VEGETABLE MATRIX AS A SOURCE OF NUTRITIONAL AND MICROBIAL VALUE FOR HEALTHY FOOD

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VEGETABLE MATRIX AS A SOURCE OF NUTRITIONAL AND MICROBIAL VALUE FOR HEALTHY FOOD

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The rising demands in maintaining human wellness through diet have greatly promoted the interest in plant-based or vegetarian diets all over the world. Several government agencies, health/nutrition organizations, and health professionals are emphasizing that regular consumption of fruits and vegetables may provide health benefits and weight management. Fruits and vegetables are recognized as rich in nutritional components, such as fiber, protein, healthy fat, and micronutrients including vitamins, minerals, and phytochemicals. A growing body of scientific evidence supports that phytonutrients may play positive roles in preventing certain diseases, mainly aging-associated diseases. Furthermore, several benefits are associated with the consumption of vegetable-based fermented foods such as cereals, fruits and starchy root crops. It is noteworthy that microbial activity increases organic acids, decreases some toxic and anti-nutritional factors, and reduces amounts of sugars, resulting in a lower glycemic index. Microbial fermentation plays also a crucial role in safety traits of foods and beverages enhancing their sensory properties and extending their shelf life.

Vegetable waste, which contains proteins, fats, natural colorants, enzymes, antimicrobials and antioxidants, represents a relevant source of natural food additives or supplements with high nutritional value. Furthermore, complex value-added chemicals such as phytochemicals, prebiotics, polysaccharides and polypeptides can be obtained via microbial, in an eco-friendly way.

This Research Topic aims to present high-qualified scientific achievements on the impact of fruit, vegetable and/or novel plant based matrices on human health, sharing both successes and failures of original research and meta-analyses studies.

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Viability of *Lactobacillus plantarum* on Fresh-Cut Chitosan and Alginate-Coated Apple and Melon Pieces

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There is an increasing trend toward foods with probiotics; the awareness of healthy diet and wellbeing is the leading cause of this increase. As a result, food producers and stakeholders require new probiotic products. The increased incidence of lactose intolerance and the new lifestyles (vegan and vegetarian styles) have led to a renewed interest in non-dairy probiotic carriers. The use of biopolymeric matrices to develop active food packaging carrying probiotics has been studied and proposed as an alternative method to design new solutions.

The main topic of this paper was the design of fresh-cut fruits (apples and melons) as carriers for a promising *Lactobacillus plantarum*; fruit pieces were coated with either alginate or chitosan. Apple (Granny Smith) and melon pieces (*Cucumis melo*, var. *Cantalupensis*) were preliminary treated with an anti-browning solution (citric and ascorbic acids). Then, fruit pieces were dipped in a solution containing *L. plantarum* c19 (9 log cfu/ml) and coated with alginate or chitosan. Samples without probiotic and/or coatings were used as controls.

All samples were stored at 4°C for 14 days under air or modified atmosphere (65% N_2, 30% CO_2, and 5% O_2); the following analyses were done: pH, color, O_2, and CO_2 in the head space, microbiology (mesophilic bacteria, lactic acid bacteria, yeasts, and molds).

The most important results can be summarized as follows: (a) Alginate coating showed better performances than chitosan-coating, as it did not affect the viability of *L. plantarum*. (b) The inoculation of probiotics in the controls negatively affected the color, but the coating was able to counteract this effect. This paper supports the combination of edible coatings and probiotic as a promising way to design new fruit-based functional foods; further investigations are required to study the effect of this combination on the sensory scores.

Keywords: probiotic, fresh-cut, edible coating, shelf life, fruit pieces

INTRODUCTION

A probiotic is "a live microorganism that, when administered in adequate amounts, confers a health benefit on the host" (FAO/WHO, 2001). Many microbes and applications fit to this definition, as they possess the three main requisites of a probiotic: microbial status, viability, and benefits to host. Lactobacilli represent a significant part of human microbiota; moreover, they possess/exert some important effects: antimicrobial activity, enhancement of immunity, and antitumorigenic activities. The most important lactobacilli of gut are *Lactobacillus acidophilus*, *L. salivarius*, *L. casei*, *L. plantarum*, *L. fermentum*, and *L. brevis*. *L. plantarum* is also the predominant species in many fermented foods, both of animal, and vegetal origin; in particular, the species *L. plantarum* was recovered in fermented Italian green olives (Bevilacqua et al., 2010). Bevilacqua et al. (2010) isolated from Italian table olives "Bella di Cerignola" *L. plantarum* c19, a strain with some promising functional traits (growth at pH 4.0–9.0, survival in presence of 10% NaCl, antimicrobial activity against *Escherichia coli* O157:H7, adhesion to IPEC-J2 cells line and survival during the simulation of the transit into the gut) (Bevilacqua et al., 2010; Altieri et al., 2011). Nevertheless, this strain showed technological robustness and a prolonged viability in vegetable matrices (Perricone et al., 2010) and a strong antimicrobial activity toward some yeasts, usually found on vegetables (Bevilacqua et al., 2013). Thus, it was chosen as a model "functional" microorganism to design a new kind of probiotic carriers.

Probiotics are usually carried by means of fermented milks, dairies, or pharmaceuticals. However, the increased incidence of lactose intolerance, allergies to milk' proteins, some concerns on cholesterol and the worldwide spreading of new life-styles (vegans and vegetarians) are the leading causes for an increased interest toward non-dairy probiotic foods, like the vegetal vehicles for probiotics (table olives, salted gherkins, and sauerkraut) (Granato et al., 2010; Vijaya Kumar et al., 2015).

The term "fresh cut" usually means vegetables ready to use (washed, cut, and fresh packaged). The International Fresh-cut Produce Association (IFPA) defines as "fresh cut" a fruit or vegetable, cut, and packaged, 100% eatable, with high nutritional value, ever ready and similar in taste to the fresh one (Lamikanra, 2002).

Fresh cut fruits show more problems than the whole ones; in fact, whole fruits are protected from microbial contamination for the presence of wax, peel, and others protective parts. Microorganisms are located on the surface and comprise bacteria (*Pseudomonas*, *Erwinia*, *Enterobacter*, *Lactobacillus* spp., etc.), molds (*Rhizopus*, *Aspergillus*, *Penicillum*, *Eurotium*, and *Wallemia*), yeasts (*Saccharomyces*, *Zygosaccharomyces*, *Hanseniaspora*, and *Candida*), viruses and parasites (Kalia and Gupta, 2006). Nevertheless, several intrinsic and extrinsic factors, may affect microbial population on fruit, influencing shelf life and/or allowing pathogen growth; for example, many *Salmonella* and *E. coli* O157:H7 infections are documented (Food and Drug Administration [FDA], 2010). Thus, fresh cut fruits have a lower shelf life, because of operations damaging tissues (peeling, cutting, and stoning), enhancing browning, softening, and spoilage (Chien et al., 2007).

Many authors proposed fruit pieces (apple, melon, pear, cashew, papaya, passion fruit, and fruit salads) as carriers for lactic acid bacteria and yeasts for either food fermentation or probiotic delivery with a focus on the viability of the

TABLE 1 | Dipping solutions.

Dipping solutions	Contents
AB	Citrate (0,2% w/v), ascorbate (1% w/v), sterile distilled water
(anti-browning for every kind of sample)	
P (9 log cfu/ml probiotic)	*Lactobacillus plantarum* culture was centrifuged (3000 g for 15 min at 4°C) (centrifuge ALC 4239R, ALC, Milan, Italy). Cells were harvested and resuspended into sterile distilled water
A (alginate coating)	Alginate powder (2% w/v) melted into sterile distilled water at 80°C
$CaCl_2$ (hardening)	$CaCl_2$ (0.5% w/v)
CH (chitosan)	chitosan (1% w/v) melted into acid solution AB
AP	A + P
CHP	CH + P

TABLE 2 | Sample preparation. The ratio fruit pieces/solution was 5 pieces in 200 ml of dipping solution.

	AB	P	A	AP	$CaCl_2$	CH	CHP	Packaging in air	Packaging in mod. atm.
O	Yes	–	–	–	–	–	–	Yes	–
PO	Yes	Yes	–	–	–	–	–	Yes	–
AO	Yes	–	Yes	–	Yes	–	–	Yes	–
APO	Yes	–	–	Yes	Yes	–	–	Yes	–
CHO	–	–	–	–	–	Yes	–	Yes	–
CHPO	–	–	–	–	–	–	Yes	Yes	–
M	Yes	–	–	–	–	–	–	–	Yes
PM	Yes	Yes	–	–	–	–	–	–	Yes
AM	Yes	–	Yes	–	Yes	–	–	–	Yes
APM	Yes	–	–	Yes	Yes	–	–	–	Yes
CHM	–	–	–	–	Yes	–	Yes	–	Yes
CHPM	–	–	–	–	–	Yes	Yes	–	Yes

starter/probiotic cultures, and on the sensory attributes (Kourkoutas et al., 2005; Tsakiris et al., 2006; Corbo et al., 2013; Martins et al., 2013; Gallo et al., 2014; Santos et al., 2017; Nikolau et al., 2019).

The market request is for a lower and lower presence of chemical preservatives in food, and food industry is responding by using more and more natural preservatives,

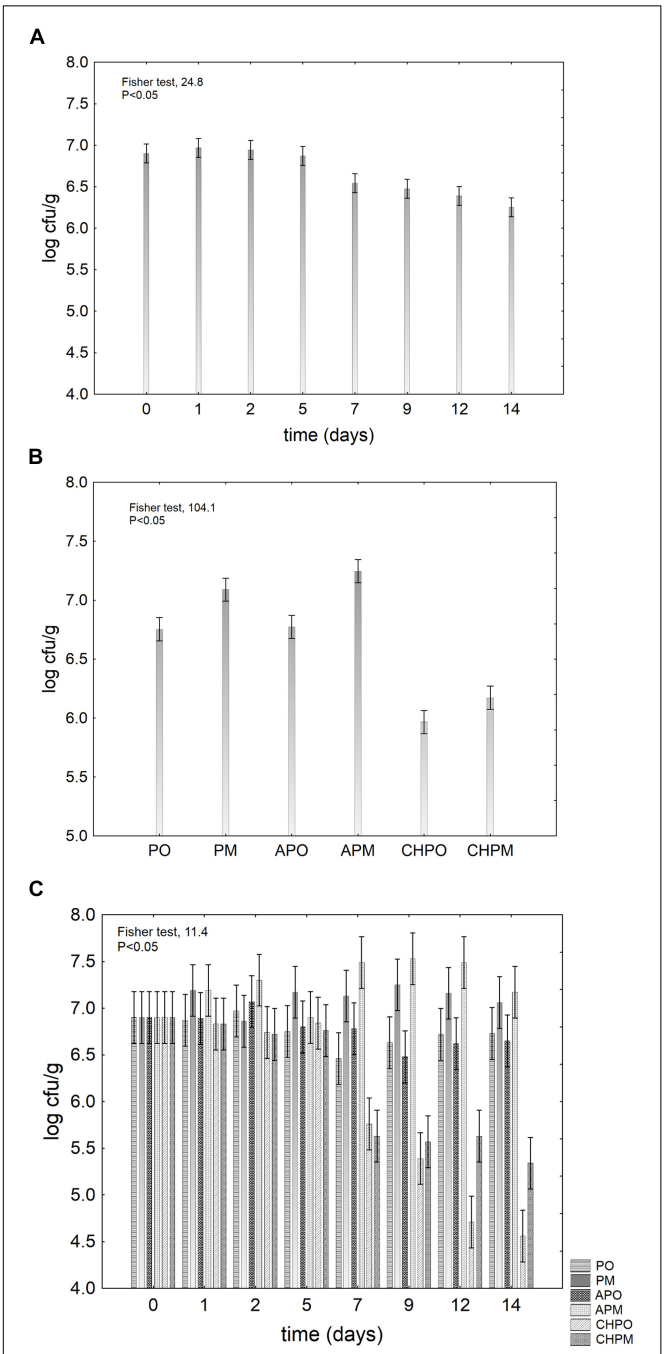

FIGURE 1 | Decomposition of the statistical hypothesis for the effect of the kind of treatment and the storage time on the viability of *Lactobacillus plantarum* c19 on apple pieces. Vertical bars denote 95% confidence intervals. **(A)** effect of the storage time; **(B)** effect of the treatment; **(C)** interaction time/treatment. For the acronyms of samples see Materials and Methods.

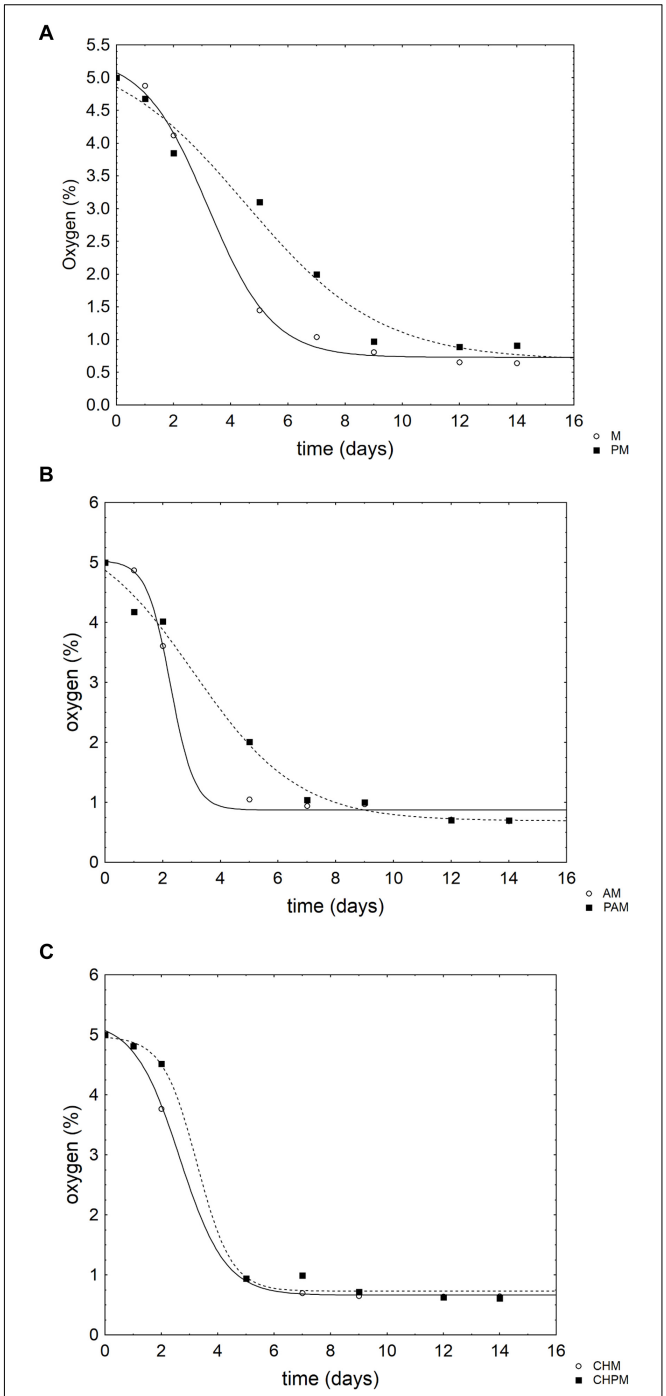

FIGURE 2 | Evolution of oxygen (v/v) in the head space of the samples of apple pieces packed in the modified atmosphere. The points represent the mean of two replicates. The lines represent the best fit through a negative Gompertz equation. For the acronyms of samples see Materials and Methods. **(A)** Apple pieces; **(B)** Apple pieces with alginate; **(C)** Apple pieces with chitosan.

able to control microbial growth. Several natural compounds have been used on fresh cut fruits, as well as phenols, chitosan, organic acids, and aldeids (Lanciotti et al., 2004), essential oils (González-Aguilar et al., 2010), applied by coating or by dipping (Lanciotti et al., 2004; Allende et al., 2006; Chien et al., 2007; Rico et al., 2007; Campaniello et al., 2008; González-Aguilar et al., 2010; D'Amato et al., 2010), and also using modified atmospheres (MAP), physical treatments, as well as high temperatures, irradiations, etc.

A promising way to improve the nutritional and the sensory traits of fruit and fruit pieces is the use of edible coatings, based on different polymers and materials (among others alginate, pectins, gellan, chitosan, caseinate, and k-carrageenate) (Campaniello et al., 2008; Moreira et al., 2015; López Aguayo et al., 2016; Sendurk Parreidt et al., 2018). However, to the best of our knowledge few data are available on the combination probiotics/edible coatings on fruit pieces and on the use of coatings as a way to overcome some drawbacks in the inoculation of probiotics in fruits.

Therefore, the aim of this work is to study and suggest a non-dairy probiotic carrier, combining the use of either apple or melon and an edible coating (chitosan or alginate), by focusing on the viability of probiotics, as a function of the coating and of the packaging atmosphere, as well as on the suitability of coatings to delay the changes on color throughout time.

MATERIALS AND METHODS

Strain

Lactobacillus plantarum c19 (Bevilacqua et al., 2010) was at −20°C in MRS broth (Oxoid, Milan, Italy) +33% of sterile glycerol (J.T. Baker, Milan, Italy). Before each experiment, the

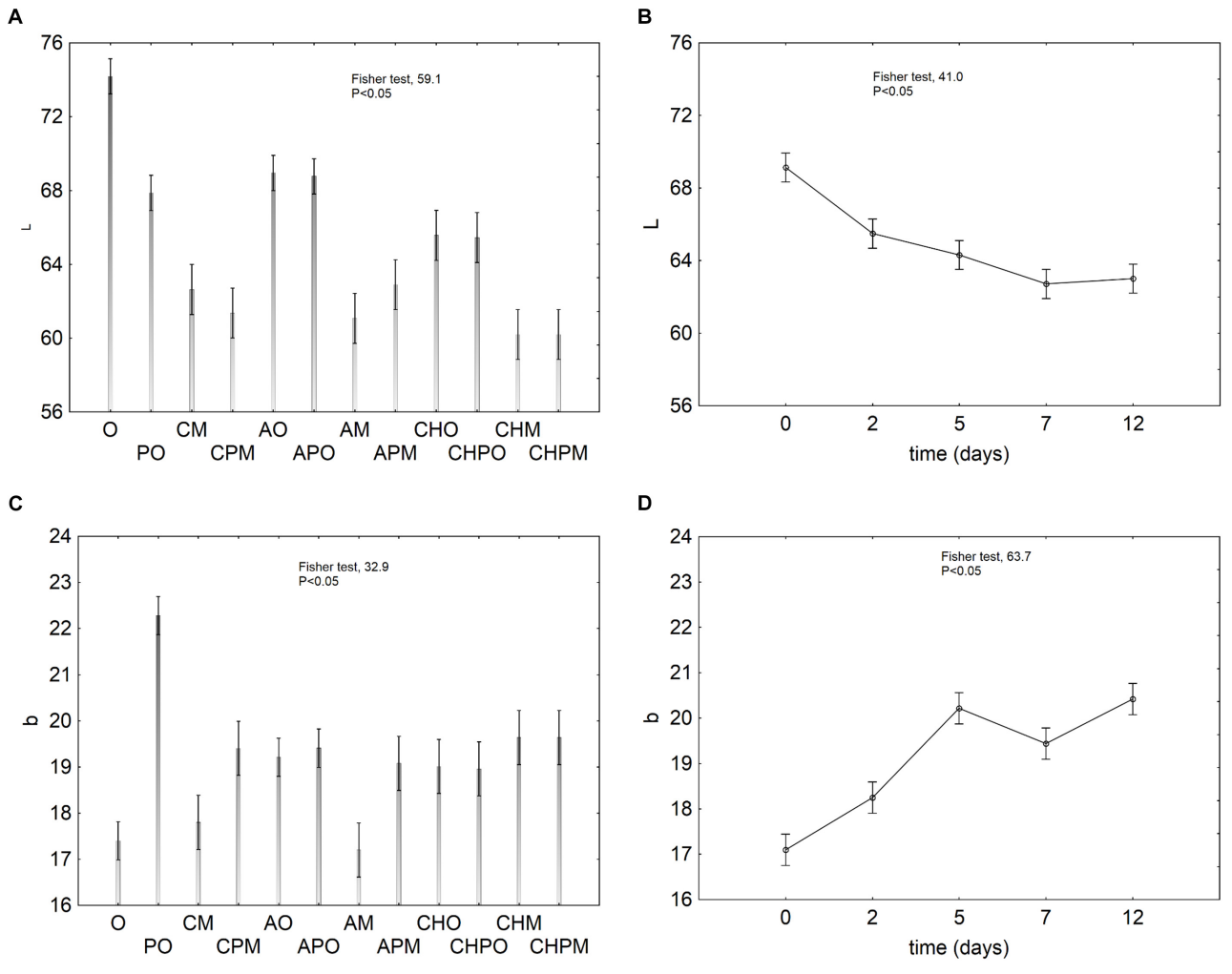

FIGURE 3 | Decomposition of the statistical hypothesis for the effect of the kind of treatment and the storage time on the instrumental color. Vertical bars denote 95% confidence intervals. For the acronyms of samples see Materials and Methods. **(A)** effect of the treatment on L; **(B)** effect of the storage time on L; **(C)** effect of the treatment on b; **(D)** effect of the storage time on b.

strain was cultured in MRS broth (37°C for 24 h under anaerobic conditions).

Sample Preparation

Apples var. Granny Smith were washed by hand under drinkable water (containing 0.2 mg/l free clorine, according to Italian law), peeled and cut (ca. 2 cm × 3 cm). Melons (sp. *Cucumis melo*, var. *Cantalupensis*) were peeled, sliced, and cut (ca. 2 cm × 3 cm).

Dipping solutions for the different treatments are reported in **Table 1**; the treatments are in **Table 2**. All samples were dipped in the AB solution (anti-browning solution) (15 min), except for chitosan-coated samples, because the anti-browning compounds were dissolved into the chitosan solution. After the anti-browning treatment, the samples were treated with the solutions reported in **Table 2** and air-dried for 15 min. Chitosan coating was used only for apple pieces.

Sample Packaging

After the preparation, the samples were packed in high-barrier plastic bags [nylon/polyethylene, 102 μm (Tecnovac, San Paolo D'Argon, Bergamo, Italy)]. The packaging was done through a S100-Tecnovac equipment. The technical characteristics of the bags were as follows: 170 mm × 250 mm; CO_2 and O_2 permeability of respectively, 3.26×10^{-19} and 9.23×10^{-19} mol m m^{-2} s^{-1} Pa^{-1} and water vapor transmission rate of 1.62×10^{-10} kg m^{-2} s^{-1}. The samples were packaged in air (O) or in MAP (M: 65% N_2, 30% CO_2, and 5% O_2).

The samples were stored at 4°C for 14 days and microbiological, and physico-chemical analyses were performed. All analyses were performed twice on two independent samples.

Microbiological Analyses

The first homogenate was prepared with 25 g of fruits and 225 ml sterile saline solution (0.9% NaCl) in a Stomacher bag (Seward, London, United Kingdom) through a Stomacher Lab Blender 400 (Seward). The following analyses were done: (a) total bacterial count on Plate Count Agar (PCA, Oxoid, Milan, Italy) (32°C for 48 h); (b) psychrotrophic bacteria (PCA, 5°C for 1 week); (c) lactic acid bacteria MRS Agar 0.17 g/l of cycloheximide (Sigma-Aldrich, Milan) (37°C for 4 days, anaerobic conditions); (d) yeasts and molds on Sabouraud Dextrose Agar (Oxoid, Milan, Italy) incubated at 28°C for 2–5 days, respectively.

Five to ten colonies were randomly selected from MRS Agar from the samples inoculated with *L. plantarum* c19; DNA was extracted and PCR was run as reported by Bevilacqua et al. (2010).

pH

Determination of pH was performed by a pH-meter Crison, model micro pH 2001 (Crison, Barcellona, Spain) on the homogenate prepared for the microbiological analyses.

Colorimetric Analysis

Color was measured using a tristimulus colorimeter CR-300 Minolta Chromameter-2 Reflectance (Minolta, Japan). Data were expressed according to CIELAB scale: L (luminescence), a (red/green coordinate), and b (yellow/blue coordinate). The calibration of colorimeter was done on standard white (L^* = 97,03, a^* = +0,01, and b^* = +1,63).

Head Space Analysis

Quantitative analysis of O_2 e CO_2 in head space was performed using a PBI Dansensor (Checkmate 9900, Ringsted, Denmark), with test volumes of 10 cm^3.

Statistic

The results were analyzed by a two-way ANOVA and Tukey's test as the *post hoc* comparison test ($P < 0.05$).

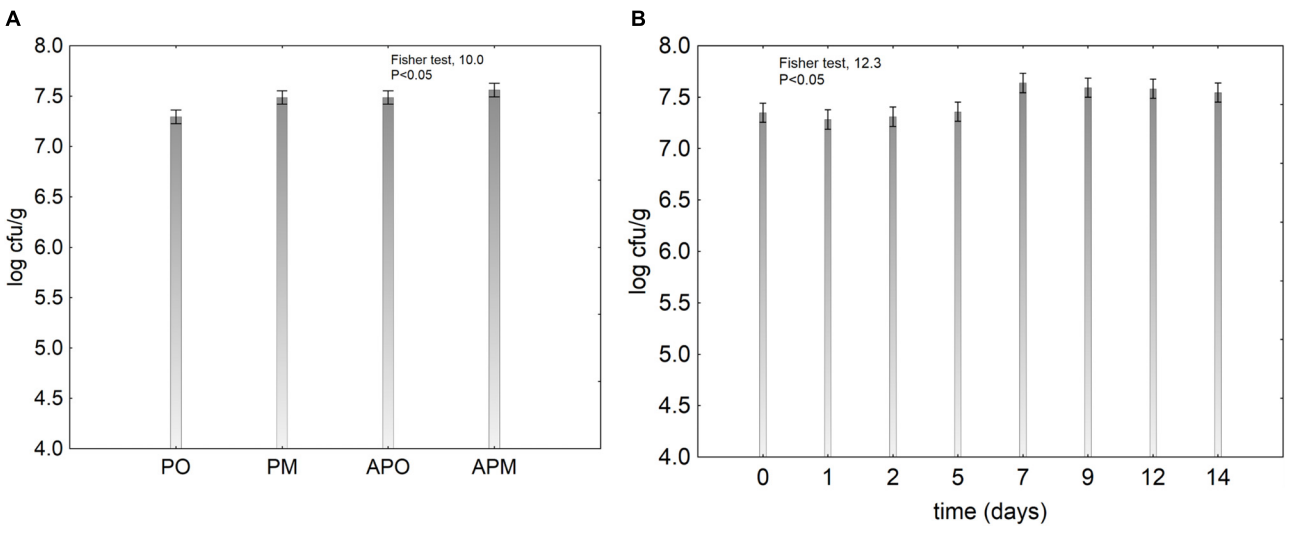

FIGURE 4 | Decomposition of the statistical hypothesis for the effect of the kind of treatment and the storage time on the viability of *L. plantarum* c19 on melon pieces. Vertical bars denote 95% confidence intervals. For the acronyms of samples see Materials and Methods. **(A)** effect of the treatment; **(B)** effect of the storage time.

RESULTS

LAB were below the detection limit on the uninoculated samples (controls: samples O, AO, CHO, M, AM, and CHM), while the identification of some isolates from inoculated samples (samples PO, APO, CHPO, PM, APM, and CHPM) confirmed that LAB population was composed by *L. plantarum* (data not shown).

The results for the viable count of *L. plantarum* c19 on apple pieces were analyzed by two-way ANOVA, using the treatment (kind of samples) and the time (duration of storage) as categorical predictors. The quantitative output of this statistic can be found in the graphs of the decomposition of the statistical hypothesis (**Figure 1**).

As expected, the storage time negatively acted on probiotic, as it caused a decrease of 1 log cfu/g (**Figure 1A**). In addition, the different treatments affected the viability of *L. plantarum* in a different way and the coating with chitosan, both in air and under MAP, caused a mean decrease of the target of 1 log cfu/g (**Figure 1B**). Both **Figures 1A,B** do not show actual values, but they only offer an insight on the quantitative effect of each factor of the design.

The effective trends of *L. plantarum* c19 are in **Figure 1C**; at the beginning, the viable count of the probiotic was 6.8 log cfu/g. Then, it experienced a strong decrease in chitosan coated-apple pieces up to 4.5 log cfu/g in air and 5.3 log cfu/g under MAP.

The legal break point for probiotics in food has been set to 10^7 cfu/g or 10^9 per day (Rosburg et al., 2010; Italian Ministry of Health, 2013). However, the main goal of this step was not to assess a "functional" shelf life (time to main the viability of the probiotic to an acceptable level), but to assess the suitability of coatings and to choose the best combination probiotic/coating. However, the low level of probiotic (<7 log cfu/g) suggests that the inoculation should be further improved to increase the concentration of *L. plantarum* on the inoculated samples.

Concerning the other microbiological data, moulds, yeasts, and psychrotrophic bacteria were below the detection limit for the whole storage time (data not shown).

Apple pieces were also analyzed in relation to physico-chemical parameters. Concerning the gases in the head-space, the samples packed in MAP showed a CO_2-trend like a negative sigmoid; therefore, the results were modeled with a negative Gompertz equation. In the controls (apple pieces without coating) (**Figure 2A**) and in those coated with alginate (**Figure 2B**), the probiotic exerted a significant effect on the rate of oxygen consumption and decreased it (from 0.88 to 0.42% O_2/day in the controls and from 1.03 to 0.55% O_2/day in alginate coated-apple pieces). This effect was not found in the chitosan coated samples (**Figure 2C**).

In the samples packed in MAP there was a linear decrease of O_2 up to 7–10% without significant differences amongst the samples (data not shown). CO_2 increased to 10–15% in the samples packed in air and to 35–36% for the samples packed in MAP (data not shown).

The effect of the probiotic on color was analyzed by the evaluation of the decomposition of the statistical hypothesis on the parameters L (luminescence) and b (yellow/blue coordinate). The effect of the treatment on L was variable and relied upon the kind of treatment itself (**Figure 3A**). In the controls packed in air (O and PO), the inoculation of *L. plantarum* c19 caused a strong decrease of L; in the samples packed in MAP (M and PM) the decrease of L was independent from the inoculation of probiotic and was probably due to the atmosphere. A higher value of L was found in alginate-coated apple pieces packed in air. As expected, the storage time negatively acted on L because of enzymatic browning (**Figure 3B**).

Figures 3C,D show the effect of the treatment and storage time on the parameter b. The inoculation of *L. plantarum* caused an increase of b in the controls (see the difference between O and PO); on the other hand, the increase was less pronounced in the samples coated with alginate and chitosan (**Figure 3C**). As expected, b increased within the storage because of the enzymatic browning (**Figure 3D**).

A second phase of this research was aimed at assessing the effect of probiotic and coating on melon pieces. Chitosan was not used due to the strong effect on the viability of *L. plantarum*, as aforementioned.

The effect of the treatment of the viability of probiotic was less significant (**Figure 4A**), whereas time exerted a significant effect

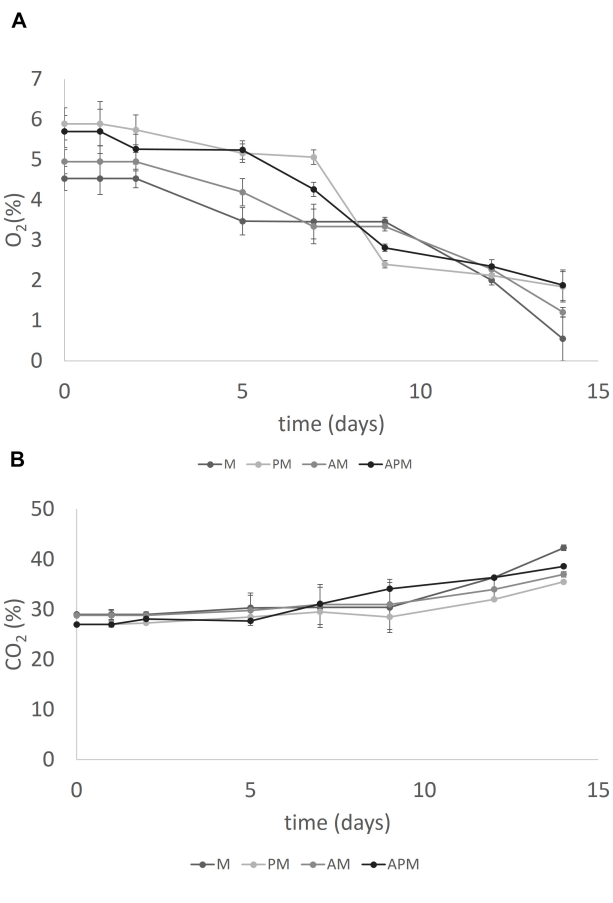

FIGURE 5 | Evolution of O_2 **(A)** and CO_2 **(B)** in the head-space of melon pieces inoculated with *L. plantarum* c19 and packed in modified atmosphere. Mean ± standard deviation. For the acronyms of samples see Materials and Methods.

as probiotic increased throughout the storage (**Figure 4B**). In the samples packaged in air and in MAP without coating, the pH decreased and the probiotic enhanced this effect: at the beginning the pH was 6.8 and after 14 days 5.0 in the controls and 4.5–4.3 in the samples with probiotics. The use of alginate-coating controlled this phenomenon and the final pH was 6.5 (data not shown).

Oxygen levels decreased and carbon dioxide increased without significant differences amongst the samples. As an example, **Figures 5A,B** show the level of gasses in the samples packed in MAP.

DISCUSSION

The demand for new food-carriers for probiotics is only one of the reason of the emerging trend toward non-dairy food products, such as those based on fruits (Martins et al., 2013), with potential benefits for human health.

Consumers require more and more ready-to-eat and convenience foods and this is why their market increased in the last decade (Rojas-Graü et al., 2011). In this increasing trend, it is important to offer foods with the dual functionality of vegetable origin and probiotics.

The high preference of consumers for probiotic foods containing fruits has been reported by Espírito-Santo et al. (2011); moreover, fruits represent a good substrate for probiotics since they have nutrients (Soccol et al., 2010) and possess morphological structures favoring microbial growth (Martins et al., 2013). Another benefit is the lack of allergenic substances of dairy products.

The results obtained in the present study underlined that the presence of the tested probiotic strain on the apple pieces was well supported. However, the inoculation of probiotics in foods often requires special technologies; probiotics, in fact, retain an active metabolism and could cause an over-acidification of the product (Bevilacqua et al., 2016; Racioppo et al., 2017), with a worsening of the sensory trait. This effect was reported by Martins et al. (2015) on apple pieces containing *L. plantarum* and was confirmed in this research on apple pieces inoculated with the probiotic but without coating (for example the sample PO). Different strategies can be used to counteract this effect; hereby we propose the use of an edible coating, based either on alginate and chitosan and combined with MAP.

Nevertheless, the study evidenced that the kind of coating significantly affected the probiotic viability; in particular, chitosan reduced the viability of lactobacilli, as one could expect from the wide antifungal and antibacterial abilities (Altieri et al., 2005; Campaniello et al., 2008; Raafat and Sahl, 2009; Campaniello and Corbo, 2010).

On the other hand, alginate coating showed better performances by preserving the viability of *L. plantarum*; moreover, it was able to counteract the bad effect of the probiotic on apple pieces, both in air, and in MAP and achieved the primary goal of this research: to design a probiotic carrier and avoid the negative effects of lactobacilli.

The second phase of the study was focused on melon. First de Oliveira et al. (2014) proposed melon pieces as probiotic carriers and this research supports their suitability.

In this phase, the chitosan coating was not tested in the light of results obtained with apple samples. Alginate exerted a positive effect whatever the storage atmosphere and also counteracted the negative effect of lactobacilli on pH.

CONCLUSION

The use of fruit-pieces (i.e., apple and melon), with a good combination of packaging atmosphere and coating can be considered a promising way to design new carriers for probiotic bacteria. In particular, alginate coating did not affect the viability of the model microorganism and to some extent could counteract the negative effect of probiotication on color.

Fresh-cut fruits are well accepted by consumers, indicating that are marketable products, having all the benefits provided by probiotic functional food, with the advantage that everybody can consume it. However, more studies and clinical trials are needed in order to evaluate id the probiotic carried out by vegetable foods exert their role in the gut.

Further investigations are also required to study the effect of probiotication on the sensory scores, as well as to increase the level of probiotics in order to fit to the legal requirements and drive the research from lab to market.

AUTHOR CONTRIBUTIONS

MC and MS conceived the study and funded the research. BS, DC, and CA designed the experiments. BS and DC performed the experiments. AB performed the statistic. CA and AB wrote the manuscript. All the authors interpreted the results and reviewed the paper.

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Exploring K2G30 Genome: A High Bacterial Cellulose Producing Strain in Glucose and Mannitol Based Media

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Demands for renewable and sustainable biopolymers have rapidly increased in the last decades along with environmental issues. In this context, bacterial cellulose, as renewable and biodegradable biopolymer has received considerable attention. Particularly, acetic acid bacteria of the *Komagataeibacter xylinus* species can produce bacterial cellulose from several carbon sources. To fully exploit metabolic potential of cellulose producing acetic acid bacteria, an understanding of the ability of producing bacterial cellulose from different carbon sources and the characterization of the genes involved in the synthesis is required. Here, K2G30 (UMCC 2756) was studied with respect to bacterial cellulose production in mannitol, xylitol and glucose media. Moreover, the draft genome sequence with a focus on cellulose related genes was produced. A pH reduction and gluconic acid formation was observed in glucose medium which allowed to produce 6.14 ± 0.02 g/L of bacterial cellulose; the highest bacterial cellulose production obtained was in 1.5% (w/v) mannitol medium (8.77 ± 0.04 g/L), while xylitol provided the lowest (1.35 ± 0.05 g/L) yield. Genomic analysis of K2G30 revealed a peculiar gene sets of cellulose synthase; three *bcs* operons and a fourth copy of *bcsAB* gene, that encodes the catalytic core of cellulose synthase. These features can explain the high amount of bacterial cellulose produced by K2G30 strain. Results of this study provide valuable information to industrially exploit acetic acid bacteria in producing bacterial cellulose from different carbon sources including vegetable waste feedstocks containing mannitol.

Keywords: ***Komagataeibacter xylinus*, bacterial cellulose, glucose, mannitol, xylitol, gluconic acid, genome sequencing**

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INTRODUCTION

Demands for renewable and sustainable biopolymers have rapidly increased in the last decades along with environmental issues. In this context, bacterial cellulose (BC), as biocompatible, renewable and biodegradable biopolymer has received considerable attention.

The primary structure of BC consists of a β-1,4-glucan chain which undergo aggregation events to form a ribbon-like structure (Saxena et al., 1994; Brown, 1996). These ribbons form the secondary

structure that generate the well-structured 3D network, characteristic of BC (Koyama et al., 1997). The tertiary structure, as a result of intermolecular hydrogen bonds and van der Waals forces, stabilizes the entire structure through an intramolecular hydrogen bond network by hydroxyls and ring oxygen among glucose residues. Each repeating unit has a directional chemical asymmetry with respect to its molecular axis (a hemiacetal unit and hydroxyl group) (Koyama et al., 1997). The fibrous network forms a hydrogel film at air surface of culture media.

Differently from its equivalent produced by plants, BC does not contain lignin and hemicellulose, it has higher degrees of purity, polymerization, crystallinity, tensile strength, water holding capacity, and biological adaptability (Costa et al., 2017; Gullo et al., 2018). Structurally, BC differs from plant cellulose on the basis of cellulose I_α and I_β content (Atalla and Vanderhart, 1984). These two allomorphs coexist always in nature and differ for the crystal system: the I_α is characterized by triclinic system, whereas I_β by a monoclinic system. Cellulose I_α is the predominant allomorph in BC, ranged from 60 to 80%. On the other hand, the allormorph I_β is the predominant form of plant cellulose (Nishiyama et al., 2002, 2003).

All BC properties are strictly linked to both intracellular biosynthesis and extracellular self-assembling mechanism. It is widely accepted that BC is synthesized within the bacterial cell as individual molecules. The biosynthesis occurs on the periplasmic space by cellulose synthase (CS), a membrane protein complex formed by a series of subunits: BcsA, BcsB, BcsC, and BcsD, of which BcsA and BcsB represent the catalytic core of CS (Gullo et al., 2018). The CS genes are organized in an operon that, based on the genus, are divided into three classes, which differ in content and structure. The first class is represented by the operon originally described in the *Komagataeibacter xylinus* species. The second one, described for the first time in *E. coli,* contains also a divergent locus, which includes genes involved in natural BC modification (Thongsomboon et al., 2018). Finally, the third class of BC operon, described in *A. tumefaciens,* consists of two convergent operons. The first three genes are ortholog of *bcsA, bcsB,* and *bcsZ* from *K. xylinus,* while the others are typical of *A. tumefaciens* (Matthysse et al., 2005). Similar operons are also found in members of *Actinobacteria* and *Firmicutes phyla* (Römling and Galperin, 2015).

As material generally recognized as safe (GRAS) by the United States Food and drug administration (FDA) in 1992, BC can be utilized as a fiber for different applications in biomedical, cosmetics and food (Shi et al., 2014). Main biomedical uses include supports as substitute artificial skin, hemostatic materials, wound healing scaffolds, and controlled drug delivery (Pavaloiu et al., 2014; Picheth et al., 2017). Recently very interestingly insights were obtained using BC as a biocarrier of dihydroxyacetone, in masking the symptoms of vitiligo by providing skin pigmentation (Stasiak and Płoska, 2018).

In cosmetic field, BC is an ingredient for facial mask creams and as a powder in facial scrubs products in association with other natural materials (as olive oil, Vitamin C, *Aloe vera* extract, and powdered glutinous rice).

In food, BC can be used as dietary fiber and as adjuvant thanks to the ability to acquire flavors and colors. It occurs in the manufacturing of nata de coco, a Philippine dessert produced from fermented coconut water, and in Kombucha tea, a fermented beverage obtained from alcoholic and acetic fermentation of sugared tea (Gullo et al., 2018).

BC production was described for different bacterial species, comprising *Rhizobium leguminosarum*, *Burkholderia* spp., *Pseudomonas putida*, *Dickeya dadantii*, *Erwinia chrysanthemi*, *Agrobacterium tumefaciens*, *Escherichia coli*, and *Salmonella enterica* species (Chawla et al., 2009; Jahn et al., 2011). Within acetic acid bacteria (AAB), different genera were reported as BC producers including *Acetobacter*, *Gluconacetobacter* and *Komagataeibacter* (Mamlouk and Gullo, 2013). AAB are considered a very versatile group of bacteria involved in a wide range of industrial process for the production of different compounds, such as acetic acid in vinegar production, gluconic acid, 2-keto-L-gluconic acid, 5-keto-L-gluconic acid, 2-keto-gulonic acid, and dihydroxyacetone (Stasiak and Blazejak, 2009; La China et al., 2018). In vinegar production, other than acetic acid they can also form BC which is considered as a disadvantage because it negatively affects the process and the sensorial properties of the product (Gullo and Giudici, 2008; Gullo et al., 2016). On the other hands, vinegar has been used as an appropriate substrate for studying the mechanism of BC synthesis by AAB (Gullo et al., 2018). Species of the genus *Komagataeibacter* are widely detected in vinegar such as *K. europaeus* and its closely related species *K. xylinus,* which is considered as a model organism for BC synthesis (Römling, 2002).

Given the wide range of use of BC and the increasing of the market of bacteria cellulose-based materials, which is expected to exceed 500 million US dollars by 2023 (based on market research report[1]), there is a need to link the knowledge of science to its industrial scale-up. Although a number of studies have highlighted great potential of application, others demonstrate limitations in term of process and economic sustainability (Gullo et al., 2017; Islam et al., 2017; Basu et al., 2018).

Main issues in BC production arise from the organism and the cultivation conditions, which affect the implementation of advantageous industrial processes. However, a number of works aiming at selecting robust wild strains and obtaining engineered strains are available. These studies mainly focuses *Komagataeibacter* species (*K. xylinus* and *K. hansenii*), tested in different culture conditions (Hwang et al., 1999; Kuo et al., 2010; Costa et al., 2017; Gullo et al., 2017). The most widely system of production is the static regime by which layers of different form and thickness are obtained, according to the ratio surface/volume (S/V) of vessels. Also the production by agitated cultivation system is reported, but it seems to provide a lower yield and generally BC is formed as spheres (Gullo et al., 2018).

Regarding the raw materials for producing BC, both the need to reduce costs and the need to provide more sustainable productions, encouraged the use of vegetable waste feedstocks containing suitable carbon sources (Kuo et al., 2010;

[1]https://www.marketresearchreports.com

Cheng et al., 2017; Costa et al., 2017). Promising results were provided from lignocellulosic materials such as wheat straw, sugarcane bagasse, rice straw, hydrolysate fiber sludge, and corn steep liquor (Hwang et al., 1999; da Silva Filho et al., 2006; Cavka et al., 2013; Narh et al., 2018). Carbon sources mainly contained in these products are glucose, sucrose, and polyols as mannitol and xylitol, that are also naturally found in fruits, other vegetables, and also produced by bacteria and yeasts (Song and Vieille, 2009).

Focusing, on genes related to BC synthesis, in this work we present the genome sequencing of K2G30 (UMCC 2756), an AAB strain from Unimore Microbial Culture collection, previously selected as highly BC producing strain (Gullo et al., 2017). We also tested the BC production ability of K2G30 in two alternative carbon sources (mannitol and xylitol), which usually occur in waste vegetables.

MATERIALS AND METHODS

Bacterial Strain and Culture Conditions

K2G30 strain used in this study was previously isolated from pellicle fraction of Kombucha tea (Mamlouk, 2012) and safely deposited at the Unimore Microbial Culture Collection (UMCC) under the collection number UMCC 2756. The strain was cultivated in aerobic conditions at 28°C in GY broth (glucose 5.0% w/v and yeast extract 1.0% w/v; pH 4.46 (at time 0 of cultivation), when appropriate, agar (0.8% w/v) was supplemented. Bacterial cellulose was produced in GY, mannitol medium (1.5% mannitol (w/v), 2% yeast extract (w/v) and 0.5% polypeptone (w/v); pH 5.75 at time 0 of cultivation) (Oikawa et al., 1995b) and xylitol medium (5% xylitol (w/v), 1% yeast extract (w/v) and 1% tryptone (w/v); pH 5.65 at time 0 of cultivation) (Singhsa et al., 2018). Glucose, mannitol and xylitol were purchased from Merck KGaA, Darmstadt, Germany; yeast extract from Thermo Fisher Scientific Inc., All the media were sterilized by autoclaving at 121°C for 15 min prior the use.

Bacterial Cellulose Production in Glucose, Mannitol, and Xylitol Broths

K2G30 was first cultivated in GY broth at 28°C for 5 days under static conditions. Aliquots were used to prepare preinocula in mannitol and xylitol broths, respectively. Triplicate assays were conducted in 500 mL beakers (diameter 8.7 cm) containing 150 mL of the respective broths inoculated with 5% (v/v) of the preinoculum culture. Cultures were incubated at 28°C for 9 days under static conditions. Moreover, cultivation in agitated conditions (130 rpm/28°C/5 days) was performed in 100 mL flasks using 40 mL of each broth inoculated with 5% (v/v) of the preinoculum.

Bacterial Cellulose Harvesting, Purification, and Weighing

BC pellicles collected at 3, 6, and 9 days of cultivation were washed with deionized water and treated with NaOH 1 M at 80°C for 40 min to remove bacterial cells; they were washed several times with deionized water to reach neutral pH, and finally dried at 37°C until constant weight. After perming experiments in triplicate, meaningful values were determined. The BC weight was expressed as previously reported (Gullo et al., 2017).

Analytic Determinations

D-glucose and D-Gluconic acid were measured by enzymatic kits (Megazyme Ltd., Bray, Ireland) according to the manufacturer's instructions. pH was measured using an automatic titrator (TitroLine®EASY SCHOTT Instruments GmbH, Mainz, Germany), equipped with an SI Analytics electrode (SI Analytics, GmbH, Mainz, Germany). The samples were obtained collecting 5 mL of each culture medium in three-day intervals.

Genomic DNA Extraction and Sequencing

Genomic DNA (gDNA) extraction from K2G30 was performed on a total 40 ml of GY culture, after 5 days of incubation at 28°C. The liquid culture was centrifuged at 10,000 × g/4°C/10 min. gDNA was extracted as previously described (Gullo et al., 2006) and it was quantified using Qubit 2.0 (Invitrogen, Carlsbad, CA, United States). A total 100 ng was used for sequencing. The whole genome of *K. xylinus* K2G30 was sequenced by Admera Health LCC (South Plainfield, NJ, United States) using Nextera XT DNA Sample Preparation Kits (Illumina) and sequenced using the Illumina HiSeq X.

De novo Genome Assembly and Annotation

The primary quality check was performed using FastQC and Trimmomatic v0.36 tool (Bolger et al., 2014) which was used to remove bases with a Phred score <20. Spades v1.10.1 (Bankevich et al., 2012) was used for *de novo* genome assembly using careful option and kmer size of 21, 33, and 55. The quality of consensus sequences was evaluated using Quast v4.5 (Gurevich et al., 2013) and reads with length lesser than 1 Kbp were discarded. Resulted contigs were used for genome annotation. Putative coding regions were identified by Prodigal v2.6.3 (Hyatt et al., 2010), while tRNA and rRNA were predicted using tRNAscan-SE v1.3.1 and RNAmmer (Lowe and Eddy, 1997; Lagesen et al., 2007). Functional annotation of translated coding regions was performed using Blastp v2.7.1 (McGinnis and Madden, 2004) against NCBI non-redundant database and Uniprot, setting E-value threshold as 1E-5. Hmmscan v3.1b2 (Mistry et al., 2013) was used for protein domain annotation via Pfam database and protein family definitions via Tigrfam databases. Cluster of orthologous groups (COG) were retrieved using EggNog-mapper v1.0.3 (Huerta-Cepas et al., 2017). These data sources were combined and manually curated using Arthemis genome browser (Carver et al., 2012) to assert a product description for each predicted protein. Genome ideogram reconstruction was performed using Circos v0.69 (Krzywinski et al., 2009). The genome version discussed in this study is the version K2G30_v1.0. Fastq files and genome assembly

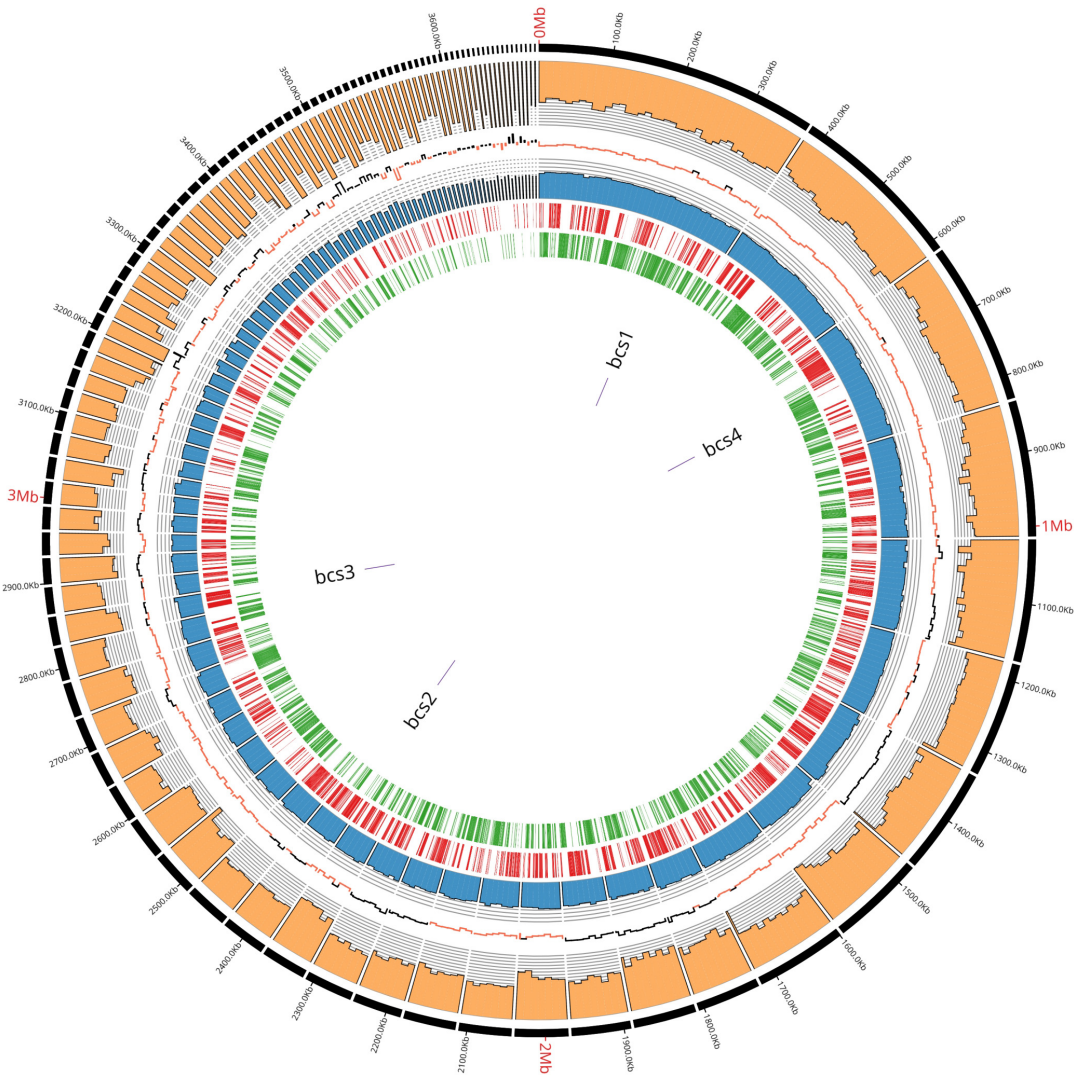

FIGURE 1 | K2G30 genome circular representation. From outside to inside: contigs with relative lenghts expressed in bp; coverage; GC skew expressed in positive changes in GC content (black) and negative (red); GC content expressed in percentage; forward CDS; reverse CDS; bcs operons genome localization.

fasta file are available at GeneBank, under the accession number QQBI00000000.

Phylogenetic Genome Reconstruction

16S and 23S rRNA sequences and genome-to-genome sequence similarity analysis were performed. The related 16S and 23S rRNA sequences were downloaded from Silva SSU and LSU databases and from Joint Genome Institute (JGI) using IMG. Both 16S and 23S were separately aligned using Muscle aligner and then concatenated using MEGAX (Edgar, 2004; Kumar et al., 2018). From concatenated alignment, a maximum likelyhood phylogenetic tree was generated using Tamura-Nei model (Tamura and Nei, 1993) with 1000 replicated, setting gamma distribution option. We used also alternative method to recognize K2G30 phylogeny, as digital DNA-DNA hybridization analysis. A total of 19 genomes of *Komagataeibacter* genus (taxid 1434011) retrieved from NCBI were used for analysis. The average nucleotide identity values using BLAST method (ANIb) and tetranucleotide usage patterns (TETRA) for all pairwise comparisons of 19 *Komagataeibacter* genomes were determined. ANIb and TETRA were calculated by the use the pyani module (Pritchard et al., 2016).

RESULTS AND DISCUSSION

K2G30 Genome Features and Bacterial Cellulose Synthase Genes

In order to describe the genetic organization of BC production related genes, the genome of K2G30 was sequenced, annotated and explored. The genome of K2G30 consists of 101 contigs with a total length of 3.63 Mbp and a coverage average of 700X

(**Figure 1**). A total of 22 contigs were more or equal than 50 Kbp in size and N50 and L50 were 86.67 Kbp and 11, respectively. The G + C content was 62.77%, which is near to the median G + C content in sequenced genomes of *K. xylinus* strains.

One operon of rRNA genes was retrieved including one copy of 5S, 16S, and 23S, respectively. The number of predicted coding DNA sequences (CDS) were 3380. The genome properties and statistics are summarized in **Table 1**. More than 75% of CDS were assigned to cluster of orthologous-group classification and functional categories (COG) (**Table 2**).

TABLE 1 | K2G30 Genome features.

Properties	Value
Contigs	101
Lenght	3.63 Mbp
N50	86.67 Kbp
L50	11
Depth of coverage	700X
CDS	3380
rRNA	3
tRNA	51
tmRNA	1
Uniprot	64.70%
Pfam	87.66%
COG	78.04%
KEGG	50.5%

TABLE 2 | Genome cluster of orthologous-group classification and functional categories.

Categories	Functional group	Percentage (%)
C	Energy production and conversion	6.55
D	Cell cycle control, cell division, chromosome partitioning	0.96
E	Amino acid transport and metabolism	7.34
F	Nucleotide transport and metabolism	2.88
G	Carbohydrate transport and metabolism	5.31
H	Coenzyme transport and metabolism	3.81
I	Lipid transport and metabolism	2.28
J	Translation, ribosomal structure and biogenesis	5.31
K	Transcription	6.2
L	Replication, recombination and repair	7.55
M	Cell wall/membrane/envelope biogenesis	6.27
N	Cell motility	0.14
O	Posttranslational modification, protein turnover, chaperones	3.81
P	Inorganic ion transport and metabolism	6.98
Q	Secondary metabolites biosynthesis, transport and catabolism	1.17
S	Function unknown	26.81
T	Signal transduction mechanisms	2.67
U	Intracellular trafficking, secretion, and vesicular transport	2.49
V	Defense mechanisms	1.46

Based on Uniprot alignment, in K2G30 genome, three copies of *bcs* operons were annotated, of which only one (*bcs1)* contains the full enzymatic set of CS related genes (BcsA, BcsB, BcsC, and BcsD) and the accessory proteins CMCax, ccpAx, and BglAx (**Figure 2**). *Bcs*1 operon was localized in contig 1, at the genomic position 256838-270633 bp. The full set of CS genes is represented by *bcsA, bcsB* (the catalytic core of CS), *bcsC, bcsD,* preceded by *cmcax,* and *ccpax* genes, in upstream position, while in downstream position *bglAx* was found. The sequences similarity span from 32.82% to 98.72% with and minimum e-value score of $2e^{-19}$.

Cmcax gene encodes for endo-β-1,4-glucanase, while the *ccpax* role is not clear. However, it was hypothesized that it can be involved in the structural organization of the terminal complex, cooperating with BcsD (Sunagawa et al., 2013). The downstream *bglax* gene encodes for β-glucosidase and seems to have also the glucosyltransferase activity (Tajima et al., 2001).

A second *bcs* operon (*bcs*2) was found in contig 19, located from 5579 to 16126 bp. This operon displays the classical architecture of *bcs2* described in other *Komagataeibacter* strains (Römling and Galperin, 2015). Differently from *bcs1* operon, in *bcs*2, the genes that encode for BcsA and BcsB subunits are fused, named *bcsAB.* No gene codifying for BcsD was detected, while the gene encoding for BcsC was detached from *bcsAB* by other two genes (*bcsX* and *bcsY*), generally described as peculiar in *bcs2* operon. The sequence similarity based on aminoacids sequences alignment was 78.79% for *bcsAB* (e-value of 0.0), instead for *bcsC* the sequence similarity was 70.24% (e-value of 0.0). The similarity of *bcsX* and *bcsY* were 92.83% and 80.05%, respectively.

bcs3 operon is located in contig 28 at position 28895-37149 bp and contains genes that encode for BcsA, BcsB and BcsC subunits. Also in this operon, the genes that codify for BcsA and BcsB were fused. In addition, in K2G30, a fourth copy of *bcsAB* genes was retrieved in contig 3, having an aminoacid sequence similarity of 67.45% (e-value score of 0.0). The high number of CS catalytic subunits can explain the high BC yield previously obtained from K2G30 (23 g/L) (Gullo et al., 2017). Only in another genome of *K. xylinus* species the fourth copy of *bcsAB* gene was described (Liu et al., 2018). Moreover, a variable copy number of *bcs* operon was previously reported for cellulose-producing AAB (Gullo et al., 2018). *K. hansenii* species were described to contain three copies of *bcs* operon (Iyer et al., 2010; Florea et al., 2016). In *K. xylinus*, the presence of one *bcs* operon was reported for a not BC producer strain (NBRC 3288) (Ogino et al., 2011), whereas two copies were retrieved in *K. xylinus* E25 (Kubiak et al., 2014).

Phylogenetic Analysis

Individual alignment of 16S and 23S rRNA genes of 19 *Komagataeibacter* species were checked manually and clipped at the same length. A phylogenetic tree was generated from concatenated rRNA genes with a total length of 3687 nucleotides (nt): 16S (1118 nt) and 23S (2569 nt).

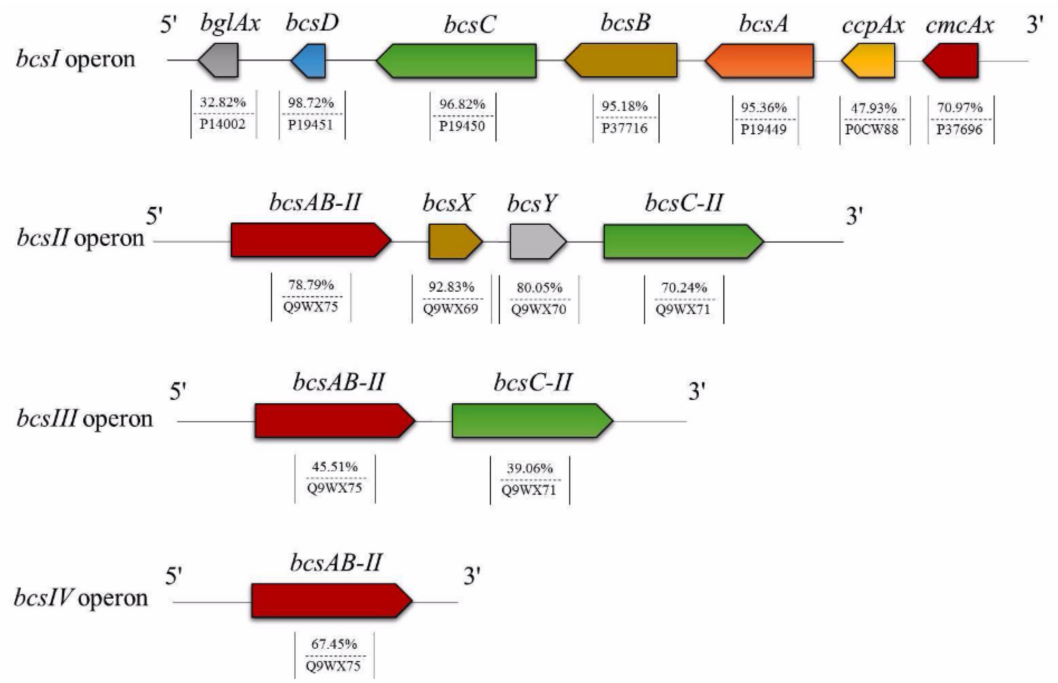

FIGURE 2 | The arrangement of *bcs* operons and BC related genes organization in K2G30.

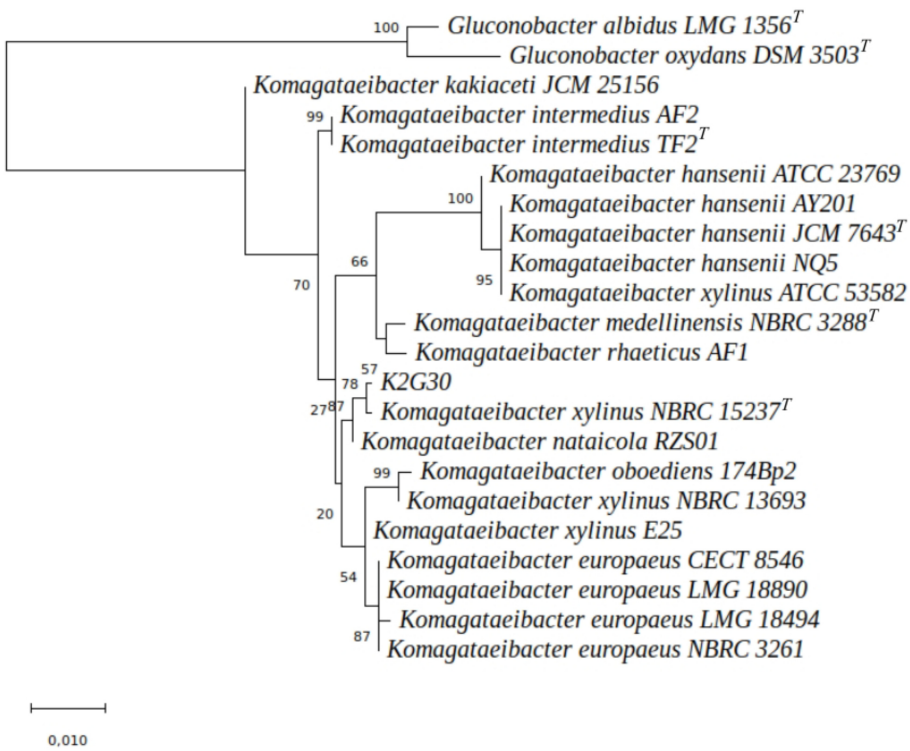

FIGURE 3 | ML tree representing the phylogenetic distances among 19 *Komagataeibacter* genomes. The node numbers indicate the bootstrap values. The branch length was expressed in 0.010 unit.

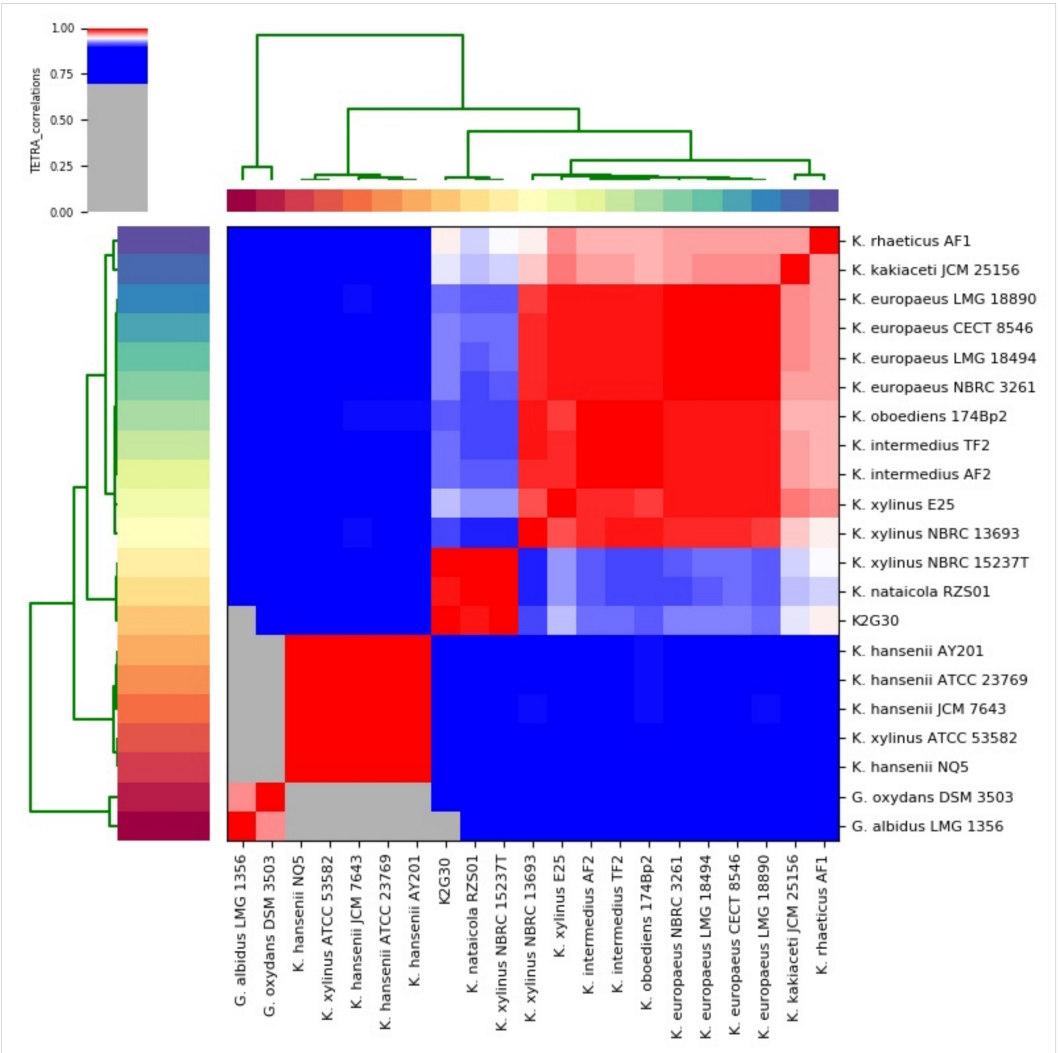

FIGURE 4 | TETRA heatmap of 21 *Komagataeibacter* genomes sequences (derived from **Supplementary Table S2**). TETRA values are represented in the central bi-color gradient heatmap (red gradients ≥ 96%; white = 95%; blue gradients ≤ 94%).

The rRNA genes of two species of *Gluconobacter* genus (*G. albidus* LMG 1356^T and *G. oxydans* DSM 3503^T), were included in the dataset and used as outgroup. The ML tree (**Figure 3**) displays that *Komagataeibacter* species clustered in three major groups, the *K. hansenii,* the *K. europaeus* and the *K. intermedius* species. *K. medellinensis* and *K. rhaeticus* were closed in a single clade. K2G30 was clustered with the type strain (*K. xylinus* NBRC 15237^T) and *K. nataicola* RZS01, with a bootstrap percentage of 78%. The information gained from the phylogenetic analysis provides suitable depiction of the evolutionary position of K2G30 strain, but does not translate directly into the overall similarity of the genomes. Here, we used two approaches of digital DNA-DNA hybridization, ANIb and TETRA, that are considered two of the traditional "gold standard" for circumscribing bacterial species (Teeling et al., 2004; Konstantinidis and Tiedje, 2005). The required threshold to ascribe one species using ANIb (94% of genome sequence similarity) and TETRA (0.997) were previously defined (Richter and Rosselló-Móra, 2009).

We used the genome sequences of the species considered for ML tree reconstruction to produce the ANIb and TETRA distance matrices. As shown in **Supplementary Table S1**, the genome sequence similarity from the comparison of K2G30 and *K. xylinus* NBRC 15237^T was 93.38%, just below the minimum threshold required to define a bacterial species. ANIb heatmap (**Supplementary Figure S1**) confirms the clustering order of the phylogenetic tree (**Figure 3**), showing the same three clades. The data from TETRA heatmap (**Figure 4**) were congruent with ANIb analysis and the ML tree, showing the same three large clades. The TETRA correlation value between K2G30 and *K. xylinus* NBRC 15237^T was 0.9973, while the correlation value for the pairwise K2G30 and *K. nataicola* RSZ01 is 0.9952, lower than the threshold.

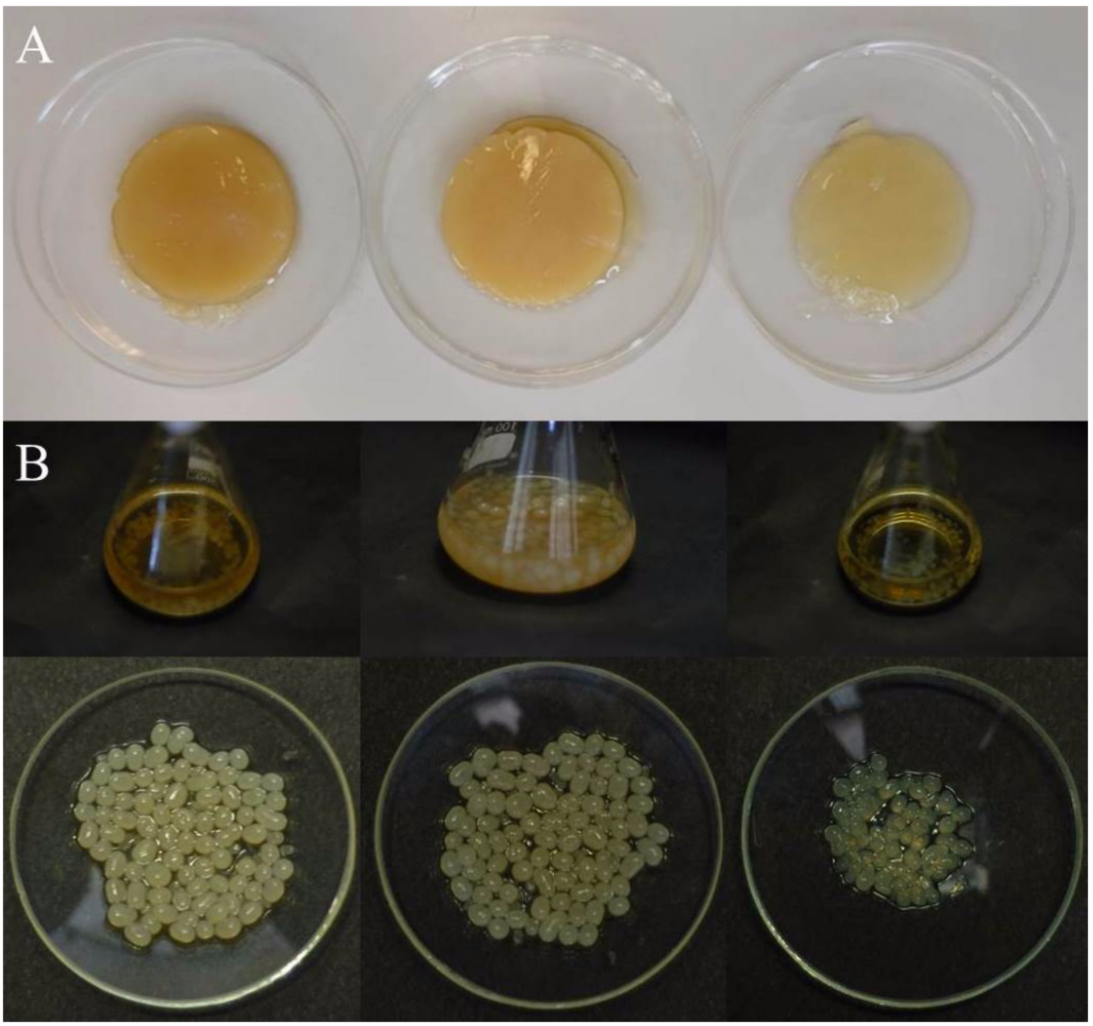

FIGURE 5 | BC produced by K2G30 in static **(A)** and agitated **(B)** conditions. From left to right: BC produced in mannitol, glucose, and xylitol, respectively.

It is clear from literature and from **Figures 3**, **4** and **Supplementary Figure S1** of this study, that several strains attributed to the species *K. xylinus* do not associate with the type strain of the species (NBRC 15237^T), but they cluster with other species of the genus (Lisdiyanti et al., 2006). Due to these misinterpretations, a critical revision of *K xylinus* strains published and available in public culture collections is advisable.

Interestingly, in our analysis in which only sequenced genomes of *Komagataeibacter* are represented, just the strain we characterized (K2G30), clusters with *K. xylinus* type strain NBRC 15237^T and could be confidently attributed to this species. For all those strains reported in literature as high BC producers that have been attributed *K. xylinus* species in the absence of genomic data, a critical re-examination and an identification using genomic data is needed. This would allow to verify if high BC production is indeed a specificity of *K. xylinus* or also of other species within the genus, which in former years and in absence of appropriate resolution tools, have been attributed to *K. xylinus,* but are indeed representatives of different species.

Glucose, Mannitol, and Xylitol as Carbon Sources for Producing Bacterial Cellulose

With the aim to select the best cultivation system for obtaining BC pellicles from the strain K2G30, cultures were grown on GY broth, in both static and agitated conditions, and on GY agar medium. Well compacted BC membranes were obtained in static cultivation, whereas spheres of different size were produced in agitated cultivation system (**Figures 5A,B**). When we cultivated K2G30 on GY agar medium, we did not observe single colonies but a soft layer on the surface of the plates. An increase of BC yield has been obtained by different strategies including the use of double carbon sources e.g., glucose and ethanol, modified sugars and polyols (Oikawa et al., 1995a,b; Canilha et al., 2008; Singhsa et al., 2018).

TABLE 3 | Bacterial cellulose production and pH values (S/V of vessel 0.40 cm^{-1})[1].

	Glucose (5% w/v)		Mannitol (1.5 w/v)		Xylitol (5% w/v)	
Time (days)	**BC (g/L)**	**pH**	**BC (g/L)**	**pH**	**BC (g/L)**	**pH**
0	0.0	4.46 ± 0.05	0	5.75 ± 0.03	0	5.65 ± 0.39
3	1.149 ± 0.001	3.81 ± 0.09	0.188 ± 0.003	5.71 ± 0.09	0.048 ± 0.001	5.57 ± 0.02
6	3.805 ± 0.001	3.24 ± 0.03	2.368 ± 0.001	5.39 ± 0.04	0.527 ± 0.001	5.51 ± 0.03
9	6.167 ± 0.024	3.10 ± 0.02	8.766 ± 0.043	5.58 ± 0.83	1.356 ± 0.050	5.23 ± 0.08

[1]*The data are averages based on three trials, reporting the standard error values.*

TABLE 4 | Gluconic acid (GlcA) production and pH values during BC production in glucose, mannitol and xylitol media (S/V of vessel 0.40 cm^{-1})[1].

	Glucose		Mannitol		Xylitol	
Time (days)	**GlcA (g/L)**	**pH**	**GlcA (g/L)**	**pH**	**GlcA (g/L)**	**pH**
0	1.353 ± 0.741	4.46 ± 0.05	0.927 ± 0.017	5.75 ± 0.03	0.530 ± 0.044	5.65 ± 0.39
3	12.887 ± 0.027	3.81 ± 0.09	1.121 ± 0.021	5.71 ± 0.09	1.182 ± 0.029	5.57 ± 0.02
6	19.580 ± 0.091	3.24 ± 0.03	1.109 ± 0.033	5.39 ± 0.04	1.144 ± 0.028	5.51 ± 0.03
9	29.779 ± 0.046	3.10 ± 0.02	1.243 ± 0.014	5.58 ± 0.83	1.193 ± 0.060	5.23 ± 0.08

[1]*The data are averages based on three trials, reporting the standard error values.*

In this study, BC production by K2G30 was tested using two polyols (mannitol and xylitol), as alternative carbon sources to glucose, by static cultivation system at 28°C for a total of 9 days, according to Oikawa et al., 1995b and Singhsa et al., 2018, respectively. Assays were performed in vessels (500 mL beakers) having a S/V ratio of 0.40 cm^{-1}. BC pellicles were visible on the surface of all performed trials within 2 days of cultivation. However, different BC yields were reached in glucose broth (reference medium), mannitol and xylitol broths. The highest BC yield was observed in mannitol (1.5% (w/v) initial mannitol content) whereas xylitol broth (5% (w/v) initial xylitol content) was less performing in BC synthesis (**Table 3**). Within strains of the species *K. xylinus* the ability to produce BC from xylitol could be a variable traits, as previously observed by Singhsa et al., 2018, which tested five strains that produced low or moderate amount of BC, except for one strain that produced BC in higher amount (more than 1.0 g/L during 7 days cultivation).

In glucose broth (5% w/v of initial glucose content), the BC yield obtained was considerable, but less compared to the amount produced in mannitol broth (**Table 3**). From these results, it was shown that using mannitol the BC yield was increased of 43% than using glucose and that xylitol was not a preferred carbon sources of K2G30 for producing BC.

In the BC production process, the accumulation of organic acids in the culture broth, such gluconic acid and acetic acid, due to the oxidative metabolism of AAB, induces pH decreases far below the optimal value for BC production, that is estimated higher than 4 (Gullo and Giudici, 2008; Giudici et al., 2016). As previously reported the pH reduction at values below 4 is suboptimal for BC synthesis (Jonas and Farah, 1998; Gullo et al., 2017). In this study, a negligible production of gluconic acid, which induced a limited pH reduction, was observed both in mannitol and xylitol broths; whereas in glucose broth, BC formation was followed by glucose oxidation to gluconic acid and a pH decrease below 4 (**Table 4**).

As in mannitol, also in xylitol broth the gluconic acid production was very low. This can be explained considering pentose and glucuronate metabolism (pathway ID KO00040), in which through different steps xylitol is converted into UDP-glucose, the precursor of BC.

Considering the metabolic pathways of mannitol and xylitol based on Kegg pathways maps, D-mannitol was converted to D-fructose and used to produce BC via galactose metabolism (pathway ID KO00051-KO00052). The candidate enzyme for this conversion seems to be D-mannitol dehydrogenase (EC 1.1.1.67). Differently from the membrane-bound enzyme polyol dehydrogenase (EC 1.1.5.2) that has low substrate specificity, D-mannitol dehydrogenase is a soluble enzyme characterized by high specificity for its substrate (Oikawa et al., 1997).

Contrary to D-mannitol, the production of BC from xylitol is a more complex pathway, involving several steps, which result less advantageous energetically for the cell.

CONCLUSION

In the present study, K2G30 genome was sequenced and annotated in order to describe the key gene sets involved in BC synthesis. From genome analysis, four copies of the enzymatic core of CS and three copies of *bcs* operons, were retrieved, explaining the high BC yield obtained from K2G30. From phylogenetic analysis, also the need of a re-examination within the *Komagataeibacter* genus was emphasized.

K2G30 is able to grow and to produce BC using different carbon sources, this is an additional attribute that highlights the versatility of the strain.

The choice of the carbon sources is one of the most important point in BC production, particularly for the obtainable yields.

Effective BC production from glucose is questionable, due to the oxidative metabolism leading to gluconic acid production and concomitant pH decrease. Our evidences confirm the limit of using glucose as a carbon source and the suitability of other carbon sources, like mannitol, by which gluconic acid production is negligible. Considering that for industrial BC production, raw material remains a weighty production cost, the evaluation of waste feedstocks is a challenge. Since mannitol is found in a number of vegetal wastes, their use in producing BC could be promising. We therefore proposed a strategy that integrate information deriving from technological and genomic data as a platform for selecting strains and for optimizing bioprocessing for BC large-scale production.

AUTHOR CONTRIBUTIONS

MG designed the research, supervised the work and wrote the manuscript. SL performed laboratory experiments, bioinformatic analysis, and participated in writing the manuscript. GP drafted the work and critically revised the manuscript. SD reviewed and edited the manuscript. PG contributed to the interpretation of data and critically revised the manuscript. All the authors read and approved 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.2019.00058/full#supplementary-material

FIGURE S1 | ANIb heatmap of 21 *Komagataeibacter* genomes sequences (derived from **Supplementary Table S1**). ANIb values are represented in the central bi-color gradient heatmap (red gradients ≥ 96%; white = 95%; blue gradients ≤ 94%).

TABLE S1 | ANIb pairwise similarity values for all *Komagataeibacter* genome sequences (n = 21).

TABLE S2 | TETRA pairwise similarity values for all *Komagataeibacter* genome sequences (n = 21).

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Patè Olive Cake: Possible Exploitation of a By-Product for Food Applications

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Patè Olive Cake (POC) is a new by-product derived from recently introduced new decanters in the olive oil production process. POC, is essentially composed of water, olive pulp and olive skin, and is rich in several valuable bioactive compounds. Moreover, it still contains about 8–12% residual olive oil. We characterized the main bioactive compounds in POC from black olives (cv. *Leccino* and *Cellina di Nardò*) and also verified the biotechnological aptitude of selected yeast and lactic acid bacteria from different sources, in transforming POC into a new fermented product. The strategy of sequential inoculum of *Saccharomyces cerevisiae* and *Leuconostoc mesenteroides* was successful in driving the fermentation process. In fermented POC total levels of phenols were slightly reduced when compared with a non-fermented sample nevertheless the content of the antioxidant hydroxytyrosol showed increased results. The total levels of triterpenic acids, carotenoids, and tocochromanols results were almost unchanged among the samples. Sensory notes were significantly improved after fermentation due to the increase of superior alcohols, esters, and acids. The results reported indicate a possible valorisation of this by-product for the preparation of food products enriched in valuable healthy compounds.

Keywords: bioactive compounds, fermentation, functional product, patè olive cake, starter

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INTRODUCTION

The olive oil industry has a great global importance and economic impact especially for Mediterranean countries, which contribute to about 92% of the world's olive oil production (about 3 million tons seasons 2017–2018). EU contributes 66% to global olive oil production and Spain, Italy and Greece are the main EU producers contributing to 38, 13, and 10%, respectively (1). The high quantity of olives processed by the milling industry results also in the production of a large amount of various by-products that must be properly managed to limit serious environmental impact. For these reasons at the present time these by-products represent a relevant cost for the milling industry. In particular the two-phase milling process produces a large amount of olive pomace also called olive cake that contains olive pulp, skin, stone, and water and is known to be a phytotoxic and environmental pollutant. Nevertheless, it is also known that olive pomace is a rich source of valuable compounds that can be recovered (2, 3). Olive drupes are indeed very rich in phenolic compounds although most of these compounds (about 98%) are lost in the olive mill by-products (4–6).

The health properties as well as the biological activities of these compounds, particularly polyphenols have been highlighted in several studies (7–9). Many reports are available indicating the protective effects of polyphenols toward atherosclerosis, diabetes, obesity, and many other chronic diseases (10, 11). Moreover, the European Food Safety Authority stated *"Olive oil polyphenols contribute to the protection of blood lipids from oxidative stress"* (12).

Due to the presence of high valuable compounds, olive mill by-products have attracted a great deal of attention for long time and many attempts have been made to exploit them in various industrial sectors including the feed and food industries (13, 14). Phenolic compounds, pectic polysaccharides and fibers of lignocellulose from olive by-products have been proposed for potential applications as a source of oligo-, di-, and monosaccharides, as antioxidants, in the development of gelling and stabilizing agents, food packaging and preservation and food fortification.

In particular, olive pomace extracts were added to soy oil for fried potatoes enrichment of several bioactive compounds (15, 16) and for the enrichment of nutritional traits of fermented milk (17).

However, up to now, real successful and commercial production has been very limited. One of the main drawbacks in the use of olive pomace for food and feed applications is the presence of lignin (resulting from crushed stones) that contributes to the poor digestibility. De-stoned olive cake added into a concentrate-based diet for lambs resulted in an improvement of the oxidative stability and the nutritional quality of meat by increasing vitamin E content in muscle (18).

Recently a new technology for olive oil extraction has been proposed that adopts an innovative two-phase decanter (Leopard, Pieralisi, Jesi, Italy). This decanter, besides producing a dehydrated husk, also produces a novel by-product named "patè" or "patè olive cake" (POC) consisting of a semisolid de-stoned olive cake that includes olive pulp, olive skin, olive mill wastewater while it is very poor in lignin. Due to the presence of residual olive oil (8–12%) POC contains fatty acids (palmitic acid, oleic acid, and polyunsaturated fatty acids). Moreover, it contains triterpenic acids (mainly oleanolic acid and maslinic acid) and several phenolics including hydroxytyrosol, tyrosol, secoiridoids derivatives, verbascoside, and many phenolic acids (19, 20). POC also contains carotenoids and lignans. Consequently POC has been suggested for various food and feed applications. Recently Padalino et al. (20) developed a spaghetti enriched with 10% POC which resulted in an increased total of polyphenol content and also showed a PUFA/SFA ratio higher than the control. Moreover, Cecchi et al. (21) reported anti-aging effects of POC on human fibroblast cells, while the preparation of olive pomace-based polyphenol rich extracts encapsulated in cyclodextrins to be used as food antioxidants has been successfully tested by Cepo et al. (22).

Starter driven fermentation has been extensively studied for table olives (23–28), their derived products (29) and for the bioremediation of olive mill wastewaters (30–36). At present, no applications of this approach have been developed for POC.

In this paper, for the first time, a production method of fermented POC, obtained from *Cellina di Nardò* and *Leccino* olives, using the Leopard decanter during the production of extra virgin olive oil, was studied. For this purpose, key steps for the selection of microorganisms as possible starters for POC fermentation were determined. Process parameters and the protocol of a sequential inoculum approach using, firstly, a selected strain of *Saccharomyces cerevisiae* and then a selected strain of *Leuconostoc mesenteroides* were adopted. Chemical and microbiological characterizations of the fermented products were carried out.

MATERIALS AND METHODS

Patè Olive Cake Samples (POC)

Olives (cv *Cellina di Nardò* and *Leccino)* were harvested from organic orchards in Salento Area (South Apulia Region, Italy) during seasons 2014–2015, 2015–2016, and 2016–2017. Five hundred kilo gram olives for each cultivar were manually harvested at a maturity index of about 3–4 (skin turning black or fully black). Olives were immediately transported to the mill, washed twice and selected by caliber and color. Olive were then milled using a Multi-Phase Decanter technology (Leopard, Pieralisi, Jesi, Italy). Aliquots of Patè Olive cake (POC) were sterilized at 121°C for 4 min to reduce/block possible spontaneous fermentations without affecting matrix composition.

Yeast strains used in this study were: a wine yeast *Saccharomyces cerevisiae* strain ENARTIS FERM SC (ESSECO, Trecate, Italy) (WSC), two brew yeasts [Australian bitter and pale ale malt (YPA), Coopers, Australia] (YAB), a commercial baker's yeast (BY), *Candida boidinii* ISPA-LE-A5y (A5y) isolated from olive mill wastewaters, *Saccharomyces cerevisiae* ISPA-LE-KI 30-1 (KI-30-1) isolated from fermented table olives, *S. cerevisiae* ISPA-LE-LI 60-17 (LI-60-17) from fermented table olives. Lactic acid bacteria strains used in this study were: *Lactobacillus plantarum* ISPA-LE-C-11 (C-11), *L. plantarum* ISPA-LE-C-34 (C-34), *Leuconostoc mesenteroides* ISPA-LE-K-1 (K-1), *L. mesenteroides* ISPA-LE-B-T3-35 (BC-T3-35). All these bacterial strains were previously isolated from fermented table olives.

Microbiological Analyses

Microorganisms present in fermented POC samples were analyzed by serial dilutions with 0.1% (w/v) peptone water. After dilution, samples were applied to agar plates containing: Man, Rogosa, and Sharpe Agar (MRS, LABM, UK) for LAB isolation and Plate count Agar (PCA, LABM, UK) for total bacterial count in presence of 0.05 g/L of nystatin and incubated at 30°C for 48–72 h; Violet Red Bile Glucose Agar (VRBGA, LABM, UK) for *Enterobacteriaceae* identification (37°C for 18–24 h); Baird Parker Agar Base (Oxoid) for the enumeration of coagulase positive Staphylococci incubated at 37°C for 24–48 h; Violet Red Bile Agar (VRBA, Oxoid) for the detection and enumeration of coli-aerogenes bacteria incubated at 37°C for 24–48 h; Mannitol Salt Agar (MSA, Oxoid) for the isolation of pathogenic Staphylococci incubated at 37°C for 18–72 h;

Sulphite-Polymyxin-Sulphadiazine Agar (SPS, Oxoid) for the detection of *Clostridium perfringens* incubated at 35–37°C for 18–48 h under anaerobic conditions; Sabouraud Dextrose Agar (LABM, UK) (yeasts identification) in presence of 0.05 g/L of kanamycin and 0.1 g/L of ampicillin by incubation for 2–4 days at 25°C.

LAB and Yeast Selection

As a first step of this work, the highest amount of lyophilized POC in growth medium that can be tolerated by yeast and LAB strains without affecting their growth was determined. To this scope, YPD and MRS media were supplemented with different amounts (0, 10, 25, 50, 75, 80, 90, 100% w/v) of lyophilized POC. The pH of the media was adjusted to 4–4.5 before thermal treatment of 10 min at 110°C. Yeasts and LAB were grown on YPD and MRS broth, respectively and adjusted to 10^7 CFU mL^{-1} final concentration (corresponding 600 nm of ≈ 1.0 optical density). Fifteen microliters of each yeast and LAB inoculum were spotted onto YPD and MRS agar media containing the different amounts of POC. The agar plates were incubated for 5–7 days at 28°C. Each assay was carried out in triplicate. Each experiment was performed twice.

In the second step, laboratory-scale fermentations were carried out for the selection of microbial starters able to ferment POC. Yeasts and LAB strains were firstly grown as a pre-adaptation period in ½ strength YPD (yeast extract 0.5% w/v, peptone 1% w/v, glucose 1% w/v) and half strength MRS (27.6 g L^{-1}, VWR, Belgium), respectively, containing 25% w/v POC and subsequently inoculated at a concentration of 5×10^6-10^7 CFU mL^{-1} in a liquid medium containing half strength YPD for yeasts, and a liquid medium containing half strength MRS for LAB isolates, both containing 50% w/v OPC. Fermentations were performed using 500 ml glass bottles with 250 ml of YPD-POC and MRS-POC at 28°C for 60 days. The fermentative activities of each yeast and LAB strains were followed by monitoring consumption of main sugars, evolution of most important organic acids, phenols and volatile compounds.

Pilot-Scale Fermentation

Fermentations were performed in plastic vessels (10 kg capacity). Sterilized POC samples of *Cellina di Nardò* and *Leccino* were diluted 1:1 with distilled water. After addition of 0.5% (w/v) yeast extract, 0.5% (w/v) peptone, 0.5% (w/v) glucose, vessels were maintained at ambient temperature (18–22°C).

Starter cultures were inoculated by a sequential inoculation strategy: *Cellina di Nardò* and *Leccino* POC samples were first inoculated with *Saccharomyces cerevisiae* ISPA-LE-KI 30-1 and then (after 30 days) with *Leuconostoc mesenteroides* ISPA-LE-B-T3-35.

About 10^7 CFU/ml yeast and LAB starter strains were used to inoculate, OPC samples. Yeasts starter firstly drove the fermentation process. Throughout the fermentation pH, sugars consumption and organic acids production were monitored. At the end of the yeast fermentations LAB were added. This time point was determined by the appearance of specific compounds (higher alcohols, terpenes, etc.), the whole process was considered terminated when compounds such as esters and acetate esters were detected (23, 24).

Detection and Quantification of Sugars, Organic Acids, and Alcohols

The soluble sugars were extracted from dried olive paste samples according to Eris et al. (37) with some adjustments. Two mililiter of ethanol: water (80:20 v/v) were added to POC powders (0.05 g). The mix, after stirring, was incubated at 85°C for 1 h in a water bath. The ethanolic phases were collected and evaporated until dry at 55°C. Organic acids were determined according to Ergönül and Nergiz (38), using a mixture of water: methanol solution (75:25 vol/vol) as solvent. The supernatant was recovered by centrifugation at 5,000 × g for 10 min. This extraction was repeated three times and the collected supernatants were evaporated until dry.

Polyphenols Extraction and Analysis

The polyphenol extraction was carried out as reported by Servili et al. (39) with minor changes. Five grams of each sample were homogenized with 50 mL of methanol (80%, v/v) containing 20 mg/L of butylated hydroxytoluene. The methanol was evaporated and the aqueous extract was used for solid-phase extraction (SPE) of phenols. The SPE procedure was performed by loading 1 mL of the aqueous extract into a 1,000 mg Bond Elut Jr-C18 cartridge (Agilent Technologies, USA), using 50 mL of methanol as the eluting solvent. The solvent was evaporated under vacuum at 30°C, the residue was dissolved in 1 mL of methanol. Quali-quantitative analysis of polyphenols was carried out as described by Servili et al. (39) using an HPLC system 1100 Series (Agilent Technologies, California, USA) equipped with a A C18 Spherisorb column ODS-1 250 x 4.6 mm with a particle size of 5 μm (Waters, Milford, MA, USA). Lignans [(+)-pinoresinol] were detected by using the FLD at an excitation wavelength of 280 nm and emission at 339 nm, whereas the other compounds were detected by using the DAD with a wavelength of 278 nm and 360 nm for rutin. The tyrosol (*p*-HPEA), vanillic acid, *p*-coumaric acid and rutin were purchased from Fluka (Milan, Italy), while the hydroxytyrosol (3,4-DHPEA) was obtained from Cabru s.a.s. (Arcore, Milan, Italy) (Arcore, Milan, Italy). Verbascoside was purchased from Extrasynthese (France). Oleacein, oleocanthal and (+)-pinoresinol were obtained from PhytoLab GmbH & Co. (Germany). Hydroxytyrosol acetate and isoverbascoside were quantified using the response factors of hydroxytyrosol and verbascoside, respectively.

Volatile Compound Extraction From POC

Volatile compounds were extracted according to Bleve et al. (24) HS-SPME analyses were carried out using a gas chromatograph (Agilent 6890N) coupled to a mass spectrometer (5973, Agilent Technologies, USA). The Separation, identification and quantification of compounds were performed according to Bleve et al. (24).

Triterpenic Acids Extraction and Analysis

Triplicate aliquots (1 g) of each sample were extracted with 4 mL of methanol/ethanol (1:1, v /v) in a Labsonic LBS1-10

ultrasonic bath [Falc Instruments, Treviglio (Bg), Italy] at room temperature for 1 min and centrifuged at 4,000 × g for 10 min, this procedure was repeated for six times (40). The extracts were combined and evaporated to dryness, and the residues were re-dissolved in 1 mL of methanol. Triterpenic acids were analyzed according to Durante et al. (40) using an 1,100 Series HPLC system (Agilent Technologies, California, USA) equipped with a Luna column (5 μm, 250 × 4.6 mm) (Phenomenex, Torrance, CA, USA). Samples were filtered before analysis through a 0.45 μm syringe filter (Millipore Corporation, Billerica, MA, USA).

Isoprenoids Extraction and Analysis

Isoprenoids (tocochromanols and carotenoids) were extracted essentially as reported by Padalino et al. (20). Triplicate aliquots (1 g) of each sample was incubated with 5 mL acetone containing 0.05% (w/v) butylated hydroxytoluene (BHT), 2 mL ethanol (95% v/v), 1 mL sodium chloride (1% w/v) and 2 mL potassium hydroxide (60% w/v) at 60°C for 30 min. After cooling, 15 mL sodium chloride (1% w/v) was added. Samples were then extracted twice with 15 mL n-hexane/ethyl acetate (9:1 v/v), and the upper phases were collected, dried under nitrogen flux, dissolved in 1 mL ethyl acetate, filtered through a 0.45 μm syringe filter (Millipore Corporation, Billerica, MA, USA) and assayed by HPLC according to Durante et al. (41). Absorbance was recorded by DAD at 474 nm and 290 nm for carotenoids and tocochromanols, respectively. Peaks were identified and quantified by comparison to authentic standards.

Lipid Extraction

Triplicate aliquots (1 g) of each sample were mixed with 5 mL of n-hexane and stirred with a mechanical stirrer (300 rpm) at 4°C for 16 h. Samples were centrifuged at 6,000 × g for 5 min. The organic phase was evaporated until dry and stored at −20°C until analysis.

Fatty Acids Analysis

Fatty acids were derivatized according to Durante et al. (42). Dried extracts were solubilized with 3 mL KOH methanolic solution (0.5 M). Samples were incubated at 100°C for 5 min. After cooling, 2 mL boron trifluoride in methanol (12% w/v) were added, and samples were incubated for 30 min at 100°C in a water bath and then cooled in an ice bath before the addition of 1 mL of *n*-hexane and 1 mL sodium chloride (0.6% w/v). Samples were shaken (30 s) on a vortex-stirring for 5 min and centrifuged at 600 x g for 10 min. The organic upper phase was collected and 3 μL aliquots were directly injected into GC-MS as described by Durante et al. (42) using an Agilent 5977E GC/MS system equipped with a DB-WAX column (60 m, 0.25 mm i.d., 0.25 mm film thickness, Agilent).

Statistical Analysis

The data are shown as mean values of replicate measurements ($n = 3$) with standard deviation. Student's *t*-test was applied to the polyphenols, triterpenic acids, isoprenoids, and fatty acids and a one-way analysis of variance (ANOVA) and the Tukey HSD *post hoc* test were applied to the volatile compounds to establish significant differences between means ($p < 0.05$). All statistical comparisons were performed using SigmaStat version 11.0 software (Systat Software Inc., Chicago, IL).

Principal component analysis (PCA) was used to compare possible correlated variables, such as volatile and phenolic compound content, of the POC samples during fermentations. All statistical analyses were carried out using the STATISTICA 7.0 software (StatSoft software package, Tulsa, OK, USA).

RESULTS

Selection of Yeasts and LAB Candidates as Starters

In the first step selection, all yeast and LAB isolates, inoculated in YPD and MRS agar media added with different amounts (0, 10, 25, 50, 75, 80, 90, 100% w/v) of lyophilized and sterilized POC, showed the ability to grow in a way similar to the controls when a concentration ≤ 50% (w/v) POC was used (data not shown).

In order to identify yeast and LAB strains as candidate starter for POC fermentation, lab-scale fermentations in media containing 50% (w/v) POC were set up. Metabolic activities of candidate starters were followed by monitoring some key chemical parameters, such as sugars (glucose and fructose), alcohols (ethanol), phenols, organic acids, and volatiles compounds associated with the microbial metabolic activities. All yeast and LAB strains were able to almost completely consume sugars. At the end of fermentation (60–days), the glucose initial content (16.51 ± 0.41 mg/g), was greatly reduced (up to 0.6–5.7% of the initial content) in POC samples inoculated with yeast as well as in POC samples inoculated with LAB isolates (up to 0–12% of the initial content). Concerning fructose, the initial concentration, corresponding to 7.21 ± 0.41 mg/g in both media, was reduced to 0–7.2% initial content in samples containing yeast isolates and to 0–9.8% in samples inoculated with LAB isolates. No substantial differences were observed among samples fermented by yeast and LAB isolates in relation to citric, tartaric, malic, lactic, and acetic acids.

The consumption of sugars and the corresponding increase of organic acid levels revealed the progress of fermentation in all POC samples inoculated with starters.

It is worth noting that, after fermentation, the levels of the important antioxidant compound hydroxytyrosol increased in all POC samples.

Analysis of Volatile Compounds in POC Fermented by Yeasts and LAB

In **Figures 1**, **2** concentrations (expressed in %) of volatile compounds identified in POC fermented by different yeast and LAB strains, respectively, were reported. The chemical classes of volatiles identified, were: alcohols, esters, terpenes, hydrocarbons, aldehydes, volatile acids, and volatile phenols.

Fermentation process, performed by different yeast and LAB strains, produced significant differences in terms of volatile profiles in POC samples.

Among POC samples fermented by yeasts (**Figure 1** and **Table S1**), as expected, alcohols represented the most

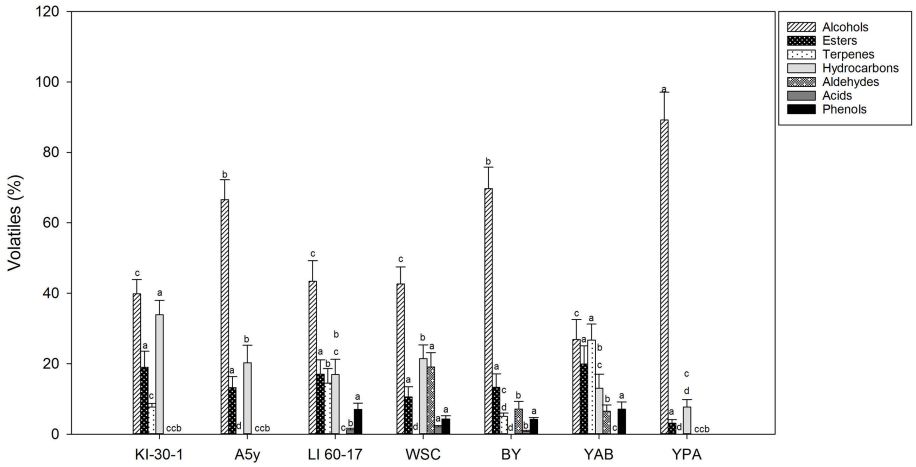

FIGURE 1 | Volatile compound classes associated to YPD-POC fermented by different yeast isolates. Different letters indicate significant differences among stage of fermentation in the same volatile classes ($p < 0.05$).

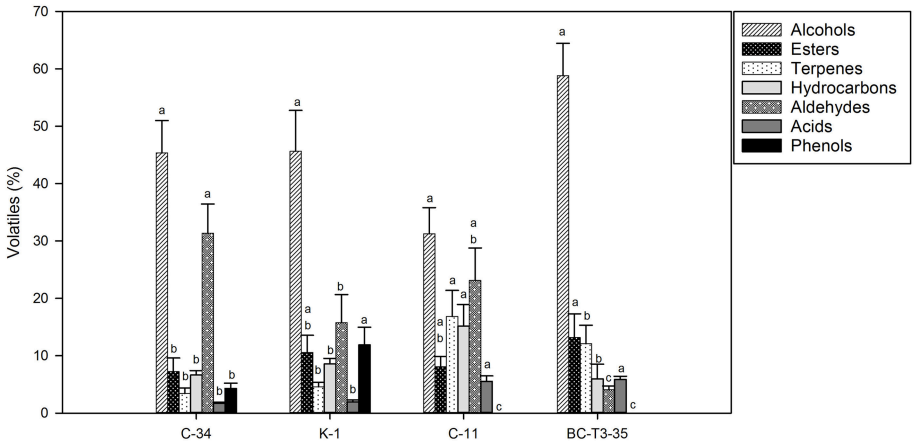

FIGURE 2 | Volatile compound classes associated with MRS-POC fermented by different LAB isolates. Different letters indicate significant variations depending on the fermentation stage in the same volatile classes ($p < 0.05$).

representative class of the volatile compounds. The highest value was detected in POC samples inoculated with YPA (89.21%) followed by BY (69.71%), A5y (66.54%), LI-60-17 (43.37%), WSC (42.60%), KI-30-1 (39.83%), and YAB (26.84%). In POC samples fermented by LAB (**Figure 2** and **Table S2**), the highest concentration of alcohols was observed in sample inoculated with BC-T3-35 (58.81%) followed by K-1 (46.35%), C-34 (45.34%), and C-11 (31.27%).

Isoamyl alcohols, benzyl-alcohol and phenyl-ethanol were the most abundant alcohols in almost all samples, with higher concentrations observed in POC samples fermented by yeasts. Phenyl-ethanol (2-PE) ranged from 6.04 to 27.47% (YAB and YPA, respectively) (**Table S1**). Lower values were observed in POC samples fermented by LAB (**Table S2**). In these samples the percentage of 2-PE ranged from 7.64% in C-11 to 15.06% in BC-T3-35. Isoamyl-alcohol was present in higher values in A5y (32.96%), and YPA (30.02%) while lower values were revealed in samples fermented by LAB (3.65–16.62%) (**Table S2**). 1-hexanol and 3-hexen-1-ol were detected in a few samples. In particular, 1-hexanol was identified in yeasts in only WSC and A5y strains, while 3-hexen-1-ol was found in BY, YPA and A5y strains. Alcohols have an important role in the production of esters, responsible for fruity notes. Among esters isoamyl acetate was the most representative in yeast-treated samples whereas ethyl octanoate was present in all LAB-treated ones. We also observed the presence of the sesquiterpene, farnesene, and furaneol. In POC fermented by yeasts, the total content of terpenes ranged from 5% in BY and 26.68% in YAB (**Figure 1** and **Table S1**) while in bacteria fermented samples, we observed values ranging from 3.41% (C-34) to 16.84% (C-11) (**Figure 2** and **Table S2**).

Principal component analysis was applied to main phenolic compounds and to volatile classes produced at the end of

the fermentations. In the bi-plot concerning fermented POC with different yeast strains, the total variance of the two main components was 69.68% (**Figure 3A**). PC2 separates terpens, hydroxycarbons and esters from phenols, alcohols, aldeydes and acids, while PC1 separates tyrosol and OH-tyrosol from the other components. The sample treated with the KI-30-1and YAB yeast isolates were located in the portion of the plane characterized by the presence of esters, terpenes and hydrocarbons (styrene). The sample inoculated with WSC isolate was associated with a greater production of acids (acetic acid) and aldehydes. The other samples treated with BY and LI-60-17 grouped together and volatile phenols and alcohols mainly characterize them. YPA and A5Y grouped in the plane mainly associated with tyrosol and hydroxytyrosol.

Samples treated with LAB showed a total variance of 83.85%, which is explained by two main components PC1 and PC2 (**Figure 3B**). The four samples were scattered in three main groups in the plane. The first one was represented by the C-34-inoculated sample which was associated with the presence of higher levels of tyrosol and aldehydes in the final product; the second one (C-11) was mainly characterized by a higher production of volatile acids, hydroxytyrosol and hydrocarbons, while BC-T3-35 and K-1 were positioned in the portion of the plane characterized by terpenes, phenols, alcohols and esters.

On the basis of the above reported results, yeast KI-30-1 and LAB BC-T3-35 isolates were selected as starters for pilot scale fermentations of POC.

Pilot-Scale POC Fermentations

Leccino and *Cellina di Nardò* sterilized POC were inoculated with *Saccharomyces cerevisiae* ISPA-LE-KI 30-1 (KI-30-1) and *Leuconostoc mesenteroides* ISPA-LE-B-T3-35 (BC-T3-35). In both starter-driven fermentations, at the end of the process, yeasts were revealed throughout the process and their count corresponded to 1–3 × 10^5 CFU/ml. *Enterobacteriaceae*,

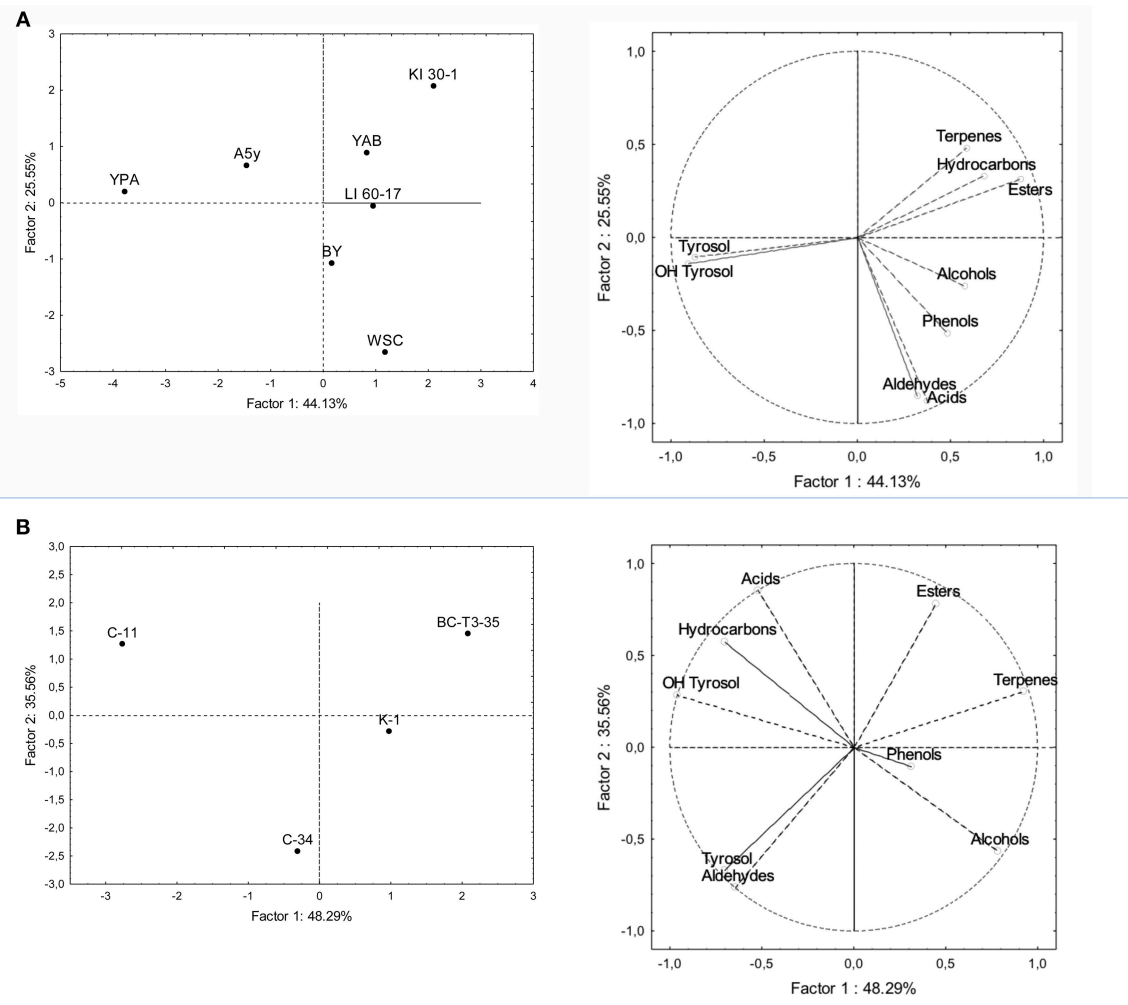

FIGURE 3 | PCA of volatile compounds associated with **(A)** POC inoculated with yeast isolates and **(B)** POC inoculated with LAB isolates. PCA variables were the data obtained from the concentration and presence of volatile compounds and of main phenols and the different microbial isolates. The figure displays the sample scores and variable loadings in the planes formed by PC1–PC2.

Staphylococci, coliforms, *Clostridia* was undetectable in starter-driven fermentations during the entire process (data not shown). After 30 days of yeast fermentation LAB isolate was inoculated and at the end of the process (50th day) LAB concentration resulted 5–7 $\times$ 10^6 CFU/ml. pH values remained constant (4.8–4.9) within the first 30 days of fermentation and, after the 50th, they resulted 4.14 for POC from *Cellina di Nardò* and 4.21 for POC from *Leccino*. Glucose and fructose were almost totally consumed in both *Cellina di Nardò* and *Leccino* fermented POC samples, whereas sucrose was not detected in any of the samples. At the end of fermentation of the *Cellina* di *Nardò* POC the organic acid results were: lactic acid concentration was 7.51 $\pm$ 0.31 mg/g, tartaric acid 3.05 $\pm$ 0.13 mg/g, succinic acid 3.5 $\pm$ 0.16 mg/g; in *Leccino* fermented POC lactic acid concentration was 7.00 $\pm$ 0.26 mg/g, tartaric acid 2.25 $\pm$ 0.23 mg/g, malic acid 3.16 $\pm$ 0.14 mg/g, citric acid 4.02 $\pm$ 0.20 mg/g.

Analysis of Volatile Compounds in POC Fermented by Selected Yeast and LAB Strains

Volatile compounds were analyzed in *Cellina di Nardò* and *Leccino* fermented POC, using the SPME-GC/MS technique (**Table 1**). When POC samples were fermented, firstly by yeast starter KI-30-1 and then by LAB isolate BC-T3-35, volatile profiles were significantly modified in comparison to non-fermented samples (NF). The volatile profile was characterized by high concentrations of alcohols, esters, terpenes and acids followed by minor compounds such as sulfur compounds, norisoprenoids, hydrocarbons, volatile phenols and aldehydes. Yeast metabolic activities induced a statistically increase of some volatile classes such as alcohols, esters, terpenes, hydrocarbons ($p < 0.05$). In *Cellina* di *Nardò* POC yeast fermented sample, showed a significant increase of volatile acids ($p = 0.026$) and a decrease of aldehydes ($p = 0.013$), whereas in *Leccino* POC samples treated with yeast there was a no significant change in aldehydes content and acids were not detectable. Among alcohols, 2-methyl propanol, isoamyl alcohols, and phenylethanol increased.

After fermentation by LAB isolate BC-T3-35, *Cellina* di *Nardò* POC sample revealed a statistically significant increase in esters ($p = 0.039$) and acids ($p = 0.003$) classes in comparison to the same sample fermented only by yeast, whereas aldehydes were further reduced ($p = 0.044$). In the corresponding sample of *Leccino*, LAB metabolism was responsible of a significant increase of terpens ($p = 0.004$) and of the appearance of acids (when compared to the same sample treated with yeast).

Ester production increased during fermentation reaching a total level (at the end of the process) of 26.83 μg/kg in *Leccino* and 45.39 μg/kg in *Cellina di Nardò*. In *Leccino* fermented POC, ethyl acetate, ethyl octanoate and methyl 2 furoate were the major representative species of ester class, while in *Cellina di Nardò* fermented POC, the most abundant esters were ethyl hexanoate, ethyl octanoate, ethyl decanoate, methyl 2 furoate and methyl salycilate. As far as aldehydes, benzaldehyde was the major compound in fermented *Leccino* POCs, whereas the *Cellina di Nardò* sample contained higher a concentration of furfural, non-anal and decanal. Different terpenes were identified in the fermented POC, in particular in *Cellina di Nardò*. The total amount of terpenes increased from 2.30 μg/kg to 8.86 μg/kg, while in fermented Leccino POC the total amount of terpenes increased from 1.01 to 8.04 μg/kg.

Chemical Characterization of POC Principal Nutritional Profile Constituents

Table 2 shows the chemical characterization of the principal constituents of the unfermented or fermented *Leccino* and *Cellina di Nardò* POC samples. The POC is characterized by the presence of several bioactive compounds such as polyphenols, triterpenic acids, tocochromanols, and carotenoids. The fermentation processes modified the bioactive phenols. A reduction of verbascoside and Oleacin (3,4-DHPEA-EDA) and a corresponding increase of hydroxytyrosol, mainly derived from the hydrolytic activities of lactic acid bacteria, were revealed in both the cultivar studied. Maslinic acid was higher than oleanoic acid, in all samples. Maslinic acid content was about 1.16 $\pm$ 0.05 mg/g FW, oleanoic acid ranged from 0.32 $\pm$ 0.04 mg/g FW (*Leccino*) to 0.43 $\pm$ 0.07 mg/g FW (*Cellina di Nardò*), these values were not significantly different ($p > 0.05$) when compared with the corresponding fermented POC samples.

The content of tocochromanols (tocopherols and tocotrienols) and carotenoids did not change significantly ($p > 0.05$) after the fermentation process. Among tocochromanols, α-tocopherol (α-T) and ß-tocotrienol (ß-T3) forms were detected. α-T content ranged from 21.10 $\pm$ 1.20 μg/g FW to 23.20 $\pm$ 0.65 μg/g FW. In turn, ß-T3 showed a minor tocochromanol component ranging from 1.25 $\pm$ 0.20 μg/g FW to 1.74 $\pm$ 0.11 μg/g FW).

Carotenoids were detected in small quantities, lutein was the most abundant (from 1.97 $\pm$ 0.11 μg/g FW to 2.21 $\pm$ 0.31 μg/g FW), followed by ß-carotene (from 0.55 $\pm$ 0.01 μg/g FW to 0.84 $\pm$ 0.04 μg/g FW), α-carotene (from 0.25 $\pm$ 0.05 μg/g FW to 0.35 $\pm$ 0.06 μg/g FW), 13 *cis* ß-carotene (from 0.32 $\pm$ 0.06 μg/g FW to 0.40 $\pm$ 0.07 μg/g FW) and zeaxanthin (from 0.05 $\pm$ 0.01 μg/g FW to 0.07 $\pm$ 0.01 μg/g FW).

The fatty acid profiles were characterized in both unfermented and fermented POC samples. Results showed that mono-unsaturated fatty acid (MUFA) was about 65% of total fatty acids, saturated fatty acids (SFA) was about 20% and polyunsaturated fatty acids (PUFA) were about 14%. As expected, the most abundant fatty acid was oleic acid followed by palmitic, linoleic and stearic acids. Other fatty acids were found in small quantities. In comparison with the unfermented POCs, the total SFA resulted in higher levels and PUFA showed lower levels, while the level of MUFAs remained unchanged.

The analyses of main nutritional parameters (fibers, fats content, humidity, ashes, total carbohydrates, total nitrogen, etc.) of both non-fermented and fermented POC samples were reported in **Table S3**.

DISCUSSION

POC, obtained by the two-phase centrifugal system, is thought to be a worthy by-product for further investigation. It offers a valuable resource for the formulation of new food, food

TABLE 1 | Volatile compounds associated with Cellina di Nardò and Leccino Patè Olive cake (POC) unfermented (NF), fermented by selected yeast strain KI-30-1 (FY, 30 days) and fermented by yeast (FY, KI-30-1) and LAB (BC-T3-35) selected strains (FYL, 50 days).

Volatile compounds	Cellina di Nardò POC			Leccino POC		
	NF	FY	FYL	NF	FY	FYL
	μg/Kg ± sd	μg/Kg ± sd	μg/Kg ± sd	μg/Kg ± sd	μg/Kg ± sd	μg/Kg ± sd
ESTERS						
Ethyl acetate	0.95 ± 0.24^{a}	1.58 ± 0.44^{a}	2.76 ± 0.44^{b}	0.68 ± 0.16^{a}	3.87 ± 0.76^{b}	4.56 ± 0.94^{b}
Isomyl acetate	0.55 ± 0.11^{a}	1.38 ± 0.16^{b}	2.25 ± 0.36^{c}	nd	2.67 ± 0.56 ^{a}	3.82 ± 0.44^{a}
Methyl Hexanoate	nd	nd	0.55 ± 0.11	nd	0.15 ± 0.005^{a}	0.22 ± 0.07^{a}
Ethyl hexanoate	nd	3.87 ± 0.94^{a}	5.65 ± 1.56^{a}	nd	0.56 ± 0.08^{a}	0.71 ± 0.23^{a}
Hexyl acetate	nd	2.10 ± 0.56^{a}	2.29 ± 0.43^{a}	nd	0.18 ± 0.006^{a}	0.27 ± 0.06^{b}
Methyl octanoate	nd	nd	2.90 ± 0.55	nd	1.67 ± 0.07 ^{a}	2.70 ± 0.54^{b}
Ethyl octanoate	2.10 ± 0.43^{a}	6.18 ± 1.74^{ab}	10.57 ± 2.90^{b}	1.94 ± 0.34^{a}	5.37 ± 0.76^{ab}	7.95 ± 2.33^{b}
Methyl decanoate	nd	nd	0.31 ± 0.06	nd	nd	0.34 ± 0.06
Methyl 2 furoate	nd	3.26 ± 0.85^{a}	4.32 ± 0.54^{a}	1.95 ± 0.26^{a}	3.44 ± 0.73^{ab}	4.34 ± 0.74^{b}
Ethyl decanoate	nd	2.96 ± 0.43^{a}	4.86 ± 0.65^{b}	nd	0.25 ± 0.07^{a}	0.41 ± 0.06^{b}
Methyl salycilate	nd	3.06 ± 0.76^{a}	6.13 ± 2.56^{b}	nd	2.44 ± 0.48^{a}	3.80 ± 0.84^{a}
Phenyl acetate	0.87 ± 0.13^{a}	1.89 ± 0.47^{b}	2.80 ± 0.44^{b}	nd	1.95 ± 0.11^{a}	2.27 ± 0.54^{a}
Total	4.47 ± 0.91	26.68 ± 6.35	5.39 ± 10.60	4.57 ± 0.76	22.55 ± 3.63	26.83 ± 5.91
ALCOHOLS						
2 Methylpropanol	0.71 ± 0.11^{a}	3.97 ± 0.66^{b}	4.17 ± 0.15^{b}	1.20 ± 0.07^{a}	3.77 ± 0.85^{b}	4.17 ± 0.85^{b}
Isoamylalcohols	nd	19.55 ± 4.17^{a}	28.4 ± 5.10^{a}	nd	20.67 ± 5.15^{a}	27.64 ± 4.77^{a}
Hexanol	nd	0.85 ± 0.14^{a}	1.71 ± 0.16^{a}	nd	nd	1.60 ± 0.45
3 Hexenol (Z)	2.10 ± 0.05^{b}	1.87 ± 0.35^{b}	1.12 ± 0.06^{a}	3.06 ± 0.65^{b}	2.93 ± 0.55^{b}	2.16 ± 0.46^{a}
1 Octen 3 ol	3.10 ± 0.44^{b}	1.84 ± 0.37^{a}	1.02 ± 0.06^{a}	3.78 ± 0.78^{b}	1.77 ± 0.54^{a}	1.23 ± 0.26^{a}
1 Heptanol	nd	nd	2.94 ± 0.46	nd	1.16 ± 0.05^{a}	2.99 ± 0.75^{b}
1 nonanol	nd	nd	1.77 ± 0.32	nd	nd	3.10 ± 0.55
Benzylalcohol	nd	nd	3.40 ± 0.48	nd	nd	2.94 ± 0.65
Phenylethanol	3.10 ± 0.93^{a}	17.05 ± 3.67^{b}	20.46 ± 4.06^{b}	nd	8.37 ± 2.15^{a}	10.35 ± 2.64^{a}
Total	9.01 ± 1.53	45.13 ± 9.36	64.99 ± 10.85	8.04 ± 1.50	38.67 ± 9.29	56.18 ± 11.38
ALDEHYDES						
2 Heptanone	nd	nd	0.22 ± 0.05	nd	nd	nd
2 Hexenal	3.17 ± 0.44^{b}	0.56 ± 0.15^{a}	nd	nd	nd	nd
Octanal	0.78 ± 0.25^{b}	0.74 ± 0.18^{b}	0.34 ± 0.05^{a}	0.88 ± 0.20^{b}	0.38 ± 0.12^{a}	0.25 ± 0.07^{a}
cis 2-Decenal	nd	nd	nd	0.95 ± 0.25^{b}	0.46 ± 0.08^{a}	0.34 ± 0.05^{a}
Furfural	0.44 ± 0.06^{a}	1.28 ± 0.05^{b}	1.45 ± 0.15^{b}	nd	nd	nd
2 Octanone	3.10 ± 0.45^{b}	2.67 ± 0.46^{b}	0.32 ± 0.06^{a}	nd	2.26 ± 0.43^{a}	3.62 ± 0.47^{b}
Octanal	3.20 ± 0.55^{b}	0.56 ± 0.07^{a}	0.17 ± 0.04^{a}	2.86 ± 0.95^{c}	1.55 ± 0.63^{b}	0.21 ± 0.06^{a}
Nonanal	3.86 ± 0.48^{b}	2.15 ± 0.07^{a}	1.76 ± 0.16^{a}	3.10 ± 0.76^{b}	0.34 ± 0.07^{a}	nd
Decanal	4.28 ± 1.15^{b}	2.94 ± 0.45^{b}	1.06 ± 0.04^{a}	3.76 ± 0.56^{c}	1.93 ± 0.35^{b}	0.96 ± 0.06^{a}
Benzaldehyde	4.11 ± 0.83^{c}	2.50 ± 0.36^{b}	0.81 ± 0.23^{a}	3.93 ± 0.65^{a}	7.12 ± 2.84^{b}	10.82 ± 2.65^{c}
trans 2-Decenal	3.66 ± 0.46^{c}	1.94 ± 0.28^{b}	0.86 ± 0.34^{a}	3.54 ± 0.94^{c}	2.77 ± 0.73^{b}	1.38 ± 0.04^{a}
Cinnamaldehyde	0.35 ± 0.07^{a}	0.68 ± 0.32^{b}	0.81 ± 0.27^{b}	nd	nd	1.25 ± 0.12
Total	26.95 ± 4.74	16.02 ± 2.39	7.80 ± 1.39	19.02 ± 4.31	16.81 ± 5.25	18.83 ± 3.52
TERPENES						
Citrale	0.06 ± 0.03^{a}	0.38 ± 0.06^{b}	0.59 ± 0.16^{b}	nd	nd	3.12 ± 0.55
Limonene	0.16 ± 0.05^{a}	0.40 ± 0.06^{b}	0.49 ± 0.17^{b}	0.14 ± 0.05^{a}	0.22 ± 0.05^{a}	0.30 ± 0.07^{b}
Trans-β-ocimene	0.95 ± 0.28^{a}	3.90 ± 0.45^{b}	4.13 ± 0.65^{b}	0.87 ± 0.15^{a}	3.56 ± 0.84^{b}	4.16 ± 0.64^{b}
Farnesene	0.37 ± 0.15^{a}	0.77 ± 0.14^{b}	0.87 ± 0.43^{b}	nd	nd	0.46 ± 0.15
Cis linalolox	nd	nd	0.41 ± 0.11	nd	nd	nd
Linalol	0.76 ± 0.23^{a}	1.83 ± 0.17^{b}	2.37 ± 0.43^{b}	nd	nd	nd
Total	2.30 ± 0.74	7.28 ± 0.88	8.86 ± 1.95	1.01 ± 0.20	3.78 ± 0.89	8.04 ± 1.41

(Continued)

TABLE 1 | Continued

Volatile compounds	Cellina di Nardò POC			Leccino POC		
	NF	FY	FYL	NF	FY	FYL
	μg/Kg ± sd	μg/Kg ± sd	μg/Kg ± sd	μg/Kg ± sd	μg/Kg ± sd	μg/Kg ± sd
HYDROCARBONS						
Styrene	0.66 ± 0.15^{a}	2.98 ± 0.94^{b}	3.055 ± 1.05^{b}	1.20 ± 0.16^{a}	2.34 ± 0.66^{b}	2.60 ± 0.25^{b}
SULFUR COMPOUNDS						
Dimethyl sulfide	0.55 ± 0.11^{b}	0.32 ± 0.08^{a}	0.24 ± 0.07^{a}	0.76 ± 0.12^{b}	0.67 ± 0.15^{b}	0.43 ± 0.12^{a}
Methionol	nd	0.55 ± 0.10^{a}	0.74 ± 0.22^{a}	nd	nd	0.70 ± 0.22
Total	0.55 ± 0.11	0.87 ± 0.18	0.98 ± 0.29	0.76 ± 0.12	0.67 ± 0.15	1.13 ± 0.35
NORISOPRENOIDS						
β-damascenon	0.66 ± 0.004^{a}	1.56 ± 0.23^{b}	1.70 ± 0.17^{b}	0.054 ± 0.007^{a}	0.58 ± 0.08^{b}	0.60 ± 0.16^{b}
VOLATILE PHENOLS						
Guaiacol	nd	nd	0.085 ± 0.015	nd	nd	0.50 ± 0.15
ACIDS						
Acetic acid	nd	nd	4.21 ± 0.45	nd	nd	2.10 ± 0.44
Hexanoic acid	0.76 ± 0.25^{a}	2.17 ± 0.56^{b}	2.89 ± 0.37^{b}	nd	nd	3.21 ± 0.83
Octanoic acid	0.44 ± 0.15^{a}	1.93 ± 0.77^{b}	1.50 ± 0.15^{b}	nd	nd	0.92 ± 0.15
Total	1.20 ± 0.40	4.10 ± 1.33	8.60 ± 0.97			6.23 ± 1.42

Data is the mean of 3 replicate measurements ± standard deviation. nd, not detected. Within the same cultivar for each compound, different letters indicate significant differences within the same row (not fermented vs. fermented) (One-Way ANOVA, $p < 0.05$).

supplements and feed considering its interesting compositional traits (19, 21). In order to verify its possible use in human food preparations a wide characterization of chemical composition in terms of main bioactive compounds and fatty acids was carried out. Moreover, in this study fermentation by selected microorganisms was used in order to produce a new valuable product from POC. The idea was to use already characterized yeast and LAB isolates able to survive and grow in hard niches such as wine, beer, olive mill wastewaters, and table brines characterized by the presence of difficult constraints, i.e., polyphenols, salt, alcohols, etc. On yeasts, one commercially available wine starter, two beer starters, one baker's starter together with two selected isolates for table olive preparation and an isolate able to detoxify OMW were tested. LAB isolates were all derived from fermented table olives.

In the first part of this study, the metabolic activity of each yeast and LAB isolates in the presence of 50% lyophilized and sterilized POC were tested. Beside sugar consumption and organic acid evolution during fermentation, the levels of the main and healthy important phenolic compounds were followed. The level of the highly valuable hydroxytyrosol increased in fermented POC samples. This was most likely due to microbial glucosidase and esterase activity (43, 44). Volatile compounds produced after fermentation by each isolate were studied. All compounds were identified in POC samples, i.e., alcohols, esters, hydrocarbons, terpenes, acids, and volatile phenols, were already described as associated with table olives *Cellina di Nardò* and *Leccino* (23), *Kalamàta* and *Conservolea* (24), *Nocellara del Belice* (45), *Manzanilla*, *Gordal*, and *Hojiblanca* (46), "alcaparras stone" from Portugal (47).

The PCA analysis, carried out on polyphenols and volatile compounds identified in POC samples inoculated with different yeasts and LAB isolates, gave the possibility to select KI-30-1 and BC-T3-35 isolates as the more promising microorganisms as candidate starters to be used in pilot-scale fermentation. KI-30-1 and BC-T3-35 isolates were able to develop main important volatile classes (i.e., esters and terpens) as well as to produce high levels of tyrosol and hydroxytyrosol in POC fermented samples. For these reasons these two isolates were chosen for pilot-scale experiments.

In pilot-scale fermentations, a sequential inoculation strategy was adopted. *Leccino* and *Cellina di Nardò* POC samples were first inoculated with the strain *S. cerevisiae* KI-30-1 and then with the strain *L. mesenteroides* BC-T3-35. This strategy has already been described on table olive fermentation, where yeasts were demonstrated to play a substantial role throughout the process and LAB, carried out lactic fermentation in the last part of fermentation (23–25). The trends of sugar consumption and the evolution of organic acids (lactic, citric, tartaric and acetic acid) in all starter-inoculated fermentations were in accordance with results reported for green and black olive fermentations (23–25, 48–50). In this work, the use of starters enabled the decrease of pH value in all fermented samples to about 4.2, which is adequate for fermented products such as black olives (25, 50). Beside the low pH value, the safety of the process and the final products were ensured by the ascertained absence of microbial contaminants. In POC samples yeast metabolic activities induced an increase in some volatile molecules such as alcohols, terpenes, hydrocarbons and volatile acids compared with un-inoculated samples. With regards to alcohols, 2-phenyl-ethanol (2-PE) is the aromatic alcohol, responsible for rose

TABLE 2 | Chemical composition of the main bioactive compounds (polyphenols, triterpenic acids, tocochromanols, and carotenoids) and fatty acids profile of Patè olive cake (POC) from the cultivar *Leccino* and *Cellina di Nardò*, before and after the fermentation process.

	Leccino POC			Cellina di Nardò POC		
	Not fermented	**Fermented**	***P*-value$^{\Omega}$**	**Not fermented**	**Fermented**	***P*-value$^{\Omega}$**
POLYPHENOLS (mg/g FW)						
Hydroxytyrosol (3,4-DHPEA)	1.8 ± 0.1^{b}	2.5 ± 0.1^{a}	0.001	6.2 ± 0.2^{b}	8.8 ± 0.5^{a}	0.001
Tyrosol (p-HPEA)	0.7 ± 0.06^{a}	0.6 ± 0.04^{a}	0.074	0.8 ± 0.02^{a}	0.8 ± 0.05^{a}	1
Vanillic acid	0.8 ± 0.02^{a}	0.2 ± 0.01^{b}	< 0.001	0.5 ± 0.02^{a}	0.3 ± 0.02^{b}	< 0.001
hydroxytyrosol acetate	0.7 ± 0.01^{a}	0.5 ± 0.01^{b}	< 0.001	0.5 ± 0.0001^{a}	0.5 ± 0.01^{a}	1
p-coumaric acid	0.3 ± 0.02^{a}	0.3 ± 0.01^{a}	1	0.2 ± 0.01^{a}	0.15 ± 0.01^{b}	0.004
Verbascoside	0.7 ± 0.03^{a}	0.6 ± 0.01^{b}	0.006	3.7 ± 0.18^{a}	1.9 ± 0.05^{b}	< 0.001
Isoverbascoside	0.6 ± 0.04^{a}	0.5 ± 0.01^{b}	0.014	0.4 ± 0.01^{a}	0.3 ± 0.02^{b}	0.002
Oleacein (3,4-DHPEA-EDA)	1.4 ± 0.09^{a}	0.4 ± 0.03^{b}	< 0.001	5.0 ± 0.2^{a}	1.9 ± 0.1^{b}	< 0.001
Oleochantal P-HPEA-EDA	0.15 ± 0.01^{a}	0.10 ± 0.01^{b}	0.004	0.4 ± 0.001^{a}	0.2 ± 0.001^{b}	< 0.001
Rutin	0.002 ± 0.001^{a}	0.001 ± 0.002^{a}	0.482	0.06 ± 0.001^{a}	0.05 ± 0.001^{b}	< 0.001
(+)-Pinoresinol	nd	nd		0.3 ± 0.1^{a}	0.3 ± 0.1^{a}	1
Total	7.1 ± 0.4^{a}	5.7 ± 0.2^{b}	0.04	18.1 ± 0.7^{a}	15.2 ± 0.9^{b}	0.012
TRITERPENIC ACIDS (mg/g FW)						
Maslinic acid	1.16 ± 0.05^{a}	1.27 ± 0.12^{a}	0.217	1.16 ± 0.15^{a}	1.31 ± 0.09^{a}	0.212
Oleanoic acid	0.32 ± 0.04^{b}	0.48 ± 0.09^{a}	0.048	0.43 ± 0.07^{a}	0.56 ± 0.08^{a}	0.102
Total	1.48 ± 0.09^{a}	1.74 ± 0.21^{a}	0.12	1.59 ± 0.21^{a}	1.87 ± 0.17^{a}	0.147
TOCOCHROMANOLS (μg/g FW)						
α-T	23.20 ± 0.70^{a}	21.10 ± 1.20^{a}	0.059	21.90 ± 0.92^{a}	22.01 ± 0.51^{a}	0.865
β-T3	1.74 ± 0.11^{a}	1.54 ± 0.08^{a}	0.064	1.25 ± 0.20^{a}	1.37 ± 0.14^{a}	0.443
Total	24.94 ± 0.81^{a}	22.64 ± 1.28^{a}	0.058	23.15 ± 1.12^{a}	23.30 ± 0.65^{a}	0.851
CAROTENOIDS (μg/g FW)						
Lutein	2.21 ± 0.31^{a}	1.89 ± 0.02^{a}	0.149	1.97 ± 0.11^{a}	2.20 ± 0.30^{a}	0.281
Zeaxanthin	0.06 ± 0.01^{a}	0.07 ± 0.01^{a}	0.288	0.05 ± 0.01^{a}	0.07 ± 0.01^{a}	0.07
α-Carotene	0.25 ± 0.05^{a}	0.35 ± 0.06^{a}	0.091	0.26 ± 0.01^{a}	0.28 ± 0.10^{a}	0.748
β-Carotene	0.74 ± 0.14^{a}	0.55 ± 0.01^{a}	0.079	0.84 ± 0.04^{a}	0.84 ± 0.07^{a}	1
13 cis β-Carotene	0.32 ± 0.06^{a}	0.40 ± 0.07^{a}	0.207	0.32 ± 0.02^{a}	0.39 ± 0.05^{a}	0.053
Total	3.58 ± 0.57^{a}	3.26 ± 0.17^{a}	0.404	3.44 ± 0.19^{a}	3.78 ± 0.53^{a}	0.305
FATTY ACIDS (%)						
Palmitic (C16:0)	17.94 ± 0.69^{a}	17.23 ± 0.65^{a}	0.264	16.73 ± 0.59^{b}	18.40 ± 0.43^{a}	0.017
Palmitoleic (C16:1)	1.82 ± 0.08^{b}	2.08 ± 0.04^{a}	0.007	1.86 ± 0.06^{b}	2.33 ± 0.01^{a}	< 0.001
Stearic (C18:0)	2.79 ± 0.02^{b}	5.12 ± 0.89^{a}	0.011	3.05 ± 0.07^{b}	3.78 ± 0.05^{a}	< 0.001
Oleic (C18:1 n−9)	63.01 ± 0.05^{a}	62.46 ± 1.2^{a}	0.472	63.11 ± 0.44^{a}	62.11 ± 1.21^{a}	0.250
Linoleic (C18:2 n−6)	13.62 ± 0.02^{a}	13.11 ± 0.11^{b}	0.002	14.47 ± 0.14^{a}	13.38 ± 0.32^{b}	0.006
Linolenic (C18:3 n−3)	0.91 ± 0.01	nd		0.78 ± 0.31	nd	
SFA	20.73 ± 0.71^{a}	22.35 ± 1.54^{a}	0.173	19.78 ± 0.66^{b}	22.18 ± 0.48^{a}	0.007
MUFA	64.83 ± 0.13^{a}	64.54 ± 1.24^{a}	0.708	64.97 ± 0.50^{a}	64.44 ± 1.22^{a}	0.525
PUFA	14.53 ± 0.03^{a}	13.11 ± 0.11^{b}	< 0.001	15.25 ± 0.45^{a}	13.38 ± 0.32^{b}	0.004
PUFA/SFA	*0.71*	*0.59*		*0.77*	*0.60*	

The data represents the mean ± standard deviation of three replicate measurements (n = 3). nd, undetected; FW, fresh weight. Within the same cultivar for each compound, different letters indicate significant differences ($^{\Omega}$Student's test, p < 0.05) within the same row (not fermented vs. fermented).

notes, produced from L-phenylalanine (51). Yeast species are responsible for the production of 2-PE and isoamyl-alcohols, the most representative molecules of alcohol class, thus these compounds can be indicative of yeast metabolism (25, 51, 52). The production of higher alcohols (isoamyl alcohols) is linked to microbial catabolism of amino acids (53). Higher alcohols are secondary products of alcoholic fermentation driven by yeasts, and can be influence with positive and negative notes, the aroma and flavor of fermented food. Higher concentrations of higher alcohols can produce a strong, pungent smell and taste whereas optimal levels can be responsible of fruity notes (54, 55). Formation of ethyl esters is mainly ascribable to yeast fermentation and to ethanolysis of acylCoA derived from fatty acid synthesis or degradation (56). Both esters and acetates

can positively influence aroma by their characteristic grape-like odor, sweet-fruity and sweet-balsamic notes (52, 57). The key representative species of ester in fermented POCs were ethyl acetate, ethyl decanoate, ethyl hexanoate, ethyl octanoate, and methyl 2 furoate. The presence of these compounds has also been reported in table olives (25, 58) as well as in other fermented products (59, 60). Very interestingly, the methyl salycilate, revealed in yeast and LAB fermented *Cellina di Nardò* POC, has already been reported as an important volatile flavor compound to differentiate semi- and fully-fermented teas (61, 62). Concerning aldehydes, according to Malheiro et al. (63), these compounds are present in high concentrations in all studied olive cultivars. In green olives aldehyde content can extend to 50% of all volatile classes and even 75% in black olives (64). Terpenes and norisoprenoids are generally present as glycosylated precursors and can be released by enzymatic hydrolysis during fermentation (24, 65). The increase of terpenes and norisoprenoids in fermented POC could be ascribable to glycosidase activities of inoculated microbial starters.

POCs, either fermented or not, represent a good source of triterpenic acids. Several studies have indicated that maslinic and oleanoic acids have anti-inflammatory, antitumoral, antihyperglicemic, hepatoprotective, cardioprotective, and antimicrobial effects (66). These bioactives are also of interest to food and cosmetic industries (14, 67). Maslinic and oleanoic acids are the main triterpenic acids found in table olive, olive oils and olive pomace oils (20, 40, 68). According to Padalino et al. (20), in POCs the content of maslinic acid were higher than oleanoic acid. Also the carotenoid profiles were similar to those obtained by Padalino et al. (20). Within tocochromanols, α-T is known to be the most biologically active, with a role in preventing lipid peroxidation and scavenging of lipid peroxyl radicals (69). α-T content observed in POCs was in agreement with the results found by Nunes et al. (14) in olive pomace from a two-phase extraction process.

The procedure for the production of fermented POC, based on sequential inoculums of a yeast LAB strains, allowed the standardization of the process and, obtaining a final product in a very limited period of time (50 days). The process can also be monitored following specific chemical descriptors suitable to describe the evolution of fermentative activities of yeasts and LAB, as already demonstrated for table olives (24, 25).

Experiments are now in progress to verify the possibility of further exploiting POC in the formulation of enriched food products (i.e., bakery products) as well as to characterize their nutritional and sensorial traits. Large-scale experiments are also now planned to improve and validate the use of these autochthonous starter cultures.

AUTHOR CONTRIBUTIONS

MT, MD, and GV conduction of the experiments, acquisition, and interpretation of the results. GB, MS, AT, and GM work design, data discussion, and manuscript preparation.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2019.00003/full#supplementary-material

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Effect of Molasses and Dried Orange Pulp as Sheep Dietary Supplementation on Physico-Chemical, Microbiological and Fatty Acid Profile of Comisana Ewe's Milk and Cheese

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The use of agro-industrial by-products for ruminant feed represents both an economical and environmental convenient way for reducing waste discharge and waste management costs for food industries. Large amounts of waste from citrus processing industries are available in Sicily, Italy. In the present study, the effect of dried citrus pulp as sheep dietary supplementation was evaluated on physico-chemical, microbiological and fatty acid composition of resulting milk and cheese. Pelleted feed integrated with molasses and blond orange pulp, replacing cane molasses, beet pulp and part of the maize and sunflower in ration, were administrated to ewes as an experimental treatment The experiment involved sixty Comisana breed sheep divided into two groups and two feeding trials (experimental and control). Ewe's milk and cheese samples were collected from January to April and analyzed for physico-chemical, microbiological and fatty acid profile composition. Results suggested that both the experimental milk and cheese were different from the controls. In particular, an increase of experimental milk yield and fat content were registered whilst the cheese samples exhibited a significant decrease of pH values and an increase in fat and protein contents. In addition, an increase of conjugated linoleic acids as well as of the oxidative stability were observed indicating the beneficial effect of dietary supplementation. Furthermore, among the main microbial groups, the experimental and control samples, no differences were detected. However, with the exception of streptococci, which was found higher in experimental cheeses, and staphylococci, which was significantly reduced by experimental feed. Moreover, the application of culture-independent methods highlighted the dominance of *Lactobacillus rhamnosus/casei* group in the experimental cheese, suggesting a driving role of the dietary supplementation in the cheese microbiota composition. The present study demonstrated that the inclusion of citrus by-products in the diet of small dairy ruminants is a promising feeding, which could positively affect milk composition and cheese manufacture.

Keywords: citrus by-products, sheep milk yield, milk coagulation properties, sheep cheese, lactobacilli

INTRODUCTION

The use of agro-industrial by-products for ruminant feed is both an economical and environmental way to reduce waste discharge and decrease waste management cost (1). Large amounts of waste from citrus juice extraction are produced in Spain and Sicily, the main citrus fruit producer in the Mediterranean region, accounting for 1.6 million of tons of citrus per year (2). The residues of the juice extraction comprise of peel, pulp, rag and seeds. These components, either individually or in various combinations, are source materials from which by-product feedstuffs can be obtained (3, 4). In Sicily, an average of 34% of citrus fruits are processed into juices leaving about a half of its weight as waste, that presents high energy content, due to high soluble carbohydrate contents, and rapidly degradable Neutral Detergent Fiber (NDF), made up of cellulose and pectin. For its nutrient content can partly replace cereal grains in animal rations, as dehydrated fed, fresh fed or as silage (4) and its use, in formulated feeds, largely depends on its availability and its relative cost-effectiveness, when compared with other alternative raw materials. It has been already demonstrated that these components have fewer negative effects than supplementation with starch or sugar-rich feeds on the rumen ecosystem (5). The high content of pectin involves to a faster rumen fermentation allowing the release of energy for a rapid microbial growth (6) and contributes to create better rumen conditions for fiber fermentation. Its high level of potential degradable dry matter provides a high total digestible nutrient content (7). Moreover, the inclusion of citrus by-products in the diet of small dairy ruminants had some effects on milk yield, composition and properties and on the quality of derived products (8). It has been well established that the addition of orange pulp into the goat's diet affected milk quality and cheese composition resulting in a lower pH and water activity, high fat and NaCl content that contribute to improve the sensory characteristics of derived ripened cheese (1). Although some researches have investigated the effects of different feeding systems on the quality and composition of milk and dairy products, up to now, no information is available on the influence of agro-industrial by-products, such as orange pulp, as sheep dietary supplementation on Sicilian ewe's milk and cheese.

In Sicily, a Southern region of Italy, Comisana sheep milk production is mainly transformed into Protected Denomination of Origin (PDO) Pecorino cheese. Typically, cheese-making is homemade, without addition of any starter cultures and cheese is ripened for at least 6 months before sale. During cheese production and ripening, several microbial and biochemical processes take place that contributing together with milk composition, to the characteristics and the quality of the final product.

The aim of this study was to investigate the effect of replacement of cane molasses and beet pulp, used in conventional fed, with molasses and dried orange pulp on physico-chemical, microbiological and fatty acid composition of Comisana sheep's milk and cheese manufacturing.

MATERIALS AND METHODS

Experimental Design, Animals and Diets

The experimental study was conducted in an organic farm, located in the Enna's province (Sicily, Italy) at an altitude of 600 m above sea level, with about 1,000 sheep of Comisana breed for the production of "Ricotta" and "Pecorino" cheese types. The applied procedures were in compliance with the European guidelines for the care and use of animals in research (Directive 2010/63/EU). The trial ran from 20 days before lambing and was performed on sixty multiparous Comisana ewes at the 5th month of gestation. The animals were randomly divided into two groups of thirty ewes each, homogeneous for age (3–6 years), body weight (49.7 $\pm$ 2.7 kg), body condition score (BCS 2.76 $\pm$ 0.33) and parity (3 $\pm$ 1), named control (C) and experimental (E). During the experimental period a commercial pelleted feed was administered to the control group (C) while a pelleted feed integrated with 4% (DM basis) of molasses and 10% (DM basis) of blond orange pulp were offered daily to ewes of the experimental group (E) in replacing the cane molasses and beet pulp present in the pelleted feed of the control group (**Table 1**). The mean sugar content of both molasses and orange pulp is reported in **Table 2**. The pelleted feed of both groups were specifically produced by a feed factory (Di Pasquale Mangimi, Ragusa, Italy) for the trial. The groups were housed in two adjacent free stall pens (60 m^2/Group) with concrete floor and equipped with feed manger, drinker and covered paddock (100m^2/Group). The two dietary treatments were isoenergetic and isonitrogenous and were administered for a period of 180 days (from-20 days before at + 160 days post-partum). Their chemical composition, expressed in percentage, is reported in **Table 3**. Moreover, the sugar composition during the trial, ewes received 500 grams per head per day of pelleted feed and 1.5 kg per head per day of vetch hay divided between morning (05:00 a.m.) and evening (05:00 p.m.) during the milking. In addition, both groups were granted the same natural pasture from 8:00 a.m. (after morning milking) to 2:00 p.m. (before evening milking). The most common botanical families were: Cruciferae (Raphanus spp., Capsella bursapastoris, Sinapis spp., Brassica spp.), Compositae (Carduus spp., Cychorium spp., Calendula aruensis), Graminaceae (Bromus spp.), Leguminosae (Trijiolium spp.), and Malvaceae (Malua neglecta). The above mentioned species were nearly always distributed in very complex mixes.

Feed, Milk and Cheese Sampling

For each pelleted feed bag, both control and experimental, elementary aliquots were collected, in order to obtain a global sample of 500 g. The same amount of hay was sampled and analyzed following the procedures of the AOAC (9) to determine the concentrations of dry matter (DM, 934.01), ash (942.05), crude protein (CP, 2001.11) and ether extract (EE, 920.39). Concentrations of Neutral Detergent Fiber (NDF) (aNDFom, 2002.04), Acid Detergent Fiber (ADF) (ADFom, 973.18) and Acid Detergent Lignin (ADL) (973.18) were determined according to Van Soest (10), using heat-stable

TABLE 1 | Proportion and ingredient of the pelleted complete feed (% of DM).

	Control	Experimental
Degerminate corn meal	32.50	33.00
Sunflower pellet	29.00	29.10
Beet pulp	10.00	–
Wheat middling	8.80	10.00
Dry orange pulp	–	10.00
Broad bean	7.80	8.70
Lemon molasses	–	4.00
Sugar cane molasses	3.50	–
Corn germ	2.80	2.80
Calcium carbonate	1.90	0.10
Carob pulp	1.10	1.00
Sodium chloride	0.95	0.95
Lysine	0.20	0.20
Magnesium oxide	0.20	0.20
Mineral- vitamin premix	0.15	0.15

TABLE 2 | Mean sugar content and composition, expressed as g/100 g, of both orange pulp and molasses.

	Orange pulp	Molasses
Glucose	2.52	8.80
Fructose	1.97	7.00
Lactose	–	–
Sucrose	5.05	2.35
Maltose	–	–

amylase and expressed exclusive of residual ash. Non-fiber carbohydrate (NFC) content was calculated as (100–[CP +EE+ ash+ aNDF]).

The record 0 was taken at 40 ± 5 days lactating (weaning period). After this period individual milk yield of the two daily milking samples (05:00 a.m. and 05:00 p.m.) was recorded and, at the same time, individual milk samples (250 ml) were collected and analyzed to measure fat, protein, casein, lactose, total solids, non-fat solids, urea and titration acidity (Soxhlet-Henkel/ SH), using Fourier Transform InfraRed (Milkoscan FT2, Foss Electric, Sweden), calibrated with appropriate sheep milk standards. Moreover, pH (Orion EA 940), clotting properties, according to the r (clotting time), k_{20} (curd firming time), a_{30} (curd firmness) parameters, using rennet Hansen Standard (200 μl/10 ml of milk) and Formagraph instrument (Foss Electric Hillerod, Denmark) according to A.S.P.A. method (1995) were detected.

Cheese production was performed in a small-scale dairy plant, located in the Enna area of Sicilian region (Italy). According to the protocol of production [(11), p. 295], the raw sheep's milk was processed without adding any starter cultures. The production was carried out in four consecutive manufactures, from January to April 2017, and the ripening was monitored monthly until 60 days. Cheese samples obtained from milk originating from

TABLE 3 | Chemical composition of the pelleted complete feed and vetch hay (% as fed).

	Control	Experimental	Vetch hay
Dry matter	89.50	89.30	87.00
Crude protein	18.90	18.93	14.30
Crude fiber	10.82	10.38	25.93
Neutral detergent fiber	28.53	26.83	43.28
Acid detergent fiber	15.99	16.61	30.47
Acid detergent lignin	4.35	4.41	5.68
Starch	30.89	31.45	—
Non-fiber carbohydrate	41.60	43.74	—
Ether extract	2.87	3.02	0.91
Ash	8.06	7.45	8.6
UFL (kg/DM)	0.99	0.99	0.74

UFL,Milk Forage Unit; energy unit of the INRA system corresponding to 1.70 Mcal of net energy for lactation estimated at maintenance level.

animals fed with control (C) and experimental (E) diets and for each making month, after 60 days of ripening, were collected and subjected to physico-chemical analyses. Overall, eight samples from control (C) and experimental (E) cheeses were obtained. For microbiological analyses, cheese samples were transported to the laboratory of Food Microbiology, at the Department of Agriculture, Food and Environment, University of Catania, in refrigerated conditions and analyzed within 4 h.

Physico-Chemical Analyses

The pH values of cheese samples were determined by pHmeter (H19017, Microprocessor, Hanna Instruments). Water activity (a_w), determined by a special apparatus (Aqualab Series 3TE dewpoint electronic water activity meter), with an accuracy of ±0.003, was measured at 21°C on grated cheese (1.5 g), in triplicate. Moreover, cheese samples were analyzed for moisture, fat, protein and salt content, using Near Infrared Spectroscopy in Transmittance (FoodScanTM Dairy Analyser; FOSS, Italy).

Cholesterol Determination

Cholesterol determination in cheese samples was performed according to the Official Method 994.10-Cholesterol in food-Direct saponification Gas Chromatographic Method (AOAC). In detail, 2 g of test portion were saponified at high temperature with ethanolic KOH solution (40 ml 95% ethanol and 8 ml 50% KOH solution). The unsaponifiable fraction containing cholesterol was extracted with toluene. The cholesterol was derivatizated to trimethylsilyl (TMS) ethers by hexamethyldisilane (HMDS) and trimethylchlorosilane (TMCS). The derivatizeted cholesterol was analyzed by GC-FID (Agilent Technologies 6890 N, Palo Alto, CA, USA) with a split/splitless injector, a flame ionization detector and a fused silica capillary column HP5, 30m × 0.32 mm I.D., 0.25 μm film thickness (Agilent J&W GC columns). The column temperature was programmed at 260°C (60 min). Temperature of the injector and detector was 280°C. Injection volume was 1.0 μl. The carrier gas used was helium (1 ml/min), and the split ratio was 1:50. Cholesterol was identified by

comparing the relative retention times with standards from Supelco. Chromatogram peak areas were acquired and calculated by Chemstation software (Agilent). Concentration of cholesterol was calculated by external standard method and expressed as mg/100 g.

Thiobarbituric Acid-Reactive Substances Determination

The thiobarbituric acid-reactive substances (TBARS) assay was performed as described by Luciano et al. (12). In brief, an aliquot of 2.5 g of cheese was finely minced and homogenized with 12.5 ml of distilled water. Trichloroacetic acid (12.5 ml; 10%, w/v) was added to precipitate proteins. Samples were filtrated and 4 ml of the filtrate were mixed with 1 ml of 0.06 M aqueous thiobarbituric acid. Samples were incubated in a water bath at 80°C for 90 min and the absorbance at 532 nm was measured. The assay was calibrated using standard solutions of 1,1,3,3,-tetra-ethoxypropane in trichloroacetic acid (5% w/v). Results were expressed as mg of malondialdehyde (MDA)/kg of cheese. Three replicates ($n = 3$) were run per sample. TBARS were determined at 0, 10, 20, 30, and 40 days of storage at 4°C.

Fatty Acids Analysis

Lipids were extracted using a mixture of chloroform/methanol (2:1, v/v), and fatty acids methyl esters of cheese fat were prepared by direct transesterification with sulfuric acid/methanol (1:9, v/v) of a weighed portion (15 mg) of the total lipids and analyzed using the high resolution gas chromatography technique. The fatty acid methyl esters (FAME) were analyzed by GC-FID (Agilent Technologies 6890 N, Palo Alto, CA, USA) with a split/splitless injector, a flame ionization detector and fused silica capillary column Omegawax 250, 30 m × 0.25 mm I.D., 0.25 μm film thickness (Supelco, Bellefonte, PA, USA). The column temperature was programmed as follows: initial isotherm of 160°C (6 min), increment of 3°C/min and a final isotherm of 250°C (30 min). Temperature of the injector and detector was 250°C. Injection volume was 1.0 μl. The used carrier gas was helium (1 ml/min), and the split ratio was 1:50. Fatty acids were identified by comparing the relative retention times of FAME peaks from samples with standards from Supelco. Chromatogram peak areas were acquired and calculated using Chemstation software (Agilent). Concentration of each fatty acid (FA) was expressed as g/100 g, considering 100 g as the summation of the areas of all FAME identified. For each sample, the chromatographic analysis was repeated three times. Regarding the fatty acid profiles of cheese samples, the saturated (SFA), Monousaturated (MUFA), and Polyunsaturated (PUFA) fatty acids were analyzed. In addition, to relate the profile of fatty acids with the risk of cardiovascular disorders, the atherogenicity index (AI) and thrombogenicity (TI) indices were calculated, as proposed by Ulbricht and Southgate (13) through the equation:

$$AI = [(4\times C14:0) + C16:0 + C18:0]/(\Sigma MUFA + \Sigma PUFA) \quad (1)$$

$$TI = (C14:0 + C16:0 + C18:0)/(0.5\times MUFA + 0.5\times PUFA - n6 + 3\times PUFA - n3 + PUFA - n3/PUFA - n6) \quad (2)$$

AI indicates the relationship between the sum of the main saturated FAs and the main classes of unsaturated FAs (14). TI expresses the tendency to form clots in the blood vessels. It is defined as the relationship between the prothrombogenetic (saturated) and the anti-thrombogenetic fatty acids (MUFAs, PUFAs - n6 and PUFAs - n3) (15).

Microbiological Analyses

Microbiological analyses of cheese samples at different sampling periods were performed in triplicate. In detail, an aliquot (25 g) of control (C) and experimental (E) cheeses, including the cheese core and the surface, were blended for 3–5 min with sterile saline solution, using a Stomacher Lab Blender 400 (International PBI S.p.A Milan, Italy), and then serially diluted into the same sterile solution. Microbiological counts were performed using the following media and conditions: Plate Count Agar (Sigma, Milan, Italy), incubated at 30°C for 72 h, for mesophilic aerobic bacteria; De Man, Rogosa and Sharp agar (Oxoid, Italy), anaerobically incubated, at 32°C for 48 h for lactobacilli; LM17 agar (Oxoid, Italy), with 0.17 g/l of cycloheximide (Oxoid, Italy), incubated at 32°C and 45°C for lactococci and streptococci, respectively; Violet Red Bile Glucose Agar (Difco, Italy), aerobically incubated at 37°C for 24 h, for *Enterobacteriaceae*; Mannitol Salt Agar, incubated at 37°C for 24–48 h, for staphylococci; Sabouraud Dextrose Agar, incubated at 25°C for 72 h, for yeast and mold. The results were expressed as $\log_{10}$ colony forming unit (CFU) per ml or g (log CFU/g-ml), the average of three replicates with standard deviation.

Total DNA Extraction and PCR-DGGE Analysis

Control and experimental cheese samples, at different sampling times, were collected for direct DNA extraction, as previously reported by Randazzo et al. (16). The concentration and purity of DNA were assessed by measuring optical density using Fluorometer Qubit (Invitrogen, Carlsbad, CA, USA). Extracted DNA was used as template for initial PCR targeting the V2 to V3 region of 16S rDNA, using the universal primers HDA1-GC (CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGGACTCCTACGGGAGGCAGCAGT-3′) and HDA2 (5′-GTATTACCGCGGCTGCTGGCAC- 3′). The PCR reaction mixture (25 μl) included PCR master mix 2X (Biotechrabbit), 75 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM Mg^{2+}, 10 mM each of the four deoxynucleoside triphosphates (dNTP), 1.255 U/ml of Taq polymerase, 10 pMol of each primer, and 1 μl of appropriately diluted template DNA. The PCR conditions and DGGE analysis of PCR amplicons were the same as previously described (17).

Statistical Analysis

Statistical analyses of cheese parameters were performed using the GLM procedure in SAS 9.3 (2011) with "Month of cheese making" and "diet" as fixed factors. When a statistically significant effect ($p \leq 0.05$) of the diet was detected, means

were compared using p-values adjusted, according to the Tukey-Kramer multiple comparisons test. All microbiological statistical analyses were performed using XLSTAT statistical software. The statistical significance among groups at different sampling periods was evaluated by one-way analyses of variance (ANOVA). In addition, the XLSTAT statistical software was used in order to visualize possible correlations between the different microbial groups and physico-chemical parameters of cheese samples.

RESULTS

Physico-Chemical Composition of Bulk Milk and Cheese

Physico-chemical composition of bulk milk and experimental and control cheese samples through the lactation period is reported in **Table 4**. Administration of experimental feed influenced the milk yield that was significantly higher in the E group ($p \leq 0.05$). The significantly highest fat and protein amount (g/d) ($p \leq 0.05$) were observed in E group. A significant ($p \leq 0.05$) increase was observed for clotting time (RCT) in the E group, while no significant differences were observed for curd firming time (k_{20}) parameter. Milk urea content was lower for E group, compared to C group ($p \leq 0.05$), resulting in a better nitrogen utilization in presence of citrus by-products supplementation. The readily fermentable molasses and orange pulp could have helped ewes to use the N diet more efficiently and increased the response. This gives the rumen microbial population a possibility to match the inflow of protein with carbohydrates. Overall, significant differences ($p \leq 0.05$) between control and experimental samples were detected, indicating that the experimental fed significantly affected all the parameters studied, except a_w. In detail, the experimental cheese samples presented lower pH values and moisture content during the whole lactation period (**Table 4**). Significant differences ($p \leq 0.05$) in salt content were observed, with values ranging from 1.84 g/100g to 2.45 g/100 g. The diet significantly affected cheese fat content, observing a higher content in experimental cheese samples than in control samples. An increase of protein level was also registered in experimental samples, with values ranged from 31.26 g/100 g, in March to 34.52 g/100 g, in February (**Table 4**).

Cholesterol and Fatty Acids Content in Cheese Samples

The total cholesterol content, expressed as mg 100/g of fat, was significantly lower in experimental cheese, mostly in February, March and April (−16.58%, −21.74% and −6.05%, respectively, **Table 4**). The fatty acids (FA) composition and nutritional indices of experimental cheeses are shown in **Table 5**. In general, fatty acid profiles of cheeses were qualitatively similar, especially regarding to unsaturated fatty acids (MUFA). The saturated fatty acid (SFA) dominated the fatty acid profiles of control samples produced in January and in March, while the replacement of the cane molasses and beet pulp by molasses and blond orange pulp produced a significant decrease of these compounds ($p \leq 0.05$) in experimental cheese in the same period. The lowest content of SFA was observed in experimental cheese produced in March (58.14%), whereas the highest ratio of these fatty acids was revealed in control samples produced in January (77.04%).

Regarding the MUFAs, nothing different was observed between the two cheese groups, except for control cheese manufactured in January, which showed the lowest registered value. Similar trend of polyunsaturated fatty acid (PUFA) content was observed in the whole experimental period except for experimental sample of March, in which the content was the highest ($p \leq 0.05$) registered (**Table 5**). The content of ω-3 fatty acid was significant lower ($p \leq 0.05$) in experimental cheese produced in January and February, while the content of ω-6 acid in this group was the highest during the whole experimentation period (**Table 5**). Because of different fatty acid compositions, cheeses were characterized by significant ($p \leq 0.05$) different health lipid indices, such as index of atherogenicity (AI), index of thrombogenicity (IT) (**Table 5**). Due to the lowest content of USFAs (22.31%), control cheese produced in January was characterized by the most unfavorable AI and TI values. On the contrary, the health lipid indices reported above were observed in experimental cheese produced in March (AI 2.34; TI 1.70). The conjugated linoleic acids (CLAs) content revealed that the experimental cheeses was high in all months of cheese making and significantly higher than in control cheeses ($p \leq 0.05$) in February, March and April (**Table 5**).

Thiobarbituric Acid-Reactive Substances

The influence of dietary supplementation with citrus by-products on lipid oxidation in cheese is presented in **Table 6**. The TBARs value of the control samples was higher than that found in experimental samples (**Table 6**), after 40 days of retention and after the 60 days of ripening ($p \leq 0.05$).

Microbiological Data

Results of microbiological analyses carried out on both control and experimental cheese samples at different sampling periods, expressed as an average of three replicates with standard deviation are shown in **Table 7**. Overall, no difference among the main groups was detected. Lactic acid bacteria (LAB) achieved an average value of about 7.30 log cfu/g in control samples and of 7.40 log cfu/g in experimental cheese, with a slight dominance of thermophilic lactobacilli in both cheese samples. Statistical data revealed that the experimental fed significantly ($p \leq 0.05$) influenced the level of streptococci, which showed higher values, and the level of staphylococci that was significantly reduced by the addition of citrus by-products (**Table 7**). Regarding streptococci group, the average value ranged from 6.15 log cfu/g to 7.44 log cfu/g in control cheese. The highest value was revealed in experimental cheese. Staphylococci group suffered a decrease of about 2 log units for coagulase positive staphylococci and of about 1 log unit for coagulase negative in experimental cheese samples, reaching a final value of about 1 log CFU/g. *Enterobacteriaceae*, yeast and mold and total mesophilic bacteria showed similar value in both samples, reaching

TABLE 4 | Physico-chemical and coagulation parameters of both control and experimental ewe's milk and cheese during at each sampling time.

	January		February		March		April		SEM	*P*-value
	Control	Experimental	Control	Experimental	Control	Experimental	Control	Experimental		
MILK										
Yield (g/d)	717.4^{a}	1,149^{b}	808.8^{a}	992.4^{b}	573.2^{a}	829.2^{b}	407.5^{a}	523.8^{b}	0.65	≤0.01
Fat (%)	6.51^{a}	5.58^{b}	6.68	6.59	6.01^{a}	6,58^{b}	6.31^{a}	5.95^{b}	0.18	≤0.05
Fat (g/d)	46.70^{a}	64.11^{b}	54.03^{a}	65.40^{b}	34.45^{a}	54.56^{b}	25.71^{a}	31.17^{b}	1.13	≤0.01
Protein (%)	5.20	4.92	5.97	6.09	5.66	5.96	5.73	5.58	0.04	0.44
Protein (g/d)	37.30^{a}	56.53^{b}	48.28^{a}	60.43^{b}	32.44^{a}	49.42^{b}	23.35^{a}	29.23^{b}	1.15	≤0.01
Casein (%)	5.50	5.18	5.25	5.35	5.81	5.26	4.85	4.86	0.15	0.53
Lactose (%)	4.26	4.37	4.23	4.26	3.93	4.06	3.64	3.94	1.09	0.34
Total solids (%)	18.50^{a}	16.50^{b}	18.34^{a}	17.85^{b}	20.07^{a}	18.09^{b}	17.69	17.19	0.23	≤0.05
Solids non-fat (%)	11.77	11.47	11.45	11.93	11.97	12.31	10.94	10.88	1.10	0.67
Urea (mg/100 ml)	25.55^{a}	20.23^{b}	21.31^{a}	19.73^{b}	23.94^{a}	19.43^{b}	25.79^{a}	21.27^{b}	0.90	≤0.01
Somatic cell count ($\log_{10}$)	4.94	4.97	5.08	5.21	4.60	4.55	4.77	4.84	1.10	0.08
pH	6.72	6.76	6.72	6.71	6.88	6.86	6.78	6.90	0.90	0.10
r (min)	17.30^{a}	19.16^{b}	16.73^{a}	17.89^{b}	20.86^{a}	21.69^{b}	19.80	19.94	0.34	≤0.05
a_{30} (mm)	64.38	64.43	57.96	60.52	55.48^{a}	48.66^{b}	56.23^{a}	48.74^{b}	0.42	≤0.05
K_{20} min	1.49	1.51	1.57	1.67	1.57	1.55	1.61	1.64	0.35	0.78
CHEESE										
pH	5.71^{a}	5.58^{b}	5.65^{a}	5.56^{b}	5.70	5.68	5.76^{a}	5.65^{b}	0.09	≤0.05
aw	0.90	0.90	0.92	0.91	0.92	0.91	0.91	0.91	0.01	0.15
Moisture (g/100 g)	30.49^{a}	28.24^{b}	29.96^{a}	31.05^{b}	33.16^{a}	32.53^{b}	29.57^{a}	28.88^{b}	0.02	0.19
Protein (g/100 g)	31.09^{a}	31.57^{b}	30.10^{a}	34.52^{b}	29.87^{a}	31.26^{b}	31.23^{a}	33.25^{b}	0.06	≤0.05
Fat (g/100 g)	29.54^{a}	30.26^{b}	29.71^{a}	31.24^{b}	30.27^{a}	31.57^{b}	30.28^{a}	31.85^{b}	0.05	≤0.01
Cholesterol (mg/100 g)	74.10	76.92	93.11^{a}	77.67^{b}	94.91^{a}	74.28^{b}	69.06^{a}	64.88^{b}	0.15	≤0.05
Salt (g/100 g)	2.43	2.45	1.55^{a}	1.94^{b}	2.11^{a}	2.03^{b}	1.72^{a}	1.84^{b}	0.01	≤0.05

^{a,b}*Mean values with different letter in superscript within rows indicates significant differences ($p \leq 0.05$) due to fed. SEM, standard error of least square means. r, clotting time, min; a_{30}, curd firmness, mm; K_{20}, curd firming time, min.*

average values of 4.05, 3.11, and 5.50 log cfu/g, respectively (**Table 7**).

PCR-DGGE

PCR-DGGE results of the 16S rDNA of bacterial population of control and experimental cheeses at different sampling periods are reported in **Figure 1**. In order to identify the dominant species, the DGGE profiles generated from control and experimental cheeses were compared to those of strains, previously isolated from Pecorino cheese and identified (data not shown). Overall, the microbial community of both cheese samples remained quite stable with several bands in common. In detail, the bands A and B, dominated all cheese samples and showed an identical profile of *Enterococcus faecalis* and *Streptococcus thermophilus*, respectively (**Figure 1**). In addition, the weak bands C and D were detected in all cheese samples analyzed, and were even more pronounced in the control cheese. They showed identical profile to *Enterococcus durans* and *Enterococcus faecium*, respectively. It is interesting to note the appearance of several bands, E, F, and H only in the experimental cheeses, which were identified as *Lactobacillus rhamnosus/casei* group, with the exception of the band G, correspondent to *Lactobacillus fermentum* species, which was not detected in the samples produced in March (line 7).

DISCUSSION

The present study investigated the effect of citrus by products as sheep dietary supplementation on physico-chemical, microbiological and fatty acid composition of Comisana sheep's milk and cheese. Despite several studies have investigated the effect of including in various forms olive by-products (18–20) or tomato wastes (21–23) in the diet of ruminants, to our knowledge there is no information available on feeding dairy Comisana sheep with fodder made from a mixture of pelleted feed integrated with molasses and blond orange pulp. Overall, results showed that experimental fed significantly affected the physico-chemical and microbiological parameters, in contrast with findings reported by Fegeros et al. (24) who, evaluating the use of dry citrus pulp (11%) in the ration of dairy ewes, did not observe any significant effect on the composition of milk. Results of the present study firstly demonstrated that the experimental feeding system significantly decreased pH and moisture of the experimental cheeses. The low pH value could be related to the high occurrence of LAB at 60 days of ripening. It is noteworthy

TABLE 5 | Cheese fatty acid composition (g/100 g of different group of FA) of control and experimental feed at each sampling time.

	January		February		March		April		SEM	*P*-value
	Control	Experimental	Control	Experimental	Control	Experimental	Control	Experimental		
ΣSFA	77.04[a]	75.18[b]	66.94	66.44	61.13[a]	58.14[b]	64.72	64.45	0.08	0.07
ΣMUFA	18.83[a]	29.30[b]	28.69	28.42	29.95	30.85	27.12	27.37	0.12	0.06
ΣPUFA	3.48	3.53	5.52	5.36	7.23[a]	9.68[b]	6.18	6.14	0.15	0.09
n−3	1.08[a]	0.98[b]	1.67[a]	1.45[b]	2.17	2.18	2.21	2.14	0.01	0.06
n−6	2.40	2.55	3.85[a]	3.92[b]	5.06[a]	5.50[b]	4.04[a]	4.53[b]	0.06	0.08
CLAs	0.65	0.68	0.93[a]	1.14[b]	1.34[a]	1.40[b]	0.98[a]	1.10[b]	0.04	≤ 0.05
AI	4.49[a]	4.09[b]	2.76[a]	2.86[b]	2.51[a]	2.34[b]	2.89	2.84	0.03	≤ 0.05
TI	3.52	3.28	2.26[a]	2.44[b]	1.90[a]	1.70[b]	2.18	2.17	0.02	≤ 0.05

[a,b]Mean values with different letter in superscript within rows indicates significant differences ($p \leq 0.05$) due to fed. SEM. standard error of least square means.
SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; n−3, omega 3 fatty acid series; n−6, omega 6 fatty acid series; CLAs, Conjugated linoleic acids.
AI, atherogenic index; TI, thrombogenic index.

TABLE 6 | Lipid oxidative markers (TBARs) of control and experimental feed at each sampling time (values are expressed in mg MDA/kg) at 60 days of cheese ripening.

Time	January		February		March		April		SEM	*P*-value
	Control	Experimental	Control	Experimental	Control	Experimental	Control	Experimental		
0	0.07	0.07	0.05	0.03	0.07	0.06	0.04	0.05	0.03	0.94
10	0.07	0.03	0.05	0.04	0.05	0.05	0.05	0.05	0.01	0.06
20	0.05	0.08	0.06	0.05	0.08	0.07	0.06	0.07	0.03	0.07
30	0.07	0.11	0.08	0.06	0.06	0.06	0.08	0.07	0.07	0.27
40	0.24[a]	0.14[b]	0.12[a]	0.08[b]	0.09[a]	0.05[b]	0.11[a]	0.08[b]	0.06	≤ 0.05

[a,b]Mean values with different letter in superscript within rows indicates significant differences ($p \leq 0.05$) due to fed. SEM. standard error of least square means.
Time, days of storage at 4°C.
Means of six cheeses for each group with five replicates of measurements at the stated time period.
TBARs, thiobarbituric acid-reactive substances; MDA, malonaldehyde.

that LAB produce large amount of lactic acid, as a consequence of their metabolism (25), which could explain the pH lowering in the experimental cheese. In addition, the pH decrease could be related to the loss of colloidal calcium phosphate from casein submicelles with a progressive dissociation of submicelles into smaller casein aggregates, leading to a greater proteolytic effect (26). In cheese, proteolysis can have a significant effect on several attributes, including development of texture and flavor (27, 28), which highly affect the overall acceptability of the final products. In the present study, it clearly showed that the experimental feed improved the microbial diversity in derived cheese samples, particularly in terms of high occurrence of lactobacilli. In fact, the application of culture-independent approach, such as DGGE, lead to the detection of *L. rhamnosus/casei* group and *L. fermentum* species in the experimental 60 days ripened cheese, confirming their importance in the cheese proteolysis and lipolysis. It is already well established that the peptidase activity of lactobacilli could increase the levels of free amino acids (FFA), affecting the development of flavor properties of cheese (28).

One of the most important results of the present study concerns the enhancement of the daily milk production and the fat content in experimental samples, in accordance with a recent study (29). It is well known that milk fat quality is affected by the composition of feed diets (30), and that feed ingredients influence the composition of milk fatty acids (FA) (31). In this context, nutritional quality is becoming a major issue in food choices because of rising consumer awareness of the link between diet and health, increasing market demand for functional foods. An overall improvement of the nutritional composition of experimental cheese, compared to controls was achieved. Our data showed a significant increase of CLAs, which are considered bioactive compounds, with beneficial effect on human health, as demonstrated in several studies (32, 33). Yang et al. (34) reviewed the array of benefits associated with CLA, including positive effects on immune function and protective effects against cancer, obesity, diabetes, and atherosclerosis in animal and human cell line studies. However, no significant difference was revealed for MUFA, PUFA, n-3 and n-6 for the whole trial period, even if an increasing trend was observed in several sampling time. The nutritional composition of the experimental cheese was also improved by the low thrombogenicity anti-atherogenic index scores, which enhanced the health benefit of the product. In addition, our data clearly demonstrated a positive effect of the experimental feed on cholesterol content, which could be related to the antioxidant properties of citrus by-product (35). The reduction of cholesterol in foods is of nutritional interest because its high level in human plasma

TABLE 7 | Microbial counts expressed as $\log_{10}$ CFU/mL or CFU/g and standard deviation (SD) of the main microbial groups detected on sheep's milk and 60 days old cheese samples at different manufactures times.

Microbial groups	January		February		March		April		Mean	
	Control	Experimental	Control	Experimental	Control	Experimental	Control	Experimental	Control	Experimental
MILK										
Mesophilic lactobacilli	6.15 ± 0.02	6.20 ± 0.03	6.28 ± 0.07	6.20 ± 0.04	6.21 ± 0.05	6.19 ± 0.08	6.14 ± 0.07	6.11 ± 0.06	6.20 ± 0.06	6.18 ± 0.04
Thermophilic lactobacilli	5.54 ± 0.06	5.55 ± 0.06	5.56 ± 0.03	5.51 ± 0.09	5.54 ± 0.10	5.58 ± 0.07	5.55 ± 0.09	5.54 ± 0.04	5.55 ± 0.01	5.55 ± 0.03
Mesophilic lactococci	5.71 ± 0.05	5.68 ± 0.05	5.69 ± 0.04	5.64 ± 0.05	5.60 ± 0.02	5.64 ± 0.08	5.60 ± 0.06	5.65 ± 0.03	5.65 ± 0.06	5.65 ± 0.02
Streptococci	5.19 ± 0.07^{a}	5.28 ± 0.03^{b}	5.14 ± 0.02^{a}	5.33 ± 0.07^{b}	5.16 ± 0.07^{a}	5.33 ± 0.07^{b}	5.18 ± 0.05^{a}	5.35 ± 0.02^{b}	5.17 ± 0.02^{a}	5.32 ± 0.03^{b}
Enterobacteriaceae	3.16 ± 0.04^{b}	3.05 ± 0.05^{a}	3.17 ± 0.05^{b}	3.00 ± 0.07^{a}	3.16 ± 0.07^{b}	3.00 ± 0.02^{a}	3.14 ± 0.04^{b}	3.04 ± 0.06^{a}	3.14 ± 0.01^{b}	3.02 ± 0.03^{a}
Coagulase positive staphylococci	3.10 ± 0.05^{b}	2.95 ± 0.05^{a}	3.14 ± 0.06^{b}	3.00 ± 0.01^{a}	3.19 ± 0.01^{b}	2.92 ± 0.03^{a}	3.19 ± 0.02^{b}	2.96 ± 0.03^{a}	3.16 ± 0.04^{b}	2.96 ± 0.03^{a}
Coagulase negative staphylococci	2.01 ± 0.02	1.95 ± 0.01	2.04 ± 0.06	1.93 ± 0.05	2.05 ± 0.03	1.96 ± 0.04	2.08 ± 0.04	1.94 ± 0.09	2.05 ± 0.03	1.95 ± 0.01
Yeast and molds	3.14 ± 0.02^{c}	2.91 ± 0.04^{a}	3.05 ± 0.07^{b}	2.90 ± 0.02^{a}	3.14 ± 0.03^{c}	2.80 ± 0.08^{a}	3.09 ± 0.07^{b}	2.94 ± 0.02^{a}	3.11 ± 0.04^{b}	2.89 ± 0.06^{a}
Total mesophilic bacteria	5.06 ± 0.06	5.00 ± 0.08	5.05 ± 0.05	5.08 ± 0.06	5.06 ± 0.04	5.02 ± 0.04	5.04 ± 0.06	5.05 ± 0.05	5.05 ± 0.01	5.04 ± 0.04
CHEESE										
Mesophilic lactobacilli	7.29 ± 0.03	7.35 ± 0.01	7.25 ± 0.02	7.18 ± 0.03	7.26 ± 0.04	7.30 ± 0.02	7.39 ± 0.06	7.52 ± 0.09	7.30 ± 0.06	7.34 ± 0.14
Thermophilic lactobacilli	7.39 ± 0.04	7.50 ± 0.08	7.47 ± 0.03	7.72 ± 0.07	7.44 ± 0.06	7.46 ± 0.04	7.28 ± 0.04	7.38 ± 0.07	7.40 ± 0.08	7.52 ± 0.15
Mesophilic lactococci	7.40 ± 0.07	7.45 ± 0.12	7.25 ± 0.04	7.21 ± 0.06	7.26 ± 0.03	7.28 ± 0.07	7.38 ± 0.05	7.40 ± 0.04	7.32 ± 0.08	7.34 ± 0.11
Streptococci	6.17 ± 0.04^{a}	7.34 ± 0.05^{b}	6.07 ± 0.06^{a}	7.38 ± 0.04^{b}	6.13 ± 0.04^{a}	7.51 ± 0.05^{c}	6.24 ± 0.02^{a}	7.53 ± 0.08^{c}	6.15 ± 0.08^{a}	7.44 ± 0.09^{b}
Enterobacteriaceae	4.02 ± 0.05	4.00 ± 0.05	4.00 ± 0.12	4.17 ± 0.05	4.04 ± 0.04	4.00 ± 0.05	4.06 ± 0.04	4.11 ± 0.04	4.03 ± 0.03	4.07 ± 0.08
Coagulase positive staphylococci	3.40 ± 0.05^{d}	1.25 ± 0.03^{b}	3.32 ± 0.03^{c}	1.03 ± 0.06^{a}	3.43 ± 0.02^{d}	1.03 ± 0.02^{a}	3.36 ± 0.03^{c}	1.04 ± 0.01^{a}	3.38 ± 0.05^{c}	1.09 ± 0.11^{a}
Coagulase negative staphylococci	2.21 ± 0.06^{b}	1.00 ± 0.01^{a}	2.20 ± 0.03^{b}	1.01 ± 0.07^{a}	2.26 ± 0.03^{b}	1.25 ± 0.04^{a}	2.15 ± 0.03^{b}	1.06 ± 0.02^{a}	2.21 ± 0.05^{b}	1.08 ± 0.12^{a}
Yeast and molds	3.05 ± 0.07	3.00 ± 0.02	3.10 ± 0.04	3.11 ± 0.08	3.24 ± 0.06	3.13 ± 0.05	3.09 ± 0.03	3.14 ± 0.06	3.12 ± 0.08	3.10 ± 0.06
Total mesophilic bacteria	5.57 ± 0.01	5.40 ± 0.05	5.60 ± 0.03	5.42 ± 0.09	5.47 ± 0.02	5.39 ± 0.04	5.52 ± 0.03	5.64 ± 0.06	5.54 ± 0.08	5.46 ± 0.12

^{a,b,c,d}*Mean values with different letter in superscript within rows indicates significant differences ($p \leq 0.05$).*

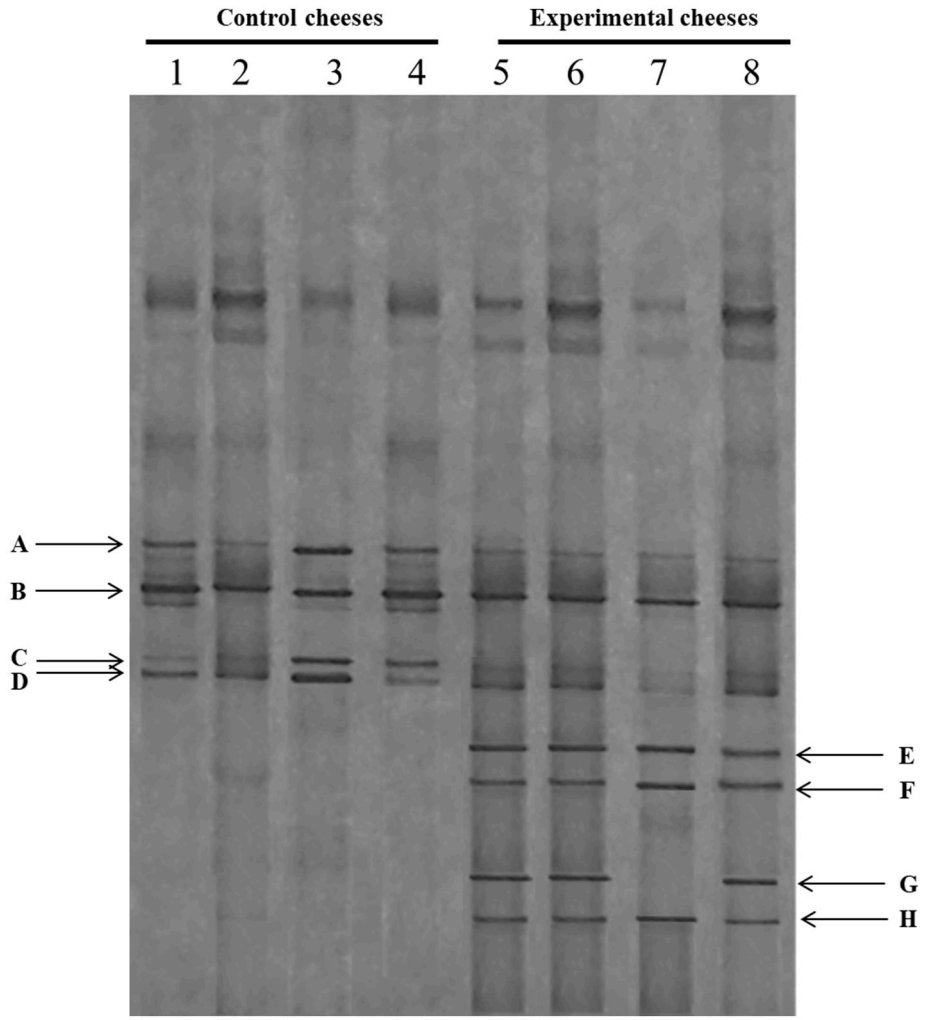

FIGURE 1 | DGGE profiles of the V2-V3 region of the 16S rRNA gene of control (C) and experimental cheese (E) samples. Lanes 1–4: C samples at different manufactures time; lanes 4–8: E samples at different manufactures time. A: *Enterococcus faecalis;* B: *Streptococcus thermophilus;* C: *Enterococcus durans*; D: *Enterococcus faecium*; E, F, H: *Lactobacillus rhamnosus/casei* group; G: *Lactobacillus fermentum*.

is associated with an increasing risk of cardiovascular disease (36, 37). The lipid oxidation in cheese, expressed by the TBARs value, was higher in control cheese, and this could lead to the formation of flavor defects and nutritional quality losses (38, 39).

Citrus fruit waste is rich in biologically active compounds, including natural antioxidants, such as phenolic acids and flavonoids (40), that show several therapeutic properties and act as antioxidant, anticancer, antitumor, and anti-inflammatory agents. Hence, the inclusion of citrus by-products in the diets for dairy ewes could increase the intake of total polyphenols, which modifying rumen fermentation (30, 41). In the present study, it has been found that LAB population clearly dominated, while staphylococci were detected at lower level in the experimental cheese. This is probably due both to the high acidification rate of LAB population and to the antimicrobial activity of polyphenols.

CONCLUSION

Citrus by-products are cheap raw material, widely available in Mediterranean countries and their potential as a feed ingredient is promising especially for sheep and cattle which are the dominant farm animal species in the area. In the present study the dietary mixture of pelleted feed integrated with molasses and blond orange pulp is a promising strategy in the diet of lactating sheep, which could improve milk and cheese nutritional quality and, therefore, enhancing the health properties of final products.

AUTHOR CONTRIBUTIONS

LL, NR, AZ, and ARDR performed the experiments. LL and CR analyzed data. LL, CR and CC wrote the manuscript. LL, CR, CC and VC designed the study and contributed to data interpretation. All authors revised the manuscript.

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

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In the present study, the β-glucosidase positive strain *Lactobacillus plantarum* F3. 3 was used as starter during the fermentation of Sicilian table olives (Nocellara Etnea cultivar) at two different salt concentrations (5 and 8%), in order to accelerate the debittering process. The latter was monitored through the increase of hydroxytyrosol compound. In addition, the potential probiotic *Lactobacillus paracasei* N24 strain was added after 60 days of fermentation. Un-inoculated brine samples at 5 and 8% of salt were used as control. The fermentation was monitored till 120 days through physico-chemical and microbiological analyses. In addition, volatile organic compounds and sensorial analyses were performed during the process and at the end of the fermentation, respectively. Lactic acid bacteria and yeasts were, in depth, studied by molecular methods and the occurrence of the potential probiotic N24 strain in the final products was determined. Results highlighted that inoculated brines exhibited a higher acidification and debittering rate than control ones. In addition, inoculated brines at 5% of salt exhibited higher polyphenols (hydoxytyrosol, tyrosol, and verbascoside) content compared to samples at 8% of NaCl, suggesting a stronger oleuropeinolytic activity of the starter at low salt concentration. Lactobacilli and yeasts dominated during the fermentation process, with the highest occurrence of *L. plantarum* and *Wickerhamomyces anomalus,* respectively. Moreover, the potential probiotic *L. paracasei* N24 strain was able to survive in the final product. Hence, the sequential inoculum of beta-glucosidase positive and potential probiotic strains could be proposed as a suitable technology to produce low salt Sicilian table olives.

Keywords: NaCl reduction, microbial debittering, starter cultures, healthy olives, molecular approach

INTRODUCTION

Among fermented vegetables, table olives are widespread in the Mediterranean area with increasing consumption in both European and non-European countries (International Olive Council, 2016). Olives are intrinsically health thanks to the high content of fiber, vitamins, and polyphenols which play a very important role, exhibiting pharmacological properties and antioxidants effects. In particular, hydroxytyrosol scavenges free radicals, inhibits human low-density lipoprotein (LDL) oxidation, inhibits platelet aggregation, and discloses anticancer activity by means of pro-apoptotic mechanisms (Raederstorff, 2009; Allouche et al., 2011; Buckland and Gonzalez, 2015).

In Sicily, table olives fermentation is mainly performed under traditional methods exploiting the fermentative action of the autochthonous microbiota. Olives are directly brined without previous debittering treatment; therefore, the indigenous microorganisms and the effect of the physico-chemical conditions of brine (pH, salt, presence/absence O_2, etc.) are mainly responsible of the hydrolysis of the oleuropein and of other β-glucosides. The oleuropein, a β-glucoside compound lending the strong "bitterness" aroma to the olive fruit, is hydrolyzed by β-glucosidases enzymes with the release of glucose and aglycones which are degraded, by an esterase, in the no-bitter phenols hydroxytyrosol, and elenolic acid (Bianchi, 2003). The spontaneous debittering is time consuming and not predictable, and it is strongly influenced by physico-chemical parameters, by the presence of fermentable substrates, and by the autochthonous microbiota. Starter cultures with oleuropein degrading activity were extensively applied in order to reduce the debittering time and to control the fermentation process (Panagou et al., 2003, 2008; Marsilio et al., 2005; Servili et al., 2006; Bevilacqua et al., 2013; Bonatsou et al., 2015), and, among them, the use of β-glucosidase positive strains could be promising (Ghabbour et al., 2011; Tataridou and Kotzekidou, 2015).

Recently, salt intake consumption hypertension and cardiovasular diseases [U.S. Dept. of Agriculture and U.S. Dept. of Health and Human Services, 2010; World Health Organization (WHO), 2012].

Recently, high attention was paid to salt intake since its overmuch intake is considered a risk factor for the onset of hypertension and cardiovascular diseases [U.S. Dept. of Agriculture and U.S. Dept. of Health and Human Services, 2010; World Health Organization (WHO), 2012]. Indeed, the set-up of table olives with low NaCl content is an issue of great interest for the sector (Bautista-Gallego et al., 2013a). However, a complete removal of salt may lead to an increased risk in the survival/ growth of spoilage or food pathogen microorganisms and may also alter food flavor, causing important economic losses. Hence, several studies evaluated the use of KCl, $CaCl_2$, and $ZnCl_2$ as NaCl replacers. It is well-demonstrated that table olives dealt with NaCl reduction and partial substitution with other salts have a more equilibrated mineral composition, enhancing the consumers' acceptance (Bautista-Gallego et al., 2010, 2011a, 2013b). Nevertheless, the effect of NaCl replaces on sensorial aspects is still controversial and strongly influenced by the concentration of the salt mixture used (Zinno et al., 2017). Recently, Pino et al. (2018a) demonstrated that the reduction of NaCl content to 5%, without any NaCl replacers, did not negatively affect the Nocellara Etnea table olives fermentation, obtaining a microbiologically safe product with appreciate sensorial traits.

Another challenge for vegetable product industry is to satisfy the increasing consumer demand for healthier products. Numerous studies demonstrated that table olives are promising carrier for probiotic strains being able to support their survival, probably thanks to the release of prebiotic substances from fruits. Additionally, their microstructure, in terms of roughness of olive surface, promotes the formation of biofilm that seem to protect probiotic bacteria from stressful conditions (such as acidic environment), favoring their survival through the human gastrointestinal tract (De Bellis et al., 2010; Arroyo-López et al., 2012a; Blana et al., 2014; Randazzo et al., 2017; Rodríguez-Gómez et al., 2017). According to that, the present study was aimed to evaluate the effect of a sequential inoculum of a β-glucosidase positive strain and probiotic bacteria on brine fermentation in order to set up a low salt Sicilian table olives.

MATERIALS AND METHODS

Pilot Scale Olives Processing

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

The experimental fermentation design comprised 8 treatments: 4 fermentations at 5% of NaCl with (F5A; F5B) and without (F5C; F5D) the addition of *L. plantarum* F3.3 strain, and 4 fermentations at 8% of NaCl with (F8A; F8B) and without (F8C; F8D) the addition of *L. plantarum* F3.3. Brine samples F5A; F5B; F8A; F8B were inoculated with the *L. plantarum* starter culture, to a final cell density of 7 log cfu/ml, directly after brining. The potential probiotic *L. paracasei* N24 strain was inoculated in F5B; F5D; F8B; F8D samples after 60 days of brining (at final cell density of 9 log cfu/ml). All fermentations were done at room temperature (18 ± 2°C), and followed up to 120 days. Marine salt was periodically added to maintain the initial concentration and fresh brine was supplied to keep olives totally dipped. Each fermentation was carried out in triplicate.

Physico-Chemical Analysis

The pH of the brines was detected by using a MettlerDL25 pHmeter (MettlerDL25, Mettler-Toledo International Inc.). Titratable acidity was determined by titring brine samples with 0.1 N NaOH and was expressed as lactic acid (g/100 ml).

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

Microbiological Analyses

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

LAB Isolation and Identification

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

Isolation and Genotypic Identification of Yeasts

From each brine samples at each sampling time, as previously reported, 200 colonies were randomly isolated from YM medium, purified, and microscopically examined prior to storing in liquid culture using 20% (v/v) glycerol at −80°C. For the yeasts characterization, DNA was extracted according to

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

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

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

Ruiz-Barba et al. (2005) and subjected to repetitive element palindromic (rep)-PCR analysis by using GTG_5 primer. The PCR reaction was carried out in a final volume of 25 μl, containing: 5 μl of DNA, 5 μl 5X PCR Buffer, 1 μl of primer GTG_5 (5-GTGGTGGTGGTGGTG-3), 13.9 μl of filtered water on 0.1 μl of Taq polymerase (Invitrogen, Italy). The amplification program was as follows; an initial denaturation (95°C, 5 min) followed by 30 cycles of denaturation (95°C, 30 s), annealing (40°C, 1 min), and extension (65°C, 8 min) with a single final extension (65°C, 16 min). PCR products were electrophoresed in a 2 % agarose gel in 1X TAE buffer, stained with ethidium bromide (30 min) and visualized under ultraviolet light. The resulting fingerprints were digitally captured and analyzed with the Bionumerics 6.6 software package (Applied Maths, Kortrijk, Belgium). Dendrogram for clustering comparison was built with UPGMA (Unweighted Pair Group Method) method and Pearson correlation.

To validate the clustering analysis and for identification of strains, the 26S rDNA gene of all isolates was further sequenced. The gDNA amplification was performed according to Porrua et al. (2018). PCR products were resolved by electrophoresis on agarose gel (1% w/v) stained with ethidium

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

	Days of fermentation				
	1	**30**	**60**	**90**	**120**
HYDROXYTYROSOL					
F5A	4.77 ± 0.01^{c}	104.13 ± 0.78^{e}	128.14 ± 0.36^{c}	199.81 ± 0.69^{c}	267.34 ± 5.56^{f}
F5B	5.24 ± 0.11^{d}	95.68 ± 0.34^{d}	101.38 ± 3.86^{a}	166.81 ± 0.79^{b}	180.12 ± 0.63^{d}
F5C	3.14 ± 0.04^{b}	86.86 ± 0.16^{c}	112.14 ± 0.23^{b}	137.87 ± 1.38^{a}	174.32 ± 1.37^{cd}
F5D	3.25 ± 0.06^{b}	84.93 ± 0.17^{bc}	113.47 ± 0.41^{b}	151.95 ± 0.49^{ab}	160.50 ± 3.87^{ab}
F8A	4.97 ± 0.01^{c}	94.16 ± 2.28^{d}	121.63 ± 1.75^{c}	191.93 ± 0.11^{c}	192.66 ± 0.30^{e}
F8B	4.98 ± 0.02^{c}	89.57 ± 1.67^{cd}	112.21 ± 1.99^{b}	153.63 ± 0.47^{ab}	172.98 ± 0.15^{cd}
F8C	3.05 ± 0.08^{b}	56.89 ± 3.17^{a}	112.21 ± 0.49^{ab}	142.40 ± 0.47^{a}	167.71 ± 2.05^{bc}
F8D	2.54 ± 0.07^{a}	80.65 ± 0.88^{b}	102.57 ± 2.00^{a}	147.98 ± 0.69^{ab}	152.78 ± 0.90^{a}
TYROSOL					
F5A	0.0 ± 0.0	8.89 ± 0.01^{d}	10.29 ± 0.06^{b}	14.77 ± 0.80^{e}	19.56 ± 0.16^{d}
F5B	0.0 ± 0.0	8.32 ± 0.37^{d}	10.28 ± 1.12^{b}	14.17 ± 0.09^{de}	13.52 ± 0.01^{bc}
F5C	0.0 ± 0.0	7.36 ± 0.02^{c}	9.37 ± 0.05^{ab}	11.29 ± 0.16^{a}	13.62 ± 0.14^{c}
F5D	0.0 ± 0.0	7.45 ± 0.01^{c}	9.66 ± 0.02^{ab}	12.58 ± 0.04^{bc}	12.66 ± 0.30^{abc}
F8A	0.0 ± 0.0	6.79 ± 0.27^{bc}	9.35 ± 0.06^{ab}	13.56 ± 0.24^{cd}	13.51 ± 0.35^{bc}
F8B	0.0 ± 0.0	6.62 ± 0.21^{b}	8.03 ± 0.36^{a}	10.98 ± 0.03^{a}	12.08 ± 0.06^{a}
F8C	0.0 ± 0.0	4.80 ± 0.06^{a}	8.58 ± 0.08*a*	11.29 ± 0.04^{a}	12.57 ± 0.45^{ab}
F8D	0.0 ± 0.0	6.78 ± 0.04^{bc}	8.32 ± 0.05^{a}	11.80 ± 0.04^{ab}	11.66 ± 0.24^{a}
OLEUROPEIN					
F5A	0.0 ± 0.0	16.07 ± 2.85^{b}	21.97 ± 0.39^{e}	35.60 ± 1.61^{c}	50.62 ± 0.59^{d}
F5B	0.0 ± 0.0	15.41 ± 2.12^{b}	11.51 ± 0.71^{bc}	20.47 ± 0.93^{ab}	21.41 ± 0.18^{ab}
F5C	0.0 ± 0.0	9.08 ± 0.22^{a}	13.51 ± 0.36^{d}	19.86 ± 2.14^{ab}	23.43 ± 0.62^{abc}
F5D	0.0 ± 0.0	8.30 ± 0.11^{a}	12.01 ± 0.33^{bc}	15.09 ± 0.45^{a}	18.81 ± 1.30^{a}
F8A	0.0 ± 0.0	9.06 ± 0.51^{a}	12.26 ± 0.05^{bcd}	26.99 ± 3.80^{b}	28.48 ± 1.22^{bc}
F8B	0.0 ± 0.0	8.32 ± 0.18^{a}	9.99 ± 0.15^{a}	22.38 ± 2.03^{ab}	24.64 ± 0.50^{abc}
F8C	0.0 ± 0.0	7.55 ± 0.10^{a}	12.79 ± 0.03^{cd}	19.22 ± 0.56^{a}	31.94 ± 3.60^{c}
F8D	0.0 ± 0.0	9.13 ± 0.24^{a}	11.19 ± 0.01^{b}	18.84 ± 1.29^{a}	24.02 ± 1.27^{abc}
VERBASCOSIDE					
F5A	0.0 ± 0.0	31.69 ± 2.39^{cd}	32.36 ± 1.95^{ab}	50.73 ± 0.17^{bc}	65.39 ± 0.41^{d}
F5B	0.0 ± 0.0	31.61 ± 1.10^{cd}	28.91 ± 3.11^{a}	54.53 ± 0.63^{cd}	58.57 ± 1.78^{c}
F5C	0.0 ± 0.0	30.03 ± 0.21^{cd}	31.72 ± 0.06^{ab}	33.32 ± 0.42^{a}	44.04 ± 0.16^{a}
F5D	0.0 ± 0.0	29.41 ± 0.25^{bc}	37.59 ± 0.71^{bc}	55.40 ± 0.93^{de}	52.57 ± 0.68^{b}
F8A	0.0 ± 0.0	32.32 ± 0.91^{cd}	36.45 ± 3.38^{bc}	59.66 ± 0.90^{e}	57.66 ± 1.39^{c}
F8B	0.0 ± 0.0	33.91 ± 1.27^{d}	40.85 ± 1.22^{c}	59.75 ± 1.56^{e}	56.92 ± 1.75^{bc}
F8C	0.0 ± 0.0	18.37 ± 0.40^{a}	32.29 ± 0.23^{ab}	35.25 ± 2.31^{a}	42.53 ± 1.64^{a}
F8D	0.0 ± 0.0	25.26 ± 0.41^{b}	31.93 ± 0.18^{ab}	47.92 ± 0.81^{b}	45.75 ± 0.74^{a}

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

TABLE 3 | Microbial counts expressed as $\log_{10}$ CFU/ml of 3 replicates ± standard deviation of the main microbial groups detected in experimental brine samples during the fermentation process.

Microbial groups	Days of fermentation				
	1	30	60	90	120
MESOPHILIC BACTERIA					
F5A	7.34 ± 0.03^{f}	7.14 ± 0.03^{d}	7.44 ± 0.03^{f}	6.79 ± 0.05^{e}	5.86 ± 0.04^{c}
F5B	7.28 ± 0.02^{bd}	6.99 ± 0.10^{d}	7.95 ± 0.03^{g}	7.43 ± 0.02^{f}	6.43 ± 0.01^{e}
F5C	6.90 ± 0.07^{f}	6.57 ± 0.10^{c}	6.35 ± 0.03^{d}	5.82 ± 0.03^{c}	5.63 ± 0.02^{d}
F5D	6.93 ± 0.02^{be}	6.44 ± 0.07^{c}	6.12 ± 0.06^{c}	6.15 ± 0.08^{d}	6.94 ± 0.06^{f}
F8A	6.72 ± 0.07^{acd}	5.84 ± 0.08^{ab}	5.76 ± 0.06^{b}	5.35 ± 0.03^{ab}	4.95 ± 0.03^{a}
F8B	6.87 ± 0.03^{bc}	5.72 ± 0.03^{a}	6.86 ± 0.02^{e}	6.94 ± 0.03^{e}	6.47 ± 0.09^{e}
F8C	6.58 ± 0.10^{a}	5.95 ± 0.02^{b}	5.38 ± 0.07^{a}	5.26 ± 0.05^{a}	5.21 ± 0.05^{b}
F8D	6.74 ± 0.03^{acde}	5.64 ± 0.11^{a}	5.51 ± 0.06^{a}	5.51 ± 0.08^{b}	5.61 ± 0.06^{d}
LACTOBACILLI					
F5A	7.11 ± 0.07^{eg}	7.73 ± 0.11^{h}	7.44 ± 0.06^{c}	6.92 ± 0.05^{d}	6.26 ± 0.13^{a}
F5B	7.20 ± 0.06^{fg}	7.74 ± 0.11^{h}	8.83 ± 0.07^{ef}	8.49 ± 0.09^{h}	8.29 ± 0.04^{c}
F5C	6.09 ± 0.06^{a}	6.97 ± 0.06^{cfg}	6.78 ± 0.06^{b}	6.21 ± 0.42^{ac}	6.02 ± 0.11^{a}
F5D	6.22 ± 0.06^{ab}	6.73 ± 0.08^{deg}	8.64 ± 0.10^{d}	7.86 ± 0.06^{ef}	7.16 ± 0.09^{b}
F8A	7.68 ± 0.18^{h}	6.69 ± 0.05^{bcd}	7.03 ± 0.08^{ab}	6.38 ± 0.09^{bc}	6.18 ± 0.06^{a}
F8B	7.02 ± 0.08^{def}	6.74 ± 0.01^{bef}	8.14 ± 0.09^{fg}	7.98 ± 0.09^{fg}	7.10 ± 0.10^{b}
F8C	6.59 ± 0.15^{bc}	6.25 ± 0.10^{a}	6.56 ± 0.06^{a}	6.00 ± 0.07^{ab}	5.87 ± 0.03^{a}
F8D	6.68 ± 0.07^{cd}	6.12 ± 0.05^{a}	8.58 ± 0.12^{eg}	7.63 ± 0.38^{eg}	6.94 ± 0.10^{b}
YEASTS					
F5A	4.04 ± 0.04^{de}	4.53 ± 0.05^{ce}	5.03 ± 0.04^{b}	7.22 ± 0.10^{d}	8.39 ± 0.12^{c}
F5B	4.37 ± 0.12^{e}	4.56 ± 0.10^{c}	6.80 ± 0.04^{d}	7.14 ± 0.12^{d}	7.07 ± 0.13^{b}
F5C	3.77 ± 0.10^{cd}	4.11 ± 0.14^{be}	6.12 ± 0.12^{c}	6.42 ± 0.09^{c}	7.11 ± 0.14^{b}
F5D	3.47 ± 0.10^{bc}	6.06 ± 0.08^{f}	5.79 ± 0.04^{c}	5.55 ± 0.05^{a}	6.93 ± 0.09^{b}
F8A	3.04 ± 0.04^{a}	3.16 ± 0.12^{a}	4.37 ± 0.03^{a}	5.64 ± 0.03^{a}	7.06 ± 0.12^{b}
F8B	3.05 ± 0.03^{ab}	4.03 ± 0.03^{b}	6.13 ± 0.14^{c}	6.51 ± 0.03^{bc}	6.04 ± 0.04^{a}
F8C	3.35 ± 0.14^{abc}	4.64 ± 0.04^{c}	6.06 ± 0.06^{c}	6.65 ± 0.03^{bc}	7.22 ± 0.10^{b}
F8D	3.24 ± 0.12^{ab}	3.42 ± 0.13^{a}	4.66 ± 0.13^{ab}	5.33 ± 0.03^{a}	6.24 ± 0.05^{a}
STAPHYLOCOCCI					
F5A	4.08 ± 0.07^{a}	4.40 ± 0.13^{c}	4.62 ± 0.03^{a}	4.25 ± 0.11^{c}	3.74 ± 0.20^{bc}
F5B	4.03 ± 0.09^{a}	4.70 ± 0.13^{d}	4.80 ± 0.10^{ab}	3.42 ± 0.21^{ab}	3.04 ± 0.06^{a}
F5C	4.15 ± 0.20^{a}	4.30 ± 0.24^{a}	4.41 ± 0.15^{a}	3.68 ± 0.52^{ab}	3.21 ± 0.08^{a}
F5D	4.06 ± 0.14^{a}	4.80 ± 0.05^{e}	4.79 ± 0.20^{ab}	3.27 ± 0.27^{a}	3.07 ± 0.11^{a}
F8A	4.85 ± 0.08^{b}	5.28 ± 0.22^{f}	4.83 ± 0.16^{ab}	4.81 ± 0.10^{d}	4.28 ± 0.14^{d}
F8B	4.21 ± 0.10^{a}	4.85 ± 0.07^{b}	5.03 ± 0.15^{b}	4.77 ± 0.17^{d}	3.98 ± 0.03^{cd}
F8C	4.80 ± 0.23^{b}	5.38 ± 0.10^{b}	5.01 ± 0.09^{b}	3.76 ± 0.14^{b}	3.51 ± 0.29^{ab}
F8D	4.31 ± 0.10^{a}	4.71 ± 0.24^{b}	5.08 ± 0.12^{b}	4.47 ± 0.20^{cd}	3.48 ± 0.19^{ab}
ENTEROBACTERIA					
F5A	2.66 ± 0.31^{a}	1.19 ± 0.15^{a}	<1	<1	<1
F5B	2.29 ± 0.09^{a}	1.49 ± 0.31^{a}	<1	<1	<1
F5C	2.28 ± 0.17^{a}	1.36 ± 0.41^{a}	<1	<1	<1
F5D	4.01 ± 0.11^{cd}	3.38 ± 0.29^{c}	2.18 ± 0.09^{a}	1.63 ± 0.15^{a}	1.55 ± 0.19^{a}
F8A	3.31 ± 0.21^{b}	3.06 ± 0.10^{bc}	2.16 ± 0.15^{a}	1.38 ± 0.27^{a}	1.21 ± 0.15^{a}
F8B	3.88 ± 0.16^{bc}	3.32 ± 0.19^{bc}	2.57 ± 0.23^{a}	1.14 ± 0.10^{a}	1.63 ± 0.14^{a}
F8C	3.41 ± 0.15^{bd}	2.72 ± 0.39^{b}	2.00 ± 0.18^{a}	1.31 ± 0.17^{a}	1.27 ± 0.17^{a}
F8D	3.43 ± 0.11^{bd}	2.75 ± 0.11^{b}	2.04 ± 0.06^{a}	1.28 ± 0.20^{a}	1.24 ± 0.14^{a}

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

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

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

Rep-PCR genomic fingerprinting was performed on 79 *L. paracasei* strains, isolated from F5B, F5D, F8B, and F8D brine samples at 120 days of fermentation, using the $(GTG)_5$-primer, as described by Versalovic et al. (1994). PCR was carried out in a 20 µl reaction mixture containing 1x Thermo Green buffer (Thermo Scientific, Waltman, MA, USA), 3.0 mM $MgCl_2$, 200 µM of each dNTP (Fermentas), 1 U of Taq polymerase (Thermo Scientific, Waltman, MA, USA), 2 µM $(GTG)_5$ primer and 50 ng gDNA. Amplifications were performed in a MyCycler thermal cycler (BioRad, Hercules, CA). The PCR cycling parameters and gel running conditions were set according to Solieri et al. (2012). The only modification was the change of annealing temperature from 40 to 45°C. The GeneRuler 100 bp Plus DNA Ladder (Thermo Scientific, Waltman, MA, USA) was used as a molecular size marker. BioDoc Gel Analyzer device (Biometra GmbH, Germany) was used to capture DNA fingerprint images which were then processed through the BioNumerics software v3.0 (Applied Maths, Sint-Martens-Latem, Belgium). Repeatability of rep-PCR was assessed using the inoculated strain N24 as internal control. Pearson's correlation similarity coefficient was chosen to calculate bands patterns similarity matrix with optimization and curve smoothening values at 1%. Unweighted pair group method with arithmetic mean (UPGMA) analysis was exploited to build the $(GTG)_5$-based dendrogram.

Analysis of Volatile Organic Compounds (VOCs)

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

Sensory Evaluation of Table Olives

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

Statistical Analysis

Microbiological and chemical (i.e., single phenol compounds, pH and acidity) data were analyzed by ANOVA (One-way Analysis of Variance) using Tukey's *post-hoc* test, in order to assess the overall differences among treatments. The reference level of significance was 0.01 for chemical assay and 0.05 for

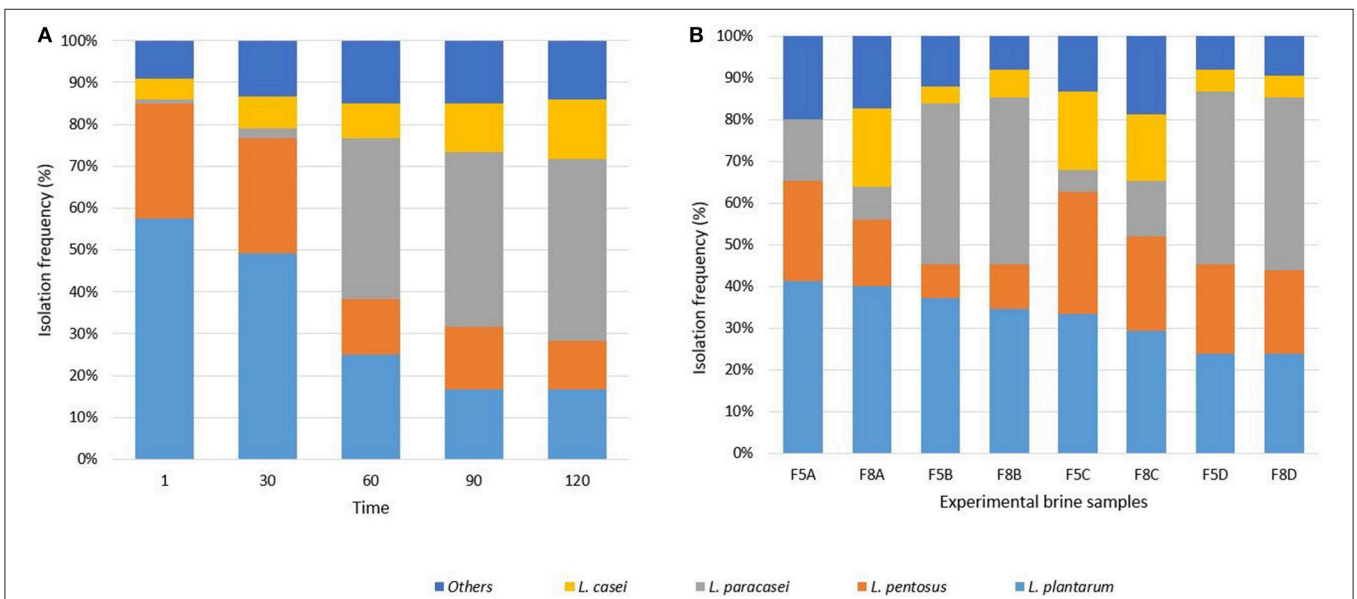

FIGURE 1 | Isolation frequency, expressed in percentage, of LAB species throughout the fermentation **(A)** and in the experimental brine samples **(B)**.

VOCs and microbiolgical assays. All statistical analyses were performed using MATLAB software (MathWorks, version 8.5.0), while sensory data were analyzed using the software package Statgraphics® Centurion XVI (Statpoint Technologies, INC.) setting samples as treatments. Data correlations between brine samples differently treated and VOCs were computed using XLStat software (version 2016.1).

RESULTS

Physico-Chemical Data

In **Table 1**, the results of pH and titratable acidity detected in brine samples analyzed thought the fermentation are shown. At the beginning of fermentation, pH values ranged from 5.8 to 6.3, and then they decreased after 30 days. Differences among samples become more appreciable after 60 days of fermentation with the lowest values showed by F5B and F8C samples. At the end of fermentation (120 days) the pH fitted the hygienic limit of 4.3 in all samples (**Table 1**).

The titratable acidity values exhibited an increasing trend in all samples with the exception of F8A and F8B brines, which slightly decreased in acidity at 120 days. The highest acidity values were detected in brines at 5% of NaCl (F5A, F5B, F5C, and F5D). In addition, a significant increase in acidity was recorded after the addition of N24 strain (90 days) mainly in F5B and F5D samples (**Table 1**).

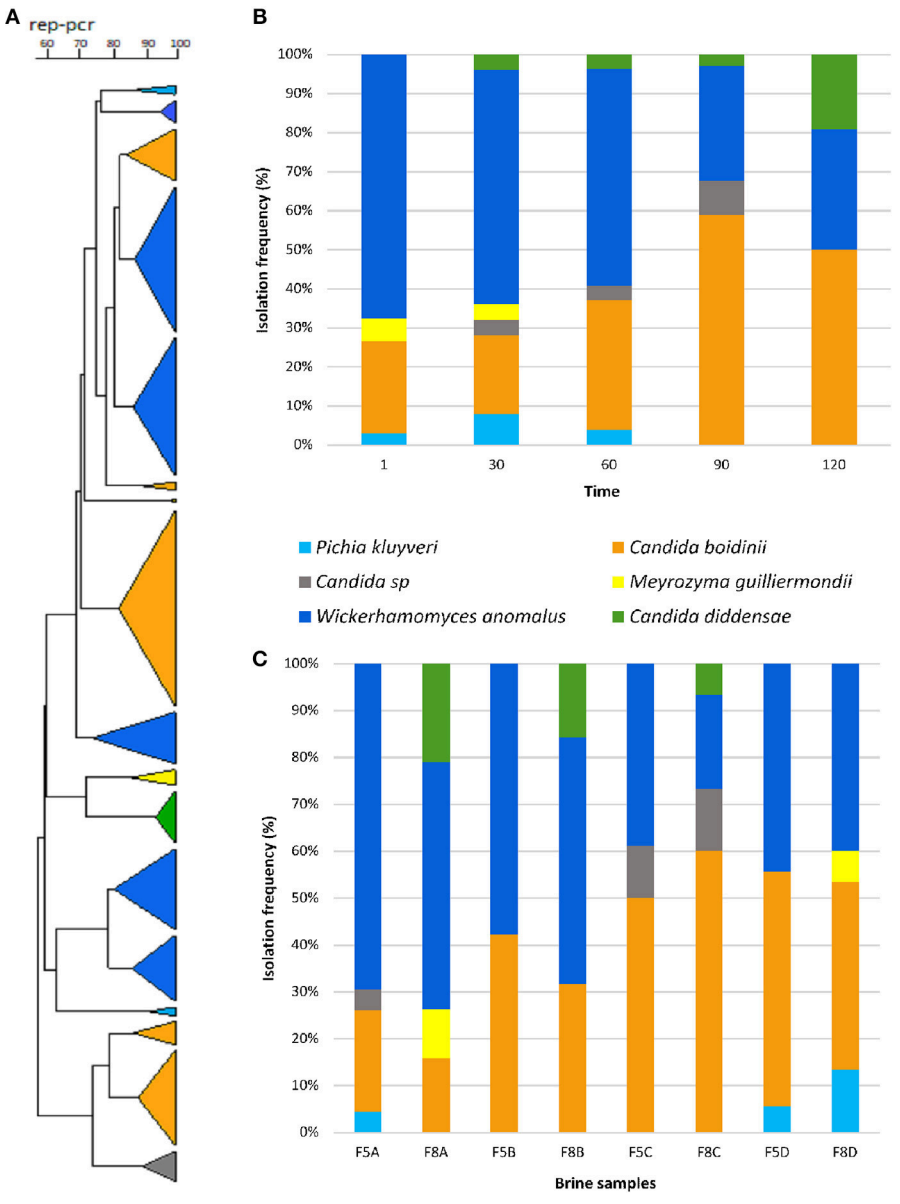

FIGURE 2 | Dendrogram generated after bioinformatics analysis with Bionumerics 6.6 software package of the rep-PCR profiles obtained with GTG_5 **(A)**. Isolation frequency (%) of yeasts during the fermentation time of Nocellara Etnea table olives **(B)**. Distribution of yeast species in different experimental brine samples **(C)**.

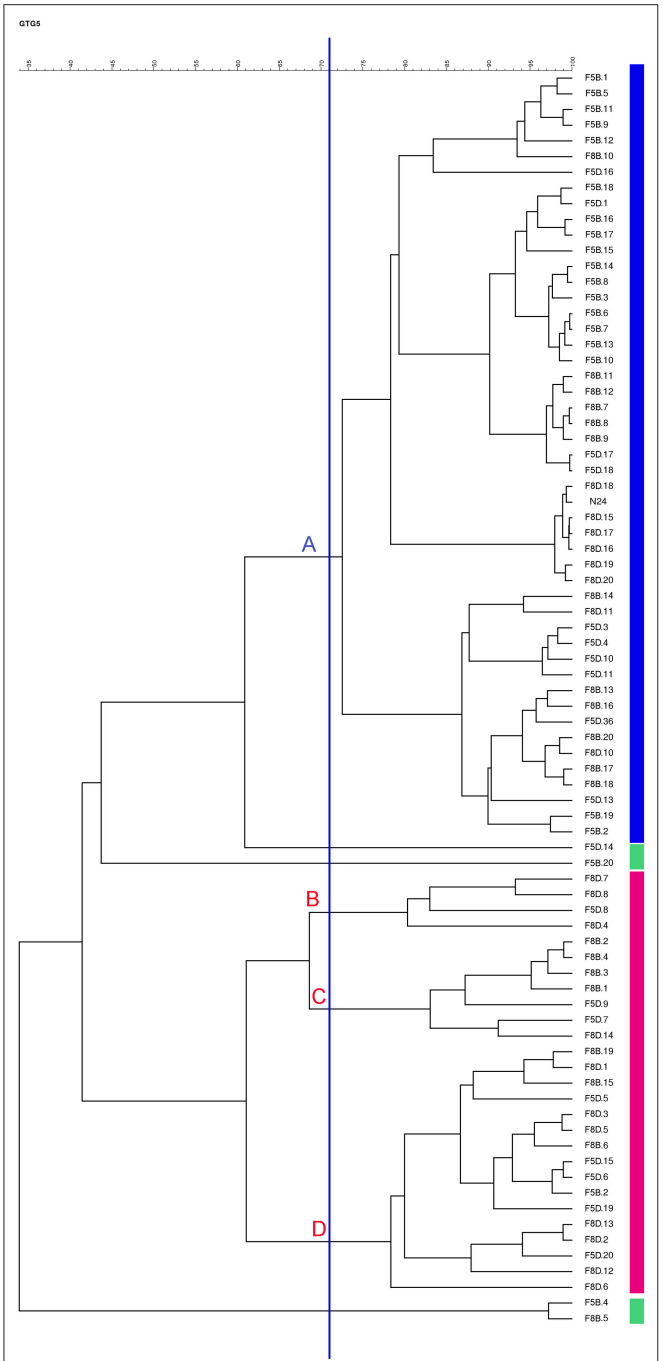

FIGURE 3 | Dendrogram generated after cluster analysis of the digitized GTG_5-PCR fingerprints of the *Lactobacillus paracasei* strains isolated from F5B, F5D, F8B, and F8D brine samples at 120 days of fermentation.

Results of hydroxytyrosol, tyrosol, oleuropein, and verbascoside quantification are shown in **Table 2**. Overall, all polyphenols analyzed showed an increasing trend during the fermentation mainly in 5% NaCl brine samples inoculated with starter culture. The highest values of hydroxytyrosol, tyrosol, and verbascoside were mainly recorded in brines inoculated with *L. plantarum* F3.3 starter culture already at 30 days. Similar behavior was observed for oleuropein.

Microbial Count

Table 3 shown microbial counts of brine samples at both 5 and 8% of NaCl, which is expressed as log cfu/ml. Viable mesophilic bacteria showed different trend among samples. In detail, brines at 5% of NaCl (F5A–F5D), from an initial average value of 7.11 log unit, exhibited a steady trend during the fermentation with slight decrease of cell density after 60 days. Similar behavior was observed for brine samples at 8% of NaCl (F8A–F8D) which showed a mean initial value of 6.73 log unit and a final mean value of 5.56 log unit (**Table 3**). Regarding LAB population, all brine samples inoculated with starter culture (F5A, F5B, F8A, F8B) presented, at the beginning of fermentation, higher cell density than spontaneous ones (F5C, F5D, F8C, F8D). From 60 to 120 days, LAB reached the highest values in samples inoculated with the potential probiotic N24 strain (F5B, F5D, F8B, F8D). Yeasts were present at an initial level of about 3 log cfu/ml in all experimental brines with the exception of F5A and F5B samples which exhibited initial value of 4.04 and 4.37 log cfu/ml, respectively (**Table 3**). The yeasts cell densities significantly increased thought the fermentation process, achieving, at 120 days, an average value of 7.37 log unit and 6.64 log unit in brine samples at 5 and 8% of NaCl, respectively. Regarding the staphylococci count, only coagulase negative staphylococci, forming red colonies in the medium, were enumerated and their level, at the beginning of the fermentation, was quite similar among all samples. After a slight increase till 60 days, a decrease to final average values of 3.0 and 3.8 log cfu/ml was achieved in 5 and 8% brine samples, respectively. Similar behavior was observed for enterobacteria counts, which significantly decreased through the fermentation process. At the end of fermentation (120 days) this microbial group was detected, at value below 2 log, in brine samples at 8% of NaCl and below the detection limit in samples at 5% of NaCl, with the exception of the F5D sample. *E. coli* was never detected in any brine samples analyzed (data not shown).

Molecular Identification of LAB

Six hundred isolates from MRS agar plates were considered LAB based on their positive Gram reaction, non-motility, absence of catalase activity and spore formation, as well as rod or coccal shape. *Rec*A and *Tuf* gene species-specific PCRs revealed the presence of strains belonging to *L. plantarum*, *Lactobacillus pentosus*, *L. paracasei*, and *Lactobacillus casei* species. The distribution of lactobacilli at different sampling times is reported in **Figure 1A** and their occurrence in the different brine samples is showed in **Figure 1B**. Isolates not identified at species level were indicated as "others." Evaluating the distribution of LAB strains through the fermentation, results indicated that *L. plantarum* and *L. pentosus* represented the dominant species at the beginning of the process (till 30th days) and a high occurrence of *L. paracasei* species was detected up to 60 days. Whereas, the highest occurrence of *L. casei* strains was achieved at 120 days (**Figure 1A**). Zooming on the fermentation at different salt content (5 and 8%) differently inoculated, results

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

Compounds	rt	T0	F5A 60	F5A 120	F5B 60	F5B 120	F5C 60	F5C 120	F5D 60	F5D 120	F8A 60	F8A 120	F8B 60	F8B 120	F8C 60	F8C 120	F8D 60	F8D 120
Acids		**0.00**	**48.46**	**92.24**	**70.48**	**150.75**	**74.29**	**124.08**	**49.57**	**40.69**	**57.77**	**35.47**	**81.06**	**111.28**	**58.14**	**135.11**	**35.60**	**50.30**
Acetic acid	24.78	0.00^{a}	26.43^{b}	58.74^{e}	46.26^{d}	77.75^{f}	72.30^{f}	96.15^{g}	44.02^{d}	39.67^{c}	29.76^{b}	35.47^{c}	50.02^{de}	62.01^{e}	58.14^{e}	106.25^{h}	35.60^{c}	34.45^{c}
Propionic acid	29.74	0.00^{a}	4.15^{d}	3.62^{d}	5.22^{e}	32.50^{f}	1.01^{b}	2.31^{c}	0.00^{a}	1.02^{b}	5.84^{e}	0.00^{a}	2.06^{c}	0.65^{b}	0.00^{ha}	3.14^{d}	0.00^{a}	0.96^{b}
Isobutyric acid	31.43	0.00^{a}	0.91^{b}	0.25^{b}	1.28^{bc}	3.10^{c}	0.98^{b}	1.98^{c}	0.00^{a}	0.00^{a}	1.02^{b}	0.00^{a}	6.56^{d}	2.01^{c}	0.00^{a}	2.26^{c}	0.00^{a}	0.00^{a}
Butanoic acid	35.01	0.00^{a}	0.32^{b}	0.62^{b}	0.90^{b}	4.37^{c}	0.00^{a}	0.15^{b}	5.55^{c}	0.00^{a}	0.62^{b}	0.00^{a}	1.05^{b}	10.60^{d}	0.00^{a}	0.96^{b}	0.00^{a}	0.00^{a}
Hexanoic acid	36.19	0.00^{a}	8.96^{b}	27.89^{f}	7.61^{b}	31.99^{f}	0.00^{a}	13.67^{cd}	0.00^{a}	0.00^{a}	10.54^{c}	0.00^{a}	18.21^{e}	34.12^{f}	0.00^{a}	12.63^{c}	0.00^{a}	14.89^{a}
2-Ethyleptanoic acid	57.20	0.00^{a}	7.69^{d}	1.12^{b}	9.21^{e}	1.04^{b}	0.00^{a}	9.82^{e}	0.00^{a}	0.00^{a}	9.99^{e}	0.00^{a}	3.16^{c}	1.89^{b}	0.00^{a}	9.87^{e}	0.00^{a}	0.00
Alcohols		**63.03**	**519.08**	**287.57**	**494.50**	**437.32**	**914.38**	**1662.74**	**276.30**	**152.56**	**539.05**	**312.90**	**962.57**	**995.99**	**960.57**	**1842.36**	**229.37**	**191.88**
Ethanol	3.33	6.77^{a}	290.25^{c}	151.13^{b}	315.24^{c}	162.54^{b}	582.12^{d}	1325.14^{e}	164.48^{b}	83.96^{b}	305.17^{c}	164.48^{b}	639.44^{d}	669.21^{d}	615.98^{d}	1485.32^{e}	152.36^{b}	125.87^{b}
Isoamylalcohol	11.58	0.00^{a}	79.84^{d}	2.61^{a}	48.91^{c}	69.71^{d}	78.83^{d}	118.13^{e}	22.14^{b}	20.16^{b}	83.78^{d}	3.01^{a}	75.12^{d}	78.65^{d}	78.01^{d}	129.58^{e}	1.03^{a}	23.04^{b}
1-Hexanol	19.23	0.00^{a}	9.52^{bc}	8.44^{b}	12.47^{c}	15.86^{d}	13.15^{c}	20.14^{e}	13.52^{c}	11.33^{c}	10.03^{c}	7.55^{b}	18.39^{e}	15.19^{d}	13.84^{c}	21.06^{e}	3.69^{a}	3.52^{a}
cis Hexen 1 ol	20.84	0.00^{a}	21.01^{d}	22.63^{d}	29.82^{e}	28.56^{e}	59.62^{g}	83.22^{i}	14.25^{c}	7.86^{b}	22.97^{d}	31.24^{e}	71.98^{h}	52.12^{f}	61.24^{g}	89.61^{i}	5.12^{b}	1.14^{a}
3-Octenol	25.13	0.00^{a}	0.00^{a}	0.96^{a}	0.86^{a}	1.99^{b}	2.05^{b}	2.63^{bc}	0.00^{a}	0.00^{a}	0.00^{a}	0.62^{a}	3.92^{d}	1.84^{b}	1.16^{a}	2.98^{c}	0.00^{a}	0.00^{a}
1-Eptanol	25.79	2.98^{b}	3.10^{b}	3.54^{b}	5.01^{c}	5.03^{c}	12.13^{e}	5.14^{c}	1.07^{a}	0.93^{a}	2.44^{b}	2.58^{b}	8.52^{d}	3.01^{b}	9.84^{d}	6.23^{c}	0.00^{a}	0.00^{a}
1-Octanol	31.12	19.58^{f}	68.11^{i}	34.99^{h}	2.84^{b}	11.84^{d}	23.14^{g}	3.21^{c}	0.86^{a}	0.51^{a}	65.28^{i}	37.37^{h}	9.68^{c}	8.72^{c}	23.69^{g}	3.02^{b}	14.32^{e}	0.00^{a}
1-Nonanol	35.91	4.11^{c}	0.00^{a}	1.05^{b}	0.00^{a}	0.00^{a}	23.96^{d}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	22.10^{d}	0.00^{a}	0.00^{a}	0.00^{a}
Benzyl alcohol	47.77	0.00^{a}	7.42^{c}	14.26^{d}	9.63^{c}	13.65^{d}	13.68^{d}	1.01^{a}	4.42^{b}	3.63^{b}	8.12^{c}	9.08^{c}	29.63^{g}	22.13^{f}	16.54^{e}	0.00^{a}	0.00^{a}	0.00
Phenylethyl alcohol	50.96	0.00^{a}	39.83^{c}	47.96^{cd}	69.72^{d}	128.14^{ef}	102.56^{e}	103.89^{e}	55.56^{d}	24.18^{b}	41.26^{c}	56.97^{d}	105.89^{e}	145.12^{f}	114.21^{e}	104.56^{e}	52.85^{d}	38.31^{a}
1-U<1ecanol	53.54	27.49^{c}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	3.14^{b}	0.23^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	3.96^{b}	0.00^{a}	0.00^{a}	0.00^{a}
Esters		**4.09**	**181.46**	**200.51**	**283.60**	**421.49**	**334.35**	**448.16**	**284.96**	**363.63**	**274.22**	**358.87**	**287.75**	**433.17**	**378.62**	**579.82**	**306.71**	**308.86**
Ethyl acetate	2.75	0.00^{a}	140.11^{b}	145.91^{b}	129.71^{b}	149.52^{b}	147.22^{b}	248.78^{d}	136.82^{b}	298.72^{e}	205.62^{c}	244.42^{d}	132.71^{b}	178.12^{c}	205.61^{c}	325.16^{e}	222.53	195.93
Ethyl propanoate	3.56	0.00^{a}	5.71^{b}	1.49^{a}	0.00^{a}	7.02^{b}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	7.69^{b}	7.71^{b}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}
Ethyl butanoate	4.07	0.00^{a}	0.00^{a}	2.50^{a}	1.88^{a}	9.02^{c}	3.96^{b}	1.02^{a}	0.00^{a}	0.00^{a}	7.53^{c}	0.00^{a}	2.48^{a}	10.86^{c}	8.88^{c}	0.00^{a}	0.00^{a}	0.00^{a}
Methyl 2-methylbutanoate	5.44	0.00^{a}	0.00^{a}	8.37^{b}	15.12^{c}	91.13^{g}	12.33^{c}	61.20^{e}	11.72^{c}	0.00^{a}	0.00^{a}	22.94^{d}	6.42^{b}	83.25^{g}	25.84^{d}	70.58^{f}	10.83^{bc}	14.18^{c}
Methyl 3-methylbutanoate	5.85	0.00^{a}	0.00^{a}	0.72^{a}	12.09^{e}	61.05^{g}	1.04^{a}	8.52^{d}	0.00^{a}	1.14^{a}	0.00^{a}	16.24^{f}	6.23^{c}	16.14^{f}	2.51^{b}	10.58^{e}	0.00^{a}	15.42^{f}
Isoamylacetate	7.47	0.00^{a}	8.52^{c}	0.44^{a}	8.13^{c}	10.28^{c}	105.31^{l}	39.18^{f}	96.21^{i}	25.47^{e}	16.18^{d}	38.55^{f}	5.62^{b}	18.12^{d}	3.46^{b}	44.01^{g}	54.99^{h}	57.92^{h}
Methyl hexanoate	9.90	0.00^{a}	0.00^{a}	2.43^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.21^{a}	0.00^{a}	0.47^{a}	14.83^{b}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}
Ethyl hexanoate	12.29	0.00^{a}	0.00^{a}	0.46^{a}	7.58^{c}	4.02^{b}	3.65^{b}	3.68^{b}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	11.13^{e}	9.06^{d}	9.01^{d}	5.12^{b}	0.00^{a}	0.00^{a}
Ethyl lactate	18.49	0.00^{a}	0.00^{a}	6.14^{b}	29.34^{e}	39.42^{f}	10.97^{c}	31.74^{e}	0.00^{a}	5.11^{b}	0.00^{a}	0.00^{a}	32.67^{e}	52.36^{h}	23.12^{d}	45.49^{g}	0.00^{a}	0.00^{a}
Methyl 2-hydroxy-3-methylbutanoate	23.10	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	3.96^{b}	0.21^{a}	7.76^{c}	0.00^{a}	0.00^{a}	0.00^{a}	11.09^{d}	0.00^{a}	4.05^{b}	0.00^{a}	8.23^{c}	0.00^{a}	0.00^{a}
Ethyl octanoate	23.44	0.00^{a}	0.00^{a}	2.95^{c}	1.87^{b}	0.26^{a}	1.48^{b}	1.02^{b}	0.00^{a}	4.60^{c}	0.00^{a}	0.00^{a}	3.02^{c}	0.75^{b}	9.09^{d}	1.81^{b}	0.00^{a}	0.00^{a}
Ethyl-3-hydroxybutyrate	28.92	0.00^{a}	18.04^{d}	9.20^{c}	12.47^{c}	6.99^{b}	0.00^{a}	0.80^{a}	0.00^{a}	8.17^{b}	0.00^{a}	0.00^{a}	3.82^{b}	6.31^{b}	0.00^{a}	1.63^{b}	0.00^{a}	10.85^{c}
Ethyl 2-hydroxy-4-methylpentanoate	30.00	0.00^{a}	1.50^{a}	10.49^{e}	6.15^{c}	7.54^{d}	3.96^{b}	7.14^{d}	9.38^{e}	4.67^{c}	3.60^{b}	5.06^{c}	11.36^{f}	12.12^{f}	6.77^{cd}	14.75^{g}	3.54^{b}	5.60^{c}
Methyl decanoate	32.95	2.95^{b}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.23^{a}	0.00^{a}	0.00^{a}
Ethyl decanoate	35.77	0.00^{a}	0.00^{a}	0.00^{a}	1.24^{b}	0.33^{a}	1.97^{b}	2.23^{b}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	1.68^{b}	1.46^{b}	1.86^{b}	4.96^{c}	0.00^{a}	0.00^{a}
Ethyl benzoate	36.74	0.00^{a}	0.00^{a}	0.95^{b}	13.82^{d}	3.68^{c}	1.45^{b}	1.88^{b}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	14.58^{d}	5.02^{c}	2.41^{b}	5.12^{c}	0.00^{a}	0.00^{a}
Acetic acid, 2 phenylethyl	43.01	0.00^{a}	0.00^{a}	1.68^{b}	7.28^{d}	2.13^{b}	0.68^{a}	1.63^{b}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	1.73^{b}	2.36^{b}	1.72^{b}	4.16^{c}	0.00^{a}	0.00^{a}
Methyl hydrocinnamate	45.91	1.14^{b}	0.00^{a}	3.65^{c}	0.00^{a}	7.61^{e}	5.23^{d}	2.22^{b}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	10.03^{f}	12.13^{f}	10.36^{f}	5.16^{d}	0.00^{a}	0.00^{a}

(Continued)

TABLE 4 | Continued

Compounds	rt	T0	F5A 60	F5A 120	F5B 60	F5B 120	F5C 60	F5C 120	F5D 60	F5D 120	F8A 60	F8A 120	F8B 60	F8B 120	F8C 60	F8C 120	F8D 60	F8D 120
Ethyl dodecanoate	46.20	0.00^{a}	0.00^{a}	0.00^{a}	8.36^{c}	0.48^{a}	0.71^{a}	9.04^{c}	6.03^{b}	0.00^{a}	8.55^{c}	0.00^{a}	12.12^{d}	1.58^{a}	1.12^{a}	21.85	0.00	0.00
Ethyl hydrocinnamate	48.97	0.00^{a}	7.58^{c}	3.12^{b}	28.56^{l}	17.05^{g}	34.18^{m}	20.11^{h}	24.80^{j}	15.28^{f}	10.22^{cd}	12.86^{e}	32.15^{m}	19.48^{h}	66.86^{n}	10.98^{d}	14.82^{f}	8.96^{c}
Aldheydes		**149.90**	**87.40**	**62.52**	**36.83**	**72.14**	**44.29**	**50.71**	**32.71**	**1.40**	**120.28**	**117.41**	**53.09**	**34.44**	**42.84**	**71.32**	**45.34**	**16.55**
Octanal	14.81	14.12^{e}	10.58^{d}	6.70^{c}	6.52^{c}	5.98^{bc}	6.48^{c}	5.12^{b}	0.00^{a}	0.61^{a}	9.21^{d}	10.64^{d}	13.58^{e}	5.86^{b}	4.47^{b}	9.36^{d}	0.00^{a}	0.00^{a}
Nonanal	20.78	65.32^{l}	40.78^{h}	4.25^{b}	11.93^{d}	31.85^{g}	14.56^{d}	18.17^{e}	13.25^{d}	0.00^{a}	32.76^{g}	52.07^{i}	19.63^{e}	9.86^{c}	14.02^{d}	25.12^{f}	24.46^{f}	1.76^{b}
3-Octanal	21.64	0.00^{a}	0.00^{a}	1.90^{b}	1.58^{b}	1.96^{b}	2.36^{b}	2.36^{b}	1.74^{b}	0.79^{a}	0.00^{a}	26.47^{e}	2.56^{b}	1.02^{a}	2.36^{b}	2.15^{b}	12.78^{d}	5.27^{c}
Decanal	26.85	68.82^{h}	30.75^{f}	41.89^{g}	6.78^{b}	18.44^{d}	9.57^{c}	9.99^{a}	7.46^{b}	0.00^{a}	24.09^{e}	0.00^{a}	5.69^{b}	5.68^{b}	11.00^{c}	18.96^{d}	0.00^{a}	7.47^{b}
Benzaldehyde	28.08	1.64^{b}	5.29^{c}	7.78^{d}	10.02^{e}	13.91^{f}	11.32^{e}	15.07^{f}	10.26^{e}	0.00^{a}	54.22^{h}	28.23^{g}	11.63^{e}	12.02^{e}	10.99^{e}	15.73^{f}	8.10^{d}	2.05^{b}
Phenols		**0.00**	**102.59**	**49.90**	**21.94**	**144.69**	**116.02**	**146.22**	**26.95**	**72.45**	**137.38**	**147.21**	**7.80**	**43.19**	**112.46**	**110.56**	**5.61**	**2.24**
Guaiacol	47.25	0.00^{a}	0.00^{a}	2.99^{b}	0.00^{a}	29.08^{d}	57.16^{e}	61.12^{f}	12.85^{c}	3.58^{b}	0.00^{a}	0.00^{a}	0.00^{a}	11.02^{c}	56.23^{e}	53.01^{e}	0.00^{a}	0.00^{a}
Creosol	52.57	0.00^{a}	102.59^{h}	43.69^{e}	10.50^{b}	98.65^{g}	17.89^{bc}	44.32^{e}	12.54^{a}	52.36	95.11	106.62	3.16	23.14	19.67	28.15	0.00	0.00
Phenol	55.36	0.00^{a}	0.00^{a}	0.63^{a}	1.33^{b}	3.56^{c}	5.69^{d}	9.75^{e}	1.56^{b}	2.58^{c}	0.00^{a}	0.00^{a}	0.23^{a}	1.02^{b}	4.50^{d}	10.42^{e}	0.00^{a}	0.00^{a}
4-Ethyl phenol	63.20	0.00^{a}	0.00^{a}	2.59^{b}	10.11^{d}	13.40^{e}	35.28^{g}	31.03^{h}	0.00^{a}	13.93^{e}	42.27^{h}	40.59^{h}	4.41^{c}	8.01^{d}	32.06^{g}	18.98^{f}	5.61^{c}	2.24^{b}
Total		217.02	938.99	692.74	907.35	1226.39	1483.33	2431.91	670.49	630.72	1128.70	971.86	1392.27	1618.07	1552.63	2739.17	622.63	569.83

a–h: for each compound different superscript letters in the same raw are significantly different ($P < 0.05$). Bold value indicates VOCs class and the total of them.

showed the dominance of *L. plantarum* strains in samples inoculated with starter, the highest occurrence of *L. casei* and *L. pentosus* in spontaneous samples (F5C, F8C, F5D, F8D) and of *L. paracasei* in samples inoculated with the probiotic strain N24 (**Figure 1B**).

Isolates not identified initially with *rec*A and *Tuf* gene primer pairs were subjected to PCR-RFLP analysis of the 16SrDNA and clustered into four different groups (data not shown). One representative isolate for each cluster was identified by 16S rRNA gene sequencing and were deposited in the GenBank database. The species attribution and the accession numbers of the sequenced strains were as follows (isolates code in parentheses): *Leuconostoc mesenteroides* MK085109 (F5C.1), *Lactococcus lactis* MK085110 (F5B.38), *Lactobacillus brevis* MK085111 (F5D.44), and *Enterococcus faecium* MK085112 (F5A.21).

Molecular Identification of Yeasts

Two-hundred yeast isolates were randomly obtained during the fermentation process. The dendogram generated by rep-PCR with primer GTG_5 showed that the isolates formed 17 groups clearly differentiated. The most numerous groups belonged to the *Wickerhamomyces anomalus* and *Candida boidinii* species, although representatives of *Candida diddensiae*, *Pichia kluyveri* and *Meyerozyma guillermondii* were also identified (**Figure 2A**).

The evolution of the different yeast species throughout the fermentative process is presented in **Figure 2B**. At this regard, *W. anomalus* and *C. boidinii* formed a stable dual species consortium through the fermentation, since they were both detected more frequently than others species, with a mean frequency of 49 and 37%, respectively. Indeed, these species were dominant in all brine samples differently treated (**Figure 2C**). The rest of the species were isolated at very low mean frequencies; in particular, *M. guillermondii* (1.97%) was detected only in samples at 8% of NaCl till 30th days of fermentation, whereas, *P. kluyveri* (2.98%) in samples F5A, F5D, and F8D till 60 days and *C. diddensiae* (5.87%) was detected starting from the 30th day of fermentation only in brines at 8% of NaCl (**Figure 2C**).

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

The presence of inoculated strain N24 was assessed at 120 days of fermentation by rep-PCR with primer GTG_5 on a pool of 79 *L. paracasei* isolates from samples F5B, F5D, F8B, and F8D. Preliminarily, the rep-PCR repeatability was evaluated using gDNA from strain N24 as internal control in four different gels, obtaining a similarity of 74.3% (data not shown). Accordingly, this value was retained as similarity threshold to establish the identity of isolates compared to the rep-PCR profile of promising probiotic strain N24. The dendrogram generated using the GTG_5-based patterns of *L. paracasei* isolates revealed the presence of four major clusters (from A to D) and four singleton *L. paracasei* isolates below 74.3% similarity (**Figure 3**; **Supplementary Figure 1**). The cluster analysis showed that the major cluster A grouped both the inoculated strain N24 and 48 out of 79 isolates, indicating that these isolates were assimilated

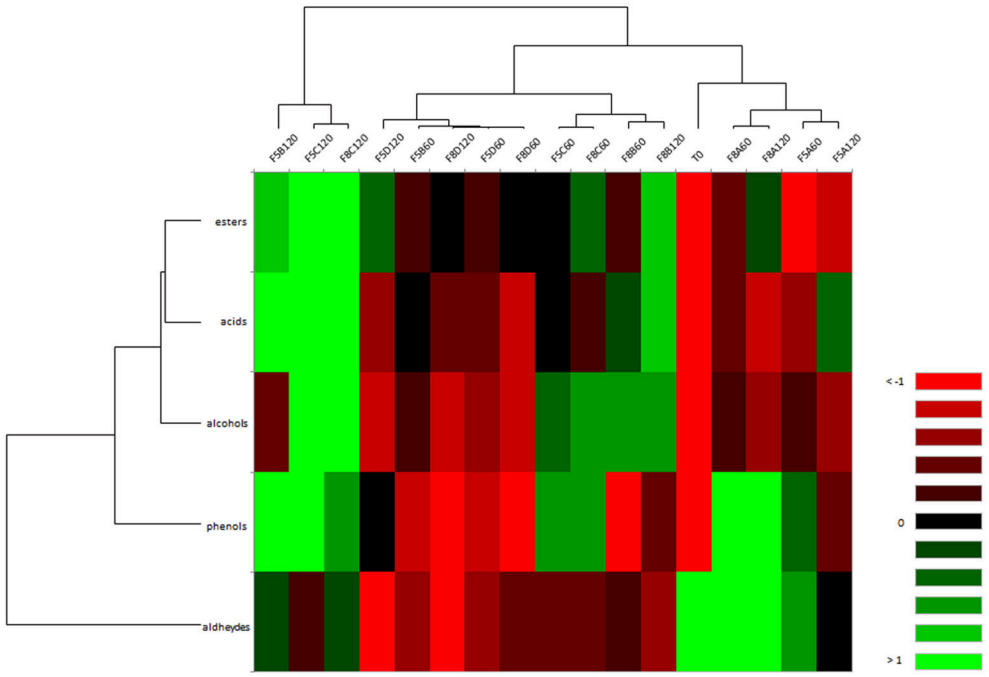

FIGURE 4 | Heat map of correlation between VOCs and F5 (A-D) and F8 (A-D) brine samples at 60 and 120 days of fermentation. The colors of the scale bar denote the nature of the correlation, with 1 indicating a perfectly positive correlation (green) and −1 indicating a perfectly negative correlation (red)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

to the N24 strain profile. The majority of them were isolated from samples F5B and F8B, over the indigenous *L. paracasei* isolates. In detail, out of 48, 18 strains were isolated from F5B, 12 from F8B, 10 from F5D, and 8 from F8D. The remaining isolates belonged to clusters B, C, and D.

Volatile Organic Compounds (VOCs)

Results of VOCs of different brine samples at 60 and 120 days of fermentation are reported in **Table 4**. Overall, 46 compounds as acids, alcohols, esters, aldehydes, and phenols were identified, exhibiting a growing trend through the fermentation, with the exception of samples F5A, F5D, F8A, and F8D. The highest value was registered in samples F8C, with a value of 2,739.17 μg/l. Alcohols were the most abundant compounds, followed by esters and acids, whereas aldehydes and phenols were detected at lower concentrations. Focusing on each compound, among alcohols, ethanol dominated the fermentation process showing an increase only in spontaneous samples (F5C and F8C), followed by isoamylalcohol and phenylethylalcohol, which registered a variable trend through the fermentation (**Table 4**). Ethyl-acetate and methyl 2-methylbutanoate were the main detected esters. Among acids, the acetic acid was the most abundant compound, with the highest value in F5C and F8C samples. The most abundant aldehydes and phenols were nonanal, benzaldehyde and creasol, respectively (**Table 4**).

Figure 4 shows correlation between VOCs and brine samples differently treated. Overall, it is possible to point out that the salt concentration did not influence the VOCs formation through fermentation in brine samples, which were mainly grouped based on the treatment (starter and/or probiotic addition and spontaneous). In detail, samples inoculated with starter (F5A and F8A) were clustered together, showing a negative correlation with alcohol and ester compounds; spontaneous brine samples inoculated with the probiotic strain N24 (F5D and F8D) were negatively correlated to phenols, aldehydes and alcohols. Different correlations were detected for samples inoculated with both starter and probiotic strains (F5B and F8B). In particular, sample F8B at 60 and 120 days of fermentation were grouped together, exhibiting a positive correlation with alcohols and acids and a negative correlation with phenols and aldehydes (**Figure 4**). Evaluating sample F5B, it is possible to assert that VOCs formation was strongly influenced by the fermentation time. In fact, the sample F5B at 120 days revealed a distinct VOCs profile, displaying a positive correlation with esters, acids, and phenols. Similarly, spontaneous fermentation samples (F5C and F8C) were grouped based on fermentation time, showing a divergent VOCs profile through the fermentation.

Sensory Data

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

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

	Descriptors														
	Abnormal fermentation						Gustatory sensations			Kinaesthetic sensations			Overall acceptability
Samples	Musty	Rancid	Cooking effect	Soapy	Metallic	Earthy	Winey-vinegary	Acidity	Saltiness	Bitterness	Hardness	Fibrousness	Crunchiness	
F5A	1.1 ± 0.18^{a}	0.6 ± 0.29^{a}	0.6 ± 0.22^{a}	0.2 ± 0.14^{a}	0.6 ± 0.26^{a}	1.6 ± 0.12^{a}	1.3 ± 0.24^{a}	2.7 ± 0.18^{a}	5.4 ± 0.11^{a}	2.3 ± 0.09^{a}	6.4 ± 0.24^{b}	2.1 ± 0.06^{a}	7.2 ± 0.26^{a}	7.5 ± 0.04^{a}
F5B	1.1 ± 0.37^{a}	0.5 ± 0.43^{a}	0.6 ± 0.16^{a}	0.3 ± 0.11^{a}	0.8 ± 0.21^{a}	1.5 ± 0.29^{a}	1.2 ± 0.28^{a}	2.5 ± 0.26^{a}	5.3 ± 0.33^{a}	2.0 ± 0.14^{a}	6.0 ± 0.21^{a}	2.8 ± 0.45^{a}	7.5 ± 0.19^{a}	9.8 ± 0.41^{b}
F5C	1.3 ± 0.21^{a}	0.6 ± 0.34^{a}	0.8 ± 0.24^{a}	0.4 ± 0.21^{a}	0.6 ± 0.09^{a}	1.6 ± 0.26^{a}	1.0 ± 0.31^{a}	7.9 ± 0.12^{b}	5.4 ± 0.25^{a}	6.7 ± 0.24^{b}	5.9 ± 0.33^{a}	2.2 ± 0.28^{a}	7.6 ± 0.21^{a}	6.9 ± 0.11^{a}
F5D	1.1 ± 0.38^{a}	0.5 ± 0.26^{a}	0.7 ± 0.26^{a}	0.3 ± 0.04^{a}	0.7 ± 0.19^{a}	1.6 ± 0.61^{a}	0.8 ± 0.21^{a}	2.9 ± 0.21^{a}	5.8 ± 0.34^{a}	6.9 ± 0.29^{b}	6.3 ± 0.25^{a}	2.5 ± 0.36^{a}	7.7 ± 0.28^{a}	6.7 ± 0.32^{a}
F8A	1.1 ± 0.26^{a}	0.4 ± 0.22^{a}	0.5 ± 0.18^{a}	0.3 ± 0.04^{a}	0.5 ± 0.24^{a}	1.4 ± 0.58^{a}	1.2 ± 0.39^{a}	2.7 ± 0.61^{a}	5.4 ± 0.10^{a}	2.5 ± 0.09^{a}	6.2 ± 0.18^{a}	2.7 ± 0.29^{a}	7.6 ± 0.35^{a}	7.6 ± 0.09^{a}
F8B	1.3 ± 0.24^{a}	0.6 ± 0.28^{a}	0.8 ± 0.23^{a}	0.2 ± 0.35^{a}	0.5 ± 0.22^{a}	1.4 ± 0.29^{a}	0.9 ± 0.15^{a}	2.6 ± 0.86^{a}	5.6 ± 0.06^{a}	2.6 ± 0.32^{a}	5.9 ± 0.24^{a}	2.1 ± 0.43^{a}	7.5 ± 0.32^{a}	9.3 ± 0.45^{b}
F8C	1.4 ± 0.27^{a}	0.4 ± 0.21^{a}	0.5 ± 0.26^{a}	0.4 ± 0.25^{a}	0.7 ± 0.13^{a}	1.5 ± 0.54^{a}	1.3 ± 0.44^{a}	8.8 ± 0.37^{c}	5.7 ± 0.21^{a}	7.3 ± 0.43^{b}	6.2 ± 0.05^{a}	2.7 ± 0.51^{a}	7.6 ± 0.33^{a}	6.4 ± 0.42^{a}
F8D	1.4 ± 0.32^{a}	0.3 ± 0.35^{a}	0.7 ± 0.28^{a}	0.4 ± 0.23^{a}	0.8 ± 0.31^{a}	1.4 ± 0.61^{a}	1.3 ± 0.43^{a}	2.9 ± 0.53^{a}	5.7 ± 0.34^{a}	7.2 ± 0.32^{b}	6.4 ± 0.09^{a}	2.5 ± 0.44^{a}	7.8 ± 0.31^{a}	6.8 ± 0.38^{a}

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

(F5C, F5D, F8C, and F8D). Finally, F5B and F8B samples received higher scores for the overall acceptability descriptor.

DISCUSSION

A current challenge in the processing technology of table olives is the selection of starter cultures able to fasten and safely drive the fermentation process. In contrast to industrial starter cultures, autochthonous strains, that naturally dominate spontaneous fermentation, tend to have high metabolic capacities, which can beneficially affect the quality of the final product. In addition, it was already established that the microbial dynamics through fermentation is influenced by the technology applied (e.g., salt reduction). One of the most widely employed strategies to reduce sodium content in table olives is the use of NaCl substitutes, which can be added alone or in combination with other salts (Bautista-Gallego et al., 2013a). Few studies have evaluated the possibility to setup low NaCl table olives without any salt replacement. Based on our previously reported data (Randazzo et al., 2017; Pino et al., 2018a), in the present study a wild β-glucosidase positive strain was used both as debittering and as driven agent during olive fermentations at lowered salt content (5%). It is well-known that β-glucosidase enzyme is important for oleuropein hydrolysis and, among lactobacilli, *L. plantarum* species has been successfully used as starter for its strong ability to break the glycosidic bond of oleuropein (Ciafardini et al., 1994; Tataridou and Kotzekidou, 2015) and for its high versatility. Overall, our data revealed that all brine samples reached a pH value $\leq$4.3 and exhibited a good acidification rate, indicating the success of the fermentation and ensuring the microbiological safety of the final product, in accordance to other researches (Corsetti et al., 2012; Martorana et al., 2017). In particular, samples inoculated with the β-glucosidase positive strain exhibited a more pronounced reduction of the fermentation time, with a higher content of hydroxytyrosol, tyrosol, and verbascoside compounds from 30 days of fermentation than un-inoculated ones, according to other studies (Romero et al., 2004; Ben Othman et al., 2009; Pistarino et al., 2013; Kaltsa et al., 2015). In addition, the autochthonous strain exhibited a better adaptation/growth rate in brine samples at 5% of salt. It is well-established that autochthonous strains are generally more adapted to harsh conditions of raw material than allochthonous ones, and, therefore, to dominate the microbiota, driving the fermentation and counteracting spoilage microorganisms (Di Cagno et al., 2008; Bevilacqua et al., 2015). Microbiological data indicated a significant reduction of *Enterobacteriaceae* starting from 30 days of fermentation, with an improvement of the safety of the final product, as previously reported (Pino et al., 2018a). It is interesting to point out that results revealed a high occurrence of yeasts as part of table olive natural microbiota, coexisting with LAB during the whole fermentation process (Arroyo-López et al., 2008, 2012a,c), which could be related to the geographic area, and cultivars (Bleve et al., 2014). Our data registered an unusual yeast count at the end of the process, higher than Spanish-style and Sicilian-style table olives, which generally reached value of 4-5- log CFU/ml. This could be linked to the processing

technology applied in the present study. Yeasts favor bacteria growth, enhancing lactic acid production to inhibit spoilage microorganisms and affect flavor and texture of the final products (Arroyo-López et al., 2008, 2012b; Bevilacqua et al., 2013). As reported in a recent review, *Candida boidinii*, *Debaryomyces hansenii*, and *Pichia membranifaciens* were revealed as the most geographically diffused species (Campus et al., 2018). Evaluating yeast behavior, although it is noteworthy that yeast development is related to high salt level and phenolic compounds or low pH. In the present study, *W. anomalus,* and *C. boidinii* were the species mainly detected in brines processed at 5% of salt, whereas *C. diddensae* and *M. guilliermondii* were mainly revealed in brines at 8% NaCl. Several studies reported strong β-glucosidase activity for *W. anomalus* species (Bautista-Gallego et al., 2011b; Arroyo-López et al., 2012c; Romero-Gil et al., 2013; Bonatsou et al., 2015), and strong lipase and esterase activities for *C. boidinii* species, which positive impacts to fruity and olive flavor (Hernández et al., 2007; Bautista-Gallego et al., 2011b; Arroyo-López et al., 2012c; Pereira et al., 2015). In contrast to previously published data (Hurtado et al., 2008; Bautista-Gallego et al., 2011b), *C. diddensae,* which is generally associated to the early stage of fermentation, was detected at the highest frequency at 120 days. Focusing on LAB population, *L. plantarum, L. pentosus,* and *L. paracasei* were the main species found in all brine samples, confirming both their key role in table olive fermentation and biofilm formation with yeasts. In addition, a high survivability of the potential probiotic *L. paracasei* N24 strain was depicted in the final products, mainly in samples at 5% of NaCl. This evidence confirms its suitability to growth in harsh environment, such as brines, and that table olives are able to support probiotic survival (Lavermicocca et al., 2005; Rodríguez-Gómez et al., 2017; Pino et al., 2018a). In fact, nutrients and prebiotics released into the brines favor the biofilm formation, protecting bacteria from acidic environment and enhancing their passage through 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). It is interesting to point out that the addition of the potential probiotic strain at 60 days of fermentation in brine samples processed at low salt content and with starter, significantly modified the VOCs pattern. In particular, compounds responsible for floral and fruity notes, such as phenylethyl alcohol and methyl 2-methylbutanoate, highly increased, while ethanol and isoamyl-alcohol significantly deceased compared to un-inoculated samples. The high content of alcohols in un-inoculated brine samples could be related to yeast metabolic activities (Bleve et al., 2014; Randazzo et al., 2017). This evidence was in accordance to sensory data since panelists attributed the higher score to the bitterness and acid descriptors in un-inoculated samples. Finally, data obtained from correlation between VOCs and brine samples differently treated revealed that the VOCs formation was mainly influenced by the starter and/or probiotic addition instead of salt content.

CONCLUSION

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

AUTHOR CONTRIBUTIONS

AP, FR, AT, AV, and LS performed the experiments and analyzed the data. AP, JB-G, and CR wrote the manuscript. CR, JB-G, and CC designed the study. CR, JB-G, and FA-L contributed to data interpretation. All authors revised the manuscript.

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SUPPLEMENTARY MATERIAL

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Wholegrain Durum Wheat Bread Fortified With Citrus Fibers: Evaluation of Quality Parameters During Long Storage

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The aim of this work was to evaluate the effect of the addition of citrus fibers, from blood orange and lemon peels to produce a functional durum wheat bread. Breads fortified in fiber were packaged under a modified atmosphere (MAP) and stored at 25°C up to 120 days. No significant differences were observed with respect to the specific volume and weight, internal structure, pH and titratable acidity among the bread samples obtained using different types and percentages of fibers. Storage time, at 30 up to 90 days, affected significantly the bread firmness and caused significant differences in 5-hydroxymethylfurfural (HMF) levels in all bread samples. In fortified breads with citrus fibers the yeast and mold counts showed values of approximately 1 log10 cfu/g for the first 30 days and 3.5 log10 cfu/g at the end of storage. The results of the sensory evaluation highlight that loaves enriched with blood orange and lemon fibers showed a citrus flavor but had a similar overall evaluation respect to control bread produced without addition of citrus fiber. The results of this study showed that the addition up to 2% of blood orange and lemon fibers in wheat whole durum flour is a possible strategy to produce "high fibre" bread.

Keywords: durum wheat bread, citrus fibers, storage, microbiological assay, HMF, sensory attributes

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INTRODUCTION

Dietary fiber have an important role in maintaining good health and prevention of disease. The increase of the dietary fiber intakes was associated with the reduction of cardiovascular disease and the incidence of type 2 diabetes (1–4) and with the prevention of excessive weight gain, thanks to the role played on the regulation of appetite (5, 6).

The majority of the world's population consumes bread daily and it represents the ideal food to act as a vehicle for healthy substances. The addition of dietary fiber, generally modified the physicochemical characteristics both of dough and bread (7). Several authors reported the successful use of fruit and vegetable fiber in bakery products (8–11). In southern Italy, particularly in Sicily, citrus industry produces approximately 500,000 t/year of "pastazzo," a by-product derived from the industrial squeezing of citrus fruit, which currently presents serious disposal problems.

After numerous washings and purifications, it is possible to obtain citrus flour rich in dietary fiber, which can be added to food products.

Several authors used citrus fiber to fortify baked goods. A decrease of bread volume without significant worsening of the crumb texture and various detrimental effects on dough handling and bread quality with the replacement of flour with dietary fiber was reported (12, 13). Nassar et al. (14), suggested that 15% orange peel and pulp could be incorporated in biscuits, as a source of dietary fiber containing bioactive compounds (flavonoids, carotenoids, etc.). Biscuits of good technological quality, good level of acceptance and a decreased energy value, were obtained by replacing up to 15 g/100 g of the wheat flour with extruded orange pulp (15). A reduction both of energy content and *in vitro* protein digestibility was reported for cookies added with apple or lemon or wheat bran fiber (16); the partial replacement of wheat flour with a dietary fiber-rich orange bagasse allowed to produce baked products containing high levels of total dietary fiber and indigestible fraction and a decrease of glycemic index of the products (17). During baking process, the heat transmission favors, mainly in the bread crust, the formation of Maillard reaction products, that improve the flavor, color and texture of food products (18). 5-Hydroxymethylfurfural (5-HMF) is a product both of the Maillard reaction and of the acid-catalyzed thermal dehydration of hexose. In several processed foods is used as index of thermal abuse (19–21) and in baking products HMF levels make possible to monitor the heating processes (22, 23). Estimated dietary intakes range between 4 and 350 mg per person and day, and among foods, cereal and cereal products, in particular bread, may serve as a major source of HMF exposure. In cereal products the average concentrations of HMF ranged from 14 to 53 mg/kg (24, 25) and the average intake can vary greatly with regard to low or high consumption. Even if, seems, that the toxic potential of HMF is rather low several strategies were used to reduce the HMF level in bakery products (26–28).

The aim of this work was to evaluate the effect of the addition of different level of citrus fiber on the physicochemical, microbiological, and sensory properties of durum wheat whole bread. Moreover, the evolution both of the quality parameters and the sensory attributes were studied during a long storage time under MAP conditions.

MATERIALS AND METHODS

Materials

Durum wheat whole semolina was provided by the "Valle del Dittaino" Agricultural Cooperative Society a.r.l. (Assoro, Enna, Italy), a local industrial bakery. The bread ingredients were food grade. The citrus fibers were derived from blood oranges (*C. sinensis* L. Osbeck) and lemons (*C. limon* L. Burm f.) and were kindly donated by Ortogel S.p.A., Caltagirone, Italy. The compressed yeast (Mauri Extra Classic) was obtained from "AB Mauri Italy S.p.A.," Pavia, Italy and the NaCl was from "Mulino S. Giuseppe," Catenanuova, Italy.

Methods

Physicochemical Analysis of Durum Wheat Whole Flour

Ash content was obtained following the ISO method 2171 (2007). The gluten index was determined using a Glutomatic 2200 apparatus (Perten Instruments AB, Huddinge, Sweden) according to the method UNI 10690 (1979). The α-amylase activity was determined using the Falling Number 1500 apparatus (Perten Instruments AB, Huddinge, Sweden), following the method UNI EN ISO 3093 (2010).

Total, soluble and insoluble dietary fiber content was determined using a K-TDFR kit from Megazyme (Megazyme International, Bray, Ireland) (29). The protein content and color indexes were determined according to the methods described for durum wheat whole flour (30).

Rheological dough properties were evaluated by mixograph (National Mfg. Co, Nebraska, USA) and farinograph curves (Brabender instrument, Duisburg, Germany) (31). All the analyses were conducted in triplicate.

Chemical Characterization of Citrus Fiber

Total, soluble and insoluble dietary fiber content was determined as already reported above (29). The moisture content was determined according to the ISO method 712:2009. The ash content was obtained according to the ISO method 2171:2007. The alkalinity of the total ash was carried out following ISO method 5520:1981 and was expressed as the alkalinity index (the number of milliliters of 1 N acid solution required to neutralize 1 g of the ash obtained from the sample). The protein amount was determined by the Kjeldhal method. The sugars were determined by using HPLC with a refractive index detector (32). The pectin content was titrimetrically quantified as galacturonic acid according to the legal pectin specification (33). The water binding capacity (WBC), the water tendency to associate with hydrophilic substances, was determined (34). All the analyses were conducted in triplicate.

Bread-Making Process and Storage Conditions

The breads were produced in an industrial bakery ("Valle del Dittaino"—Agricultural Cooperative Society a.r.l., Assoro, Italy). For each dough, 68 kg of durum wheat whole flour was mixed with tap water (78 ± 3%), compressed yeast (0.5%), NaCl (2.6%) and blood orange (OF) and/or lemon fibers (LF). In the control dough no citrus fiber were added; whereas in the other four doughs citrus fiber were added in variable amount: 1.5 and 2.0% of orange fiber (code samples 1.5OF and 2.0OF, respectively) or lemon fiber (code samples 1.5LF and 2.0LF, respectively) or a mix of orange and lemon fiber (1.0 + 1.0%) (code samples 1.0 + 1.0OLF). These levels were chosen to obtain breads with a total dietary fiber content higher than 6 g/100 g, which is the minimum required limit to indicate that a food is "high in fibre" (35). All the ingredients were mixed for 13 min using a mixer with beater arms (IBT 300, Pietroberto S.p.A, Marano Vicentino, Italy). The tap water temperature was 22°C, and at the end of mixing, the dough temperature was 25.9 ± 0.4°C. All the other ingredients were expressed as a % of the weight of whole wheat durum flour.

Afterward, the dough was broken into approximately 1,150 g pieces using volumetric dividing (Omega, Pietroberto S.p.A, Marano Vicentino, Italy), obtaining approximately 100 loaves for each dough. The loaves were rounded in a conical rounder (CO 3000, 1500 pieces/h, Turri F.lli S.r.l., Costa di Rovigo, Italy) and leavened in a tray proofing cell (Pavailler Engineering S.r.l., Galliate, Italy) for 2 h and 45 min at 32°C with a relative humidity (RH) of 65%.

The loaves were baked for 1 h at 210°C in a continuous oven (Pavailler Engineering S.r.l., Galliate, Italy). After 2 h of cooling in a conditioned room at 20°C with a RH of 60%, the breads were packaged under a modified atmosphere in T6011B film (275 μm thickness) (Sealed Air, Cryovac, Italy), and T9250B film (125 μm thickness) (Sealed Air, Cryovac, Italy) was used to cover and seal the package. The containers were vacuumed, filled with mixed gas and sealed with a Multivac R-230 packaging machine (Germany) equipped with a modified atmosphere (MAP) mix 9,000 gas-mixer (PBI–Dansensor A/S, Ringsted, Denmark). The trays were packed using 70% N_2:30 % CO_2 gas combinations. All packaged bread samples were stored at 25 ± 2°C and 60 ± 2% RH for 4 months and were periodically (0, 30, 60, 90, and 120 days of storage) sampled. Nine loaves for each formulation were withdrawn each month and immediately analyzed. The CO_2 and O_2 concentrations in the samples were measured using Check Point (Dansensor PBI, Ringsted, Denmark) gas analyzing equipment.

The following properties were tested for each bread sample during each sampling: volume, height, weight, diameter, crumb porosity, internal structure, loaf firmness, crust thickness, crust and crumb color, moisture, pH, acidity, HMF content, microbiological, and sensory analysis. All the analyses were conducted in triplicate.

Bread Quality Evaluation

Determination of the Physicochemical Properties of Breads

The physicochemical properties of bread samples were evaluated according to Spina et al. (31).

The volume was determined according to the rapeseed displacement in a loaf volume meter, and the specific volume (cubic meters per gram) was calculated as a ratio of the loaf volume and the bread weight. The internal structure was visually estimated, and the crumb porosity was estimated using the Mohs scale. Loaf crust thickness was measured using a digital caliper (Digi-MaxTM, Scienceware®, NJ, U.S.A.), on the loaf basis of three central slices, after removing the crumb. The loaf firmness was measured using a texture analyser (Zwick Z 0.5 Roëll, Germany) equipped with an aluminum 8-mm- diameter cylindrical probe. The resulting peak force was measured in kilograms per cubic centimeter.

The CIE L*a*b* color parameters were measured for the crumb, in the transversely cut bread and on the crust surface, averaging ten distinct points in each case, using a Chroma Meter (CR-200, Konica Minolta, Osaka, Japan) with illuminant D65.

The moisture content was determined in triplicate by gravimetric analysis. The bread samples were ground in a home grinder (La Moulinette, Moulinex, 2002), and then portions of the ground bread sample were placed in an oven at 105°C until the dry weight was constant.

The pH and the total titratable acidity (TTA) were measured in triplicate, using a pHmeter (Mettler Toledo, MP 220). The TTA results were expressed, for the dry matter, as milliliters of 0.1 M NaOH consumed (36).

Total, soluble and insoluble dietary fiber content was measured in 1 g of dried sample using a K-TDFR kit from Megazyme (Megazyme International, Bray, Ireland) (29).

HMF Extraction and HPLC Analysis

The HMF was determined according to Spina et al. (31). The bread samples were ground in a home grinder (La Moulinette, Moulinex, 2002); subsequently, an aliquot of the ground sample (5 g) was transferred into a volumetric flask (50 ml), and 25 ml of water was added (JT. Baker, Deventer, Holland). The solution was stirred for 10 min, and then the sample was diluted up to 50 ml with water (JT. Baker, Deventer, Holland) and centrifuged for 45 min at 5,000 rpm. An aliquot of the supernatant was filtered through a 0.45-μm filter (Albet) and injected into an HPLC system (Shimadzu Class VP LC-10ADvp) equipped with a DAD (Shimadzu SPD-M10Avp). The column was a Gemini NX C18 (150 × 4.6 mm, 5 μm) (Phenomenex), fitted with a guard cartridge packed with the same stationary phase. The HPLC conditions were the following: isocratic mobile phase, 90% water at 1% of acetic acid and 10% methanol; flow rate, 0.7 ml/min; injection volume, 20 μl (20). All of the solvents used were HPLC purity grade: water from J.T. Baker, and acetic acid and methanol from Merck. The wavelength range was 220–660 nm, and the chromatograms were monitored at 283 nm.

HMF was identified by splitting the peak of the HMF from the bread-solution sample with a standard of HMF ($P > 98\%$ Sigma-Aldrich, St. Louis, Mo., U.S.A.) and by comparison of the UV spectra of the HMF standard with that of the bread samples. All analyses were performed in duplicate, including the extraction procedure, and the reported HMF concentration is therefore the average of four values. The results were expressed as mg of HMF per kilogram of the bread dry matter.

Microbiological Analysis

The microbiological analysis of the bread samples was performed in triplicate at 0, 30, 60, 90, and 120 days of storage, according to Spina et al. (31). Ten grams of each loaf were aseptically weighed and homogenized using a Stomacher (Brinkmann, Westbury, NY, USA) for 5 min and were serially diluted in a sterile physiological solution (0.9% NaCl). Serial dilutions of the suspension were pour-plated in duplicate onto the following media: plate count agar (PCA) (Oxoid) for total viable count; violet red bile glucose agar (VRBGA) (Oxoid) for Enterobacteriaceae; and Saboraud dextrose agar for yeasts and molds. The VRBGA plates were incubated at 32°C for 48 h under aerobic conditions. The PCA plates were incubated at 30°C for 4 days, and the yeast-count plates were incubated at 25°C for 4 days.

Sensory Evaluation

The sensory profile was determined by applying the UNI 10957 (2003) method and was defined by a panel of 12 judges (six females and six males) recruited among student and University staff that have chosen to participate to the research and signed the informed consent as our institution do not have an ethics committee for the taste and food quality evaluation studies. The judges were submitted to training over 4 weeks to generate attributes using handmade and industrial breads (31, 37, 38) and to familiarize themselves with the scales and procedures (UNI EN ISO 8586, 2012). The judges, using a scale between 1 (absence of the sensation) and 9 (extremely intense), evaluated the intensity of the eighteen sensory attributes selected on the basis of frequency (%) (39) (**Table 1**). The evaluation sessions, which were performed at 0, 30, 60, 90, 120 days of storage, were conducted in an equipped laboratory (UNI EN ISO 8589, 2010) from 11:00 to 12:00 a.m. in individual booths illuminated with white light. All data were acquired by a direct computerized registration system (FIZZ Biosystemes, ver. 2.00 M, Couternon, France). The data reported were expressed as the mean ± standard deviation.

Statistical Analysis

Statistical analysis of the results was performed using the STATSOFT 6.0 program (Vigonza, Italy). The significant differences were evaluated by Two-Way analysis of variance (ANOVA), and the mean separation was determined using the Tukey test with respect to time x thesis factor. The sensory data for each attribute were submitted to ANOVA using the software package Statgraphics® *Centurion XVI* (Statpoint Technologies, INC.) using the samples as factors. Significant differences were determined at the alpha level of 0.01 and 0.05 (only for citrus fibers protein and sensory parameters).

RESULTS AND DISCUSSION

Physicochemical Characterization of the Durum Wheat Whole Flour

Chemical characteristics of whole flour were: moisture 13.15 ± 0.07 g/100 g, ash 1.75 ± 0.10 g/100 g, protein 12.5 ± 0.10 g/100 g. These quality parameters fulfilled the legal requirements (40). Total dietary fiber value was 6.45 ± 0.6 g/100 g, which was constituted by 3.93 ± 0.4 g/100 g of insoluble dietary fiber and 2.52 ± 0.2 g/100 g of soluble dietary fiber. Dry gluten content was 8.4 ± 0.2 g/100 g. Gluten index value was 95.9 ± 5.0 and the value of amylase activity at falling number was low (633 ± 2.0 s). With regard to dry gluten content and relative qualitative index, the whole flour sample exhibited regular gluten quantities but a very high gluten tenacity. As regards color parameters, the values were: brown index (100—L*) 15.8 ± 0.2, red index (a*) 0.12 ± 0.01, yellow index (b*) 18.03 ± 0.04. As for dough rheological characteristics at Mixograph apparatus they were high: mixing time 360 ± 6.0 s and peak dough height 8.0 ± 2.0 M.U. (Mixograph Unit). Consequently, the classification scale (1-8) was good (7 ± 0.0). At Farinograph apparatus the quantity of water absorbed (%) at a 500 B.U. (Brabender Unit) dough consistency (64.6 ± 0.02 %) indicates that the whole flour had a high ability to absorb water due to high fiber content. The values of dough development time 270 ± 6.0 s and dough stability 1,080 ± 11 s were very long due to the good whole flour quality and were in agreement with those reported by other authors (41).

Characterization of Citrus Fiber

Table 2 reports the chemical characterization of the lemon and blood orange fibers used for the baking process. Both lemon and blood orange had highest levels of total dietary fiber (about 70%), with a balanced content of soluble (about 32%) and insoluble fibers (about 37.5%), differently from data reported

TABLE 1 | Descriptive terms used for sensory profiling of bread.

	Attributes	Definition	Scale anchors	
Crumb appearance	Crumb color	Color intensity of crumb	Whitish	Light yellow
	Alveolar structure	Porosity of crumb	Fine and uniform	Coarse and poorly homogeneous
Visual-tactile	Humidity	Humidity perceived at the surface of bread crumb	Dry	Humid
Aroma/Flavor	Bread	The typical aroma/flavor of bread just taken out of the oven	Weak	Strong
	Yeasty	The aroma/flavor of a fermented yeast-like	None	Strong
	Citrus	The typical aroma/flavor of citrus	None	Strong
	Off-odor/ Off-flavor	Aroma/Flavor unpleasant, not characteristic of bread perceived through taste and smell when swallowing	None	Strong
Taste	Sweet	A basic taste factor produced by sugars	None	Strong
	Salty	A basic taste factor produced by sodium chloride	None	Strong
	Sour	A basic taste factor produced by acids	None	Strong
	Bitter	A basic taste factor produced by caffeine	None	Strong
Mouthfeel	Astringent	Sensory perception in the oral cavity that may include drying sensation and roughing of the oral tissue	None	Strong
Texture	Softness	Force required to compress the product with the molars	Hard	Soft
	Overall	An overall assessment expressed by considering all the attributes	Low	High

TABLE 2 | Chemical characterization of citrus (lemon and blood orange) fiber used in bread making.

Parameter	Citrus fibers	
	Lemon	Blood orange
Total dietary fiber (g/100 g)	70.7 ± 1.0n.s.	70.5 ± 1.0n.s.
Soluble dietary fiber (g/100 g)	31.9 ± 0.05b	33.2 ± 0.05a
Insoluble dietary fiber (g/100 g)	38.8 ± 0.05a	37.3 ± 0.05b
Moisture (g/100 g)	8.84 ± 0.02a	7.85 ± 0.02b
Ash (g/100 g)	6.62 ± 0.15a	5.66 ± 0.15b
Alkalinity of total ash (index)	55.5 ± 0.08a	52.7 ± 0.06b
Protein (g/100 g)	5.69 ± 0.26B	6.48 ± 0.25A
Sugars (g/100 g)	4.21 ± 0.22n.s.	4.01 ± 0.20n.s.
Pectin (g/100 g)[b]	2.15 ± 0.10a	1.60 ± 0.10b
WBC (%)	803 ± 0.00b	804 ± 0.00a

Data expressed as the mean ± standard deviation. Different letters in the same row are significantly different lower case, $p < 0.01$; upper case, $p < 0.05$; n.s., not significant. WBC, water binding capacity.

for grapefruit, lemon and orange fibers by other authors (12), who reported lower levels of soluble fibers. Citrus fruit are characterized by distinctive concentrations of different minerals, mainly K, Ca, and P (42). Thus, the technological process applied for the preparation of the citrus fibers produced dried flours with 6.63 ± 0.15 g/100 g and 5.67 ± 0.15 g/100 g ash content with a relevant alkalinity index of 55.5 ± 0.08 and 52.7 ± 0.06 for the lemon and blood orange fibers, respectively. The moisture, total protein and sugar values were approximately in lines with values reported by other authors (12, 17).

Dietary Fiber Content in Breads

Table 3 reports total, soluble, and insoluble dietary fiber content in bread samples after baking. The highest level of total dietary fiber of citrus fiber (**Table 2**) had the advantage that small addition to the whole flour did not modify the technological properties of the resulting dough. Furthermore, it resulted in a significant increase in total fiber level in bread. The highest values of total, soluble, and insoluble fiber were recorded in the bread with 2.0% of citrus fibers, even if these thesis did not differ statistically from the others.

All the theses with citrus fiber exceeded 6.0% of total dietary fiber content, which is the minimum required limit to indicate that a food is "high in fibre," as required by Regulation (EC) No 1924/2006 of the European Parliament and of the Council on nutrition and health claims made on foods (35).

Therefore, addition of lemon and/or blood orange citrus fibers used in this study as ingredients to produce fiber-enriched bread can be helpful and interesting in integrating the whole flour with valuable and balanced sources of both soluble and insoluble dietary fiber fractions, including pectins.

Quality Parameters of Breads and the Evolution During Storage

Table 4 reports the *p*-values for all physico-chemical parameters studied on the breads with respect to storage time x thesis factor,

TABLE 3 | Dietary fiber content after baking in bread samples.

Sample code	Total dietary fiber (g/100 g)	Soluble dietary fiber (g/100 g)	Insoluble dietary fiber (g/100 g)
Control	5.93 ± 0.10b	2.32 ± 0.04b	3.61 ± 0.06b
1.5LF	7.10 ± 0.22a	2.86 ± 0.06a	4.24 ± 0.16a
2.0LF	7.41 ± 0.04a	2.99 ± 0.06a	4.42 ± 0.08a
1.5OF	7.01 ± 0.08a	2.87 ± 0.06a	4.14 ± 0.02a
2.0OF	7.33 ± 0.06a	2.96 ± 0.03a	4.37 ± 0.03a
1.0 + 1.0OLF	7.32 ± 0.02a	2.99 ± 0.07a	4.33 ± 0.05a

1.5LF, 2.0LF sample codes for breads with 1.5 or 2% of lemon fiber, respectively; 1.5OF 2.0OF sample codes for breads with 1.5 or 2% of blood orange fiber, respectively; 1.0 + 1.0OLF sample code for bread with 1 + 1% of lemon and blood orange fiber. Data expressed as the mean ± standard deviation. Different letter in the same column indicates significant difference ($p < 0.01$).

while **Tables 5**, **6** showed the results of the physical and chemical properties of the industrial breads in the MAP conditions during 120 days of storage, respectively. The specific volumes and weights of the loaves were not significant for any of the 3 factors of variability (thesis (A), storage time (B), and their interaction (A × B) (**Table 4**). No significant differences in the specific volumes and weights were shown among the bread samples and during the storage time, whatever the type and level of citrus fibers, probably due to the use of wheat whole durum flour. These findings are not in agreement with those already reported (12). Wheat white bread is bulkier and less heavy than that made with durum wheat whole semolina, and this fact strongly and negatively impacted the contributions of the orange fiber. The ratio between the height and diameter of the loaves is used in the baking industry to parameterize any failure of the dough (**Table 5**). The h/d ratio was significant respect to all the 3 factors of variability, even if with different *p* levels ($p \leq 0.001$ for theses, $p \leq 0.01$ for storage time and their interaction) (**Table 4**). During the storage, the control showed the greatest h/d ratio (approximately 3.4) due to the absence of citrus flour that leads to a high dough tenacity. The other bread samples, as expected, exhibited a lower ratio, even if they are not statistically different from the control, with the exception of sample 1 + 1OLF after 30 days of storage (**Table 5**).

After baking (t0), the 50% of the thesis showed a better developed crumb porosity. Starting from 30 days of storage, the porosity performance of 1.5 and 2.0 OF bread samples decreased (value 7), whereas the 2.0 LF sample maintained good characteristics until 90 days of storage (**Table 5**).

As regards the bread internal structure was significant both respect to the theses factor ($p \leq 0.05$) and for the other two factors of variability ($p \leq 0.01$) (**Table 4**). Only the samples 1.5 LF and 1.0 + 1.0OLF showed an irregular structure both after baking and during storage. The addition of blood orange fiber decreased the loaf volume but did not deteriorate the crumb characteristics (43) (**Table 5**).

Regarding the crust thickness, this parameter was significant respect to theses ($p \leq 0.001$), times ($p \leq 0.01$) and their interaction ($p \leq 0.05$) (**Table 4**). Only the sample 2.0 OF showed a minor crust thickness after 60 days of storage (**Table 5**).

TABLE 4 | Analysis of variance of the physico-chemical, color and microbiological parameters studied on the loaves (*p*-values).

Factors of variability	Degrees of freedom	Specific volume	Specific weight	h/d ratio	Porosity	Internal structure	Crust thickness	External firmness	Moisture	pH	TA	HMF	Crust			Crumb			Yeast and mold	Total viable count
													L*	a*	b*	L*	a*	b*		
Thesis (A)	5	0.123	0.132	0.000	0.000	0.049	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.085	0.000	0.321	0.000	0.000	0.000	0.000
Storage time (B)	4	0.174	0.139	0.003	0.000	0.003	0.009	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
A x B	20	0.834	0.977	0.003	0.000	0.003	0.010	0.000	0.000	0.000	0.000	0.000	0.000	0.035	0.000	0.000	0.005	0.000	0.000	0.000

Concerning to the external firmness it was significant respect to the all factors of variability (**Table 4**). The storage time significantly affected the external firmness. After baking (t0), all bread samples showed values ranging from 2.08 to 2.42 kg/cm^2. At 30 days of storage and up to 60 days, a slight reduction was observed in all samples. Then, the values of the external firmness increased in all bread samples. From 30 days after baking and up to 60 days, the moisture in the loaf moved centripetally, causing a softening of the crust and underlying the crumb, producing a decrease in the firmness of the loaves. Then, the moisture balanced again in different parts of the loaves and the texture gradually increased, reaching a high value after 120 days (**Table 5**).

Table 6 reports the moisture content, pH, TA and HMF content of the bread samples during the entire storage time. The moisture content was significant respect to the all factors of variability (**Table 4**). The moisture content ranged from approximately 38.5–41.6% at the beginning, but no significant differences were found between the control bread and the other bread samples. At 60 days of storage, the moisture content decreased up to approximately 30% in the control and in the bread with lemon fiber. Bread samples produced with orange fiber and with the mixed fiber highlight the lowest moisture content (about 20%) but only 2.0 OF and 1.0+1.0OLF were significantly different from the control bread. At 90 and 120 days of storage, no remarkable differences were evident between the bread samples, with the exception of 120 days in the 2.0 LF sample, which had the lowest moisture level (**Table 6**).

The effect of the addition of citrus fiber in bread samples on pH and on titratable acidity seems to be more related to the storage time rather than the recipe. No significant differences in pH were found between the control bread and other bread samples, except for 2.0LF at 90 and 120 days of storage. The pH of this bread sample remains constant throughout the storage time.

Concerning to the TA it was significant at the highest level respect to the all 3 factors of variability (**Table 4**). The TA ranged from approximately 7.4–8.8 at the beginning. After it decreased gradually up to 90 days of storage while increasing up to the initial levels, afterwards (**Table 6**).

For the first time the HMF level in durum wheat whole semolina bread was reported. In control bread the HMF was higher (about 65.4 mg/kg dry matter) than those determined in durum wheat bread (39.7 ± 2.22) (31). Differences both in recipe and in chemical characteristics of durum wheat whole semolina are responsible for the different HMF level (44). The HMF content was significant for all 3 factors of variability (**Table 4**).

The HMF content (**Table 5**) ranged from approximately 44.8–85.9 mg/kg at the beginning, and significant differences were found between the control bread and the 1.5 LF and 2.0 OF bread. Storage caused significant differences in the HMF levels in all bread samples. HMF decreased with storage up to 60 days, increasing up to 120 days of storage, showing a similar trend respect to TA. This trend, is the result of formation and degradation reactions of HMF (45) influenced by the pH, the temperature and the presence and concentration of sugars and aminoacids (26, 46, 47). The HMF behavior was similar to those observed for TA and

TABLE 5 | Evaluation of properties of bread samples produced using different levels of lemon (LF) and blood orange (OF) fibers, during storage.

Days of storage	Samples	Specific volume (cm^3/g)	Specific weight (g/cm^3)	h/d ratio	Porosity (1–8)[a]	Internal structure (1–2)[b]	Crust thickness (mm)	External firmness (kg/cm^2)
0	Control	2.48 ± 0.10	0.40 ± 0.02	3.40 ± 0.08ab	7a	1	4.67 ± 0.58abc	2.39 ± 0.52abc
	1.5LF	2.31 ± 0.05	0.43 ± 0.01	3.43 ± 0.09ab	7a	2	4.51 ± 0.02abc	2.36 ± 0.17abcd
	2.0LF	2.60 ± 0.40	0.39 ± 0.05	3.35 ± 0.10abc	6ab	1	5.00 ± 0.01a	2.42 ± 0.31ab
	1.5OF	2.61 ± 0.03	0.38 ± 0.00	3.43 ± 0.02ab	6ab	1	4.95 ± 0.13ab	2.08 ± 0.10abcdefg
	2.0OF	2.59 ± 0.14	0.39 ± 0.02	3.17 ± 0.07abc	6ab	1	4.33 ± 0.59abc	2.27 ± 0.28abcde
	1.0+1.0OLF	2.58 ± 0.52	0.40 ± 0.07	2.93 ± 0.08bc	7a	2	4.34 ± 0.58abc	2.12 ± 0.26abcdefg
30	Control	2.56 ± 0.09	0.39 ± 0.01	3.57 ± 0.11a	7a	1	4.34 ± 0.57abc	1.49 ± 0.16ghij
	1.5LF	2.41 ± 0.08	0.42 ± 0.01	3.10 ± 0.16abc	7a	2	4.17 ± 0.29abc	1.88 ± 0.08abcdefg
	2.0LF	2.43 ± 0.05	0.41 ± 0.01	3.12 ± 0.02abc	6ab	1	5.00 ± 0.03a	1.77 ± 0.26bcdefg
	1.5OF	2.57 ± 0.04	0.39 ± 0.01	3.23 ± 0.25abc	7a	1	4.66 ± 0.56abc	1.69 ± 0.06cdefgh
	2.0OF	2.47 ± 0.16	0.41 ± 0.03	3.12 ± 0.27abc	7a	1	4.34 ± 0.60abc	1.59 ± 0.09efghij
	1.0+1.0OLF	2.33 ± 0.09	0.43 ± 0.02	2.77 ± 0.08c	7a	2	4.01 ± 0.01abc	0.97 ± 0.08j
60	Control	2.72 ± 0.18	0.37 ± 0.02	3.16 ± 0.16abc	7a	1	4.34 ± 0.61abc	1.42 ± 0.11hij
	1.5LF	2.55 ± 0.07	0.39 ± 0.01	3.06 ± 0.20abc	7a	2.	4.01 ± 0.02abc	1.53 ± 0.14fghij
	2.0LF	2.64 ± 0.13	0.38 ± 0.02	3.04 ± 0.21abc	6ab	1	5.00 ± 0.01a	1.62 ± 0.26efghij
	1.5OF	2.65 ± 0.03	0.38 ± 0.00	2.90 ± 0.16bc	7a	1	4.35 ± 0.57abc	1.59 ± 0.05efghij
	2.0OF	2.54 ± 0.12	0.39 ± 0.02	3.10 ± 0.40abc	7a	1	3.68 ± 0.58bc	1.67 ± 0.05defghi
	1.0+1.0OLF	2.55 ± 0.05	0.39 ± 0.01	3.17 ± 0.15abc	7a	2	4.00 ± 0.01abc	1.01 ± 0.17ij
90	Control	2.52 ± 0.05	0.40 ± 0.01	3.43 ± 0.15ab	7a	1	3.99 ± 0.10abc	1.61 ± 0.18efghij
	1.5LF	2.52 ± 0.03	0.40 ± 0.00	2.97 ± 0.20bc	7a	2	4.01 ± 0.02abc	2.54 ± 0.06a
	2.0LF	2.58 ± 0.26	0.39 ± 0.01	2.97 ± 0.12abc	6ab	1	4.67 ± 0.58abc	2.03 ± 0.11abcdefg
	1.5OF	2.68 ± 0.07	0.37 ± 0.01	3.13 ± 0.08abc	7a	1	4.01 ± 0.02abc	1.79 ± 0.15bcdefg
	2.0OF	2.52 ± 0.06	0.40 ± 0.01	2.99 ± 0.11abc	7a	1	3.73 ± 0.31bc	2.24 ± 0.02abcdef
	1.0+1.0OLF	2.50 ± 0.54	0.41 ± 0.01	3.07 ± 0.25abc	7a	2	3.99 ± 0.01abc	2.11 ± 0.05abcdefg
120	Control	2.51 ± 0.01	0.40 ± 0.00	3.37 ± 0.14abc	7a	1	4.01 ± 0.02abc	2.07 ± 0.09abcdefg
	1.5LF	2.53 ± 0.09	0.40 ± 0.01	3.38 ± 0.07ab	7a	2	4.00 ± 0.02abc	1.76 ± 0.30*bcdefg*
	2.0LF	2.55 ± 0.08	0.40 ± 0.09	3.15 ± 0.15abc	7a	1.	4.42 ± 0.58abc	2.17 ± 0.28abcdef
	1.5OF	2.53 ± 0.04	0.40 ± 0.01	2.99 ± 0.18abc	7a	1	3.95 ± 0.23abc	1.94 ± 0.11abcdefg
	2.0OF	2.54 ± 0.20	0.40 ± 0.03	3.07 ± 0.14abc	7a	1	3.50 ± 0.51c	1.93 ± 0.06abcdefg
	1.0+1.0OLF	2.07 ± 0.60	0.41 ± 0.00	2.92 ± 0.14bc	7a	2	3.84 ± 0.29ab	2.37 ± 0.27abcd

1.5LF, 2.0LF sample codes for breads with 1.5 or 2% of lemon fiber, respectively; 1.5OF 2.0OF sample codes for breads with 1.5 or 2% of blood orange fiber, respectively; 1.0 + 1.0OLF sample code for bread with 1% +1 % of lemon and blood orange fiber. Data expressed as the mean ± standard deviation. Different letter in the same column indicates significant difference ($p < 0.01$).

[a] *1, most porous; 8, least porous.*

[b] *1, regular; 2, irregular.*

the two parameters shows a good correlation ($r^2 = 0.5200$). It is known that during the Maillard reaction several acids (i.e., formic, acetic) were formed as degradation products (48). Similar result was highlighted during storage of durum wheat bread (31).

Table 7 reports the color parameters of the crust and crumb of bread samples produced using various doses of blood orange and lemon citrus fibers. The crust lightness was significant respect to the all factors of variability (**Table 4**). At time 0, the 1.0+1.0OLF bread samples had the darkest crust values, whereas the other bread samples had the greatest L* values. At times 30 and 60, the L* crust values were lower for all bread samples. A similar trend was reported for durum wheat bread fortified with pectins and flavonoids (49). At time 90, the crust lightness values remained equal, whereas after 120 days, all of the bread samples showed the greatest L* values.

The crust redness it was significant for storage time and the interaction between theses and storage time, instead crust yellowness was significant respect to all the factors of variability (**Table 4**).

No significant differences were found in the a* and b* parameters after baking or at time 30 between the crust color of the control and other bread samples. Therefore, low values were shown in the 1.0+1.0OLF crust samples. At times 60 and 90, the a* and b* parameters demonstrated a lowering trend for all of the bread samples. After 120 days of storage, an increase in the a* and b* values was observed, with intermediate values between 0 and 30, 60 and 90 days.

TABLE 6 | Evaluation of the chemical characteristics of the bread samples produced using different levels of citrus fiber during storage.

Days of storage	Samples	Moisture (g/100 g)	pH	TA[a] (ml NaOH N/10)	HMF (mg/kg dry matter)
0	Control	40.57 ± 0.07ab	5.88 ± 0.06efghij	7.72 ± 0.59abcdefg	65.4 ± 5.09b
	1.5LF	39.38 ± 0.09abc	5.96 ± 0.04abcde	7.42 ± 0.03bcdefghi	85.9 ± 0.56a
	2.0LF	38.53 ± 0.05abcd	5.96 ± 0.04abcde	8.43 ± 0.12ab	65.6 ± 4.49b
	1.5OF	39.43 ± 0.01abc	5.84 ± 0.01ijkl	7.88 ± 0.44abcde	60.0 ± 1.25bc
	2.0OF	40.03 ± 0.14abc	5.90 ± 0.01defghi	8.56 ± 0.33ab	47.4 ± 1.65def
	1.0+1.0OLF	41.58 ± 0.25a	5.85 ± 0.01hijkl	8.78 ± 0.02a	44.8 ± 2.10b
30	Control	39.47 ± 0.09abc	5.93 ± 0.01bcdefg	7.00 ± 0.22cdefghilm	16.9 ± 4.72hil
	1.5LF	38.37 ± 0.07abcd	5.98 ± 0.03abc	7.72 ± 0.02abcdefg	49.9 ± 1.23cdef
	2.0LF	34.19 ± 0.27abcdef	6.00 ± 0.01ab	8.12 ± 0.60abcd	51.0 ± 4.95cde
	1.5OF	32.43 ± 7.14cdefgh	5.93 ± 0.02bcdefg	7.69 ± 0.35abcdefgh	22.9 ± 2.09hi
	2.0OF	39.49 ± 0.12abc	5.90 ± 0.01defghi	8.12 ± 0.13abcd	44.4 ± 0.99ef
	1.0+1.0OLF	37.29 ± 4.24abcde	5.86 ± 0.04fghijk	8.27 ± 0.43abc	39.8 ± 3.38ef
60	Control	30.30 ± 1.54efghi	5.99 ± 0.01abc	6.93 ± 0.01defghilm	9.99 ± 1.17l
	1.5LF	30.30 ± 1.54efghi	5.99 ± 0.01abc	6.05 ± 0.09mn	8.21 ± 1.43l
	2.0LF	27.84 ± 0.26fghij	6.02 ± 0.03a	6.50 ± 0.18ghilmn	13.2 ± 4.28il
	1.5OF	24.80 ± 0.34hijk	5.96 ± 0.01abcd	7.00 ± 0.10cdefghilm	15.6 ± 0.06hil
	2.0OF	19.98 ± 3.57k	5.83 ± 0.03ijkl	7.39 ± 0.10bcdefghil	14.7 ± 2.56hil
	1.0+1.0OLF	22.46 ± 0.76jk	5.95 ± 0.00abcde	6.58 ± 0.36fghilmn	22.7 ± 11.3hi
90	Control	28.81 ± 2.90fghij	5.87 ± 0.01fghijk	5.40 ± 0.09n	10.3 ± 1.04l
	1.5LF	27.91 ± 0.25fghij	5.93 ± 0.02bcdefgh	6.09 ± 0.09lmn	40.3 ± 0.18ef
	2.0LF	23.22 ± 1.83ijk	6.00 ± 0.01abc	5.59 ± 0.29n	37.9 ± 11.9fg
	1.5OF	23.64 ± 0.33ijk	5.86 ± 0.02ghijk	6.39 ± 0.10hilmn	12.6 ± 0.75il
	2.0OF	25.70 ± 1.34ghijk	5.94 ± 0.01bcdef	6.38 ± 0.20ilmn	9.37 ± 1.89l
	1.0+1.0OLF	26.93 ± 5.02fghijk	5.92 ± 0.01cdefgh	6.64 ± 0.29efghilmn	26.1 ± 2.29gh
120	Control	33.16 ± 1.81bcdefg	5.80 ± 0.01kl	8.18 ± 0.01abcd	57.4 ± 2.01bcd
	1.5LF	31.63 ± 0.07defgh	5.78 ± 0.04l	7.95 ± 0.50abcd	42.9 ± 1.57ef
	2.0LF	23.22 ± 1.83ijk	6.00 ± 0.01abc	7.52 ± 0.11abcdefghi	38.6 ± 0.94f
	1.5OF	31.53 ± 0.56defgh	5.80 ± 0.02kl	7.85 ± 0.32abcdef	11.4 ± 0.53il
	2.0OF	32.87 ± 0.67bcdefg	5.81 ± 0.02jkl	8.39 ± 0.12ab	48.5 ± 4.81cdef
	1.0+1.0OLF	32.84 ± 0.10cdefg	5.81 ± 0.01jkl	8.54 ± 0.12ab	17.2 ± 0.84hil

1.5LF, 2.0LF sample codes for breads with 1.5 or 2% of lemon fiber, respectively; 1.5OF 2.0OF sample codes for breads with 1.5 or 2% of blood orange fiber, respectively; 1.0 + 1.0OLF sample code for bread with 1 +1% of lemon and blood orange fiber. Data expressed as the mean ± standard deviation. Different letter in the same column indicates significant difference ($p < 0.01$).

[a] *Titratable acidity expressed on dry matter.*

The crumb lightness was not significant respect to one factor of variability (theses), but was significant for storage time and their interaction (**Table 4**). The effect of the addition of citrus fibers on the L* parameter of crumbs after baking and after 30 days of storage were not significant. At time 60, the L* value was reduced. The control and 1.5LF bread sample had the greatest values.

The a* and b* crumb color parameters were significant respect to the all factors of variability (**Table 4**). After time 60 the a* values decreased and then increased again to 120 days of storage (**Table 7**).

As browning index parameter has been considered 100–L* (22). In this study, no linear correlation was found between HMF and 100–L* ($r^2 = 0.348$) according to results reported for durum wheat bread (31), but in contrast with those reported for dough baked at various times even if product with the same colors can have different HMF levels (23).

Microbiological Assay

Table 8 reports microbial dynamics of the different groups throughout the storage period. The values of CO_2 and O_2 in the ATM packaged samples remained unchanged for the entire storage period (data not shown). The yeast, mold and total viable count were significant respect to all the factors of variability (**Table 4**).

No mold growth was visible in well-sealed packages. At the end of storage, the greatest total viable count (TVC) and yeast counts were achieved. The yeast and mold counts exhibited values of approximately 1 $\log_{10}$ cfu/g for all of the bread samples prior to 30 days of storage. From 30 through 120 days of storage, a gradual increase in the yeast and mold counts was detected in all samples, achieving the greatest values of 3.79 $\log_{10}$ cfu/g for sample 1.0+1.0 OLF, whereas the control sample had the lowest at 2.47 $\log_{10}$ cfu/g. A similar trend was reported for TVC, with values of 4.51 and 4.46 $\log_{10}$cfu/g for samples 2.0 OF and 1.0+1.0

TABLE 7 | Color parameters of breads produced using various doses of blood orange and lemon citrus fibers.

Days of storage	Samples	Crust			Crumb		
		L*	a*	b*	L*	a*	b*
0	Control	45.36 ± 3.21abcde	26.44 ± 1.59a	31.40 ± 2.57abcde	78.31 ± 1.96ab	7.25 ± 0.46e	33.04 ± 1.18a
	1.5LF	45.28 ± 0.40bcde	26.06 ± 0.78ab	30.89 ± 1.11abcd	77.77 ± 1.05ab	7.80 ± 0.30cde	33.14 ± 0.56a
	2.0LF	48.85 ± 1.09ab	26.46 ± 0.69a	32.19 ± 1.65abc	78.68 ± 0.22ab	7.91 ± 0.50cde	33.10 ± 1.11a
	1.5OF	47.38 ± 1.49abc	26.37 ± 0.55ab	32.28 ± 1.72abc	77.45 ± 1.63ab	7.59 ± 0.38de	33.01 ± 0.61a
	2.0OF	49.71 ± 0.14a	25.75 ± 0.58ab	32.99 ± 0.34ab	79.78 ± 0.38a	7.24 ± 0.30e	32.74 ± 0.03ab
	1.0+1.0OLF	39.05 ± 0.81ghijkl	25.50 ± 0.67ab	27.93 ± 0.73cde	77.46 ± 0.45ab	8.36 ± 0.26bcde	33.80 ± 0.67a
30	Control	41.65 ± 0.20defg	27.11 ± 0.37a	30.37 ± 0.54abcd	74.39 ± 1.08abcd	8.74 ± 0.15abcd	34.53 ± 0.70a
	1.5LF	45.55 ± 1.53abcd	26.03 ± 0.34ab	31.91 ± 1.11abc	74.45 ± 3.18abcd	9.16 ± 0.14abc	34.84 ± 1.39a
	2.0LF	40.32 ± 1.04fghij	24.78 ± 1.24abc	28.29 ± 1.04bcde	73.66 ± 0.46bcde	8.88 ± 0.16abcd	33.54 ± 0.29a
	1.5OF	40.99 ± 0.56efgh	24.78 ± 1.24abc	34.29 ± 0.47a	74.31 ± 0.91abcd	8.66 ± 0.29abcd	28.29 ± 1.04cde
	2.0OF	41.62 ± 2.00defg	24.67 ± 0.89abc	28.39 ± 1.99bcde	74.34 ± 4.18abcd	8.60 ± 0.23abcde	33.40 ± 0.63a
	1.0+1.0OLF	35.61 ± 0.79klmn	24.46 ± 0.03abc	27.56 ± 0.59cde	75.49 ± 1.02abc	7.96 ± 0.27cde	33.99 ± 0.35a
60	Control	36.09 ± 1.10ijklmn	16.51 ± 0.63h	16.79 ± 0.55j	71.45 ± 1.57cdef	3.58 ± 0.38g	28.43 ± 0.28cde
	1.5LF	33.64 ± 1.19no	17.79 ± 1.44gh	15.90 ± 0.73j	71.28 ± 0.96cdef	4.70 ± 0.40fg	29.51 ± 0.41cd
	2.0LF	40.37 ± 0.98fghi	17.46 ± 0.42gh	18.82 ± 0.68hij	67.65 ± 4.17fgh	4.46 ± 0.63fg	27.05 ± 1.41cdef
	1.5OF	37.82 ± 0.72ghijklmn	18.75 ± 0.85efgh	18.13 ± 0.08hij	67.81 ± 0.46efgh	4.78 ± 0.05fg	27.75 ± 0.57cdef
	2.0OF	34.85 ± 0.28lmno	17.83 ± 1.54gh	17.00 ± 0.33ij	68.10 ± 0.35efgh	4.54 ± 0.76fg	27.26 ± 1.09cdef
	1.0+1.0OLF	30.65 ± 0.79o	17.88 ± 0.46gh	15.08 ± 0.15j	67.57 ± 1.27fgh	4.99 ± 0.30f	27.59 ± 0.57cdef
90	Control	36.07 ± 1.21ijklmn	18.23 ± 1.15fgh	17.57 ± 0.66ij	66.86 ± 0.17fghi	4.37 ± 0.29fg	27.08 ± 0.43cdef
	1.5LF	38.12 ± 0.48ghijklm	18.70 ± 0.29efgh	18.56 ± 0.68hij	66.68 ± 0.87fghij	5.26 ± 0.29f	27.41 ± 0.32cdef
	2.0LF	33.54 ± 0.27no	17.87 ± 1.06gh	16.19 ± 0.65j	66.16 ± 0.70fghij	5.16 ± 0.46f	27.21 ± 0.78cdef
	1.5OF	36.98 ± 1.14hijklmn	17.48 ± 0.13gh	17.57 ± 0.19ij	62.80 ± 1.20ghij	4.90 ± 0.25fg	26.08 ± 0.33ef
	2.0OF	34.13 ± 2.46mno	16.76 ± 0.72h	15.88 ± 0.67j	61.25 ± 1.32ij	5.02 ± 0.15f	24.83 ± 0.32f
	1.0+1.0OLF	35.97 ± 0.68jklmn	18.73 ± 0.42efgh	17.55 ± 0.58ij	68.26 ± 0.50efgh	5.05 ± 0.46f	28.09 ± 0.48cde
120	Control	39.52 ± 1.08ghijk	23.17 ± 1.10bcd	26.71 ± 2.43defg	61.59 ± 2.70ij	8.79 ± 0.59abcd	27.30 ± 2.23cdef
	1.5LF	45.70 ± 0.21abcd	21.90 ± 1.01cde	26.94 ± 2.37def	64.08 ± 1.76ghij	9.78 ± 0.04a	29.90 ± 0.85bc
	2.0LF	35.24 ± 1.33klmn	21.73 ± 1.11cde	22.62 ± 2.91fgh	62.36 ± 1.37hij	9.11 ± 0.22abc	26.32 ± 0.64ef
	1.5OF	43.95 ± 1.52cdef	21.11 ± 2.37def	26.49 ± 2.23defg	66.54 ± 0.90fghij	9.78 ± 0.80a	29.12 ± 1.03cde
	2.0OF	35.91 ± 0.60klmn	21.79 ± 0.17cde	23.82 ± 1.03efg	68.49 ± 0.46defg	9.14 ± 0.31abc	29.78 ± 0.23bc
	1.0+1.0OLF	37.56 ± 0.22ghijklmn	20.50 ± 1.73defg	21.81 ± 1.95ghi	60.86 ± 0.48j	9.39 ± 0.09ab	26.67 ± 0.95def

1.5LF, 2.0LF sample codes for breads with 1.5 or 2% of lemon fiber, respectively; 1.5OF 2.0OF sample codes for breads with 1.5 or 2% of blood orange fiber, respectively; 1.0 + 1.0OLF sample code for bread with 1 +1% of lemon and blood orange fiber. Data expressed as the mean ± standard deviation. Different letters in the same column indicates significant difference ($p < 0.01$).

OLF, respectively. In the control bread, the yeast, mold and TVC remained slightly lower during all storage times possibly due to the absence of fiber, although the differences are not significant up to 60 days of storage.

Trials on freshly baked bread enriched with potato flour with 10 days of storage showed similar trends, with mold values of approximately 1–2 $\log_{10}$ cfu/g and bacteria of approximately 5 $\log_{10}$ cfu/g for bread enriched with 10% Irish potato flour (50).

Aerobic plate counts ($\log_{10}$ cfu/g) of bread enriched with plantain flour after baking ranged from 3.20 to 4.50 (51). These counts were minimal and would not constitute health hazards, but are greater compared with the data obtained in this study. This is the first time that microbiological data on industrial bread enriched with fiber have been reported after a long storage period. Confirming the importance of packaging conditions and good practices during industrial production to prevent contamination and microbial proliferation after processing.

Sensory Evaluation

For the first time the sensory profiles of a durum wheat whole semolina bread and durum wheat whole semolina bread with citrus fiber were determined.

The sensory profile both of control and of the fortified bread with citrus fiber was peculiar due to the ingredients used, such as durum wheat whole semolina and citrus fiber. A previous study (52) reported that durum wheat breads, have peculiar sensory features, different from those of common wheat breads (38).

Table 9 reports the mean scores of all attributes for t0, while for the other days of storage only the statistically significant attributes. From the ANOVA results of the sensory data, the freshly baked samples were significant different for the attributes humidity, bitter, overall and citrus aroma and flavor.

Panelist of bread samples containing up to 30% of fine bran, with a fiber level ranging from 1.5 to 3% did not find significant differences in crumb color, texture and flavor when

TABLE 8 | Mean log_{10} of microbial population counts of breads produced using various doses and types of citrus fibres during storage.

Days of storage	Samples	Yeast and mold	Total viable count
0	Control	1.08 ± 0.06ij	0.96 ± 0.01k
	1.5LF	1.14 ± 0.13ij	1.08 ± 0.40k
	2.0LF	1.11 ± 0.15ij	1.08 ± 0.26k
	1.5OF	1.02 ± 0.15i	2.00 ± 0.15i
	2.0OF	1.22 ± 0.26ij	1.78 ± 0.28ij
	1.0+1.0OLF	1.27 ± 0.09hij	1.29 ± 0.06jk
30	Control	1.72 ± 0.23ghij	2.05 ± 0.08i
	1.5LF	1.87 ± 0.14fghi	2.07 ± 0.02i
	2.0LF	2.07 ± 0.05efgh	2.31 ± 0.04hi
	1.5OF	1.72 ± 0.08ghij	2.69 ± 0.08gh
	2.0OF	2.36 ± 0.04defg	3.42 ± 0.17def
	1.0+1.0OLF	2.53 ± 0.15cdefg	2.64 ± 0.07gh
60	Control	2.09 ± 0.19efg	2.86 ± 0.11fgh
	1.5LF	2.59 ± 0.06cdef	2.80 ± 0.23gh
	2.0LF	2.74 ± 0.11bcde	3.06 ± 0.13efg
	1.5OF	2.78 ± 0.10bcde	3.08 ± 0.10efg
	2.0OF	2.86 ± 0.13bcde	3.86 ± 0.15cd
	1.0+1.0OLF	3.07 ± 0.11abcd	2.75 ± 0.30gh
90	Control	2.15 ± 0.02efg	3.12 ± 0.18efg
	1.5LF	3.11 ± 0.17abcd	3.83 ± 0.21cd
	2.0LF	3.30 ± 0.08abc	3.71 ± 0.21cd
	1.5OF	3.14 ± 0.22abcd	3.72 ± 0.22cd
	2.0OF	3.16 ± 0.21abcd	4.15 ± 0.14abc
	1.0+1.0OLF	3.33 ± 0.12abc	3.88 ± 0.21bcd
120	Control	2.47 ± 0.16defg	3.61 ± 0.37cde
	1.5LF	3.43 ± 0.31ab	4.12 ± 0.23abc
	2.0LF	3.69 ± 0.49a	4.12 ± 0.18abc
	1.5OF	3.33 ± 0.05abc	3.99 ± 0.05abcd
	2.0OF	3.49 ± 0.18ab	4.51 ± 0.23a
	1.0+1.0OLF	3.79 ± 0.28a	4.46 ± 0.14ab

1.5LF, 2.0LF sample codes for breads with 1.5 or 2% of lemon fiber, respectively; 1.5OF 2.0OF sample codes for breads with 1.5 or 2% of blood orange fiber, respectively; 1.0 + 1.0OLF sample code for bread with 1 + 1% of lemon and blood orange fiber.Data expressed as the mean of microbial log counts (log_{10} cfu/g) ± standard deviation. Different letters in the same column indicates significant difference ($p < 0.01$).

compared with the control (whole wheat flour) (53). Results reported in this study showed that bread samples containing up to about 7.3% of total dietary fiber, due to fortification with citrus fiber, did not show changes in crumb color, alveolar structure, softness and bread flavor, suggesting citrus fiber as a good source of dietary fiber that did not significantly change the main sensory attributes of durum wheat whole semolina bread.

The samples 1.5LF and 1+1OLF had the low intensity of citrus aroma; the sample 2.0OF showed the high intensity of bitter while the addition up to 1.5% of orange fiber improves the overall intensity scores.

At 30 days of storage, the samples were significant different for the attributes crumb color, humidity, softness, overall, citrus aroma, and flavor. Samples with citrus fiber had the same intensity of citrus aroma and flavor. The bread sample 1.5OF had the highest intensity of softness due to its highest humidity intensity score.

At 60 days, the bread samples were significant different for the attributes humidity, sweet, softness, overall, bread flavor, citrus aroma, and flavor. Samples 1.5 LF and 2.0LF showed the high intensity of citrus aroma, while the sample 1.5OF and control had the highest intensity of softness strictly related to their highest humidity intensity score. Moreover, the control bread showed the highest intensity of bread flavor and overall.

At 90 days of storage, the sensory profile of samples changes. In fact, on the nine sensory attributes, four attributes, such as alveolar structure, sour, astringent and yeasty flavor, for the first time were statistically significant. Other significant different attributes were crumb color, humidity, citrus aroma and flavor and overall. The samples with orange fiber had an alveolar structure similar with control bread. Also the astringent intensity was similar in control and 1.5OF bread samples. Bread sample 1.5OF had the highest intensity of yeasty flavor. The overall scores of bread samples were similar.

At 120 days, the samples were significant different only for the attributes citrus aroma and flavor, yeasty flavor and overall, suggesting a leveling of the samples' sensory profile. The bread sample with the highest addition of lemon fiber (2.0LF) showed the highest intensity of citrus flavor and the lowest of yeasty flavor, while the sample 1.5OF had the highest intensity of overall. For the attribute citrus aroma all the bread samples fortified with citrus fiber showed the same intensity. The control sample had the highest intensity of yeasty flavor. The attribute of yeasty flavor differentiated the samples after 90 days of storage. This is probably due to bread aging, ascribed to a reduction of the intensity of citrus aroma and flavor. These changes were more evident in control bread produced without citrus fiber. Moreover, it is important to highlight that bread samples, during storage, did not develop off-odor and off-flavor, and up to 30 days of storage overall scores of bread samples were similar independently to recipe.

CONCLUSIONS

For the first time in this paper, was studied the possibility to add citrus fiber to produce a wheat whole durum bread fortified in dietary fiber. The addition of citrus fiber up to 2% allowed to produce, generally, wheat whole durum bread with quality parameters, similar to control bread. No deep differences were highlighted between bread samples. Long storage time in MAP conditions significantly affected the bread characteristics independently from the addition of citrus fiber and allowed to maintain the microbiological safety of the bread samples.

This study showed that the addition of citrus industry by-product fibers in durum wheat whole semolina is a possible strategy and a good prospect to produce "high fibre" wholemeal durum wheat bread to increase the nutritional value and the dietary fiber intake. These results, moreover, should be interesting for the possibility to produce a "high in fibre" durum wheat bread with a long shelf-life.

TABLE 9 | Mean scores of the sensory attributes at the different days of storage.

Days of storage	Attributes[a]	Control	1.5OF	2.0OF	1.5LF	2.0LF	1.0+1.0OLF
0	Crumb color	6.58 ± 1.73	6.33 ± 1.61	7.00 ± 1.81	6.33 ± 1.61	6.41 ± 1.51	7.16 ± 1.59
	Alveolar structure	5.25 ± 2.22	5.16 ± 1.70	4.41 ± 1.44	5.25 ± 2.30	5.75 ± 1.60	5.00 ± 2.09
	Humidity	6.58 ± 1.44*b*	6.16 ± 1.80ab	6.08 ± 2.11ab	6.00 ± 1.48ab	4.91 ± 2.27a	5.83 ± 2.41ab
	Bread aroma	5.83 ± 1.85	5.58 ± 2.07	5.58 ± 1.73	6.25 ± 1.54	5.58 ± 1.88	6.00 ± 1.86
	Yeasty aroma	4.41 ± 2.19	4.41 ± 2.11	3.91 ± 2.23	4.83 ± 1.75	5.16 ± 1.64	5.50 ± 1.68
	Citrus aroma	1.00 ± 0.00a	3.83 ± 2.37c	3.66 ± 2.15c	2.08 ± 0.90ab	2.83 ± 1.47bc	2.16 ± 1.34ab
	Off-odor	2.50 ± 1.62	3.16 ± 2.76	3.25 ± 1.82	3.00 ± 1.60	2.50 ± 1.57	2.91 ± 1.83
	Sweet	3.75 ± 2.18	2.58 ± 1.56	3.25 ± 1.82	3.50 ± 1.73	3.75 ± 2.26	3.50 ± 1.45
	Salty	4.16 ± 2.25	4.33 ± 2.27	4.33 ± 1.37	4.91 ± 1.38	4.58 ± 2.47	5.16 ± 1.70
	Sour	3.41 ± 2.31	4.25 ± 2.09	4.08 ± 2.23	3.00 ± 1.71	3.66 ± 2.64	3.66 ± 2.31
	Bitter	2.91 ± 1.51a	3.58 ± 2.15ab	5.33 ± 2.46b	3.58 ± 2.23ab	3.75 ± 2.53ab	3.91 ± 2.07ab
	Astringent	4.66 ± 2.15	4.25 ± 2.73	4.16 ± 2.41	4.16 ± 2.55	4.50 ± 2.20	4.41 ± 2.71
	Softness	5.66 ± 1.61	4.58 ± 2.15	4.58 ± 2.71	5.16 ± 1.95	4.08 ± 2.11	5.08 ± 2.11
	Bread flavor	5.58 ± 2.23	4.33 ± 2.06	4.50 ± 1.62	4.66 ± 2.31	4.91 ± 2.15	4.83 ± 2.33
	Yeasty flavor	3.66 ± 1.87	3.75 ± 1.48	3.25 ± 1.48	4.08 ± 2.64	3.91 ± 1.68	4.16 ± 1.59
	Citrus flavor	1.00 ± 0.00a	3.50 ± 2.50b	2.83 ± 2.50*b*	2.83 ± 2.15b	3.25 ± 2.00b	2.91 ± 2.01b
	Off-flavor	2.41 ± 2.34	1.91 ± 2.92	2.33 ± 1.81	3.33 ± 2.26	3.08 ± 2.84	2.50 ± 2.67
	Overall	4.08 ± 1.81ab	5.25 ± 1.78b	4.00 ± 2.19a	3.83 ± 1.71a	3.91 ± 1.70a	4.00 ± 2.18a
30	Crumb color	6.16 ± 1.64ab	5.25 ± 1.82a	7.00 ± 1.76b	6.50 ± 1.51ab	7.16 ± 1.75b	6.75 ± 1.42b
	Humidity	3.83 ± 1.85ab	4.75 ± 1.66b	3.41 ± 1.16ab	3.75 ± 1.96ab	3.75 ± 1.66ab	3.25 ± 1.96*a*
	Citrus aroma	1.00 ± 0.00a	3.08 ± 1.98b	2.91 ± 2.02b	2.75 ± 1.86b	3.00 ± 1.86b	2.41 ± 1.62b
	Softness	3.25 ± 1.14a	4.33 ± 1.30b	2.33 ± 0.98a	3.25 ± 1.48a	3.00 ± 1.28a	2.66 ± 1.07a
	Citrus flavor	1.00 ± 0.00a	3.50 ± 2.15b	2.83 ± 1.95b	2.83 ± 1.99b	3.25 ± 2.09b	2.91 ± 1.68b
	Overall	4.08 ± 1.73ab	5.25 ± 1.60b	4.00 ± 1.13a	3.83 ± 1.59a	3.91 ± 1.51a	4.00 ± 1.48a
60	Humidity	3.83 ± 1.47b	4.00 ± 1.76b	3.58 ± 1.31ab	2.33 ± 0.98a	2.91 ± 1.44ab	3.00 ± 2.04ab
	Citrus aroma	1.00 ± 0.00a	3.58 ± 2.23bcd	4.00 ± 2.04d	2.33 ± 1.23b	2.66 ± 1.15bc	3.66 ± 1.97cd
	Sweet	4.00 ± 1.65ab	3.08 ± 1.38ab	4.25 ± 1.14b	4.00 ± 1.81ab	3.08 ± 1.00ab	3.00 ± 1.65a
	Softness	3.58 ± 1.56b	3.58 ± 1.51b	3.33 ± 1.56ab	2.16 ± 1.19a	2.75 ± 1.14ab	3.33 ± 2.06ab
	Bread flavor	5.00 ± 1.71b	3.75 ± 1.82ab	4.08 ± 1.73ab	3.75 ± 1.29ab	2.83 ± 1.11a	3.66 ± 1.61a
	Citrus flavor	1.00 ± 0.00a	4.75 ± 2.45b	4.58 ± 2.35b	3.58 ± 2.07b	4.08 ± 1.73b	5.00 ± 2.30b
	Overall	4.91 ± 1.56b	4.08 ± 1.38ab	3.91 ± 1.38a	3.41 ± 1.24a	3.00 ± 1.28a	3.91 ± 1.88ab
90	Crumb color	5.08 ± 1.00ab	4.58 ± 0.79a	5.83 ± 0.58bc	5.41 ± 1.31bc	5.25 ± 1.06abc	5.91 ± 0.79c
	Alveolar structure	3.83 ± 1.27b	4.16 ± 1.34b	4.08 ± 1.68b	2.58 ± 1.16a	3.66 ± 1.23ab	3.33 ± 1.44ab
	Humidity	2.58 ± 1.16a	3.75 ± 0.62b	3.25 ± 1.06ab	2.66 ± 1.30a	3.25 ± 0.97ab	2.75 ± 1.22a
	Citrus aroma	1.00 ± 0.00a	2.33 ± 1.07bc	3.00 ± 1.48c	2.00 ± 0.95b	2.41 ± 1.08bc	2.58 ± 1.38bc
	Sour	2.58 ± 1.31abc	3.00 ± 1.13bc	1.83 ± 0.83a	3.25 ± 1.29c	2.33 ± 0.89bc	2.50 ± 1.00abc
	Astringent	3.41 ± 1.44b	3.33 ± 1.07b	2.16 ± 1.19a	3.08 ± 1.31ab	2.83 ± 1.40ab	3.25 ± 1.54ab
	Yeasty flavor	2.58 ± 0.79a	4.00 ± 1.76b	2.58 ± 1.00a	3.00 ± 0.85a	3.00 ± 1.04a	2.50 ± 1.00a
	Citrus flavor	1.00 ± 0.00a	2.91 ± 0.90b	4.16 ± 1.59c	3.08 ± 1.88b	3.25 ± 1.54bc	2.83 ± 1.03b
	Overall	3.00 ± 0.74a	3.16 ± 0.94a	4.66 ± 1.37b	3.91 ± 1.38ab	3.75 ± 1.06ab	3.33 ± 1.15a
120	Citrus aroma	1.00 ± 0.00a	2.50 ± 1.45b	3.16 ± 1.95b	2.41 ± 1.62b	2.25 ± 1.48b	2.58 ± 1.51b
	Yeasty flavor	4.25 ± 1.96b	3.25 ± 1.54ab	3.50 ± 1.88ab	3.50 ± 1.78ab	2.83 ± 1.11a	3.91 ± 1.78ab
	Citrus flavor	1.00 ± 0.00a	2.91 ± 1.83b	3.25 ± 1.91bc	2.75 ± 1.60b	4.50 ± 2.47c	2.91 ± 2.02b
	Overall	3.58 ± 1.51ab	4.50 ± 1.51b	3.50 ± 1.17ab	3.16 ± 0.72a	3.75 ± 1.54ab	3.33 ± 1.50a

1.5LF, 2.0LF sample codes for breads with 1.5 or 2% of lemon fiber, respectively; 1.5OF 2.0OF sample codes for breads with 1.5 or 2% of blood orange fiber, respectively; 1.0 + 1.0OLF sample code for bread with 1 + 1% of lemon and blood orange fiber.

[a]For t0 all attributes are shown, for the other days of storage only the statistically significant attributes.

Data expressed as the mean ± standard deviation. Values marked with different letters in the same row are significant difference ($p \leq 0.05$).

Finally, the use of citrus fibers in bread making can be considered as an environmental-friendly alternative for the reuse and valorisation of citrus processing wastes and by-products.

AUTHOR CONTRIBUTIONS

AS and EA planned the experiments, surveyed laboratories, interpreted and discussed the results, and co-wrote the manuscript. SM performed laboratory flour analysis, acquired bread data during storage, performed the microbiological assay, and the statistical analyses. AM performed sensorial analysis, interpreted, and discussed sensory results. SB performed laboratory chemical analysis and acquired bread data during storage. SF performed laboratory citrus fiber analysis and participated in the critical revision of manuscript. BF and PR conceived the idea, designed the study and participated in the critical revision of manuscript.

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Hydroxytyrosol Improves Obesity and Insulin Resistance by Modulating Gut Microbiota in High-Fat Diet-Induced Obese Mice

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Obesity is a common chronic metabolic disease that is harmful to human health and predisposes the affected individuals to a cluster of pathologies. Insulin resistance (IR) is one of the most frequent complications of obesity. Hydroxytyrosol (HT) may reduce obesity and IR in high-fat diet (HFD)-fed mice; however, the mechanism underlying is still unknown. Systemic low-grade inflammation and intestinal dysfunction are thought to be associated with obesity and IR. In this study, we found that HFD feeding for 8 weeks altered the intestinal microbiota, injured intestinal barrier function, increased endotoxin release into the blood, enhanced the expression of inflammatory factors (TNF-α, IL-1β, IL-6) and lipid accumulation in liver, caused obesity, and aggravated IR via the JNK/IRS (Ser 307) pathway in HFD mice. We also found that HT gavage could reverse those effects and the beneficial effects of HT were transferable through fecal microbiota transplantation. Our data indicate that HT can improve obesity and IR by altering the composition of the intestinal microbiota and improving integrity of the intestinal wall. We propose that HT replenishment may be used as a dietary intervention strategy to prevent obesity and IR.

Keywords: hydroxytyrosol, fecal microbiota transplantation, obesity, inflammation, insulin resistance, liver steatosis

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INTRODUCTION

Childhood obesity is a major public health problem and is becoming increasingly prevalent in most regions of the world. The worldwide prevalence of childhood obesity has risen from 4% in 1975 to 18% in 2016, and recent studies have shown that more than 340 million children and adolescents are overweight or obese (Orlando et al., 2018). Obesity is often accompanied by chronic low-grade inflammation, disorders of glucose and lipid homeostasis, and metabolic diseases such as type 2 diabetes, cardiovascular disease, and hepatic steatosis (Hotamisligil, 2017). Childhood obesity may increase the likelihood of obesity and metabolic disorders in adulthood, greatly decreasing the individual's quality of life (Bass and Eneli, 2015).

Abbreviations: BAT, brown adipose tissue; eWAT, epididymal white adipose tncipal coordinates analysis; FMT, fecal microbiota transplantation; HFD, high-fat diet; HOMA-IR, homeostasis model assessment of insulin resistance; HT, hydroxytyrosol; HTF, HT fecal microbiota transplantation group; IR, insulin resistance; MD, Mediterranean diet; rWAT, perirenal white adipose tissue; scWAT, subcutaneous white adipose tissue; SMT, selective fecal transplant experiment.

It is widely accepted that unhealthy diets, sedentary lifestyles, and sleep deprivation are associated with an increased risk of obesity (Baranowski and Taveras, 2018). In addition, a growing number of studies have shown that changes in gut microbiota in early life may also play a role in the development of obesity. There are trillions of microorganisms in the human body (Tanaka et al., 2009; Dominguez-Bello et al., 2010), which undergo dynamic changes during an individual's life. Environmental factors (Zhang et al., 2010), including diets (Siddharth et al., 2013), are the main contributors to the composition of microorganisms rather than genetic factors. Most studies have shown that the fetus's intestinal tract is sterile during pregnancy (Makino et al., 2013), and the initial bacterial colonization occurs during birth. It has been suggested that the methods of birth and feeding determine the type of bacteria which are colonized into the baby's gastrointestinal tract (Munyaka et al., 2014). Moreover, the timing of supplementary food addition also affects the composition of the gut microbiota, after which the gut microbiota remains stable until old age (Villanueva-Millan et al., 2015).

The "Mediterranean diet" (MD), a diet rich in fruits and vegetables, fish, whole grains, beans, and olive oil, is considered to be one of the healthiest diets (Del Chierico et al., 2014). Numerous studies have shown that people in the Mediterranean region are far less likely to suffer from cardiovascular disease, diabetes, colon cancer, and rectal cancer than people in other European and American countries. Studies have shown that the MD increases *Bacteroides* and *Clostridium*, reduces *Proteus* and *Bacillus* populations in the gut microbiota (Marlow et al., 2013). A recent study showed that the MD could increase the gut microbiota diversity in cynomolgus monkeys (Nagpal et al., 2018), increase the abundance of *Lactobacillus* and *Clostridium*, and reduce the abundance of *Ruminococcus* and *Coprococcus*. The current literature on the role of the MD in childhood is lacking. Olive oil, an important component of the MD, is beneficial due to its abundance of monounsaturated fatty acids and antioxidants (Hamden et al., 2009). HT, the main component of olive oil, has been shown to have no toxic effects on cells and animals within a certain dose range (Auñon-Calles et al., 2013a,b; D'Angelo et al., 2001). Previous experiments have shown that HT prevents obesity, inflammation, hyperglycemia, and IR induced by a HFD (Carito et al., 2013; Cao et al., 2014; Voigt et al., 2015; Wang et al., 2018). HT may decrease lipid deposits by downregulating the SREBP-1c/FAS pathway in the liver and skeletal muscle tissues in HFD-fed mice (Cao et al., 2014). Additionally, HT regulates Toll-like receptor 4 (TLR-4)-dependent inflammation through a NF-κB-independent pathway to relieve oxidative stress (Takeda et al., 2014). Our recent research has revealed that HT improves inflammation, IR, and hepatic steatosis by reducing endoplasmic reticulum (ER) stress and by regulating the JNK/IRS pathway in HFD-induced obese mice (Wang et al., 2018). However, little research has been done on the effect of HT on the gut microbiota. In the present study, we hypothesized that HT may prevent obesity and IR by altering the composition of the intestinal microbes and alleviating inflammatory status. Therefore, we examined the effect of HT supplementation on HFD-induced obese mice in early life and explored the mechanism of action of the gut microorganisms through bacterial fecal transplantation experiments.

MATERIALS AND METHODS

Animal and Dietary Intervention

Twenty-eight three-week-old male C57BL/6J mice were raised at the Specific Pathogen Free Animal Experimental Center of Dalian Medical University (The Ethical approval number of C57BL/6J mice is AEE17057) and were divided into 4 groups: control group (chow), HFD group, HT group, and HTF. Seven mice per cage were housed in the following conditions: 12/12 hour light/dark cycle, maintained temperature of 23°C ± 2°C, and a humidity of 60%, with free access to food and water. The chow group was fed a normal diet as a reference, and the other three groups were fed an HFD (45% kcal fat content, MD12032, Medicience Ltd.). The intragastric interventions were performed for eight weeks as follows: the chow group and HFT group were gavaged with distilled water (10 ml/kg/day), the HT group was gavaged with HT (50 mg/kg/day, dissolved in distilled water, purity ≥ 98%, APP-ChemBio, China), the HTF group was subjected to fecal transplantation (fecal transplant donor from the HT group, 100 mg/1 mL sterile saline).

Fecal Collection and Transplantation

One hundred milligrams of fresh feces (collected during the experiment every day) from the HT group were dissolved in 1 mL of sterile saline in an autoclaved tube, vortexed with a turbine for 10 s, and then centrifuged at a speed of 800 *g* for 3 min (Biofuge Primo R, Thermo). The prepared solution was used within 10 min as described previously (Chang et al., 2017).

Oral Glucose Tolerance Tests and Insulin Tolerance Tests

After 8 weeks of feeding, the mice were fasted for 10 h overnight without restrictions on drinking water and gavaged with glucose solution [2.0 g/kg body weight (bw)] for OGTT. For ITT, the mice were fasted for 5 h and injected intraperitoneally with insulin (0.75 unit/kg bw, FosunPharm). Tail vein blood was collected, blood glucose was measured using a glucometer (Yue Zhun Type II 560, Yuyue), and the area under the curve (AUC) was calculated as described previously (Wang N. et al., 2017).

Sample Collection and Preservation

After the mice were sacrificed, the liver, scWAT, BAT, rWAT, epididymal white adipose tissue (eWAT), and ileum were removed, weighed, and wrapped in aluminum foil. The blood samples were divided into two parts: one part was centrifuged (4°C, 3000 rpm, 15 min) for serum, and another part was centrifuged (4°C, 3000 rpm, 15 min) for plasma in a test tube containing heparin. All samples were placed in liquid nitrogen for quick freezing and stored at −80°C for further use.

Hematoxylin and Eosin Staining

A portion of the scWAT was fixed in 10% formalin overnight and was then dehydrated and embedded in paraffin. Sections

(thickness of 4–5 μm) were stained with hematoxylin and eosin and were then visualized with a microscope (Olympus BX72, Japan). Cell size was analyzed using Image J software (National Institutes of Health).

Oil Red O Staining

The liver tissue was successively placed in 10, 20, and 30% sucrose solutions for gradient dehydration. The dehydrated liver was processed into frozen sections (thickness of 6 μm), stained with oil red (WLA055a, Wanleibio) for 10 min, and observed using a microscope. The size of the lipid droplets was analyzed using Image J software.

Biochemical Analysis

Plasma lipopolysaccharide (LPS) levels were quantified using a Chromogenic Endpoint Tachypleus Amebocyte Lysate (CE TAL, Xiamen Bioendo Technology Co., Ltd., China) assay according to the manufacturer's instructions. The concentrations of TNF-α, IL-1β, and IL-6 in liver were analyzed using commercial kit (MB-2868A, MB-2776A, MB-5737A, Meibiao, China). Fasting serum insulin concentrations were determined using a commercial ELISA kit (ER1113, FineTest, China). IR was assessed using the index of HOMA-IR: fasting blood glucose (mmol/L) × fasting blood insulin (mU/L) / 22.5.

Gut Microbiota Analysis

Before the mice were sacrificed, stool samples were collected for gut microbiota analysis using an autoclaved tube. Fecal DNA was extracted from the stool samples using the E.Z.N.A Soil DNA Kit (Omega Bio-tek, United States) according to the manufacturer's protocol. The hypervariant region V3–V4 of the bacterial 16S rRNA gene was amplified with the primers 338F (5′-ACTCCTACGGGAGGCAGCA-3′) and 806R (5′-ACTCCTACGGGAGGCAGCA-3′) by PCR. The PCR product was recovered using a 2% agarose gel, purified using an AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, United States), eluted with Tris-HCl, and detected by 2% agarose electrophoresis. Quantification detection was performed using Quantifluor-ST (Promega, United States). Sequencing was performed using Illumina's MiSeq PE300 platform (Shanghai Meiji Biomedical Technology Co., Ltd., China). UPARSE software was used to perform operational taxonomic unit (OTU) clustering of sequences based on 97% similarity (version 7.1[1]). The OTU was subsampled for further analysis. Chimeras were eliminated using UCHIME software. Each sequence was compared to the Silva database (SSU123) for species classification annotation using the RDP classifier[2], with the alignment threshold set to 70%. The Wilcoxon rank-sum test was used to compare differences between the two groups.

The R language tool was used to make the graph of the Rank-Abundance curve, the Venn diagram, and the community histogram/heatmap. Rarefaction curve and alpha-diversity indexes were analyzed by Mothur. Sample hierarchical clustering and principal coordinates analysis (PCoA) were performed using the Bray-Curtis distance algorithm, and the graphics were created with R language. Community comparison was evaluated using the UniFrac Server followed by a Wilcoxon rank sum-test.

[1]http://drive5.com/uparse/

[2]http://rdp.cme.msu.edu/

Western Blotting

Total proteins were extracted from the liver and ileum using total protein extraction kits (KGP1100, KeyGEN BioTECH). The concentration of the extracted proteins was quantified using a Pierce BCA Protein Assay Kit (Thermo). Equal amounts of protein from each sample were transferred to a polyvinylidene fluoride membrane after polyacrylamide gel electrophoresis. After blocking with 10% skim milk for 1 h at 37°C in a water bath, the blot was incubated with primary antibody (a suitable concentration diluted with TBST) overnight at 4°C. After being washed with TBST (5 times, 5 min at a time), the blot was incubated with secondary antibody for 2 h at room temperature. We used a Bio-Rad ChemiDoc MP imaging system to detect the bands of target proteins, and the relative density of the bands was analyzed using Image J software.

Antibodies

Primary antibodies: internal control β-actin (1:1000; TA-09, ZSGB-BIO), GAPDH (1:1000; 10494–1-AP, Proteintech), IL-1β (1:1000; WL00896, WanleiBio), IL-6 (1:1000; WL01678, WanleiBio), TNF-α (1:1000; WL02770, WanleiBio), JNK (1:500; WL01295, WanleiBio), phosphorylated JNK (Thr183 Tyr185) (1:500; WL01813,WanleiBio), AKT (1:1000; 10176-2-AP, Proteintech), phosphorylated AKT (Ser473) (1:500; WLP001a, WanleiBio), TLR4 (1:500; WL00196, WanleiBio), Tight junction protein 1 (ZO-1) (1:500; WL03419, WanleiBio), Occludin (1:1000; WL01996, WanleiBio), IRS-1 (insulin receptor substrate-1, 1:1000; #2382, CST), phosphorylated IRS-1 (serine 307, 1:500; WH081658, ABClone). Secondary antibodies: peroxidase-conjugated goat anti-mouse IgG (1:5000; ZB-2305, ZSGB-BIO) or peroxidase-conjugated goat anti-rabbit IgG (1:5000; ZB-2301, ZSGB-BIO).

Statistical Analysis

The data are expressed as the means ± standard error of mean (SEM) and were analyzed and plotted with GraphPad Prism 6 (GraphPad Software, United States). Data sets that involved more than two groups were assessed by one-way ANOVA followed by Tukey's honest significant difference *post hoc* tests. P-value ≤ 0.05 was considered statistically significant.

RESULTS

HT and HTF Reversed HFD-Induced Obesity in Mice

To determine whether HT supplementation or fecal transplant from HT-supplemented mice would reduce obesity in HFD mice, we recorded the body weights of the mice and the excised adipose tissue. HFD induced significant increases in final body weight, body weight gain, liver weight, and tissue mass of the

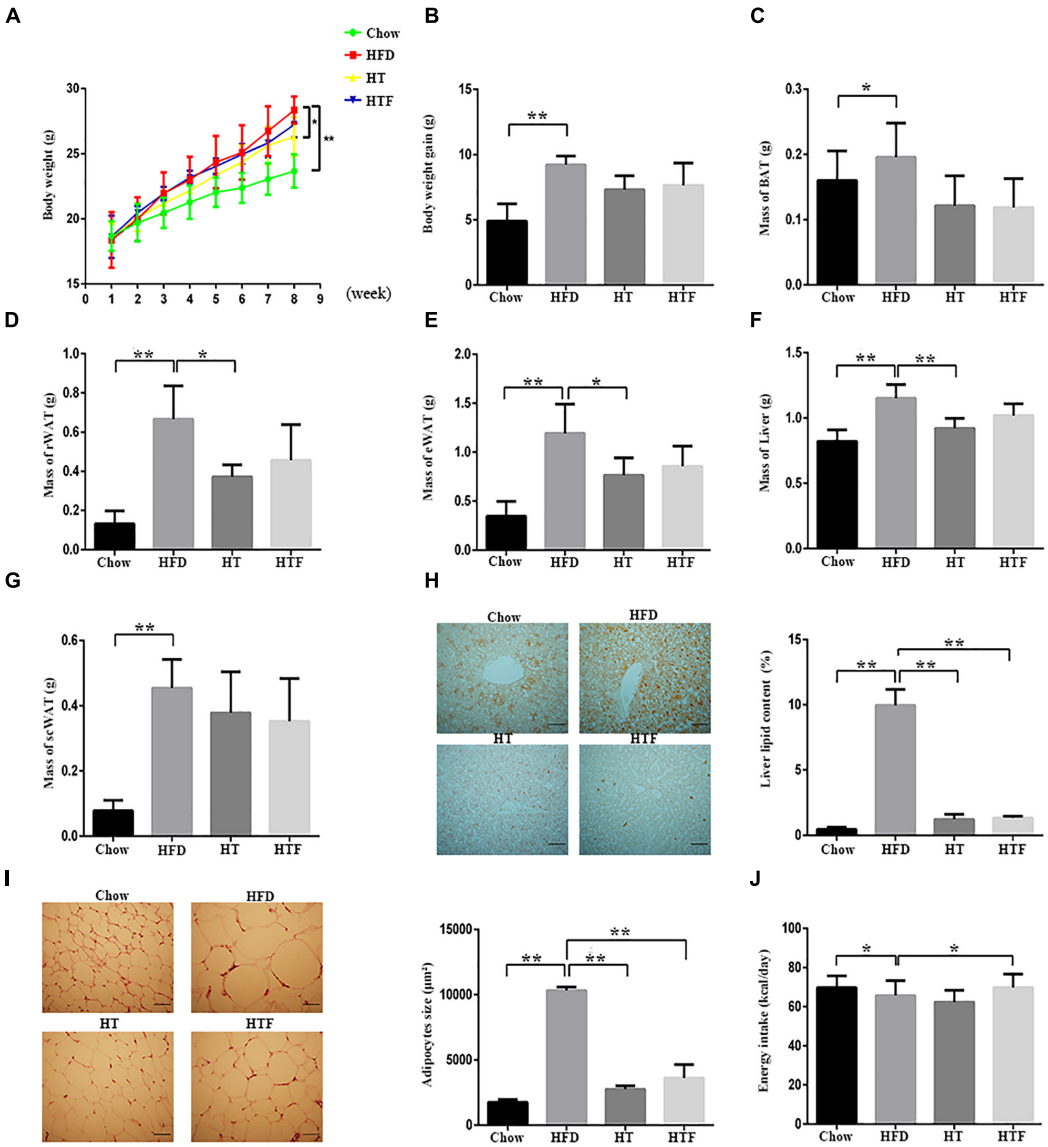

FIGURE 1 | HT and HTF reduces body weight and fat accumulation in HFD-fed mice. HFD-fed mice were treated daily with HT (50 mg/ kg) or HTF (100 mg/1 ml) by intragastric gavage for 8 weeks (n = 7 for each group). Effects of HT and HTF treatment on body weight **(A)** body weight gain **(B)** brown fat **(C)** perirenal fat **(D)** epididymal fat **(E)** liver weight **(F)** subcutaneous fat **(G)** oil red O staining **(H)** subcutaneous adipocyte size **(I)** and energy intake **(J)**, are shown. In **(H)**, Liver lipid content was assessed using the Image J software. Scale bar, 50 μm. In **(I)**, adipocyte size was estimated using the Image J software (lower panel). Scale bar, 50 μm. Graph bars with statistically significant results ($P \leq 0.05$) based on one-way ANOVA analysis followed by Tukey's honest significant difference *post hoc* tests. Data are shown as mean ± s.e.m (*$P \leq 0.05$, **$P \leq 0.01$).

BAT, perirenal adipose tissue (rWAT), epididymal white adipose tissue (eWAT), and the scWAT compared with the chow group. HT supplementation significantly reduced the final body weight, mass of the rWAT, eWAT mass, and liver mass compared with the HFD group. The body weight gain and the weights of the BAT and scWAT tended to decrease, but there were no statistically significant differences (**Figures 1A–G**). All of the above parameters also declined in the HFT group compared to the HFD group, but none reached significance (**Figures 1A–G**). Consistently, we observed evident lipid deposition in the hepatocytes and increases in adipocyte size in the HFD group compared to the chow group, which were reversed by HT supplementation and HFT (**Figures 1H–I**). Interestingly, HFD mice consumed less energy than the chow group, and the HFT mice consumed more energy than the HFD mice (**Figure 1J**).

HT and HTF Promoted Intestinal Integrity and Alleviated Inflammation in HFD Mice

To examine whether HT and HTF can improve intestinal integrity, we measured the concentration of lipopolysaccharide (LPS) in the plasma and the protein expressions of ZO-1 and

occludin in the ileum. As shown in **Figures 2A–D**, HFD increased the plasma LPS levels and disrupted the intestinal barrier, whereas HT and HTF were able to reduce the plasma LPS concentration and prevent intestinal barrier damage. Thus, HT and HTF may reduce the release of LPS into the blood by promoting intestinal barrier integrity. Additionally, intestinal integrity can also affect inflammation levels. As expected, the protein expressions of TLR-4, TNF-α, IL-1β, IL-6, and p-JNK in the mouse livers increased significantly in the HFD group compared to the chow group (**Figures 2E–J**). We further discovered that the concentration of the liver's IL-1β and IL-6 declined significantly after HT and HTF intervention, the concentration of TNF-α have only a downward trend (**Figures 2G–I**). Simultaneously, the expression of IκB-α, significantly decreased compared to the chow group (**Figure 2K**). The expression of TLR-4, TNF-α, IL-1β, IL-6, and p-JNK were significantly decreased in the HT and HTF groups (**Figures 2E–J**). The expression of IκB-α was significantly decreased in the HT group, but did not change significantly in HTF group (**Figure 2K**). Previous studies have shown that the TLR4 signaling pathway promotes the production of pro-inflammatory cytokines through the activation of JNK and NF-κB. Increased phosphorylation of JNK and NF-κB ultimately triggers IR via phosphorylation of IRS-1 on serine-307 and deactivation of AKT.

HT and HTF Decreased IR in HFD-Fed Mice

To evaluate insulin sensitivity, we performed oral glucose tolerance tests (OGTT) and insulin tolerance tests (ITT), tested fasting blood glucose and insulin, and calculated the index of HOMA-IR (**Figures 3A–G**). Specifically, after 8 weeks of a HFD, the OGTT area under the curve (OGGT-AUC) (**Figure 3A**), fasting blood glucose concentration (**Figure 3E**), fasting insulin concentration (**Figure 3F**), and HOMA-IR index (**Figure 3G**) were enhanced significantly in the HFD group compared to the chow group. In addition, the ITT-AUC (**Figure 3D**) tended to increase. The fasting blood glucose concentration, fasting insulin concentration, and HOMA-IR index were decreased significantly in HT group; and the OGTT-AUC, fasting insulin concentration, and HOMA-IR index were decreased significantly in HTF group. Although the other indicators also decreased with HT and HTF, no significant differences were found. These results indicate that HT supplementation and HTF alleviated IR induced by HFD. Furthermore, we measured the expression of key proteins related to IR (**Figure 3H**). As shown in **Figures 3I–J**, after 8 weeks of HFD, the expression of p-IRS (**Figure 3I**) was elevated significantly compared to the chow group, and the expression of p-AKT (**Figure 3J**) was significantly decreased. The expression of p-IRS and p-AKT (**Figures 3I–J**) significantly decreased in HT and HTF groups.

HT and HTF Modulated Gut Microbiota Composition and Reversed HFD-Induced Gut Dysbiosis

To explore changes to the gut microbiota after HFD, HT, and HTF, we performed 16S rRNA gene analysis of fecal samples

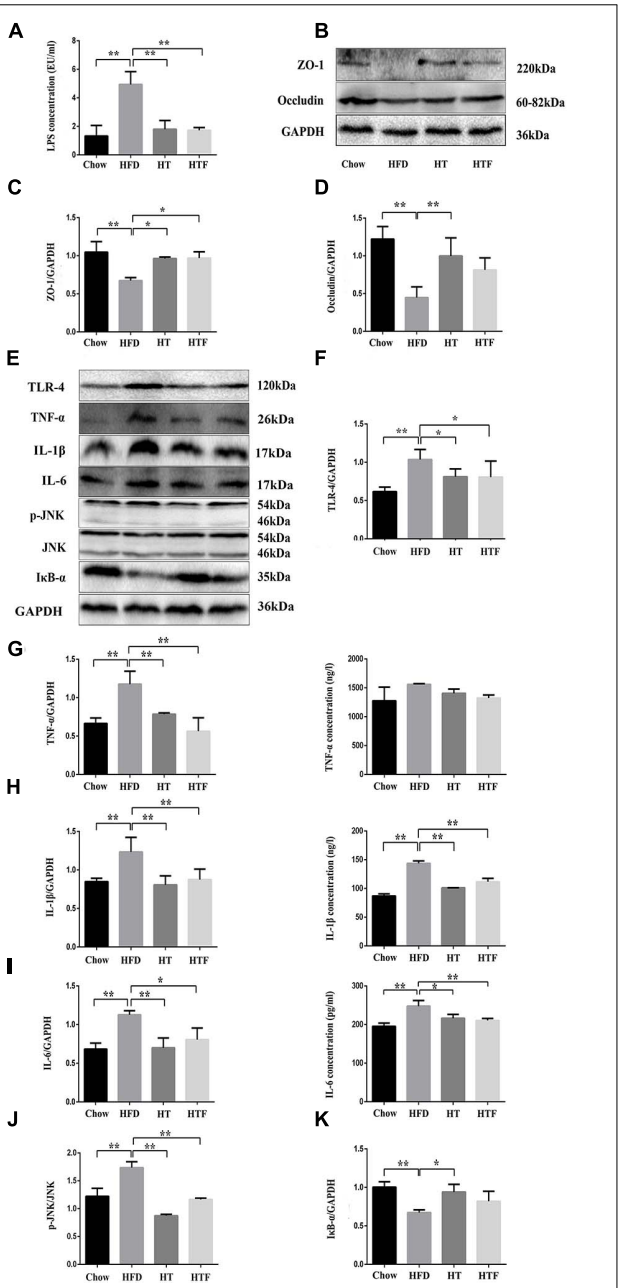

FIGURE 2 | HT and HTF protected intestinal integrity, decreased concentration of plasma LPS and alleviated inflammation in HFD mice. Effects of HT and HTF treatment on plasma endotoxin using a Chromogenic End-point Tachypleus Amebocyte Lysate **(A)** The expression of Occludin, ZO-1 and GAPDH in ileum was assessed using western blot **(B)**. Bar graphs represent normalized data of Occludin /GAPDH **(C)** and ZO-1 /GAPDH **(D)** $n = 3$. The expression of Occludin, TLR-4, TNF-α, IL-1β, IL-6, p-JNK, IκB-α and GAPDH in liver was assessed using western blot **(E)**. Bar graphs represent normalized data of TLR-4/GAPDH **(F)**, TNF-α/ GAPDH and the concentration of TNF-α in liver **(G)**, IL-1β/GAPDH and the concentration of IL-1β in live **(H)**, IL-6/GAPDH and the concentration of IL-6 in live **(I)**, p-JNK/JNK **(J)**, IκB-α/GAPDH **(K)** $n = 3$. Molecular weight markers were indicated as kilodaltons (kDa), Graph bars with statistically significant results ($P \leq 0.05$) based on one-way ANOVA analysis followed by Tukey's honest significant difference *post hoc* tests. Data are shown as mean ± s.e.m (*$P \leq 0.05$, **$P \leq 0.01$).

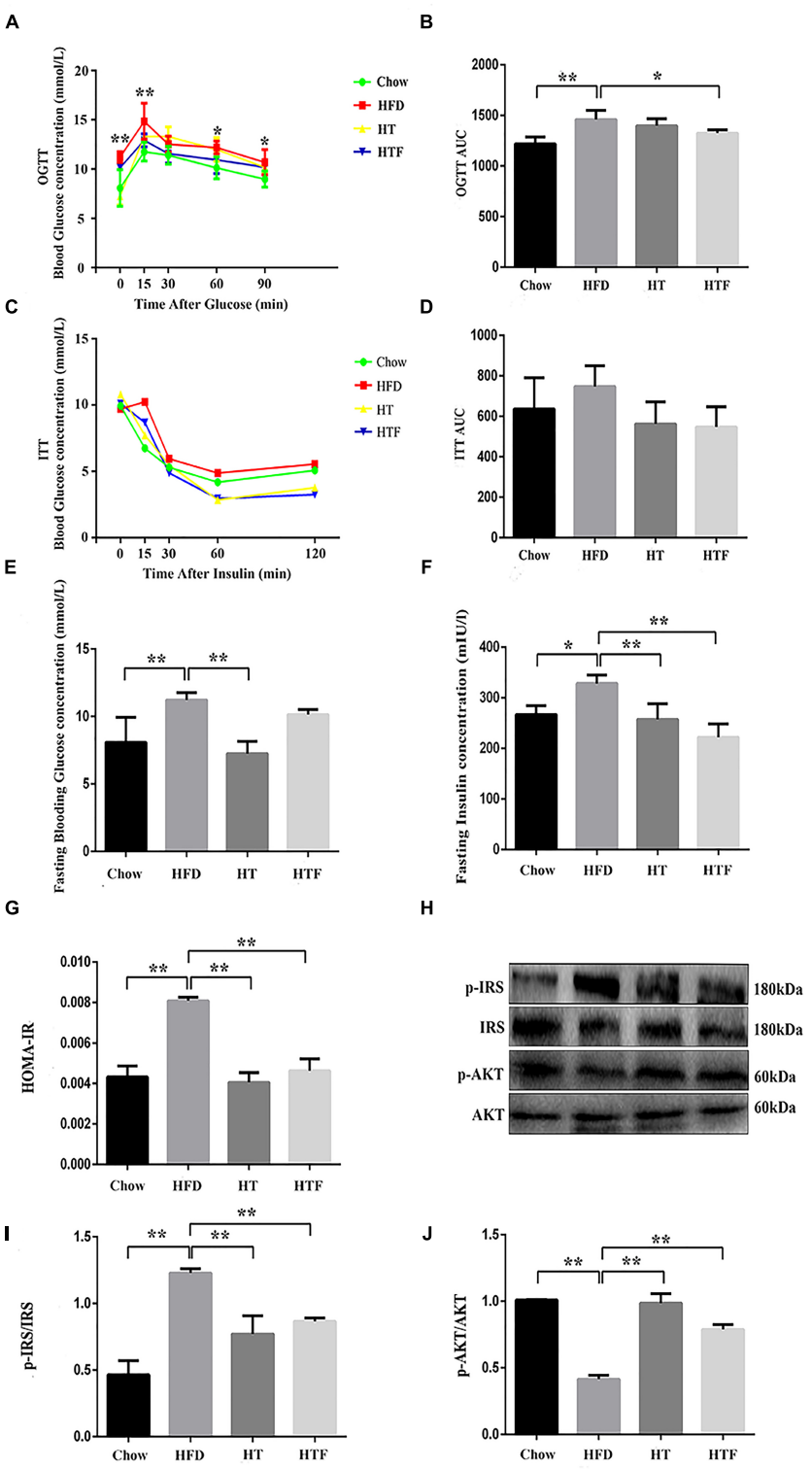

FIGURE 3 | HT and HTF improved IR in HFD-fed mice. Oral Glucose tolerance test (OGTT, 2 g/kg bw) after 8 weeks of intervention $^{*}P < 0.05$, $^{**}P < 0.01$ HFD vs. Chow **(A)** and the area under the curve (AUC) calculation **(B)**, Insulin tolerance test (ITT, 0.75 U/kg bw) after 8 weeks of intervention **(C)** and the AUC calculation **(D)**, Fasting blood glucose levels of each group **(E)**, Fasting blood insulin of each group **(F)** and HOMA-IR index after 8 weeks of intervention **(G)**. The expression of p-IRS and p-AKT in liver was assessed using western blot **(H)**. Bar graphs represent normalized data of p-IRS/IRS **(I)** and p-AKT/AKT **(J)** $n = 3$. Molecular weight markers were indicated as kilodaltons (kDa), Graph bars with statistically significant results ($P \leq 0.05$) based on one-way ANOVA analysis followed by Tukey's honest significant difference *post hoc* tests. Data are shown as mean ± s.e.m ($^{*}P \leq 0.05$, $^{**}P \leq 0.01$). HOMA-IR: homeostasis model assessment of insulin resistance.

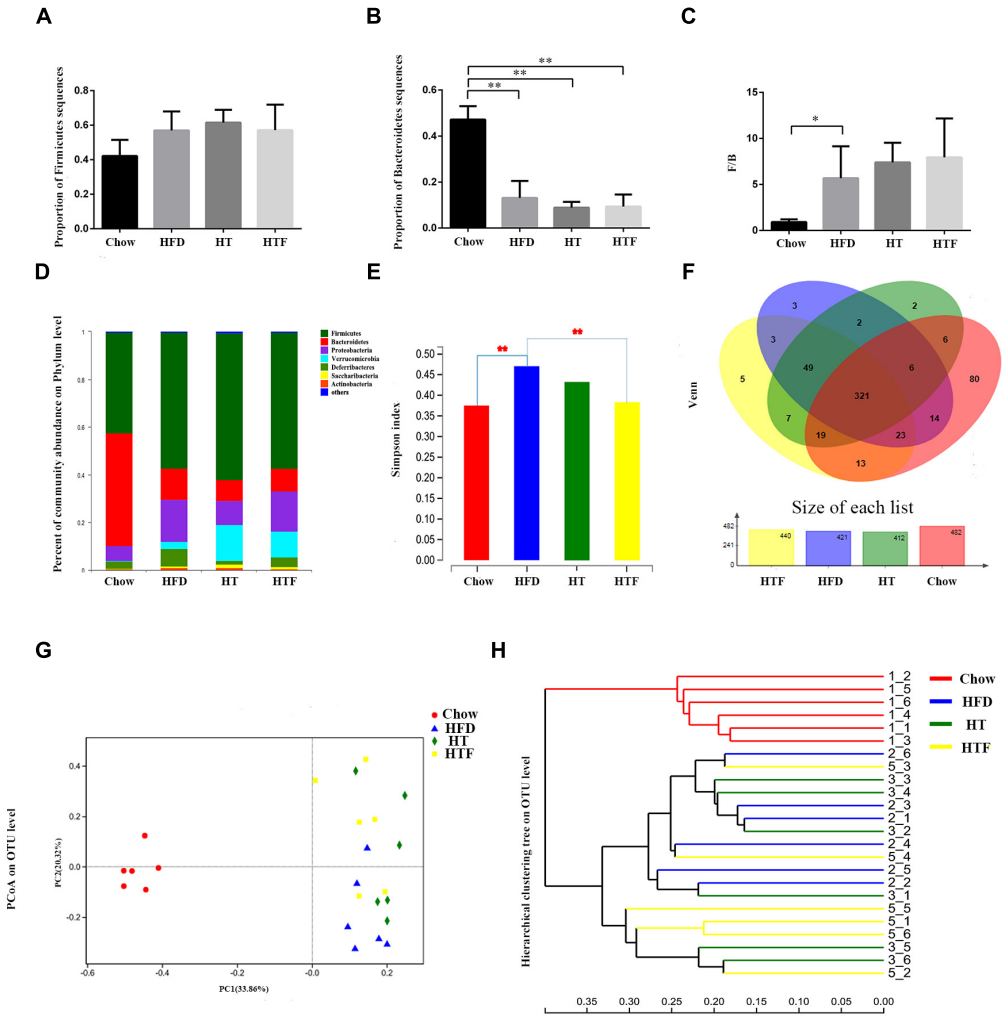

FIGURE 4 | HT and HTF alters microbiota structure in HFD-fed mice. Microbiota composition in feces of chow-fed mice and HFD mice treated with HT and HTF were analyzed using next generation sequencing (n = 6 for each group). Proportion of Firmicutes **(A)**, Proportion of Bacteroidetes **(B)**, F/B **(C)**, Graph bars with statistically significant results ($P \leq 0.05$) based on one-way ANOVA analysis followed by Tukey's honest significant difference *post hoc* tests. Plots shown were generated using the weighted version of the bray curtis-based Bacterial taxonomic profiling in the phylum level of intestinal bacteria from each group **(D)** Simpson index **(E)** were analyzed using unpaired two-tailed Student's t-test. Venn chart **(F)**, PCoA **(G)** and Hierarchical clustering tree on OTU level **(H)** (*$P \leq 0.05$; **$P \leq 0.01$).

from the mice. High-throughput sequencing yielded 1574700*2 original sequences from 24 samples (**Supplementary Table S1**), of which 1,090,437 were valid sequences (33,690 ± 4,147 per sample) (**Supplementary Table S2**). The coverage index was 1 ± 0.00001, indicating adequate species coverage during sequencing (**Supplementary Table S3**).

We found no significant differences in *Firmicutes* between the four groups (**Figure 4A**). However, the number of *Bacteroides* in the chow group was significantly higher than the HFD-fed groups (**Figure 4B**). The Chow group had the lowest ratio of *Firmicutes* to *Bacteroides* (F/B) compared to the other three groups (**Figure 4C**). Neither the number of *Bacteroides* nor the ratio of F/B were altered after HFD, HT, and HTF (**Figures 4B–C**). Notably, taxonomic profiling showed that supplementation with HT and HTF reduced the numbers of *Proteobacteria* and *Deferribacteres* in HFD-fed mice (**Figure 4D**).

The Simpson Index was proposed by Edward Hugh Simpson in 1949 to quantify the biodiversity of a region and serves as an indicator of microbial diversity. Larger Simpson Index values indicate lower diversity in the community. The Simpson index of HFD group was significantly higher than that of the chow group and was decreased after HT and HTF treatment, albeit no statistical significance was observed in the HT group (**Figure 4E**).

All sequences were classified into 553 operational taxonomic units (OTUs) at 97% similarity after subsampling. The Venn diagrams in **Figure 4** showed that the OTU of the chow group was the highest, reaching 482, followed by 440 in the HTF group, 421 in the HFD group, and 412 in the HT group. 553 OTUs were found in all of the groups. In this study, there were 80 OTUs unique to the chow group, 3 OTUs unique to the HFD group, 2 OTUs unique to the HT group, and 5 OTUs unique to the HFT group (**Figure 4F**).

We used principal coordinates analysis (PCoA) plots (**Figure 4G**) and sample-level clustering analysis (**Figure 4H**) to reflect the similarities and differences in the composition of the microbial community. In the PCoA plots, the chow group is distributed on the left side, the HFD group is mostly distributed in the lower right corner, the HTF group is mostly distributed in the upper right corner, and the HT group is evenly distributed on the right side. The results showed a clear difference in the gut microbiota composition between the chow group and the other three groups, and the microbiota composition of HT and HTF groups were similar.

To further explore bacterial species changes in obese mice undergoing HT or HTF treatment, we performed a significant difference comparison between the groups. Since a large number of bacteria were found in this study, statistical differences at the species, genus and family levels were calculated. At the species level, we found that 2 species were altered by HT and/or HTF. *Lactobacillus johnsonii* was significantly increased after HT supplementation and HTF, while *Anaerotruncus sp. G3 (2012) was* significantly decreased after HTF supplementation (**Figure 5A**). At the genus level, four representative genera were altered by HT and/or HTF treatment. *Lactobacillus* significantly increased after HT and HTF, while *Rikenella* was significantly decreased after HT and HTF. *Desulfovibrio* and *Ruminiclostridium* were decreased after HT and HTF, but the decreases were not statistically significant (**Figure 5B**). At the family level, *Ruminococcueae* and *Christensenellaceae* were decreased after HT and HTF, but the decreases were not statistically significant (**Figure 5C**).

DISCUSSION

Previous studies have shown that HT prevents inflammation, hyperglycemia, hyperlipidemia, IR, and obesity. Some studies have shown that HT supplementation is beneficial to HFD-induced obese mice, while other studies have shown that HT has no effect on obesity (Cao et al., 2014; Voigt et al., 2015; Wang et al., 2018). Specifically, Voigt et al. (2015) found that HT (20 mg/kg/day, 21 days) reduced weight gain in HFD mice, but there have statistical difference only on the 15th and 18th days. A previous study has showed (Jemai et al., 2015) that HT treatment (8 mg/kg, 16 mg/kg, 4 weeks) has no effect on the body weight of diabetic rats, but can improve plasma glucose, liver glycogen and total cholesterol levels. At the same time, high doses significantly improved plasma glucose and total cholesterol levels compared to low doses. In addition, Zheng et al. (2015) found that the neurons factor of the cerebral cortex were lost in db/db mice compared with the control mice. HT supplements (10 mg/kg, 50 mg/kg, 8 weeks) improved the neuronal survival of brain in db/db mice. Of note, high doses significantly improved neuronal survival compared to the low doses. The differences in these results may be due to a difference in HT concentration and intervention time. In the present study, HT gavage at a concentration of 50 mg/kg/day for 8 weeks was able to maintain the integrity of intestinal barrier, alter the composition of gut microbiota, reduce the release of endotoxin into the blood, and reduce inflammation, ultimately preventing HFD-induced obesity and IR.

Jumpertz et al. (2011) found that the gut microbiota is associated with energy metabolism (Bäckhed et al., 2004; Fleissner et al., 2010; Velagapudi et al., 2010). Our results demonstrated that mice fed regular chow consumed more energy than HFD-fed mice, but the body weight of the chow group was lower than that of the HFD mice, indicating that HFD induces body weight increase by regulating energy utilization. In addition, we observed that the body weight of the HTF mice was lower and the energy intake was higher than that of the HFD mice, suggesting that changes in the gut microbiota caused by HT may affect energy absorption and metabolism. Interestingly, the energy intake of the HT mice was lower than that of the HTF group, and more metabolic benefits were seen in the HT group. It is possible that the concentration used for the fecal transplantation was not enough, or that HT may benefit HFD mice through a mechanism which isn't dependent upon gut microbiota.

Previous studies have highlighted that HFDs reduce microbial diversity (Turnbaugh et al., 2008; Turnbaugh et al., 2009). In contrast, Kasai et al. (2015) found that the bacterial diversity in obese individuals was significantly higher than that in lean individuals in clinical trials. We observed that HT and HTF restored bacterial diversity which had been decreased by HFD. Our results suggest that bacterial diversity is inversely proportional to obesity. Moreover, one of the characteristics of the gut microbiota in obese individuals is a high ratio of F/B (Ley et al., 2005; Ley et al., 2006; Turnbaugh et al., 2006). We found that HFD mice had a significantly increased F/B, which was not altered by HT or HTF intervention. This indicates that HT may be beneficial for metabolism but does not regulate F/B.

We found that HT supplementation significantly changed the composition of the gut microbiota. Consistent with the findings of Kim et al. (2012), we found that *Ruminococcaceae* were significantly decreased by HT supplementation at the family level. In addition, *Proteobacteria* and *Ferribacter* were increased in the HFD-fed mice, consistent with previous studies (Hu et al., 2015; Wang C. C. et al., 2017). The proportions of these two species decreased after HT intervention but did not reach statistical significance. This suggests that HT may prevent obesity partially through regulation of *Proteobacteria* and *Ferribacter*. In addition to these bacteria, other bacteria may also help to prevent obesity. It has been reported that oral administration of *Parabacteroides goldsteinii* reduced the body weight of HFD-induced obese mice, enhanced adipose tissue heat production, enhanced intestinal barrier integrity, and mitigated inflammation and IR (Wu et al., 2018). In the present study, we found that HT reversed the decrease of *Parabacteroides* after a HFD, suggesting that *Parabacteroides* may play an anti-obesity role. Moreover, clinical studies by Million et al. (2012) revealed that some *Lactobacillus* species were associated with normal body weight. We found that HT reversed the alteration of *Lactobacillus johnsonii* by HFD. Furthermore, *Christensenellaceae*, which belong to the *Firmicutes*, are enriched in low BMI individuals compared to those with a high-BMI (Goodrich et al., 2014) and are negatively correlated with obesity. Conversely, in our

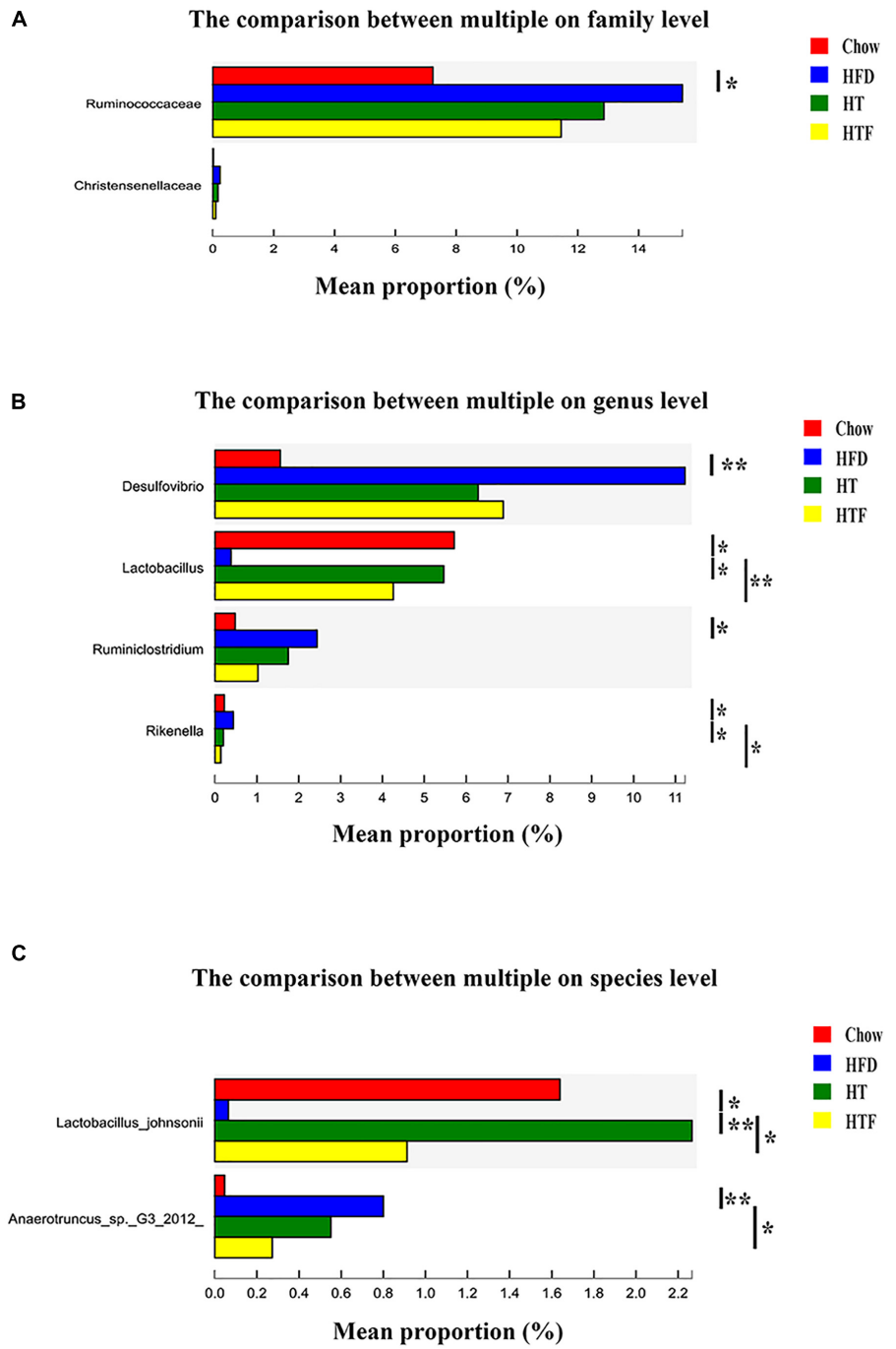

FIGURE 5 | The analysis of species difference in different groups. The comparison between HFD and the other three groups in family **(A)**, genus **(B)** and species **(C)** level based on wilcoxon rank sum test (*$P \leq 0.05$; **$P \leq 0.01$).

study, HT reversed the rise of *Christensenellaceae* caused by HFD. Finally, we found that HT reversed the decrease of *Rikenella* induced by HFD. HT enhanced a variety of bacterial species that are negatively associated with obesity and reduced species that are positively associated with obesity. In summary, HT may prevent HFD-induced obesity by regulating a variety of different gut microbiota.

The successes of FMT serve as direct evidence of the interaction between the gut microbiota and many diseases (Zhang et al., 2018). Previous studies have shown that FMT can affect body weight and some metabolic indicators. For example, Maria et al. found that fecal transplantation from chow-fed mice could increase gut microbiota species diversity and abundance in recipient HFD-fed mice without changing

body weight (Kulecka et al., 2016). *Ridaura et al.* found that FMT recipients receiving a transplant from an obese individual gained more weight than those receiving a transplant from a non-obese individual (Ridaura et al., 2013). There is still a lack of research on the mechanism by which HT improves obesity. In order to investigate whether the gut microbiota played a role, we transplanted the fecal bacteria from mice fed HT to HFD mice. The PCoA and the hierarchical clustering tree indicate that the gut microbiota of the HT group and the HTF group were similar but not identical. This result reflects the effectiveness and reliability of the gut microbiota transplantation.

We found that FMT from mice supplemented with HT (HTF) also resulted in beneficial effects similar to those seen in HT-gavaged mice, including an increase in the integrity of the intestinal barrier, prevention of inflammation, and alleviation of obesity and IR induced by a HFD. Furthermore, HFT altered the intestinal microbiota in a manner consistent with HT supplementation. It is worth mentioning that most of the beneficial effects seen after HTF intervention were weaker than those seen after HT, which may be the result of an insufficient concentration of fecal bacteria. These results indicate that HT may benefit obese individuals by changing the composition of the intestinal flora.

In addition to analyzing changes in microbiota, our *in vivo* experiments showed that administration of HT reduced the expression of proteins involved in inflammation and reduced IR in HFD-fed mice. Our results suggest that a HFD induced intestinal dysfunction and increased the permeability of the intestinal mucosa in mice, which resulted in increased endotoxin release into the blood. Endotoxins trigger inflammation by activating the TLR-4 receptor and the NF-κB pathway, which induces IRS-1 phosphorylation at serine 307 and ultimately leads to IR and obesity (Wang N. et al., 2017). In the present study, HT and HTF restrained the activation of the TLR-4 and NF-κB pathways, reduced the expression of pro-inflammatory factors and IRS-1 phosphorylation at serine 307, and enhanced the phosphorylation of AKT. Thus, HT may alleviate IR through the TLR-4 and NF-κB signaling pathways.

There were a few limitations in our experiments. Although numerous bacteria related to the improvement of obesity have been identified, no further SMTs have been carried out. Additionally, our study was exclusively performed in animals. Future work should verify the effects of specific gut bacteria on obesity and IR, study the synergy of various gut bacteria by SMT, and explore the application of SMT in the treatment of obesity in humans. Overall, our results suggest that HT reduces weight gain, chronic inflammation, and IR in HFD-induced obese mice, partially through regulation of specific gut microbiota.

Overall, our results suggested that HT reduced weight gain, chronic inflammation and IR in HFD-induced obesity mice, partially by beneficial changes in specific gut microbiota.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the **Supplementary Files**.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of Animal Ethics Committee of the Institute of Genome Engineered Animal Models for Human Disease, Dalian Medical University. The protocol was approved by the Animal Ethics Committee of the Institute of Genome Engineered Animal Models for Human Disease, Dalian Medical University.

AUTHOR CONTRIBUTIONS

DW gave a general research direction. ZL completed the experimental design, data analysis, and manuscript writing. NW and YM conducted article language retouching. All authors reviewed 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.2019.00390/full#supplementary-material

TABLE S1 | Original sequences information.

TABLE S2 | Valid sequences information.

TABLE S3 | Coverage index.

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frontiers in Microbiology

ORIGINAL RESEARCH
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Beneficial Effects of Pomegranate Peel Extract and Probiotics on Pre-adipocyte Differentiation

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The beneficial effects of pomegranate are due to the ellagitannins and anthocyanins content, which are protective toward a wide variety of diseases including inflammatory diseases. Many investigators have reported that pomegranate waste (peel and seeds) extracts, made from waste product of industrial processing, show free radical scavenger and a potent antioxidant capacity. Pomegranate extracts (PEs) were also reported to possess noteworty antibacterial, antiviral, hypolipidemic, and anti-inflammatory bioactivities thanks to the polyphenolic compounds content, which includes punicalagins, gallic acid, and ellagic acid derivatives. The focus of the present manuscript was to study the prebiotic potentiality of a PE, soluble in water, and characterized through HPLC–PDA–ESI/MS^n for its phenolic content. Moreover, since it has been reported that pomegranate extracts decreased the level of lipids in the blood and that a number of probiotic strains have been shown to affect adipogenesis in cell culture, this study was also performed to test the *in vitro* effects of PE and probiotic *L. rhamnosus* GG ATCC 53103 strain (LGG) on 3T3-L1 cell line. PE and probiotics substantially reduced the triglyceride content and intracellular lipid increase, compared to the control group. However, the combination treatment of PE and LGG filtered spent broth (SB) was the most effective in reducing triglyceride content and intracellular lipid accumulation. The mRNA expression levels of the main transcriptional factors implicated in adipocyte differentiation were substantially lower in 3T3-L1 cells treated with PE and LGG filtered SB. These results evidenced that a synergistic effect of probiotics and polyphenols contained in PE may affect *in vitro* adipogenesis and may contribute in development of new nutraceutical/probiotic-based remedies to prevent and to treat obesity.

Keywords: lactobacilli, antimicrobial activity, pomegranate extract, adipocyte differentiation, combining foods

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INTRODUCTION

Pomegranate is a fruit whose positive health effects have been extensively studied. This fruit is rich in bioactive compounds such as ellagitannins and anthocyanins content, which are protective toward degenerative diseases. Pomegranate fruit, because of its high nutritive value, health benefits, and antioxidant bioactive compounds, is considered as a food medicine. In fact, pomegranate has

been considerably used in herbal medicine for several pathologies including flu and infections of the upper respiratory tract. All parts of the pomegranate fruit, i.e., peel and seeds, considered as waste products, can be processed for value-added products having industrial, medicinal, and cosmetic value (Dhumal et al., 2014).

Pomegranate wastes are produced in all the phases of fruits life cycle, i.e., during agricultural production, industrial manufacturing, and processing. It is possible to take advantage of pomegranate by-products as they are a rich source of bioactive compounds such as flavonoids, phenolic acids, and tannins. Moreover, many researchers have described that pomegranate extracts, made from by-products of the processing factories, have an effective free radical scavenging activity and antioxidant capacity (Lee et al., 2010; Panichayupakaranant et al., 2010; Fischer et al., 2011).

Furthermore, the pomegranate extracts act as natural inhibitors of pathogens, bacteria, and fungi (Al-Zoreky, 2009; Tehranifar et al., 2011; Romeo et al., 2015).

Pomegranate ellagitannins are hydrolyzed by gut microbiota to smaller phenolics, such as ellagic acid. Ellagic acid is then absorbed into the blood circulation, while ellagitannins are not absorbed and are metabolized into urolithins.

It has been reported that pomegranate by-products and punicalagins significantly are able both to inhibit the growth of pathogenic *Escherichia coli*, *Pseudomonas aeruginosa*, *Clostridia*, and *Staphylococcus aureus* (Reddy et al., 2007; Bialonska et al., 2009) and to increase the growth on beneficial bacteria including *Bifidobacterium* spp. and *Lactobacillus* spp. (Reddy et al., 2007; Bialonska et al., 2010).

Pomegranate extracts were also reported to decrease the level of lipids in the blood and to have significant anticancer, antiviral, and anti-inflammatory activities (Li et al., 2006; Hossin, 2009; Lin et al., 2013; Bassiri-Jahromi, 2018).

These potential beneficial effects are attributed to the polyphenolic compounds that the pomegranate extracts contain including punicalagins, gallic acid, and ellagic acid derivatives (Vanella et al., 2013a,b,c; Romeo et al., 2015).

Because obesity is one of the main public health problems, new preventive strategies are necessary (Smith and Smith, 2016).

Adipocyte plays a major role in the begin or development of metabolic complications associated to obesity, such as metabolic syndrome and diabetic complications (Kim and Plutzky, 2016).

The interest of the researchers in the identification of natural products obtained from dietary plants that have anti-obesity activities has increased. It has been reported that the xanthigen and fucoxanthin, natural compounds of pomegranate seed oil, significantly suppressed adipocyte differentiation and lipid accumulation (Lai et al., 2012).

The focus of the present manuscript was firstly to study the antioxidant and antimicrobial activities, and the prebiotic potential of a PE rich in phenolic compounds. Moreover, the enriched standardized PE, containing high percentages of pomegranate natural antioxidants, was chemically characterized through HPLC–PDA–ESI/MSn. Secondly, since pomegranate juice (Les et al., 2018) and different types of pomegranate extracts (PEs), including extract prepared from the whole fruit (Li et al., 2015) and extract derived from pomegranate peel (Neyrinck et al., 2013), and a number of probiotic strains (Moon et al., 2012; Park et al., 2014) have been shown to affect adipogenesis, this study was carried out to test the *in vitro* effects of PE, probiotic *L. rhamnosus* GG ATCC 53103 (LGG) preincubated with PE, alone or in combination, on 3T3-L1 cell differentiation.

MATERIALS AND METHODS

Chemicals

The powdered pomegranate extract (Dermogranate®) employed in this study was provided by Medinutrex (Catania, Italy). Briefly, the extract was prepared from dried and grinded pomegranate fruits mixed with hydroalcoholic solutions (food grade) and then filtered. The filtrate was concentrated and then spray-dryed to obtain the standardized extract. The Dermogranate® extract had the following chemical composition: total polyphenols (16%), punicalagins (8%), ellagic acid, and derivatives (8%).

Folin–Ciocalteu reagent (FCR), sodium carbonate (Na_2CO_3), gallic acid, punicalin (mixture of anomers), punicalagin, and ellagic acid were purchased from Sigma-Aldrich (Milan, Italy). Granatin B was purchased from LGC Standards (London, United Kingdom). HPLC–MS grade solvents (Merck KgaA, Darmstadt,Germany) were used for chromatography and all other reagents were of analytical grade.

Determination of Total Polyphenols Content

The Folin–Ciocalteu assay (Singleton et al., 1999) was used for the determination of total polyphenols content with slight modifications. 0.1 mg/ml of extract was dissolved in distilled water. Then, 5 ml of 10% FCR and 4.5 ml of Na_2CO_3 solution (7.5% w/v) were added to 500 μl of sample. The final solution was agitated for 2 h in the dark and then the Abs at $\lambda = 765$ nm was measured. Analyses were carried out in triplicate and the concentration of total polyphenols was expressed as g of gallic acid equivalents (GAEs)/100 g of extract.

HPLC–PDA–ESI/MS^n Analysis of Phenolic Compounds

Separation and quantification of phenolic compounds were performed as previously described (Romeo et al., 2015). For the identification of phenolic compounds, the retention times (RTs), spectra, and MS data in negative ESI mode were compared to those of authentic standards. Quantification of each phenolic compound was performed using the corresponding standard as external standard. Quantification was carried out at 280 nm for gallic acid. Punicalins, granatin B, punicalagins, and ellagic acid were quantified at 378 nm; the same wavelength was used for the quantification of ellagic acid derivatives using ellagic acid as reference standard. Analyses were carried out in triplicate and the results were expressed as g of compound/100 g of extract.

Quenching of DPPH

The free radical-scavenging capacity of different concentrations of PE extract (3.4–1.7–0.85–0.56–0.42–0.34–0.21–0.17–0.11–0.085–0.028 mg/ml) was measured by 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH)-free radical method as previously reported (Salerno et al., 2012). Results are expressed as percentage of inhibition rate ± *SD*.

Scavenger Effect on Superoxide Anion

The superoxide anion-scavenging capacity of different concentrations of PE extract (3.4–1.7–0.85–0.56–0.42–0.34–0.21–0.17–0.11–0.085–0.028 mg/ml) was measured as previously reported (Salerno et al., 2012). Results are expressed as percentage of inhibition rate ± SD.

3T3-L1 Murine Pre-adipocytes Cell Viability

3T3-L1 murine pre-adipocytes were bought from American Type Culture Collection (Rockville, MD, United States). Cells were plated at a concentration of 2×10^5 cells per well of a 96-well microplate and cultured at 37°C in incubator with 5% CO_2 for 48 h in the absence and presence of the different concentrations of PE reported above. Cell viability was measured by MTT assay as previously reported (Di Giacomo et al., 2015). MTT, a yellow tetrazole, is reduced to purple formazan in living cells. Results are expressed as percentage of formazan produced in treated 3T3-L1 murine pre-adipocytes cells compared to untreated cells.

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Bacterial Cultivation

The commercial pathogen strains *E. coli* ATCC 25922, *S. aureus* ATCC 29213, *Listeria innocua* ATCC 33090, and *Salmonella enterica* ATCC 14028 were used. *E. coli* strain was grown in Luria-Bertani (LB) broth at 37°C overnight; *S. aureus*, *S. enterica*, and *L. innocua* were routinely grown overnight, in Tryptone Soya Broth (TSB) at 37 and 30°C, respectively. All media and supplements were provided by Oxoid (Milan, Italy).

The commercial probiotic strains LGG, *Bifidobacterium animalis* BB12, *B. longum* BB536, and the wild strain *Lactobacillus paracasei* N 24, isolated from Pecorino crotonese cheese, were cultured in deMan–Rogosa–Sharpe (MRS) broth at 37°C overnight.

Overnight bacterial culture was incubated at 37°C for 24 h, under anaerobic condition, until they reached a cell density of approximately 1.0×10^9 cfu/ml.

Growth Rate Determination

Based on results obtained on free radical scavenger activity of PE and on 3T3-L1 murine pre-adipocytes cell viability experiments, different concentrations were used for treatment of probiotic or pathogen strains as described below.

Antimicrobial activity of the PE was evaluated against the commercial pathogen strains mentioned above. Overnight pathogen cultures were co-cultured at 37°C for 24 h with PE at different concentrations (1.7–0.34–0.17 mg/ml), and the antimicrobial activity was evaluated by plating count of live bacteria and expressed as cfu/ml.

The effect of PE on the growth of probiotic strains mentioned above was evaluated inoculating in co-culture the probiotic strains at a cell density of approximately 1.0×10^9 cfu/ml with the PE at different concentrations (0.085–0.042–0.028 mg/ml). The effect of the extract on growth of probiotic strains was evaluated after incubation at 37°C for 24 h under anaerobic conditions by plating count of live bacteria and expressed as cfu/ml.

All experiment was conducted in duplicate and results were expressed as mean values and standard deviation. Based on preliminary results LGG was chosen for the subsequent analyses.

Fresh broth cultures were centrifuged at 5000 rpm for 10 min at 4°C, and the supernatant was decanted to collect the spent broth (SB), which was filtered (FSB), using a 0.22-μm filter and then used for further analyses.

The bacterial pellet was resuspended in 1 ml of PBS and sonicated five times at 44% amplitude for 2 min with 6 min of rest. The sonicate was then centrifuged at $1100 \times g$ for 15 min at 4°C. The supernatant was collected, filtered through a 0.22-μm filter, and labeled bacterial cell extract (CE). The CE was used for further analysis.

Cell Culture and Adipocyte Cell Differentiation

3T3-L1 murine pre-adipocytes were resuspended in Dulbecco's Modified Eagle Medium (DMEM), containing 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, United States) and 1% antibiotic/antimycotic solution (Invitrogen, Carlsbad, CA, United States) and seeded in a 75-cm^2 flask at a density of 1 to 2×10^4 cells. Adipocyte cell differentiation was obtained as previously reported (Waldman et al., 2016).

Differentiating 3T3-L1 pre-adipocytes were treated for 7 days with PE (0.028 mg/ml), LGG CE (25 μg/ml), and LGG filtered SB (10 μg/ml) from overnight bacterial culture incubated with or without PE (0.028 mg/ml).

Lipid Content Quantification

To quantify lipid accumulation, Oil Red Staining was performed as previously reported (Barbagallo et al., 2017). Formation of lipid drops was measured with an inverted multichannel LED fluorescence microscope (Evos, Life Technologies, Grand Island, NY, United States).

RNA Extraction and qRT-PCR

Expressions of adiponectin, PPAR-γ, SREBP, FAS, IL-6, and IL-10 were evaluated by real-time PCR. RNA was extracted and quantified as previously reported (Raffaele et al., 2018). Appropriate primer sequences were used (**Table 1**). The relative mRNA expression level was measured by the threshold cycle (Ct) value of each PCR product and normalized with that of GAPDH by using comparative $2^{-\Delta\Delta Ct}$ method.

Statistical Analyses

Statistical analyses of multiple comparisons were performed by the Fisher method. *P*-values lower than 0.05 were accepted as

TABLE 1 | PCR primers used in this study.

Gene	Forward primer	Reverse primer
Adiponectin	GAAGCCGCTTATGTGTATCGC	GAATGGGTACATTGGGAACAGT
IL-6	TTCCTCTCTGCAAGAGACTTCC	AGGAGAGCATTGGAAATTGGGG
FAS	GGAGGTGGTGATAGCCGGTAT	TGGGTAATCCATAGAGCCCAG
GAPDH	AGCTTCGGCACATATTTCATCTG	CGTTCACTCCCATGACAAACA
IL-10	GCTGGACAACATACTGCTAACC	ATTTCCGATAAGGCTTGGCAA
SREBP-1	GATGTGCGAACTGGACACAG	CATAGGGGGCGTCAAACAG
PPAR-γ	TCGCTGATGCACTGCCTATG	ACCTGATGGCATTGTGAGACAT

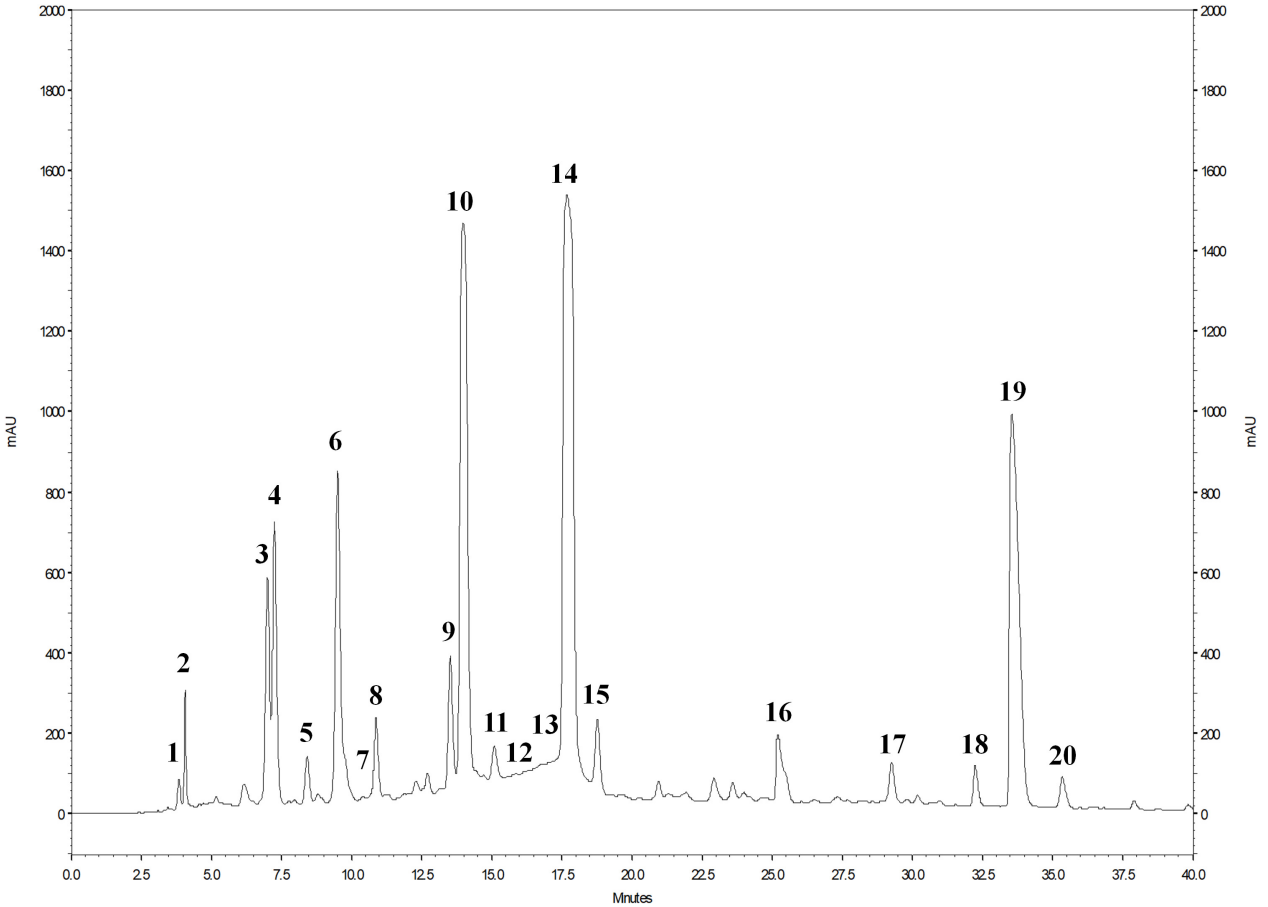

FIGURE 1 | HPLC chromatogram of phenolic compounds of PE detected at 378 nm. For the identification of the peaks, see **Table 2**.

significant. Data were analyzed using either single-factor analysis of variance (ANOVA) for multiple groups, or the unpaired *t*-test for two groups, and the results are presented as mean ± SD.

RESULTS

HPLC–PDA–ESI/MS^n Analysis of PE

The phenolic profile of PE (**Figure 1**) included the determination of 1 hydroxybenzoic acid and 19 ellagitannins. The main peaks corresponded to punicalin (peak 4), granatin B (peak 6), punicalagin A and B (peaks 10 and 14), and ellagic acid (peak 19) (**Figure 1**). The presence of gallic acid (peak 1) and ellagic acid derivatives (peaks 2, 3, 5, 7–9, 11–13, 15–18, and 20) was also revealed. As shown in the chromatogram, ellagitannins are the predominant class of phenolic compounds in pomegranate peel and marc (a by-product made up of seeds and peels), since they represent over the 99% of the total content of pomegranate phenolics. Punicalagins, the major ellagitannins of pomegranate by-products, accounted for 47.6% of the total phenolics content in PE (**Table 2**). Ellagic acid derivatives, ellagic acid, and other minor phenolic compounds (punicalin, granatin B, and gallic acid), accounted for 38.4, 10.2, and 3.8% of the total phenolics content in PE, respectively (**Table 2**).

TABLE 2 | Peak list and quantification of the phenolics in PE.

Peak number[a]	RT (min)	λ_{max} (nm)	$[M–H]^-$ (*m/z*)	MS^n (*m/z*)	Phenolic compounds	g/100 g[b]
1	3.9	269,310	169	125	Gallic acid	0.07 ± 0.02
2	4.1	255,364	–	301	Ead[d]	0.03 ± 0.01
3	7.0	255,363	–	301	Ead[d]	0.41 ± 0.04
4	7.3	263,364	781	601	Punicalin	0.27 ± 0.04
5	8.4	264,366	–	301	Ead[d]	0.19 ± 0.09
6	9.5	260,365	951	933/613	Granatin B	0.28 ± 0.03
7	10.4	259,361	–	301	Ead[d]	0.02 ± 0.01
8	10.9	257,360	–	301	Ead[d]	0.05 ± 0.01
9	13.5	256,362	–	301	Ead[d]	1.23 ± 0.04
10	14.0	258,378	1083	781/601	Punicalagin A	3.05 ± 0.05
11	15.1	257,363	–	301	Ead[d]	0.13 ± 0.09
12	15.9	257,361	–	301	Ead[d]	0.95 ± 0.03
13	16.8	258,360	–	301	Ead[d]	2.69 ± 0.11
14	17.7	257,378	1083	781/601	Punicalagin B	4.77 ± 0.21
15	18.8	257,362	–	301	Ead[d]	0.12 ± 0.01
16	25.2	256,363	–	301	Ead[d]	0.17 ± 0.09
17	29.3	254,361	–	301	Ead[d]	0.17 ± 0.01
18	32.2	255,360	–	301	Ead[d]	0.08 ± 0.03
19	33.6	256,367	301	229/185	Ellagic acid	1.68 ± 0.01
20	35.4	248,362	–	301	Ead[d]	0.08 ± 0.02
Total polyphenols[c]						16.48 ± 2.49
Punicalagins						7.82
Ellagic acid derivatives						6.31
Ellagic acid						1.68
Other phenolic compounds						0.62
Total						16.43

[a] *The numbering is according to* ***Figure 1***. [b] *Results are expressed as the mean ± standard deviation.* [c] *Expressed as g GAE/100 g.* [d] *Ellagic acid derivative (Ead).*

The mass spectrometric properties of the 20 phenolic compounds identified (peaks 1–20) are shown in **Table 2**. As previously reported (Romeo et al., 2015), two isomeric forms (A and B) of punicalagins (peaks 10 and 14) were observed, as well as the presence of granatin B (peak 6) was highlighted. Furthermore, these compounds were also characterized by direct infusion-negative ion ESI/MS^n analysis of standard compounds. Peak 4 was identified as punicalin (*m/z* 781) while peaks 2, 3, 5, 7–9, 11–13, 15–18, and 20 were identified as ellagic acid derivatives according to their UV–Vis and mass characteristics (λ_{max} around 370 nm and MS^1 fragment at *m/z* 301 corresponding to ellagic acid).

Free Radical Scavenging Activity of PE

Antioxidant activity of PE was tested by their ability to reduce the stable DPPH radical.

Particularly, the percentage of inhibition of DPPH resulted up to 75% at concentrations lower to 0.21 mg/ml (76,78, 79, and 80% respectively, at concentration of PE of 0.17- 0.11- 0.085- and 0.028 mg/ml). At concentrations higher to 0.21 mg/ml the percentage of inhibition of DPPH resulted lower (**Figure 2**).

Pomegranate extract inhibited superoxide anion formation in a dose-dependent manner (**Table 3**). As a general trend, in this test PE resulted more effective than in the previous one. This might be due to the smaller size of superoxide anion compared to DPPH radical.

Effect of PE on 3T3-L1 Cell Viability

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay was carried out to evaluate 3T3-L1 cell viability. Results show a significant reduction of cell viability with high concentrations of PE (3.4, 1.7, 0.85, 0.56, and 0.42 mg/ml), whereas lower (0.34, 0.21, 0.17, 0.11, and 0.085 mg/ml) concentrations had a moderate inhibitory effect and 0.028 mg/ml concentration had no significant effect on 3T3-L1 murine pre-adipocytes cell viability (**Figure 3**).

Microbiology

Antimicrobial Activity of the PE on Pathogen Strains

Data of co-culture assay have shown that pathogens were differently sensitive to the PE. In detail, as showed in **Figure 4**, the extract at the concentration of 1.7 mg/ml showed the highest antimicrobial activity against all pathogens, with a significant decrease of *L. innocua* (proximally 4 log unit). At the concentration of 0.34 mg/ml the extract was efficacy against *E. coli*, *L. innocua*, and *S. aureus*, exhibiting a reduction of cell density of 1 and 2 log units, respectively. When the PE was tested at 0.17 mg/ml concentration, *S. aureus*, *S. enterica*, and *L. innocua*

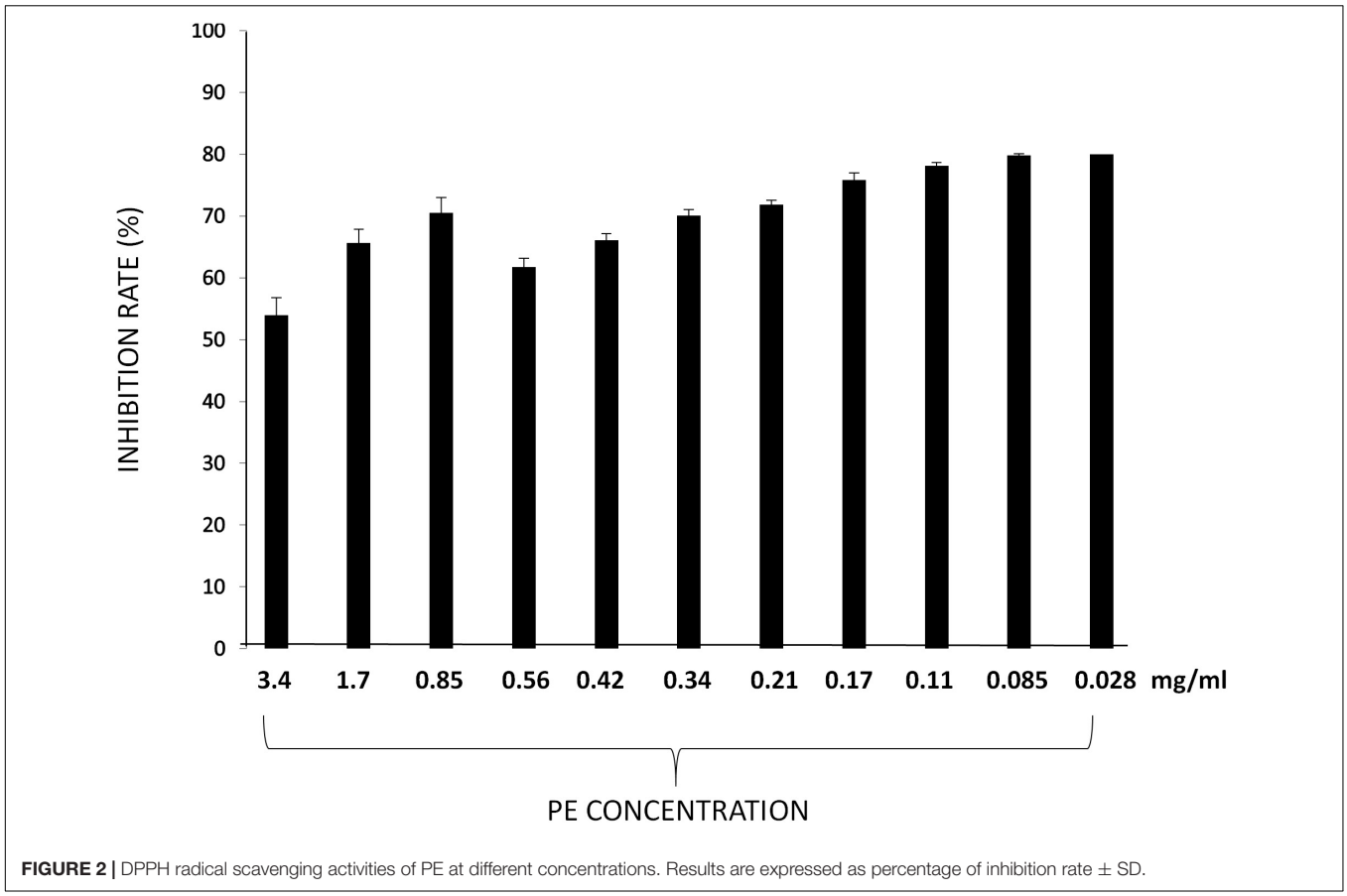

FIGURE 2 | DPPH radical scavenging activities of PE at different concentrations. Results are expressed as percentage of inhibition rate ± SD.

TABLE 3 | Superoxide ion scavenging activities of different concentrations of PE.

PE concentrations	% of inhibition	PE concentrations	% of inhibition
PE (3.4 mg/ml)	65 ± 2	PE (0.21 mg/ml)	78 ± 2
PE (1.7 mg/ml)	73 ± 3	PE (0.17 mg/ml)	80 ± 1
PE (0.85 mg/ml)	75 ± 1	PE (0.11 mg/ml)	85 ± 3
PE (0.56 mg/ml)	70 ± 2	PE (0.085 mg/ml	88 ± 4
PE (0.42 mg/ml)	68 ± 1	PE (0.028 mg/ml)	95 ± 1
PE (0.34 mg/ml)	75 ± 4		

Results are expressed as percentage of inhibition rate + SD.

strains were still inhibited, while the growth of *E. coli* was not significantly affected.

Effect of the PE on Growth of Probiotic Strains

Results shown in **Table 4** highlighted that PE, at all tested concentrations, did not have any inhibitory activity on growth of probiotic strains tested. A slight increase in growth was observed for LGG co-cultured with PE at concentration of 0.028 mg/ml (**Table 4**).

Effect of PE on Lipid Content

Pomegranate extract- and LGG-filtered SB significantly decreased the triglyceride content compared with the control group (**Figures 5A,B**). However, the effect of filtered SB derived from cells incubated with PE (LGG-T1) or without (LGG-T0) was similar. The simultaneous treatment of 3T3-L1 murine pre-adipocytes with PE- and LGG-filtered SB significantly decreased the triglyceride content compared with the treatment of LGG-filtered SB alone. Moreover, these data evidenced that combination treatment of PE and LGG-T1 was the most effective in reducing triglyceride content and intracellular lipid accumulation.

The treatment of 3T3-L1 murine pre-adipocytes with LGG cellular extract (CE), derived from cells incubated with PE (CE+PE) or without (CE), had no effect on intracellular lipid accumulation compared with the control group (Control: ABS 490 nm = 0.200 ± 0.07; CE: ABS 490 nm = 0.187 ± 0.09; CE+PE = ABS 490 nm = 0.190 ± 0.05).

Effect of PE on Adipogenic Markers

The mRNA expression levels of the main transcriptional factors involved in adipocyte differentiation were significantly less expressed in 3T3-L1 cells treated with PE- and LGG-filtered SB. Particularly PE, LGG-T0, and LGG-T1 were able to decrease gene levels of Adiponectin, PPAR-γ, SREBP, FAS, and IL-6 and to increase gene levels of IL-10 (**Figures 6A–F**).

The simultaneous treatment of 3T3-L1 murine pre-adipocytes with PE- and LGG-filtered SB (LGGT0 and LGGT1) significantly decreased mRNA expression levels of the main transcriptional

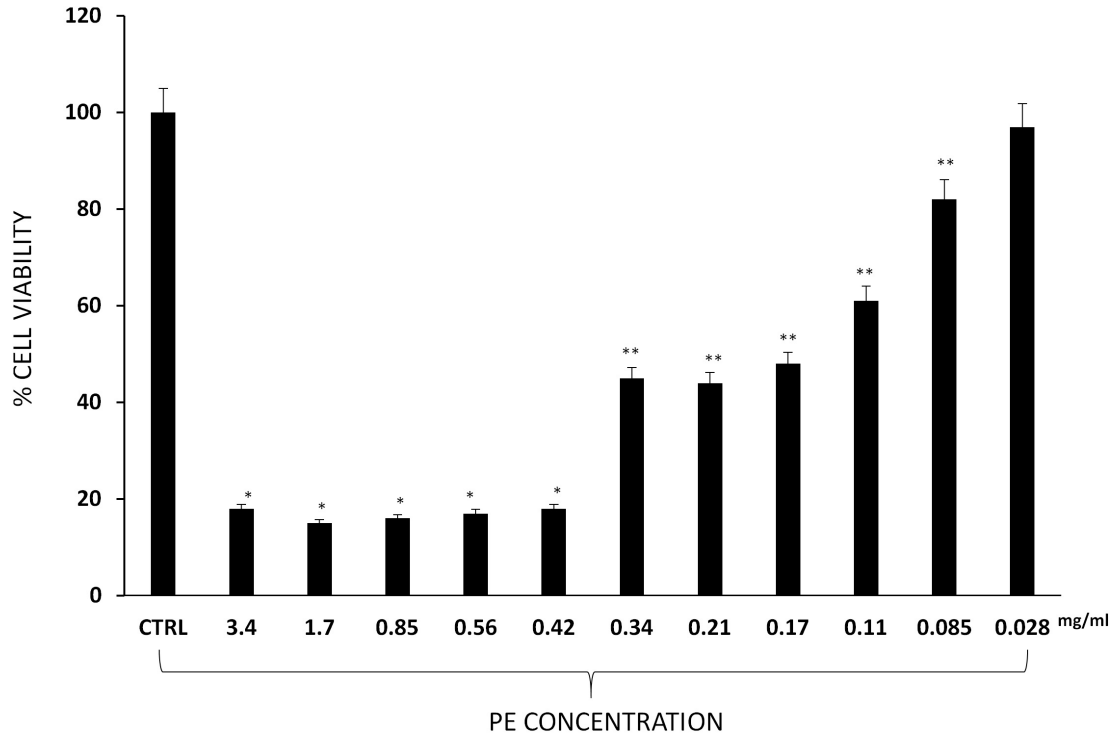

FIGURE 3 | Percentage of 3T3-L1 murine pre-adipocytes survival in the presence of PE at different concentrations. Results are expressed as the means ± SD of four experiments performed in triplicate. Significant vs. untreated controls: $*p < 0.005$; $**p < 0.05$.

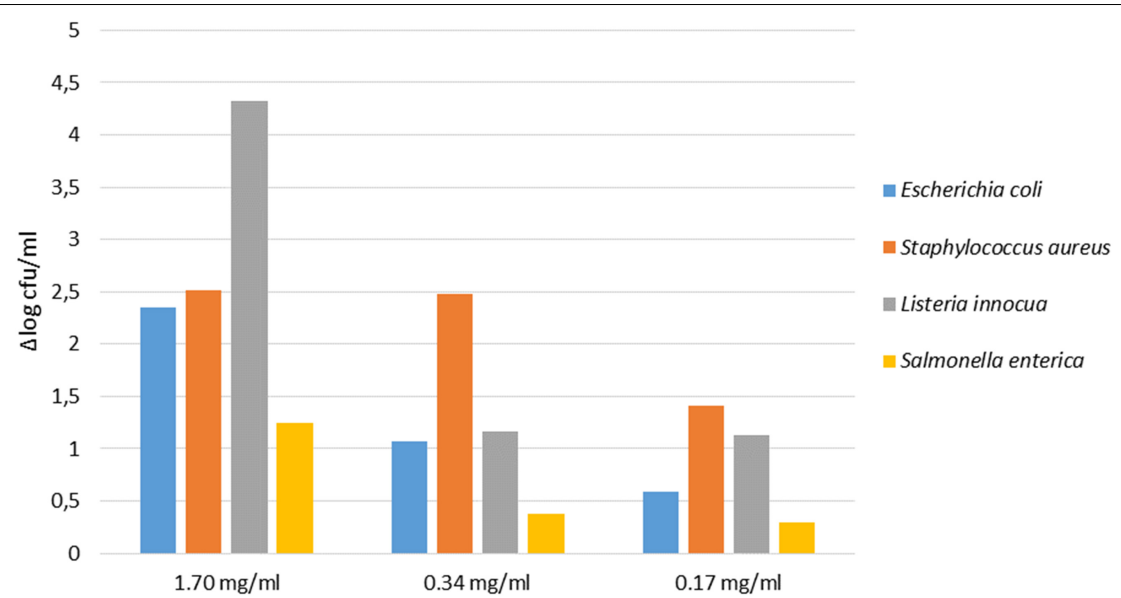

FIGURE 4 | Antimicrobial activity of different concentration of PE on pathogens detected at T24 (24 h of inoculum). The cell density is expressed as Δlog cfu/ml.

factors involved in adipogenesis, compared with the treatment of LGG-filtered SB (LGGT0 and LGGT1) alone (**Figures 6A–E**).

The simultaneous treatment of 3T3-L1 murine pre-adipocytes with PE- and LGG-filtered SB (LGGT0 and LGGT1) significantly increased mRNA expression levels of IL10, compared with the treatment of LGG-filtered SB (LGGT0 and LGGT1) alone (**Figure 6F**). Moreover, these data evidenced that combination treatment of PE+LGG T1 was the most effective in reducing mRNA expression levels of Adiponectin, IL-6, FAS, and in upregulating IL-10 (**Figures 6A,D–F**).

TABLE 4 | Bacterial counts expressed as log_{10} cfu/ml of three replicates ± SD of *L. rhamnosus* GG ATCC 53103, *Bifidobacterium animalis* BB12, *B. longum* BB536, and the wild strain *Lactobacillus paracasei* N 24 after incubation with PE at different concentrations.

		Log_{10}
L. rhamnosus GG	Baseline	8.83 ± 0.09^a
ATCC 53103	PE (0.085 mg/ml)	9.20 ± 0.10^b
	PE (0.042 mg/ml)	9.24 ± 0.07^b
	PE (0.028 mg/ml)	9.26 ± 0.04^b
Lactobacillus paracasei	Baseline	8.65 ± 0.15^a
N24	PE (0.085 mg/ml)	8.70 ± 0.08^a
	PE (0.042 mg/ml)	8.79 ± 0.10^a
	PE (0.028 mg/ml)	8.74 ± 0.09^a
Bifidobacterium animalis	Baseline	9.15 ± 0.05^a
BB12	PE (0.085 mg/ml)	9.15 ± 0.12^a
	PE (0.042 mg/ml)	9.44 ± 0.10^b
	PE (0.028 mg/ml)	9.55 ± 0.07^b
B. longum	Baseline	9.77 ± 0.03^a
BB536	PE (0.085 mg/ml)	9.54 ± 0.10^a
	PE (0.042 mg/ml)	9.57 ± 0.12^a
	PE (0.028 mg/ml)	9.44 ± 0.16^a

DISCUSSION

The beneficial effects of pomegranate fruit and/or juice consumption have received considerable scientific interest (Basu and Penugonda, 2009). Many investigators have reported that PEs, made from a waste product of the processing factories, have a free radical scavenging and potent antioxidant capacity (Panichayupakaranant et al., 2010; Fischer et al., 2011). PE used in the present study was soluble in water, and characterized through HPLC–PDA–ESI/MS^n for its phenolic and anthocyanin content. In agreement with previous reports (Fischer et al., 2011; Qu et al., 2012; Romeo et al., 2015), ellagitannins are the predominant class of phenolic compounds in pomegranate peel and marc (a by-product made up of seeds and peels), since they represent over the 99% of the total content of pomegranate phenolics. The major ellagitannins of pomegranate by-products, as well as pomegranate products (fruit and juice), are punicalagins (Gil et al., 2000; Fischer et al., 2011; Qu et al., 2012).

Pomegranate extract contains high percentages of phenolic compounds and showed antioxidant activities in a concentration-dependent manner as shown for both the DPPH and superoxide anion scavenging assay.

It has been reported that dietary plant polyphenols are able to selectively modulate the growth of susceptible microorganisms (Tabasco et al., 2011). Plant extracts commonly inhibit bacterial growth, but the magnitude of the effect depends on the composition of the extract and the type of bacterial strain.

Results obtained in our experimental conditions demonstrated that high concentrations of pomegranate polyphenols exert antimicrobial activity on some pathogen strains such as *L. innocua* and *S. aureus.* These results are in agreement with studies of Panichayupakaranant et al. (2010), Fawole et al. (2012), and Su et al. (2012).

However, at concentrations of PE lower to 0.34 mg/ml, although none inhibitory activity concentrations was detected against the probiotic strains, only a slight increase in growth of LGG was evaluated. These results are not in agreement with studies of Li et al. (2015) and of Neyrinck et al. (2013) that demonstrated that pomegranate polyphenols may potentially work as prebiotics.

Obesity is a condition in which the lipids have accumulated leading to expansion of the adipose tissue that acts as a metabolic and endocrine organ. The molecular mechanisms that modulate pre-adipocytes growth, differentiation, and lipogenesis of fat cells have been subjected to extensive studies (Vanella et al., 2012; Stechschulte et al., 2014; Moseti et al., 2016; Palmeri et al., 2016; Waldman et al., 2016; Carpene et al., 2018).

During adipocyte differentiation, preadipocytes differentiate into mature adipocytes (Lefterova and Lazar, 2009). Increased fat accumulation is strongly correlated with cell number and/or size of adipocytes (Jiang et al., 2008).

It has been reported that the degree of obesity is related to the differentiation of preadipocytes in adipocytes and with enlarged adipocytes in adipose tissues (Wang and Jones, 2004).

Other authors reported that PEs were able to suppress preadipocyte differentiation and adipogenesis and to ameliorate fatty liver in the rats with obesity and type 2 diabetes (Xu et al., 2009).

In agreement with data of Moon et al. (2012) and Park et al. (2014) in our experimental conditions PE, LGG-T0, and LGG-T1 resulted in a significant reduction in lipid accumulation in 3T3-L1 cells during differentiation into adipocytes suggesting that PE, LGG-T0, and LGG-T1 are able to suppress adipocyte differentiation. However, the treatment with LGG-filtered SB derived from cells incubated with PE (LGG-T1) or without (LGG-T1) was similar. In our experimental conditions, in fact, it was not observed the prebiotic effect demonstrated by other authors. Moreover, the combination treatment of PE and LGG-T1 was the most effective in reducing intracellular lipid accumulation. These data demonstrate that, even if in our experimental conditions it was not observed a prebiotic effect, filtered SB obtained from LGG incubated with PE (LGG-T1), might contain, besides the beneficial bacterial secreted bioactive compounds, also small amounts of PE-derived bioactive compounds. The latter, present in small amounts in LGG-T1, would not be able to exert higher beneficial effects than LGG-T0, but in combination with PE could have potential synergistic health benefits.

Adipocyte-specific peroxisome proliferator-activated receptor-γ (PPARγ) is involved in the early stage of adipocyte differentiation (Rosen et al., 2000) regulating the expression of adipogenic genes such as fatty acid synthase (FAS) and sterol regulatory element-binding proteins (SREBP) and then triggering the accumulation of fat in the cells (Kawada et al., 2001; Berger and Moller, 2002).

Adipose tissue is not only a primary fat reservoir, but it is also an endocrine organ which controls lipid homeostasis. Altered levels of adipose tissue-derived adipokines can contribute in developing of inflammation, resulting in impaired lipid metabolism (Armani et al., 2010). In chronic inflammation,

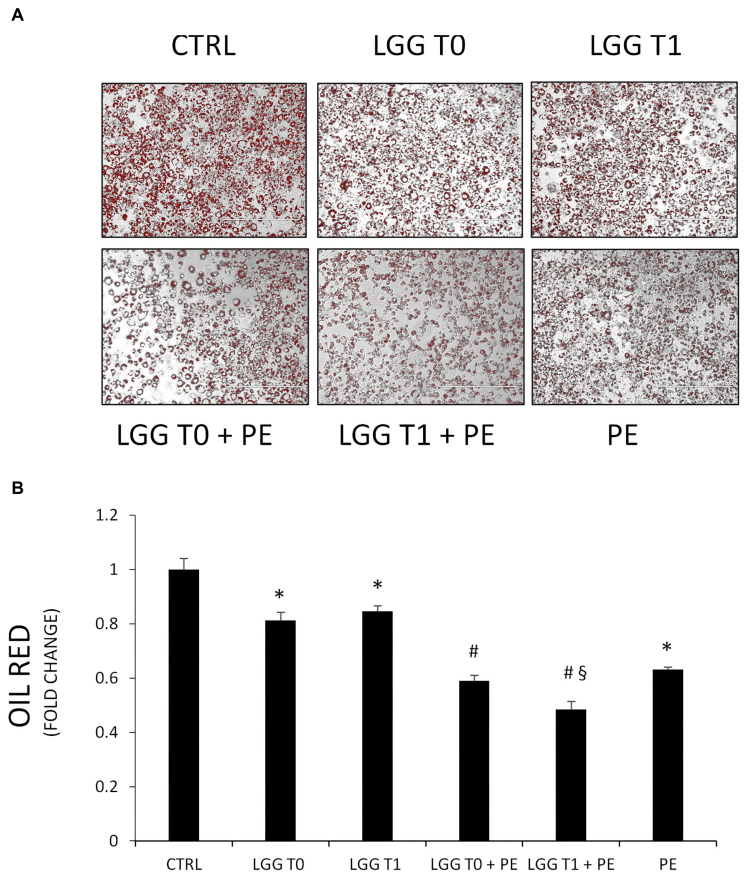

FIGURE 5 | (A) Representative Oil red O staining of 3T3-L1 cells in absence and in presence of PE, LGG T0, LGG T1, LGG T0 + PE, and LGG T1 + PE. **(B)** Lipid content was quantified with Oil Red O staining (mean ± *SD*, $^{*}p < 0.05$ versus control; $^{\#}p < 0.05$ versus LGG T0 and LGG T1; § $p < 0.05$ versus LGG T0+PE).

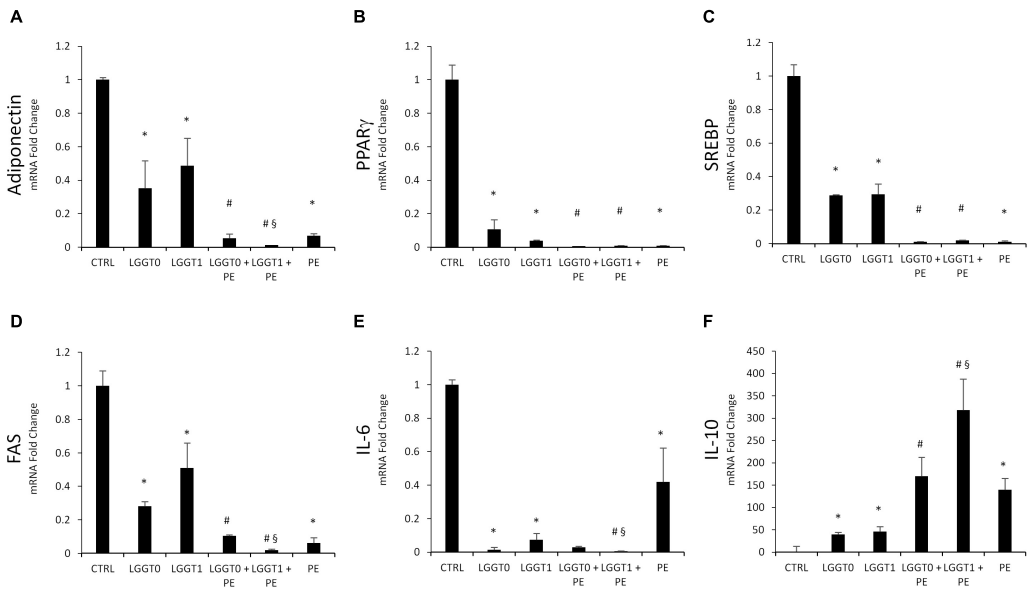

FIGURE 6 | (A–F) Analysis of gene expression by qRT-PCR of lipogenic pathway in 3T3-L1 cells in absence and in presence of PE, LGG T0, LGG T1, LGG T0+PE, and LGG T1+PE. Results are expressed as the means ± SD of four experiments performed in triplicate ($^{*}p < 0.05$ versus control; $^{\#}p < 0.05$ versus LGG T0 and LGG T1; § $p < 0.05$ versus LGG T0+PE).

proinflammatory cytokines such as IL-6 are upregulated while antiflammatory cytokines such as IL-10 are downregulated (Kershaw and Flier, 2004; Bradley et al., 2008; Lira et al., 2012; Liu et al., 2018).

Our results demonstrate that the combination treatment with PE+LGG-T1 significantly downregulated the mRNA levels of adiponectin, FAS, and IL-6 and upregulated IL-10.

We can conclude that the combination treatment with PE+LGG-T1 possesses anti-inflammatory properties and it is able to inhibit the adipocyte differentiation by modulating the expression levels of key adipogenic transcription factors involved in adipogenesis.

CONCLUSION

Pomegranate extract- and LGG-filtered SB significantly decreased intracellular lipid accumulation. A synergistic effect of probiotics and polyphenols contained in PE was observed. Moreover, our results evidenced that combination treatment of PE+LGG T1 was the most effective in reducing mRNA expression levels of Adiponectin, IL-6, FAS, and in upregulating IL-10.

These results evidenced that probiotics and polyphenols contained in PE may affect adipogenesis *in vitro*. Moreover, our results demonstrate that the synergistic properties of combining foods such as pomegranate and probiotics may exert combined health benefits.

Then pomegranate and probiotics such as LGG strain may contribute in development of new nutraceutical/probiotic-based remedies to prevent and to treat obesity.

AUTHOR CONTRIBUTIONS

VS, LV, CR, CC, FR, and GB collected research articles, conceived the experiments, analyzed the results, and wrote the manuscript. CR, MR, SF, and NT conducted the experiments.

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Insights Into the Cultivable Microbial Ecology of "Manna" Ash Products Extracted From *Fraxinus angustifolia* (*Oleaceae*) Trees in Sicily, Italy

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Microbial communities characterizing a specific food matrix, generally, strongly contribute to both its composition, and properties for food applications. To our knowledge, this is the first study to investigate the cultivable microbial ecology of Sicilian "Manna" ash products in order to acquire new information on the hygienic quality, shelf-life and potential application of this traditional food. To this purpose, several manna samples belonging to different commercial categories were collected and subjected to the analysis of bacteria, yeasts, and filamentous fungi. Furthermore, an investigation of the sugar content and physicochemical parameters was performed. The results of our study followed the trend generally reported for other sugary foods. Conversely, as regards microbiological analyses, in the present study, the presence of microorganisms at high levels confirmed their survival in stressing conditions characterizing this food matrix in a viable and cultivable form. Most species were osmophilic, endophytic bacteria, antagonistic of fungi pathogen of plants. Yeasts were the most abundant microbial populations and a total of six species were identified: *Candida aaseri*, *Candida lactis-condensi*, *Citeromyces matritensis*, *Lachancea thermotolerans*, *Saccharomyces cerevisiae,* and *Zygosaccharomyces bailii*. Filamentous fungi included five genera, which were considered common contaminants of honey and of other foods due to their xerophilic characteristics. Interestingly, our results suggest that the strains of *L. thermotolerans* isolated in this study might be evaluated for their potential to act as starters either singly or in multi-combination for food applications.

Keywords: bacteria, yeasts, filamentous fungi, microbial ecology, manna ash, osmotic environment

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INTRODUCTION

Manna is the product obtained from several species of *Fraxinus* sp. (Schicchi et al., 2007; Yücedag and Sen, 2008). During the summer season, the trunk and the main branches of the tree are notched by incision with cutters and phloem sap, a cerulean liquid, is collected. When in contact with the air, this liquid quickly thickens and forms a light crystalline whitish layer that represents manna. The dripping liquid forms a whitish stalactite of various length, in connection with the natural inclination of the trunk and the application of specific artificial tools placed on the bark (knives, metal foils, etc.). This stalactite is called "cannolo," "manna in cannolo," or "manna eletta" and represents the most

refined part of the product. In recent times, to facilitate the formation of the cannolo, a small metal logline has been inserted under the line of the incision. The part of the sap that thickens along the trunk is instead called "manna in *rottame,*" The cultivation of ash trees in Sicily is typical of restricted areas characterized by high temperatures, low temperature range, and low air humidity, that represent the best conditions for manna production (Oieni, 1953). Current Sicilian manna production is basically obtained from *Fraxinus angustifolia* Vahl and *F. ornus* L. In recent years, the price of manna has substantially increased, becoming profitable enough to allow a recovery of the ash tree cultivation. Paradoxically, despite several new prospects available for trading in the industry of officinal biological, pharmaceutical and confectionery production, there has not yet been a significant increase in manna production. This is mainly due to the fact that the current modest production cannot meet the recent market demand which is directed to the manna in cannolo. The productions are very variable and depend on the type of product but also on the seasonal agro-climatic conditions. On average a plant in the best years produces between 3 and 4 kg of raw product.

The chemical composition of manna is very complex and variable, depending upon the species and the cultivars from which it is extracted. The most abundant active substance is the mannite or D-mannitol, a hexavalent colorless and odorless alcohol with a sugary taste, also known as "manna sugar" (Caligiani et al., 2013). Many other substances are present, such as glucose, fructose, mannotriose, mannotetraose, mineral elements, organic acids, water, and other minor components that are yet to be fully identified (Lazzarini and Lonardoni, 1984). Manna represents a pharmacologically important substance because it is employed to treat different pathologies. It is mainly used to counteract constipation problems and as a purgative with no side effects, both in childhood and adulthood (Lentini et al., 1983). In case of poisoning, the mannite produces an increase of dieresis and facilitates the removal of toxic substances in the organism through the kidneys. It is used in hypertonic solutions to remove pulmonary or cerebral edema. Manna is also recommended for the expulsion of intestinal parasites. In moderate doses, it stimulates the secretion of the biliary system. Furthermore, it is well-tolerated by diabetic patients and it can therefore be used as a food sweetener (Mazzola et al., 2016).

Manna possesses a high hygroscopic power. Therefore, it swells and undergoes processes of fermentation that are responsible for the formation of gas bubbles, and a nauseating smell of brewer's yeast (Ilardi, 1988). To our knowledge, no information is available on the microbial ecology (yeasts, bacteria, and filamentous fungi) of this product. Hence, the aim of the present work was to characterize the cultivable microorganisms associated with the several products obtained during manna processing. To this purpose, several manna samples, collected within woods located within Palermo province, were subjected to microbiological investigations. Specifically, the study included the following objectives: (i) enumeration and isolation of total bacteria, total lactic acid bacteria, osmophilic bacteria, members of *Enterobacteriaceae* family, and *Clostridium* spp., total (osmophilic and osmotolerant) yeasts and total filamentous fungi; (ii) characterization of bacterial and fungal isolates by phenotypic analysis; (iii) genotypic grouping of isolates and species identification by sequencing of specific genomic regions. The sugar content of selected samples and some physicochemical parameters were also investigated.

MATERIALS AND METHODS

Sample Collection and Microbiological Analysis

A total of 35 manna samples (**Table 1**), classified as *cannolo*, *rottame,* and *liquid* samples, were collected from manna production throughout the Madonie area in Palermo province (37°53′N 14°01′E/37.883333°N 14.016667°E37.88333). All samples were transported to the laboratories of SAAF Department (University of Palermo) and stored in dark conditions at room temperature until analysis.

Microbiological analyses were carried out to investigate the main microbial groups associated with manna production. Osmophilic microorganisms were counted after homogenization of samples (25 g) in a 30% (w/v) and glucose solution (sample/diluent 1:9) following the indications ISO 21527-2 to avoid shock of cells and to recover sub-lethally injured cells. The first dilution of manna samples was obtained with a stomacher (BagMixer 400, Interscience, Saint Nom, France) for 2 min at the highest speed. Cell suspensions were spread-plated and incubated as follows: total (osmophilic and osmotolerant) yeasts (TY) on tryptone glucose yeast extract agar (TGY), incubated aerobically at 25°C for 7 days (Beuchat et al., 2001); osmophilic bacteria on De Whalley Agar (DWA) supplemented with cycloeximide (170 ppm) and biphenyl (1 g/L) to inhibit the growth of yeasts and molds, incubated aerobically at 25°C for 6 days as indicated by Justé et al. (2008); osmotolerant yeasts on De Whalley Agar (DWA) supplemented with chloramphenicol (0.1 g/L) to inhibit bacteria growth, incubated aerobically at 25°C for 7 days.

All other microorganisms were recovered by homogenization of samples (25 g) in ringer solution (0.9% NaCl). Cell suspensions were plated and incubated as follows: total mesophilic count (TMC) spread on plate count agar (PCA), incubated aerobically at 30°C for 72 h; filamentous fungi (FF) spread on potato dextrose agar (PDA), incubated aerobically at 25°C for 21 d; lactic acid bacteria (LAB) poured on MRS (generic for rod LAB) and glucose (5 g/L) M17 (GM17) agar (generic for coccus LAB), incubated anaerobically with the AnaeroGen AN25 system at 30°C for 72 h; *Enterobacteriaceae* poured on violet red bile glucose agar (VRBGA), incubated aerobically by overlay agar at 37°C for 24 h; clostridia on reinforced clostridial medium (RCM) by 3 × 3 Most Probable Number (MPN) procedure (BAM, 2006).

Except VRBGA, all media used for bacterial growth were supplemented with cycloeximide (170 ppm) to inhibit the growth of yeasts and filamentous fungi, while all media used for fungal growth were supplemented with chloramphenicol (0.1 g/L) to inhibit bacterial growth. Media were purchased from Oxoid (Basingstoke, UK) and chemicals from Sigma-Aldrich (Milan, Italy). The enrichment procedure was performed for

TABLE 1 | Sample of manna subjected to microbiological and physic-chemical analyses.

Sample code	Source	Site of sampling (Province of Palermo)
1–10	Cannolo[a]	Pollina contrada Vallata
2–6	Cannolo	Castelbuono contrada Boscamento
3–8	Cannolo	Castelbuono contrada Boscamento
4–11	Cannolo	Castelbuono
5–15	Cannolo	Castelbuono "Consorzio Manna"
6–16	Cannolo	Castelbuono "Consorzio Manna"
7–10b	Cannolo	Pollina contrada Vallata
8–6b	Cannolo	Castelbuono contrada Boscamento
9–11b	Cannolo	Castelbuono
10–15b	Cannolo	Castelbuono "Consorzio Manna"
11–16b	Cannolo	Castelbuono "Consorzio Manna"
12–10c	Cannolo	Pollina contrada Vallata
13–6c	Cannolo	Castelbuono contrada Boscamento
14–11c	Cannolo	Castelbuono
15–15c	Cannolo	Castelbuono "Consorzio Manna"
16–16c	Cannolo	Castelbuono "Consorzio Manna"
17–5	Rottame[b]	Castelbuono IPSA
18–7	Rottame	Castelbuono
19–12	Rottame	Castelbuono
20–13	Rottame	Castelbuono
21–17	Rottame	Castelbuono "Consorzio Manna"
22–18	Rottame	Castelbuono "Consorzio Manna"
23–5b	Rottame	Castelbuono IPSA
24–12b	Rottame	Castelbuono
25–17b	Rottame	Castelbuono "Consorzio. Manna"
26–17b	Rottame	Castelbuono "Consorzio Manna"
27–18b	Rottame	Castelbuono "Consorzio Manna"
28–5c	Rottame	Castelbuono IPSA
29–12c	Rottame	Castelbuono
30–17c	Rottame	Castelbuono "Consorzio. Manna"
31–17	Rottame	Castelbuono "Consorzio Manna"
32–18c	Rottame	Castelbuono "Consorzio Manna"
33–9	Liquid[c]	Pollina contrada Vallata
34–9b	Liquid	Pollina contrada Vallata
35–9c	Liquid	Pollina contrada Vallata

[a] *Cannolo, manna sample without impurity and undamaged.*
[b] *Rottame, manna sample with impurity and damaged.*
[c] *Liquid, manna sample collected at liquid phase.*

all microbial groups. All samples (10 g) were inoculated in 10 mL of different enrichment media for 48 h in relation to the same group of microorganisms: Nutrient Broth (NB) for total mesophilic microorganisms; De Whalley broth (DWB) for osmophilic bacteria (30°C); MRS broth (Oxoid, Italy) for rod LAB (30°C); M17 broth (Oxoid, Italy) for cocci LAB (30°C); YPD broth (BD Difco™, Italy) for yeasts and fungi (25°C); Buffered Peptone Water (Biolife, Italy) for *Enterobacteriaceae* (ISO 21528-1: 2017; 37°C); Cooked Meat Medium (Oxoid, Italy) for Clostryridial groups (37°C). The microbiological analyzes were conducted even after 5 days of enrichment. Microbial loads (Log CFU/g) were expressed as an average of three replicates.

Isolation, Grouping, and Genotypic Identification of Bacteria

After growth, at least five colonies per morphology were randomly collected from the GM17 agar plates of each samples and purified to homogeneity after several sub-culturing steps onto isolation medium agar.

Presumptive *Enterobacteriaceae* and clostridia were not isolated. The isolates were purified by successive sub-culturing and the purity was checked microscopically. Presumptive LAB were phenotypically characterized by cell morphology (cocci and rods), Gram reaction (KOH method), and catalase (determined by transferring fresh colonies from a Petri dish to a glass slide and adding H_2O_2 5%, v/v).

Applying the strategy described by De Angelis et al. (2001), ∼30% of the isolates representing each phenotypic group for each sample were processed by RAPD analysis, with three primers (M13, AB111, and AB106) used singly by means of Thermal cycler (Swift™ MaxPro, Esco Technologies, Inc., USA). The amplified products were separated by electrophoresis, then visualized, and acquired by the KODAK Gel Logic 100 System (Kodak, Rochester, USA). The analysis of the RAPD patterns was performed with the Gelcompar II software, version 6.5 (Applied-Maths, Sint-Martens-Latem, Belgium).

One or more representative cultures for each group were identified by 16S rRNA gene sequencing. PCR reactions were performed as described by Weisburg et al. (1991) using the primers rD1 (5′-AAGGAGGTGATCCAGCC-3′) and fD1 (5′-AGAGTTTGATCCTGGCTCAG-3′). The PCR mixture (50 µL total volume) included 62.5 ng of target DNA, 1 × Taq DNA polymerase buffer with 2 mM $MgCl_2$ (ThermoFisher Scientific, Monza, Italy), 250 µM of each dNTP, 0.2 µM of each primer and 2.5 U of Taq DNA polymerase (ThermoFisher Scientific, Monza, Italy). PCR conditions were as follows: initial denaturing step at 95°C for 3 min; 30 cycles (1 min at 94°C, 45 s at 54°C, 2 min at 72°C); and an additional final chain elongation step at 72°C for 7 min. The amplicons corresponding approximately to 1,400 bp were purified using the Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA). Sequences were manually corrected and assembled using Crhomas 2.6.2. (Technelysium Pty Ltd, Australia).

The PCR products were visualized by UV transillumination on a 2% (w/v) agarose gel (Gibco BRL, Cergy Pontoise, France), stained with SYBR® Safe DNA gel stain (Molecular Probes, Eugene, OR, USA). The GeneRuler 100 bp Plus DNA Ladder (M-Medical S.r.l, Milan, Italy) was used as a molecular weight marker. The resulting DNA was sequenced by AGRIVET (University of Palermo, Italy) using the same primers employed for the PCR amplifications.The identities of the sequences were determined by BlastN search against the NCBI non-redundant sequence database located at NCBI web site[1] http://www.ncbi.nlm.nih.gov and those of the sole type strains within the database EzTaxon, located at the EzTaxon web site[2]

[1] http://www.ncbi.nlm.nih.gov
[2] http://www.ezbiocloud.net/eztaxon

Isolation and Identification of Yeasts

As for the bacterial and yeasts characterization, five colonies were collected of different morphology with Petri dishes inoculated with the highest dilutions of each sample. Yeasts were collected for all morphologies observed on TGY and DWA-y mediums. The isolates were purified by successive sub-culturing on the same media and their purity was verified under an optical microscope (Carl Zeiss Ltd.). All isolates were subjected to overnight growth in broth media (TGY and DW broth) at the optimal temperatures. Cells were harvested, and DNA was extracted as reported by Ruzauskas et al. (2015). DNA extraction of 30% isolates was performed for the differentiation of yeasts that were obtained by RFLP of the region spanning the internal transcribed spacers (ITS1 and ITS2) and the 5.8S rRNA gene. The DNA fragments were amplified with the primer pair ITS1 (5′-TCCGTAGGTGAACCTTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (Esteve-Zarzoso et al., 1999) by means of T1 Thermocycler (Biometra, Göttingen, Germany) and subsequently digested with the endonucleases CfoI, HaeIII and HinfI (MBI Fermentas) at 37°C for 8 h. ITS amplicons and their restriction fragments were analyzed twice on agarose gel using at first 1.5% (w/v) agarose and then 3% (w/v) agarose in 1 × TBE buffer and visualized as reported above. Standard DNA ladders were GeneRuler 50 pb plus DNA Ladder (MMedical s.r.l., Milan, Italy). The 3% of isolate per group was further processed by sequencing the D1/D2 region of the 26S rRNA gene to confirm the preliminary identification obtained by RFLP analysis. D1/D2 region was amplified with primers NL1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL4 (5′-GGTCCGTGTTTCAAGACGG-3′) (Kurtzman and Robnett, 1998). The PCR mixture (30 μ L total volume) included 100 ng of target DNA, 1 × Taq DNA polymerase buffer with 2 mM $MgCl_2$ (ThermoFisher Scientific, Monza, Italy), 250 μM of each dNTP, 0.2 μM of each primer and 2.5 U of Taq DNA polymerase (ThermoFisher Scientific, Monza, Italy). PCR conditions were as follows: initial denaturing step at 95°C for 5 min; 30 cycles (1 min at 95°C, 45 s at 52°C, 1 min at 72°C); and an additional final chain elongation step at 72°C for 7 min. The amplicons corresponding ∼550 bp were purified using the Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA). Sequences were manually corrected using Crhomas 2.6.2. (Technelysium Pty Ltd, Australia). The reaction of DNA sequencing and the identities of sequences were determined as reported above.

Isolation and Identification of Filamentous Fungi

Filamentous fungi colonies were analyzed onto PDA. All colonies were identified at the genus level through observed macroscopic and microscopic characters by light microscopic analysis and dicotomous key, including color, texture, diffusible pigments, exudates, growth zones, aerial, and submerged hyphae, growth rate, and topography (Barnett and Hunter, 1998).

Determination of Sugars

About 5 mg of samples were dissolved in 2 ml of a 1,000 ppm solution of phenyl-β-D-glucopyranoside in DMF and then sylanized with 0.5 ml TMCS and 1.0 ml HMDS. The final solution was heated at 50°C for 40 min before injection. Standard solutions of maltose, galactose, mannose, mannitol, myo-inositol, xylitol, glucose, fructose, saccharose, and gluconic acid were prepared with the same protocol. Samples were extracted with 1 ml hexane and injected (split mode) into GC–MS instrument (HP GC6890, Hewlett Packard, Palo Alto, CA) and a MS detector (HP MS5973) equipped with a Supelco SLB-5MS capillary column; (length 30 cm; internal diameter 0.25 mm; film thickness 0.25 μm) in the following chromatographic conditions: The injector temperature was 280°C. The oven starting temperature was 60°C, and after 3 min it was increased at a rate of 20°C/min until 280°C, and then held at constant temperature for 6 min. The transfer line was held at 280°C. The ion source, an electron-impact ionization (EI) type, setup at 70 eV, was held at 230°C, quadruple at 150°C, acquisition mode: scan (m/z 45–500) and calibration was done by auto-tuning. A ChemStation data system (G1701CA, Hewlett Packard, Palo Alto, CA) was used for data processing. The response factors relative to the internal standard method were calculated using the relation Ki = grI/grIS × areaIS/areai, where Ki is the response factor for the i-th species, grI is the weight of the i-th species, grIS is the weight of the internal standard, areai is the peak area of the i-th species, and areaIS is the peak area of the internal standard. All the experimental data were obtained from three replicated independent samples.

Determination of Physicochemical Parameters

Manna samples were tested for moisture and water activity (a_w).

Moisture content was determined by automatic moisture analyzer (Gibertini Elettronica, Novate Milanese (MI), ITALIA) at 110°C, while, Aw was determined by AquaSorp Isotherm Generator (Decagon Devices Inc., Pullman, WA, USA) at 25°C. Analyses were carried out in triplicate.

Statistical Analysis

ANOVA test was applied to identify significant differences among the microbial counts and chemical-physical parameters. The *post-hoc* Tukey's method was applied for pairwise comparison of microbial counts and chemical-physical parameters. Statistical significance was attributed to $p < 0.05$. In addition, an explorative multivariate approach analysis by principal component analysis (PCA) has been employed, in order to investigate relationships among total sugar and bacteria/yeast population from the different manna samples.

Statistical data processing and graphic construction were performed with the XLStat software version 2014.5.03 (Addinsoft, NewYork, USA) for excel.

RESULTS

Microbial Counts

The viable counts of the microbial groups examined in this study are reported in **Table 2**, **Tables S1**, **S2**. On PCA, the highest microbial count levels were observed in the 6–16 Manna sample (5.46 Log CFU/g). In addition, 11 samples (n. 3 of cannolo and

TABLE 2 | Microbial loads (CFU/g or mL) of manna samples before the enrichment procedure.

Sample code	Source	Media							
		Bacteria					Yeasts		FF
		PCA	GM17	DWA-b	VRBGA	RCM[a]	TGY	DWA-y	PDA
1–10	Cannolo	$<2^{m}$	1.60 ± 0.20^{def}	$<2^{f}$	$<1^{c}$	0.00	$<2^{h}$	$<2^{k}$	$<2^{f}$
2–6	Cannolo	2.48 ± 0.21^{hijkl}	$<1^{g}$	$<2^{f}$	$<1^{c}$	1.39	$<2^{h}$	$<2^{k}$	$<2^{f}$
3–8	Cannolo	4.40 ± 0.24^{bc}	1.70 ± 0.10^{cdef}	2.95 ± 0.30^{cd}	$<1^{c}$	0.00	$<2^{h}$	2.90 ± 0.50^{ghi}	$<2^{f}$
4–11	Cannolo	2.00 ± 0.00^{l}	$<1^{g}$	$<2^{f}$	$<1^{c}$	0.00	$<2^{h}$	$<2^{k}$	$<2^{f}$
5–15	Cannolo	2.60 ± 0.40^{ghijkl}	2.00 ± 0.30^{cd}	$<2^{f}$	$<1^{c}$	1.15	$<2^{h}$	2.48 ± 0.20^{hij}	$<2^{f}$
6–16	Cannolo	5.46 ± 0.50^{a}	2.15 ± 0.20^{cd}	2.00 ± 0.00^{e}	1.48 ± 0.20^{a}	0.00	$<2^{h}$	$<2^{k}$	$<2^{f}$
7–10b	Cannolo	2.15 ± 0.12^{kl}	1.60 ± 0.20^{def}	$<2^{f}$	$<1^{c}$	0.00	$<2^{h}$	$<2^{k}$	$<2^{f}$
8–6b	Cannolo	3.47 ± 0.30^{defg}	$<1^{g}$	$<2^{f}$	$<1^{c}$	0.00	$<2^{h}$	$<2^{k}$	$<2^{f}$
9–11b	Cannolo	2.11 ± 0.14^{l}	1.70 ± 0.10^{cdef}	$<2^{f}$	$<1^{c}$	1.21	$<2^{h}$	2.15 ± 0.14^{ij}	$<2^{f}$
10–15b	Cannolo	3.26 ± 0.52^{efgh}	$<1^{g}$	$<2^{f}$	1.25 ± 0.20^{b}	0.00	$<2^{h}$	$<2^{k}$	$<2^{f}$
11–16b	Cannolo	3.09 ± 0.43^{fghij}	2.00 ± 0.30^{cd}	$<2^{f}$	$<1^{c}$	0.00	$<2^{h}$	$<2^{k}$	$<2^{f}$
12–10c	Cannolo	2.35 ± 0.35^{ijkl}	2.05 ± 0.30^{cd}	$<2^{f}$	$<1^{c}$	1.08	$<2^{h}$	$<2^{k}$	$<2^{f}$
13–6–1c	Cannolo	2.49 ± 0.46^{hijkl}	2.00 ± 0.10^{cd}	2.14 ± 0.10^{e}	$<1^{c}$	0.00	$<2^{h}$	2.22 ± 0.13^{ij}	$<2^{f}$
14–11c	Cannolo	$<2^{m}$	$<1^{g}$	$<2^{f}$	$<1^{c}$	0.00	$<2^{h}$	$<2^{k}$	$<2^{f}$
15–15c	Cannolo	$<2^{m}$	1.78 ± 0.30^{cde}	$<2^{f}$	$<1^{c}$	0.00	$<2^{h}$	2.18 ± 0.10^{ij}	$<2^{f}$
16–16c	Cannolo	2.22 ± 0.20^{jkl}	1.15 ± 0.14^{ef}	$<2^{f}$	$<1^{c}$	0.00	<2	$<2^{k}$	$<2^{f}$
17–5	Rottame	$<2^{m}$	$<1^{g}$	2.00 ± 0.00^{e}	$<1^{c}$	0.00	$<2^{h}$	$<2^{k}$	2.50 ± 0.20^{d}
18–7	Rottame	2.00 ± 0.3^{l}	$<1^{g}$	$<2^{f}$	$<1^{c}$	0.00	2.60 ± 0.30^{efg}	2.00 ± 0.00^{ij}	$<2^{f}$
19–12	Rottame	3.00 ± 0.30^{ghijk}	2.04 ± 0.40^{cd}	2.00 ± 0.00^{e}	$<1^{c}$	2.04	4.14 ± 0.20^{bc}	4.23 ± 0.20^{f}	$<2^{f}$
20–13	Rottame	3.18 ± 0.61^{efghi}	$<1^{g}$	2.78 ± 0.30^{d}	1.20 ± 0.10^{b}	1.59	2.78 ± 0.30^{def}	3.26 ± 0.40^{gh}	$<2^{f}$
21–17	Rottame	4.04 ± 0.20^{bcde}	2.86 ± 0.50^{b}	2.78 ± 0.30^{d}	$<1^{c}$	1.44	5.11 ± 0.40^{a}	5.30 ± 0.10^{abc}	2.00 ± 0.00^{e}
22–18	Rottame	3.89 ± 0.40^{cdef}	2.11 ± 0.10^{cd}	3.56 ± 0.30^{ab}	$<1^{c}$	0.00	3.30 ± 0.70^{de}	4.48 ± 0.30^{def}	$<2^{f}$
23–5b	Rottame	$<2^{m}$	2.31 ± 0.30^{bc}	3.01 ± 0.20	$<1^{c}$	1.20	3.20 ± 0.10^{de}	3.21 ± 0.10^{gh}	$<2^{f}$
24–7b	Rottame	$<2^{m}$	1.21 ± 0.10^{ef}	2.03 ± 0.02^{e}	$<1^{c}$	0.00	4.21 ± 0.50^{b}	4.28 ± 0.20^{ef}	2.22 ± 0.20^{de}
25–17b	Rottame	4.00 ± 0.20^{bcde}	3.84 ± 0.20^{a}	$<2^{f}$	$<1^{c}$	1.05	$<2^{h}$	5.00 ± 0.60^{bcdef}	3.44 ± 0.50^{ab}
26–17b	Rottame	$<2^{m}$	$<1^{g}$	$<2^{f}$	1.54 ± 0.10^{a}	2.22	2.66 ± 0.30^{efg}	$<2^{k}$	$<2^{f}$
27–18b	Rottame	3.02 ± 0.20^{fghijk}	2.22 ± 0.30^{bcd}	3.08 ± 0.40^{bcd}	$<1^{c}$	1.59	3.44 ± 0.20^{cd}	2.33 ± 0.10^{ij}	$<2^{f}$
28–5–1c	Rottame	2.81 ± 0.30^{ghijkl}	1.74 ± 0.40^{cdef}	3.44 ± 0.20^{bc}	$<1^{c}$	1.44	$<2^{h}$	3.37 ± 0.30^{g}	$<2^{f}$
29–12d	Rottame	$<2^{m}$	1.10 ± 0.10^{f}	$<2^{f}$	$<1^{c}$	0.00	$<2^{h}$	4.74 ± 0.30^{cdef}	$<2^{f}$
30–13c	Rottame	$<2^{m}$	1.74 ± 0.40^{cdef}	3.08 ± 0.50^{bcd}	$<1^{c}$	0.00	5.47 ± 0.40^{a}	5.11 ± 0.40^{abcd}	2.13 ± 0.10^{de}
31–17d	Rottame	$<2^{m}$	$<1^{g}$	3.50 ± 0.20^{bc}	$<1^{c}$	1.33	2.01 ± 0.01^{g}	5.33 ± 0.60^{abc}	$<2^{f}$
32–18c	Rottame	$<2^{m}$	1.10 ± 0.10^{f}	4.11 ± 0.10^{a}	$<1^{c}$	1.40	3.31 ± 0.50^{de}	$<2^{k}$	$<2^{f}$
33–9	Liquid	4.20 ± 0.50^{bcd}	$<1^{g}$	3.30 ± 0.30^{bcd}	$<1^{c}$	0.00	2.33 ± 0.24^{fg}	5.85 ± 0.40^{a}	3.80 ± 0.30^{a}
34–9g	Liquid	4.52 ± 0.20^{bc}	$<1^{g}$	2.93 ± 0.40^{cd}	$<1^{c}$	0.00	4.97 ± 0.30^{a}	5.76 ± 0.10^{ab}	2.97 ± 0.10^{c}
35–9–1b	Liquid	4.87 ± 0.30^{ab}	$<1^{g}$	3.21 ± 0.10^{bcd}	$<1^{c}$	0.00	5.25 ± 0.20^{a}	5.04 ± 0.30^{bcde}	3.22 ± 0.40^{bc}
Statistical significance[b]		***	***	***	**		***	***	***

*[a]As estimated by MPN. FF, filamentous fungi; PCA, plate count agar for total mesophilic bacteria; GM17, glucose M17 added with cycloeximide for lactic acid bacteria counts; DWA-b, De Whalley agar added with cycloeximide for osmophilic bacteria counts; VRBGA, violet red bile glucose agar for Enterobacteriaceae counts; RCM, reinforced clostridial medium; TGY, tryptone glucose yeast extract agar added with chloramphenicol for total (osmophilic and osmotolerant) yeasts; DWA-y, De Whalley agar added with chloramphenicol for osmotolerant yeast counts; PDA, potato dextrose agar for filamentous fungi counts. Results indicate mean values with standard deviation. Data within a column followed by the same letter are not significantly different according to Tukey's test. [b]P-value:*** $P < 0.001$; ** $P < 0.01$.*

n. 8 rottame) showed values of microbial count less than the limit of detection. The liquid manna samples (33–9, 34–9g and 35–9–1b Manna) showed TMC values in the range of 4.20–4.87 Log CFU/ml. After 48 h of incubation on PCA, 1–10 Manna sample showed TMC values of 3.48 Log CFU/ml. After 5 days, TMC values of 6.10 Log CFU/ml were detected in 17–5 Manna.

The highest microbial count values of LAB populations were observed in the 25–17b Manna sample (3.84 Lof CFU/g). A total of 13 samples (n. 5 cannolo, n. 5 rottame and n. 3 liquid) showed values lower than the limit of detectability and then underwent an enrichment procedure. The enrichment procedure after 48 h of incubation allowed for detection of the LAB at levels between

1.22 and 5.70 Log CFU/ml. No LAB was found after 5 days of incubation throughout the enrichment procedure.

The microbial count values of osmophilic bacteria were between 2.00 and 4.11 Log CFU/g or ml. Seventeen samples showed values lower than the limit of detection and after enrichment procedure for 48 h, 10 samples (n. 9 from cannolo and n. 1 from rottame) showed in DWA-b values in the range of 2.12–5.69 Log CFU/ml. Seven manna samples were negative due to the presence of osmophilic bacteria after 5 days of enrichment.

The presence of *Enterobacteriaceae* was detected in four samples (6–16, 10–15b, 20–13, and 26–17b Manna) with microbial count values in the range of 1.20–1.54 Log CFU/g. The presence of presumptive *Clostridium* spp. on RCM medium was observed in n. 4 samples of manna from cannolo and n. 9 samples of manna from rottame. The enrichment procedures did not increase the concentration of *Enterobacteriaceae* and Clostridial group in all samples after both 2 and 5 days of incubation.

The levels of detected yeast populations were different in relation to the source of the samples and the culture medium used. Before the enrichment procedure, all the samples coming from cannolo in TGY (n. 17) showed values lower than the detection limit. While 12 samples of manna from scrap showed total yeast (TY) levels in the range of 2.01–5.47 CFU/g. The presence of TY in the liquid manna samples in TGY was between 2 and 5 logarithmic cycles. After 48 h of incubation through an enrichment procedure, all the cannolo samples, except the 7–10b and 14–11c samples, showed microbial count levels between 2.20 and 8.04 Log CFU/ml. The presence of osmotolerant yeast count has reached higher levels (2.00–5.85 Log CFU/g or ml) in samples from rottame and liquids. While the cannolo manna samples showed a microbial concentration around two logarithmic cycles. After the enrichment procedure, of the 14 samples (11 cannoli and 3 scrap) only four samples showed values lower than the detection limit. The enrichment procedure up to 5 days allowed to obtain osmotolerant yeasts only in the sample 1–10 on DWA-y (8.25 Log CFU/ml).

Isolation and Identification of Bacteria

A total of 3,175 pure cultures were isolated and purified from count plates, specifically 1,505 isolates were obtained on DWA-b, while 1,670 isolates had come from GM17. After the Gram characterization and catalase test, no presumptive LAB were found within the isolates from GM17. In MRS, all the manna samples that were also subjected to enrichment procedure showed values of microbial counts that were lower than the limits of detection. The phenotypic group of bacteria isolates from manna samples were reported in **Table 3**. The results of the identification process are better detailed in **Table 4**. About 30% of the isolates sharing the phenotypic characteristics were analyzed by RAPD-PCR. As reported in the dendrogram (**Figure 1**), the cultivable bacterial community associated to the different manna samples collected. All 18 strains were identified by sequencing of the 16S rRNA gene and were deposited in GenBank (Acc. No.MK509926–MK509943). The bacteria were represented by 11 species: *Bacillus amyloliquefaciens*, *Bacillus halotolerans*, *Bacillus mojavensis*, *Bacillus safensis*, *Bacillus subtilis*, *Bacillus tequilensis, Bacillus vanillea*, *Clavibacter michiganensis*, *Erwinia tasmaniensis*

TABLE 3 | Phenotypic grouping of bacteria isolated from manna samples.

Characteristic	Phenotype of cluster		
	I (n = 2,291)	II (n = 837)	III (n = 47)
Morphology	Rods	Cocci	Rods
Gram	+	+	−
Catalase	+	+	+

Staphylococcus epidermidis, *Staphylococcus succinus* subsp. *Casei,* and *Staphylococcus warneri*.

Isolation and Identification of Yeasts and Molds

A total of 2,888 pure cultures were isolated and purified from count plates for yeasts on TGY (n. 1238) and DWA-y (n. 1650). Eight hundred and seventy isolates were clustered into 10 groups according to the RFLP profile. The genetic analysis allowed the identification of the following species: *Candida aaseri* (n = 54), *Candida lactis-condensi* (n = 72), *Citeromyces matritensis* (n = 67), *Lachancea termotolerans* (n = 616), *Saccharomyces cerevisiae* (n = 33), and *Zygosaccharomyces bailii* (n = 28). The majority of yeasts belonged to the species *Lachancea termotolerans* (**Table 5**).

With regards to the filamentous fungi obtained from manna just after the collection and enrichment, based on the morphological traits, five main groups (genera) were recognized: *Alternaria*, *Aspergillus*, *Cladosporium*, *Mucor,* and *Penicillium*. The distribution of filamentous fungi among samples are reported in **Table 6**. A total of 251 filamentous fungus isolates were obtained. The most representative genus is *Cladosporium* spp. with 95 isolates.

Sugar Content and Physicochemical Parameters

The results of the sugar content, aw and RH% of the manna samples are shown in **Table 7**.

Globally, the quali-quantitative sugar profile of the samples included mannitol, mannotriose, fructose, stachyose, and glucose as the most abundant saccharides.

In the manna samples classified as cannolo, the sugars present in greater quantities were mannitol and mannotriose. Samples 3–8 showed the highest values of mannitol (60.27/100 g), while the highest level of mannotriose was obtained in the 11–16b samples (26.49/100 g).

Similar situation was observed in the manna samples coming from the rottame. In particular, an analysis of average values showed that the sugar composition is mainly represented by mannitol (28.06–37.33/100 g) and fructose (3.66–33.64/100 g). A similar situation was observed for the liquid manna samples.

Globally, the UR% and a_w values ranged between 0.57–11.03 and 0.520–0.801, respectively. The highest values of a_w (0.801) and UR% (11.03) e were observed in samples 3–8.

In order to better evaluate the correlations among manna samples, the data concerning sugar content and yeast/bacterial contamination were processed by PCA analysis. The biplot

TABLE 4 | Molecular identification by PCR amplified products of 16S rDNA of manna bacteria strains.

Manna source	Species	% similarity (accession no. of closest relative) by		Sequence lenght (bp)	Acc. No.
		BLAST	EzTaxon		
Rottame	*Bacillus safensis*	99 (NR_148787.1)	99.66 (FO-36b)	1480	MK509926
Rottame	*B. safensis*	99 (NR_113945.1)	99% (FO-36b)	1477	MK509927
Cannolo	*Staphylococcus succinus* subsp. *casei*	99 (NR_037053.1)	99.45 (SB72)	1480	MK509928
Rottame	*Clavibacter michiganensis*	98% (NR_152027.1)	99.79 (LPPA 982)	1423	MK509929
Cannolo	*Bacillus amyloliquefaciens*	99 (NR_112685.1)	99.86 (DSM 7)	1449	MK509930
Cannolo	*Staphylococcus epidermidis*	99 (NR_113957.1)	99.32 (NCTC 11047)	1477	MK509931
Liquid	*S. succinus* subsp. *casei*	99 (NR_037053.1)	99.31 (SB72)	1476	MK509932
Cannolo	*Staphylococcus warneri*	99 (NR_025922.1)	99.66 (ATCC 27836)	1477	MK509933
Rottame	*Bacillus mojavensis*	100 (NR_112725.1)	99.93 (RO-H-1)	1436	MK509934
Rottame	*B. amyloliquefaciens*	99 (NR_112685.1)	99.38 (DSM 7)	1478	MK509935
Rottame	*Bacillus halotolerans*	99 (NR_115063.1)	99.72 (ATCC 25096)	1447	MK509936
Rottame	*St. succinus* subsp. *casei*	99 (NR 037053.1)	99.79 (SB72)	1449	MK509937
Rottame	*St. succinus* subsp. *casei*	99 (NR_037053.1)	99.79 (SB72)	1438	MK509938
Rottame	*Bacillus subtilis* subsp. *subtilis*	99 (NR_102783.2)	99.72 (NCIB 3610)	1435	MK509939
Cannolo	*Bacillus tequilensis*	99 (NR_104919.1)	100 (KCTC 13622)	1457	MK509940
Liquid	*Erwinia tasmaniensis*	99 (NR_074869.1)	98.81 (Et1/99)	1427	MK509941
Rottame	*Bacillus vanillea*	99 (KF986320.1)	99 [XY18(T)]	1363	MK509942
Rottame	*B. subtilis*	99 (NR_113265.1)	99.73 (KCTC 13429)	1473	MK509943

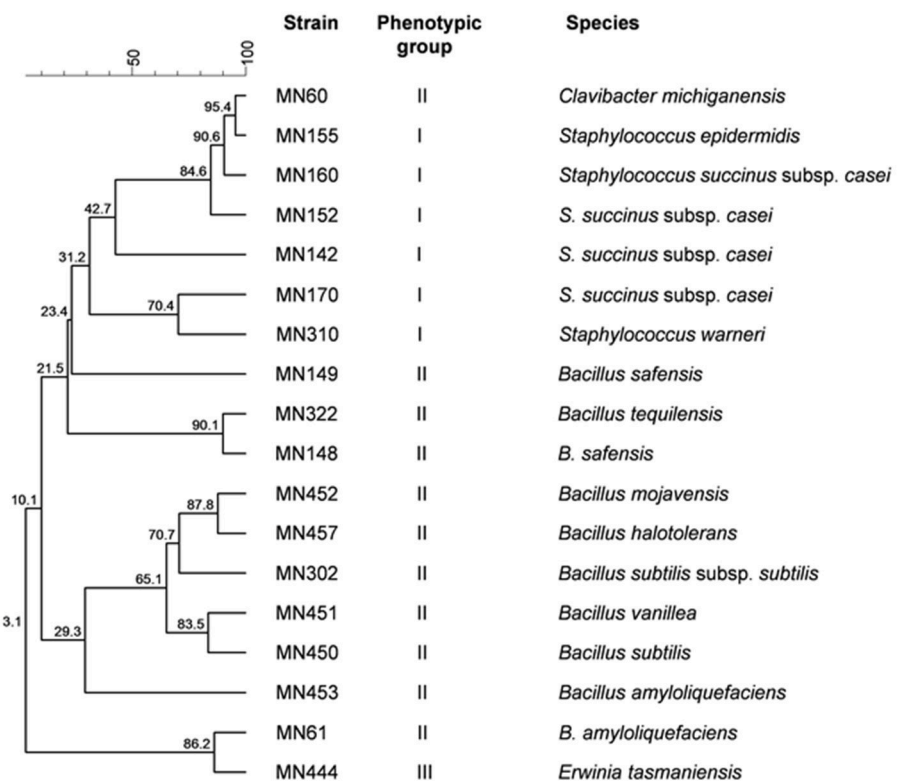

FIGURE 1 | Dendrogram of bacterial strains obtained with combined RAPD-PCR patterns with primers M13, AB106, and AB111.

illustrated in **Figure 2** highlights the distribution of the different manna samples in relation to sugar composition, microbial concentrations of bacteria (GM17 and DWA-b) and yeast population (TGY and DWA-y). For the F1 factor, there is a positive correlation between sugar content and the values of microbial counts detected on TGY, DWA-y, and DWA-b, while

TABLE 5 | Length in pb of the PCR amplified products of 5.8S rDNA-ITS region, ITS-RFLP, and identified species of yeast isolated from manna samples.

Manna Source	Size amplicons 5.8S-ITS (bp)	Size of restriction fragment[a]			Species	% similarity (accession no. of closest relative) by BLAST[b]	Sequence lenght (bp)	Acc. No.
		*Cfo*I	*Hae*III	*Hinf*I				
Rottame	680	260 + 240	300 + 260 + 120	275	*Lachancea termotolerans*	100 (XR 002432231.1)	531	MK513712
Rottame	790	260 + 240	700	275	*Zygosaccharomyces bailii*	100 (NG 055054.1)	554	MK513713
Rottame	680	260 + 240	300 + 260 + 120	275	*L. termotolerans*	100 (XR 002432231.1)	543	MK513714
Cannolo	680	260 + 240	300 + 260 + 120	275	*L. termotolerans*	100 (XR 002432231.1)	540	MK513715
Cannolo	680	260 + 240	300 + 260 +120	275	*L. termotolerans*	100 (XR 002432231.1)	540	MK513716
Rottame	800	260 + 240	300 + 260 + 120	275	*L. termotolerans*	100 (XR 002432231.1)	525	MK513717
Rottame	700	320 + 280	n.c.	290 +265	*Citeromyces matritensis*	97 (KY 106923.1)	555	MK513718
Rottame	700	320 + 280	n.c.	290 +265	*C. matritensis*	99 (KY 106921.1)	555	MK513719
Rottame	680	260 + 240	300 + 260 + 120	275	*L. termotolerans*	100 (DQ 655683.1)	553	MK513720
Rottame	880	250 + 230	n.c.	260	*Saccharomyces cerevisiae*	99 (KY 471391.1)	552	MK513721
Cannolo	680	n.c.	550	285	*Candida aaseri*	100 (EU807900.1)	496	MK513722
Cannolo	600	n.c.	550	285	*C. aaseri*	100 (KY 106270.1)	490	MK513723
Cannolo	680	210 + 200	600	275	*L. termotolerans*	100 (DQ 655683.1)	542	MK513724
Cannolo	680	210 + 200	600	275	*L. termotolerans*	100 (DQ 655683.1)	548	MK513725
Rottame	680	590	600	275	*L. termotolerans*	100 (KT922994.1)	529	MK513726
Rottame	680	210 + 200	n.c.	275	*L. termotolerans*	100 (KT922390.1)	547	MK513727
Cannolo	680	210 + 200	600	275	*L. termotolerans*	100 (KT922994.1)	543	MK513728
Rottame	680	210 + 200	n.c.	275	*L. termotolerans*	100 (KT922994.1)	548	MK513729
Rottame	680	210 + 200	n.c.	275	*L. termotolerans*	100 (KT922994.1)	546	MK513730
Rottame	680	320 + 280	n.c.	275	*L. termotolerans*	100 (KT922994.1)	541	MK513731
Cannolo	680	320 + 280	n.c.	275	*L. termotolerans*	100 (KT922994.1)	533	MK513732
Liquid	680	320 + 280	n.c.	275	*L. termotolerans*	99 (KT922994.1)	543	MK513733
Rottame	680	320 + 280	n.c.	275	*L. termotolerans*	99 (KT922994.1)	545	MK513734
Rottame	680	320 + 280	n.c.	275	*L. termotolerans*	100 (KT922994.1)	542	MK513735
Rottame	680	320 + 280	n.c	275	*L. termotolerans*	100 (KT922994.1)	546	MK513736
Cannolo	680	320 + 280	n.c.	275	*L. termotolerans*	100 (KT922994.1)	553	MK513737
Rottame	700	320 + 280	n.c.	290 + 265	*C. matritensis*	100 (EF550346.1)	579	MK513738
Liquid	500	175 + 100	300	180 + 170	*Candida lactis-condensi*	100 (JN248611.1)	463	MK513739
Cannolo	500	175 + 100	300	180 + 170	*C. lactis-condensi*	100 (JN248611.1)	462	MK513740

[a] *Values refer to the number of base pairs per fragment.*

[b] *Results of Blast comparison of the D1/D2 region of the 26S rRNA gene into Genbank database.*

TABLE 6 | Genus of filamentous fungi.

Code	*Cladosporium* spp.	*Mucor* spp.	*Aspergillus* spp.	*Alternaria* spp.	*Penicillium* spp.
1–10	•	•		•	•
16–16c	•		•		•
17–5 ☐					
21–17 ☐					
24–7b ☐					
25–17b ☐					
30–13c ☐					
31–17d	•	•	•	•	
33–9 ☐					
34–9 ☐					
35–9–1b ☐					

☐ *Manna just after the collection.*

• *Manna after 48 h of enrichment.*

for F2 factor, the correlation concerns only GM17. Some samples of manna (1–10, 2–6, 6–16, 7–10b, 12–10c, 13–6c, 14–11c, 15–15c, 21–17, 22–18, 26–17b, 30, 17c, 32–18c, 34–9b, and 35–9c) clustered into one main group that was statistically correlated with TGY, DWA-y, DWA-b, and sugar concentrations. On the other quadrant of biplot, manna samples (4–11, 17–5, 18–7, 29–12c, 31–17 and 33–9) were associated to GM17.

DISCUSSION

The aim of the present study was to evaluate the microbial communities hosted on manna samples extracted by different cultivars of *Fraxinus angustifolia* planted in the Madonie area (Palermo province). We also investigated sugar content and some physicochemical parameters characterizing different samples. The work was born from the need of the producers of manna consortia in the above mentioned

TABLE 7 | Physicochemical parameters of manna samples.

Sample code	Source	Mannitol	Sorbitol	Myoiositol	Fructose	Glucose	Galactose	Mannose	Saccharose	Mannotriose	Stachiose	UR%	a_w
1–10	Cannolo	48.07^{efgh}	0.55^{efghi}	0.17^{klm}	16.47^{i}	2.47^{pq}	0.07^{lm}	0.14^{ef}	0.78^{cde}	23.26^{e}	3.80^{q}	7.47^{d}	0.595^{bcd}
2–6	Cannolo	51.12^{b}	0.73^{cd}	0.05^{nop}	9.12^{rs}	2.81^{kl}	0.01^{m}	0.02^{ijk}	0.02^{k}	16.48^{ij}	1.81^{rs}	8.33^{c}	0.744^{abc}
3–8	Cannolo	60.27^{a}	0.89^{ab}	0.03^{op}	8.76st	3.02^{hi}	0.00^{m}	0.00^{k}	0.00^{k}	15.34^{lm}	1.90^{r}	11.03^{a}	0.801^{a}
4–11	Cannolo	47.25^{ghi}	0.54^{fghi}	0.22^{hijk}	9.67^{pqr}	2.16st	0.72^{ghi}	0.01^{jk}	0.49^{gh}	25.32^{bc}	10.05^{g}	4.48^{kl}	0.569^{bcd}
5–15	Cannolo	33.48^{nop}	0.51^{ghij}	0.51^{b}	23.22^{e}	4.51^{b}	1.21^{b}	0.37^{c}	0.76^{cde}	15.26^{lm}	11.53^{d}	4.63^{jk}	0.554^{bcd}
6–16	Cannolo	48.65^{defg}	0.58^{defgh}	0.18^{jk}	9.74^{pq}	2.22^{s}	0.70^{ghi}	0.00^{k}	0.75^{de}	25.12^{cd}	9.87^{g}	3.19^{q}	0.539^{d}
7–10b	Cannolo	50.86^{b}	0.74^{bc}	0.04^{op}	8.98st	2.65^{mn}	0.01^{m}	0.01^{jk}	0.00^{k}	15.46^{klm}	1.75^{rs}	10.64^{b}	0.754^{ab}
8–6b	Cannolo	46.21^{i}	0.61^{cdefgh}	0.24^{ghij}	10.11^{op}	2.42^{qr}	0.76^{fgh}	0.01^{jk}	1.01^{b}	24.63^{d}	7.11^{l}	2.99^{r}	0.609^{abcd}
9–11b	Cannolo	48.96^{cdef}	0.58^{defgh}	0.11^{mn}	9.05st	3.22^{g}	0.25^{k}	0.12^{efgh}	0.07^{k}	12.04^{s}	6.01^{n}	0.57^{v}	0.520^{d}
10–15b	Cannolo	48.78^{def}	0.67^{cdef}	0.05^{nop}	11.34^{m}	2.88^{jk}	0.00^{m}	0.00^{k}	0.00^{k}	14.48^{no}	1.67^{s}	6.42^{ef}	0.548^{cd}
11–16b	Cannolo	46.82^{hi}	0.73^{cd}	0.25^{fghi}	10.56^{no}	2.59^{no}	0.67^{ghi}	0.00^{k}	0.54^{fgh}	26.49^{a}	6.36^{m}	6.55^{e}	0.535^{d}
12–10c	Cannolo	49.11^{cde}	0.70^{cde}	0.04^{op}	12.45^{kl}	2.93^{ij}	0.01^{m}	0.02^{ijk}	0.00^{k}	13.33^{q}	1.70^{rs}	5.39^{h}	0.541^{cd}
13–6c	Cannolo	48.88^{def}	0.91^{a}	0.03^{op}	12.12^{kl}	2.72^{lm}	0.01^{m}	0.00^{k}	0.00^{k}	14.29^{op}	1.75^{rs}	5.39^{h}	0.585^{bcd}
14–11c	Cannolo	50.34^{bc}	0.92^{a}	0.04^{op}	12.54^{k}	2.76^{l}	0.00^{m}	0.02^{ijk}	0.01^{k}	13.82^{pq}	1.82^{rs}	3.14^{qr}	0.598^{abcd}
15–15c	Cannolo	49.76^{bcd}	0.63^{cdefg}	0.19^{ijk}	11.02^{mn}	2.56^{nop}	0.78^{efg}	0.00^{k}	1.21^{a}	23.78^{e}	9.98^{g}	4.44^{l}	0.553^{bcd}
16–16c	Cannolo	47.57^{fghi}	0.48^{ghijkl}	0.18^{jkl}	9.11st	2.06^{t}	0.68^{ghi}	0.00^{k}	0.69^{ef}	25.69^{b}	8.47^{j}	4.46^{kl}	0.549^{cd}
17–5	Rottame	37.33^{jk}	0.35^{klmno}	0.34^{d}	22.48^{f}	3.65^{f}	1.19^{b}	0.35^{c}	0.89^{bcd}	16.71^{hi}	11.07^{e}	3.28^{q}	0.578^{bcd}
18–7	Rottame	36.17^{kl}	0.37^{jklmn}	0.32^{de}	21.22^{g}	3.78^{e}	1.09^{bcd}	0.33^{c}	0.94^{bc}	15.98jk	11.13^{e}	5.83^{g}	0.561^{bcd}
19–12	Rottame	30.21^{rs}	0.41^{ijklm}	0.00^{p}	4.41^{v}	1.54^{v}	0.00^{m}	0.00^{k}	0.00^{k}	8.12^{v}	0.87^{t}	2.28^{s}	0.545^{cd}
20–13	Rottame	34.07^{mno}	0.19^{pq}	0.31^{def}	20.93^{g}	5.13^{a}	0.93^{def}	0.06^{hijk}	0.42^{hi}	9.06^{u}	11.68^{d}	1.43^{u}	0.573^{bcd}
21–17	Rottame	35.17^{lm}	0.60^{cdefgh}	0.04^{op}	15.76^{j}	1.65^{u}	0.41^{jk}	0.70^{a}	0.93^{bcd}	10.63^{t}	8.84^{i}	6.26^{f}	0.545^{cd}
22–18	Rottame	35.31^{lm}	0.19^{pq}	0.44^{c}	9.15^{rs}	1.72^{u}	1.17^{bc}	0.39^{c}	0.44^{hi}	14.21^{op}	14.42^{a}	2.00^{t}	0.574^{bcd}
23–5b	Rottame	32.98^{opq}	0.33^{lmnop}	0.65^{a}	11.94^{l}	3.65^{f}	0.71^{ghi}	0.59^{b}	0.63^{efg}	6.50^{x}	9.13^{h}	1.31^{u}	0.546^{cd}
24–12b	Rottame	34.47^{mn}	0.36^{jklmno}	0.06^{nop}	25.85^{c}	4.40^{c}	1.04^{bcd}	0.35^{c}	0.50^{gh}	15.20^{lm}	4.27^{p}	3.88^{n}	0.562^{bcd}
25–17b	Rottame	36.08^{kl}	0.41^{ijklm}	0.31^{def}	27.12^{b}	4.16^{d}	1.16^{bc}	0.65^{ab}	0.78^{cde}	12.60^{r}	0.11^{u}	3.21^{q}	0.563^{bcd}
26–17b	Rottame	28.06^{t}	0.30^{mnopq}	0.08^{no}	9.23^{qrs}	3.00^{i}	1.43^{a}	0.64^{ab}	0.06^{k}	15.70^{k}	3.87^{q}	4.07^{m}	0.537^{d}
27–18b	Rottame	34.92^{lmn}	0.19^{pq}	0.01^{p}	7.12^{u}	3.25^{g}	0.56^{hij}	0.08^{fghi}	0.28^{ij}	13.36^{q}	13.87^{b}	4.34^{l}	0.581^{bcd}
28–5c	Rottame	35.92^{kl}	0.50^{ghijk}	0.27^{efgh}	3.66^{w}	2.14st	1.19^{b}	0.07^{ghij}	1.02^{b}	15.50^{kl}	9.31^{h}	3.82^{no}	0.578^{bcd}
29–12c	Rottame	33.49^{nop}	0.35^{klmno}	0.30^{defg}	8.56^{t}	2.15st	0.68^{b}	0.13^{efg}	0.45^{ghi}	19.28^{g}	13.03^{c}	3.69^{op}	0.556^{bcd}
30–17c	Rottame	29.54^{s}	0.24^{nopq}	0.31^{def}	33.64^{a}	3.11^{h}	0.66^{ghi}	0.35^{c}	0.12^{jk}	16.68^{hi}	4.78^{o}	4.76^{ij}	0.552^{bcd}
31–17	Rottame	31.63^{qr}	0.21^{opq}	0.34^{d}	24.03^{d}	2.52^{opq}	1.06^{bcd}	0.25^{d}	0.45^{ghi}	14.93^{mn}	10.27^{f}	7.42^{d}	0.550^{bcd}
32–18c	Rottame	32.24^{pq}	0.49^{ghijk}	0.23^{hijk}	19.35^{h}	3.81^{e}	1.19^{b}	0.15^{e}	0.48^{gh}	21.46^{f}	3.81^{q}	4.91^{i}	0.547^{cd}
33–9	Liquid	38.72^{j}	0.47^{hijkl}	0.51^{b}	16.45^{i}	0.88^{w}	0.65^{ghi}	0.06^{hijk}	0.93b^{cd}	17.09^{h}	7.36^{k}	7.49^{d}	0.574^{bcd}
34–9b	Liquid	32.20^{pq}	0.16^{q}	0.02^{op}	33.49^{a}	2.35^{r}	0.54^{ij}	0.38^{c}	0.10^{jk}	10.50^{t}	8.38^{j}	3.64^{p}	0.556^{bcd}
35–9c	Liquid	38.00^{j}	0.17^{q}	0.16^{lm}	23.19^{e}	3.82^{e}	0.97^{cde}	0.39^{c}	0.36^{hi}	7.31^{w}	8.84^{i}	3.90^{mn}	0.525^{d}
S.E.		1.40	0.04	0.03	1.32	0.15	0.08	0.04	0.06	0.92	0.71	0.40	0.01
Statistical significance		***	*	*	***	**	*	*	*	***	***	***	*

UR, relative humidity; a_w, water activity; S.E., standard error.
The sugar content is expressed as g/100 g.
*Data within a columns followed by the same letter are not significantly different according to Tukey's test. *P < 0.05; **P < 0.01; ***P < 0.001.*

area that have turned to the University of Palermo with a specific request: to characterize the manna products for cultivable microorganisms.

Manna represent very traditional food products obtained by expert hands and without addition of chemical preservatives, thus manna still retains a natural image as high-quality food and/or food adjuvant. Considering this, an increase in manna consumption could result in the increase in demand from novel consumers interested in the re-discovery of traditional food products (Souza et al., 2006; Settanni and Moschetti, 2014).

In order to better characterize the microbial stability of manna, the microbial populations and sugar content were analyzed in this study. The microorganisms present in different types of manna products (*cannolo* and *rottame*) might provide new information regarding the hygienic quality and potential application of these traditional foods. Furthermore, a deep investigation of microorganisms hosted by manna and associated with production environment might improve quality and shelf-life of final products. To this purpose, several manna samples belonging to different commercial categories were collected and

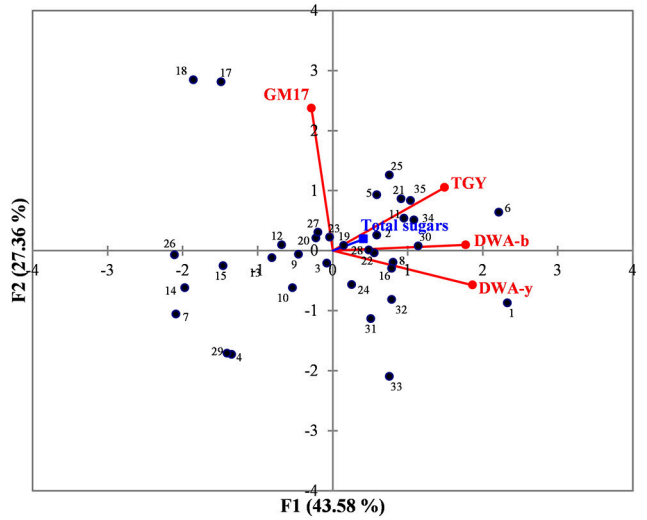

FIGURE 2 | Biplot graph showing the distribution of manna samples in relation to the content of total sugar, bacteria, and yeast contaminations. GM17, glucose M17 added with cycloeximide for lactic acid bacteria counts; DWA-b, De Whalley agar added with cycloeximide for osmophilic bacteria counts; TGY, tryptone glucose yeast extract agar added with chloramphenicol for total (osmophilic and osmotolerant) yeasts; DWA-y, De Whalley agar added with chloramphenicol for osmotolerant yeast counts. Numbers in the biplot: 1, 1–10; 2, 2–6; 3, 3–8; 4, 4–11; 5, 5–15; 6, 6–16; 7, 7–10b; 8, 8–6b; 9, 9–11b; 10, 10–15b; 11, 11–16b; 12, 12–10c; 13, 13–6c; 14, 14–11c; 15, 15–15c; 16, 16–16c; 17, 17–5; 18, 18–7; 19, 19–12; 20, 20–13; 21, 21–17; 22, 22–18; 23, 23–5b; 24, 24–12b; 25, 25–17b; 26, 26–17b; 27, 27–18b; 28, 28–5c; 29, 29–12c; 30, 30–17c; 31, 31, 17c; 32, 32–18c; 33, 33–9; 34, 34–9b; 35, 39,9c.

subjected to the analysis of bacteria, yeasts and filamentous fungi, and physical-chemical parameters. To our knowledge, there is currently no published data on this topic, and the only few papers available on manna products focused on its chemical composition.

Concerning manna sugar content, no detailed/specific works on Arabian samples showed them to contain sucrose, melezitose, and trehalose. Our data suggested a high content of mannitol, mannotriose, and sucrose for both *cannolo* and *rottame* saples. A positive correlation between UR% and a_w parameters was observed.

According to the other high content sugary foods (Snowdon and Cliver, 1996; Feás et al., 2010; Sinacori et al., 2014), the microbial loads, although variable, are generally $<10^3$cfu/g. This is due to their high content of sugar, which creates stressful conditions for the growth and survival of non-osmo-tolerant microorganisms (Iurlina and Fritz, 2005). Manna products are food matrices with a sugar content of about 85% (w/w). However, several microbial isolates were collected from the samples analyzed in this study.

As reported by Sinacori et al. (2014) for honey, yeasts on high-sugary foods were mainly non-*Saccharomyces*, while the group of bacteria included several *Bacillus* species (Iurlina and Fritz, 2005; Alippi and Reynaldi, 2006; Sinacori et al., 2014). The results of our study followed the trend generally reported for other sugary foods. Presumptive LAB were not detected in any sample and only two samples turned out positive for the presence of *Enterobacteriaceae* family members, whereas the presumptive osmophilic bacteria were identified as *B. amyloliquefaciens, B. halotolerans, B. mojavensis, B. safensis, B. subtilis, B. tequilensis,* and *B. vanilla.* Most of these species are endophytic bacteria and are antagonist of fungi pathogen of plants (Bacon and Hinton, 2002), whereas *B. safensis*, that was *first* isolated in spacecraft, colonizes habitats with stringent condition for the survival of some microorganisms (Satomi et al., 2006; Lateef et al., 2015). Furthermore, within the bacteria found on DWA plates, *Clavibacter michiganensis,* and *four* species of *Staphylococcus* spp. were also identified. Staphylococci are widespread in nature and are found consistently on birds (Place et al., 2002), therefore, their presence in manna depends on the environmental contamination, as well as to the production process. *Staphylococcus epidermidis, St. haemolyticus,* and *St. warneri* are considered unsafe because some strains showed decreased susceptibility to vancomycin (Center et al., 2003), in contrast to *St. succinus* subsp. *casei* (Place et al., 2002). Although, at low levels clostridia were observed; similar findings were previously reported for honey (Sinacori et al., 2014).

In the present study, yeasts were the most abundant microbial populations and a total of six species were identified: *Candida aaseri*, *Candida lactis-condensi*, *Citeromyces matritensis*, *Lachancea termotolerans*, *Saccharomyces cerevisiae,* and *Zygosaccharomyces bailii*. The species *L. termotolerans* was the most frequently isolated. *L. thermotolerans*, like all non-*Saccharomyces* yeasts, is a widespread and applied cosmopolitan species as a starter in oenology (Hranilovic et al., 2018). It is a yeast that is able to positively influence the sensorial profile of the wine. In winemaking environments, it is to be considered a robust starter as it resists up to 13.6% (v/v) of ethanol. This species is usually applied as a starter culture to ferment several alcoholic beverages and mainly in co-fermentation with *Saccharomyces cerevisiae* to improve the overall quality of wine (Gobbi et al., 2013). Several applications of *L. termotolerans* are also reported as a starter to produce beer (Domizio et al., 2016).

Generally, *L. thermotolerans* is not known in literature as a yeast capable of resisting high sugar concentrations. Manna, like honey and other sugar matrixes, is characterized by low water activity caused by a high concentration of sugar. In literature, it is known that different species of yeasts are osmotolerant and mainly belong to the following genera: *Candida*, *Metschnikowia*, *Millerozyma* and *Zigosaccharomyces* (Kurtzman et al., 2011). The isolates of *L. thermoltolerans* found on the manna represent a particular case, but given the enormous adaptation of this species of yeast to different habitats (Hranilovic et al., 2017), this work could provide a better starting point to further study the complexity of the structure of the population, ecology and evolution of this non-*Saccharomyces* yeast.

Citeromyces matritensis is a yeast generally found in sugar syrups (Marvig et al., 2014). *Zygosaccharomyces bailii* is a spoilage yeast, known for its extreme resistance to preservatives and ability to grow in excess of legally-permitted concentrations of

preservatives (Stratford et al., 2013), while *Candida aaseri* is a novel species, never detected in food matrices (Brandt and Lockhart, 2012).

Filamentous fungi included five genera: *Cladosporium*, *Mucor*, *Aspergillus*, *Alternaria,* and *Penicillium*. All genera are considered common contaminants of honey and of other foods due to their xerophilic characteristics (Nasser, 2004; Kacániová et al., 2012; Yoder et al., 2013).

CONCLUSIONS

This work represents the first investigation on the microbial ecology of manna. Despite the stringent conditions due to high sugar content of manna, osmophilic bacteria, yeasts, and fungi filamentous were found in several samples. Plate counts on manna samples after the enrichment procedures, showed the presence of these microorganisms at high levels, confirming their survival in such stressing conditions in a viable and cultivable form.

However, the microbial ecology of manna was mainly represented by *Bacillus* and non-*Saccharomyces* yeast genera. The presence of several contaminant microorganisms, belonging to *Staphylococcus* spp., *Zygosaccharomyces,* and *Candida* genera, were also revealed. To this purpose, higher attention should be paid to the microbiological safety of manna as food.

Although more studies are needed, our results suggest that the strains of *L. thermotolerans* isolated in this study might be evaluated *in situ* for their potential to act as starters in single or in multi-combination for food applications. Further investigations should be carried out to analyze the presence and cell densities of the potential pathogenic microorganisms vectored by manna.

AUTHOR CONTRIBUTIONS

RS has been involved in the sampling of vegetable matrices. RGa, AA, FC, GM, RGu, and NF contributed with laboratory work, data analyses, and text writing. AT performed all the chemico-physical analyses.

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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.2019.00984/full#supplementary-material

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Hazelnut as Ingredient in Dairy Sheep Diet: Effect on Sensory and Volatile Profile of Cheese

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The opportunity of replacing expensive feedstuffs with agro-industrial by-products in the diet of food producing animals is raising increasing interest while addressing global concern for the scarcity of natural resources and environmental impact of livestock farming. Hazelnut peels, rich in fiber and vitamins and characterized by a high concentration of fats, is considered a suitable ingredient to be included in the diet of ruminants. The aim of this research was to assess the effect of dietary hazelnut peels on the chemical and sensory properties of sheep cheese during refrigerated storage. To this purpose, 20 Comisana lactating ewes were randomly assigned to two experimental groups, control (C) and hazelnut peels (HP), balanced for parity, milk yield and body weight. Bulk milk collected from the 2 groups was used to produce 5 Pecorino cheeses for each group. After 40 d of aging, each cheese of each experimental group was divided into 3 pieces: 1 piece was sampled for analyses (C0, HP0) and 2 were wrapped in PVC film, simulating the condition of pre-wrapped products, and analyzed after 7 (C7, HP7) and 14 days of storage (C14, HP14) at 8°C with 80% moisture. The cheeses were analyzed for chemical and fatty acid composition, sensory analysis, odor active compounds and SmartNose. As expected, HP cheeses presented a higher lipid content compared to C, a lower content in SFA and PUFA, and a greater content in MUFA. A triangle test revealed a clear distinction between the 2 groups ($\alpha = 0.01$) The sensory profile showed a significant effect on holes ($P < 0.05$) and a marginal production of off-flavors linked to spicy and acid attributes for HP cheeses The volatile profile of C and HP cheese samples showed a good similarity, partially explained by the short ripening time and the absence of 2-nonanone in HP7, suggesting a higher antioxidant protection grade of this cheese compared to the others. These results were confirmed by Smart Nose analysis. Further studies on vitamin content should be conducted in order to investigate the interactions between the presence of antioxidant volatile compounds and the oxidative stability of ewe cheese.

Keywords: dairy sheep, hazelnut, sensory properties, diet supplement, sheep cheese

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INTRODUCTION

The global concern for the scarcity of natural resources dramatically increased over the last decades. For this reason, FAO and EU promote the principles of 3R (Reduce, Reuse, and Recycle) for a sustainable development in all the productive sectors, including livestock production. Animal feedstuffs production, processing and transport are highly demanding in terms of natural resources. Moreover, livestock farming greatly impacts on the environment. In this context, the inclusion of agro-industrial by-products in the diet of food producing animals as a replacer of conventional, and more expensive feedstuffs, is under investigation to mitigate the impact of livestock production (1). Interestingly, most of the food-derived by-products are rich of bioactive molecules that can exert positive effects both on animal welfare and product quality (2).

Hazelnut (*Coryllus Avellana* L.) is worldwide consumed and the global production accounts for more than 1 million tons per year. Hazelnut can be consumed as such, but the biggest part of the production is destined to pastry and chocolate industry. Hazelnut peel (HP) is the by-product that results from the roasting phase during the industrial processing of the hazelnut and represents about 2.5% of total hazelnut kernel weight (3). Indeed, hazelnut peel is the perisperm of the kernel and need to be removed from the fruit to prevent food off-flavors and off-colors due to the great content of phenolic compounds (4).

Thanks to its chemical composition, HP could be considered a suitable ingredient to be included in the diet of ruminants. In particular, HP is rich in fiber (∼30%), fat (∼20%) and even at lower percentage can afford protein (∼7%) (5). The oil fraction is characterized by the high content of unsaturated fatty acids (UFA; principally oleic and linoleic acid). Also, HP is rich of vitamins, mostly represented by vitamin E. Lastly, HP is a natural source of polyphenols. Specifically, del Rio et al. (6) report that among the total phenolic compounds (675 mg/100 g DM) gallic acid, procyanidin dimers and trimers, flavan-3-ols, flavonols and hydrolizable tannins as glansreginin A, B, and C represent the main part.

The bioactive molecules contained in the HP, or their derivatives, could be transferred to ruminant products affecting their nutritive value and sensory properties (7). Indeed, it is well-accepted that dietary unsaturated fatty acids can be used to improve the healthiness of meat, milk and cheese (8). However, increasing the proportion of lipids highly sensitive to oxidation can play a role in the development of the aroma in repined products such as cheese (9). Also, vitamins and phenolic compounds, besides adding value to the healthy properties of ruminant products, could act as antioxidant (or modulators of the bacterial activity) thus influencing the maturation processes of the fermented products (10).

In the literature, the use of dietary plant extracts rich in polyphenols, such as condensed or hydrolysable tannins, has been investigated to improve quality traits of ruminant products (7, 11). However, the use of plant extract could increase the cost of the animal diet. In the literature the effect of the inclusion of hazelnut peel in animal diet on the quality of ruminant products is lacking. In this context, recycling hazelnut peel as a source of bioactive substances for feeding livestock could represent an innovation in the field of research in animal production. Additionally, the chemical composition of hazelnut peel may justify a high level of inclusion in the animal diet, which would allow a more effective exploitation of this by-product as a replacer of traditionally used feedstuffs.

Considering the all above, the aim of this research was to assess, for the first time, the effect of dietary hazelnut peel on the chemical and the sensory properties of sheep cheese during refrigerated storage.

MATERIALS AND METHODS

Animals and Diets

The trial was carried out at the Experimental Farm of the Department of Agricultural, Food and Environmental Science of the University of Perugia. Twenty multiparous Comisana lactating ewes with 89 ± 10 days in milk were randomly assigned to two experimental groups ($n = 10$), namely control (C) and hazelnut (HP), balanced for parity (2.3 vs. 2.5), current milk yield (824 vs. 804 g/d) and BW (62.6 vs. 64.0 kg). The animals were kept in two separate boxes (one per group) with sawdust bedding. The experimental trial lasted 28 days after 14-day adaptation period during which the animals were gradually adapted to the respective diet composed by chopped alfalfa hay offered *ad libitum* (particle size >4 cm in length) and 800 g/ewe/day of a pelleted concentrate containing 370 g/kg DM of dried beet pulp (C) or 360 g/kg DM of hazelnut peels (HP). The concentrate, formulated to cover the nutrition requirements of a sheep weighing 68 kg producing 1 kg milk per day with 6.5% fat (12), was given individually at the morning and afternoon milking (400 g DM at each milking) until complete consumption.

Representative samples of the offered feeds were analyzed for neutral detergent fiber (NDF) according to Van Soest et al. (13). Furthermore, crude protein, crude fat (ether extract) and ash were analyzed according to methods 976.06, 920.39, and 942.05, respectively (14). In addition, lipids from the individual feeds were extracted and converted to fatty acid methyl esters (FAME) with a 1-step procedure using chloroform (15) and 2% (v/v) sulfuric acid in methanol (16) to determine the fatty acid profile. Non-adecanoic acid was used as an internal standard. Gas chromatographic analysis was carried out as later described for the analysis of cheese fatty acids. **Table 1** reports the ingredients and the chemical composition of the offered feeds.

The research activity reported in this paper treated the supplementation fed to animals by including either beet pulp or hazelnut peel in the concentrate. Therefore, this project is not regulated by the Directive 2010/63/EU art. 1, point 4, letter f, on the protection of animals used for scientific purposes, according to which the directive does not apply to the practices not likely to cause pain, suffering, distress or prolonged damage equivalent or superior to that caused by the insertion of a needle according to the good veterinary practices. The feeding trial followed the ordinary practices of dairy sheep farms. Therefore, approval was not needed according to institutional and national guidelines. Nevertheless, all the experimental procedures adopted

TABLE 1 | Ingredients and chemical composition of diets used in the experiment including concentrates containing either beet pulp (C) or hazelnut peel (HP).

			Experimental concentrates	
	Hay	**Hazelnut peel**	**C**	**HP**
INGREDIENTS (G/KG DRY MATTER)				
Hazelnut peel			-	360.2
Barley			345.1	329.5
Wheat bran			98.6	97.0
Soybean meal			140.8	168.2
Dried beet pulp			369.8	-
Molasses			25.4	25.0
Calcium carbonate			5.0	5.0
Sodium bicarbonate			5.0	5.0
Dicalcium phosphate			5.0	5.0
Sodium chloride			5.0	5.0
CHEMICAL COMPOSITION (G/KG DRY MATTER)				
Crude protein	149.9	78.6	157.7	162.7
Ether extract	15.8	226.3	16.3	91.5
NDF	527.7	510.7	302.2	358.3
ADF	429.3	387.7	135.4	225.8
ADL	95.7	202.8	15	75.6
Ash	75.6	24.8	63.9	51.8
PROTEIN FRACTIONS				
A	39.1	1.8	21.5	8.2
B1	6.9	3.7	5.9	19.0
B2	71.0	18.1	100.0	73.1
B3	19.3	1.6	24.1	32.7
C	13.7	53.3	6.1	29.7
FATTY ACIDS (G/100G DM)				
14:0	0,007	0,015	0,004	0,007
16:0	0,193	1,041	0,374	0,663
18:0	0,039	0,383	0,033	0,17
cis-9 18:1	0,044	11,067	0,268	4,485
cis-9 cis-12 18:2	0,145	2,018	0,883	1,497
cis-9 cis-12 cis-15 18:3	0.245	0.031	0.087	0.078

agree with the European Union guidelines about experimental animals (Gazzetta Ufficiale 61, 2004).

Cheese Production and Shelf-Life Evaluation

Bulk milk from each of the two experimental groups was collected during last week of the experimental period and stored at −30°C until the quantity of 40 kg was reached. The milk was thawed in cold condition at 5°C. The cheese-making procedure was performed according to a traditional technique reported by Mughetti et al. (17), with modifications. Briefly, the milk was heated at 39°C and a mixed-strain starter culture (MW039S SACCO) was added and incubated for 10 min. Then, a liquid calf-lamb rennet was added (22g/100 L). After 20 min the curd was turned on the surface and broken in 3cm large square to let the whey bleed. After 5 min the curd was further broken until the corn grain dimension was reached. Finally, the curd was put on plastic basket and the whey was left bleeding until the pH reached 5.5 level. A total of 10 cheeses were produced (5 per experimental group). After 24 h refrigeration at 7°C, all the cheeses were put in brine for 12 h and aged in a cold room for 40 days.

After the aging, each cheese was divided into 3 pieces, each representing a subsample. One of the three subsamples of each cheese was destined to the analyses described below without storage (C0 and HP0). The other two subsamples were wrapped using a PVC film, simulating the method adopted in the stores for the pre-wrapped products, and analyzed after 7 (C7, HP7) or 14 days of storage (C14, HP14) at 8°C with 80% moisture.

CHEESE ANALYSES

Chemical and Fatty Acid Composition

Cheese samples were analyzed to determine moisture according to the method proposed by Bradley and Vanderwarn (18), lipid content according to the Gerber - Van Gulik method (ISO 1975)

and protein content (total nitrogen x 6.38) determined using the Kjeldhal method. Cheese fatty acid composition was determined by gas-chromatography. Fat was extracted from 5 g of finely minced cheese using a mixture of chloroform and methanol (2:1, v/v) as described by Folch et al. (19) and 30 mg of lipids were converted to FA methylesters (FAME) by base catalyzed transesterification (20) using 0.5 mL of sodium methoxide in methanol 0.5 N and 1 mL of hexane containing 19:0 as an internal standard. Gas chromatographic analysis was performed on a Trace Thermo Finnigan GC system (ThermoQuest, Milan, Italy) equipped with a flame-ionization detector and a 100 m fused silica capillary column (0.25 mm i.d., 0.25-μm film thickness; SP-24056; Supelco Inc., Bellefonte, PA) and helium as the carrier gas (1 mL/min). FAME profile in a 1-μL sample volume (split ratio 1:80) was determined according to the temperature gradient program described by Valenti et al. (21). The oven temperature was programmed at 50°C and held for 4 min, then increased to 120°C at 10°C/min, held for 1 min, then increased up to 180°C at 5°C/min, held for 18 min, then increased up to 200°C at 2°C/min, held for 15 min, and then increased up to 230°C at 2°C/min, held for 19 min. The injector and detector temperatures were at 270 and 300°C, respectively. FAME identification was based on a commercial mixture of standard FAME (Nu-Chek Prep Inc., Elysian, MN, USA), individual standard FAME (Larodan Fine Chemicals, Malmo, Sweden). Fatty acids were expressed as percentage of total fatty acids.

Sensory Analysis

The cheese for the groups C and HP were evaluated through a discriminant triangle test (ISO 4120:2004) by comparing the two treatments at each storage time (C0 vs. HP0, C7 vs. HP7, C14 vs. HP14). Seventeen assessors were involved and the significance level was set at 0.01. The pieces of cheeses were served at room temperature using white plastic dishes marked each using a 3-digit code. The tasting station was lighted in order to prevent the perception of differences in colors of the samples. The samples were also described by qualitative descriptive analysis (QDA), according to Stone et al. (22). Attribute terms for evaluation of cheeses were developed by the 17 panelists using QDA methodology. Briefly, ballot development and panelist training were accomplished during seven working sessions. The descriptive terms developed for each major sensory attribute category are reported in **Table 3**. Each attribute was presented as a separate unstructured line scale that recorded panelist responses in increments of 0.1 between 1 (leftmost position) and 15 (rightmost position). The cheese samples were cubed (∼1 cm each side) and were presented on white paperboard plates. The panelists also had available an entire transverse slice of each cheese for evaluating appearance attributes. The samples were identified using random 3-digit codes and were at room temperature at the time of testing.

Extraction and Detection of Odor Active Compounds (OACs)

Qualitative analysis was performed in order to study and compare the aroma profile of C and HP cheeses. Odor active volatile compounds (OACs) extraction was realized by using static headspace solid-phase microextraction (SPME) using a DVB/CAR/PDMS fiber (Supelco, Bellefonte, PA, USA). Ten grams of cheese samples were put into a 22-mL vial and conditioned in a water bath at 40°C for 30 min. Further 40 min of fiber exposition time was required for the static headspace extraction. The fiber was preconditioned before initial use by inserting it into the injector port of a gas chromatography instrument for 1 h at 270°C, and was reconditioned between extractions at the same temperature for 5 min, followed by 10 min at room temperature. Solid phase micro-extraction shows many advances due to the fact that it is a solventless technique and non-artifact forming method as a consequence. It requires a minimal manipulation and small amount of sample (23). Nowadays SPME is commonly used for dairy products flavor extraction such as Camembert cheese (24), Cheddar cheese (25), smear-ripened cheese (26), Trachanas fermented cheese (27), milk proteins (28). Solid phase micro-extraction has been also used for the isolation of odor active compounds of several animal feeds for small ruminants (29).

The detection of the extracted volatile compounds was performed by Gas Chromatography Olfactometry (GC/O) using the single sniff method as described by Marin et al. (30). The OACs were desorbed and separated into a modified Hewlett Packard 6890 GC (Datu, Inc., Geneva, NY, USA) using a fused-silica HP-5 capillary column (30 m × 0.25 mm ID × 0.25 μm film thickness, Agilent Technologies, USA). The chromatographic conditions were as follows: splitless injection at 250°C; oven temperature programme: 35°C for 3 min, 6°C min^{-1} to 190°C, then 30°C min^{-1} to 225°C and 225°C for 3 min; He carrier gas, column flow rate 1.9 mL min^{-1}. The eluted compounds were mixed with a stream of humidified air using a method described by Acree and Barnard (31) and the "sniffer" was exposed to this source continuously for 30 min. The response time for each perceived individual odor was recorded by Charmware software (v.1.12, Datu, Inc., Geneva, NY, USA). Retention times (RT) for each OAC were converted into retention indices (RI) and were displayed by the software as a series of peaks in an "aromagram". RI values were calculated relative to a series of normal alkanes (C7–C18) previously injected into the flame ionization detector (FID) port of the same gas chromatograph. The identification of OACs were carried out by using a GC/Mass Spectrometer (Hewlett Packard 6890), under the same extraction and chromatographic conditions used for GC/O analysis.

This procedure permitted direct comparisons between RI and RT values obtained from GC/O and GC/MS, respectively.

Each odor compound detected by GC/O analysis was defined by a RI value and an odor description. Odor active compounds were also identified using the Flavornet internet database (32), containing over 550 VOCs identified using GC/O techniques. The extraction and detection of flavor profile were performed twice at the same operative conditions in order to confirm odor perception results.

Smart Nose

Smart Nose is an electronic nose, that allows the direct analysis by MS of volatile organic components from liquid and solid samples without separation of the headspace components.

The analysis was performed with a Smart Nose system incorporating an autosampler CTC Analytics AG (CTC Combi Pal with the Cycle Composer software), a high-sensitivity quadrupole mass spectrometer (Inficon AG) with an ionic mass detection, ranging from 1 to 200 amu, and a user-friendly multivariate analysis software (Smart Nose 1.51) for data acquisition.

Four milliliter of milk were filled into 20-ml vials (adapted for the Combi Pal autosampler).The samples were analyzed in triplicate and randomly placed in the autosampler trays to avoid biases due to external factors.

The main operating conditions were as follow: incubation temperature, 60°C; incubation time, 30; injector temperature, 160°C; purge gas, nitrogen; purge flow, 200 ml/min; Syringe purge time, 1 min. Mass spectrometer scan speed; Mass range, 10–160 amu; speed scan: 2 mass/s; SEM voltage 1540.

STATISTICAL ANALYSES

Data on cheese chemical and fatty acid composition were analyzed using a one-way ANOVA to test the effect of the dietary treatment (C vs. HP). Data on cheese sensory characteristics were analyzed using a GLM mixed model to test the effect of the dietary treatment (C vs. HP) and of the time of storage (0, 7, 14), while the variable panelist was considered as random effect. Student's *t*-tests ($\alpha = 0.05$) were used to determine differences between diet and storage time means when significant differences for that effect were found. SMart Nose data set were transformed using the software supplied with the SMart Nose. First, the mean value of the second and third cycle was calculated. Then the processed data set was normalized using the atomic ion of argon ($m/z = 40$) from air. This mass to charge ratio is subject to practically no contamination from other compounds and the concentration of this gas in the headspace can be considered as constant. Then a Principal Components Analysis (PCA) was applied. Principle Components Analysis (PCA) is a multivariate statistical analysis that allows to correlate several variables in bi-dimentional plot. In detail, Smart Nose is set in a range of 10–160 amu of ion fragments. PCA allocates a Principal Component (PC) to a single ion fragment and it gives an order from 1 to n ion fragments. PC1 represents the PC with the highest explained variability, the successive (PC2, 3, 4.…) represent PC with lower explained variability.

RESULTS

Feed Composition

As reported in **Table 1**, the HP chemical composition is characterized by a high content of crude fat (226 g/kg DM) and fiber fractions (510, 388 and 203 g/kg DM for NDF, ADF and ADL, respectively). As regard fatty acids, HP was characterized by the prevalence of oleic acid (11 g/kg DM), and linoleic acid (2 g/kg DM). Except for the protein content, planned to be similar between the two concentrates, the chemical composition of HP concentrate reflects the composition of the hazelnut peel. In particular, the 36% hazelnut peel inclusion in the HP concentrate increased crude fat (91 vs. 16 g/kg D;), oleic (4.5 vs. 0.3 g/kg DM) and linoleic acid (1,5 vs. 0.9 g/kg DM), and all the fiber fractions in comparison with the C concentrate.

TABLE 2 | Chemical and fatty acid composition of cheeses produced with milk of ewes fed with Control (C) or Hazelnut Peels (HP) diets.

Parameter	Diet		SEM	*P*-value
	C	HP		
CHEMICAL COMPOSITION (G/KG DRY MATTER)				
Moisture	40.02	38.87	1.55	0.272
Fat	26.51^{b}	31.94^{a}	0.82	<0.001
Ash	3.76^{a}	3.29^{b}	0.10	<0.001
Protein	25.29	24.26	1.29	0.241
FATTY ACIDS (G/100G TOTAL FATTY ACIDS)				
SFA	73.94^{a}	56.07^{b}	0.36	<0.001
MUFA	15.87^{b}	34.48^{a}	0.26	<0.001
PUFA	6.81^{a}	6.45^{b}	0.09	0.007
OBCFA	5.31^{a}	3.40^{b}	0.04	<0.001

^{ab}*means with superscripts differ significantly ($P < 0.05$) within that effect.*

Cheese Composition

The feeding treatment affected the chemical composition of cheese **(Table 2)**. Specifically, HP cheese was greater in lipid (31.9 vs. 26.5 %; $P < 0.001$) but showed a lower ash content (3.29 vs. 3.76%, $P < 0.001$). As regard lipid profile, the percentage of total saturated (SFA; 56.1 vs. 73.9, $P < 0.001$), polyunsaturated (PUFA; 6.45 vs. 6.81, $P = 0.007$) and odd and branched-chain fatty acids (OBCFA; 3.4 vs. 5.31, $P < 0.001$) were lower in HP cheese as compared to C cheese. On the contrary, a significant increase of monounsaturated fatty acids (MUFA, $P < 0.001$) was found in HP cheeses.

Sensory Analysis

For all times of conservation (0, 7, and 14 days) the triangle test was significant with $\alpha = 0.01$. At time 0, 13 out of 17 answers were correct, whereas after 7 and 14 days 15 out of 17 were correct. The mean attribute ratings from sensory analysis of Control (C) and Hazelnut Peels (HP) cheeses at different times of storage (0, 7, and 14 days) at 8°C and 80% humidity are reported in **Table 3**. Diet had a significant effect on holes ($P < 0.05$) and a marginal effect on spicy and acid attribute ($P < 0.10$). In particular, HP cheeses showed fewer holes (4.21 vs. 3.71) and were evaluated less spicy (1.92 vs. 1.35), but were more acid (3.03 vs. 3.70) compared to C cheeses. Time of storage had a significant impact on rim color and oiliness ($P < 0.05$) and a marginal effect on rind thickness ($P < 0.10$). All these attributes decreased with increasing time of storage. The interaction between diet and time of storage effect was not significant.

Odor Active Compounds by Gas Cromatography/Olfactometry and Mass Spectrometry

A total of six C and HP cheese batches were analyzed by Gas Cromatography/Olfactometry (GC/O) and GC/Mass spectrometry (GC/MS). In general, C and HP cheese samples

TABLE 3 | Mean attribute ratings and standard error from sensory analysis of Control (C) and Hazelnut Peels (HP) cheeses at different storage times (0, 7, and 14 days) at 8°C and 80% humidity.

Attribute	Diet			Storage time (days)			
	C	HP	St err	0	7	14	St err
APPEARANCE							
Rind color	3.17	3.35	0.28	3.00	3.12	3.66	0.32
Rind thickness	2.32	2.19	0.25	2.57	2.34	1.85	0.28*
Rim color	3.66	3.75	0.27	4.42[a]	3.40[b]	3.29[b]	0.33**
Rim thickness	3.20	3.16	0.25	3.52	3.06	2.96	0.29
Color	3.15	3.25	0.28	3.50	2.96	3.14	0.32
Homogeneity	4.89	4.38	0.26	4.69	4.48	4.73	0.32
Holes	4.21[a]	2.71[b]	0.30**	3.11	3.70	3.56	0.36
Moistness	3.18	3.63	0.26	3.59	3.46	3.16	0.31
AROMA							
Overall intensity	4.94	4.97	0.28	5.09	4.99	4.79	0.33
Fresh milk	4.03	4.51	0.32	4.33	4.41	4.07	0.38
Vegetal	3.11	3.08	0.32	2.80	3.36	3.14	0.37
Floral	2.14	1.81	0.35	1.95	1.93	2.04	0.38
Fruity	2.04	1.69	0.35	1.85	1.64	2.10	0.39
Roasted	2.26	1.86	0.29	2.14	2.00	2.04	0.34
Spicy	1.92	1.35	0.28*	1.52	1.66	1.72	0.32
TASTE							
Sweet	3.07	2.61	0.28	2.63	3.09	2.80	0.32
Salty	3.25	3.32	0.33	3.59	2.91	3.35	0.38
Acid	3.03	3.70	0.33*	3.69	3.34	3.06	0.38
TEXTURE							
Consistency	4.27	4.41	0.29	3.95	4.44	4.63	0.34
Homogeneity of consistency	4.77	5.14	0.30	4.49	5.43	4.95	0.35
Chewiness	5.07	5.42	0.31	5.29	5.28	5.16	0.36
Solubility	4.35	4.74	0.35	4.82	4.52	4.29	0.39
Hardness	3.31	2.96	0.30	3.12	3.41	2.89	0.36
Friability	3.14	3.29	0.36	3.06	3.56	3.03	0.42
Adhesiveness	2.43	2.21	0.27	2.52	2.28	2.15	0.32
Graininess	3.08	3.28	0.27	3.40	3.21	2.93	0.33
Elasticity	3.43	3.65	0.31	3.61	3.68	3.33	0.36
Oiliness	4.04	4.30	0.33	4.90[a]	3.84[b]	3.76[b]	0.38**
Pungency	2.71	2.78	0.28	3.01	2.69	2.53	0.34
Astringency	1.58	1.92	0.28	2.12	1.66	1.46	0.33
Aftertaste	4.39	4.23	0.31	4.04	4.21	4.68	0.36
Persistence	4.68	5.11	0.30	5.13	4.71	4.85	0.36

*[ab]means with superscripts differ significantly (P<0.05) within that effect. *P < 0.10 **P < 0.05.*

showed a poor and very similar volatile profiles (**Table 4**). No significant difference in number and type of volatile compounds between C and HP groups were found. Some volatile compounds were found in all cheeses. In detail, two esters ethyl butyrate and ethyl octanoate, three ketons diacetyl, 1-octen-3-one and 2-nonanone and one terpene, β-carene were detected as common compounds for both C and HP cheese batches. Moreover, the monitored storage at 0 to 14 days did not affect volatile profile during the shelf life of cheeses. At 7 days of storage, HP cheese showed relevant differences for specific volatile compounds. In detail, HP7 group showed the highest number of esters, two of which were detected only in this sample, and two sulfo-organic compounds also revealed as unique.

Volatile Fingerprint by SMart Nose

Principal Component Analysis from SMart Nose data showed a clear separation among hazelnut peel cheese vs. control cheese at 0 and 7 days (PC1 95,64%; PC2 2,46%). In detail, PC1 separated C0, C7 and C14 from HP0 and HP7. PC1 also explained similar volatile profile of C and HP at 14 days that resulted overlapped in the score plot (**Figure 1**). Data concerning PC2 highlighted similar volatile profile for C and HP groups at 0 days that

TABLE 4 | Odor active compounds in control (C) and Hazelnut Peels (HP) cheeses at different storage times (0, 7, and 14 days) at 8°C and 80% humidity extracted by solid-phase microextraction (SPME).

Compound	Chem class	Odor perception	LRI[a]	Ident[b]	C0	HP0	C7	HP7	C14	HP14
pentanal	aldehyde	pungent, apple	725	PI,MS		x				x
2-hexenol	alcohol	fruit	881	PI	x					
ethyl butyrate	ester	apple	790	PI,MS	x	x	x	x		x
2-methylbutyl acetate	ester	fruit	891	PI				x		
ethyl isohexanoate	ester	orange	957	PI				x		
ethyl octanoate	ester	wine	1186	PI,MS	x	x	x	x	x	x
diacetyl	ketone	butter	638	PI	x	x	x	x	x	x
1-octen-3-one	ketone	mushroom	970	PI,MS	x	x	x	x	x	x
2-nonanone	ketone	milk	1090	PI,MS	x	x	x		x	x
dimethyl pyrazine	pyrazina	roasted,toasted	911	PI						x
thiophene	sulfur	garlic	665	PI,MS				x		
methylfuranthiol	sulfur	broth	858	PI				x		
3-isothiocyanato-1-propene	sulfur	garlic	886	PI	x				x	
β-carene	terpene	orange	993	PI,MS	x	x	x	x	x	x
Total					**8**	**7**	**6**	**9**	**6**	**8**

[a] *LRI, Linear Retention Index wihHP-5 column.*

[b] *Identification: PI published Index on flavornet data base (http://www.flavornet.org/flavornet.html); MS, mass spectrometry.*

were separated from C and HP groups at 7 and 14 days. SMart Nose also revealed major differences for HP7 group than the other cheeses.

DISCUSSION

Cheese Chemical Composition

The chemical composition of HP used in the present study is consistent with previous studies describing the nutritive characteristics of hazelnut by-products (5, 33). The animal diet is one of the most important factors affecting the chemical composition of ruminant products. In particular, it is known that providing a diet reach in fat usually increases the fat percentage in milk and cheese (34). Therefore, not surprisingly HP cheese showed a greater percentage of fat in comparison with C cheese. Similarly, the fatty acid composition of ruminant derived products can be partially manipulated by the diet (8). Therefore, the greater percentage of MUFA found in the HP cheese and the contemporary reduction of SFA could be due to the fatty acid composition of the concentrate supplied to HP ewes. In human, the consumption of MUFA is positively associated with the reduction of both plasma LDL cholesterol and triacylglycerol concentrations, thus reducing the risk of cardiovascular diseases (35). Moreover, HP cheese had a lower SFA to UFA ratio in HP cheese. Literature reports that the intake of unsaturated fatty acid should be increased at expense of SFA. Therefore, the administration of dietary improved the nutritive value of the HP cheese.

Sensory Profile

The triangle test used as a discriminant tool to assess whether trained assessors were able to distinguish between the 2 differently treated samples confirmed that the HP and C cheeses' overall sensory profiles were significantly different one each other, because, 13 and 15 persons, after 7 and 14 days of storage, respectively, correctly answered to the test, hence, a number higher than the minimum one (11) necessary to conclude that significant differences existed between the two samples compared, according to the ISO 4120:2004, and the α risk selected (0.01). Regarding the QDA results, the statistical analysis of the attribute ratings collected from 17 trained panelists evidenced a significant reduction of holes and spiciness and an increase of acidity in HP compared to C cheeses. These results agree with the study reported in Torri et al. (36) based on consumers' acceptability of cheeses enriched with 0.8, 1.6, and 2.4% of grape skin powder from 2 different vines (Barbera and Chardonnay), added during cheese-making. The addition of grape skin powder highly influenced sensory properties of such innovative products, above all in terms of appearance and texture. White color, homogeneity and elasticity of the paste and the presence of lactic odor positively influenced consumers' preference. Conversely, the appearance of marbling, granularity, sandyness, acidity, saltiness, and astringency negatively affected the acceptability of the cheese when the quantity of added grape skin powder exceeded 0.8 and 1.6% for Barbera and Chardonnay, respectively. However, the higher intensity of acidity found in this study for HP cheeses was marginal. Presumably the higher presence of holes mostly determined the significance of the triangle test. Contrasting results, on the contrary, were obtained by several authors about the effect of using milk obtained from cows or goats fed with flax extruded, on possible off-flavors products due to lipid oxidation.

Dubreouq et al. (37) and Gaborit et al. (38) have shown how the addition of flax extruded in the diets of dairy cows and goats not integrated with antioxidants, besides improving the acidic composition of the lipid fraction of the cheese, important for a

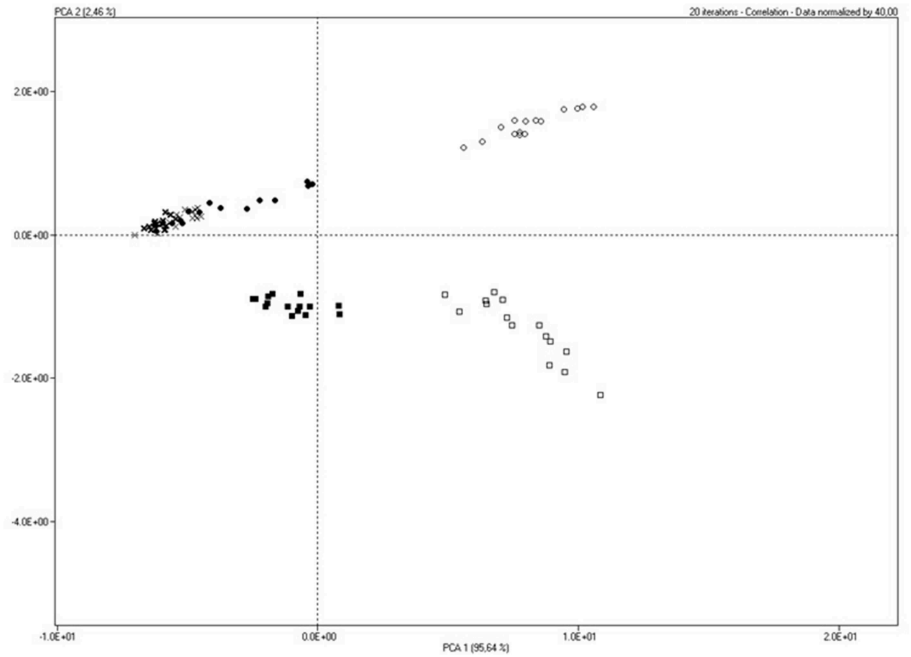

FIGURE 1 | Smart Nose score plot of cheese samples produced with milk from sheeps fed with Control (C) or Hazelnut Peels (HP) diet at different storage time (0, 7, or 14 days) at 8°C and 80% humidity.

health point of view, determined the production of off-flavors (metallic and oxidized flavor). The production of off-flavors was not observed by Lerch et al. (39) in aged Saint-Nectaire cheese. However, according to the authors themselves, the problem could arise for long-ripened cheeses in which an important role is played by lipolysis levels that could increase susceptibility to the oxidation of PUFAs. The cheeses examined in this study were sampled right after aging for 40 days, thus they can be considered fresh cheeses. Further investigation would help to understand the effect of the inclusion of polyphenols extracts in longer maturation periods.

ODOR Active Compounds and Volatile Fingerprint by GC/O and SMart Nose

The volatile profile of C and HP cheese samples showed a good similarity. These results could be explained by the short ripening time (time 0 = 48 days). Cheese ripening is a complex process in which three flavor generating pathways, glycolysis, lipolysis and proteolysis, are initially involved, leading to secondary metabolites production responsible for flavor precursors origin (40). As a consequence, ripening time plays an important role in flavor development (41).

Looking into every single cheese batch, in C group, 2-hexenol was detected only in C0, degrading in C7 and C14. The alcohol, moreover detected as unique compound among all cheeses, was likely processed to secondary products. Two ester compounds were found in C groups: ethyl butyrate was detected in C0 and C7, whereas ethyl octanoate was found in all C cheese samples. Three ketones (diacetyl, 1-octen-3-one and 2-nonanone) were also detected as common in all C group. A sulfur compound, 3-isothiocyanato-1-propene, was detected at 0 and 14 days of storage but not at 7 days.

In HP group, HP0 and HP14 showed the same profile, except for dimethyl pyrazine, detected as unique compounds in HP14. Pyrazine could derive from the hazelnut feeding treatment.

The esters form through free fatty acids and alcohols reaction, giving an important impact to cheese flavor profile (42–46). In this study, ester was detected as the main representative chemical class, conferring fruity notes to the cheeses.

Two sulfur compounds, thiophene and methyl furanthiol, were identified as unique in HP7 cheese group. In general, sulfur compounds originate from the catabolism of sulfur-amino acids (42). Beside the well-known antimicrobial activity (47), recent studies on antioxidant protection grade of cheese report an increasing antioxidant effect in cheese during ripening time in parallel with thiophene and methyl furanthiol production (48, 49). The unique aldehyde extracted by SPME was detected only in HP0 and HP14 cheeses. Moreover, 2-nonanone was found in all cheeses except for HP7. The aldehyde and 2-nonanone are considered fat oxidation products. The absence of these volatile compounds in HP7 suggests a higher antioxidant protection grade of this cheese than the others.

Further studies on vitamin content should be conducted in order to investigate the interactions between the presence of antioxidant volatile compounds and the oxidative stability of ewe cheese by calculating the Degree of Antioxidant Protection (DAP).

Results from SMart Nose were confirmed by GC/O analysis. SMart Nose score plot (**Figure 1**) showed a clear separation of HP7 group from the others, suggesting a different volatile profile.

The separation of HP7 group was supported by GC/O results, in fact, in HP7 cheeses, two unique esters (2-methylbutyl acetate and ethyl isohexanoate) and two unique sulfur compounds (thiophene and ethylfuranthiol) were detected, whereas, pentanal and 2-nonanone, this latter revealed in all the other cheeses, were absent. The olfactive responses could be responsible of HP7 behavior in the SMart Nose score plot.

CONCLUSIONS

The experiment presented in this paper aimed at the evaluation of the inclusion of hazelnut peels in the diet of dairy ewes on the chemical and sensory characteristics of cheeses. Sensory analysis revealed a clear distinction between the 2 experimental groups, whereas the sensory profile showed a marginal production of off-flavors linked to spicy and acid attributes for the cheeses produced with milk of ewes fed with the addition of hazelnut peels. However, lipolysis levels in longer ripening period could increase susceptibility to the oxidation of PUFAs, therefore further investigation would help to understand the effect of the inclusion of polyphenols extracts in longer maturation periods. The volatile profile showed the absence of some volatile compounds in HP cheeses, suggesting a higher antioxidant protection grade linked to the inclusion of HP in the diet. Further studies on vitamin content should be conducted in order to investigate the interactions between the presence of antioxidant volatile compounds and the oxidative stability of ewe cheese.

AUTHOR CONTRIBUTIONS

GL and MP designed and conducted the experiment. BV and AP carried out fatty acid composition analysis and mainly contributed to the conception and structure of this manuscript. VM interpreted results from the antioxidant point of view. GB contributed reagents, materials, tools, and interpretation of results for SmartNose analysis. TR detected odor active compounds and contributed to the manuscript for the aroma and SmartNose results and discussion. SE, AT, and MS performed sensory analysis. MC analyzed, interpreted, and discussed sensory analysis data and coordinated and complemented authors' contributions to the manuscript. All authors have read and approved the final manuscript.

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