) (**Table 1**). The presence of sucrose and glucose was relatively low, and both sugars were entirely consumed soon (**Figures 1A,B**). Regarding the other sugars, fructose was also used in a high proportion and the final residue, regardless of the treatment,

TABLE 1 | Overall changes over the period of study in the total sugar content, fructose, and mannitol[1] according to treatments.

Treatment	Total sugars		Fructose		Mannitol	
	Initial	Final	Initial	Final	Initial	Final
Control	0.951 (0.054)^{c2}	0.180 (0.004)^{a1}	0.427 (0.017)^{b2}	0.089 (0.004)^{ab1}	0.155 (0.019)^{a2}	0.091 (0.001)^{a1}
0.050%	0.846 (0.021)^{bc2}	0.198 (0.022)^{a1}	0.391 (0.019)^{ab2}	0.085 (0.003)^{ab1}	0.138 (0.010)^{a1}	0.113 (0.021)^{a1}
0.075%	0.751 (0.055)^{ab2}	0.180 (0.008)^{a1}	0.390 (0.032)^{ab2}	0.077 (0.014)^{a1}	0.133 (0.010)^{a1}	0.103 (0.008)^{a1}
0.100%	0.610 (0.009)^{a2}	0.220 (0.006)^{a1}	0.350 (0.005)^{a2}	0.111 (0.006)^{b1}	0.127 (0.014)^{a1}	0.109 (0.009)^{a1}

[1]*Values are averages in g/L, followed by standard error in parenthesis. Sucrose and glucose were omitted from the table since no final residues of them were found at the end of the period of study. Values in the same column followed by different letter superindexes are significantly different at $p < 0.05$. Values within compounds with different number superindixes for initial and final values are significantly different at $p < 0.05$.*

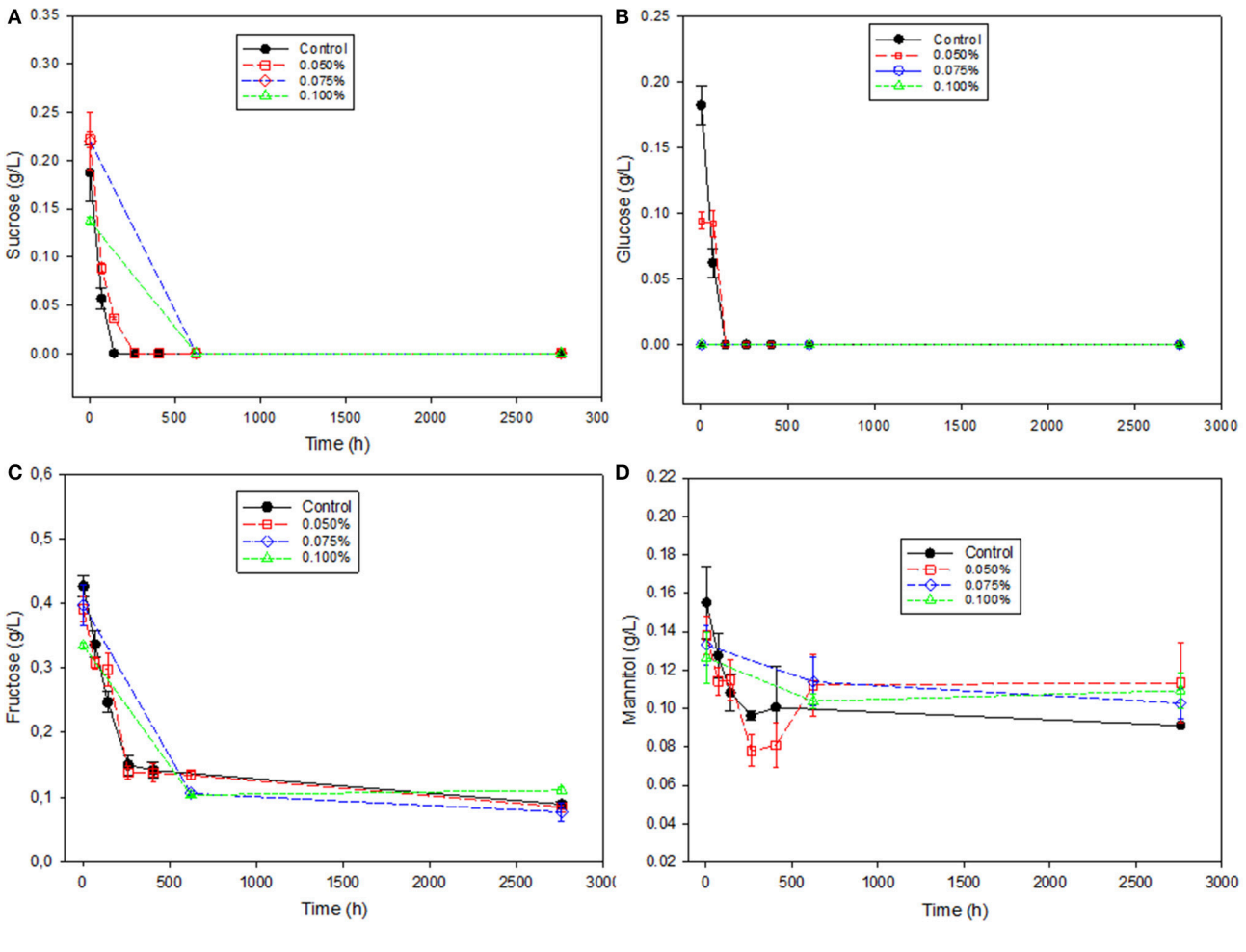

FIGURE 1 | Changes in sugar concentrations in natural black Manzanilla table olive brines over time, according to $ZnCl_2$ levels (%, w/v). **(A)** Sucrose; **(B)** Glucose; **(C)** Fructose; and **(D)** Mannitol.

significantly decreased at the end of the study. However, mannitol consumption was slower than any other initially present sugar and was used in lower proportion since the decrease in mannitol during the studied period was significant only in the Control (**Table 1** and **Figures 1C,D**). This indicates that the presence of Zn can hardly improve the manitol consumption. Overall, the total sugar residues at the end of the study was still high but without significant differences among treatments, indicating that the Zn salts were as efficient as the preservatives used by the industry to prevent the sugars' use (except mannitol). In "seasoned" Manzanilla-Aloreña packaging, fructose and mannitol also decreased slowly, with the levels of the last one being unusually high in some treatments (31). The apparent effect of the zinc salt in this work was also in agreement with the results obtained in cracked table olives where the $ZnCl_2$ presence led to higher concentrations of residual sugars in brine

(due to its selective microbial inhibition) but also to similar trends in their consumptions (10). In general, the residual sugars in the packages represent a source of instability, even in the case of using preservatives and reduce the shelf life of the products (8). The problem is also relevant in the case of cracked Manzanilla-Aloreña table olives, which is an EU DOP, since such instability reduces its commercialization to the area close to their production, particularly when prepared as a fresh-packaged "seasoned" presentation (31).

pH, Titratable Acidity, and Organic Acids

The trends followed by these variables were similar in all treatments, although with clear differences among them (**Figure 2**), with the control showing consistent significantly ($p < 0.05$) higher levels after 1,248 h. Initially, there was a relevant general pH increase (∼0.5 units), which reached its maximum at about 250 h after packaging (**Figure 2A**), simultaneously accompanied by a decrease in titratable acidity, which reached its minimum slightly earlier (**Figure 2B**). Therefore, the initial changes in pH and titratable acidity were just a matter of equilibrium between the packaging brine and the olive flesh. The phenomenon is normal in olive packaging, where the storage of the final products for 7–15 days before distribution is habitual to allow not only the equilibrium of the physicochemical conditions

TABLE 2 | Overall changes over the period of study in the acetic and lactic concentrations[1] according to treatments.

Treatment	Acetic acid		Lactic acid	
	Initial	Final	Initial	Final
Control	4.594 (0.125)^{c1}	5.276 (0.136)^{b2}	4.594 (0.126)^{c1}	5.276 (0.136)^{a2}
0.050%	2.950 (0.020)^{b1}	3.194 (0.135)^{a1}	2.156 (0.064)^{ab1}	7.125 (0.485)^{b2}
0.075%	2.529 (0.044)^{a1}	3.352 (0.076)^{a2}	2.552 (0.122)^{b1}	5.005 (0.199)^{a2}
0.100%	2.467 (0.026)^{a1}	3.203 (0.050)^{a2}	1.708 (0.176)^{a1}	4.311 (0.124)^{a2}

[1] *Values are averages in g/L, followed by standard error in parenthesis. Values in the same column followed by different letter superindexes are significantly different at $p < 0.05$. Values within compounds with different number superindixes for initial and final values are significantly different at $p < 0.05$.*

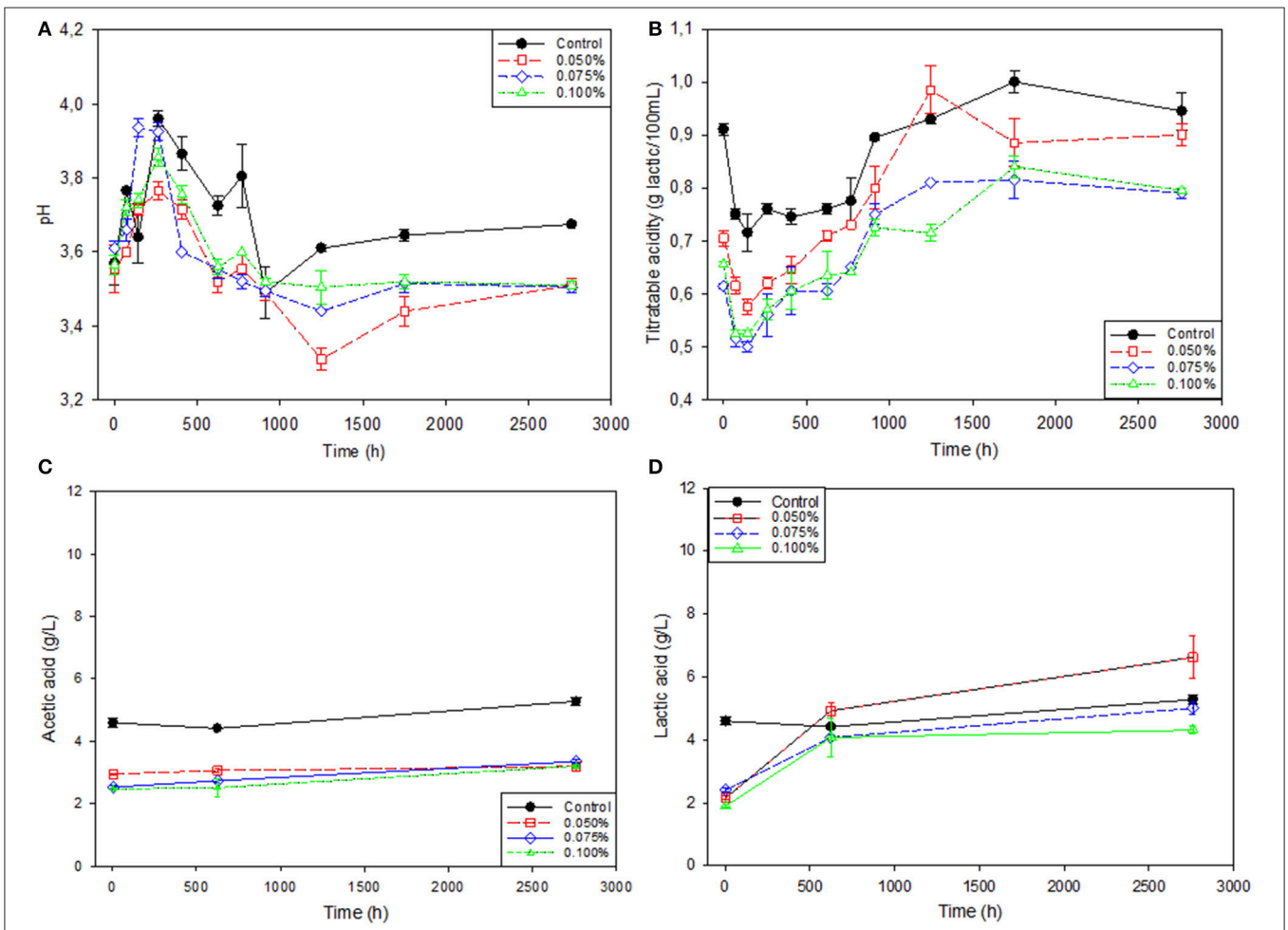

FIGURE 2 | Evolution of pH, titratable acidity and acetic, and lactic acid concentration in natural black Manzanilla table olive brines over time, according to $ZnCl_2$ levels (%, w/v). **(A)** pH; **(B)** Titratable acidity; **(C)** Acetic acid; and **(D)** Lactic acid.

but also the weight, due to the different brine concentrations used for storage/fermentation and at packaging (8). However, the effect is hardly observable in cracked olives where, due to the fast diffusion of compounds throughout the interior of the olive flesh, the equilibrium is rapid (31). Once equilibrated, there was a relatively fast production of titratable acidity which reached their maxima after ~1,250 h for treatments 0.050 and 0.075% $ZnCl_2$ or ~1,750 h for the control and 0.100% $ZnCl_2$, with a limited acid formation after these periods. The acidity evolution in the Control was usually significantly ($p > 0.05$) above those in treatments containing Zn (except for 0.050% $ZnCl_2$ at 1,248 and 2,760 h), due to its relevant initial sugar content, followed by the treatment containing 0.050% $ZnCl_2$. The treatments with higher Zn presence showed similar trends but the lowest (significant at $p < 0.005$) levels at 1,248 h and final titratable acidities (**Figure 2B**). Therefore, the delay effect on sugar leaching by the progressive presence of Zn in the cover brines was also reflected on a lower titratable acidity. The formation of titratable acidity during shelf life was also observed in previous works and was associated with the presence of LAB whose growth was favored by the reduction of the yeast population by preservatives. However, the use of zinc in Manzanilla-Aloreña enlarged the period of titratable acidity production, possibly due to the high contents of residual nutrients (10). In any case, the excessive LAB presence might not be necessarily favorable since it has also been associated with instability in not lye treated olives (31).

In this work, the titratable acidity was composed of acetic and lactic acids which concentrations were initially most abundant (significant at $p < 0.05$) in the Control than in the treatments containing Zn (**Table 2** and **Figures 2C,D**). The differences in acetic acid between the control and the packages containing Zn scarcely changed during the 4 months due to its similar net increment regardless of treatments (**Table 2** and **Figure 2C**). On the contrary, the lactic acid had a remarkable (significant at $p < 0.05$) initial increment during the first days after brining only in treatments containing Zn (**Figure 2D**). Later, the production was very slow, except when containing 0.050% $ZnCl_2$, which reached the final highest level (significant at $p < 0.05$), followed by the control (**Table 2**). Therefore, only 0.050% $ZnCl_2$ presence stimulated the lactic acid formation.

Combined Acidity, NaCl, and Olive Firmness

The combined acidity is a measure of the buffer capacity of the brine (8). The highest level was always found in the Control (**Figure 3A**) with significant ($p < 0.05$) differences at most sampling periods. Hence, the presence of Zn in the brine not only reduced the sugar leaching but also decreased the solubilization of the organic compounds responsible for the combined acidity. In mixtures of chloride salts (KCl, NaCl, and $CaCl_2$), the decrease in this parameter was associated with the presence of Ca (also a divalent cation) and its interaction with the olive flesh (32). The Zn may have a different response against olive flesh than Ca, but it can still exert a specific affinity for its components with the retention in the flesh of, at least, a fraction of their hydro soluble compounds. In turn, the reduced combined acidity levels resulted in the lowest pH values (**Figure 2A**), which can be favorable for the packaged product safety (8).

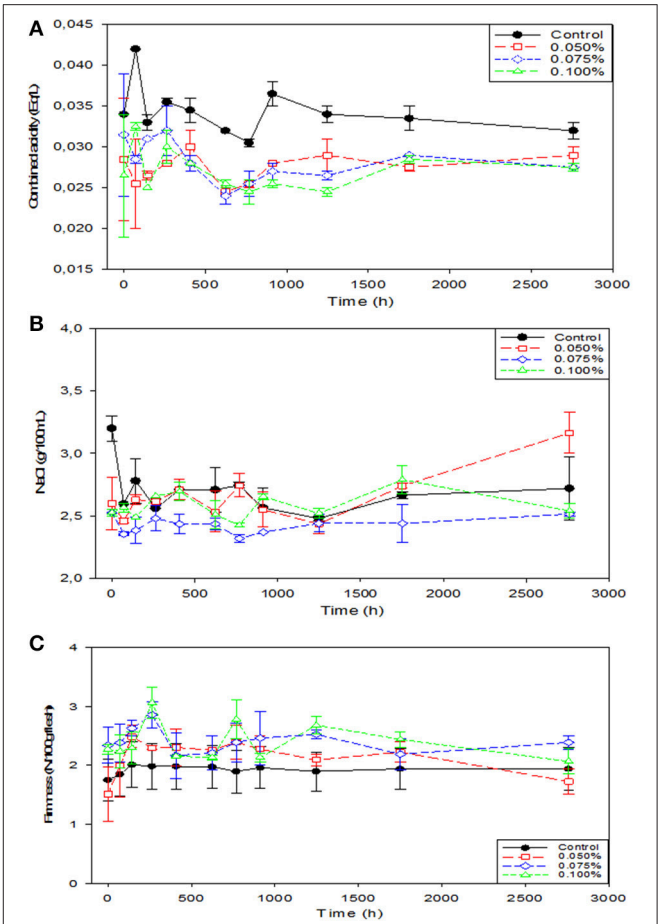

FIGURE 3 | Changes in combined acidity, NaCl concentration and firmness in natural black Manzanilla table olive brines over time, according to $ZnCl_2$ levels (%, w/v). **(A)** Combined acidity; **(B)** NaCl; and **(C)** Firmness.

Concentrations of NaCl, regardless of treatments, followed similar trends (significant difference at $p < 0.05$ only in the initial sampling) and were habitually around 2.5% during the whole period (**Figure 3B**). This stability is reasonable due to the inorganic nature of salt. Panagou et al. (33) and Panagou (34) did not find either any significant change of this parameter in dry-salted olives of the Thassos variety when the fruits were stored under modified atmospheres at 4 and 20°C.

The firmness of the treatments containing Zn was mainly above that of the Control and in some cases significantly ($p < 0.05$) higher (**Figure 3C**), although at the end of the period this had an intermediated value. Therefore, the Zn, as a divalent cation, in addition to preventing the organic matter solubilization, formed stable complexes with the wall structure of the olive cells and increased the product hardness (~0.5 kN/100 g olive flesh), at least during most of the packaging period. Interestingly, firmness of control scarcely changed with time despite the acid medium. This trend is in contrast with the progressive degradation of firmness in seasoned olives, reported by Fernández-Bolaños et al. (35). In this case, the effect was attributed to both the enzymes coming from the constituents

of the dressing products (garlic, lemon, etc.) and those from the endogenous olive flesh since the influence was noticed in either the presence or absence of seasoning materials. The authors related the changes in texture to the degradation of polysaccharides, caused by α-cellulase and polygalacturonase present in the flesh which were not so active as in the case of natural black Manzanilla olives.

Color

The changes in color were followed by studying color index and the CIE L*, a*, b*. Initially, the effect of zinc was outstanding on the color index which was rather high (significant at $p < 0.05$) and similar in samples containing 0.075 and 0.100% $ZnCl_2$; later, slightly decreased and stabilized. On the contrary, the color index in the Control and 0.050% $ZnCl_2$ increased over time up to ≈1750 h. After this time, they became stable and followed a similar trend (without significant individual changes) than the other two Zn treatments (**Figure 4A**). The gradation of the color index, in ascendant value order, was: Control, 0.050%, 0.075–0.100% $ZnCl_2$, with significant ($p < 0.05$) differences over most of the studied period. Therefore, in general, Zn presence led to higher color indexes.

The influence of $ZnCl_2$ was also observed on the CIE* parameters which followed similar trends with the control always below (in some cases significantly at $p < 0.05$) the treatments with Zn (**Figures 4B–D**), and final values above those showed at packaging but without significant differences. There was a period (between 250 and 500 h) where, apparently, the Zn effect was more intense (significant at $p < 0.05$) on L^* and b^* which values, on the contrary, always decreased at the end of the period. Changes in a^* followed a similar trend, although the average values of Control were among the lowest during most of the studied period. The effect on both L^* and b^* paramenters should be attributed to the presence of Zn since the levels pH (high) and acidity (low) would have influenced on opposed direction. According to Arroyo-López et al. (31), in cracked Manzanilla-Aloreña, the only parameter affected was a^* which had a marked increase due to surface color change from green to more or less intense yellow. This transformation was caused by the degradation of chlorophylls and carotenoids into colorless or

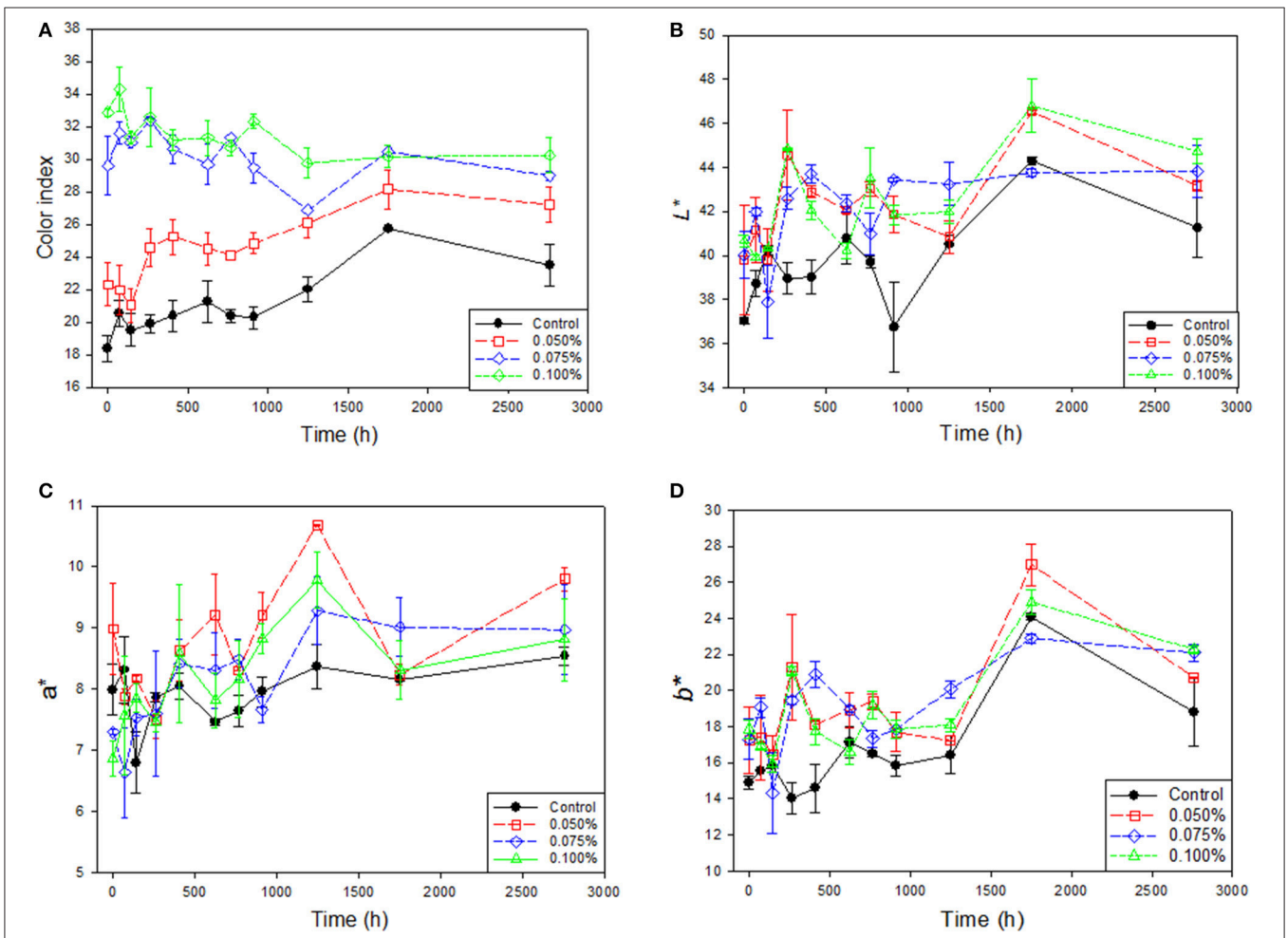

FIGURE 4 | Evolution of color index, L^*, a^*, and b^* (unweighted means) in natural black Manzanilla table olive brines over time, according to $ZnCl_2$ concentrations (%, w/v). **(A)** Color index; **(B)** L^*; **(C)** a^*; and **(D)** b^*.

brownish compounds (36). In this work, the main visual change in the natural black Manzanilla olive color was from purple to pink due to the degradation of anthocyanins.

Changes in the Microbiological Populations

The study of the packaged olive microbiota was based on total viable counts, LAB, and yeasts (**Figure 5**) since no *Enterobacteriaceae* were found. Total viable counts had an initial decrease, but the population grew soon to reach a level close to 8 log_{10}CFU/mL which remained practically stable throughout the 4 months, independently of treatment (**Figure 5A**). To notice that the effect of zinc on total viable counts was similar to that observed for preservatives (sorbate + benzoate) in the Control. This microbial population was mainly composed of LAB and yeasts. LAB population was remarkable high (∼5–6 log_{10}CFU/mL) from the beginning and increased after a short period to reach 7.5–8 log_{10}CFU/mL, in treatments containing Zn, but only around 7 log_{10}CFU/mL, in the Control. Later, their counts remained stable during the rest of the period, although maintaining the differences (**Figure 5B**). Therefore, the LAB trends were quite similar, but the average populations in the Control were always below those treatments containing Zn, although the differences were mostly non-significant due to the high variability between replicate. The presence of the metal stimulated the LAB growth slightly but did not encourage simultaneously the titratable acidity production since its average levels were below those in the Control throughout the period, except in one sampling point (**Figure 2B**). Molecular analysis at the end of the period revealed that LAB population was mainly composed of *L. pentosus* (39%) and *L. plantarum* (61%), but their distribution was not related to the $ZnCl_2$ contents.

The yeast population in the Control was always similar or only slightly below than in those treatments containing zinc (except in one sampling point), without any systematic difference based on the Zn concentration (**Figure 5C**). Their populations had a small initial decrease but soon recovered and stabilized around 3–4 log_{10}CFU/mL with a slight decrease in the last counting. Therefore, the presence of $ZnCl_2$ had an approximately inhibitory similar effect on yeast than the preservatives.

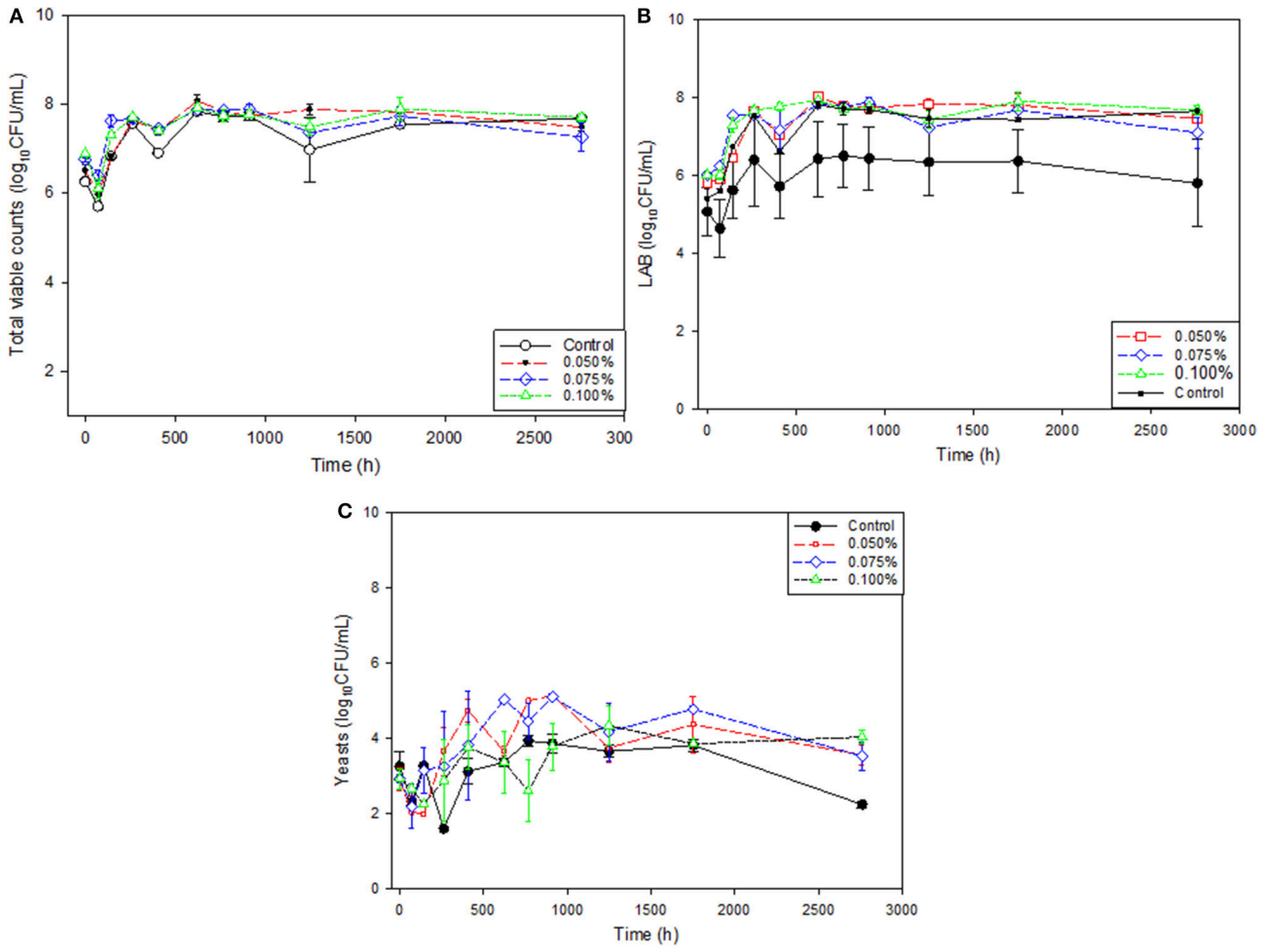

FIGURE 5 | Changes in microbial populations (unweighted means) in natural black Manzanilla table olive brines over time, according to $ZnCl_2$ concentrations (%, w/v). **(A)** total counts; **(B)** LABs; and **(C)** Yeasts.

Sensory Analysis and Nutritional Implications

The results of the sensory analysis by the panel were subjected to both panel performance and characterization of products. In a first step, the data for each attribute were subjected to an ANOVA considering the effects of treatments as fixed and those of assessors, sessions and their interaction as random. Only bitter and overall scores in the Control and 0.075% $ZnCl_2$ treatment resulted significantly different at $p \leq 0.05$ (**Figure 6A**). A procrustean analysis of the scores, using XLSTAT, showed that, in general, the panelists segregated the treatments correctly (**Figure 6B**). However, the interest was mainly focused on the characterization of the products according to the descriptors used in the sensory evaluation. Such a task was accomplished by observing the adjusted mean values from the corresponding ANOVA test (**Table 3**). The highest overall score (in blue) was given to the product containing 0.075% $ZnCl_2$, the lowest corresponded to control (in red), while the other treatments had intermediate scores. The highest scores for acid and bitter were obtained for the Control. On the contrary, the lowest values for fibrousness were found in treatment with only 0.050% $ZnCl_2$.

The contributions of descriptors to discriminate among treatments agreed with the previous observations (**Figure 6A**) and indicated that only overall score and bitter had significant discriminant powers. However, for more extensive product characterization, fibrousness and acid should also be considered (although they had a lower significance, $p \leq 0.100$) since they

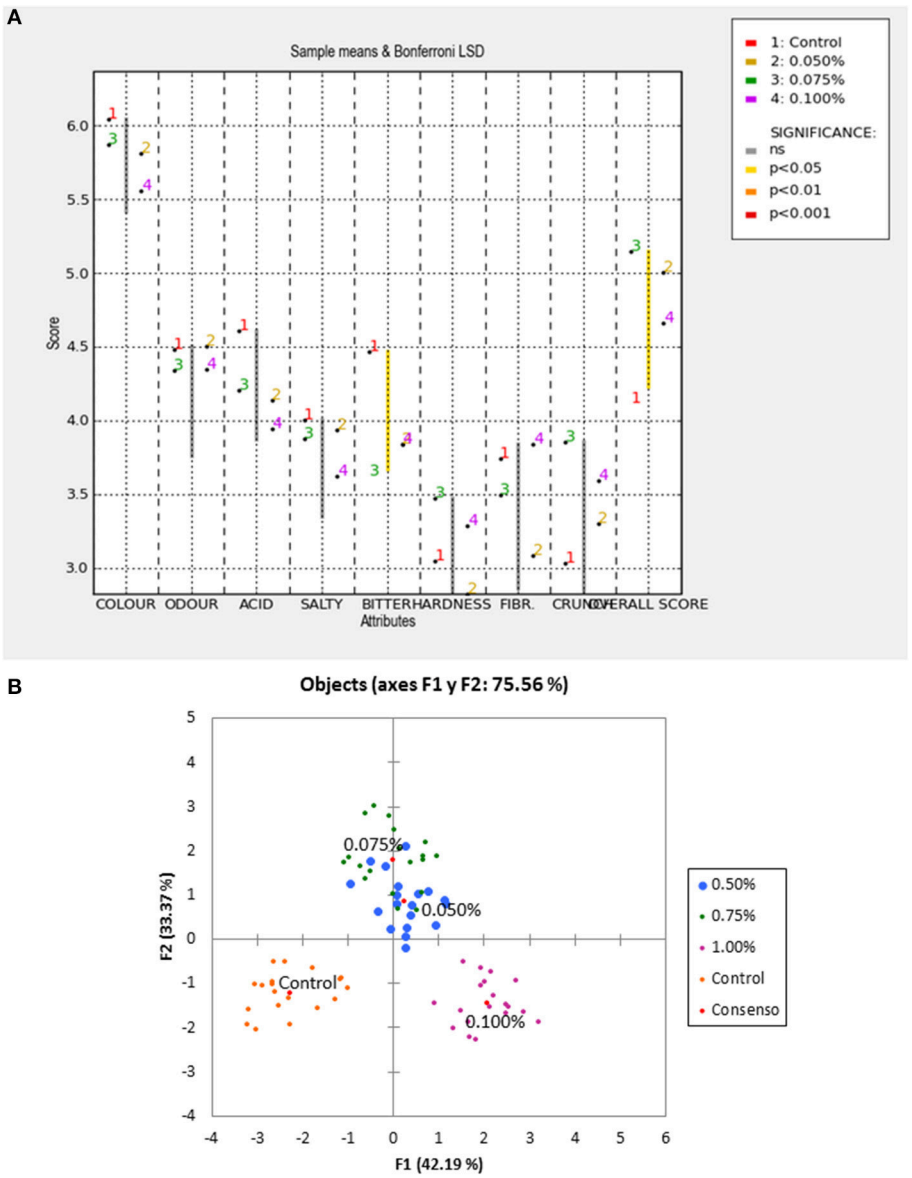

FIGURE 6 | Sensory analysis of natural black Manzanilla table olive, according to $ZnCl_2$ concentrations (%, w/v) in the packaging brines **(A)** significant descriptors and **(B)** segregation of treatments by panelists.

TABLE 3 | Average scores of descriptors adjusted for products after ANOVA application to the original data.

	Crunch	Overall score	Hardness	FIBR	Color	Salty	Odor	Acid	Bitter
0.100%	3.600	4.663	3.289	3.846	5.563	3.626	4.351	3.949	3.840
0.075%	3.861	5.146	3.475	3.496	5.874	3.881	4.340	4.205	3.625
0.050%	3.307	5.009	2.826	3.086	5.814	3.940	4.505	4.142	3.844
Control	3.040	4.118	3.054	3.746	6.046	4.004	4.486	4.611	4.470

CRUNCH, crunchiness; FIBR, fibrousness. The highest (in blue) and the lowest (in red) overall score.

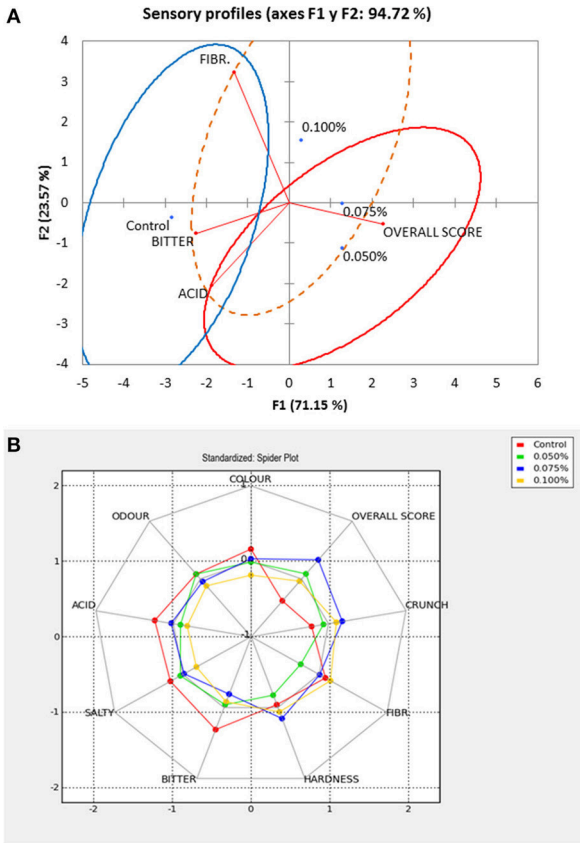

FIGURE 7 | Sensory analysis of natural black Manzanilla table olive, according to $ZnCl_2$ concentrations (%, w/v) in the packaging brines **(A)** Projection of the different treatments scores on the first two Principal Components (sensory profiles) and their ellipse confident limits ($p = 0.05$). **(B)** Spider plot, based on all descriptors, to characterize the diverse products, using standardized average scores.

had relevant low and high scores, respectively. Applying PCA, a projection of the loadings of the attributes (filtered for only those significant at $p \leq 0.100$) and the treatment scores on the two first components, three groups and their 95% confidence limits were obtained (**Figure 7A**). The first corresponded to the control and was mainly positively associated to bitter as well as to acid and negatively to the overall score. The second group consisted of treatments using 0.050 and 0.075% $ZnCl_2$ which were linked to the highest overall scores and the lowest bitter and fibrousness levels. Finally, treatment with 0.100% $ZnCl_2$, was characterized by low acid and bitter scores, intermediate fibrousness and overall scores; however, it was not well segregated from the other groups with which overlapped extensively. A characterization of the diverse products based on all descriptors, using standardized average scores, was also obtained by the spider plot (**Figure 7B**). The control had the highest average scores in color, odor, acid, salty, bitter (mainly), and fibrousness.

On the contrary, the treatment containing 0.075% $ZnCl_2$ had the highest hardness, crunchiness and overall scores. Therefore, the sensory analysis showed that the presence of 0.075% $ZnCl_2$ markedly improved the sensory quality of the product. Notably, the addition of the Zn contributed to the reduction of the bitter taste which was a sensation strongly and negatively related to overall score (**Figure 7A**).

The treatments were also pairwise compared, applying a methodology similar to that used by the h-index estimation. The test consisted of two essays: the first was performed with the aim of knowing the discriminant power of the assessors while the second had the objective of analyzing their preferences. In the first case, the control was considered different from treatments containing 0.100, 0.075, and 0.050% $ZnCl_2$ by 95, 84, and 95% of the assessors; therefore, the products containing Zn were differentiated from the control (with preservatives). When the comparison was made between presentations containing Zn, treatment with 0.050% $ZnCl_2$ was considered different from 0.075 to 0.100% by 79 and 95% of the panelists, respectively. The difference between 0.075 and 0.100% was also perceived by 84% of the assessors. Therefore, it may be concluded that the treatments containing Zn are different from control and among them.

Concerning preferences, when the treatments containing progressive lower contents were compared to the Control, 53, 79, and 58% of the assessors preferred those containing 0.050, 0.075, and 0.100% $ZnCl_2$, respectively. The significance of the responses, evaluated by a χ^2 test ($\chi^2 = 0.277$, $p = 0.599$; $\chi^2 = 33.524$, $p < 0.0001$; and $\chi^2 = 6.927$, $p = 0.122$, respectively), showed that only the treatment containing 0.075% $ZnCl_2$ was significantly preferred against the Control. Therefore, in general, the treatments added with Zn were differentiated from the control, but only the treatment added with 0.075% $ZnCl_2$ was statistically preferred over it. Treatments containing $ZnCl_2$ were also compared among them. The treatment containing 0.075% was significantly preferred against that with 0.100% by 68% ($\chi^2 = 13.572$, $p = 0.0002$) but only by 47% vs. that with 0.050% ($\chi^2 = 0.277$, $p = 0.599$). However, there was no statistical difference between 0.050 and 0.100% treatments. The comparison among Zn treatments was not as clear as that against the control but showed that olives containing 0.075% $ZnCl_2$ were significantly preferred against those with 0.100% but not against 0.050% $ZnCl_2$. The results obtained in this work agree with those previously observed in "seasoned" *Aloreña de Málaga* packaged in brines added with Zn in which the packages containing 0.075% $ZnCl_2$ were also preferred to any other treatment. Furthermore, in the experiment with *Aloreña de Málaga*, the highest scores in the absence of Zn were also given to bitter,

but the values decreased as the element proportion in treatments increased (10).

Also working with Manzanilla *Aloreña de Málaga*, the correlations between bitter scores and Zn contents in flesh were negative. Besides, when the sensory data were subjected to PLS analysis, the projections of attribute loadings and treatments scores on the first two PCs positively related the control to bitter and the kinesthetic sensations while the product with 0.075% $ZnCl_2$ was associated with high titratable acidity and acid scores (11). In a first approach for the preparation of green Spanish-style olive packaging containing $ZnCl_2$, however, no effect of Zn content was observed, possibly due to the lye treatments used for debittering (12). Further comparisons between packaged green olives using $ZnCl_2$ and potassium sorbate or sodium benzoate showed that the panelists preferred the olives packaged with Zn against those containing the classical preservatives but no relationship with bitter was found (private communication). Therefore, regarding sensory characteristics, the presence of $ZnCl_2$ has demonstrated a clear mitigating effect on the bitter perception mainly in directly brined olives, in which the residual sensation of this attribute is still notable.

The inclusion of this important nutritional element in such presentations could lead to new and healthier table olive presentations. The product will increase the Zn intake in ∼8–20 mg Zn/100 g olive flesh (considering its equilibrium between brine and flesh moisture). In the case of the most appropriate concentration (0.075% $ZnCl_2$ in cover brine), the content would be ∼15 mg/100 g olive flesh which, for a reasonable service of about 7 olives, will represent a contribution of about half the current recommended Zn daily intake for an adult (10 mg/day) (37). However, polyphenols are among the compounds that decrease the Zn absorption (38). As the concentrations of polyphenols are particularly high in directly brined natural black olives (7), a study of the bioavailability of Zn in this product would be convenient before a more accurate estimation of the potential nutritional improvement of natural black olive zinc fortified presentations could be established.

CONCLUSIONS

This work has developed a novel Zn fortified natural black Manzanilla olive preparation. The presence of Zn decreased sugar diffusion from olives, had a comparable effect on yeasts than preservatives, stimulated LAB growth slightly and lactic acid production (at moderate Zn concentration), led to high color index, and improved sensory characteristics of the product considerably. Although other factors like pH, acidity or microbial growth might also have been influenced the above-mentioned parameters, their changes were, in turn, related to the presence of Zn and, therefore, indirectly associated to the presence of Zn. Treatments with zinc were differentiated and preferred against the control since the mineral element reduced the bitter perception of the packaged olives. Considering that bitter is one of the primary negative table olive attributes for consumers, the addition of a moderate proportion of $ZnCl_2$ to these products will improve sensibly not only their healthy characteristics but also their appreciation and, as a result, contribute to the expansion of the natural table olive market.

AUTHOR CONTRIBUTIONS

JB-G, FR-G, VR-G, and AB-C performed the experiments, participated in the acquisition, analysis and interpretation of the data, and approved the final version of the paper. JB-G, FA-L, and AG-F supervised the laboratory work, participated in the analysis and interpretation of the data, drafted the manuscript, and approved the final version of the paper.

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REFERENCES

1. IOC (International Olive Council). *World Table Olive Figures* (2017). Available online at: http://www.internationaloliveoil.org/estaticos/view/132-world-table-olive-figures (Accessed June 2017).
2. Georgoudis N. Greek table olives in the international market. In: *Quality Greek Food, Wines and Spirit International Conference.* Kerasma (2006).
3. Romero C, Brenes M, Yousfi K, García P, García A, Garrido A. Effect of cultivar and processing method on the contents of polyphenols in table olives. *J Agri Food Chem.* (2004) 52:479–84. doi: 10.1021/jf030525l
4. Romero C, García A, Medina E, Ruíz-Méndez MV, de Castro A, Brenes M. Triterpenic acids in table olives. *Food Chem.* (2010) 118:670–4. doi: 10.1016/j.foodchem.2009.05.037
5. Alexandraki V, Georgalaki M, Papadimitriou K, Anastasiou R, Zoumpopoulou, G., Chatzipavlidis I, et al. Determination of triterpenic acids in natural and alkaline-treated Greek table olives throughout the fermentation process. *LWT Food Sci Technol.* (2014) 58:609–13. doi: 10.1016/j.lwt.2014.04.005
6. Sánchez-Quesada C, López-Biedma A, Warletta F, Campos M, Beltrán G, Gaforio JJ. Bioactive properties of the main triterpenes found in olives, virgin olive oil, and leaves of *Olea europaea*. *J Agr Food Chem.* (2013) 61:12173–82. doi: 10.1021/jf403154e
7. Romero C, García A,Brenes M, García A, Garrido A. Polyphenol changes during the fermentation of naturally black olives. *J Agr Food Chem.* (2004) 52:1973–9. doi: 10.1021/jf030726p
8. Garrido-Fernández A, Fernández Díaz M, Adams R. *Table Olives*: *Production and Processing.* London, Chapman & Hall (1997).
9. Bautista-Gallego J, Romero-Gil V, Garrido-Fernández A, Arroyo-López FN. Modelling the inhibitory effects of zinc chloride on table olive related yeasts. *Food Control* (2012) 23:499–505. doi: 10.1016/j.foodcont.2011.08.020
10. Bautista-Gallego J, Arroyo-López FN, Romero-Gil V, Rodríguez-Gómez F, Garrido Fernández A. Evaluating the effect of Zn chloride as a preservative in cracked table olive packing. *J Food Prot.* (2011) 74:2169–76. doi: 10.4315/0362-028X.JFP-11-201

11. Bautista-Gallego J, Moreno-Baquero JM, Garrido-Fernández A, López-López A. Development of a novel Zn fortified table olive product. *LWT Food Sci Technol.* (2013) 50:264–71. doi: 10.1016/j.lwt.2012.05.017
12. Bautista-Gallego J, Arroyo-López FN, Romero-Gil V, Rodríguez Gómez F, Garrido-Fernández A. The effect of $ZnCl_2$ on green spanish-style table olive packaging, a presentation style dependent behavior. *J Sci Food Agric.* (2014) 95:1670–7. doi: 10.1002/jsfa.6870
13. Romero-Gil V, Rejano-Zapata L, Garrido-Fernández A, Arroyo-López FN. Effect of zinc formulations, sodium chloride, and hydroxytyrosol on the growth/no-growth boundaries of table olive related yeasts. *Food Microbiol.* (2016) 57:71–80. doi: 10.1016/j.fm.2016.01.007
14. Kaur K, Gupta R, Saraf SA, Saraf SK. Zinc: the metal of life. *Compr Rev Food Sci Food Safety* (2014) 13:358–76. doi: 10.1111/1541-4337.12067
15. Directive 2002/46/EC. *Directive 2002/46/EC of the European Parliament and of the Council of 10 June 2002 on the Approximation of the Laws of the Member States Relating to Food Supplements.* Official Journal of the European Communities, 12.7.2002, L183/51-57 (2002).
16. Rodríguez-Gómez F, Bautista-Gallego J, Romero-Gil V, Arroyo-López FN, Garrido-Fernández A. Effects of salt mixtures on Spanish green table olive fermentation performance. *LWT Food Sci Technol.* (2012) 46:56–63. doi: 10.1016/j.lwt.2011.11.002
17. Sánchez AH, Rejano L, Montaño A. Determinación del color en las aceitunas verdes aderezadas de la variedad Manzanilla. *Grasas y Aceites* (1985) 36:258–61.
18. Bautista-Gallego J, Arroyo-López FN, López-López A, Garrido Fernández A. Effect of chloride salt mixtures on selected attributes and mineral content of fermented cracked Aloreña olives. *LWT Food Sci Technol.* (2011) 44:120–9. doi: 10.1016/j.lwt.2010.06.027
19. Torriani S, Felis GE, Dellaglio F. Differentiation of *Lactobacillus plantarum*, *L. pentosus,* and *L paraplantarum* by *recA* gene sequence analysis and multiplex *PCR* assay with recA gene-derived primers. *Appl Environ Microbiol.* (2001) 67:3450–4. doi: 10.1128/AEM.67.8.3450-3454.2001
20. Moreno-Baquero JM, Bautista-Gallego J, Garrido-Fernández A, López-López A. Mineral content and sensory characteristics of Gordal green table olives fermented in chloride salt mixtures. *J Food Sci.* (2012) 77:S107–14. doi: 10.1111/j.1750-3841.2011.02573.x
21. Moreno-Baquero JM, Bautista-Gallego J, Garrido-Fernández A, López-López A. Mineral and sensory profile of seasoned cracked olives packed in diverse salt mixtures. *Food Chem.* (2013) 138:1–8. doi: 10.1016/j.foodchem.2012.10.027
22. IOC (International Olive Council). *Sensory Analysis of Table Olives.COI/OT/N° 1/Rev 1 February 2010.* Madrid: IOC (2010).
23. Stone H, Sidel JL. Descriptive analysis. In: Caballero B, Trugo LC, Finglas, P, editors. *Encyclopaedia of Food Science and Nutrition.* London: Academic Press (2003). p. 5152–61.
24. IOC (International Olive Council). *Guidelines for Tasters and Leader Training in the Sensory Assessment of Table Olives and Panel Management According to Standard COI/OT/N1/Rev 1 February* 2010. Madrid: IOC (2010).
25. Mailgaard M, Civille GV, Carr BT. *Sensory Analysis Techniques.* Boca Ratón, FL: CRC Press (1991).
26. Lee J, Chambers D. Descriptive analysis and U.S. consumer acceptability of 6 green tea samples from China, Japan, and Korea. *J Food Sci.* (2010) 75:141–47. doi: 10.1111/j.1750-3841.2009.01503.x
27. Lee HS, Van Hout D. Quantification of sensory and food quality: the R-index analysis. *J Foos Sci.* (2009) 74, R57–64. doi: 10.1111/j.1750-3841.2009.01204.x
28. Kowalski BR, Bender CF. Pattern Recognition. A powerful approach to interpreting chemical data. *J Am Chem Soc.* (1972) 94:5632–9. doi: 10.1021/ja00771a016
29. Hibbert B. Chemometric analysis of sensory data. In: Brown SD, Tauler R, Walczak, B, editors. *Comprehensive Chemometrics. Chemical and Biochemical Data Analysis.* Amsterdam: Elsevier (2009). p. 377–424.
30. Jollife IT. *Principal Component Analysis.* New York, NY: Springer Verlag (1986).
31. Arroyo-López FN, Bautista-Gallego J, Segovia-Bravo KA, García García P, Durán Quintana MC, Romero C, et al. Instability profile of fresh-packed "seasoned" Manzanilla-Aloreña table olives. *LWT Food Sci Technol.* (2009) 42:1629–39. doi: 10.1016/j.lwt.2009.06.004
32. Bautista-Gallego J, Arroyo-López FN, Durán-Quintana MC, Garrido-Fernández A. Fermentation profiles of Manzanilla-Aloreña cracked green table olives in different chloride salts. *Food Microbiol.* (2010) 27:410–2. doi: 10.1016/j.fm.2009.11.015
33. Panagou EZ, Tassou CC, Katsaboxakis KZ. Microbiological, physicochemical and organoleptic changes in dry salted olives of Thassos variety stored under different modified atmospheres at 4 and 20°C. *Int J Food Sci Technol.* (2002) 37:635–41. doi: 10.1046/j.1365-2621.2002.00590.x
34. Panagou EZ. Greek dry-salted olives: monitoring the dry-salting process a subsequent physicochemical and microbiological profile during storage under different packaging conditions at 4 and 20°C. *LWT Food Sci Technol.* (2006) 39:322–9. doi: 10.1016/j.lwt.2005.02.017
35. Fernández Bolaños J, Rodríguez R, Saldaña C, Heredia A, Guillén R, Jiménez A. Factors affecting the changes in texture of dressed ("aliñadas") olives. *Eur Food Res Technol.* (2002) 214:237–41. doi: 10.1007/s00217-001-0439-0
36. Gallardo-Guerrero L, Gandul-Rojas B, Moreno-Baquero JM, López-López A, Bautista-Gallego J, Garrido-Fernández A. Pigment, physicochemical, and microbiological changes related to the freshness of cracked table olives. *J Agric Food Chem.* (2013) 61:3737–47. doi: 10.1021/jf400240e
37. Regulation (EU) 1169/2011 of the European Parliament and of the Council. *Regulation (EU) 1169/2011 of the European Parliament and of the Council of 25 October 2011 on the Provision of Food Information to Consumers.* Official Journal of the European Union 22.11.2011, L 304/18.
38. Lim KH, Riddell LJ, Nowson CA, Booth AO, Szymlek-Gay EA. Iron and Zinc nutrition in the economically-developed world: a review. *Nutrients* (2013) 5:3184–211. doi: 10.3390/nu5083184

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frontiers in Microbiology

REVIEW
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Table Olive Wastewater: Problem, Treatments and Future Strategy. A Review

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The table olive industry produces a high quantity of wastewater annually. These wastewaters are very problematic because of their characteristics of high organic matter, high phenolic content, high salinity and conductivity. The quantities in which they are produced are also a serious problem. The worldwide production of table olives reached 2,550,000 tons in the last five campaigns, with the European Union contributing to 32% of total production. The problem of these wastewaters is focused on the Mediterranean area where the highest quantity of table olives is produced and to a lesser extent on the United States and South America. Countries like Spain produce around 540,000 tons of these wastewaters. At present, there is no standard treatment for these wastewaters with acceptable results and which is applied in the industry. Currently, the most common treatment is the storage of these wastewaters in large evaporation ponds where, during the dry season, the wastewater disappears due to evaporation. This is not a solution as the evaporation ponds depend completely on the climatology and have a high number of associated problems, such as bad odors, insect proliferation and the contamination of underground aquifers. Different studies have been carried out on table olive wastewater treatment, but the reality is that at the industrial level, none has been successfully applied. New and promising treatments are needed. The current review analyzes the situation of table olive wastewater treatment and the promising technologies for the future.

Keywords: table olive wastewaters, advanced oxidation processes, biological treatments, bioremediation technologies, added value compounds

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INTRODUCTION

The recent worldwide production of table olives was around 2.5–2.6 million tons (average data corresponding to harvest seasons 2011/2012 to 2016/2017, last season's data are provisional) with a prediction for the 2017/2018 season of around 2.8 million tons. The table olive industry is an economic activity which is widely extended throughout the Mediterranean countries. The countries belonging to the European Union produce 886,500 tons (season 2015/2016) and other countries like Egypt, Turkey, Algeria, Syria, and Morocco have a total production of 1,223,500 tons (season 2015/2016), making them the main producers of table olives. High productions are also achieved by the United States (70,500 tons in season 2015/2016) and some South American countries like Argentina, Mexico and Peru with a production of 151,500 tons (season 2015/2016) (International Olive Council [IOC], 2017).

Figure 1 shows the global forecast for table olive production (in percentage of the overall predicted production of 2.8 million tons for season 2017/2018) by producing countries (International Olive Council [IOC], 2017). **Figure 2** shows the forecast for table olive production in Europe (in percentage of the overall predicted production of 2.8 million tons for season 2017/2018) by countries (International Olive Council [IOC], 2017).

The elaboration process of table olives results in the generation of a high volume of wastewaters coming from the various steps of industrial elaboration. The organic charge, chemical composition and the characteristics of the different streams produced during table olive processing vary depending on the preparation type. During the elaboration process of table olives different chemicals are used, e.g., NaOH, NaCl, lactic acid, etc., and high amounts of clean water are used for the de-bittering step, the different rinses, the brining and the packing step. The chemical characteristics and the volume of these wastewaters make them a huge environmental problem.

Olives which use lye for their preparation require multiple washes and can use up to five times the amount of potable water compared with natural methods. Furthermore, table olive processing using lye treatments has higher requirements of energy and labor costs (Kailis and Harris, 2007), but table olives elaborated in this way have shorter processing times than those elaborated by natural methods, and are in high demand around the world.

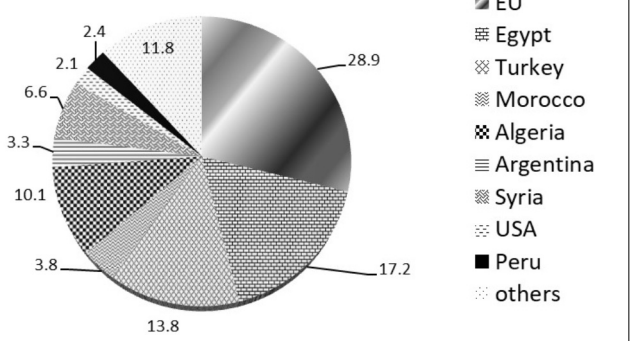

FIGURE 1 | Global prediction for table olive production (in percentage of the overall predicted production of 2.8 million tons for season 2017/2018) by countries. Source: International Olive Council [IOC], 2017.

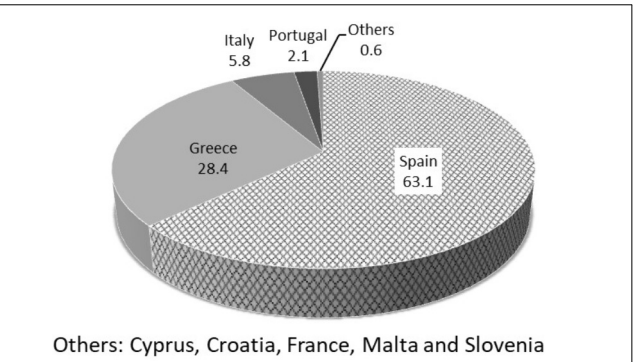

FIGURE 2 | Prediction for table olive production in the EU (in percentage of the overall predicted production of 2.8 million tons for season 2017/2018) by countries. Source: International Olive Council [IOC], 2017.

Generally, the effluents produced in each step of the elaboration process are mixed in one stream that is stored in evaporation ponds. This treatment technique is even more difficult than treating each effluent or wastewater separately. In addition, the storage in evaporation ponds of these wastewaters depends completely on climate and has a high number of associated problems like bad odors, insect proliferation and contamination of underground aquifers. In some countries these facilities are not allowed (Martin, 1992), but in several European countries, including Spain, they are still operational. Such practices are changing with the time in an attempt to care for the environment. In this sense, some of the solutions used in the process are now reused, such as the use or re-use of only one lye solution for de-bittering different batches of olives. Traditionally, the de-bittering step was carried out using fresh lye every time, but it has been proven that it is viable to use exhausted lye from other de-bittering steps, achieving lower environmental pollution and less water consumption (Garrido Fernández et al., 1997; Segovia-Bravo et al., 2008). With the same target in mind, the possibility of replacing the wash of the olives after the de-bittering step by re-using waters or the use of some organic and inorganic acids to neutralize the NaOH has also been studied (Garrido Fernández et al., 1997; Sánchez-Gómez et al., 2006).

Besides the fact that a huge volume of wastewater is produced, two of the main wastewaters, de-bittering and rising waters, are produced seasonally between September and November, due to the seasonal olive recollection (Ferrer-Polonio et al., 2017a). In addition, table olive processing is concentrated in narrow geographic areas where wastewater production is very high. This fact makes the situation even worse because of the huge volume of wastewaters generated in a short frame of time and place. Fermentation wastewaters from table olive processing, unlike the de-bittering and rising wastewaters, are generated during the year in the packaging plants. This fact and the very different characteristics of them, i.e., high pH and strong alkalinities in lyes and subsequent washing wastewater with acidic pH, oils in suspension, polyphenols and high salinity in brine wastewater, make it necessary to segregate these effluents from the general drainage systems in order to treat them separately (Romero-Barranco et al., 2001). A sustainable solution would be to refrain from mixing them. These practices would avoid the outlay of many liters of clean water.

The use of low concentration lyes or the re-use of fermentation brine are other practices studied (Garrido Fernández et al., 1997). Romero-Barranco et al. (2001) studied the possibility of introducing salt-free or reduced salt processes and segregation.

The direct re-use of fermentation wastewater or spent brines, coming from green or naturally black table olive processing has been studied. However, "the presence of metabolites interferes with the subsequent fermentation process" (Romero-Barranco et al., 2001). The presence of combined acidity, polyphenols, etc. does not provide the quality for safe storage and some organoleptic attributes of the olives can be damaged

(Brenes et al., 1989; Romero-Barranco et al., 2001). The re-use of fermentation wastewater or partially regenerated brines has also been studied by Romero-Barranco et al. (2001).

Other measures studied aimed to better the sustainability of table olive processing by using acidified water instead of brines in the case of Californian-style black olives (olives darkened by oxidation) (De Castro et al., 2007), thereby reducing the NaCl concentration in the wastewater. The acidification of the media favors lactic acid bacteria and makes the media incompatible with enterobacteriaceae growth, with yeasts being the prominent microorganisms in these solutions (De Castro et al., 2007; Rejano et al., 2010).

The legislation in different countries concerning environmental issues is becoming more and more strict in order to control pollution. In addition to the separation of wastewaters and the improvement of operational procedures in the industry, wastewater treatment is also necessary. To increase the sustainability and reduce the environmental impact of the traditional table olive elaboration process different treatments for the wastewaters from table olive processing (TOPW) have also been studied and applied. Among the TOPW treatments studied there are several studies that use advanced oxidation processes (AOPs) such as: ozonation (Benítez et al., 2003), Fenton's reaction (Kotsou et al., 2004), electrochemical treatments (Deligiorgis et al., 2008), TiO_2 photocatalysis (Chatzisymeon et al., 2008), electro-coagulation (García-García et al., 2011) and wet air oxidation (Katsoni et al., 2008). Biological treatments have also been explored and include anaerobic digestion (Borja et al., 1993; Beltrán et al., 2008), aerobic digestion processes (Brenes et al., 2000; Benítez et al., 2002b) and combinations of the two (Aggelis et al., 2001; Ferrer-Polonio et al., 2015).

Several works employed bioremediation technologies using microalgae to remove pollution (Serrano et al., 2017) and others which use fungi obtained promising results for chemical oxygen demand (COD) removal from wastewater (Lasaridi et al., 2010) and for de-colorization (Ayed et al., 2016). Other approaches have been to use certain wastewaters for irrigation (Murillo et al., 2000) or for the extraction and recovery of added-value products (Brenes et al., 2004; Kiai et al., 2014).

This review analyzes the current situation of the treatment for wastewater from table olive processing and gives an overview of the different strategies and treatments studied along with promising technologies for the future.

TABLE OLIVE ELABORATION PROCESS

The table olive elaboration process starts after picking the olives from the olive tree (*Olea europaea L.*) when they have a good size and color, e.g., from green to yellow. After picking, leaf removal and classification, the table olive elaboration process follows three main steps:

- *De-bittering or lye step*: in this step olive bitterness is removed by immersing the olives in a NaOH aqueous solution with concentrations between 1 and 2% w/v during 8–12–15 h (Parino et al., 2007; Cappelletti et al., 2011). The concentration of the NaOH used depends of the olive variety, the degree of ripeness of the drupe and the temperature and characteristics of the water to be used. More concentrated solutions can soften the flesh of the drupe, while more dilute solutions adversely affect the subsequent fermentation (Cappelletti et al., 2011). In this first step oleuropein is hydrolyzed to elenolic acid glucoside and hydroxytyrosol (Marsilio and Lanza, 1998; Marsilio et al., 2001).
- *Rinsing*: after the de-bittering step, the olives are washed, one or more times until all the alkali is removed (Fendri et al., 2013). This step uses large quantities of fresh water to separate the sodium hydroxide from the flesh of the olives and can vary in duration. The most commonly used method for washing is to rinse for 18–25 h with an initial short rinse of 1–2 h and two more rinses of 8–12 h each. "In this case the olives retain enough fermentable substances to ensure proper lactic fermentation" (Cappelletti et al., 2011). There are other options for longer or shorter duration rinses depending on the purpose of the olives to be washed.
- *Fermentation in brine*: after the rinsing step, the olives are submerged in a 9–10% w/v concentration NaCl. Fermentation preserves the olives and improves their organoleptic properties (Marsilio and Lanza, 1998). The addition of used brines or "mother brines," ensures the onset of a safe lactic fermentation (Cappelletti et al., 2011).

Finally, after the olives are washed with new water and after selection, avoiding damaged ones, they are packaged in 3–5% brine and pasteurized at 90°C during 1 h, following traditional elaboration processes.

There are many elaboration processes for table olives depending of the kind of olives and their specific necessities according to their natural composition, degree of ripeness, country of origin, local or regional customs, etc. Regarding standard trade preparations table olives can be sorted as shown

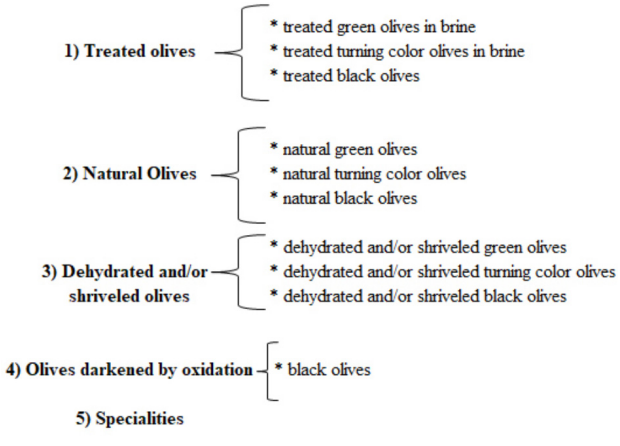

FIGURE 3 | Table olives sorted regarding standard trade preparations (International Olive Oil Council [IOOC], 2004).

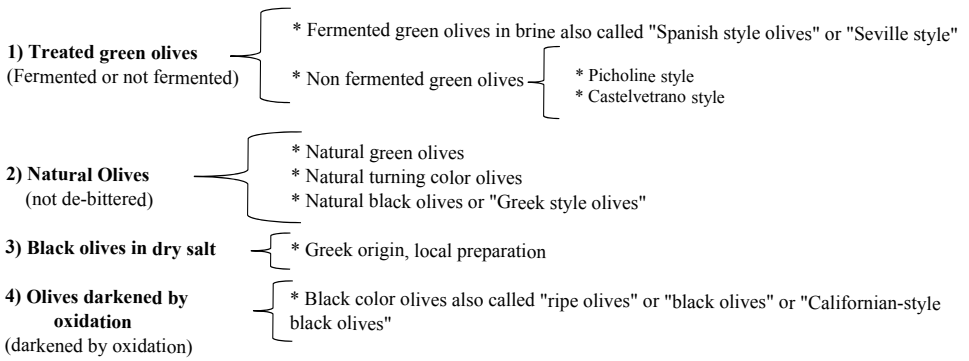

FIGURE 4 | Table olives sorted regarding the economic importance from global standpoint (adapted from Rejano et al., 2010).

in **Figure 3**. The most important processing methods regarding economic importance from a global standpoint are shown in **Figure 4** and detailed below

Treated Green Olives

"Treated olives are green olives, turning color olives or black olives that have undergone alkaline treatment, then placed in a brine where they undergo complete or partial fermentation, and are preserved or not by the addition of acidifying agents" (Sánchez-Gómez et al., 2006).

Green olives are harvested when they reach an average size, prior to color variation and are usually picked manually. The main treatments used for green olive preparation are: fermentation or "Spanish-style olives" and non-fermentation or "Picholine and Castelvetrano styles" (Sánchez-Gómez et al., 2006; Rejano et al., 2010).

Fermented Treated Green Olives or "Spanish-Style Olives"

After harvesting, transport and grading, green olives are kept in a lye solution (2.0–3.5% w/v, NaOH in water). Treatments usually take place in 10-ton capacity containers "until the lye has penetrated 2/3 of the way through the flesh" (Rejano et al., 2010). When the lye has reached this depth it is replaced by water to eliminate all the alkali in different wash steps and "to drag over soluble sugars needed for fermentation" (Rejano et al., 2010). Finally, the olives are preserved in brine (9–10% NaCl initially) which propitiates the culture media for fermentation. Brine concentration typically drops to 5% owing to the interchange between the water and the olives.

Initially, only Enterobacteriaceae, Gram-negative bacteria and Enterococci grow and they gradually become undetectable when the pH decreases "as a consequence of their own metabolism" (De Castro et al., 2002). Acid generation by these microorganisms favors the growth of lactobacilli, which is mainly responsible for fermentation (De Castro et al., 2002).

Non-fermented Treated Green Olives or "Picholine Style" and "Castelvetrano Style"

"Picholine style" for table olive preparation is used in the south of France, Morocco and Algeria. Picholine table olives undergo an initial de-bittering step with lye (2.0–2.5% NaOH w/v) for 8–12 h. After this time they are washed and placed in brine (5–6%) for 2 or 3 days, and then changed to a more concentrated brine (7%), using citric acid to keep the pH at around 4.5. The olives are ready for use after 8–10 days (Rejano et al., 2010).

"Castelvetrano style" for table olive preparation is used in the Castelvetrano region, Italy, with the olive variety "Nocellara del Belice." After harvesting, the olives are placed in a NaOH solution (1.8–2.5% w/v) with 5–8 kg of salt/140 kg of fruits 1 h after the de-bittering step begins. The olives are kept in this solution for 10–15 days. After this period, a light washing takes place before consumption (Salvo et al., 1995; Rejano et al., 2010).

Natural Olives

Natural olives are green olives, turning color olives or black olives that are placed directly in brine where they undergo complete or partial fermentation, preserved or not by the addition of acidifying agents (Sánchez-Gómez et al., 2006).

Natural olives are not de-bittered, they are placed directly in brine (9% NaCl) when they arrive to the table olive factory and they undergo a fermentation process. At the beginning of fermentation, the tanks are hermetic to avoid contact with air and to maintain anaerobic conditions. This kind of fermentation "takes a long time because the diffusion of soluble compounds through the epidermis in fruits not treated with alkali is slow" (Rejano et al., 2010). Natural olives are prepared from dark olives, but they are also prepared from green. Yeasts are the main microorganisms in this kind of brine although there are diverse microorganisms. Although the olives are not treated with lye, the brine reduces their bitterness and the olives are not packaged until the bitterness is weak enough. The color of the olives is corrected after fermentation by aeration or treatment with ferrous gluconate or lactate. "Natural black olives" are also known as "Greek style olives" (Sánchez-Gómez et al., 2006).

Black Olives in Dry Salt

This preparation uses overripe olives and has Greek origin. For this preparation, the olives are placed in baskets and covered with layers of salt (15% of the weight of the olives), and usually used for local consumption (Rejano et al., 2010).

Olives Darkened by Oxidation

Olives darkened by oxidation are green olives or turning color olives preserved in brine, fermented or not, darkened by oxidation in an alkaline medium and preserved in hermetically sealed containers subjected to heat sterilization; "they shall be a uniform black color" (International Olive Oil Council [IOOC], 2004). These are also known as "ripe olives" or "black olives" (Sánchez-Gómez et al., 2006) or "Californian-style black olives."

Californian-style black-ripe olives are olives treated and oxidized during the elaboration process to produce ranges of color from dark brown to black. These olives are picked when they are partially or completely ripe and then they are stored in brine (8–10%) during 30 days before treatment with NaOH (Sánchez-Gómez et al., 2006).

The de-bittering step is usually carried out with a NaOH (1–2% w/v) solution, applying at least three de-bittering treatments in a row, during 2 to 6 h. After each pretreatment there is a rinse step where air is bubbled into the water, producing an enzymatic reaction which causes the surface of the olives to darken (Brenes et al., 2004; Sánchez-Gómez et al., 2006). The lye treatments are between three and five. The main objective is to achieve a gradual penetration of the lye into the flesh so that it reaches the pit (Rejano et al., 2010).

Finally, the olives are washed to remove the sodium hidroxyde until a pH in the flesh of 8.0. Olives darkened by oxidation have to be sterilized to avoid pathogen growth.

TABLE OLIVE PROCESSING WASTEWATERS (TOPW): CHEMICAL COMPOSITION AND VOLUMES PRODUCED

Although there are three main streams that are produced after table olive processing, such as wastewaters coming from lye, wastewaters coming from the washes and wastewaters from fermentation brines, there are additional wastewaters from a table olive processing plant which are produced from the washing of the plant, cleaning of the vessels or containers, etc. There also are many different kinds of wastewaters produced, depending on the elaboration process, e.g., Spanish-style green olives, Californian-style, etc., the degree of maturation of the olives, the kind of water employed, and the additives used, among other factors. All of the wastewaters from table olive processing cause a serious environmental problem because of their chemical characteristics and the huge volumes produced. Lye wastewaters and the subsequent washing wastewaters are so problematic because of their high pH and strong alkalinity, and brine wastewaters because of their acidic characteristics and salinity, with high ClNa concentrations, as well as the organic charge due to the interchange of compounds with the olives during table olive processing, as in treated and fermented green olives where the content in polyphenols in the wastewaters coming from fermentation brines is rich but null in reducing sugars which are consumed in the fermentation step.

The volumes of wastewaters produced during the table olive processing methods are shown in **Table 1**. The most polluting effluents produced are those that include a lye treatment followed by exhaustive washings for the elimination of the alkali. Among them the production of Californian-style black-ripe olives has the highest pollutant potential with around 2–6 L/kg olives produced (Garrido Fernández et al., 1997; Papadaki and Mantzouridou, 2016), followed by the Californian green ripe olives and Spanish table olives with an average of 1.5–3.5 L/kg olives produced and finally, the Naturally black olives and the untreated green and turning color olives with 1 L/kg olives produced. Wastewaters from fermentation in brine represent 20% of the total volume within the global industry; it is 85% of the global wastewater pollution (Garrido Fernández et al., 1997; Moussavi et al., 2010; Ferrer-Polonio et al., 2016a).

Figure 5 shows the main characteristics of the generated wastewaters from the most economically important elaboration systems.

TABLE OLIVE PROCESSING WASTEWATER TREATMENTS

Advanced Oxidation Processes (AOPs)

Advanced oxidation processes have been presented as adequate methods for treating TOPW due to their ability to reduce the organic matter content.

The main systems used as AOPs are ozonation, UV irradiation, photocatalysis, hydrogen peroxide oxidation, Fenton's reaction, electrochemical oxidation and wet air oxidation.

These processes are characterized by the generation of highly reactive free radicals which are capable of oxidizing several organic substances, such as phenols. These compounds are able to react with carbon–carbon double bonds and thus attack the aromatic nucleus, which are typical characteristics of refractory organic compounds (Zaviska et al., 2009).

One of the main issues with these processes is their high operational costs (Comninellis et al., 2008), thus the application of AOPs for treating TOPW is only recommended when a biological process is not possible or is insufficient. **Table 2** summarizes the operating conditions, process efficiencies and benefits derived from the use of AOPs for the treatment of these wastewaters.

Ozonation

Ozonation consists of the use of ozone, a powerful oxidant which can either decompose in water, forming hydroxyl radicals that act as a stronger oxidant, or attack functional groups of organic compounds through an electrophilic mechanism (Ayed et al., 2017). The main mechanism of these advanced oxidation processes consists of the production of highly free radicals which can react with phenols through aromatic substitution and/or dipolar cyclo addition reactions (Langlais et al., 1991). Commonly, this treatment is enhanced using hydrogen peroxide as a further oxidant and UV radiation as a photocatalytic agent. Generally, these three oxidant agents can directly oxidize

TABLE 1 | Volume of the different effluents produced during table olive processing (lye, fermentation brine, washing, and preservation brine) by the different methods in Liters/kg of table olives.

	Spanish style	Untreated green and turning color olives	California green ripe olives	California black ripe olives	Naturally black olives
(1) Lye	0.5		0.5	0.5–0.25	
(2) Fermentation brine	0.5	0.5	0.5	0.5	0.5
(3) Washing	0.5–2.0		0.5–2.0	0.5–3.0	
(4) Preservation brine	0.5	0.5	0.0–0.5	0.5	0.5

Source: *Garrido Fernández et al., 1997.*

organic matter, though several studies show that some positive synergetic effects take place between them. Ozone in the presence of H_2O_2 generates hydroxyl radicals and in the presence of UV radiation ozone can be converted into more hydrogen peroxide which produces hydroxyl radicals and increases its oxidant activity. Thus, when these agents are used together the main oxidative agents are the hydroxyl radicals (Benítez et al., 2003).

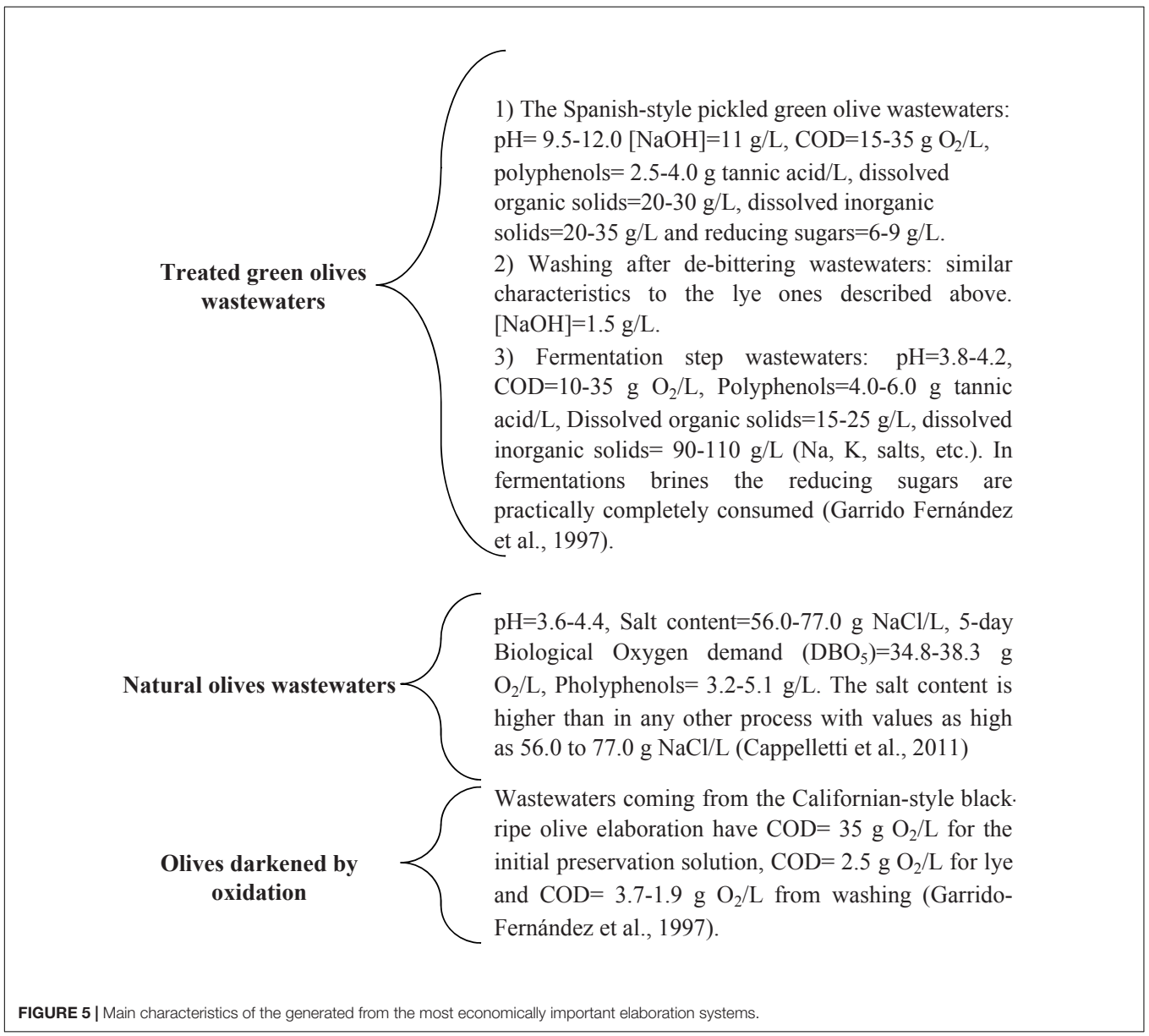

FIGURE 5 | Main characteristics of the generated from the most economically important elaboration systems.

TABLE 2 | Summary of the operating conditions, process efficiencies and benefits derived from the use of advanced oxidation processes (AOPs) for TOPW treatment.

Wastewater type	Treatment	Operating conditions	Process efficiency	Benefits	Reference
Green olive de-bittering wastewaters	Ozonation	O_3: 10g. O_3 rate: $7{\cdot}10^{-4}$ mol min^{-1}. Time: 5 h. COD_0: 20 g O_2/L.	COD removal: 50%.	The pH was stabilized from 13 to 9.6.	Beltrán et al., 1999
Black olive washing wastewaters	Ozonation	Time: 3 h. Temperature: 10–30°C.	COD removal: 24–33%.	An increase in temperature enhanced the COD and aromaticity removal.	Beltrán-Heredia et al., 2000b
Table olive processing wastewaters	Ozonation	[O_3]: 45 mg O_3/L. O_3 rate: 20 L/h. Time: 35 min.	COD removal: 80% (after aerobic digestion).	This treatment enhanced the post aerobic digestion, by reducing the pH, the phenols content and the ammonia.	Rivas J. et al., 2000
Green olive de-bittering and washing wastewaters diluted with urban wastewaters	Ozonation	O_3-O_2 rate: 0–20 L/min. Acidic and basic cycles (pH 4–10). Time: 3 h.	COD removal: 80%.		Rivas F.J. et al., 2000
Black olive washing wastewaters	Ozonation	P(O_3): 4.25 kPa. O_3-O_2 rate: 0–40 L/min. Temperature: 20°C. Time: 6 h.	COD removal: 80%.	The pH was stabilized	Benítez et al., 2001
Black olive lye-wastewaters	Ozonation	P(O_3): 2.76–4.36 kPa. Temperature: 20°C. Time: 8 h.	COD removal: 70%.	An increase in initial ozone pressure increased phenol removal	Benítez et al., 2002a
Black olive processing wastewaters	Ozonation	P(O_3): 1.04 – 4.5 kPa.	COD removal: 14–23%.	COD removal depends on the initial ozone pressure.	Benítez et al., 2003
Green olive de-bittering wastewaters	Ozonation + H_2O_2	O_3: 3–4 g. O_3 rate: $5{\cdot}10^{-4}$ mol min^{-1}. [H_2O_2]: 10^{-3} M. Time: 2 h.	COD removal: 80–90%.	Addition of H_2O_2 enhanced the COD removal from 50% to 90%.	Beltrán et al., 1999
Black olive washing wastewaters	Ozonation + H_2O_2	P(O_3): 4.25 kPa. O_3-O_2 rate: 0–40 L/min. [H_2O_2]: 0.0525–0.129 M. Temperature: 20°C. Time: 6 h.	COD removal: 92%.	Addition of H_2O_2 enhanced the COD removal from 80 to 92%.	Benítez et al., 2001
Black olive processing wastewaters	Ozonation + H_2O_2	P(O_3): 4.5 kPa [H_2O_2]: 0.2–0.5 M	COD removal: 24–29%.	Addition of H_2O_2 enhanced the COD removal from 23 to 29%.	Benítez et al., 2003
Green olive de-bittering wastewaters	Ozonation + UV	O_3: 3–4 g. O_3 rate: $6.91{\cdot}10^{-4}$ mol min^{-1}. UV: 254 nm. Time: 2 h.	COD removal: 80–90%.	UV addition enhanced the COD removal, although, using H_2O_2 the TC depletion is higher (55%).	Beltrán et al., 1999
Black olive washing wastewaters	Ozonation + UV	P(O_3): 4.25 kPa. O_3–O_2 rate: 0–40 L/min. UV: (Hanau TQ150 high-pressure mercury vapor lamp) $1.76 \cdot 10^{-5}$ einstein/s. Temperature: 20°C. Time: 6 h.	COD removal: 92%.	Using UV enhanced the aromatic compound removal when comparing with single ozonation or H_2O_2 addition.	Benítez et al., 2001

(Continued)

TABLE 2 | Continued

Wastewater type	Treatment	Operating conditions	Process efficiency	Benefits	Reference
Black olive lye-wastewaters	Ozonation + UV	$P(O_3)$: 4.41 kPa. Temperature: 20°C. Time: 8h.	COD removal: 85%.	The use of UV enhanced polyphenol removal (100%).	Benítez et al., 2002a
Black olive processing wastewaters	Ozonation + UV	$P(O_3)$: 1.04 – 4.5 kPa.	COD removal: 16–39%.	Using UV enhanced COD removal when comparing with single ozonation or H_2O_2 addition.	Benítez et al., 2003
Black olive processing wastewaters	Ozonation + H_2O_2 + UV	$P(O_3)$: 4.5 kPa. $[H_2O_2]$: 0.2–0.5 M.	COD removal: 39%.		Benítez et al., 2003
Black olive processing wastewaters	Fenton's Reagent + UV	Temperature: 20°C. Time: 8 h. $[Fe^{2+}]$: 0.025M. $[H_2O_2]$: 0.5M.	COD removal: 24%.		Benítez et al., 2003
Table olive washing and de-bittering wastewaters	Fenton's reagent	$[H_2O_2]$: 2, 4, 6, 8 g/L.	COD removal: 34%.	The pH was reduced to 2.2	Kotsou et al., 2004
Black olive washing wastewaters	Electrochemical Treatment (BDD)	COD_0: 10 g O_2/L. Current intensity: 30 A. Time: 14 h.	COD removal: 73%.	Initial pH and H_2O_2 did not show any enhancement in COD removal.	Deligiorgis et al., 2008
Meski olive washing and de-bittering wastewaters	Electrochemical Treatment (BDD)	Time: 2 h. Current density: 110 mA/m^2.	COD removal: 97%.		Gargouri et al., 2017
Black olive processing wastewaters	Electrochemical Treatment (BDD)	SBR mode: 0.5 L/min. COD_0: 7500 mg O_2/L. Time: 30–240 min. Current density: 187.5 mA/cm^2.	COD removal: 96.5%.	The pH was stabilized	Tatoulis et al., 2016
Meski olive washing and de-bittering wastewaters	Electrochemical Treatment (Led dioxide electrode)	Time: 2 h. Current density: 110 mA/m^2.	COD removal: 71%.		Gargouri et al., 2017
Green olive washing and de-bittering wastewaters	Electrochemical Treatment (Fe electrode)	$[H_2O_2]$: 17 g/L.	COD removal: 75%.	The use of $Ca(OH)_2$ enhanced the COD removal up to 98%.	Kyriacou et al., 2005
Table olive processing wastewaters	Electrochemical Treatment (Planar graphite electrode) + Anaerobic Digestion	Temperature: 35°C. Current density: 7.1 A/m^2.	COD removal: 32%. Maximum methane yield: 109 NmL CH_4/g COD.	This wastewater did not produce methane during the anaerobic digestion when the potential was not applied.	Marone et al., 2016

(Continued)

TABLE 2 | Continued

Wastewater type	Treatment	Operating conditions	Process efficiency	Benefits	Reference
Black olive de-bittering wastewaters	Photocatalysis (TiO_2)	COD_0: 1–8 g O_2/L. UV-A: 300–366 nm.	COD removal: 13–38%.	An addition of H_2O_2 enhanced the COD removal by 20%.	Chatzisymeon et al., 2008
Green olive processing wastewaters	Electro-coagulation (Al/Fe)	Temperature: 20-25°C. Time: 50 min. Current density: 25 mA/cm^2.	COD removal: 40%.	The pH was neutralized.	García-García et al., 2011
Green olive washing and de-bittering wastewaters	Wet air oxidation	$P(O_2)$: 5 MPa. Time: 6–8 h. [Cu^{2+}]: 419.4 mg/L. Temperature: 180°C.	COD removal: 59.8%.	Reducing the amount of Cu^{2+} enhanced the phenol depletion up to 95%, although, COD removal was lower (28.5%).	Rivas et al., 2001
Black olive fermentation wastewaters	Wet air oxidation	$P(O_2)$: 2.5 MPa. COD_0: 1240 mg O_2/L. Temperature: 180°C. pH: 7. Time: 2 h.	COD removal: 70%.	Reaction time, temperature and initial pH affected significantly the COD reduction.	Katsoni et al., 2008

COD_0: initial COD.

Single ozonation was applied to wastewaters derived from the de-bittering step of green table olive processing for 5 h (with a COD of 21 g O_2/L) with a COD removal of nearly 50% (Beltrán et al., 1999). After ozonation the pH was reduced from 13 to 9.6; aromatic compounds were reduced by 23% and the color was completely eliminated (Beltrán et al., 1999).

A further study by Beltrán-Heredia et al. (2000a) evaluated the kinetics of the aerobic biological processing of black table olive wastewaters using the Contois model. Results showed that a single aerobic treatment led to specific kinetic parameters for the substrate removal rate (COD and total phenols) of $4.81 \cdot 10^{-2}$ h^{-1}; a cellular yield coefficient of 0.279 g VSS/g COD (VSS: volatile suspended solids), and finally, the kinetic constant for endogenous metabolism was $1.92 \cdot 10^{-2}$ h^{-1}. When ozonation was applied prior to the aerobic treatment these parameters were: $5.42 \cdot 10^{-2}$ h^{-1}, 0.280 g VSS/g COD and $9.1 \cdot 10^{-3}$ h^{-1}, respectively. So the use of ozone as a previous step before aerobic degradation improved the kinetics of the process as well as the pollutant reduction, as reported in this previous work.

Ozonation processes for wastewater resulting from the de-bittering stage of green table olive preparation have been improved by the combination with hydrogen peroxide and/or UV radiation. For instance, an 80% or 90% COD removal was achieved with ozone doses of 3 and 4 g with the addition of hydrogen peroxide (10–3M) or UV radiation (254 nm) (Beltrán et al., 1999).

An ozone dosage of 45 mg/L (flow rate 20 L/h; 35 min) decreased the pH (from 11.5 to 7.5–8), phenol content (35%) and nitrogen as ammonia (70%) in table olive wastewater with an increase in biodegradability which allowed for an 80% COD removal after aerobic digestion (Rivas J. et al., 2000).

Rivas F.J. et al. (2000) showed that using ozonation as a first step followed by the aerobic treatment of green table olive wastewaters generated in the de-bittering and washing steps of this process and diluted with urban wastewaters (final COD: 1450 mg O_2/L) increased biodegradability by 100% (as BOD_5/COD ratio) when acidic and basic cycles (pH 4 and 10) were applied in the ozonation step. This combined treatment led to a 90% phenolic compound removal and 80% COD removal during the ozonation step, and a further reduction in COD by 38% after the aerobic process.

When the ozonation of black table olive wastewaters from the washing step was applied after aerobic digestion an 87% COD removal was obtained, which implies that aerobic pre-treatment improved subsequent ozone action due to the elimination of most of the organic matter. Thus, ozone applied after aerobic degradation acted mostly on non-biodegradable compounds while the phenolic compounds were basically removed in the previous aerobic stage (Beltrán-Heredia et al., 2000b).

When ozonation was applied to black table olive wastewater from the washing step an 80% substrate removal was reached. Moreover, a 78% aromatic compound removal and a reduction in pH from 12.62 to 8.26 were accomplished when the inlet ozone pressure was 4.25 kPa and applied for 6 h (Benítez et al., 2001). A combined ozonation with hydrogen peroxide or UV-radiation has been also studied and the results showed an enhancement in substrate and aromatic compound removals.

When combined with hydrogen peroxide a maximum substrate removal was reached (92%), although when combined with UV radiation a better aromatic compound removal (100%) and lower pH (8.54) were observed (Benítez et al., 2001). In this same study, ozonation (4.20 kPa; 20°C; 2 h) after aerobic biodegradation (3.75 days) was evaluated, and this combined treatment reduced substrate concentration by 90%, which could be enhanced when UV radiation was coupled with ozone, reaching a total substrate reduction of 93%.

Lye wastewaters produced during the de-bittering and darkening of black table olives have also been studied due to their alkali contents. Benítez et al. (2002a) showed that single ozonation of this lye wastewater (20°C, pH 13.6 and 8 h) reduced the COD by 70%. This study also showed that raising the initial pressure of the ozone from 2.76 to 4.36 kPa affected COD removal by only 2%, while polyphenol removal increased from 87 to 94%. pH was also affected during single ozonation and was reduced to 9.89 and 9.28, depending on the initial ozone pressure; while aromaticity removal was not affected by initial ozone pressure (80%). In this study some combinations of O_3 with H_2O_2 or UV radiation were also evaluated. The results showed that the best combination resulted from O_3 plus UV radiation with an initial ozone pressure of 4.41 kPa which led to an 85% COD removal, a 99% aromaticity removal, a 100% polyphenol removal and a decrease in pH to 9.32.

Benítez et al. (2003) evaluated the effect of ozonation combined with H_2O_2 and UV radiation on wastewaters from black table olive processing. This study concluded that single ozonation (1.04–4.48 kPa) reduced COD concentrations by 14–23%, depending on the initial ozone pressure and aromatic compounds by 73%, regardless of the initial O_3 pressure. The combination of O_3 (4.5 kPa) and H_2O_2 (0.2–0.5 M) resulted in a COD removal of 24–29% and an aromatic reduction of 74–75%. A combined ozone (1.04–4.50 kPa) and UV radiation treatment reached a 16–39% COD removal and aromatic reduction of 83–86%. When H_2O_2 and UV radiation were used in combination with O_3 the COD removal increased by up to 39% and the aromatic reduction to 86%. Single UV radiation reached COD and aromatic compound removals of 9 and 27%, respectively. These removals increased when 0.5 M of H_2O_2 was used (COD removal increased to 13% and the aromatic removal to 38%).

Moreover, Benítez et al. (2003) studied the effect of ozonation and ozonation/UV radiation as a pre-treatment for aerobic degradation. A single aerobic treatment (initial concentration from 0.54 to 3.55 gVSS/L; initial COD = 34.2 g/L) led to a COD removal of 66–67%, which can be enhanced when ozone (3.04 kPa; 2 h) and ozone/UV were used previously, which led to a COD removal of 71% in both cases.

In most cases, the ozonation of table olive wastewater has proven to be an excellent pre-treatment for further biological processing such as aerobic digestion due to its capacity for phenolic compound removal, alkalinity depletion and pH reduction. Moreover, COD removals of 80–90% were achieved.

However, the major limitation of this process was the high cost of ozone generation coupled with its short half-life period (Ayed et al., 2017). Another drawback was the low solubility of O_3 in these solutions which reduced its efficiency (Gogate and Pandit, 2004).

Fenton's Reaction

Fenton's reaction consists of the addition of H_2O_2 and Fe (II) salts directly into wastewaters. The oxidation mechanism consists of the catalytic decomposition of H_2O_2 into hydroxyl radicals which produce an oxidized iron (III) which can act as a coagulation and sedimentation agent for other compounds. Moreover, these new compounds can also oxidize more hydrogen peroxide as well as phenolic compounds (Rivas et al., 2003; Cañizares et al., 2007). Furthermore, iron forms can produce several organic and inorganic complexes which highly affect the reactivity of this metal over hydrogen peroxide. Thus, iron complexes from carboxylic acids have been proven to act as accelerating agents, while phosphates inhibit the oxidation process (Rivas et al., 2003).

Benítez et al. (2003) evaluated the effect of Fenton's reagent combined with UV radiation by treating wastewaters from black table olive processing. The results showed that at a temperature of 20°C during 8 h the COD removal reached 24% under the best conditions (i.e., $[Fe^{+2}] = 0.025M$; $[H_2O_2] = 0.5M$).

A combined aerobic and Fenton's reagent treatment of table olive washing waters and de-bittering processes was studied by Kotsou et al. (2004). After the aerobic stage (2-days), using *Aspergillus niger*, COD reduction was 70% and total and simple phenol depletions were 41% and 85%, respectively. During the oxidation step using Fenton's reagent the effect of H_2O_2 concentration was evaluated. It was concluded that different concentrations (2, 4, 6, 8 gH_2O_2/L) did not show any effect on phenol removal, which is the first organic compound to be oxidized (within the first 15 min). Only the lowest concentration did not reach the same phenol removal due to the complete elimination of hydrogen peroxide. Other organic compounds were reduced by the hydrogen peroxide left. After the Fenton's reagent treatment, COD removal was 34–72% (depending on H_2O_2 initial concentration), total phenolic compound removal was 64–91%, pH was reduced to 2.2 and an increase in temperature was observed to up to 34°C (Kotsou et al., 2004).

In order to reduce the operational cost of this AOP the replacement of ferrous iron by the ferric form could be an alternative (Ayed et al., 2017). One major disadvantage of this AOP is the necessity of a further treatment which may reduce the iron present in the effluent. In addition, the presence of hydrogen peroxide at the end of the treatment process can limit the efficiency of a further biological process (Gogate and Pandit, 2004). In addition, lower COD removals (24–34%) were achieved compared to Ozonation processes.

Electrochemical Treatment

The high conductivity of boron-doped diamond (BDD) and other types of electrodes makes these materials a good choice for electrochemical treatments which consist of the oxidation of H_2O at the anode to generate adsorbed hydroxyl radicals in the electrode surface. These hydroxyl radicals are capable oxidizing organic compounds near the electrode zone into CO_2 and H_2O (Alvarez-Pugliese et al., 2014).

Wastewaters from the washing process of black table olives were treated using BDD electrodes with the aim of evaluating the effect of initial COD (1340–5370 mg O_2/L), reaction time (30–120 min), current intensity (5–14 A), initial pH (3–7) and the application of hydrogen peroxide (500 mg/L) as an additional oxidant (Deligiorgis et al., 2008). After the study, it was concluded that the initial pH and the use of hydrogen peroxide did not present any effect on COD and total phenol removal. By performing a further experiment under the best obtained conditions (initial COD: 10 g O_2/L; 30 A; 14 h) a reduction of 73% COD was achieved. Certain issues arose: the current intensity was near to the maximum recommended by the fabricator (35 A) and the higher the initial COD concentration the higher the temperature, which could turn out to be impermissible for the reactor.

Gargouri et al. (2017) compared lead dioxide electrodes and BDD electrodes as the anodes when treating Meski olive wastewaters from washing and de-bittering processes. The results showed that the pollutant reduction in this wastewater when using BDD electrodes presented a higher oxidation rate than that obtained with the lead dioxide electrode. Under the best conditions (2 h, 110 mA/m^2), the BDD electrode presented a COD removal of 97% while lead dioxide electrode showed a 71% COD removal. This difference was explained on the basis of the different natures of the physio-sorbed hydroxyl radicals generated on both electrodes.

Kyriacou et al. (2005) studied the combined treatment of the wastewaters from green olive table washing and de-bittering processes by aerobic degradation and further electrochemical treatment with hydrogen peroxide. Aerobic degradation was performed using an *Aspergillus niger* strain as inoculum and ran for 3 days. The electrochemical system evaluated three different electrode types (iron, stainless steel and Ti/Pd) and several reaction times (30 and 60 min). The results showed that the aerobic degradation reduced the COD by up to 66–86% and the phenol compounds by up to 65%; pH was also reduced from 5.0 to3.5. The electrochemical treatment at laboratory-scale reduced the COD and phenol content by 97% when 2.5% H_2O_2 and an iron electrode were used for 60 min; while at pilot scale COD removal reached 75% when 1.7% H_2O_2 was used.

A combined aerobic system and electrochemical oxidation using the BDD treatment of black table olive wastewater was evaluated by Tatoulis et al. (2016). In this research the effects of the initial COD concentration (5500 – 15000 mg O_2/L) and the operation mode [batch and sequential batch reactors (SBR) with recirculation] of the aerobic process on final purification were assessed. The results showed that the best conditions were obtained with SBR mode (recirculation: 0.5 L/min) and an initial COD of 7500 mg O_2/L which resulted in a COD reduction of 96.5%, a phenol reduction of 64.5%, and a pH neutralization from 4 to 6 when an inoculum made from the original indigenous microorganism from wastewater was added at the beginning of the aerobic process. However, color could not be removed completely. When the BDD electrode was used together with the aerobic process the COD, phenol and color removal was complete with a current density of 187.5 mA/cm^2 within 30–240 min, depending on the initial COD.

A different electrochemical system was used by Marone et al. (2016), in which a planar graphite plate was selected as working electrode in a hermetic potentiostatically controlled half-cell system in combination with an anaerobic digestion system at 35°C. Using table olive brine processing wastewater without any potential applied, no methane production was recorded in its anaerobic digestion. However, when a potential was applied a maximum methane yield of 109 ± 21 NmL CH_4/g COD removed was observed with a current density of 7.1 ± 0.4 A/m^2 and a coulombic efficiency of 30%. In addition, 80% of the phenolic compounds were removed, though COD was only reduced by 32%. Furthermore, a microbial study of anodic biofilms was performed by the sequencing of bacterial 16s rDNA, and no archaea was found and within the bacterial community the *Proteobacteria* were predominant (>48%) over *Bacteroidetes* and *Firmicutes*. The most abundant anode-respiring bacteria (ARB) found in every assay performed was *Desulfuromonas desulfuromonadaceae* (23–55%). Another predominant ARB found in some assays was *Geoalkalibacter geobacteraceae* (20–40%).

The electrochemical treatments are generally high cost processes, i.e., BDD electrode electricity costs can reach 7–10€/kg COD removed (Chatzisymeon et al., 2009). They do not allow the total color and phenolic compounds removals from these wastewaters, for which further biological processes are needed. By contrast, acceptable COD reductions (75–95%) can be achieved.

TiO_2 Photocatalysis

Photocatalysis consists of the excitation by UV or Visible of a semiconductor. The semiconductor (TiO_2) transforms photon energy into chemical energy by redox reactions which produce activated sites of TiO_2 and the subsequent degradation of organic compounds due to chain reactions promoted by strong radical oxidants like the OH generated by water molecules (Linsebigler et al., 1995).

Wastewaters from the de-bittering step of black table olives were used for photocatalysis treatment after dilution with distilled water to reach an initial concentration of 1–8 gO_2/L (Chatzisymeon et al., 2008). In this study several commercial TiO_2 were evaluated due to their different particle size and specific area, although UV-A radiation between 300 and 366 nm was kept the same for each experiment. The highest COD reduction (38%) was obtained with the TiO_2 with the highest specific area, though total phenols and color reduction were almost the lowest (11 and 12%, respectively). The highest phenol removal (58%) was obtained with a TiO_2 with a medium value of specific area when COD reduction was very low (13%) but color reduction was slightly high (77%). Then, the highest color reduction (83%) was observed with the second highest specific area TiO_2. In each case, the TiO_2 form was anatase, which presented a higher photocatalysis activity than the rutile form.

In a further study, Chatzisymeon et al. (2008) compared the same anatase TiO_2 when the initial COD was changed (from 2 to 8gO_2/L). This study concluded that the lower the initial COD concentration, the higher the reduction in COD, total phenols and color. Moreover, an improvement of 20% in COD, aromatic compounds and color removal was achieved when hydrogen

peroxide was added during the treatment, although total phenol reduction remained unchanged.

The main advantage of TiO_2 is its non-toxicity, water insolubility, hydrophobicity, cheap availability and photo-corrosion stability nature. However, a great drawback is the necessity of UVA irradiation for a good photoactivation. Since solar irradiation into the earth's surface only contains 3–5% of UVA, an enhanced method needs to be studied (Comninellis et al., 2008). Moreover, the effluents obtained using this method, even when the organic content has been reduced, present less biodegradable compounds than the untreated wastewater as can be seen when table olive wastewaters are treated only aerobically or combined with a photocatalysis pre-treatment. Borja et al. (1994a) showed a COD reduction of nearly 90% after aerobic biodegradation while Chatzisymeon et al. (2008) reached only a 60–65% when aerobic biodegradation was done after a photocatalysis pre-treatment, though the time needed for the photocatalysis treatment was at least twice the order of magnitude faster than the time needed for biological degradation.

Electro-Coagulation

Electro-coagulation is an electrochemical method with a sacrificial anode which is dissolved into the wastewaters in order to generate active coagulant precursors. The main precursors used are aluminum or iron cations which react with negatively charged particles present in the wastewaters (García-García et al., 2011).

The application of electro-coagulation technology as a pre-treatment for wastewaters from green table olive processing was evaluated by García-García et al. (2011). Under the best conditions (20–25°C; 50 min; 25 mA/cm^2), 40% COD removal was obtained as well as the elimination of most of the phenol contents (78–87%) and color. pH was also neutralized (6.5–7.0). Across the several electrode combinations evaluated it was concluded that using Al in the anode and Fe in the cathode the COD, phenol and color concentrations were reduced faster than without them.

García-García et al. (2011) also studied the chemical reactions occurring on the electrode surfaces and in the bulk solution. They concluded that the pH growth was linked to the hydroxyl radicals liberated by the action of organic acids present in the wastewaters with the hydroxyl compounds of aluminum and iron after oxidation in the electrodes. Furthermore, the total consumption electricity spent was estimated at 0.76 kW $h/m^3_{wastewater}$, and the aluminum loss in the anode was 2.15 $g/m^3_{wastewater}$.

This technology has been successfully proven for color and the removal of colloidal particles, although a relatively low COD removal of 40% was reported (García-García et al., 2011).

Wet Air Oxidation

Wet air oxidation is a thermochemical AOP. The use of high temperature (200 – 320°C) and pressure (2 – 20 MPa) allow the water molecule to form hydroxyl radicals and other active oxygen species which react with organic matter producing highly oxidized compounds and eventually carbon dioxide and water (Levec and Pintar, 2007; Katsoni et al., 2008).

Rivas et al. (2001) treated diluted wastewaters from the washing and de-bittering processes of green table olives with ultrapure water (1:2). Wet air oxidation was carried out in batch mode with an initial air pressure of 1 MPa for 6–8 h. In addition, copper (II) sulfate was added as catalyst. The results showed that the higher the amount of copper added (50.8–419.4 mg/L), the higher the COD reduction. The influence of O_2 partial pressure was also analyzed, and the results showed that the higher the pressure applied (3.0–7.0 MPa) the higher the COD removal, especially the phenol reduction, which reached 95%. A first-order kinetics related to COD removal was applied and the kinetic constant under the best conditions (7.0 MPa) was $3.97 \pm 0.47 \cdot 10^{-5}$ s^{-1}. Temperature (170–210°C) was also modified in order to deduce its influence on COD removal, although no representative differences were detected. The influence of hydrogen peroxide (340–3400 mg/L) was also taken into account. Finally, it was concluded that the highest COD reduction (59.8%) was obtained at 180°C, with an oxygen partial pressure of 5 MPa and with 419.4 mg/L of Copper (II), and with these conditions phenol conversion was 76.8%. The best conditions for phenol conversion (94.9%) were at 180°C with an oxygen partial pressure of 5 MPa and 50.8 mg/L of Cu^{+2}, while COD removal was 28.5%.

Furthermore, Rivas et al. (2001) evaluated the effect of a wet air oxidation treatment prior to aerobic degradation. Due to the negative effect of Cu^{+2} on biodegradability, most of the experiments carried out used wastewater with hydrogen peroxide which was further diluted with synthetic urban waters. Thus, the use of wet air oxidizes wastewater after 10 h and at 20°C, achieving a COD reduction of between 23.4 and 77.1%.

Wastewaters from black table olive fermentation processes were used by Katsoni et al. (2008) with the aim of evaluating the influence of initial substrate concentration (1240–5150 mg COD/L), operation time (30–120 min), temperature (140–180°C), initial pH (3–7) and H_2O_2 (500 mg/L) as an additional oxidant during wet air oxidation. Wet air oxidation was performed in an autoclave and pure O_2 was fed continuously, the O_2 partial pressure was maintained at 2.5 MPa. It was observed that across the different parameters evaluated, the operation time, the temperature and the initial pH presented a higher effect on COD removal, while initial COD, reaction time and temperature showed an important influence on phenol removal. Under the best conditions (initial COD 1240 mg O_2/L; temperature of 180°C; pH 7; time 120 min and H_2O_2 0 mg/L) phenol removal was complete, de-colorization reached 90% and COD reduction was 70%.

Therefore, the phenol and COD removals in these processes were clearly influenced by oxygen partial pressure, temperature and reaction time.

Biological Treatments

Biological treatment processes utilize microorganisms to remove the organic matter contained in wastewaters. They can be classified into aerobic and anaerobic processes according to the type of microorganisms used and the operational conditions,

i.e., presence or absence of oxygen. These processes have been widely and successfully applied for the treatment and purification of many high- and medium-organic content wastewaters. However, there are not many scientific works described in the literature related to the application of biological processes for treating table olive wastewater. This is because of its elevated content in recalcitrant and phenolic compounds, which are characterized by high toxicity and antimicrobial effect (Ayed et al., 2017). Moreover, the severe pH values, high salinity and unbalanced composition of TOPW may inhibit microbial growth and metabolism when biological treatment technologies are applied (Papadaki and Mantzouridou, 2016). **Tables 3–5** summarize the operating conditions, process efficiencies and benefits derived from the use of anaerobic, aerobic and combined anaerobic-aerobic processes, respectively, for the purification of these wastewaters.

Anaerobic Treatment

Anaerobic digestion is a complex biological process in which organic raw substances are transformed to biogas, a mixture of methane (50–75%) and carbon dioxide (30–40%), and traces of other minor components(H_2, H_2S, etc.) by different groups of microorganisms which are sensitive to or completely inhibited by oxygen (Borja and Rincón, 2017). Using anaerobic digestion, it is possible to transform wastewaters from many industries into profitable by-products, mainly biogas, a useful fuel that may be used to provide heat, electrical power or combustible for transport. The transformation of organic matter to biogas occurs through four steps: hydrolysis, acidogenesis, acetogenesis and methanogenesis. The hydrolysis stage degrades both insoluble organic substances and high molecular weight compounds such as lipids, polysaccharides and proteins into soluble organic compounds. In a second stage, volatile fatty acids are generated by acidogenic or fermentative bacteria, as well as NH_3, CO_2, H_2S, and other intermediate compounds. The third stage is acetogenesis. In this step the higher organic acids and other compounds produced by acidogenesis are further digested by acetogens to generate acetic acid, CO_2 and H_2. Finally, the fourth stage, or methanogenesis, produces methane. Two groups of methanogenic microorganisms produce methane: the first group converts acetate into methane and carbon dioxide (aceticlastic methanogens) and the second group uses hydrogen as electron donors and CO_2 as acceptor to produce methane (hydrogenotrophic methanogens) (Borja and Rincón, 2017). The main advantages of the anaerobic digestion process over other forms of waste treatment are: quite a high degree of purification with high-organic-load feeds can be achieved; up to 90% reduction in space requirements; a combustible biogas is generated (around 31 m^3 of methane per 100 kg of COD, with a maximum energetic value of 108 kWh in electric energy or 308 kWh in heat); the generation of biogas enables the process to produce energy; no use of fossil fuels for treatment (saving about 0.5–1 kWh per kg of organic matter); lower biomass sludge is produced in comparison to aerobic treatment processes, specifically a decrease in excess sludge production by 90% is detected; the sludge generated (digestate) is very stable and is an enhanced fertilizer in terms of both its availability to plants and its rheology; fewer nutrient requirements are necessary with optimum C:N:P of 100:0.5:0.1, which is 10% of the nutrient demand for the adequate development of the aerobic process.

An early study demonstrated the suitability of the anaerobic digestion process to treat the wastewater generated in the manufacturing of black olives (pH: 9.1; COD: 2.5 g/L; TS: 2.3 g/L) (Borja et al., 1993). This process was performed in a 1-l, continuous flow, completely mixed reactor operating at 35°C. The reactor performed satisfactorily at hydraulic retention times in the range of 2.5–10 days removing more than 93% of the initial COD in all cases. The macroenergetic parameters of this system were determined using Guiot's kinetic model and were found to be 0.035 g VSS/g COD (biomass yield coefficient) and 0.078 g COD/(g VSS·d) (specific rate of substrate removal for cell maintenance). This study also showed that the rate of substrate removal was related with the concentration of biodegradable substrate through an equation of the Michaelis-Menten type (Borja et al., 1993). An additional research of the anaerobic digestion of black olive wastewater at the above-mentioned operational conditions using increasing influent substrate concentrations in the range of 1.4–4.4 g O_2/L showed a decrease in the biomass yield coefficient by 6 times and an increase in the specific rate of substrate uptake by 5 times. This fact may be attributed to the higher mineral (sodium and potassium ions) solids and phenolic compound concentrations present in the most concentrated influents (Borja et al., 1994b). Anaerobic digestion experiments on black olive wastewater in batch mode at mesophilic temperature (35°C) revealed the influence of the bacterial immobilization support on the methane yield, with values of 333 and 316 mL CH_4/g COD for the reactors with Sepiolite and Bentonite, respectively, as microorganism support. Average COD removal efficiencies of 95% were achieved, although a gradual decrease in the specific rate constant was observed for substrate concentrations higher than 1 g COD/L, showing the occurrence of an inhibition process (Borja et al., 1992).

The anaerobic digestion process of green table olive processing wastewater was also studied at mesophilic temperature (35°C) in batch mode (Beltrán et al., 2008). COD removal efficiencies between 81 and 94% were obtained for influent substrate concentrations in the range of 0.6–3.0 g COD/L, with a mean methane yield coefficient of 270 mL CH_4/g COD. However, the global kinetic constants, obtained with the modified Monod model, diminished from 0.067 to 0.014 h^{-1} when the influent concentration increased to between the above-mentioned values, indicating that some inhibition effects took place by the phenolic substances contained in the wastewater (Beltrán et al., 2008).

Aggelis et al. (2001) also evaluated the mesophilic anaerobic digestion process of green olive de-bittering wastewater in reactors fed in a fill and draw mode and in continuously-stirred tank reactors (CSTR), achieving a 49% maximum efficiency of organic matter reduction with a polyphenol removal of about 12%, when the reactor operated at hydraulic retention times (HRTs) in the range of 50–25 days and organic loading rates from 0.33 to 0.94 g COD/(L·d). This process was severely inhibited as suggested by the low and restricted COD removal efficiency, volatile fatty acid accumulation and low methane production.

TABLE 3 | Summary of the operating conditions, process efficiencies and benefits derived from the use of anaerobic processes for TOPW treatment.

Wastewater type	Treatment	Operating conditions	Process efficiency	Benefits	Reference
Black olive wastewater	Anaerobic	CSTR reactor at mesophilic temperature (35°C). HRTs = 2.5–10 d	COD removal: 93%.	0.035 g VSS/g COD (low biomass yield coefficient) and 0.078 g COD/(g VSS·d) (specific rate of substrate removal for cell maintenance).	Borja et al., 1993
Black olive wastewater	Anaerobic	CSTR reactor at mesophilic temperature (35°C).	COD removal: 94.5% -92.6%	A decrease was observed in the biomass yield coefficient by 6 times and an increase in the specific rate of substrate uptake by 5 times.	Borja et al., 1994b
Black olive wastewater	Anaerobic	Batch reactors (with different microorganism immobilization supports) at mesophilic temperature (35°C).	Average COD removal: 95%.	Influence of the bacterial immobilization support on the methane yield, with values of 333 and 316 mL CH_4/g COD for the reactors with sepiolite and bentonite, respectively.	Borja et al., 1992
Green table olive processing wastewater	Anaerobic	Batch mode at mesophilic temperature (35°C).	COD removal: 81–94%.	Mean methane yield coefficient: 270 mL CH_4/g COD	Beltrán et al., 2008
Green olive de-bittering wastewater	Anaerobic	Reactors fed in a fill and draw mode at mesophilic temperature (35°). OLRs from 0.33 to 0.94 g COD/(L·d).	COD removal: 49%.	The process was severely inhibited.	Aggelis et al., 2001
De-bittering and washing effluent (DWE) with cattle manure (CM) and pig manure (PM)	Anaerobic co-digestion	Batch mode at mesophilic (35°C) and thermophilic temperatures (55°C).	VS removals: 65–73% (35°C) and 70–77% (55°C)	Co-digestion of TOPW with other substrates with different characteristics improve synergic effects between the microorganisms. Methane yields of between 250–300 mL CH_4/g VS_{added} at 35°C and between 270 and 350 mL CH_4/g VS_{added} at 55°C.	Zarkadas and Pilidis, 2011

TABLE 4 | Summary of the operating conditions, process efficiencies and benefits derived from the use of aerobic processes for TOPW treatment.

Wastewater type	Treatment	Operating conditions	Process efficiency	Benefits	Reference
Black olive wastewater	Aerobic (activated-sludge system)	HRT: 10 h; SRT: 4–15 d.	COD and BOD removals: 92%.	The effluent COD concentration, specific maximum growth rate, and half-saturation constant were all dependent on the feed substrate concentration. No sludge-settling problems were detected.	Borja et al., 1994a
Green table olive wastewater	Aerobic (activated-sludge system)	HRT: 0.51–0.37 d, Dissolved oxygen: 2–3 mg/L.	COD removal: 75–85%.	Increasing the HRT from 0.37 to 0.51 days and the temperature from 10 to 32°C increased the efficiency of process. NaCl concentrations of up to 3% did not influence the COD removal efficiency of the process.	Brenes et al., 2000
Green olive de-bittering wastewater	Aerobic	Draw-and-fill mode reactor, temperature: 25°C, HRT: 10 d.	COD removal: 71.6–75.9%.	A COD/N/P ratio of approximately 100/5/1 is adequate to maintain satisfactory microbial activity in the culture.	Aggelis et al., 2001
Mixture of washing waters and de-bittering wastewaters (at a ratio 3:1 by volume)	Aerobic	Well-mixed batch reactor. Temperature: 28°C, air flow-rate: 50 L/h.	COD removal: 86%.		Benítez et al., 2002b
Green table olive wastewater	Aerobic	Batch reactor, temperature: 28°C.	COD removal: 49–67%.	Total phenolic compound removal varied between 92% and 100%.	Beltrán et al., 2008
Black olive wastewater	Aerobic	Shake-flask reactors, operating at 150 rpm and 20°C.	COD removal: 65%.	The biodegradation rate of the original effluent was three times higher than the oxidized one using TiO_2 and hydrogen peroxide.	Chatzisymeon et al., 2008
Table olive processing wastewater	Aerobic	Suspended and attached growth reactors (trickling filters) operating with influent COD of between 5500 and 15000 mg/L	COD removals of 71.7 and 82.7% were achieved after 6 and 8 days of treatment respectively.	For a feed COD concentration of 5500 mg/L, the total phenolic compound removal was 67%.	Tatoulis et al., 2016

SRT: solids retention time.

TABLE 5 | Summary of the operating conditions, process efficiencies and benefits derived from the use of anaerobic-aerobic treatment combinations for TOPW treatment.

Wastewater type	Treatment	Operating conditions	Process efficiency	Benefits	Reference
Green olive de-bittering wastewater	Anaerobic-aerobic	HRT: 50 d (anaerobic) and 5 d (aerobic).	COD removal: 83.8%	The successive anaerobic-aerobic treatment resulted in a lower amount of aerobic sludge and does not need a pH correction of the anaerobic or the aerobic influent.	Aggelis et al., 2001
Fermentation brines from table olive packaging industries	Anaerobic - aerobic	Two SBR working in parallel (SBR1 and SBR2). In SBR-1, the sludge was preliminarily acclimated to a high concentration of salt, while in SBR-2, the acclimatization of the sludge was made directly with TOPW.	COD removals: 88% (SBR1) and 73% (SBR2).	All phenols were completely removed from SBR-1 and SBR-2.	Ferrer-Polonio et al., 2015
Fermentation brines from table olive packaging industries	Anaerobic - aerobic	Different anaerobic-aerobic ratios were tested.	COD removal: 82.3%	The best anaerobic/aerobic ratio was 0/22. For a ratio of 8/14 the reactor consumed much less energy.	Ferrer-Polonio et al., 2016a
Fermentation brines from table olive packaging industries	Anaerobic - aerobic	SBRs with an optimal COD/N/P ratio of 250/5/1.	COD removal: 80%.		Ferrer-Polonio et al., 2016b
Fermentation brines from table olive packaging industries	Anaerobic - aerobic	SBRs.	COD removal: 80%.	It was observed that the increase in hydraulic retention had an effect on the decrease in organic matter.	Ferrer-Polonio et al., 2017a
Fermentation brines from table olive packaging industries	Anaerobic - aerobic	SBRs with ultrafiltration and nanofiltration.	The total integrated process gave effluents with COD < 125 mg/L.	The turbidity and the characteristic color of this type of wastewater were completely removed.	Ferrer-Polonio et al., 2017b
Table olive processing wastewater	Anaerobic - aerobic	SBRs.	COD removal: 80%.	TOPW salinity increased the reactor's conductivity over time.	Soler-Cabezas et al., 2017

High COD removals (80–95%) and methane yields were attained in anaerobic digestion processes of these wastewaters. However, these processes are sometimes inhibited by the high content of phenolic compounds and high pH and salinity present in some of these wastewaters.

Anaerobic Co-digestion of TOPW With Other Wastes

An alternative considered by some researchers to minimize the difficulties and overcome the inhibition found in the anaerobic digestion of TOPW is its co-digestion with other wastes. Co-digestion with other substrates with different characteristics would allow to compensate toxicity and nutrient imbalance and to improve synergetic effects between the microorganisms (Zarkadas and Pilidis, 2011). For instance, batch experiments carried out with different mixtures of table olive de-bittering and washing effluents (DWE) with cattle manure (CM) and pig manure (PM) resulted in ultimate methane yields of between 250 and 300 mL CH_4/g Volatile $Solids_{added}$ at a mesophilic temperature of 35°C and between 270 and 350 mL CH_4/g Volatile $Solids_{added}$ at a thermophilic temperature 55°C. The highest methane production for the thermophilic temperature was achieved for a combination of wastewater containing 35% CM, 35% PM and 30% DWE (C/N ratio of 17.6), while for mesophilic digestion the highest methane yield was found for a mixture of 50% CM, 25% PM and 25% DWE (C/N ratio of 19.3). In these cases, no inhibition was observed since there was a small lag-adaptation of 3 days at the beginning of the process. In addition, no volatile fatty acid accumulation was observed, showing that the reactors were not operating under stress-overloading conditions (Zarkadas and Pilidis, 2011).

No inhibition and better stability were observed in the co-digestion processes of DWE with other substrates with different characteristics compared with the single anaerobic digestion process of the DWE.

Aerobic Treatments

The effectiveness of the aerobic treatments for removing the polluting load of the TOPW varies considerably depending on the class of microorganisms, organic substances to be removed and the environmental factors that influence process performance. In aerobic treatments the microorganisms oxidize the dissolved and particulate carbonaceous organics into simpler compounds and new sludge (Ayed et al., 2017).

A first study revealed that both natural and diluted black olive wastewaters (700–2200 mg COD/L) were easily purified by a completely mixed activated-sludge treatment system (Borja et al., 1994a). At least 92% of the COD and BOD were reduced in this system at an HRT of 10 h and solid retention time of 4–15 days. It was observed that the effluent COD concentration, specific maximum growth rate, and half-saturation constant were all dependent on the influent substrate concentration. The multiple-substrate model of Adams et al. (1975) allowed to predict adequately the effluent COD under variable influent COD concentrations. Finally, no sludge-settling problems were detected in this aerobic treatment (Borja et al., 1994a).

Another study on the purification of green table olive wastewaters by an activated-sludge system showed COD removal efficiencies in the range of 75–85%, when the reactor operated with influent COD concentrations in the range of 2500–3250 mg/L, HRTs of between 0.51 and 0.37 days (constant cellular retention time: 3.32 days) and with dissolved oxygen varying between 2 and 3 mg/L (Brenes et al., 2000). COD removal was mainly due to the reduction in organic acids and the ethanol present in the wastewater. On the contrary, only a low portion of polyphenols was removed. These polyphenols, especially those in a polymerized state, were not removed and were responsible for the color of the solutions and the residual measured COD. The substrate removal model proposed by Grau was applied to consider the effect of influent-substrate concentration on the effluent COD concentration, with the kinetic constant obtained at 9.8 $days^{-1}$. Increasing the HRT from 0.37 to 0.51 days and the temperature in the range of 10–32°C augmented the efficiency of the sludge activated process, obtaining effluent COD values of 200–300 mg/L in all cases studied. Concentrations of NaCl up to 3% did not affect the COD removal efficiency of the process, although the sludge volume index was higher than 200 cm^3/g (Brenes et al., 2000).

An aerobic treatment of green olive de-bittering wastewater was also evaluated with an influent COD of 16500 mg/L and polyphenol concentration of 1350 mg/L (Aggelis et al., 2001). In this study, a laboratory 1-L (useful) volume stirred draw-and-fill aerobic reactor was used. The reactor operated at a temperature of 25°C, using an HRT of 10 days and organic loading rates in the range of 1.6–2.3 g COD/(L·d) with pH adjustment of the wastewater to a value below 8.5. Na_2HPO_4 and urea were added as nutrients in order to maintain a COD/N/P ratio of approximately 100/5/1, which is appropriate to maintain a high microbial activity in the culture (Aggelis et al., 2001). This aerobic treatment was more effective than its anaerobic digestion, resulting in a degradation efficiency of 71.6–75.9%. However, it hardly affected the polyphenolic compound content, with the additional disadvantages of the requirement for pH correction of the influent and the high generation of biomass due to the aerobic metabolism (Aggelis et al., 2001).

The aerobic treatment of a mixture of washing waters and de-bittering wastewaters (at a ratio 3:1 by volume) was assessed by Benítez et al. (2002b). This research was performed in a 1-l well-mixed batch reactor at a constant temperature of 28°C with an air flow-rate of 50 L/h, with an influent COD concentration of 3.85 g/L. COD and BOD diminished continuously with reaction time. The overall COD reduction was 86% at the end of the experiment (7 days). The biomass variation agreed well with the typical growth-cycle phases for batch cultures: acclimation stage (lag phase), increase in the biomass concentration (exponential growth phase), maximum size of population (stationary stage) and decline in cell numbers (death phase) (Benítez et al., 2002b).

More recently, the aerobic biodegradation of green table olive wastewater was also investigated by Beltrán et al. (2008). A batch reactor was used in this research, which operated at 28°C, with influent substrate concentrations and initial biomass concentrations in the range of 9.5–41.6 g COD/L and 0.2–2.2 g VSS/L, respectively. The total polyphenolic concentration present in the wastewater was 3.1 g caffeic acid/L. Total COD removal efficiencies ranged between 49 and 67%, while total phenolic

compound removal varied between 92 and 100%. A kinetic study allowed for determining the cellular yield coefficient ($Y_{x/s}$ = 0.057 g VSS/g COD) and the kinetic constant of cellular death phase (k_d = 0.16 d^{-1}) (Beltrán et al., 2008).

An aerobic treatment of black olive wastewater (COD: 40 g/L; total phenols: 3.6 g/L) performed in shake-flask reactors, operating with non-acclimated activated sludge at 150 rpm and 20°C revealed that this wastewater was partially biodegradable aerobically (Chatzisymeon et al., 2008). After 16 days of treatment in batch mode, COD removal was 65%. Moreover, it was found that biosorption was always less than 20%. Therefore, the observed COD removal after the mentioned time of incubation was attributed mainly to the biodegradation of the organic content of the effluent by the non-acclimated sludge. In addition, the biodegradation rate of the original effluent was three times greater than the oxidized one using TiO_2 and hydrogen peroxide (Chatzisymeon et al., 2008).

In a recent study, table olive processing wastewaters were cleansed in aerobic biological reactors using native microorganisms originating from these wastewaters (Tatoulis et al., 2016). The aerobic biological processes were performed in suspended and attached growth reactors (trickling filters) using different feed substrate concentrations of 5500, 7500, and 15000 mg COD/L. Two different operating modes were studied to determine the optimum performance of the filter, i.e., batch and SBR with recirculation. In the batch suspended-growth flask reactors, COD removals of 71.7 and 82.7% were reached after 6 and 8 days of treatment at influent concentrations of 5500 and 7500 mg COD/L, respectively. In addition, for an initial COD concentration of 5500 mg/L, the total phenolic compound removal was 67%, while for the higher influent concentrations of 7500 and 15000 mg COD/L, phenolic compounds decreased to 63 and 57%, respectively (Tatoulis et al., 2016).

Except for olive de-bittering wastewaters, which are characterized by their high pH, anaerobic treatments showed higher COD and phenolic compound removals compared with those obtained in the aerobic processes.

Anaerobic and Aerobic Treatment Combinations

The aerobic treatment of the anaerobic digestion effluent of green olive de-bittering wastewater resulted in COD and polyphenolic compound removals of 74 and 19.6%, achieving an overall depletion of 83.8 and 28%, respectively, operating at HRTs of 50 days (anaerobic stage) and 5 days (aerobic stage) (Aggelis et al., 2001). Most likely, the anaerobic pre-treatment of the original green olive wastewater hydrolyzes polyphenolic compounds, giving a more readily biodegradable compound under aerobic conditions. In addition, the successive anaerobic-aerobic treatment resulted in a lower amount of aerobic sludge and does not need a pH correction of the anaerobic or the aerobic influent (Aggelis et al., 2001).

Another option is the use of SBRs. SBRs can be defined as a system of activated sludge, the functioning of which is based on the sequence of aerobic and anaerobic treatment phases. With the combination of these two treatments, organic matter, nitrogen and phosphorus can be removed simultaneously (Pavšelj et al., 2001).

With the introduction of the anaerobic phase the release of phosphorus occurs with part of the microorganisms; in the aerobic phase the nitrification, consumption of oxygen and phosphorus take place; while the denitrification occurs in the next anoxic phase (Cárdenas et al., 2006).

Ferrer-Polonio et al. (2015) studied the effect of various start-ups of SBRs for treating TOPW. In SBR-1, the sludge was preliminarily acclimated to a high concentration of salt, but not to a high concentration of phenols. While in SBR-2, the acclimatization of the sludge was made directly with TOPW. They reported that salinity promoted the population of ɣ-proteobacteria at the expense of other microorganisms. It was also observed that the SBR-1 had more operational problems consisting of a higher de-flocculation than SBR-2 which led to high turbidity values in the effluent, and finally the organic matter removal in this reactor was lower than the organic matter reduction achieved in SBR-2. All phenols were completely eliminated from SBR-1 and SBR-2, concluding that the concentration of phenols contained in TOPW was not an inhibitory concentration for the bacteria present in these bioreactors.

An SBR treating TOPW was used by Ferrer-Polonio et al. (2016a). They tested different anaerobic/aerobic ratios and found that the ratio that best adapted to this type of water was 0/22, where they achieved a COD reduction of 82.3 and 77.9% for total phenols. However, the ratio of 8/14 was determined as the optimal ratio, since the reduction in nutrients was very similar and working in this way the reactor consumed much less energy.

Ferrer-Polonio et al. (2016b) also treated TOPW with SBR with the aim of reducing hydraulic retention times by adding extra nutrients to TOPW and discovered that a COD/N/P ratio of 250/5/1 was optimal for the biological process to work efficiently. During this experiment COD was reduced by up to 80%. They also studied the population of bacteria in their reactors, observing that the main bacteria were ɣ-proteobacteria.

In another study applying the SBR technology on TOPW, the effect of alternating aerobic/anaerobic treatment on the protist population was assessed (Ferrer-Polonio et al., 2017a). During this study the authors observed that the increase in hydraulic retention not only had an effect on the decrease in organic matter, but also favored the population of ciliates against flagellates.

In another recent study by Ferrer-Polonio et al. (2017b) related to the treatment of TOPW, a mixed SBR technology with ultrafiltration and nanofiltration was evaluated. It was reported that only with the SBR 80% of the organic matter and 71% the total phenol concentration were removed, but with the addition of ultrafiltration and nanofiltration, the COD was finally less than 125 mg/L, with a final COD removal of 45.9 ± 1.9% (the high salinity could be responsible for this lower COD reduction). In addition, the turbidity and the characteristic color of this type of wastewater were completely removed.

Soler-Cabezas et al. (2017) also applied the SBR technology to purify TOPW, finding that this system was able to reduce 80% COD and 76% total phenol concentration. The main problem was that TOPW salinity increased the reactor's conductivity over time.

The combined anaerobic-aerobic processes allow to achieve higher COD removals compared with single anaerobic or aerobic processes, which result relevant and very efficient for wastewaters with inhibitory compounds (i.e., olive de-bittering wastewaters).

Bioremediation Technologies

Use of Microalgae

Microalgae and cyanobacteria are photosynthetic microorganisms able to produce oxygen which can be used for the oxidation of organic matter and NH_4^+, saving in aeration costs; while the autotrophic and heterotrophic growth of algal and bacterial biomass lead to higher nutrient recoveries. The recent worldwide interest in the cultivation of microalgae for energy purposes, together with the need for environmentally more sustainable wastewater treatment technologies, have made microalgae wastewater treatment processes a promising alternative from economic and environmental points of view (Chinnasamy et al., 2014). In addition, the algal biomass produced can be a valuable raw material for the generation of bioenergy, biofertilizers and other valuable products.

Although the use of microalgae to treat wastewaters is widespread, it has hardly been used to treat TOPW. However, good results have been found by Serrano et al. (2017), who found removals of 69.1, 50.9, 54.3 and 71.85% for TOC, TSN (total soluble nitrogen), phosphate and total phenols respectively, when growing *Nannochloropsis gaditana* in TOPW diluted at 80%.

Use of Fungi

The treatment of wastewaters using fungi is widespread, especially nowadays when there are several studies about the ability of fungi to produce pharmaceutical products from water (Lucas et al., 2018).

Kyriacou et al. (2005) combined a biological process using *Aspergillus* strain with BDD treatment in the presence of hydrogen peroxide as a treatment for de-bittering and washing wastewaters. They reported 86 and 65% COD and total phenol removals, respectively, after the biological treatment. An improvement in the reduction of organic matter and total phenols was observed after the electrochemical treatment, achieving a total reduction of 98% for both organic matter and phenols.

Similar results have been reported by Lasaridi et al. (2010) using *Aspergillus niger* on fresh de-bittering wastewater and washing water. They used the main table olive de-bittering wastewater with NaOH and an alternative with KOH using dilutions of 100, 85, 70, 55, and 40%. All these wastewaters were inoculated with *Aspergillus niger*. These studies showed COD removal efficiencies in the range of 60–87% for NaOH and 50–87% for the KOH treatment (Lasaridi et al., 2010).

Different fungi strains were isolated from brine wastewaters by Crognale et al. (2012) with the aim of producing extracellular phenoloxidases. A total of 20 strains were isolated, although only two of them were significant effective in producing laccases and Mn-peroxidase, when grown under saline conditions (0–10% NaCl). *Citeromyces matritensis* (syn. *Candida globose*) and *Aspergillus fumigatus* 6C2 decreased the phenolic compounds of TOPW by up to 82.3%.

TABLE 6 | Summary of the operating conditions, process efficiencies and benefits derived from the use of bioremediation processes (microalgae and fungi) for TOPW treatment.

Wastewater type	Treatment	Operating conditions	Process efficiency	Benefits	Reference
Table olive processing wastewater	Microalgae growth	*Nannochloropsis gaditana* TOPW diluted at 80%.	TOC removal: 69.1%. TSN removal: 50.9%. phosphate removal: 54.3%.	Nutrient recovery and algal biomass production that can be used for bioenergy production	Serrano et al., 2017
De-bittering and washing wastewater	Biological and electrochemical treatment	*Aspergillus* strain	Biological treatment: COD removal: 86%.	High organic matter reduction	Kyriacou et al., 2005
De-bittering and washing wastewater	Biological treatment	*Aspergillus niger*. NaOH and KOH treatment in water diluted 100, 85 70, 55, and 40%.	NaOH treatment; COD removal: 60–87%. KOH treatment; COD removal: 50–87%	High organic matter reduction	Lasaridi et al., 2010
Brine wastewater	Biological treatment	*Citeromyces matritensis* and *Aspergillus fumigates* 6C2.	Total phenol reduction: 82.3%	Extracellular phenoloxidases production and high organic matter reduction	Crognale et al., 2012
De-bittering and washing wastewater	Biological treatment	*Geotrichum candidum*	COD reduction: 71%.	High organic matter reduction	Ayed et al., 2016

TSN: total soluble nitrogen.

The *Geotrichum candidum* strain was used by Ayed et al. (2016) to study its effect on color reduction in de-bittering and washing wastewaters. A significant improvement in color reduction was observed when the growth of the fungus remained constant. The extracellular peroxidases of *G. Candidum* have been able to effectively reduce phenol content in TOPW, which is responsible for its coloration. The main phenols found, i.e., coumaric acid, oleuropein, tyrosol and vanillic acid have been reduced by more than 55% in all cases. During this treatment COD, color and total phenols decreased by 71, 63, and 60%, respectively.

The use of fungi for the treatment of TOPW is mainly focused on reducing the organic matter in general and particularly phenol contents with promising results. Moreover, these microorganisms allow obtaining valuable products (i.e., enzymes) although they need a pH regulation in the wastewater.

Table 6 summarizes the operating conditions, process efficiencies and benefits derived from the use of microalgae and fungi for treatment and/or re-use of these wastewaters.

Combination of Different Treatments

Membrane bioreactors (MBRs) are reactors which contain an active sludge and different types of membranes. These reactors have received great attention in the last decade due to significant cost reductions, which leads to an increase in the use of MBR systems for treating wastewater. The main advantages of new MBR technologies are: low space requirement, flexible configurations, stability, and elimination of the problems associated with the sedimentation of the sludge. However, there are few studies using MBR technology for the treatment of TOPW, despite the above-mentioned advantages.

Patsios et al. (2016) used activated sludge from a municipal wastewater treatment plant to activate the membrane. Then, the membrane was gradually acclimated to a high salinity substrate and, finally, TOPW was used directly. A TOC removal efficiency of 91.5% and an efficiency of total phenol removal of 82.8% were reported. Despite this high elimination of phenols, the resulting waters continued to have the problem of being colored.

Another recent study using MBRs for the treatment of TOPW was carried out by Soler-Cabezas et al. (2017). The main drawback reported in this study was the high salinity of the TOPW, which was finally eliminated, causing an increase in salinity in the reactor. On the other hand, the authors stated that this type of treatment, as a low energy cost process, was capable of removing up to 80% of COD.

Other Uses

Irrigation

Agriculture consumes up to 70% fresh water. Many Mediterranean countries are suffering great periods of drought, so the reuse of industrial waters for agriculture would solve a major problem of water shortage, in addition to directly providing nutrients to the soil (Libutti et al., 2018).

Murillo et al. (2000) investigated the application of TOPW for the irrigation of olive trees. However, they found that in just 15 days of using TOPW as irrigation water, a decrease in leaf water potential and a stomatal conductance to water and photosynthesis were observed.

In addition, a reduction in the nitrogen load of the leaf was also reported. The authors assumed that this type of wastewater is totally unsuitable for agricultural proposes due to its characteristics and above all, to its high salinity concentrations.

Extraction and Recovery of Added-Value Products

Phenols are strong antioxidants which are difficult to synthesize and can be easily extracted from the fruit of the olive. The effect of three commercial membranes to concentrate phenols from TOPW was examined by Kiai et al., (2014). The use of a direct contact membrane distillation process, regardless of the membrane type tested, showed separation coefficients which were greater than 99.5%. However, membrane TF450 at 70°C gave the highest concentration factors. In addition, the most resistant membrane to the fouling phenomenon was TF200, with the lowest pore size.

The washwaters from Spanish-style green olive (Hojiblanca type) processing have also been treated by combined fermentation and evaporation systems for the recovery of phenols (Brenes et al., 2004). The fermentation step was conducted on a pilot plant scale (500 L) and stored for 9 months. These results showed that there was no significant difference when wastewaters were acidified to pH 5 and inoculated with *Lactobacillus pentosus* or acidified to pH 3.4 and without inoculation. The fermentation process reduced the COD by up to 20%. After the evaporation step (rotary evaporator under vacuum at 65°C) a large amount of high-value compounds were found in the concentrate (lactic acid and hydroxytyrosol at concentrations up to 123.7 ± 1.6 g/dm^3 and 36.4 ± 1.6 g/dm^3, respectively). Furthermore, it was observed that the fermentation step without any pH adjustment was mainly conducted by putrefactive bacteria (*Enterobacteriaceae* family), which produce a large amount of gas and strong off-odors.

An investigation on wastewaters from table olive brine reported hydroxytyrosol and tyrosol concentrations of 690 and 98 mg/g dry weight extract, respectively (Bouaziz et al., 2008). These authors also tested the phenol antioxidant power against 2 human cancer cell lines with promising results.

In another study about Megaritike, a Greek style olive oil and table olive, the authors were able to concentrate and recover phenols with an adsorption resin. They found that the main phenols present in this kind of TOPW were hydroxytyrosol-4-*O*-glucoside, 11-methyl-oleoside, hydroxytyrosol and tyrosol (Mousouri et al., 2014).

The extraction and recovery of added-value products from TOPW would allow reducing the operating costs of other treatment processes, and at the same time, this procedure would result in an improvement in the efficiency of biological processes avoiding inhibitory processes.

CONCLUSION AND FUTURE TRENDS

The legislation in different countries regarding environmental issues is becoming increasingly stricter. In order to achieve a

decrease in environmental impact, the segregation of the effluents to be treated would be necessary as proposed previously by different researchers. Moreover, the decrease in the amount of materials used (NaOH, NaCl, H_2O, etc.), the use of low concentration lyes, the reuse of fermentation brines would lead to a decrease in the polluting content and the high volumes of resulting wastewater. Different studies have been developed on the treatment and management of table olive wastewaters, but the reality is that these treatments are not applied to a great extent at the industrial level. The problem continues to be very serious and further research into new approaches to the problem is needed. Among these approaches, integrated purification processes combining a first step of chemical oxidation, with stronger advanced oxidation methods (i.e., Ozonation and Electrochemical treatments with BDD), with a second biological step are a promising alternative. Another challenge to be considered would be the management of the sludge that is produced during the biological treatment: transformation to compost and the use of the biomass generated as renewable energy sources. In addition, different combined fermentation and evaporation systems should be further studied in order to maximize phenol recovery, given the high value of these compounds as antioxidant agents. All these considerations will be focused on to achieve a circular economical model and to obtain the desired zero waste, which is of great interest for European table olive producing countries.

AUTHOR CONTRIBUTIONS

BR-L, DL-C, MF-R, and RB-P did the bibliographic search and wrote the full manuscript. BR-L planned the index and structure of the paper, coordinated the work and the final manuscript.

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REFERENCES

Adams, C. E., Eckenfelder, W. W., and Hovious, J. C. (1975). A kinetic model for design of completely-mixed activated sludge treating variable-strength industrial wastewaters. *Water Res.* 9, 37–42. doi: 10.1016/0043-1354(75)90150-5

Aggelis, G. G., Gavala, H. N., and Lyberatos, G. (2001). Combined and separate aerobic and anaerobic biotreatment of green olive debittering wastewater. *J. Agric. Eng. Res.* 80, 283–292. doi: 10.1006/jaer.2001.0732

Alvarez-Pugliese, C. E., Marriaga-Cabrales, N., and Machuca-Martínez, F. (2014). "A patent review of technologies for wastewater treatment by electrochemical oxidation with boron doped diamond electrodes," in *Evaluation of Electrochemical Reactors as a New Way to Environmental Protection*, ed. C. A. Martı nez-Huitle (Trivandrum: Research Signpost), 77–94.

Ayed, L., Asses, N., Chammem, N., and Hamdi, M. (2016). Improvement of green table olive processing wastewater decolorization by *Geotrichum candidum*. *Desalin. Water Treat.* 57, 17322–17332. doi: 10.1080/19443994.2015.1084596

Ayed, L., Asses, N., Chammem, N., Othman, N. B., and Hamdi, M. (2017). Advanced oxidation process and biological treatments for table olive processing wastewaters: constraints and a novel approach to integrated recycling process: a review. *Biodegradation* 28, 125–138. doi: 10.1007/s10532-017-9782-0

Beltrán, F. J., García-Araya, J. F., Frades, J., Álvarez, P., and Gimeno, O. (1999). Effects of single and combined ozonation with hydrogen peroxide or UV radiation on the chemical degradation and biodegradability of debittering table olive industrial wastewaters. *Water Res.* 33, 723–732. doi: 10.1016/S0043-1354(98)00239-5

Beltrán, J., Gonzalez, T., and Garcia, J. (2008). Kinetics of the biodegradation of green table olive wastewaters by aerobic and anaerobic treatments. *J. Hazard. Mater.* 154, 839–845. doi: 10.1016/j.jhazmat.2007.10.102

Beltrán-Heredia, J., Torregrosa, J., Domínguez, J. R., and García, J. (2000a). Aerobic biological treatment of black table olive washing wastewaters: effect of an ozonation stage. *Process Biochem.* 35, 1183–1190. doi: 10.1016/S0032-9592(00)00160-6

Beltrán-Heredia, J., Torregrosa, J., Domínguez, J. R., and García, J. (2000b). Ozonation of black-table-olive industrial wastewaters: effect of an aerobic biological pretreatment. *J. Chem. Technol. Biotechnol.* 75, 561–568.

Benítez, F. J., Acero, J. L., González, T., and García, J. (2001). Ozonation and biodegradation processes in batch reactors treating black table olives washing wastewaters. *Ind. Eng. Chem. Res.* 40, 3144–3151. doi: 10.1021/ie000735c

Benítez, F. J., Acero, J. L., González, T., and García, J. (2002a). Application of ozone and advanced oxidation processes to the treatment of lye-wastewaters from the table olives industry. *Ozone Sci. Eng.* 24, 105–116. doi: 10.1080/01919510208901601

Benítez, F. J., Acero, J. L., Gonzalez, T., and Garcia, J. (2002b). The use of ozone, ozone plus UV radiation, and aerobic microorganisms in the purification of some agro-industrial wastewaters. *J. Environ. Sci. Health A* 37, 1307–1325. doi: 10.1081/ESE-120005988

Benítez, F. J., Acer, J. L., and Leal, A. I. (2003). Purification of storage brines from the preservation of table olives. *J. Hazard. Mater.* 96, 155–169. doi: 10.1016/S0304-3894(02)00183-8

Borja, R., Banks, C. J., and Garrido, A. (1994a). Kinetics of black-olive wastewater treatment by the activated-sludge system. *Process Biochem.* 29, 587–593. doi: 10.1016/0032-9592(94)80023-5

Borja, R., Garrido, A., Martín, A., and Alonso, V. (1994b). Influent of substrate concentration on the macroenergetic parameters of anaerobic digestion of black-olive wastewater. *Biotechnol. Lett.* 16, 321–326. doi: 10.1007/BF00134633

Borja, R., Garrido, A., and Durán, M. M. (1992). Kinetic study of anaerobic digestion process of wastewaters from ripe olive processing. *Grasas Aceites* 43, 317–321. doi: 10.3989/gya.1992.v43.i6.1126

Borja, R., Martín, A., and Garrido, A. (1993). Anaerobic digestion of black-olive wastewater. *Bioresour. Technol.* 45, 27–32. doi: 10.1016/0960-8524(93)90139-3

Borja, R., and Rincón, B. (2017). "Biogas production," in *Reference Module in Life Sciences*, ed. B. D. Roitberg (New York, NY: Elsevier), 1–24. doi: 10.1016/B978-0-12-809633-8.09105-6

Bouaziz, M., Lassoued, S., Bouallagui, Z., Smaoui, S., Gargoubi, A., Dhouib, A., et al. (2008). Synthesis and recovery of high bioactive phenolics from table-olive brine process wastewater. *Bioorg. Med. Chem.* 16, 9238–9246. doi: 10.1016/j.bmc.2008.09.012

Brenes, M., García, P., Romero, C., and Garrido, A. (2000). Treatment of green table olive waste waters by an activated-sludge process. *J. Chem. Technol. Biotechnol.* 75, 459–463. doi: 10.1002/1097-4660(200006)75:6<459::AID-JCTB234>3.0.CO;2-D

Brenes, M., García-García, P., and Garrido-Fernández, A. (1989). Influence of reusing re-generated brines on some characteristics of the packing Green olives. *Grasas Aceites* 40, 182–189.

Brenes, M., Romero, C., and De Castro, A. (2004). Combined fermentation and evaporation processes for treatment of washwaters from Spanish-style Green olive processing. *J. Chem. Technol. Biotechnol.* 79, 253–259. doi: 10.1002/jctb.970

Cañizares, P., Lobato, J., Paz, R., Rodrigo, M. A., and Saéz, C. (2007). Advanced oxidation processes of olive-oil mills wastewaters. *Chemosphere* 67, 832–838. doi: 10.1016/j.chemosphere.2006.10.064

Cappelletti, G. M., Nicoletti, G. M., and Russo, C. (2011). "Wastewaters from table olive industries," in *Wastewater-Evaluation and Management*, eds F. Sebastian and G. Einschlag (Rijeka, FL: In Tech), 351–376. doi: 10.1016/0043-1354(94)90135-X

Cárdenas, C., Perruolo, T., Tarre, Y., Flores, K., Trujillo, A., Saules, L., et al. (2006). Removal of biological nutrients by means of a sequential batch reactor. *Interciencia* 31, 787–793.

Chatzisymeon, E., Stypas, E., Bousios, S., Xekoukoulotakis, N. P., and Mantzavinos, D. (2008). Photocatalytic treatment of black table olive processing wastewater. *J. Hazard. Mater.* 154, 1090–1097. doi: 10.1016/j.jhazmat.2007.11.014

Chatzisymeon, E., Xekoukoulotakis, N. P., Diamadopoulos, E., Katsaounis, A., and Mantzavinos, D. (2009). Boron-doped diamond anodic treatment of olive mill wastewaters: statistical analysis, kinetic modelling and biodegradability. *Water Res.* 43, 3999–4009. doi: 10.1016/j.watres.2009.04.007

Chinnasamy, S., Sood, A., Renuka, N., Prasanna, R., Ratha, S. K., Bhaskar, S., et al. (2014). Ecobiological aspects of algae cultivation in wastewaters for recycling of nutrients and biofuel applications. *Biofuels* 5, 141–158. doi: 10.4155/bfs.13.78

Comninellis, C., Kapalka, A., Malato, S., Parsons, S. A., Pulios, I., and Mantzavinos, D. (2008). Advanced oxidation processes for water treatment: advances and trends for R&D. *J. Chem. Technol. Biotechnol.* 83, 769–776. doi: 10.1002/jctb.1873

Crognale, S., Pesciaroli, L., Petruccioli, M., and D'annibale, A. (2012). Phenoloxidase-producing halotolerant fungi from olive brine wastewater. *Process Biochem.* 47, 1433–1437. doi: 10.1016/j.procbio.2012.05.014

De Castro, A., García, P., Romero, C., Brenes, C., and Garrido, A. (2007). Industrial implementation of black ripe olive storage under acid conditions. *J. Food Eng.* 80, 1206–1212. doi: 10.1016/j.jfoodeng.2006.09.010

De Castro, A., Montaño, A., Casado, F. J., Sánchez, A. H., and Rejano, L. (2002). Utilization of *Enterococcus casseliflavus* and *Lactobacillus pentosus* as starters cultures for Spanish-style green olive fermentation. *Food Microbiol.* 19, 637–644. doi: 10.1006/fmic.2002.0466

Deligiorgis, A., Xekoukoulotakis, N. P., Diamadopoulos, E., and Mantzavinos, D. (2008). Electrochemical oxidation of table olive processing wastewater over boron-doped diamond electrodes: treatment optimization by factorial design. *Water Res.* 42, 1229–1237. doi: 10.1016/j.watres.2007.09.014

Fendri, I., Chamkha, M., Bouaziz, M., Labat, M., Sayadi, S., and Abdelkafi, S. (2013). Olive fermentation brine: biotechnological potentialities and valorization. *Environ. Technol.* 34, 181–193. doi: 10.1080/09593330.2012.689364

Ferrer-Polonio, E., Carbonell-Alcaina, C., Mendoza-Roca, J. A., Iborra-Clar, A., Álvarez-Blanco, S., Bes-Piá, A., et al. (2017a). Brine recovery from hypersaline wastewaters from table olive processing by combination of biological treatment and membrane technologies. *J. Clean. Prod.* 142, 1377–1386. doi: 10.1016/j.jclepro.2016.11.169

Ferrer-Polonio, E., Pérez-Uz, B., Mendoza-Roca, J. A., Iborra-Clar, A., and Pastor-Alcañiz, L. (2017b). Biological treatment of hypersaline wastewater from table olive processing: process performance and protist population under different operating conditions. *J. Ind. Eng. Chem.* 56, 364–374. doi: 10.1016/j.jiec.2017.07.033

Ferrer-Polonio, E., García-Quijano, N. T., Mendoza-Roca, J. A., Iborra-Clar, A., and Pastor-Alcañiz, L. (2016a). Effect of alternating anaerobic and aerobic phases on the performance of a SBR treating effluents with high salinity and phenols concentration. *Biochem. Eng. J.* 113, 57–65. doi: 10.1016/j.bej.2016.05.010

Ferrer-Polonio, E., Mendoza-Roca, J. A. E., Iborra-Clar, A., Alonso-Molina, J. L., and Pastor-Alcañiz, L. (2016b). Biological treatment performance of hypersaline wastewaters with high phenols concentration from table olive packaging industry using sequencing batch reactors. *J. Ind. Eng. Chem.* 43, 44–52. doi: 10.1016/j.jiec.2016.07.046

Ferrer-Polonio, E., Mendoza-Roca, J. A., Iborra-Clar, A., Alonso-Molina, J. L., and Pastor-Alcañiz, L. (2015). Comparison of two strategies for the start-up of a biological reactor for the treatment of hypersaline effluents from a table olive packaging industry. *Chem. Eng. J.* 273, 595–602. doi: 10.1016/j.cej.2015.03.062

García-García, P., López-López, A., Moreno-Baquero, J. M., and Garrido-Fernández, A. (2011). Treatment of wastewaters from the Green table olive packaging industry using electro-coagulation. *Chem. Eng. J.* 170, 59–66. doi: 10.1016/j.cej.2011.03.028

Gargouri, B., Gargouri, O. D., Khmakhem, I., Ammar, S., Abdelhèdi, R., and Bouaziz, M. (2017). Chemical composition and direct electrochemical oxidation of table olive processing wastewater using high oxidation power anodes. *Chemosphere* 166, 363–371. doi: 10.1016/j.chemosphere.2016.09.080

Garrido Fernández, A., Adams, M. R., and Fernández Diez, M. J. (1997). *Table olives. Production and Processing*. London: Chapman & Hall. doi: 10.1007/978-1-4899-4683-6

Gogate, P. R., and Pandit, A. B. (2004). A review of imperative technologies for wastewaters treatment I: oxidation technologies at ambient conditions. *Adv. Environ. Res.* 8, 501–551. doi: 10.1016/S1093-0191(03)00032-7

International Olive Council [IOC] (2017). Available at: http://www.internationaloliveoil.org/estaticos/view/132-world-table-olive-figures

International Olive Oil Council [IOOC] (2004). *Trade Standard Applying to Table Olives. Document COI/OT/NC no.*1. Madrid: IOOC.

Kailis, S., and Harris, D. J. (2007). *Producing Table Olives*. Collingwood: Landlinks Press.

Katsoni, A., Frontistis, Z., Xekoukoulotakis, N. P., Diamadopoulos, E., and Mantzavinos, D. (2008). Wet air oxidation of table olive processing wastewater: determination of key operation parameters by factorial design. *Water Res.* 42, 3591–3600. doi: 10.1016/j.watres.2008.05.007

Kiai, H., García-Payo, M. C., Hafidi, A., and Khayet, M. (2014). Application of membrane distillation technology in the treatment of table olive wastewaters for phenolic compounds concentration and high quality water production. *Chem. Eng. Process.* 86, 153–161. doi: 10.1016/j.cep.2014.09.007

Kotsou, M., Kyriacou, A., Lasaridi, K., and Pilidis, G. (2004). Integrated aerobic biological treatment and chemical oxidation with Fenton's reagent for the processing of green table olive wastewater. *Process Biochem.* 39, 1653–1660. doi: 10.1016/S0032-9592(03)00308-X

Kyriacou, A., Lasaridi, K. E., Kotsou, M., Balis, C., and Pilidis, G. (2005). Combined bioremediation and advanced oxidation of green table olive processing wastewater. *Process Biochem.* 40, 1401–1408. doi: 10.1016/j.procbio.2004.06.001

Langlais, B., Reckhow, D. A., and Brink, D. R. (eds) (1991). *Ozone in Water Treatment: Application and Engineering*. Chelsea, MI: Lewis Publishers.

Lasaridi, K. E., Chroni, C., Fortatos, S., Chatzipavlidis, I., and Kyriacou, A. (2010). Estimating the bioremediation of green table olive processing wastewater using a selected strain of *Aspergillus niger*. *Desalinat. Water Treat.* 23, 26–31. doi: 10.5004/dwt.2010.1411

Levec, J., and Pintar, A. (2007). Catalytic wet-air oxidation processes: a review. *Catal. Today* 124, 172–184. doi: 10.1016/j.cattod.2007.03.035

Libutti, A., Gatta, G., Gagliardi, A., Vergine, P., Pollice, A., Beneduce, L., et al. (2018). Agro-industrial wastewater reuse for irrigation of a vegetable crop succession under Mediterranean conditions. *Agric. Water Manage.* 196, 1–14. doi: 10.1016/j.agwat.2017.10.015

Linsebigler, A. L., Lu, G., and Yates, J. T. (1995). Photocatalysis on TiO_2 surfaces: principles, mechanisms, and selected results. *Chem. Rev.* 95, 735–758. doi: 10.1021/cr00035a013

Lucas, D., Castellet-Rovira, F., Villagrasa, M., Badia-fabregat, M., Barceló, D., Vicent, T., et al. (2018). The role of sorption processes in the removal of pharmaceuticals by fungal treatment of wastewater. *Sci. Total Environ.* 61, 1147–1153. doi: 10.1016/j.scitotenv.2017.08.118

Marone, A., Carmona-Martínez, A. A., Sire, Y., Meudec, E., Steyer, J. P., Bernet, N., et al. (2016). Bioelectrochemical treatment of table olive brine processing wastewater for biogas production and phenolic compounds removal. *Water Res.* 100, 316–325. doi: 10.1016/j.watres.2016.05.008

Marsilio, V., Campestee, C., and Lanza, B. (2001). Sugar and polyol compositions of some European olive fruits varieties (*Olea europea* L.) suitable for table olive purposes. *Food Chem.* 72, 485–490. doi: 10.1016/S0308-8146(00)00268-5

Marsilio, V., and Lanza, B. (1998). Characterisation of an oleuropein degrading strain of *Lactobacillus plantarum*. Combined effects of compounds present in olive fermenting brines (phenols, glucose and NaCl) on bacterial activity. *J. Sci. Food Agric.* 76, 520–524. doi: 10.1002/(SICI)1097-0010(199804)76:4<520::AID-JSFA982>3.0.CO;2-I

Martin, C. J. (1992). Remediation of groundwater contaminated by the olive processing waste. *Desalination* 88, 253–263. doi: 10.1016/0011-9164(92)80118-S

Mousouri, E., Melliou, E., and Prokopios, M. (2014). Isolation of megaritolactones and other bioactive metabolites from "Megaritiki" table olives and debittering water. *J. Agric. Food Chem.* 62, 660–667. doi: 10.1021/jf404685h

Moussavi, G., Barikbin, B., and Mahmoudi, M. (2010). The removal of high concentrations of phenol from saline wastewater using aerobic granular SBR. *Chem. Eng. J.* 158, 498–504. doi: 10.1016/j.cej.2010.01.038

Murillo, J. M., López, R., Fernández, J. E., and Cabrera, F. (2000). Olive tree response to irrigation with wastewater from the table olive industry. *Irrigat. Sci.* 19, 175–180. doi: 10.1007/s002710000016

Papadaki, E., and Mantzouridou, F. T. (2016). Current status and future challenges of table olive processing wastewater valorization. *Biochem. Eng. J.* 112, 103–113. doi: 10.1016/j.bej.2016.04.008

Parino, C. S., Stalikas, C. D., Giannopoulous, T. S., and Pilidis, G. A. (2007). Chemical and physicochemical profile of wastewaters produced from the different stages of Spanish-style green olives processing. *J. Hazard. Mater.* 145, 339–343. doi: 10.1016/j.jhazmat.2006.12.061

Patsios, S. I., Papaioannou, E. H., and Karabelas, A. J. (2016). Long-term performance of a membrane bioreactor treating table olive processing wastewater. *J. Chem. Technol. Biotechnol.* 91, 2253–2262. doi: 10.1002/jctb.4811

Pavšelj, N., Hvala, N., Kocijan, J., Roš, M., Šubelj, M., Mušič, G., et al. (2001). Experimental design of an optimal phase duration control strategy used in batch biological wastewater treatment. *ISA Trans.* 40, 41–56. doi: 10.1016/S0019-0578(00)00042-2

Rejano, L., Montaño, A., Casado, F. J., Sánchez, A. H., and de Castro, A. (2010). "Table olives: varieties and variations," in *Olives and Olive Oil in Health and Disease Prevention*, eds V. R. Preedy and R. R. Watson (Orlando FL: Academic Press), 5–15. doi: 10.1016/B978-0-12-374420-3.00001-2

Rivas, F. J., Beltrán, F. J., and Gimeno, O. (2000). Join treatment of wastewater from table olive processing and urban wastewater. Integrated Ozonation – Aerobic Oxidation. *Chem. Eng. Technol.* 20, 177–181. doi: 10.1002/(SICI)1521-4125(200002)23:2<177::AID-CEAT177>3.0.CO;2-4

Rivas, J., Beltrán, F., Acedo, B., and Gimeno, O. (2000). Two-step wastewater treatment: sequential Ozonation – Aerobic Biodegradation. *Ozone Sci. Eng.* 22, 617–636. doi: 10.1080/01919510009408803

Rivas, F. J., Beltrán, F. J., Gimeno, O., and Álvarez, P. (2001). Chemical-Biological treatment of table olive manufacturing wastewater. *J. Environ. Eng.* 127, 611–619. doi: 10.1061/(ASCE)0733-93722001127:7(611)

Rivas, F. J., Beltrán, F. J., Gimeno, O., and Álvarez, P. (2003). Treatment of brines by combined Fenton's reagent-aerobic biodegradation II. Process modelling. *J. Hazard. Mater.* 96, 259–276. doi: 10.1016/S0304-3894(02)00216-9

Romero-Barranco, C., Brenes-Balbuena, M., García-García, P., and Garrido-Fernández, A. (2001). Management of spent brines or osmotic solutions. *J. Food Eng.* 49, 237–246. doi: 10.1016/S0260-8774(00)00204-1

Salvo, F., Cappello, A., and Giacalone, L. (1995). *L'Olivicoltura nella Valle del Belice. Instituto Nazionale di Economuia Agraria*. Rome: Alimentari e Forestali.

Sánchez-Gómez, A. H., García-García, P., and Rejano-Navarro, L. (2006). Elaboration of table olives. *Grasas Aceites* 57, 86–94. doi: 10.3989/gya.2006.v57.i1.24

Segovia-Bravo, K. A., García-García, P., Arroyo-López, F. N., López-López, A., and Garrido-Fernández, A. (2008). Ozonation process for the regeneration and recycling of Spanish green table olive fermentation brines. *Eur. Food Res. Technol.* 227, 71–76. doi: 10.1007/s00217-007-0742-5

Serrano, A., Contreras, C., Ruiz-filippi, G., Borja, R., and Fermoso, F. G. (2017). Sequential adaptation of *Nannochloropsis gaditana* to table olive processing water. *J. Environ. Sci. Health A* 52, 986–991. doi: 10.1080/10934529.2017.1324711

Soler-Cabezas, J. L., Luján-Facundo, M. J., Mendoza-Roca, J. A., Vicent-Vela, M. C., and Pastor-Alcañiz, L. (2017). A comparative study of the influence of salt concentration on the performance of an osmotic membrane bioreactor and a sequencing batch reactor. *J. Chem. Technol. Biotechnol.* 93, 72–79. doi: 10.1002/jctb.5321

Tatoulis, T. I., Zapantiotis, S., Frontistis, Z., Akratos, C. S., Tekerlekopoulou, A. G., Pavlou, S., et al. (2016). A hybrid system comprising an aerobic biological process and electrochemical oxidation for the treatment of black table olive processing wastewaters. *Int. Biodeterior. Biodegrad.* 109, 104–112. doi: 10.1016/j.ibiod.2016.01.013

Zarkadas, I. S., and Pilidis, G. A. (2011). Anaerobic co-digestion of table olive debittering & washing effluent, cattle manure and pig manure in batch and high volume laboratory anaerobic digesters: effects of temperature. *Bioresour. Technol.* 102, 4995–5003. doi: 10.1016/j.biortech.2011.01.065

Zaviska, F., Drogui, P., Mercier, G., and Blais, J. F. (2009). Procédés d'oxydation avancée dans le traitement des eaux et des effluents industriels: application à la dégradation des polluants réfractaires. *Rev. Sci. Eau* 22, 535–564. doi: 10.7202/038330ar

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