LACTIC ACID BACTERIA WITHIN THE FOOD INDUSTRY: WHAT IS NEW ON THEIR TECHNOLOGICAL AND FUNCTIONAL ROLE

EDITED BY: Paola Lavermicocca, Joaquin Bautista-Gallego and Cristina Reguant

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LACTIC ACID BACTERIA WITHIN THE FOOD INDUSTRY: WHAT IS NEW ON THEIR TECHNOLOGICAL AND FUNCTIONAL ROLE

Topic Editors:
Paola Lavermicocca, Italian National Research Council, Italy
Joaquin Bautista-Gallego, University of Extremadura, Spain
Cristina Reguant, University of Rovira i Virgili, Spain

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Table of Contents

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Editorial: Lactic Acid Bacteria Within the Food Industry: What Is New on Their Technological and Functional Role

Paola Lavermicocca[1], *Cristina Reguant*[2] *and Joaquin Bautista-Gallego*[3]*

[1] *Institute of Sciences of Food Production, National Council of Research of Italy, Bari, Italy,* [2] *Departament de Bioquímica i Biotecnologia, Facultat d'Enologia, Universitat Rovira i Virgili, Tarragona, Spain,* [3] *Departamento de Ciencias Biomédicas (Área de Microbiología), Facultad de Ciencias, Universidad de Extremadura, Badajoz, Spain*

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Editorial on the Research Topic

Lactic Acid Bacteria Within the Food Industry: What Is New on Their Technological and Functional Role

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Edited by:
Eugenia Bezirtzoglou, Democritus University of Thrace, Greece

Reviewed by:
Svetoslav Todorov, University of São Paulo, Brazil
Yiannis Kourkoutas, Democritus University of Thrace, Greece

***Correspondence:**
Joaquin Bautista-Gallego
joaquin.bautistagallego@gmail.com

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INTRODUCTION

The application of lactic acid bacteria (LAB) in food processing has a very long history (Leroy and De Vuyst, 2004), however the research continuously provides new insight. The biotransformation performed by LAB is implemented in many industrial processes since selected pro-technological LAB—through their lactic acid fermentative activity—can start and/or modulate the fermentation by producing acidic metabolites and other biomolecules such as enzymes, antimicrobials, and molecules contributing to aroma and texture. Through their proteolytic activity, LAB are able to degrade proteins into small peptides and free amino acids converted, in turn, to various metabolites—alcohols, aldehydes, acids, and ester compounds. Furthermore, lipolysis, glycolysis, and pyruvate metabolism performed by LAB produce many metabolites including aromatic compounds acting as flavor compounds (Leroy and De Vuyst, 2004). All these molecules contribute to the overall quality, shelf-life, and safety of foods, by enhancing their technological, sensorial, nutritional and functional features (Ravyts et al., 2012). It is worthy to note that the functional relevance of biotransformation operated by LAB relies also to their ability in modifying the bioavailability of bioactive molecules composing the food (Debelo et al., 2020). To successfully manage food processing and provide consumers with healthy foods, the genetic traits, and metabolisms of LAB have been deeply investigated in order to select strains suitable for specific applications (Giraffa, 2014).

The most common food-related LAB include species of the genera *Lactobacillus, Lactococcus, Leuconostoc, Enterococcus, Pediococcus, Streptococcus,* and *Weissella.* Within the phylum *Firmicutes*, the majority of LAB belongs to the order of *Lactobacillales*. Recently, the genus *Lactobacillus* has been reclassified into 25 genera (Zheng et al., 2020). However, to be applied in food processing LAB species must be recognized as safe (GRAS) by Food and Drug Administration (FDA) or must have achieved the Qualified Presumption of Safety (QPS) status by European Food Safety Authority (EFSA) (EFSA Panel on Biological Hazards et al., 2017).

Foods represent also the main source of LAB strains having probiotic features. Besides, food components play a role as vector in delivering probiotic populations by protecting them through the gastro-intestinal tract and by sustaining their gut colonization (Flach et al., 2018).

Probiotic bacteria—defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Hill et al., 2014)—mainly belonging to *Lactobacillus* and *Bifidobacterium* genera, are widely used in commercial products, particularly in milk-based preparations, that fall in the category of functional foods. Gut colonization by individual strains selected for beneficial functions strengthen the barrier function played by the epithelial and endothelial gut cells. Furthermore, their implantation modifies the resident microbial populations favoring the increase of those beneficial while limiting potential pathogens and, by modulating the components of the intestinal immune system, may stimulate the host immune response (Chugh and Kamal-Eldin, 2020). In the last decades, consumer's and market's interest for foods characterized by functional attributes has determined the widespread of research on probiotic foods. Particular interest is devoted to vegetable and fruit matrices rich in bioactive molecules that may act as carrier for probiotic microorganisms, producing innovative symbiotic (Flach et al., 2018).

Furthermore, the research and commercial interest in selecting strains for specific food processing, suggest the need for an update on the ongoing studies regarding metabolisms, genetic traits, fermentation performances, and functional features of LAB. Thus, the information obtained in this Research Topic can provide the food industry with rigorous scientific demonstrations for even more efficient biotransformation processes.

The Research Topic "Lactic Acid Bacteria within the Food Industry: What is New on their Technological and Functional Role" belongs to the Food Microbiology section in the Frontiers in Microbiology journal. It covers a total of 12 contributions divided in two reviews and 10 original research papers. Many of the most relevant researchers in the field have collaborated in its elaboration.

We present an overview of these papers which can be grouped under different research themes as follows: (i) probiotic and health-promoting characteristics for the production of functional foods and beverages; (ii) Interactions with other microorganisms; (iii) new technological approaches. The diversity of research displayed in this Research Topic demonstrates the important potential of these microorganisms and its impact in the food industry.

In the first group of papers, Hernández-Alcántara et al. have evaluated different probiotic properties of *L. plantarum* strains, such as their survival in the human digestive tract and *in vivo* studies using a murine model. Two strains (M5MA1-B2 and M9MG6-B2) were able to tolerate the gastrointestinal stresses and displayed *in vitro* high adhesion capacity to Caco-2 cells. Furthermore, they showed great biofilm formation characteristics which indicate a potential capability for intestine colonization, and an improvement of the surface area of the intestinal epithelium in the *in vivo* murine model. A similar study was carried out by Baliyan et al. who isolated potential probiotics microorganisms from a cereal-based traditional fermented beverage -lugri- (rice, wheat and barley) focusing in the functional and safety characteristics of this kind of beverage. *Lacticaseibacillus paracasei* LUL:01 exhibited the best performance and potential for its application in functional food formulation (milk-based formulation).

In addition, Cordeiro et al. have described the therapeutic effect in an induced colitis mice model of a Minas Frescal cheese made using the probiotic bacteria *L. lactis* NCDO 2118. They showed that this microorganism was able to limit the histopathological damages and restore intestinal barrier by increasing expression of gene related to tight junction protein and modulated cytokine production in mice. Furthermore, this functional cheese was able to produce high levels of bioactive peptides with antihypertensive, antioxidant, and antidiabetic activities.

Verni et al. studied the use of brewers' spent grain, a by-product of the brewing industry, as a source to increase the intake of antioxidant compounds. To enable the release or synthesis of these compounds, a fermentation with different selected *Lactiplantibacillus plantarum* strains and treatment with a commercial xylanase was carried out.

Cataldo et al. evaluated the production of gamma-aminobutyric acid through fermentation of strawberry and blueberry juices by *Levilactobacillus brevis* CRL 2013. A significantly higher production was reached in the case of fermented strawberry juice and it was able to modulate the expression of *cox-2* in lipopolysaccharide stimulated macrophages and exerted a remarkable anti-inflammatory effect. This study supports the potential use of this kind of fermented juice to reduce the inflammatory response of chronic inflammatory diseases.

Regarding to the interactions of LAB with other microorganisms, Medveďová et al. have studied the effect of different combinations of LAB on the growth of different *Staphylococcus aureus* and *Escherichia coli* strains during the ripening of curd cheeses. This study emphasizes the importance of the use of LAB starter cultures to improve the sensory profile and safety conditions of the final product. In the same trend, Canon et al. reviewed the different ways available at present that could be used to create positive interactions between LAB: different types of positive interactions; co-cultivation and their mechanisms to reach the positive interactions; possible strategies that could be used to assemble LAB; and the particular role of nutritional dependencies.

With concern of the new technological approaches for LAB, four original articles and one review overview different studies to their implementation. Speranza et al. have studied the optimization of a fish fermented product using two *L. plantarum* strains. Both strains reduced the fermentation time and ensured good microbiological, chemico-physical, and sensorial quality of the final product.

Liu et al. have provided an approach to investigate the molecular mechanisms of formation and metabolic pathways of flavors in rice-acid fermented with *L. paracasei* H4-11 at different time and inoculation (co-inoculation with *Kluyveromyces marxianus*) methods. At transcriptional level, they detected that the genes related to amino sugar and nucleotide sugar metabolism and starch and sucrose metabolism affected the energy required for the growth of *L. paracasei* in the early stage. Even more, a different expression of those genes

was detected in the growth of *L. paracasei* in the presence of *K. marxianus*.

Valerio et al. delved in the selection of LAB strains able to produce EPS in liquid sourdoughs based on pseudocereal flours and evaluated the effect of its composition on EPS production and protein degradation. Thus, the modulation of flour type, DY and sucrose content can stimulate the metabolic activities of *Weisella cibaria* and *L. plantarum* modulate the fermentation process, enriched in the EPS content. Furthermore, this increment in the EPS production was detected in the presence of pseudocereals amaranth or quinoa.

Kazou et al. compared the bacterial (and yeast/fungal) microbiota of different Greek kefir samples, using classical microbiological and amplicon-based metagenomics approaches. They identified mainly *Lentilactobacillus kefiri*, *Leuconostoc mesenteroides*, *Lacticaseibacillus rhamnosus*, *Streptococcus thermophilus*, *Lactococcus lactis*, and *Leuconostoc mesenteroides*. Yeasts were also isolated and identified during kefir production, being *K. marxianus, Debaryomyces hansenii,* and *Saccharomyces cerevisiae* the mostly identified yeast species. Even more, some (opportunistic) pathogens were detected at home-made kefir samples, which indicates poor hygiene practices.

Finally, Virdis et al. have reviewed the role of lactic acid bacteria in wine, focusing on the malolactic fermentation. Furthermore, they delved in the study of: (i) their contribution to the sensorial profile (citrate and glycosidase metabolism); (ii) impact on wine color (reduction of anthocyanin glucosides); (iii) production of volatile thiols; (iv) other diverse activities (reduction of the use of bentonite); and their negative effects (biogenic amines, ethyl carbamate).

The varied contributions to this Research Topic are evidence of the study undertaken by researchers that provide an updated and high-quality overview of the impact of lactic acid bacteria and their future perspectives. We hope that this Research Topic informs readers properly about the benefit of this product and the challenges that have yet to be overcome in this field.

AUTHOR CONTRIBUTIONS

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

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Effect of Amaranth and Quinoa Flours on Exopolysaccharide Production and Protein Profile of Liquid Sourdough Fermented by *Weissella cibaria* and *Lactobacillus plantarum*

Francesca Valerio, Anna Rita Bavaro, Mariaelena Di Biase, Stella Lisa Lonigro, Antonio Francesco Logrieco and Paola Lavermicocca*

Institute of Sciences of Food Production, National Research Council of Italy, Bari, Italy

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Edited by:
Eugenia Bezirtzoglou, Democritus University of Thrace, Greece

Reviewed by:
Dan Xu, Jiangnan University, China
Elena Bartkiene, Lithuanian University of Health Sciences, Lithuania

***Correspondence:**
Francesca Valerio
francesca.valerio@ispa.cnr.it

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Exopolysaccharides (EPSs) are known for their positive contribute to the technological properties of many foods, including bakery products. These molecules can be obtained performing piloted fermentation with lactic acid bacteria (LAB). In order to select strains able to produce EPS, a screening test in agar medium containing sucrose, fructose or glucose as carbohydrate source was performed on 21 LAB strains. Results allowed to select 8 *Weissella cibaria*, 2 *Weissella confusa,* and 2 *Leuconostoc* spp. strains as EPS producers only in the presence of sucrose. A further screening in liquid medium enriched with sucrose (10%) (mMRS_S) indicated the *W. cibaria* strain C43-11 as the higher EPS producer. The selected strain was used to develop liquid sourdoughs (LSs) with dough yield (DY) 500, fermented for 15 h and based on wheat flour and wheat gluten or pseudocereals (quinoa or amaranth) in ratio 1:1, in the presence or not of sucrose at 3% (w/w, LS weight), in comparison to *Lactobacillus plantarum* ITM21B, a strain not producing EPS in mMRS_S. Results indicated that the use of pseudocereals favored the EPS production. Formulations were optimized by modifying DY (500 or 250), sucrose concentration (3 or 6%) and flour ratio. LSs were characterized for the content of organic acids (lactic, acetic, phenyllactic, OH-phenyllactic), pH, TTA, EPS, viscosity, total protein degradation and protein pattern. The highest EPS production (20.79 g/kg) and viscosity (1168 mPa s) were obtained in LS (DY 250, sucrose 6%) based on quinoa flour and started with C43-11 strain. The LS was characterized by the presence of phenyllactic and OH-phenyllactic acids, protein degradation by 51.7% and proteins in the range 14–80 kDa. In these conditions, also strain ITM21B was able to produce EPS at level of 4.61 g/kg and to degrade proteins by 53.8% in LS based on wheat and quinoa flours (1:1) (DY250 and sucrose 3%). Therefore, results demonstrated that the use of selected conditions (flour type, DY, sucrose) can stimulate specific attributes of strains making them suitable for production of short fermented (15 h) LSs which can be used as bread improvers.

Keywords: pseudocereal, fermentation, lactic acid bacteria, protein degradation, chip electrophoresis, bread improvers, viscosity

INTRODUCTION

According to the World Health Organization (WHO) industries should contribute to improve human nutrition by providing high quality foods in terms of nutritional and functional attributes. The requirements for a nutritionally balanced diet have driven food producers to modify processing by exploring high nutritious raw materials and adopting sustainable methodologies that can maximize their health outcome (Norum, 2005; Galli et al., 2017). Information and methodologies are made available by the scientific community involved in research with high technology transfer level often supported by public actions, involving producers that focus at optimizing the organoleptic quality of foods while valorizing their nutrient content. Particularly, the bakery sector is currently exploring biotechnological processes, also combining alternative flours as sources of carbohydrates, proteins, vitamins and minerals to wheat flour, to improve the nutritional, functional and technological quality of products. The interest toward bakery products with reduced fat content and/or processed using protein sources such as non-wheat cereal flours other than ancient grains and legumes, is increased (Nishida et al., 2004; Gobbetti et al., 2019). Traditional cereal-based products can be produced replacing, at least part, wheat flour with pseudocereals, for instance amaranth and quinoa, because of their nutritional and textural features (Coda et al., 2014). Amaranth (*Amaranthus cruentus* or *hypochondriacus*) has a higher mineral and protein content than most cereal grains and a very balanced amino acid composition. Although amaranth grain is a high glycemic ingredient, it is a rich source of unsaturated fatty acids and polyphenols (flavonoids) with cholesterol-lowering and antioxidant activities (Singh and Singh, 2011). The cooking of amaranth improves its digestibility and the absorption of nutrients. Amaranth proteins are composed mainly of three major fractions (albumins, globulins, and glutelins) with little or no storage prolamin. Amarantin is the most important component of globulins constituting its 90% and approximately the 19% of the total grain proteins (Singh and Singh, 2011). Quinoa (*Chenopodium quinoa* Willd.) is a nutritionally well-balanced pseudocereal due to the high protein content that ranges from 12.9 to 16.5% (Bastidas et al., 2016) and essential amino acids (lysine, threonine, and methionine), as well as unsaturated fatty acids (linolenic and linoleic acids), vitamins and minerals (Wang and Zhu, 2016). These pseudocereals are widely used to produce gluten-free products, even if the lack of gluten, that contributes to produce a viscoelastic dough, limits their use in bread-making (Lynch et al., 2018).

To overcome this technological drawback several additives can be used (Houben et al., 2012; Lynch et al., 2018) including hydrocolloids, which are water-soluble, long-chain polysaccharides that bind water and modify the dough viscosity then improving the loaf volume and crumb structure of the breads (Lazaridou et al., 2007; Sciarini et al., 2010; Mir et al., 2016). Hydrocolloids can be added to the dough also as fat replacer in baked goods (Colla et al., 2018). These substances are generally added at 0.1–1% of flour basis to improve the rheological properties of dough (Rosell et al., 2001; Guarda et al., 2004). Hydrocolloids mainly originate from plants or plant seeds (pectin, locust bean gum, and guar gum), but there are similar molecules produced by bacteria, the exopolysaccharides (EPS) with the same properties (Zannini et al., 2016; Lynch et al., 2018).

Depending on their composition and biosynthesis mechanism, EPS can be divided into homopolysaccharides (HoPS) and heteropolysaccharides (HePS) (Galle and Arendt, 2014). Lactic acid bacteria (LAB) can produce both types. HoPS, generally produced by *Weissella, Leuconostoc, Streptococcus, Lactobacillus*, consist of one monosaccharide, either glucose or fructose, with the resulting EPS termed glucans or fructans, respectively (Lynch et al., 2018). Their synthesis includes intracellular and extracellular steps through the activity of glycansucrases (glycosyltransferases) (Badel et al., 2011). Among EPSs, dextran, a homopolysaccharide in which the main unit is composed by α-1-6 glycosidic linkages, can be used in foods since it has been generally recognized as safe (GRAS) by the Food and Drug Administration. As an example, the use of a *Leuconostoc mesenteroides* preparation containing dextran has been authorized by the European Commission as food ingredient in bakery products (Byme, 2001) to improve softness, crumb texture and loaf volume. The polymer dextran is generally produced by *Leuconostoc* and *Weissella* even if it has been synthesized by some *Lactobacillus plantarum* strains (Di Cagno et al., 2006; Das and Goyal, 2014).

HePS are generally produced in lower amounts by mesophilic and thermophilic LAB (*Lactobacillus lactis, Lactobacillus casei, Lactobacillus plantarum, Lactobacillus rhamnosus, Lactobacillus helveticus*, etc.) and are known for their positive role in the rheology, texture and mouthfeel of fermented dairy products (Tallon et al., 2003; Zannini et al., 2016; Şentürk et al., 2020).

A biotechnological approach combining pseudocereals and EPS producing LABs, can improve the overall quality (shelf-life, flavor, dough structure) of final products and their nutritional characteristics (Rühmkorf et al., 2012; Wolter et al., 2014). Authors demonstrated the suitability of pseudocereals as substrate for EPS production by LAB strains. Moreover, the protein hydrolysis operated by LAB determines the formation of several molecules, including amino acids, organic acids, bacteriocins, peptides, etc. (De Vuyst et al., 2009). Furthermore, pseudocereal proteins are generally more accessible to cereal and microbial proteases than wheat since their hydrophilic nature (Dallagnol et al., 2013; Wolter et al., 2014). Therefore, these studies suggest the suitability of pseudocereals to produce sourdough with high nutritional value. Moreover, due to their water holding capacity, EPS can be used to replace the fatty substances added in bakery products since they produce a structured polysaccharide network, that interacts with the gluten network, mimicking the role of fats maintaining good textural and sensorial properties (Serin and Sayar, 2017; Colla et al., 2018).

Recently, the application of pre-fermented sourdough or fermentation products started with selected technological strains, has led to obtain final products with improved nutritional, technological and sensorial quality and prolonged shelf-life (Katina et al., 2009; Galle et al., 2010; Valerio et al., 2014, 2017; De Bellis et al., 2019; Di Biase et al., 2019). In this perspective, the aim of the current research was to select LAB strains able to produce EPS in liquid sourdoughs (LSs) based on pseudocereal flours and

to study the effect of LSs composition (flour type, dough yield, sucrose) on EPS production and protein degradation.

MATERIALS AND METHODS

Raw Materials

The ingredients used in this study were soft wheat flour type 0 (protein, 14,5%; fat, 1%; carbohydrate, 68%) and wheat gluten (protein 75%; fat, 5%; carbohydrate, 15%), both supplied by Valle Fiorita Catering S.r.l. (Ostuni, Italy), organic amaranth flour (protein, 15.5%; fat, 7.1%; carbohydrate, 71.0%) (Sottolestelle srl, San Giovanni Rotondo, Italy), organic quinoa flour (protein, 11.9%; fat, 7.2%; carbohydrate, 56.3%) (Ecor, Verona, Italy) and sucrose (JT Baker, Milan, Italy).

LAB Strains and Growth Conditions

Twenty-one strains of LAB, belonging to the Culture Collection of the Institute of Sciences of Food Production, National Research Council and deposited in the Belgian Coordinated Collections of Microorganisms (BCCM/LGM, Gent, Belgium) or in the ITEM Collection, were screened for EPS production. *Lactobacillus brevis* 18F, *L. plantarum* ITM21B, *L. plantarum* 19A (Corsetti et al., 2003), *Lactobacillus hilgardii* 51B (Di Cagno et al., 2003), *Lactobacillus sanfranciscensis* C57 (Corsetti et al., 1996) were isolated from sourdough; *Lactobacillus paracasei* IMPC2.1 (LMG P-22043) and *L. paracasei* IMPC4.1 (LMG S-27068) were human isolates (Lavermicocca et al., 2005); *Lactobacillus pentosus* 15BG and *L. pentosus* 14TG were isolated from olive surface (De Bellis et al., 2010); *Leuconostoc citreum* C2-27, *Ln. mesenteroides* C43-18, *Weissella cibaria* strains C21-4, C2-5, C43-11, C3-2, C3-4, C3-19, C4-21, C2-32 and *Weissella confusa* strains C5-4 and C5-7 were isolated from wheat semolina (Valerio et al., 2009).

Strains were cultured in MRS broth (Biolife Italiana S.r.l., Milan, Italy) and incubated at 37°C (*Lactobacillus* spp.) or 30°C (*Weissella* spp.). For long-term storage, stock cultures were prepared by mixing 8 mL of a culture with 2 mL of Bacto glycerol (Difco, Becton Dickinson, Co., Sparks, MD, United States) and freezing 1 mL portions of this mixture at −80°C. Cultures were subcultured twice and incubated at the optimal growth temperature for each strain (30°C *Weissella* spp. and *Leuconostoc citreum* or 37°C *Lactobacillus* spp.) for 24 h before use.

Screening for EPS Production in Synthetic Media

To identify EPS producers, each bacterial strain was cultivated for 24 h in a modified MRS (mMRS) broth prepared by adding fresh yeast extract (5%, v/v) and 28 mM maltose to the MRS (Biolife Italiana) composition (final pH 5.6) as reported in Di Cagno et al. (2006). Cultures were inoculated on mMRS agar plates enriched with three carbon sources: 292 mM sucrose (mMRS_S), 146 mM glucose (mMRS_G) or 146 mM fructose (mMRS_F). After incubation at 30 or 37°C for 6 days, EPS producing strains were distinguished by visual appearance of mucoid colonies. To quantify the production of EPS, each positive strain was inoculated at 4% (v/v) in mMRS_S broth and cultures were incubated for 24 h at the appropriate temperatures. Liquid mMRS without additional carbohydrates was used as a control. Furthermore, to assess the production of EPS in a shorter time, cultures were also incubated for 15 h at the appropriate temperatures.

Both experiments were carried out twice and analyzed in duplicate (2 × 2).

Production of Liquid Sourdoughs (LSs)

To study the effect of sucrose on EPS production, preliminary formulations of LSs were obtained by mixing wheat flour (W) with wheat gluten (Gl) or amaranth flour (Am) or quinoa flour (Q) in a 1:1 ratio, in the presence or not of sucrose (S) (3% w/w of LS), giving a dough yield (DY) of 500 (**Table 1**). The preliminary LSs were inoculated with *W. cibaria* C43-11 (EPS-high producer strain) or *L. plantarum* ITM21B (not producing EPS in mMRS_S). Cells from 24 h cultures in mMRS_S were washed, resuspended in distilled water and inoculated in the flour mixtures at 4% (v/v). Mixtures were incubated at optimal growth temperature for each strain (30°C *W. cibaria*, C43-11 37°C *L. plantarum* ITM21B) for 15 h and the resulting fermentation products were analyzed.

A deeper study on the factors affecting EPS synthesis, metabolic activities (organic acids, protein degradation, pH, TTA) and starter viability, was carried out by formulating three different LS types distinguished for the dough yields (250 and 500), pseudocereal/wheat flour ratio (1:1 or 1:0) and sucrose content (3 and 6% LS weight). Mixtures were inoculated with strains *W. cibaria* C43-11 or *L. plantarum* ITM21B and incubated as described above. For each pseudocereal flour, six LSs were obtained as described in **Table 2**. The same formulations not containing the bacterial inoculum and added with antibiotics (100 mg kg^{-1} chloramphenicol and 50 mg kg^{-1} erythromycin) were used as controls (Co), as reported in Korakli et al. (2002).

Experiments were carried out twice and analyzed in duplicate (2 × 2).

EPS Quantification Method

Samples were prepared for the quantification assay as reported in Di Cagno et al. (2006). Briefly, liquid cultures in mMRS_S were heat treated at 100°C for 15 min to inactivate enzymes and centrifuged (9000 × *g*, 10 min, 4°C). Three volumes of chilled 96–99% (v/v) ethanol were added to the resulting supernatants

TABLE 1 | Ingredients of LSs containing wheat flour (W) with gluten (Gl) or amaranth flour (Am) or quinoa flour (Q) (ratio 1:1), in the presence or not of sucrose (S) (3% w/w) (DY 500).

Ingredients	W/Gl+S	W/Gl	W/Am+S	W/Am	W/Q+S	W/Q
Wheat flour type 0	10 g	10 g	10 g	10 g	10 g	10 g
Gluten	10 g	10 g	–	–	–	–
Amaranth flour	–	–	10 g	10 g	–	–
Quinoa flour	–	–	–		10 g	10 g
Water	76 ml	76 ml	76 ml	76 ml	76 ml	76 ml
Sucrose	3 g	–	3 g	–	3 g	–
Culture	4 ml	4 ml	4 ml	4 ml	4 ml	4 ml

TABLE 2 | Optimization of LS formulations containing pseudocereals (amaranth or quinoa) in combination or not with wheat flour (W) in ratio 1:1, with sucrose 3% or 6% LS weight, at DY 500 or DY250.

Ingredients	LS type[a]					
	DY500_3%S		DY250_3%S		DY250_6%S	
Amaranth/Quinoa flour	10 g	20 g	20 g	40 g	20 g	40 g
Wheat flour type 0	10 g	–	20 g	–	20 g	–
Water	76 ml	76 ml	56 ml	56 ml	56 ml	56 ml
Sucrose	3 g	3 g	3 g	3 g	6 g	6 g
Culture	4 ml	4 ml	4 ml	4 ml	4 ml	4 ml
DY	500	500	250	250	250	250

[a]*LS types: wheat flour (W) in combination with amaranth (Am) or quinoa (Q) and sucrose 3% LS weight, DY 500 (W/Am or W/Q_DY500_3%S); amaranth (Am) or quinoa (Q) and sucrose 3% LS weight, DY 500 (Am or Q_DY500_3%S); wheat flour (W) in combination with amaranth (Am) or quinoa (Q) and sucrose 3% LS weight, DY 250 (W/Am or W/Q_DY250_3%S); amaranth (Am) or quinoa (Q) and sucrose 3% LS weight, DY 250 (Am or Q_DY250_3%S); wheat flour (W) in combination with amaranth (Am) or quinoa (Q) and sucrose 6% LS weight, DY 250 (W/Am or W/Q_DY250_6%S); amaranth (Am) or quinoa (Q) and sucrose 6% LS weight, DY 250 (Am or Q_DY250_6%S).*

and the solutions were stored at 4°C overnight. The precipitated EPS were collected by centrifugation (11325 × *g*, 20 min), dissolved in distilled water, dialyzed (12–14 kDa) against distilled water at 4°C for 48 h, lyophilized and rehydrated with distilled water at the initial volume. Whereas, LSs were diluted 1:10 with distilled water, centrifuged (8000 × *g*, 20 min) and the supernatants treated as described above. The concentration of EPS (g/L or g/kg) was determined according to the phenol-sulfuric method (Dubois et al., 1956), using glucose as a standard (LOD 0.078 g/L or g/kg).

Viscosity Measurement of LSs

The viscosity of LS was measured on 35 mL of each sample using the sine-wave vibro-viscometer SV-10 (A&D Company, Ltd., Tokyo, Japan). Viscosity (expressed as mPa s) was obtained by detecting the driving electric current needed to resonate two sensor plates at a constant frequency of 30 Hz and amplitude of less than 1 mm and at constant room temperature (20 ± 1.0°C).

Microbiological and Physico-Chemical Analyses

Serial decimal dilutions of mMRS_S 15 h cultures or LSs in sterile NaCl (0.85% w/v) + Tween 80 (0.025%), were prepared, and 100 μl aliquot of each dilution was spread on MRS plates which were incubated for 48 h at the optimal growth temperature for the two selected strains. The total LAB count was expressed as log CFU/ml or CFU/g.

The strains inoculated in LSs were identified as reported in Valerio et al. (2017). Briefly, 20% of total colonies, randomly picked from MRS agar plates containing the two highest dilutions, were isolated and checked for purity. Bacterial DNA was extracted from each colony from overnight cultures grown in MRS broth at 30 or 37°C as previously described (De Bellis et al., 2010). The amplification products were separated by microfluidic electrophoresis using the DNA7500 LabChip kit with the Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). Chips were prepared according to the manufacturer's instructions. Data were analyzed using the 2100 Expert software provided by the same company. Genetic identification of strains was based on the comparison of the REP-PCR profile of each isolate with the specific pattern obtained from pure cultures of *L. plantarum* ITM21B and *W. cibaria* C43-11.

The pH of LSs was recorded with a portable pH-meter (type110, Eutech Instruments, Singapore) supplied with Double Pore D electrode (Hamilton, Bonaduz, Switzerland). Total titratable acidity (TTA) was measured according to AOAC Method No. 981.12 (AOAC, 1990) and expressed in mL of 0.1N NaOH required to achieve a pH of 8.3.

Determination of Organic Acids in LSs

Sample preparation and analysis of lactic, acetic, phenyllactic (PLA) and hydroxy-phenyllactic (OH-PLA) acids was performed as reported in Di Biase et al. (2019). Briefly, 10-g portions of each LS were diluted in sterile tap water (90 mL), homogenized in a Stomacher (Seward, London, United Kingdom) for 2 min, then the suspensions were centrifuged (9072 × *g*, 10 min, 4°C) and the supernatants freeze-dried. The freeze-dried samples were re-suspended in the HPLC mobile phase (0.007 mol/L H_2SO_4) (Fluka, Deisenhofen, Germany) and filtered by centrifugation (7000 × *g*, 1 h, 2°C) through a 3000 Da cut-off micro-concentrator (Ultracel-3k, Amicon, Danvers, MA, United States). The fraction containing molecules with molecular weight lower than 3000 Da was analyzed by HPLC (AKTABasic10, P-900 series pump, Amersham Biosciences AB, Uppsala, Sweden; degasser Gastorr BG-12, FLOM Corporation, Tokyo, Japan), using a Rezex ROA organic acid H+ (8%) column (7.80 mm × 300 mm, Phenomenex, Torrance, CA, United States), an injection volume of 10 μL, a 3-channel UV detector (Amersham Biosciences 900) set at 210 (lactic, acetic, PLA acids) and 220 nm (OH-PLA). The mobile phase was pumped at a flow rate of 0.7 mL/min through the column heated to 65°C. Quantification of the organic acids was performed by integrating calibration curves obtained from the relevant standards. Limit of detection (LOD) and limit of quantification (LOQ) were calculated considering a signal-to-noise ratio (S/N) of 3 and 6, respectively. LOD values were the following: lactic acid, 0.263 mmol/kg; acetic acid, 0.279 mmol/kg; PLA, 1.08 μmol/kg; OH-PLA, 0.774 μmol/kg. LOQ values corresponded to 2 × LOD. Final concentration of each organic acid in LSs was calculated considering concentration and/or dilution factors and expressed as mmol/kg or μmol/kg of product.

Protein Characterization of Flour Samples and LSs

Total proteins were extracted from flours and LSs (before and after fermentation) under reducing conditions. Briefly, 40 mg of flour – or an equivalent amount of LSs weighed on the basis of their flour content – were mixed with 200 μL of extraction solution containing 5% mercaptoethanol and 2% SDS (Džunková et al., 2011) for 3 h at 4°C. Afterward insoluble material was removed by centrifugation (12000 × *g* for 15 min)

and supernatants, after heating at 100°C for 2 min, were stored at −20°C and used for electrophoresis. Protein concentration was determined by the Bradford method (Bradford, 1976) using the Bio-Rad dye reagent (Bio-Rad Laboratories, Hercules, CA, United States) with bovine serum albumin as the standard and expressed as mg of protein per gram of flour. The total protein degradation (TPD) during fermentation was expressed as a percentage and quantified using the following equation:

$$\%\ TPD = 100 - \left[\left(\frac{TP_f}{TP_i}\right) \times 100\right] \quad (1)$$

Where TP_f represents the total protein content after fermentation and TP_i is the initial total protein content (before fermentation).

Total protein extracts were analyzed by the Lab-on-a-Chip (LoaC) capillary electrophoresis using the Protein 230 Lab Chip kit (Agilent Technologies, Waldbronn, Germany) with a molecular weight range of 14–230 kDa. Sample preparation and chip loading was performed according to manufacturer's instructions and data evaluation was carried out by the dedicated 2100 Expert software that aligns sample proteins to a molecular weight ladder using internal standards. Results were displayed for each sample as peaks (electropherogram), as bands (gel-like image) and in a tabular format that reports, for each protein peak, molecular weight (Mw), time-corrected peak area (TCA), relative concentration (RC) based on a one-point calibration to the upper marker (60 ng/mL) and protein percentage (%) calculated on total peak areas in the sample. Manual integration of peaks was performed after each run and peaks with RC <20 ng/μl were excluded from analysis as their significance is low considering the detection limit of the method. All experiments were performed twice ($n = 2$).

Statistical Analysis

All data are presented as mean values ± standard error of the mean. To evaluate the effect of formulations characterized by different DY, sucrose content, flour type and ratio and bacterial starter (ITM21B, C43-11, Co) on the physico-chemical parameters (EPS, %TPD, viscosity, pH, TTA, organic acids lactic, acetic, PLA, and OH-PLA) of LSs, data were analyzed by factorial ANOVA followed by the Tukey HSD test. Results were considered as statistically significant when the p-value was less than 0.05. All statistical analysis were performed by Statistica 13 software (Dell Inc., 2015). Data were analyzed by principal component analysis (PCA) to investigate the correlation between the physico-chemical parameters. Multivariate analysis was performed by the Unscrambler (version 10.1, CAMO, Oslo, Norway).

RESULTS

Screening for EPS Production

Twenty-one strains were tested on mMRS agar plates containing different carbon sources (glucose, fructose, or sucrose) and the *W. cibaria*, *W. confusa,* and *Leuconostoc* spp. strains resulted to be the most active EPS producers, but only in the presence of sucrose (**Supplementary Table S1**). In particular, the 10 *Weissella* spp. strains producing EPS in the presence of sucrose as carbon source and a not-producing strain (*L. plantarum* ITM21B), were grown for 24 h in liquid medium (mMRS_S) containing sucrose 292 mM (10% w/v): the higher amount of EPS (18.56 ± 2.64 g/L) was registered for strain C43-11 while for ITM21B the EPS production was not detected (**Table 3**).

In order to produce a LS in a shorter time, the two selected strains C43-11 and ITM21B were grown in mMRS_S for 15 h, an incubation time generally used to produce fermentation products suitable for bread-making (Valerio et al., 2017; Di Biase et al., 2019). Results indicated that already after 15h of incubation, strain C43-11 was able to produce EPS (16.1 ± 0.1 g/L) while ITM21B grew better (9.54 ± 0.007 vs. 8.80 ± 0.01 log CFU/ml, $p < 0.05$) determining lower pH values (3.96 ± 0.002 vs. 4.33 ± 0.01, $p < 0.05$) but comparable TTA values (21.5 ± 0.61 for C43-11 and 20.2 ± 3.80 for ITM21B, $p > 0.05$). Therefore, even if a lower EPS production was registered after 15 h respect to 24 h growth, this fermentation time was used in the further experiments using wheat and pseudocereal flours as carbon source in the presence or not of sucrose.

Effect of Sucrose on EPS Production in Preliminary Liquid Sourdough Formulations

In order to assess the contribute of sucrose on EPS production three LS formulations at DY 500 based on wheat flour with pseudocereals or wheat gluten as sources of carbohydrates, in the presence or not of sucrose (3% LS weight), were prepared. The flour mixtures were inoculated with *W. cibaria* C43-11 or *L. plantarum* ITM21B, a strain not producing EPS in the above tested conditions, and fermented for 15 h at 30 or 37°C. LSs were characterized for the pH, TTA, LAB count, EPS content and acid production. As shown in **Figure 1A**, the presence of pseudocereals favored EPS production by strain ITM21B,

TABLE 3 | Production of EPS in mMRS_S after 24 h growth by selected producer strains (*Weissella cibaria* and *Weissella confusa*) in comparison to the strain *L. plantarum* ITM21B not producing EPS in agar media.

Species	Strain	EPS g/L ± SE
W. cibaria	C21-4	13.82 ± 1.88ac
	C3-2	13.42 ± 3.45ac
	C3-4	6.21 ± 2.12ab
	C43-11	18.56 ± 2.64c
	C3-19	14.82 ± 0.66ac
	C4-21	8.20 ± 3.29abc
	C2-32	0.51 ± 0.64b
	C2-5	8.02 ± 3.36abc
W. confusa	C5-7	2.08 ± 0.03ab
	C5-4	1.08 ± 0.39ab
L. plantarum	ITM21B	<LOQ

Values (mean ± standard error of the mean) with different lower-case letters are significantly different ($p < 0.05$). LOD: 0.078 g/L.

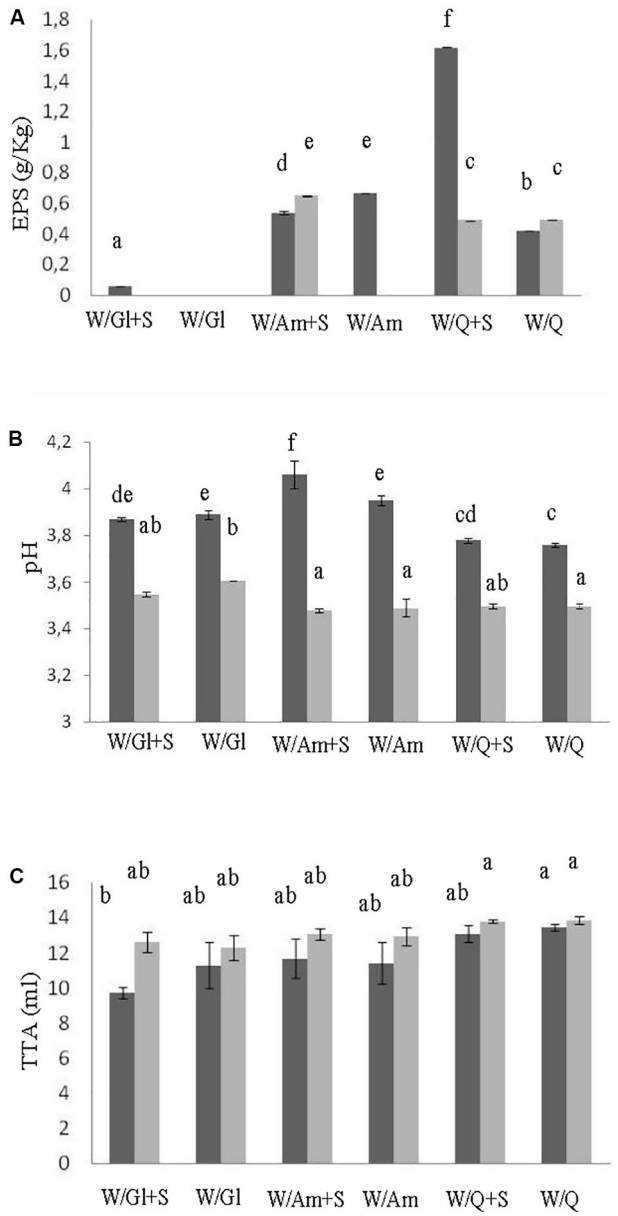

FIGURE 1 | Effect of different flour combinations on EPS content **(A)**, pH **(B),** and TTA **(C)** of liquid sourdoughs (LSs) obtained by the selected high EPS producer *W. cibaria* strain C43-11 (dark gray) and the low EPS producer strain *L. plantarum* ITM21B (light gray) after 15 h fermentation. Ingredients: wheat flour (W) with gluten (Gl) or amaranth flour (Am) or quinoa flour (Q) (ratio 1:1), in the presence or not of sucrose (S) (3% on LS weight). The data are represented as mean ± standard error. Different letters above the columns indicate significant difference among the means of EPS content **(A)**, pH **(B)**, and TTA **(C)** by Tukey HSD's test ($p < 0.05$).

even if at lower concentration respect to strain C43-11. The higher EPS production was registered in C43-11 W/Q+S LS even if at levels considerably lower (1.62 ± 0.01 g/kg, $p < 0.05$) than that observed in mMRS_S. The addition of sucrose at 3% (w/w LS weight, corresponding to the 15% on flour weight) significantly improved the EPS production by C43-11 strain only in LS containing quinoa/wheat flours. The strain count ranged between 7.0 ± 0.02 and 9.0 ± 0.02 log CFU/g for strain C43-11 and between 8.5 ± 0.01 and 10.2 ± 0.01 log CFU/g for strain ITM21B ($p < 0.05$). For each strain, a higher bacterial count was registered in formulations containing quinoa or amaranth (data not shown). Lower ($p < 0.05$) pH values and slightly ($p > 0.05$) higher acidity (TTA) and lactic acid were observed for all LSs fermented by ITM21B strain (**Figures 1B,C**). All LSs formulations including gluten, showed the lowest content of organic acids (**Figure 2**), highlighting a scarce proteolysis. The production of lactic acid, PLA and OH-PLA was related to the flour type and to the presence of pseudocereals as a high production of these metabolites was observed in all LSs containing quinoa or amaranth. In particular, the highest content of PLA and OH-PLA (60.55 ± 9.92 and 43.6 ± 6.3 μmol/kg, respectively) was observed in W/Q_S LS fermented by strain C43-11 (**Figure 2A**). These metabolites are known for their antimicrobial properties and for their contribution to the taste of salt-reduced bread (Valerio et al., 2008, 2017). Strain ITM21B

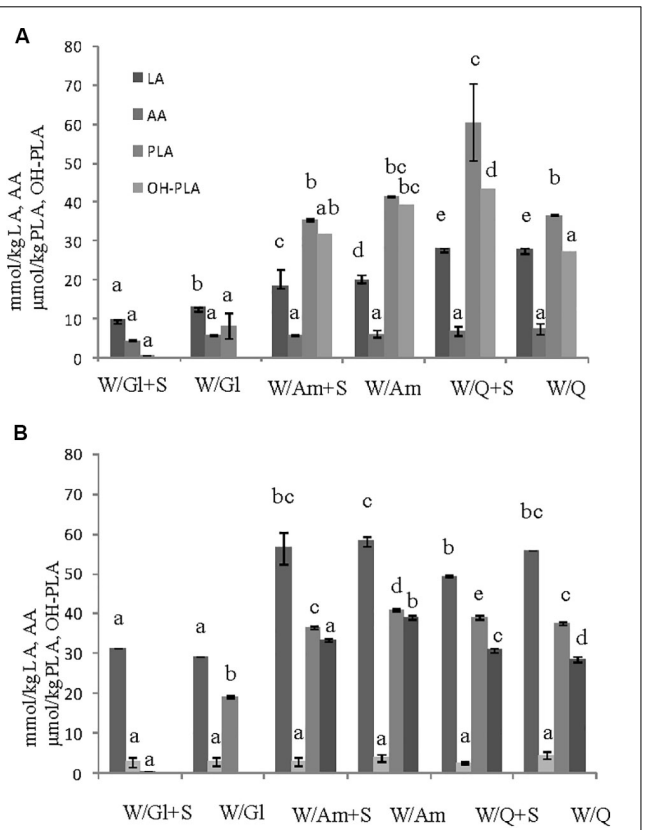

FIGURE 2 | Effect of different flours on the organic acid content (lactic, LA, acetic, AA, phenyllactic, PLA, OH-phenyllactic, OH-PLA) registered in the preliminary LSs obtained by the **(A)** *W. cibaria* C43-11 and the **(B)** *L. plantarum* ITM21B after 15 h fermentation. Ingredients: wheat flour (W) with gluten (Gl) or amaranth flour (Am) or quinoa flour (Q) (ratio 1:1), in the presence or not of sucrose (S) (3% w/w). The data are represented as mean ± standard error. Different letters above the columns indicate significant difference among the means of each organic acid by Tukey HSD's test ($p < 0.05$).

produced lactic acid at a significantly ($p < 0.05$) higher level, mainly in the presence of pseudocereals, respect to strain C43-11 while the remaining acids were comparable (**Figure 2B**). Notably, except for EPS production, all chemical and microbiological results in LSs were not affected by the presence of sucrose, thus suggesting the contribution of proteins and aminoacids contained in pseudocereals on the modifications observed for pH, TTA, and organic acids.

Effect of LS Formulation on EPS Production, Microbiological and Physico-Chemical Parameters, and Protein Profile

To study the influence of the starter strain (*W. cibaria* C43-11 or *L. plantarum* ITM21B), DY (500 or 250), flour composition and ratio (W/Am, Am, W/Q, Q) and sugar content (3 or 6% w/w of LS) on EPS production, metabolic activities (organic acids, total protein degradation, pH, TTA) and starter viability, three LS types were formulated (**Figures 3–5**). They were mainly distinguished by DY and sugar content (DY500, 3% sucrose; DY250, 3% sucrose; DY250, 6% sucrose).

Since preliminary results (**Figures 1**, **2**) suggested the use of pseudocereals to increase the EPS production, LSs at DY 500 and with sucrose 3% containing only the pseudocereal, were produced. As shown in **Figure 3A** the flour type and its proportion respect to wheat flour had a significant effect on the EPS production: *W. cibaria* C43-11 confirmed the production of EPS, in all DY500 LSs, particularly in that containing only quinoa (3.17 ± 0.78 g/kg), even if at levels considerably lower ($p < 0.05$) than those observed in mMRS_S (16.1 ± 0.1 g/L). No significant differences ($p > 0.05$) in the viscosity values among the DY500 3%_S LSs within each strain were observed, but C43-11 based LSs showed the higher values ($p < 0.05$) respect to strain ITM21B and Co. A significant increase of the bacterial populations (from a median value of 7.11 to 9.18 log CFU/g for C43-11 and from 7.88 to 9.54 log CFU/g for strain ITM21B) was registered in all inoculated LSs. Values of pH in LS fermented by strain C43-11 were quite similar (range: 3.95÷4.04, $p > 0.05$), while a significant modification was observed in LSs fermented by strain ITM21B changing the flour type: the lowest value was observed in W/Am LS (3.25 ± 0.02), followed by Am LS (3.40 ± 0.002) and quinoa based LSs (3.74 ± 0.002). Whereas, the pH of Co LSs remained unvaried at values of 6.30÷6.42.

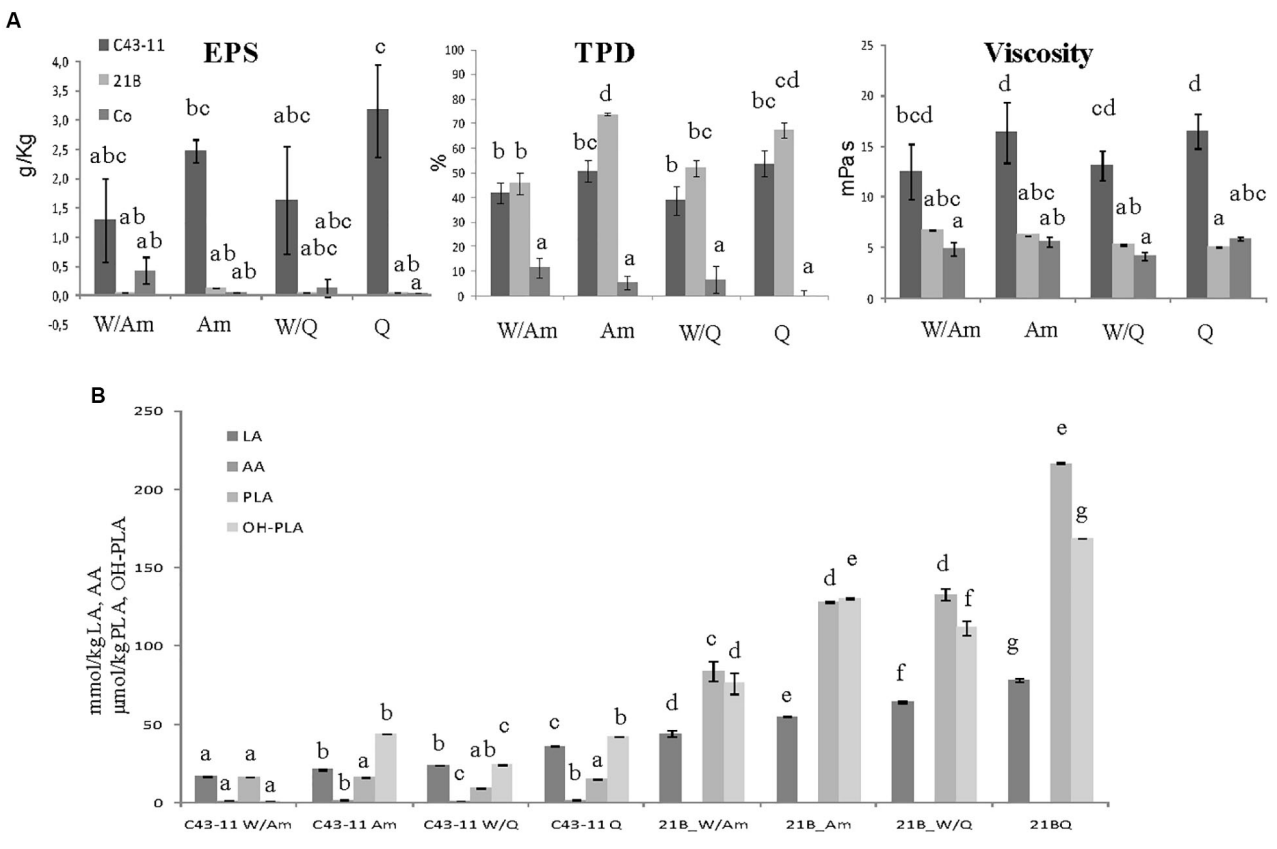

FIGURE 3 | Effect of pseudocereal flours in combination or not with wheat flour on **(A)** EPS content, viscosity, total protein degradation (TPD%) and **(B)** organic acid content in LSs obtained by *W. cibaria* C43-11, or *L. plantarum* ITM21B and in control LS after 15 h fermentation. Ingredients: wheat flour (W) in combination (ratio 1:1) or not with amaranth flour (Am) or quinoa flour (Q), in the presence of sucrose (S) (3% LS weight) at DY 500. The data are represented as mean ± standard error. Different letters above the columns indicate significant difference among the means of EPS content, pH, and TTA **(A)** and among each organic acid **(B)** by Tukey HSD's test ($p < 0.05$).

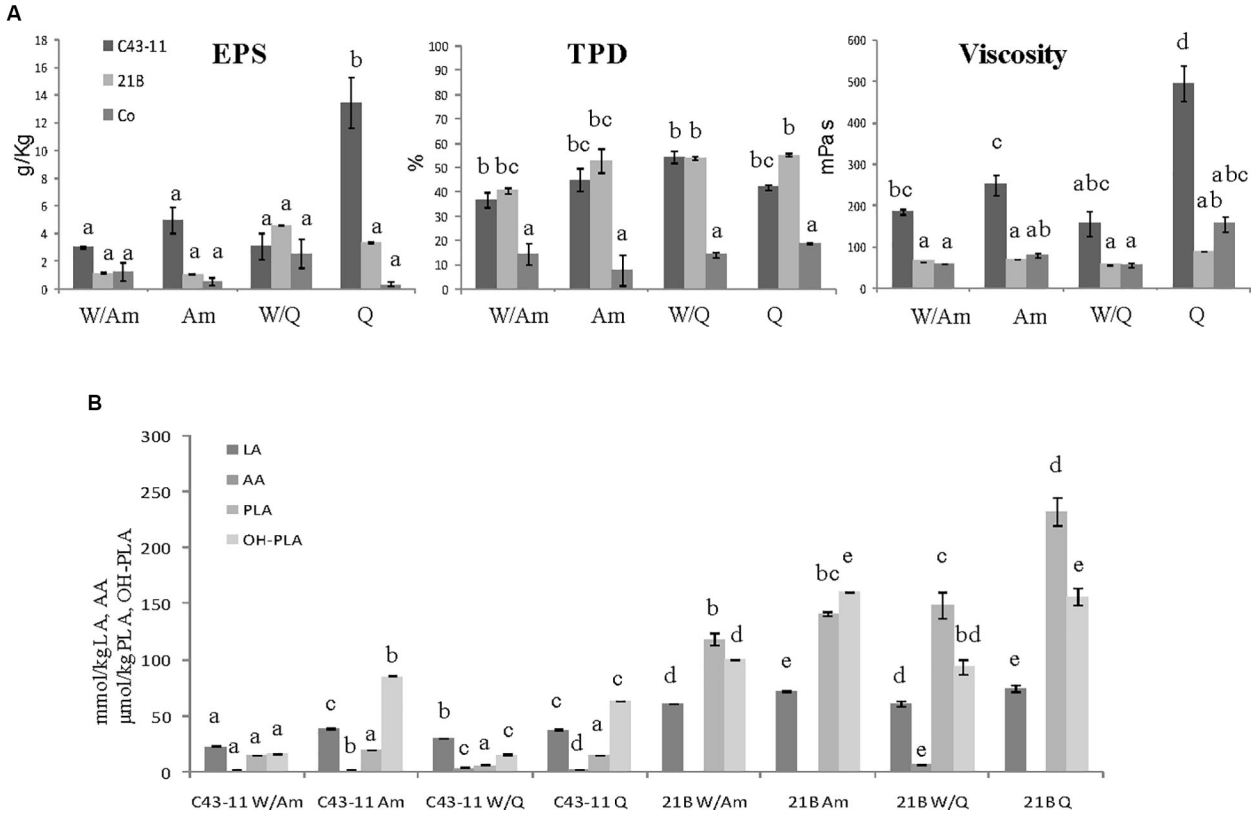

FIGURE 4 | Effect of pseudocereal flours in combination or not with wheat flour on **(A)** EPS content, viscosity, total protein degradation (TPD%) and **(B)** organic acid content in LSs obtained by *W. cibaria* C43-11, or *L. plantarum* ITM21B and in control LS after 15 h fermentation. Ingredients: wheat flour (W) in combination (ratio 1:1) or not with amaranth flour (Am) or quinoa flour (Q), in the presence of sucrose (S) (3% LS weight) at DY 250. The data are represented as mean ± standard error. Different letters above the columns indicate significant difference among the means of EPS content, pH, and TTA **(A)** and among each organic acid **(B)** by Tukey HSD's test ($p < 0.05$).

However, the TTA values were always higher in ITM21B LSs containing quinoa flour (**Table 4**). The production of organic acids was considerably higher in ITM21B based LSs, except for acetic acid which was produced at a low extent only by the strain C43-11 (**Figure 3B**). Consequently the total protein degradation (% TPD) mainly occurred in the presence of strain ITM21B, particularly in quinoa and amaranth flours, in which a 67.3 ± 3.04 and 73.7 ± 0.7% reduction was observed.

When the DY was lowered to 250, maintaining the sucrose level at 3% LS weight, the most significant changes were observed in C43-11 Q LS which showed the highest EPS production (13.43 ± 1.83 g/kg) and relevant viscosity (495.25 ± 43 mPa s) (**Figure 4A**). In addition, also strain ITM21B resulted able to produce an appreciable EPS amount (4.61 ± 0.01 g/kg LS corresponding to about 11 g/kg of flour) in W/Q LS, with a concomitant protein degradation of more than 50%, even if the viscosity remained at a low level (56.9 ± 0.1 mPa s). In the overall, TTA values significantly ($p < 0.05$) increased in all samples (**Table 5**) even if for pH values, range: 4.05÷4.29 for C43-11, 3.48÷3.97 for ITM21B, 5.88÷6.31 for Co and acetic acid content (**Figure 4B**), only a slight increase was observed. Strain ITM21B determined a significantly lower ($p < 0.05$) pH value in W/Am LS (3.48 ± 0.002) respect to the other formulations (Am 3.68 ± 0.01, W/Q 3.87 ± 0.002 and Q 3.97 ± 0.004). The same behavior was observed for strain C43-11 since the lower ($p < 0.05$) pH value was registered in W/Am LS (4.05 ± 0.01) followed by Am (4.14 ± 0.002), W/Q (4.2 ± 0.004), and Q (4.29 ± 0.01). In LSs at DY 250, the content of flour, and consequently of proteins, was twofold higher than LS DY 500 (**Supplementary Table S2**) but the protein degradation (% TDP) remained almost unvaried ($p > 0.05$) indicating that a lower DY favors proteolysis (**Figure 4A**). Interestingly, in the presence of strain C43-11, proteins were hydrolyzed at the same extent of LS fermented by *L. plantarum* ($p > 0.05$) and percentage values were slightly higher in Am (44.9 ± 4.6%) and W/Q (54.4 ± 2.4%) LSs (**Figure 4A**).

The further increase of sucrose concentration to 6% (w/w of LS), allowed to considerably enhance the EPS production and viscosity, mainly in Q and Am LSs fermented by C43-11 strain (**Figure 5A**). In particular, the EPS production by strain C43-11 reached the value of 20.79 ± 3.55 g/kg LS and a relevant viscosity of 1168.2 ± 139.4 mPa s demonstrating the special attribute of this strain in producing these molecules, while the EPS production remained unvaried for strain ITM21B. Regarding the other parameters, only slight changes were observed (**Figure 5A**). The pH value ranged between 4.07 and 4.26 for C43-11, 3.49

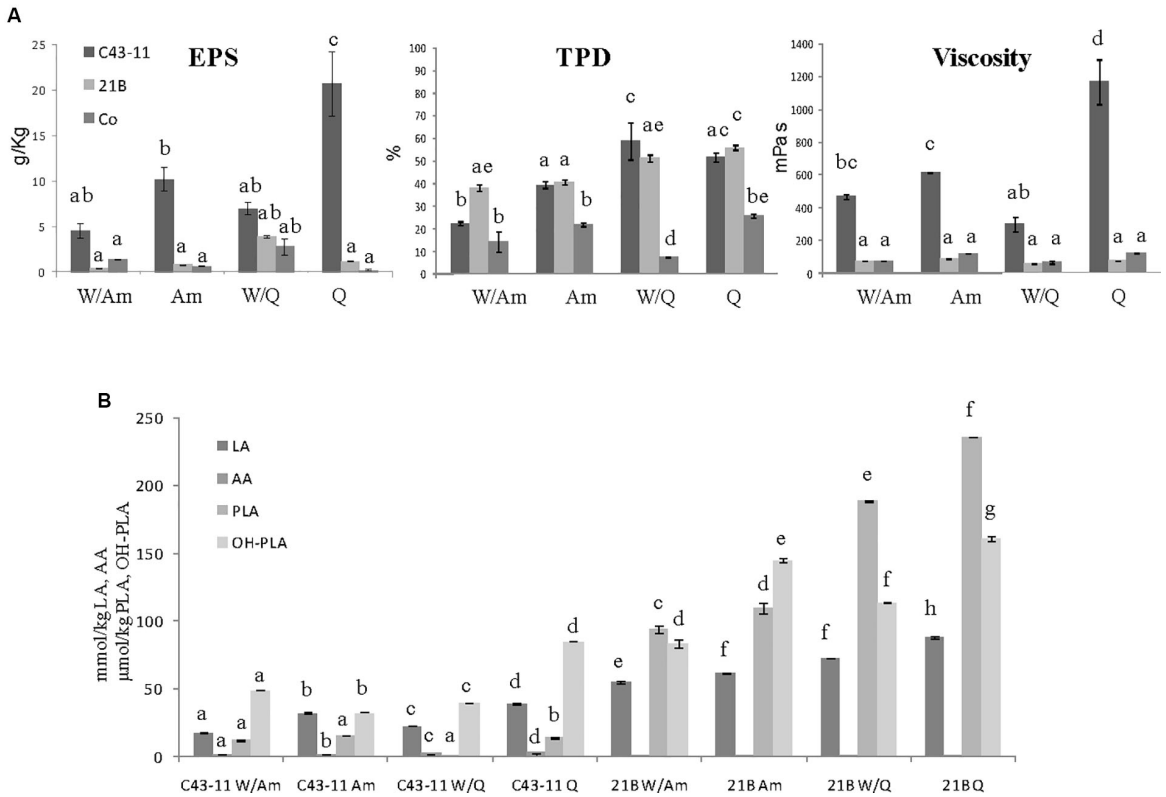

FIGURE 5 | Effect of pseudocereal flours in combination or not with wheat flour on **(A)** EPS content, viscosity, total protein degradation (TPD%) and **(B)** organic acid content in LSs obtained by *W. cibaria* C43-11, or *L. plantarum* ITM21B and in control LS after 15 h fermentation. Ingredients: wheat flour (W) in combination (ratio 1:1) or not with amaranth flour (Am) or quinoa flour (Q), in the presence of sucrose (S) (6% LS weight) at DY 250. Data are represented as mean ± standard error. Different letters above the columns indicate significant difference among the means of EPS content, pH, and TTA (A) and among each organic acid (B) by Tukey HSD's test ($p < 0.05$).

TABLE 4 | Values of TTA in optimized LSs, at DY 250 or 500, fermented by *W. cibaria* C43-11 or *L. plantarum* ITM21B in comparison to the uninoculated control (Co), after 15 h incubation.

Flour	Co	C43-11	ITM21B
DY 500, 3% Sucrose			
W/Am	1.40 ± 0.04a	6.05 ± 0.02d	9.90 ± 0.16e
W/Q	1.73 ± 0.05a	9.25 ± 0.10e	14.00 ± 0.20ghi
Am	2.15 ± 0.06a	10.25 ± 0.06ef	14.75 ± 0.10ghi
Q	2.78 ± 0.19abc	13.15 ± 0.14gh	18.00 ± 0.61jkl
DY 250, 3% Sucrose			
W/Am	2.78 ± 0.09abc	10.25 ± 0.02ef	15.75 ± 0.10hij
W/Q	5.08 ± 1.22bcd	14.85 ± 0.14ghi	23.40 ± 0.16no
Am	3.98 ± 0.21abcd	18.00 ± 0.04jkl	22.00 ± 0.41mn
Q	5.43 ± 0.38cd	20.25 ± 0.92lm	26.75 ± 0.92p
DY 250, 6% Sucrose			
W/Am	2.60 ± 0.04ab	10.35 ± 0.10ef	14.40 ± 0.12ghi
W/Q	5.28 ± 1.64bcd	12.75 ± 0.31fg	20.75 ± 0.92lmn
Am	3.55 ± 0.06abcd	16.25 ± 0.18ijk	19.55 ± 0.02lm
Q	5.40 ± 0.32cd	18.55 ± 0.18kl	26.15 ± 1.08op

Ingredients: wheat flour (W) with amaranth flour (Am) or quinoa flour (Q) (ratio 1:1) in the presence of sucrose (S) (3% or 6% LS weight). Values (mean ± standard error of the mean) with different lower case letters are significantly different among all ($p < 0.05$).

and 3.93 for ITM21B and 5.98 and 6.26 for Co and were similar ($p > 0.05$) to values of LSs at DY250 with sucrose 3%. In addition TTA values were similar or a little lower respect to those registered in LSs with DY 250 and sucrose 3% (**Figure 5B**) and the organic acid production did not significantly change. Whereas, a slightly higher protein degradation was observed in quinoa based LSs respect to the Am based LSs.

In order to better discriminate LS samples, a PCA was performed on all data (pH, TTA, EPS, viscosity, organic acids, protein degradation) (**Figure 6**). As shown in the loading plot (**Figure 6A**), a high correlation between EPS content and viscosity of LSs was registered, indicating that this parameter can be useful to follow the carbohydrate polymer production. A good discrimination among samples mainly based on the strain used to ferment flours, was observed (**Figure 6B**). Control LSs located on the right of the score plot while the C43-11 LSs were sufficiently discriminated by the ITM21B LSs. Even if at very low level, acetic acid was mainly produced by strain C43-11 while the other acidic molecules were found in LSs fermented by strain ITM21B. In particular, C43-11 LSs were mainly characterized by higher EPS content and viscosity, while ITM21B LSs contained higher amounts of acids and higher TTA and % TPD. Results clearly indicated that an appreciable EPS production (4.61 ± 0.01 g/kg

TABLE 5 | Distribution of proteins (expressed as protein percentage on total peak areas) in Mw areas (A1, A2, A3) and molecular weight ranges of proteins for each LS type, as a result of LoaC capillary electrophoretic analysis on Protein 230 Labchip, before and after fermentation with *Weissella cibaria* C43-11 or *Lactobacillus plantarum* ITM21B and in comparison to the uninoculated control (Co).

	Am				**W/Am**				**Q**				**W/Q**			
								LS DY500_3%S								
Mw area (kDa)	T0	Co	ITM21B	C43-11	T0	Co	ITM21B	C43-11	T0	Co	ITM21B	C43-11	T0	Co	ITM21B	C43-11
A1 (14–30)	40.9	56.7	96.9	88.7	25.1	27.8	40.7	40.2	20.1	41.8	100	94.3	20.4	26.2	78.9	75.4
A2:																
I (31–42)	47.8	40.1	3.1	11.3	31.4	42.9	40.7	41.7	22.1	40.6	0	5.7	18.3	21.9	4.6	13.2
II (43–55)	8.5	1.7	0	0	25.0	15.7	15.6	12.8	40.0	8.2	0	0	43.7	28.7	3.7	4.0
III (56–79)	2.2	1.5	0	0	13.6	11.0	3.0	5.3	13.7	6.9	0	0	13.6	13.7	10.0	7.4
A3 (>80 kDa)	0.6	0	0	0	4.9	2.6	0	0	4.1	2.5	0	0	4.0	9.5	2.8	0
Mw range (kDa)	14–94	14–79	14–35	14–35	14–222	14–225	14–57	14–57	14–117	14–105	14–30	14–32	14–225	14–225	14–103	14–57
								LS DY250_3%S								
A1 (14–30)	43.8	55.8	89.4	88.6	24.5	24.4	73.0	37.7	24.1	37.4	93.5	70.1	14.2	20.7	95.3	55.1
A2:																
I (31–42)	46.0	40.8	10.6	11.4	33.0	36.1	15.5	33.5	27.3	39.8	6.5	27.0	4.0	17.8	4.7	21.1
II (43–55)	7.6	1.3	0	0	25.1	29.8	10.7	16.4	32.3	10.7	0	0	48.4	34.0	0	16.5
III (56–79)	2.0	2.1	0	0	13.6	5.7	1.9	6.9	11.4	9.1	0	0.9	25.5	17.0	0	7.3
A3 (>80 kDa)	0.6	0	0	0	3.8	4.0	0	5.5	4.9	3.0	0	2.0	7.9	10.5	0	0
Mw range (kDa)	14–96	14–79	14–32	14–35	14–226	14–225	15–57	14–220	14–117	14–104	14–36	14–97	14–214	14–225	14–39	14–60
								LS DY250_6%S								
A1 (14–30)	46.2	62.4	94.2	74.2	34.8	27.3	68.8	53.2	20.4	40.3	93.8	64.1	16.0	21.0	100	53.4
A2:																
I (31–42)	42.3	33.8	5.8	25.8	21.6	33.8	16.9	26.1	21.1	41.7	6.2	33.9	13.2	18.7	0	28.0
II (43–55)	8.8	1.7	0	0	21.2	24.9	10.8	14.3	42.1	8.1	0	0	32.0	37.8	0	12.0
III (56–79)	2.0	2.2	0	0	13.9	10.0	0	0.5	13.9	7.1	0	2.0	32.1	16.2	0	5.7
A3 (>80 kDa)	0.7	0	0	0	8.5	4.0	3.5	5.9	2.5	2.8	0	0	6.7	6.3	0	0.9
Mw range (kDa)	14–96	14–79	14–43	14–41	14–217	14–219	14–123	14–130	14–117	14–105	14–37	14–57	14–230	14–220	14–21	14–88

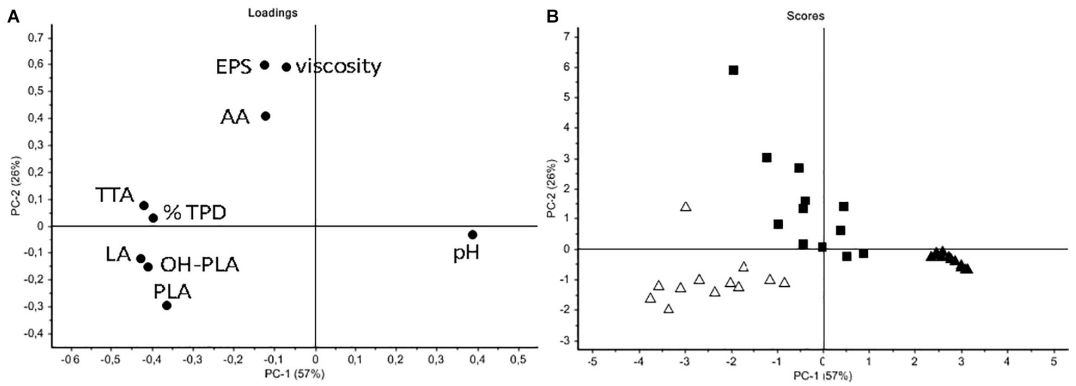

FIGURE 6 | Principal component analysis (PCA) of all data (from the optimized LSs formulated as in **Table 2**). **(A)** Loading plot indicating the correlation between variables (pH; TTA; % total protein degradation, TPD; organic acids – lactic, LA; acetic, AA; phenyllactic, PLA; hydroxy-phenyllactic OH-PLA), EPS content and viscosity. **(B)** Score plot indicating sample distribution basing on the inoculated strains (black square C43-11; open triangle ITM21B; black triangle Co).

LS corresponding to about 11 g/kg of flour) can be obtained also for the low producer strain ITM21B in W/Q LS at DY 250 and sucrose 3%, with a concomitant protein degradation of more than 50%.

Proteolysis in LSs

Total proteins of flours and modifications in LSs after fermentation were deeply investigated using LoaC capillary electrophoresis. **Figure 7** shows a representative gel-like image of the protein profile of flours highlighting the presence of about 15 protein bands ranging from 14 to 220 kDa in wheat flour and between 14 and 118 kDa in amaranth and quinoa flours. Banding patterns were characterized by common proteins and additional specific bands, i.e., 70, 143, and 180 kDa in wheat flour and 20, 31, and 78 kDa in amaranth and quinoa flours.

According to Mw, three different areas were considered in the electropherograms: A1 (14–30 kDa), A2 (31–79 kDa), and A3 (80–230 kDa) and percentages of peaks for each Mw area were gained and are summarized in **Figure 7**. The major differences were observed in A2, therefore that area was further separated in three sectors (I, II, III). In wheat flour, A2 accounted for the major protein content (about 80%) and it was characterized by main protein bands in the 43–55 kDa range.

In amaranth and quinoa flours, peaks were mainly detected in A1 (about 42%) and A2 (about 55%). In amaranth flour, the presence of double peaks at 16–20 and 31–37 kDa, and two peaks at 41 and 55 kDa, indicated the presence of amaranthin, while the major peaks detected in quinoa flour, i.e., 30–38 and 19–24 kDa are likely to represent the principal protein of quinoa, the chenopodina (Hager et al., 2012). In all flours, Mw A3 contained a low percentage of peaks, i.e., 7.9, 3.5, and 2.1% in wheat, quinoa, and amaranth flours, respectively.

In order to monitor the flour proteolysis in LSs samples, LoaC analysis was performed before and after fermentation (**Figure 8** and **Table 5**). In detail, at the beginning of fermentation (T0) in all LSs, banding patterns of about 18 protein bands in the 14-225 kDa range were observed for W/Am and W/Q LSs, respectively while about 14 protein bands were found in the 14–96 and

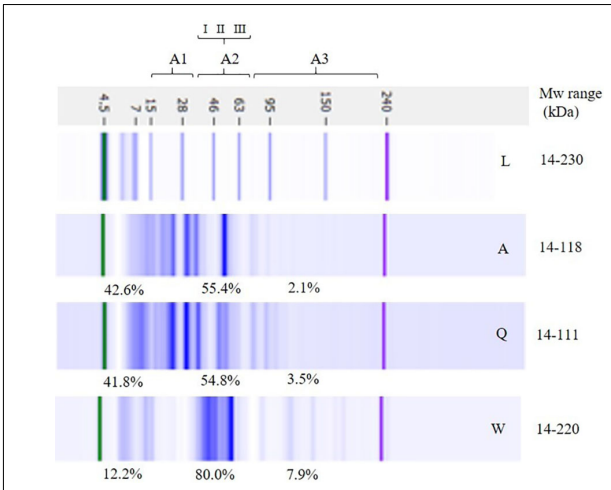

FIGURE 7 | Electrophoretic analysis (LoaC) of total proteins from amaranth (Am), quinoa (Q), and wheat (W) flours and molecular weight marker (L) shown as gel-like images on Protein230 LabChip. Brackets indicate molecular weight areas: A1 (14–30 kDa), A2 (I, 31–42 kDa; II, 43–55 kDa; III, 56–79 kDa), and A3 (>70 kDa). Molecular weight (Mw) and percentage of proteins (%) in each area are the average of two separate experiments (n = 2).

14–117 a kDa range for Am and Q LSs, respectively. As visualized in **Figure 8**, after 15 h of fermentation the pattern profile of all inoculated LSs considerably changed since high molecular weight proteins were degraded and a concomitant increase of smaller proteins was observed. In uninoculated LSs controls no change in the protein pattern respect to the beginning of fermentation was observed.

Table 5 and **Figure 8** summarize the protein changes in the Mw areas for the three LS types.

In inoculated LSs at DY 500, at the beginning of fermentation, proteins were distributed in the three Mw areas (**Figure 8A** and **Table 5**) as observed in the relevant flours. After fermentation, in Q and Am LSs, proteins were mainly found in A1 and at a lesser extent in A2, particularly in the 31–42 kDa range,

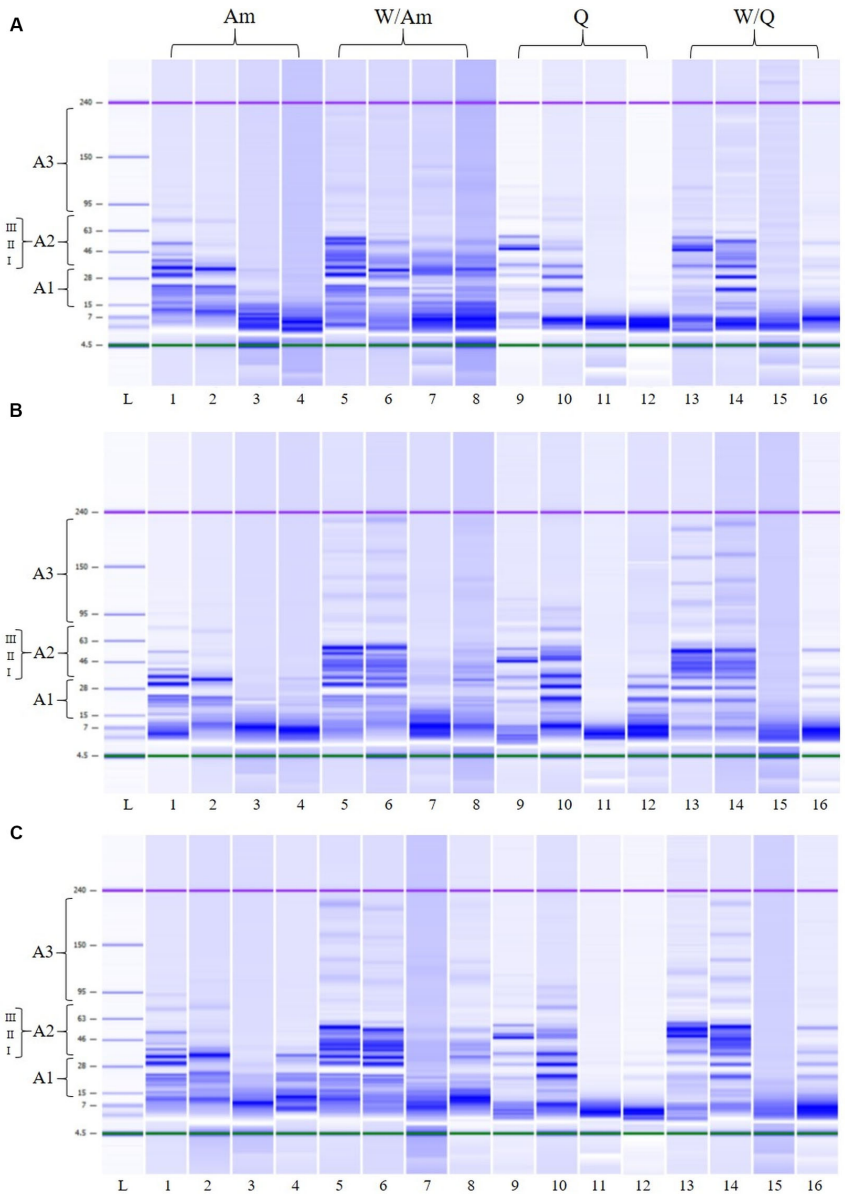

FIGURE 8 | Electrophoretic analysis (LoaC) of total proteins from amaranth (Am), wheat/amaranth (W/Am), quinoa (Q), and wheat/quinoa/ (W/Q) liquid sourdoughs (LSs) with DY500_3%S **(A)**, DY250_3%S **(B)**, and DY250_6%S **(C)** shown as gel-like images on Protein230 LabChip; Molecular weight marker (L); LS uninoculated control T0 (lanes 1, 5, 9, 13); LS uninoculated control T15 (lanes 2, 6, 10, 14); *L. plantarum* ITM21B (lanes 3, 7, 11, 15); *W. confusa* C43-11 (lanes 4, 8, 12, 16). Numbered brackets indicate molecular weight areas: A1 (14–30 kDa), A2 (I, 31–42 kDa; II, 43–55 kDa; III, 56–79 kDa), A3 (>70 kDa).

indicating the formation of small proteins due to proteolysis. In the presence of wheat, proteins were observed in the whole A2 region (31–79 kDa) at different percentages depending on the pseudocereal.

Lowering the DY to 250 (3 or 6% sucrose), no significant changes in the protein distribution were observed except for the A2 region of W/Am and W/Q LSs fermented by ITM21B (**Figures 8B,C**). In particular, proteins in the 43–55 kDa region containing the gliadin and glutenin fractions of wheat, were reduced or completely degraded in W/Am and W/Q LSs, respectively.

Finally, in all LSs, as already observed in their relevant flours, a low % of peaks was found in the A3 region and no significant changes occurred during fermentation.

DISCUSSION

The use of pseudocereals was considered since their high nutritional value and applicability in gluten-free bread. Among different sources of carbohydrates, the pseudocereals quinoa and amaranth can provide a high content of starch and

essential nutrients (Comai et al., 2011; Singh and Singh, 2011), but they don't produce a viscoelastic dough as for wheat containing gluten. EPS producer LAB strains can successfully pilot the sourdough fermentation favoring the proteolysis and production of microbial carbohydrate polymers. EPS, acting as textural improvers similarly to plant hydrocolloids, remedy the technological drawbacks due to the lack of gluten occurring in gluten-free bakery products or with reduced fat content. Furthermore, EPS production in sourdough meets producer and consumer requirements for "clean label" products.

Several studies on EPS production report the *Weissella* spp. and *Leuconostoc* spp. as high producer genera (Byme, 2001; Di Cagno et al., 2006; Katina et al., 2009; Galle et al., 2010; Wolter et al., 2014). In the current study, all *Weissella* species tested resulted to produce these metabolites in mMRS containing sucrose 10%. In particular, the *W. cibaria* C43-11 was selected as a high producer. Similarly, Zannini et al. (2013) found a *W. cibaria* strain MG1 producing high amount of EPS in similar conditions. As previously reported, the EPS production is highly related to the sucrose concentration, the incubation time and the carbon source (Katina et al., 2009; Rühmkorf et al., 2012; Yu et al., 2018).

With the aim of producing LSs enriched with *in situ* produced EPS, the high producer strain *W. cibaria* C43-11 and the low producer sourdough strain *L. plantarum* ITM21B, were used to ferment wheat flour and/or pseudocereals (quinoa and amaranth). Interestingly, the *L. plantarum* strain, not producing EPS in mMRS_S, resulted to be able to synthesize these molecules, even if at low levels, in the presence of the pseudocereal flours and this ability was strongly affected by the DY and flour type. This effect was amplified for strain C43-11 whose EPS production was optimal in quinoa based LS at DY 250 and containing sucrose 6%. In fact, in the optimal conditions strain C43-11 produced about 20.79 g/kg sourdough, a concentration considerably higher than that produced by *W. cibaria* strains in sourdoughs from different flours (Galle et al., 2012a,b; Wolter et al., 2014; Hu and Gänzle, 2018). Our results confirmed the suitability of quinoa as a substrate for EPS production as previously observed by Rühmkorf et al. (2012). Authors studied the effect of different flours, DY and sucrose addition on EPS yield after fermentation with three *Lactobacillus* species. They found the highest EPS production (20.63 g/kg flour) in quinoa sourdough (DY 250, sucrose 7.5% flour weight, corresponding to our 3% sucrose/LS weight at DY250) after 24 h fermentation. When the sucrose was increased to the 15% flour weight (corresponding to our 6% LS weight at DY250) strain *L. reuteri* TMW 1.106 produced about 25 g/kg flour in 24 h. In our study, the highest EPS yield (20.79 ± 0.2 g/kg LS corresponding to 58.75 g/kg flour) was obtained in Q LS at DY 250 in the presence of 6% sucrose after a shorter fermentation time (15 h). Interesting results on the rheological and textural properties of wheat and sorghum sourdough breads were obtained by Galle et al. (2012a,b) after addition (10 or 20% on dough weight) of EPS-enriched sourdough containing from 0.6 to 8 g/kg of EPS. Therefore, to obtain an improvement in bread quality, it can be enough to add the 1% of the C43-11 quinoa based LS (DY250, 6% sucrose) or the 10% of the 21B W/Q LS DY250 and 3% sucrose, to the bread dough.

The higher EPS production by C43-11 in LSs at DY 250 with 6% sucrose could be related to the osmotic stress generated by the presence of a high content of sucrose (Rühmkorf et al., 2012). In our study, by reducing the DY and therefore the water content, the TTA values increased, mainly in the presence of the pseudocereal alone, indicating a higher amount of undissociated acids, while the pH of inoculated LSs, slightly decreased and the content of lactic and acetic acids remained almost unvaried. The pH increase observed at lower DY (250) could be related to the lower water content and consequently to the lower diffusion of organic acids in the environment (De Vuyst et al., 2014). Moreover, in the presence of quinoa higher pH values were observed indicating the buffering capacity of the pseudocereal as suggested by other authors (Rühmkorf et al., 2012). The slower acidification observed in LSs at DY 250 could be responsible for the lower protein hydrolysis registered for these samples. In fact, as reported by Spicher and Stephan (1999), at higher DY organic acids easily diffuse into the environment and rapidly acidify it. Whereas, in sourdough with low DY the higher amount of carbohydrates available for fermentation lead to a higher buffering capacity that slows the acidification rate (De Vuyst et al., 2014). Moreover, in the presence of higher amounts of EPS the proteolysis lowered maybe since the ability of the polysaccharides to form a network hampering the hydrolysis of proteins, mainly the soluble ones: this can also be the reason of the higher pH (about 4.3) observed in C43-11 LSs at DY 250 respect to that of LS at DY500 (3.95÷4.04). However, an appreciable protein hydrolysis and EPS production was observed with the strain ITM21B, mainly in LSs at DY250 and sucrose 6%. The *L. plantarum* strain was selected during the screening test in mMRS_S as not-producer strain, but it became able to produce EPS by modifying the LS formulation ensuring a good proteolysis and therefore having a key role in improving the overall quality of sourdough bread (Gobbetti et al., 2019). In particular, proteolysis is a fundamental process in sourdough technology of gluten free bread since it positively affects the quality of final products, as reported by several authors (Moroni et al., 2009; Coda et al., 2010; Dallagnol et al., 2013; Rizzello et al., 2017). Generally, in our study a higher protein degradation was observed for both strains in LSs containing only the pseudocereal maybe for the more hydrophilic nature of their proteins which can be quickly degraded (Dallagnol et al., 2013; Wolter et al., 2014).

The LoaC capillary electrophoresis offers great advantages over classic SDS-PAGE and, due to the improved resolution of peaks, provided quantitative and reproducible results on the protein profile changes after LAB fermentation. The protein profile and overall arrangement of bands in wheat, quinoa and amaranth flours well-compared with previous studies in which the same protein profiles were observed on Protein 230 Loac or SDS gels (Hager et al., 2012; Alonso-Miravalles and O'Mahony, 2018). Wheat flour was mainly characterized by the presence of protein bands in the 43–55 kDa range which could represent gliadins and the low molecular weight glutenins (Bradová and Matějová, 2008; Juhász et al., 2015). In amaranth and quinoa flours, the presence of the typical proteins amaranthin and chenopodin, respectively, was ascertained in the A1 and A2 areas which represent the region generally modified

during fermentation (Lacaze et al., 2007). Actually, the most relevant changes in LSs fermented by C43-11 and ITM21B strains occurred in those areas where an increase of low molecular weight proteins was observed. An interesting result was the consistent degradation of proteins in the 43–55 kDa region, containing the gliadin and glutenin fractions of wheat, observed in W/Am and W/Q LSs at DY 250 (3 or 6% sucrose).

CONCLUSION

Results demonstrated that the modulation of fermentation parameters (flour type, DY, sucrose) can stimulate metabolic activities of *W. cibaria* C43-11 and *L. plantarum* ITM21B making them suitable for the production of short fermented (15 h) LSs, enriched in the EPS content, which can be applied in the bread-making process. The *W. cibaria* strain (C43-11), isolated from Italian wheat semolina, was able to produce a considerable amount of EPS mainly in the presence of pseudocereals amaranth or quinoa and to hydrolyze proteins during fermentation. A more efficacious proteolysis with degradation of proteins in the region including the glutenin and gliadin fraction of wheat, was obtained using the *L. plantarum* sourdough strain ITM21B in the wheat/quinoa formulations at DY 250.

In conclusion, the study highlighting the still unexplored potential of LAB strains in food manufacturing and their contribution in improving the rheological and functional features of bakery products.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

FV conceived and supervised the study. FV, MD, AB, and SL designed the experiments and wrote the first draft of the manuscript. MD, AB, and SL performed the experiments. PL coordinated the research. AL coordinated the funding projects. PL, FV, and SL provided a critical revision of the manuscript. All authors interpreted the results, contributed to the manuscript revision, and read and approved the submitted version.

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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.2020.00967/full#supplementary-material

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Bioprocessing of Brewers' Spent Grain Enhances Its Antioxidant Activity: Characterization of Phenolic Compounds and Bioactive Peptides

*Michela Verni[1], Erica Pontonio[1], Annika Krona[2], Sera Jacob[2], Daniela Pinto[3], Fabio Rinaldi[3], Vito Verardo[4,5], Elixabet Díaz-de-Cerio[4], Rossana Coda[6,7] and Carlo Giuseppe Rizzello[1]**

[1] Department of Soil, Plant and Food Science, University of Bari Aldo Moro, Bari, Italy, [2] Research Institute of Sweden, Gothenburg, Sweden, [3] Giuliani S.p.A., Milan, Italy, [4] Department of Nutrition and Food Science, University of Granada, Granada, Spain, [5] Institute of Nutrition and Food Technology "José Mataix", Biomedical Research Centre, University of Granada, Granada, Spain, [6] Department of Food and Nutrition Sciences, University of Helsinki, Helsinki, Finland, [7] Helsinki Institute of Sustainability Science, Helsinki, Finland

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***Correspondence:**
Carlo Giuseppe Rizzello
carlogiuseppe.rizzello@uniba.it

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Brewers' spent grain (BSG) is the major by-product of the brewing industry which remain largely unutilized despite its nutritional quality. In this study, the effects of fermentation on BSG antioxidant potential were analyzed. A biotechnological protocol including the use of xylanase followed by fermentation with *Lactiplantibacillus plantarum (Lactobacillus plantarum)* PU1, PRO17, and H46 was used. Bioprocessed BSG exhibited enhanced antioxidant potential, characterized by high radical scavenging activity, long-term inhibition of linoleic acid oxidation and protective effect toward oxidative stress on human keratinocytes NCTC 2544. Immunolabelling and confocal laser microscopy showed that xylanase caused an extensive cell wall arabinoxylan disruption, contributing to the release of bound phenols molecules, thus available to further conversion through lactic acid bacteria metabolism. To clarify the role of fermentation on the antioxidant BSG potential, phenols were selectively extracted and characterized through HPLC-MS techniques. Novel antioxidant peptides were purified and identified in the most active bioprocessed BSG.

Keywords: brewers' spent grain, bioprocessing, phenolic compounds, bioactive peptides, antioxidant activity

Abbreviations: ABTS, 2,2′-azino-di-(3-ethylbenzthiazoline sulfonate); AX, arabinoxylans; BHT, butylated hydroxytoluene; BSA, bovine serum albumin; BSG, brewers' spent grain; CLSM, confocal laser scanning microscopy; DPPH, 2,2-diphenyl-1-picrylhydrazyl; eBSG, enzyme treated brewers' spent grain; eBSG fH46, enzyme treated brewers' spent grain fermented with *L. plantarum* H46; eBSG fPRO17, enzyme treated brewers' spent grain fermented with *L. plantarum* PRO17; eBSG fPU1, enzyme treated brewers' spent grain fermented with *L. plantarum* PU1; EF30, bioprocessing protocol including simultaneous enzymatic treatment and fermentation at 30°C for 24 h; E50 + F30, Bioprocessing protocol including sequential enzymatic treatment at 50°C for 5 h and fermentation at 30°C for 24 h; FBS, fetal bovine serum; HPLC-FLD, high-performance liquid chromatography with fluorescence detection; LAB, lactic acid bacteria; ME, methanolic extracts; MRS, De Man, Rogosa, and Sharpe; MMT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OPA, *o*-phtaldialdehyde; PBS, phosphate-buffered saline; PUFAs, polyunsaturated fatty acids; rBSG, raw BSG; RP-FPLC, reversed-phase fast performance liquid chromatography; TFAA, total free amino acids; UPLC-PDA-ESI-QTOF, ultra-performance liquid chromatography with photodiode array detection electrospray ionization, quadrupole and time-of-flight mass spectrometry; WSE, water/salt soluble extracts.

INTRODUCTION

Brewers' spent grain (BSG), the major by-product of the beer-brewing industry, consists of the seed coat–pericarp–husk layers covering the original barley (*Hordeum vulgare*) grain (Lynch et al., 2016), and of insoluble residues deriving from other additional ingredients, such as raw or malted cereals, like maize (*Zea mays*) or wheat (*Triticum durum* and *Triticum aestivum*). Briefly, after extraction of the soluble compounds from the barley into the mash, the BSG is separated from the wort (liquid), which is then boiled with hop and fermented. BSG represents a very abundant by-product with an average annual global production of approximately 39 million tons, 10% of which produced in Europe only (Lynch et al., 2016).

Brewers' spent grain is essentially made of fiber (mainly hemicellulose and cellulose) which constitute half of its composition on dry basis; other important constituents are protein (up to 30%) and phenols. Arabinoxylans (AX), the main hemicellulose constituent, have a backbone of xylose residues, which can be substituted with arabinose residues (Lynch et al., 2016). BSG arabinoxylans include a significant water-extractable fraction and during enzymatic digestion they can release xylooligosaccharides with varying degree of polymerization (Wang et al., 2010).

Brewers' spent grain is a valuable source of phenolics, whose antioxidant, antiradical, but also anti-carcinogenic and anti-apoptotic properties, are widely recognized (Lynch et al., 2016). BSG phenolics are mainly contained in the barley husk, accumulated in cell walls and esterified to sugar residues, conditions that decrease their antioxidant activity since availability of free hydroxyl group is essential to stabilize free radicals (Bhanja et al., 2009). The major part of phenolic compounds in BSG are hydroxycinnamic acids, of which ferulic acid is the most abundant and in strong correlation with cereal antioxidant capacity. However, since ferulic acid is bound to the ligninocellulosic structure of BSG, it has low bioaccessibility. Bioprocessing can improve the bioaccessibility of phenolic compounds from fiber-rich cereal matrix. In a previous study, a variety of hydrolytic enzymes, mainly xylanase, β-glucanase, α-amylase, cellulase, and ferulic acid esterase coupled with yeast fermentation was used to treat wheat bran with the aim of improving the bioaccessibility of phenolic compounds in bran-containing breads. This treatment led to an increase of the bioaccessibility of ferulic acid and other phenolic acids of bran *in vitro* (Anson et al., 2009).

In addition to the potential health benefits, when added to food, antioxidants control rancidity development, maintaining the nutritional quality and shelf-life of products. Nowadays, there is still a growing interest in finding natural sources of antioxidants to effectively replace synthetic ones, which have been related to toxic and carcinogenic effects (Bouayed and Bohn, 2010).

Brewers' spent grain protein component is also of interest, thanks to the lysine abundance in comparison with other cereal-derived matrices, and for the antioxidant, anti-inflammatory and immunomodulatory potential of the proteolysis derivatives, previously investigated trough *in vitro* and *ex vivo* assays (Lynch et al., 2016). Additionally, BSG can also be a source of antioxidant peptides as previously shown for several cereal flours, including whole wheat. These bioactive peptides, released through fermentation with lactic acid bacteria (LAB), showed protection of cells from oxidation (*ex vivo*) as well as inhibition of linoleic acid oxidation (Verni et al., 2019).

Despite the high nutritional and functional potential, most of the BSG produced is used for feed or as substrate to produce bioethanol, lactic acid, xylitol and enzymes (Nigam, 2017). Yet, BSG use as food ingredient is more limited due to its complex structure and consequent poor technological performance, which requires treatments able to reduce the negative impact on texture and sensory properties of the final food product (Lynch et al., 2016). Among these, pre-treatments with xylanase were effective in improving specific volume and texture of breads supplemented with BSG and increased the amount of soluble fibers (Ktenioudaki et al., 2015). Similarly, fermentation, largely recognized as a technology able to improve sensory and technological properties of fiber-rich cereal by-products, was proven helpful to enhance nutritional and functional properties of BSG in different bread and beverages applications (Gupta et al., 2013).

The focus of this study was to understand the effect of fermentation on the antioxidant properties of BSG. To enable the release or synthesis of antioxidant compounds in BSG, a biotechnological process based on fermentation with selected LAB and treatment with a commercial xylanase was developed.

The effects of bioprocessing on the antioxidant activity were investigated through an integrated multi-step approach including *in vitro* and *ex vivo* assays, study of BSG microstructure, and characterization of phenolic compounds and peptide profile.

MATERIALS AND METHODS

Microorganisms

Thirty-three LAB strains, *Lactiplantibacillus plantarum* (formerly classified as *Lactobacillus plantarum*) LB1, C2, C48, H46, H48, T6B4, T0A10, T6C16, 18S9, MRS1, 1A7, PU1, PRO17, *Furfurilactobacillus rossiae* (formerly classified as *Lactobacillus rossiae*) LB5, T0A16, *Levilactobacillus brevis* (formerly classified as *Lactobacillus brevis*) MRS4, AM7, *Pediococcus pentosaceus* H11, T1A13, I76, I214, I02, I014, F01, OA1, S3N3, BAR4, *Pediococcus acidilactici* 10MM0, *Pediococcus* sp. I56, *Leuconostoc mesenteroides* 12MM1, I57, *Weissella confusa* KAS3, and NEY6, all belonging to the Culture Collection of the Department of Soil, Plant and Food Sciences (University of Bari, Italy) were used to ferment BSG. All the strains were previously used as starters for fermentation and characterized for pro-technological properties (growth and acidification performances, as well as the ability to increase antioxidant activity) as expressed in their own food isolation matrix (wheat, wheat germ quinoa, hop, hemp, faba bean, chickpea, carrots) (Di Cagno et al., 2008; Rizzello et al., 2010, 2016; Pontonio et al., 2015; Mamhoud et al., 2016; Nionelli et al., 2018).

The strains were routinely propagated on De Man, Rogosa, and Sharpe (MRS) (Oxoid, Basingstoke, Hampshire, United Kingdom) at 30°C. Before inoculation they were

cultivated until the late exponential phase of growth was reached (*ca*. 10 h), harvested by centrifugation at 9000 × *g* for 10 min at 4°C, washed twice in 50 mM sterile phosphate buffer (4°C, pH 7.0), resuspended in sterile distilled water and used to inoculate BSG.

Raw Material and Enzymes

The BSG employed in this study, kindly provided by Peroni brewery (Bari, Italy), is obtained from the production of a lager beer brewed with barley malt (70%) and maize (*Z. mays*) (30%) and do not contain spent yeast. Before any experiment, BSG was grinded with a laboratory mill Ika-Werke M20 (GMBH, and Co. KG, Staufen, Germany). After milling, the BSG particle size distribution was: 250–500 μm (30%), 500–750 μm (60%), 750–1000 μm (10%). BSG proximal composition was: moisture, 80 ± 0.07%; protein, 21.11 ± 0.19% of dry matter (d.m.); fat, 10.89 ± 0.01% of d.m.; cellulose, 22.51 ± 0.68% of d.m.; hemicellulose, 24.81 ± 0.97% of d.m, lignin, 15.33 ± 0.28% of d.m; ashes, 5.12 ± 0.05% of d.m.

The commercial hydrolytic enzyme, Depol^{TM} 761P (Biocatalysts, Chicago, IL, United States), a preparation derived from *Bacillus subtilis* having xylanase activity (14,670 nkat/g), was used either individually or in combination with three selected strains for BSG fermentation.

BSG Bioprocessing

Starter Selection

Lactic acid bacteria strains were singly inoculated (initial cell density ca. 7.5 cfu/g) in BSG homogenized with water at a 60:40 ratio and incubated at 30°C for 24 h. Native BSG and BSG incubated without the inoculum were used as control (Ct t0 and Ct t24). Fermentation was monitored by measuring, before and after incubation, pH and enumerating presumptive LAB using MRS (Oxoid, Basingstoke, Hampshire, United Kingdom) agar medium, supplemented with cycloheximide (0.1 g/L). Plates were incubated in anaerobiosis condition (AnaeroGen and AnaeroJar, Oxoid) at 30°C for 48 h. Yeasts, molds and total *Enterobacteria* were respectively enumerated on: Sabouraud Dextrose Agar (Oxoid) supplemented with chloramphenicol (0.1 g/L) at 25°C for 48 h; Potato Dextrose Agar (Oxoid) at 25°C for 48 h, and Violet Red Bile Glucose Agar (Oxoid) at 37°C for 24 h.

To select the strains able to induce the highest increase of the antioxidant activity, fermented BSG was subjected to methanolic and aqueous extraction, and extracts were analyzed for the radical scavenging activity, total phenols, peptide, and free amino acids concentration as described in section "Antioxidant Activity."

Set-up of the Bioprocessing Protocol

The enzymatic treatment with Depol (100 nkat/g dough) was performed contextually to fermentation with the strains *L. plantarum* PU1, H46, and PRO17 (initial cell density ca. 7.5 cfu/g) at 30°C for 24 h (EF30), or before fermentation, at 50°C for 5 h (E50 + F30) (Severini et al., 2015). Samples of BSG not inoculated with the LAB strains but added with xylanase and incubated in the conditions used for fermentation, were also prepared and used as control (eBSG). Antioxidant activity was evaluated on untreated, raw BSG (rBSG), eBSG, BSG treated with xylanase and fermented with *L. plantarum* PU1 (eBSG fPU1), H46 (eBSG fH46), and PRO17 (eBSG fPRO17). The list of treatments and codes is reported in **Table 1**.

Fermentation was monitored by measuring, before and after incubation, pH, enumerating presumptive LAB, yeasts, molds, and total *Enterobacteria* as described above.

Antioxidant Activity

Antioxidant Activity in Methanolic Extracts

Five grams of each sample were mixed with 50 mL of 80% methanol to get methanolic extracts (ME). The mixture was purged with nitrogen stream for 30 min, under stirring condition, and centrifuged at 4600 × *g* for 20 min. ME were further purged with nitrogen stream and stored at ca. 4°C before analysis. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity was determined on ME as described by Yu et al. (2003). The scavenging activity was expressed as follows: DPPH scavenging activity (%) = [(blank absorbance−sample absorbance)/blank absorbance] × 100. The value of absorbance was compared with 75 ppm butylated hydroxytoluene (BHT), which was used as the antioxidant reference. The analysis of total phenols in ME was performed according to the method of Slinkard and Singleton (1997) using gallic acid as standard.

Antioxidant Activity in Water/Salt Soluble Extracts (WSE)

Since polyphenols are not the only compounds capable of improving antioxidant properties, water/salt soluble extract (WSE) from raw or bioprocessed BSG were prepared according to the method originally described by Osborne and modified by Weiss et al. (1993). In details, 25 g of BSG slurry were suspended in 12 mL of 50 mM Tris–HCl (pH 8.8), held at 4°C for 1 h, in stirring conditions, and centrifuged at 20,000 *g* for 20 min. The supernatant was used for analyses. The radical cation [2,2′-azino-di-(3-ethylbenzthiazoline sulfonate)] (ABTS+) scavenging capacity of the WSE was measured using the Antioxidant Assay Kit CSO790 (Sigma Chemical Co.), following the manufacturer's instruction. Trolox (6-hydroxy 2,4,7,8-tetramethylchroman-2-carboxylic acid) was used as standard. The scavenging activity was expressed as Trolox equivalent.

Water/salt soluble extract were then used to analyze peptides, and total free amino acids (TFAA). Peptides concentration was determined by the *o*-phtaldialdehyde (OPA) method as described by Church et al. (1983). TFAA were analyzed by a Biochrom30 series Amino Acid Analyzer (Biochrom Ltd., Cambridge Science Park, United Kingdom) with a Na-cation exchange column (20 cm × 0.46 cm internal diameter as reported in Rizzello et al., 2010).

Inhibition of Linoleic Acid Peroxidation

One milligram of freeze-dried WSE and ME of each sample was suspended in 1.0 mL of 0.1 M phosphate buffer (pH 7.0) and added to 1 mL of linoleic acid (50 mM), previously dissolved on ethanol (99.5%). After incubation, at 60°C in the dark for 8 days, in a glass test tube tightly sealed with silicone rubber cap, linoleic acid degree of oxidation was

TABLE 1 | List of treatments and samples codes.

Code	Description
rBSG	Untreated, raw BSG
EF30: Bioprocessing protocol including addition of xylanase and simultaneous fermentation at 30°C for 24 h	
eBSG	BSG added with xylanase, not inoculated and incubated at 30°C for 24 h
eBSG fH46	BSG added with xylanase, inoculated with *L. plantarum* H46 and incubated at 30°C for 24 h
eBSG fPU1	BSG added with xylanase inoculated with *L. plantarum* PU1 and incubated at 30°C for 24 h
eBSG fPRO17	BSG added with xylanase inoculated with *L. plantarum* PRO17 and incubated at 30°C for 24 h
E50 + F30: Bioprocessing protocol including sequential treatment with xylanase at 50°C for 5 h and fermentation at 30°C for 24 h	
eBSG	BSG treated with xylanase at 50°C for 5 h, and further incubated at 30°C for 24 h (not inoculated)
eBSG fH46	BSG treated with xylanase at 50°C for 5 h, then inoculated with *L. plantarum* H46 and fermented at 30°C for 24 h.
eBSG fPU1	BSG treated with xylanase at 50°C for 5 h, then inoculated with *L. plantarum* PU1 and fermented at 30°C for 24 h.
eBSG fPRO17	BSG treated with xylanase at 50°C for 5 h, then inoculated with *L. plantarum* PRO17 and fermented at 30°C for 24 h.

determined by measuring the values of ferric thiocyanate according to the method described by Mitsuda (1966). One hundred microliters of the above sample were mixed with 4.7 mL of 75% (v/v) ethanol, 0.1 mL of 30% (w/v) ammonium thiocyanate, and 0.1 mL of 0.02 M ferrous chloride, dissolved in 1 M HCl. After 3 min, the degree of color development, representing the oxidation of linoleic acid, was measured spectrophotometrically at 500 nm. BHT (1 mg/mL) was also assayed as antioxidant references. A reference sample (without the addition of antioxidants) was included in the assay as negative control.

Microstructure Characterization and Dietary Fibers Analysis

Sample Preparation for Microscopy

Brewers' spent grain treated as described in section "Set-up of the Bioprocessing Protocol" were frozen in liquid nitrogen, embedded in PELCO Cryo-Embedding Compound (Ted Pella inc., Redding, CA, Untied States) and cut into 10 μm thick sections in a Leica CM 1900. Sections were applied to polysine coated microscopy slides.

Bright Field Microscopy

Sections were stained with light green to visualize starch and proteins. The stained sections were examined using a Nikon microphot-FXA microscope and micrographs captured with a DFK33UX264 camera (The Imaging Source Europe GmbH, Bremen, Germany) and processed with the software NIS-Elements D (Nikon Instruments Europe, Amsterdam, Netherlands).

Immunolocalization of AX by Confocal Laser Microscopy

For immunolabelling, sections were fixated for 30 min in 4% paraformaldehyde in PBS buffer (pH 7.4) and rinsed with PBS. The sections were then pre-incubated 40 min in PBS buffer with 2.5% BSA before applying the primary antibody, LM11 (Plant Probes, Leeds, United Kingdom) diluted 1:50 in PBS containing 0.5% BSA, for 2 h. Negative controls were made by replacing the primary antibody solution with PBS containing 0.5% BSA. After incubation, the sections were rinsed thoroughly with PBS and then incubated for 2 h in the dark with fluorescently labeled secondary antibody Alexa Fluor® 647 (Invitrogen, Carlsbad, CA, United States). All incubations were performed in moisturized chambers at room temperature. Sections were then rinsed with PBS and water and mounted with ProLong®Diamond (Invitrogen, Carlsbad, CA, Untied States) anti-fading reagent.

Micrographs were acquired using a confocal laser microscopy (CLSM; Leica TCS SP5, Heidelberg, Germany) A 488 nm argone laser and a 633 nm HeNe laser with a HCX PL APO lambda blue 20.0 × 0.70 IMM UV objective, zoom 1× and 3×. Emissions were collected at 500–550 and 650–700 nm. Image format 1024 × 1024 pixels, eight lines average.

Dietary Fibers Analysis

To further confirm the results obtained from microscopy analysis, insoluble and soluble dietary fibers content was determined in raw and bioprocessed BSG according to the official AOAC International (2007).

Ex vivo Assays on Human Keratinocytes

The human keratinocyte cell line NCTC 2544 was obtained from the National Institute for Cancer Research of Genoa, Italy. Cells were cultivated in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM l-glutamine, 1% penicillin (100 U/mL)/streptomycin (100 U/mL) and 0.1% of gentamicin. NCTC 2544 cells were incubated in 25 cm^2 surface culture flasks at 37°C with 5% CO_2 (Sanchez et al., 2004). When ca. 80% of confluence was reached, cells were harvested with trypsin/EDTA and seeded at a density of 5×10^4 cells per well into 96 well plates for 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide proliferation (MTT) assay, and oxidative stress tests. Medium and all chemicals were purchased from Sigma Aldrich (Italy).

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide proliferation assay was used for the determination of the viability of H_2O_2-stressed NCTC 2544 cells (Coda et al., 2012). Cells were incubated with raw and bioprocessed BSG WSE for 16 h. The concentrations were 0.1, 0.5, and 1 mg/mL; α-tocopherol (250, 500, and 1000 μg/mL) was used as the positive control. At the end of incubation, MTT assay was performed

(Mosmann, 1983). Data were expressed as the percentage of viable cells compared to negative control. Each experiment was carried out in triplicate.

Characterization of the Phenolic Profile

Extraction of Phenolic Compounds and Qualitative and Quantitative Analysis by UPLC-PDA-ESI-QTOF

Free and bound phenolics were extracted as described in Verardo et al. (2011). The analysis of BSG free and bound polyphenols was carried out with the use of an ACQUITY Ultra Performance LC system equipped with photodiode array detector with a binary solvent manager (Waters Corporation, Milford, MA, United States) series with a mass detector Q/TOF micro mass spectrometer (Waters) equipped with an electrospray ionization (ESI) source operating in negative mode at the following conditions: capillary voltage, 2300 kV; source temperature, 100°C; cone gas flow, 40 L/Hr; desolvatation temperature, 500°C; desolvatation gas flow, 11,000 L/h; and scan range, m/z 50–1500. Separations of individual polyphenols were carried out using an ACQUITY UPLC BEH Shield RP18 column (1.7 μm, 2.1 mm × 100 mm; Waters Corporation, Milford, MA, United States) at 40°C. The elution gradient was carried out using water containing 1% acetic acid (A) and acetonitrile (B), and applied as follows: 0 min, 1% B; 2.3 min, 1% B; 4.4 min, 7% B; 8.1 min, 14% B; 12.2 min, 24% B; 16 min, 40% B; 18.3 min, 100% B, 21 min, 100% B; 22.4 min, 1% B; 25 min, 1% B. The sample volume injected was 2 μL and the flow rate used was 0.6 mL/min. The compounds were monitored at 280 nm. Integration and data elaboration were performed using MassLynx 4.1 software (Waters Corporation, United States). For the quantification of phenolic compounds, solutions of ferulic acid, chlorogenic acid, catechin, and quercetin in methanol were prepared and used as standard.

Extraction and Quantification of Proanthocyanidins

Proanthocyanidins were extracted as described by Verardo et al. (2015) and analyzed by HPLC-FLD analysis, performed by an Agilent 1200 Series (Agilent Technologies, Santa Clara, CA, United States), equipped with a Luna Hilic column (150 × 2.0 mm; 3 μm) and a fluorescence detector with an excitation wavelength of 230 nm and an emission wavelength of 321 nm. The separation was performed with 98% acetonitrile and 2% acetic acid (A) and 95% methanol, 3% water, and 2% acetic acid (B) as described by Hollands et al. (2017).

Characterization of Peptide Profiles

Purification of Antioxidant Peptides

Water/salt soluble extracts obtained from BSG treated with xylanase at 50°C for 5 h and further fermented with *L. plantarum* PU1, which showed the highest radical scavenging activity, was first fractionated by ultra-filtration (Ultrafree-MC centrifugal filter units, Millipore) using membrane sizes of 50, 30, 10, and 3 kDa cut-off, following the manufacturer's instructions. Fractions were tested for the antioxidant activity on DPPH radical. The WSE fraction with a molecular mass of <3 kDa was further automatically fractionated (2 mL per fraction, 32 fractions for each run) by reversed-phase fast performance liquid chromatography (RP-FPLC), using a Resource RPC column and an ÄKTA FPLC equipment, with the UV detector operating at 214 nm (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Gradient elution was performed at a flow rate of 1 mL/min using a mobile phase composed of water and acetonitrile (CH_3CN), containing 0.05% TFA. The concentration of CH_3CN was increased linearly from 5 to 46% between 16 and 62 min, and from 46 to 100% between 62 and 72 min. Solvents were removed from collected fractions by freeze drying. Fractions were re-dissolved in sterile water, assayed for the antioxidant activity on DPPH radical and the peptide content by the OPA method as described above (Rizzello et al., 2010).

Proteolysis and Heat Stability

Water/salt soluble extracts and purified fractions, which showed the highest antioxidant activities, were subjected to sequential protein hydrolysis by digestive enzymes (pepsin, pancreatin, and trypsin) according to the method described by Pasini et al. (2001). Digested samples were heated for 5 min at 100°C and centrifuged at 12,000 × *g* for 20 min to recover the supernatants. After treatments, samples were subjected the scavenging activity on radical DPPH as described above.

Identification of Antioxidant Peptides

The peptides contained in the WSE fractions with the highest radical-scavenging activity were subjected to identification. The identification was carried out by nano-Liquid Chromatography-Electrospray Ionization-Mass Spectra/Mass Spectra (nano-LC-ESI-MS/MS), using a Finnigan LCQ Deca XP Max ion trap mass spectrometer (ThermoElectron) through the nano-ESI interface. According to manufacturer's instrument settings for nano-LC-ESI-MSMS analyses, MS spectra were automatically taken by Xcalibur software (ThermoElectron), in positive ion mode. MS/MS spectra were processed using the software BioWorks 3.2 (ThermoElectron) generating peak lists suitable for database searches. Peptides were identified using MS/MS ion search of Mascot search engine (Matrix Science, London, United Kingdom) and NCBInr protein database (National Centre for Biotechnology Information, Bethesda, MD, United States). For identification of peptides the following parameters were considered: enzyme: "none"; instrument type: "ESI-trap"; peptide mass tolerance: ±0.1% and fragment mass tolerance: ±0.5 Da. Results from peptide identification were subjected to a manual evaluation, as described by Chen et al. (2005), and the validated peptide sequences explained all the major peaks in the MS/MS spectrum.

Statistical Analysis

All the microbiological and chemical analysis were carried out in triplicate for each batch of BSG. Data were subjected to one-way ANOVA; pair-comparison of treatment means was achieved by Tukey's procedure at $P < 0.05$, using the statistical software Statistica 8.0 (StatSoft Inc., Tulsa, OK, United States). Data of the *ex vivo* assays were analyzed with a statistical software: GraphPad Prism v7.00 (GraphPad Software Inc.). Student's *t*-test with was

Mann–Whitney correction was used. *P*-values equal to or less than 0.05 were considered significant.

RESULTS

Starter Selection

Before incubation BSG had a pH of 5.87 ± 0.08 which significantly decreased after 24 h of spontaneous fermentation at 30°C (5.01 ± 0.05). After fermentation, the pH of the inoculated samples ranged from 3.87 ± 0.03 to 4.42 ± 0.04. The lowest variation was observed when BSG was fermented with *W. confusa* NEY6. On the contrary, *L. plantarum* H48, PU1, T6C16, T0A10, PRO17, *P. pentosaceus* OA1, and *W. confusa* KAS3 caused the highest pH decrease, reaching values lower than 4 at the end of fermentation.

Cell density of presumptive LAB in rBSG was lower than 2 log cfu/g, while yeasts and total *Enterobacteriaceae* were 4.76 ± 0.03, and 3.58 ± 0.03 log10 cfu/g, respectively. Molds were not found (<10 cfu/1 g). When BSG was inoculated (initial cell density ca. 7.5 log10 cfu/g) an increase of *ca.* 2 log cycles of presumptive LAB cell density was observed (final values ranged from 9.36 to 9.67 log10 cfu/g). The highest final cell density was observed for *L. plantarum* H48, 1A7, and PRO17. At the end of fermentation, yeasts and *Enterobacteriaceae* cell density was lower than 4.12 ± 0.02 and 2.10 ± 0.03 log10 cfu/g, respectively, in all the BSG samples.

The main criterion for LAB selection was the ability of fermented BSG extracts to scavenge DPPH and ABTS radicals, compared to the untreated BSG. The scavenging activity on DPPH for rBSG was 42.5 ± 2.3%. Overall, activity increments up to 7% were observed. The highest increase ($P < 0.05$) was observed for *L. plantarum* PU1 and H46 with antioxidant activity of 48 ± 0.96 and 49 ± 0.78%, respectively. Significant ($P < 0.05$) increases of the ABTS scavenging activity were also found during fermentation. BSG fermented with *L. plantarum* PRO17, *P. pentosaceus* I76 and I214 showed an increase ($P < 0.05$) exceeding 0.2 mM Trolox equivalent, more than 33% higher than BSG before fermentation (0.17 ± 0.05 mM Trolox equivalent).

The total phenol content of BSG methanol extracts was determined using the Folin–Ciocalteu method. Before incubation rBSG had total phenol content 2.09 ± 0.16 mmol/100 g. Except for the spontaneously fermented control (Ct t24), in which a decrease ($P < 0.05$) was observed, for all fermented BSG, total phenol content remained stable or increased. In BSG fermented with *L. plantarum* LB1, 18S9, MRS1, PRO17, *F. rossiae* LB5, T0A16, *Lv. brevis* MRS4, *P. pentosaceus* BAR4, OA1, S3N3 an increase ranging from 10 to 20% was observed. Fermentation with *L. plantarum* PU1, *L. mesenteroides* 12MM1, I57, *P. pentosaceus* I56, I76 and F01 allowed an increase higher than 30%, reaching values up to 2.71 mmol/100 g.

Peptides concentration before fermentation was 74.65 ± 2.25 mg/g (d.m.) and no changes were observed after spontaneous fermentation. When fermented with LAB, 12 out of 33 strains caused an increase of peptide content up to 20%, reaching 98 mg/g when fermented with *L. mesenteroides* 12MM1. Amino acids content was from two to ninefold higher than that of rBSG (215 ± 10.87 mg/kg on d.m.) in all the fermented BSG samples.

All the data obtained from the biochemical characterization of raw and fermented BSG were subjected to principal component analysis (PCA) as shown in **Figure 1**. Factor 1 and 2 represented 32.95 and 24.10% of the variance, respectively. Untreated BSG and BSG incubated without LAB clearly separated from the fermented sample; Factor 1 separated fermented BSG having lower content in peptides and amino acids from those with higher content.

Bioprocessing

Lactiplantibacillus plantarum PU1, H46, and PRO17 were used in combined bioprocessing with Depol 761P. The use of the enzyme simultaneously with LAB did not enhance the scavenging activity on DPPH compared to BSG fermented without enzyme (**Figure 2**).

On the contrary, the enzymatic treatment before fermentation at 50°C for 5 h positively influenced the antioxidant properties, increasing the radical scavenging activity up to 18% in ME and 40% in WSE. Compared to rBSG, the xylanase treatment carried out without the inoculum of the selected LAB (eBSG) led to a slight but significant decrease of the antioxidant activity when incubation was carried out at 30°C for 24 h (EF30), or to a significantly lower increase than those observed for eBSF fH46, eBSG fPU1, eBSG fPRO17 when the pre-incubation at 50°C for 5 h was carried out (**Figure 2**). In both cases, cell density of LAB ranged from 5.46 ± 0.03 to 5.71 ± 0.02 log10 cfu/g, while yeasts and *Enterobacteriaceae* were 5.42 ± 0.02 and 4.50 ± 0.03, and 2.42 ± 0.03 and 4.20 ± 0.03 log10 cfu/g, respectively in EF30 and E50 + F30 eBSG. Based on these results, the sequential use of xylanase and LAB fermentation E50 + F30 was selected as the bioprocessing option and used for further experiments.

Linoleic Acid Peroxidation

Extracts from raw and treated BSG were tested for the ability to inhibit lipid peroxidation. During linoleic acid oxidation, peroxides oxidize Fe^{2+} to Fe^{3+}. The Fe^{2+} ions form a complex with thiocyanate, which has a maximum absorbance at 500 nm. In this method, the concentration of peroxides decreases as the antioxidant activity increases (Omwamba and Hu, 2010). Compared to the reference (reaction mixture without antioxidants), the presence of all extracts inhibited linoleic acid autoxidation (**Figure 3**). The oxidation of linoleic acid was markedly inhibited by the addition of WSE and ME from bioprocessed BSG, comparable to that of BHT. There were no differences ($P < 0.05$) among bioprocessed samples whereas the WSE and ME of rBSG had a significant lower effect on linoleic acid inhibition.

Microstructure Characterization and Dietary Fibers and Analysis

Morphology Characterization of Bioprocessed BSG

Intact layers of aleurone cells and large clusters of connective endosperm proteins characterized rBSG. Xylanase treatment (eBSG), caused the extensive degradation of the aleurone cells,

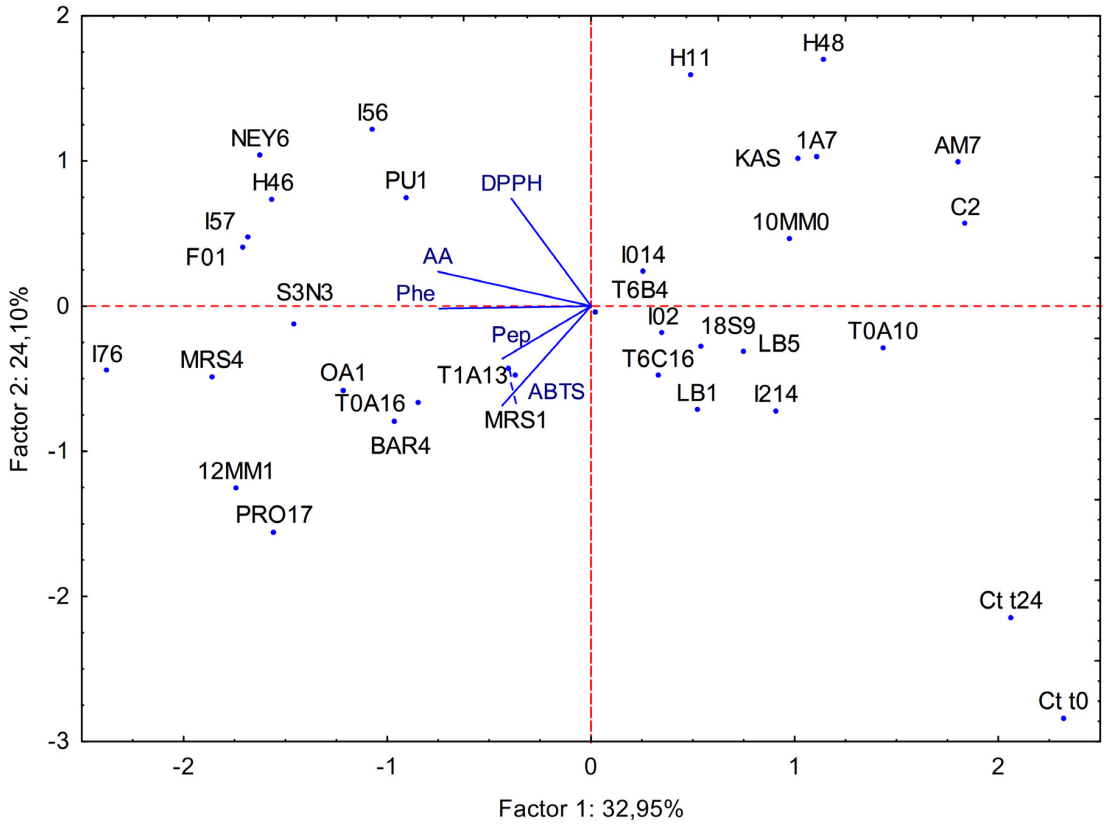

FIGURE 1 | Principal component analysis (PCA) of the biochemical characteristics of raw BSG (Ct t0), spontaneously fermented BSG (Ct t24) and BSG fermented with selected acid bacteria (name of the strains are reported in section "Microorganisms"). ABTS, antioxidant activity on ABTS; Pep, peptide concentration; AA, amino acid concentration; Phe, phenolic compounds content; DPPH, antioxidant activity on DPPH.

and the disassembling of the large protein clusters, apart for some proteins (located in the aleurone cells) still forming globular structures. When fermentation with selected starters followed the enzymatic treatment, no further distinct impact on the microstructure was observed, although the protein appeared unevenly dispersed (**Figure 4**).

Localization of AX

In rBSG, AX were mainly located in the aleurone cell walls. Small amounts were also observed in the pericarp. In eBSG, as expected, AX of the aleurone cells walls were markedly degraded and disorganized. Contrarily to rBSG, AX fragments dispersed in the matrix were observed in eBSG. Very few AX were detectable in bioprocessed samples inoculated with LAB after the xylanase treatment, both in aleurone cell walls and dispersed in the matrix, especially when *L. plantarum* PU1 and PRO17 were used as starters. Nevertheless, residual bound AX were seen in pericarp after enzymatic treatment and fermentation. Overall, the sequential bioprocessing treatments caused the protein arrangement in rows with globular structures (**Figure 5**).

Dietary Fibers Analysis

Compared to rBSG, all bioprocessed BSG also had a lower content of insoluble fibers (below 40% against the 49 ± 4% of rBSG) and higher content of soluble fibers (ranging from 4.7 ± 1.9 to 5.6 ± 0.7% against 1.5 ± 0.3% found in rBSG).

Ex vivo Assays on Human Keratinocytes

Brewers' spent grain antioxidant properties were investigated on human keratinocytes line NCTC 2544. The cells subjected to oxidative stress were grown in the presence of untreated and bioprocessed BSG. Compared to cell viability after oxidative stress (25.5 ± 3.6%), α-tocopherol, used as reference for the antioxidant activity, significantly ($P < 0.05$) increased cell survival. All the samples had a protective effect comparable to that of α-tocopherol when tested at 0.1 and 0.5 mg/mL (cell survival of ca. 50%). When tested at 1 mg/mL, survival significantly decreased up to 30% in rBSG and eBSG, whereas only fermented BSG showed protection with a vitality of 42.41 ± 3.37, 39.29 ± 4.62, and 43.30 ± 1.18% for eBSG fH46, eBSG fPRO17, and eBSG fPU1, respectively.

Characterization of the Phenolic Profile

Free and Bound Phenolic Compounds

Phenolic and other polar compounds of free and bound extracts were separated and identified by UPLC-PDA-ESI-QTOF. **Supplementary Table S1** summarizes the information related to the compounds tentatively identified: retention times,

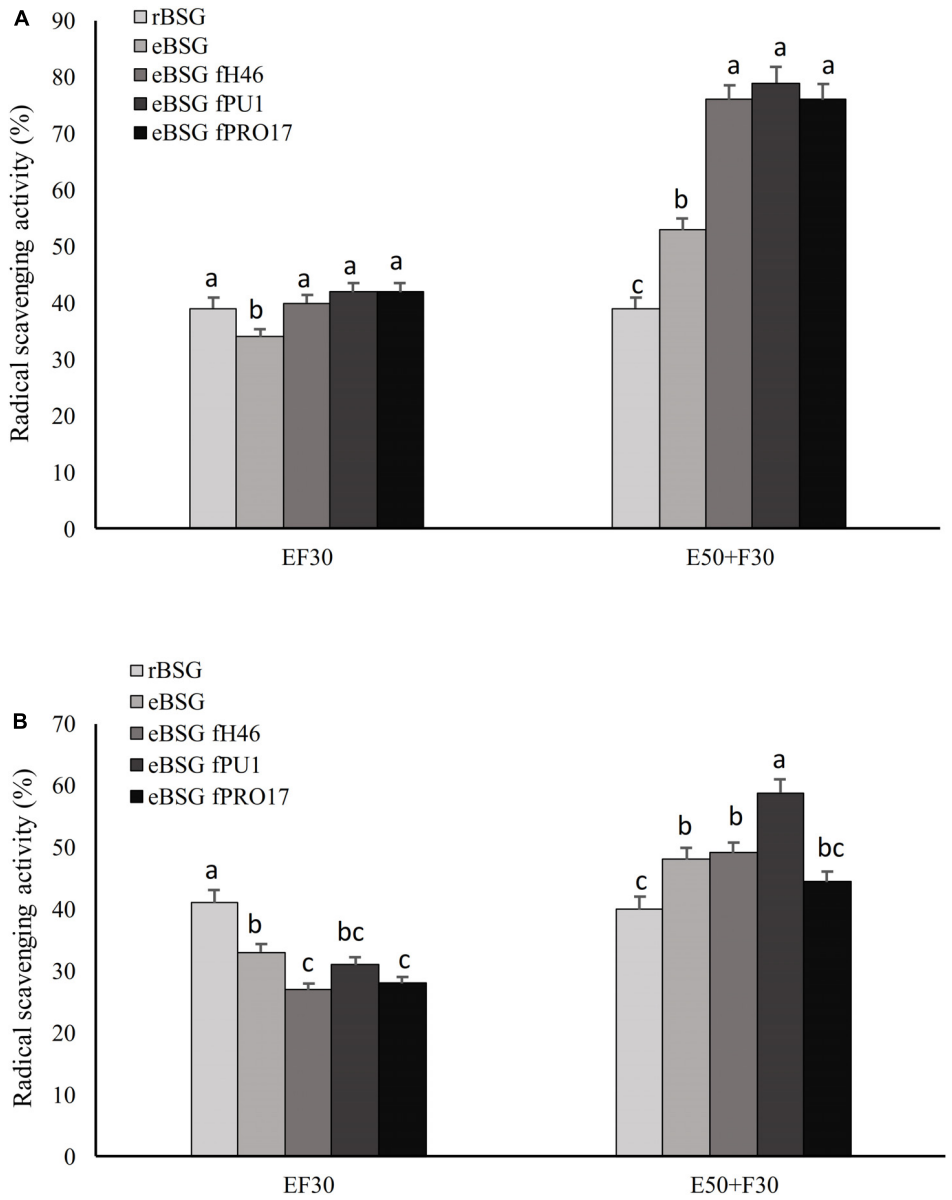

FIGURE 2 | Scavenging activity on DPPH radical of the water/salt soluble **(A)** and methanol extracts **(B)** of raw BSG (rBSG); BSG treated with xylanase and not inoculated (eBSG); BSG treated with xylanase and fermented with *L. plantarum* PU1 (eBSG fPU1), H46 (eBSG fH46), and PRO17 (eBSG fPRO17). The enzymatic treatment was performed contextually to fermentation at 30°C (EF30) for 24 h, or before fermentation at 50°C for 5 h (E50 + F30). Data are the means from three independent experiments. Bars represent standard deviation. $^{a-c}$Columns with different superscript letters, within the same protocol, differ significantly ($P < 0.05$).

experimental and calculated m/z, molecular formula, fragments, score, and error (ppm). Forty-three phenolic compounds were identified between free and bound profiles (details in **Supplementary Material**, see section "Characterization of the Phenolic Compounds").

Bound phenolic profile was characterized by several phenolic acids and their derivatives. In particular, bound phenolics in rBSG resulted more than 50-fold higher than free phenolics (1768.11 ± 61.23 against 33.40 ± 1.19 mg/kg d.m.), this difference drastically changed with the treatment with xylanase, which freed 25% of bound phenols, most of which, ferulic acid derivatives (**Table 2**). Compared to rBSG, bound phenolics concentration decreased ($P < 0.05$) in eBSG, indeed, decrease of all ferulic acid dimers, trimers and tetramers was observed. Nevertheless, compared to eBSG, ferulic acid oligomers concentrations significantly increased in all fermented samples, up to three times higher, when *L. plantarum* PU1 was used as starter. Ferulic acid dimers were detected in higher concentration than rBSG only when fermentation was carried out by *L. plantarum* PU1 and PRO17. The combined effect of enzyme and fermentation allowed an increase of the total phenols extractability from the matrix, up to 18%, therefore exceeding 2 g/kg.

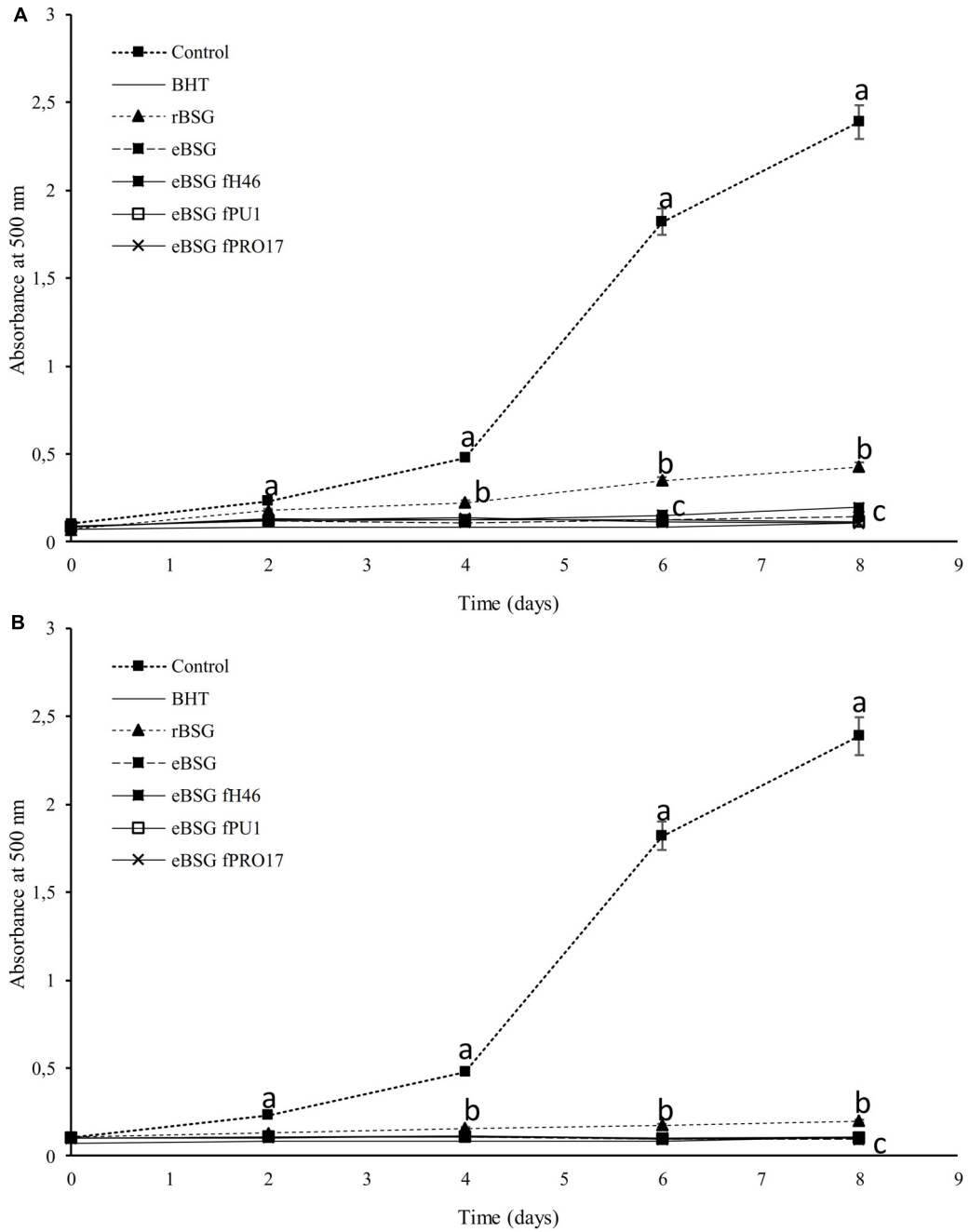

FIGURE 3 | Lipid peroxidation inhibitory activity of the WSE **(A)** and ME **(B)** in raw BSG (rBSG); BSG treated with xylanase and not inoculated (eBSG); BSG treated with xylanase and fermented with *L. plantarum* PU1 (eBSG fPU1), H46 (eBSG fH46), and PRO17 (eBSG fPRO17). The activity was measured under linoleic acid oxidation system for 8 days. BHT (1 mg/mL) was used as the positive control. The reaction mixture without the addition of the antioxidant was considered as a negative control. Data are the means from three independent experiments. Bars represent standard deviation. $^{a-c}$Points with different superscript letters differ significantly ($P < 0.05$).

A slight but significant ($P < 0.05$) decrease was observed for quercetin in eBSG compared to rBSG. As for catechin and epicatechin, a significant decrease was also observed during fermentation with *L. plantarum* PRO17. An intense metabolic activity on phenolic acids was observed. Indeed, higher concentrations of vanillic, caffeic, *o*-coumaric, and sinapic acid, and lower of ferulic acid were found in the bound fraction of the fermented BSG.

Free phenolics profile of rBSG was less complex than bioprocessed BSG (**Table 2**). Ferulic acid derivatives were not found in rBSG instead they were at concentration higher than 400 mg/kg in eBSG. A slight but significant reduction of their

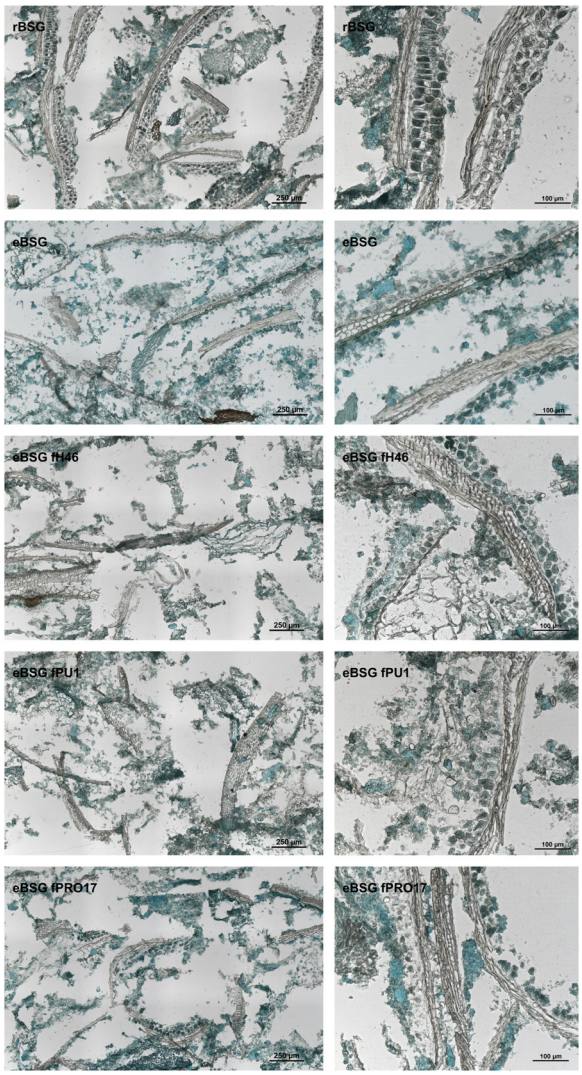

FIGURE 4 | Micrographs of raw BSG (rBSG); BSG treated with xylanase and not inoculated (eBSG); BSG treated with xylanase and fermented with *L. plantarum* PU1 (eBSG fPU1), H46 (eBSG fH46), and PRO17 (eBSG fPRO17). Proteins are shown in green and cell walls/fibers are shown in brown. Scale bars on **left** column are 250 μm and on **right** column are 100 μm.

content was observed after fermentation, probably due to a further release of ferulic acid and its consequent reduction to dihydroferulic acid, which increased up to 15-fold during fermentation. Caffeic and coumaric acid liberated from bound phenolics during the incubation were also metabolized to their respective reduced forms, dihydrocaffeic and phloretic acids which increased up to seven and threefold, respectively. The highest metabolites concentrations were found for eBSG fH46.

Free Proanthocyanidin Quantification by HPLC-FLD

Extracts from raw and bioprocessed BSG, containing the proanthocyanidin fraction of all phenolic compounds, were subjected to chromatographic analysis. Although the separation poorly allowed to distinguish among the galloylated forms, a clear separation between monomers and oligomers was obtained. Despite the similar trend among samples, proanthocyanidin monomers resulted almost 10-fold higher compared to the data of free phenolics, reaching 68 ± 3 mg/kg in rBSG. Indeed, fluorescence was proved to be more suitable than UV detection, with increased selectivity for procyanidins and reduced interferences from other absorbing compounds. Multiple studies found the detection limits using fluorescence to be nearly one thousand times lower than those observed with UV detection (Hümmer and Schreier, 2008).

When BSG was fermented after the enzymatic treatment, polymeric forms of proanthocyanidins were under the detection limit (**Supplementary Figure S1**). A slight but significant reduction of proanthocyanidin oligomers was also found in eBSG (44 ± 3 against 32 ± 2 mg/kg of rBSG), probably due to the activity of endogenous microbiota.

Characterization of Potentially Bioactive Peptides

Antioxidant activity of the WSE of bioprocessed BSG was not affected ($P < 0.05$) by enzymatic digestion and heating. All the fractions obtained from the first separation (corresponding to the permeate at cut-off 50, 30, 10, and 3 kDa) did not show decrease in radical scavenging activity. Based on these results, it was hypothesized that active compounds possessed molecular mass lower than 3 kDa. WSE permeate at <3 kDa was further purified by RP-FPLC, obtaining 33 fractions (peptide concentration in the range 0.40 ± 0.02–12.05 ± 0.06 mg/mL).

The highest antioxidant activity was found in fractions 11 and 15 (31 and 39%, respectively), while only weak activity (19–22%) was found in fractions 2 and 22. Fractions eluted at 12 and 20% of eluent B. Fractions were characterized by peptide concentration ranging from 5.11 ± 0.05 to 8.05 ± 0.06 mg/mL).

Five peptides, having 8–10 amino acid residues, were identified by nano-LC-ESI-MS/MS analysis. A mixture of peptides was identified in all the active fractions. LFGFTYLR (molecular mass of 1016.21 Da), LVLANAIYFK (1151.410 Da), and VGYVANFCK (1000.18 Da) had net charge of 1. While IFLENVIR (1003.21 Da) and EVQMDFVR (1000.18 Da) had net charge of 0 and −1, respectively. Peptides were reported in the NCBI database as encrypted into sequences of *H. vulgare* proteins: beta-amylase (LFGFTYLR), alpha-amylase/trypsin inhibitor (EVQMDFVR), and predicted protein (VGYVANFCK); *Z. mays* proteins: histone H4 (IFLENVIR) and putative serpin-Z12 (LVLANAIYFK). Identified peptides contained from 8 to 10 amino acid residues. Total hydrophobicity ratio ranged from 50 (LFGFTYLR and EVQMDFVR) to 70% (LVLANAIYFK). Peptides were rich in tyrosine (Y), leucine (L), alanine (A), isoleucine (I), valine (V), phenylalanine (F), and contained one or more aromatic amino acids.

DISCUSSION

The increasing awareness of the benefits of a healthier diet and lifestyle is encouraging the study and management of

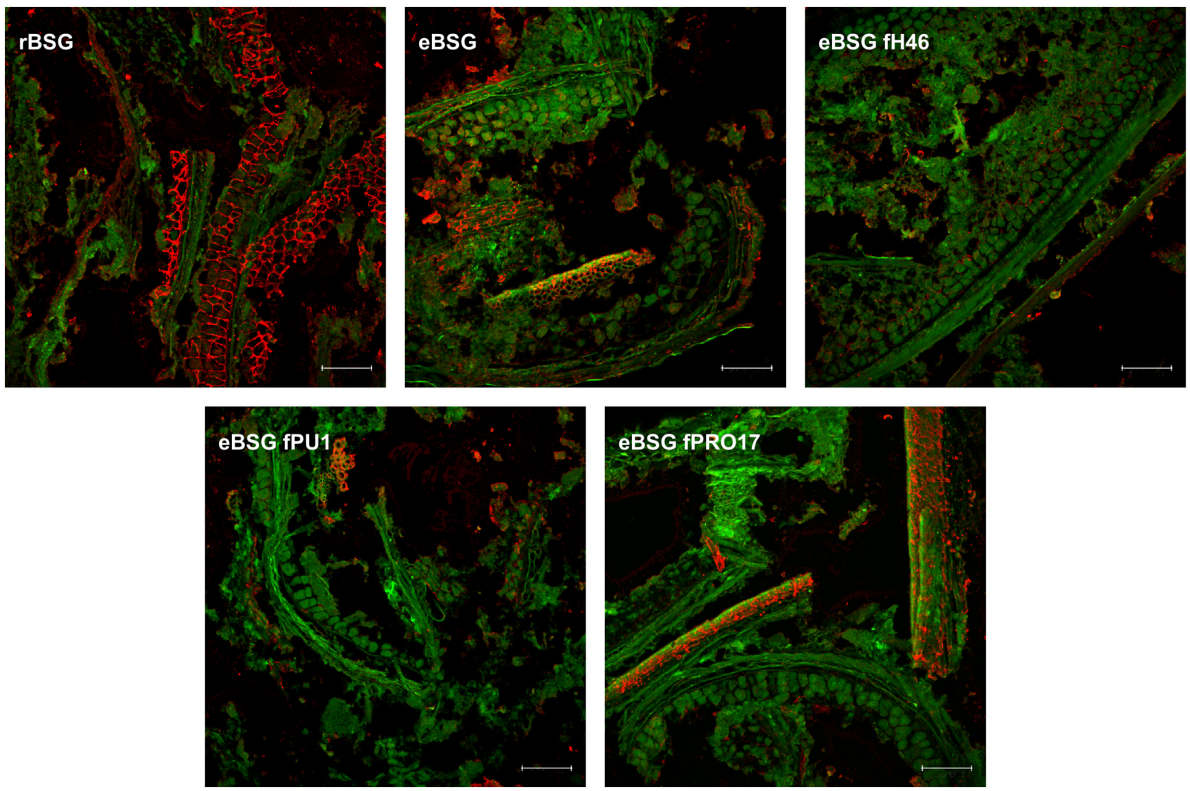

FIGURE 5 | CLSM micrographs of raw BSG (rBSG); BSG treated with xylanase and not inoculated (eBSG); BSG treated with xylanase and fermented with *L. plantarum* PU1 (eBSG fPU1), H46 (eBSG fH46), and PRO17 (eBSG fPRO17). Arabinoxylans are shown in red after labeling with LM11 antibody. BSG morphology is shown in green with autofluorescence. Scale bars correspond to 25 μm.

antioxidant compounds in food, since they represent the most feasible way of protection against free radicals. In humans, the generation of free radicals, unstable and highly reactive chemical species, increases as consequence of the exposure to different physiochemical conditions and pathological states. Free radicals are responsible for oxidative stress, associated to aging, cancer, cardiovascular and neurodegenerative diseases, atherosclerosis, and inflammatory state (Lobo et al., 2010).

In this study, BSG was bioprocessed with the aim of enhancing its antioxidant potential, foreseeing its use as functional food ingredient. The potential of food fermentation in improving antioxidant properties has been highlighted in multiple occasions (for review see Verni et al., 2019). Thanks to its high content of phenolic acids and other potentially bioactive precursors, BSG is a good substrate for the formation of compounds with high antioxidant potential. A preliminary screening of thirty-tree LAB (details in **Supplementary Material**) allowed the selection of three strains responsible for the most relevant increase of the antioxidant activity in the fermented matrix. Hence, *L. plantarum* PU1, H46 and PRO17, were selected and used for the set-up of a combined bioprocess including the use of a food-grade xylanase.

Together with the increase of the radical scavenging activity, tested on DPPH and ABTS radicals, the capacity of the strains to release phenolic compounds and protein derivatives (peptides and free amino acids) was investigated, since all these compounds are potentially responsible for the antioxidant properties of a fermented matrix (Verni et al., 2019). In addition to fermentation, xylanase was used to degrade the hemicellulose fraction (Robertson et al., 2010), therefore improving the release of phenolic compounds bound to AX of the BSG cell walls. The enzymatic pre-treatment at 50°C for 5 h markedly increased the radical scavenging activity compared to the simultaneous enzymatic/fermentative bioprocess and was therefore selected as the optimal bioprocessing option.

Bioprocessed BSG, subjected to the sequential enzymatic/fermentative treatment, was also able to inhibit lipid peroxidation during 8 days of incubation. Among the different factors involved in this effect, phenolic compounds might have played a crucial role, since they are able to scavenge lipid-derived radicals thereby breaking the free radical chain reaction of lipid peroxidation (Cheng et al., 2003). From a technological point of view, the persistence of the antioxidant activity could prevent food from oxidation, thus reducing loss of nutrients, and maintaining texture, color pigments, taste, freshness, functionality, and aroma (Verni et al., 2019).

To clarify the effects of bioprocessing on BSG, the overall morphology of samples was examined with light microscopy and both content and localization of AX, using differential staining, were visualized by CLSM. As expected, the xylanase treatment allowed an extensive cell wall disruption, contributing

TABLE 2 | Quantification (mg/kg d.w.) of the phenolic compounds identified in raw BSG (r-BSG), treated with xylanase (eBSG), treated with xylanase (5 h at 50°C) and sequentially fermented (at 30°C for 24 h) with *L. plantarum* PU1 (eBSG fPU1), H46 (eBSG fH46), and PRO17 (eBSG fPRO17) obtained by HPLC-DAD-ESI-QTOF-MS.

	rBSG	eBSG	eBSG fH46	eBSG fPU1	eBSG fPRO17
Bound phenolic compounds					
5,7-dihydroxychromone	11.36 ± 0.48^{a}	4.30 ± 0.12^{b}	4.65 ± 0.17^{b}	2.93 ± 0.12^{c}	3.08 ± 0.11^{c}
Vanillic acid	6.78 ± 0.27^{c}	8.25 ± 0.34^{b}	12.07 ± 0.29^{a}	11.66 ± 0.35^{a}	12.11 ± 0.37^{a}
Catechin	5.89 ± 0.26^{a}	5.12 ± 0.20^{b}	6.11 ± 0.18^{a}	5.80 ± 0.23^{a}	1.39 ± 0.10^{c}
Caffeic acid	34.98 ± 1.40^{ab}	28.33 ± 1.10^{c}	32.36 ± 1.17^{b}	38.70 ± 1.23^{a}	37.96 ± 1.28^{a}
Epicatechin	1.53 ± 0.06^{b}	1.41 ± 0.06^{b}	1.71 ± 0.05^{a}	1.73 ± 0.06^{a}	1.02 ± 0.03^{c}
p-cumaric acid	40.85 ± 1.78^{b}	30.43 ± 1.21^{c}	39.99 ± 1.26^{b}	42.29 ± 1.36^{b}	47.58 ± 1.68^{a}
o-cumaric acid	181.00 ± 6.74^{b}	139.13 ± 5.69^{c}	184.70 ± 6.20^{b}	195.89 ± 6.47^{ab}	211.77 ± 7.28^{a}
Ferulic acid	52.82 ± 2.22^{a}	31.60 ± 1.30^{c}	38.09 ± 1.24^{b}	22.63 ± 0.78^{d}	23.51 ± 0.82^{d}
Isoferulic acid	311.79 ± 13.08^{b}	267.18 ± 9.98^{c}	367.02 ± 13.48^{a}	373.28 ± 14.26^{a}	377.19 ± 13.74^{a}
Ferulic acid dimers	568.08 ± 20.81^{b}	382.03 ± 14.66^{c}	588.69 ± 20.96^{ab}	630.86 ± 23.46^{a}	620.79 ± 23.21^{a}
Sinapic acid	15.33 ± 0.71^{b}	15.61 ± 0.69^{b}	22.29 ± 0.78^{a}	22.69 ± 0.78^{a}	22.03 ± 0.81^{a}
Ferulic acid tetramers	449.61 ± 15.82^{c}	332.28 ± 12.62^{d}	467.73 ± 17.34^{c}	580.26 ± 20.63^{a}	535.66 ± 18.89^{b}
Quercetin	0.56 ± 0.01^{a}	0.44 ± 0.01^{b}	0.52 ± 0.02^{ab}	0.53 ± 0.01^{ab}	0.52 ± 0.02^{ab}
Ferulic acid trimers	87.52 ± 3.68^{d}	59.17 ± 2.43^{e}	111.55 ± 3.45^{c}	141.03 ± 4.57^{a}	121.62 ± 4.24^{b}
Total	1768.11 ± 61.23^{c}	1305.29 ± 53.51^{d}	1877.48 ± 51.33^{b}	2070.28 ± 79.74^{a}	2016.21 ± 77.64^{a}
Free phenolic compounds					
Dihydroferulic acid	3.35 ± 0.11^{b}	3.41 ± 0.14^{b}	52.79 ± 1.78^{a}	51.51 ± 1.65^{a}	48.37 ± 1.59^{a}
Dihydrocaffeic acid	5.09 ± 0.21^{b}	5.63 ± 0.22^{a}	36.61 ± 1.23^{c}	31.48 ± 1.46^{d}	33.56 ± 1.31^{c}
Phloretic acid	23.15 ± 0.87^{c}	22.83 ± 0.84^{c}	69.30 ± 2.39^{a}	56.09 ± 2.31^{b}	58.44 ± 2.12^{b}
Ferulic acid derivatives	–	444.79 ± 18.20^{a}	316.62 ± 11.98^{b}	294.47 ± 10.97^{b}	313.69 ± 11.23^{b}
Ferulic acid	–	1.28 ± 0.05^{c}	4.39 ± 0.12^{a}	3.39 ± 0.10^{b}	3.42 ± 0.11^{b}
Chrysoeriol	0.89 ± 0.04^{a}	0.89 ± 0.03^{a}	0.85 ± 0.02^{a}	0.79 ± 0.02^{a}	0.84 ± 0.03^{a}
Xanthohumol	0.93 ± 0.03^{a}	0.86 ± 0.02^{ab}	0.80 ± 0.01^{ab}	0.73 ± 0.02^{b}	0.77 ± 0.03^{b}
Total	33.40 ± 1.19^{c}	479.69 ± 17.71^{a}	481.36 ± 16.58^{a}	438.47 ± 15.18^{b}	459.09 ± 16.84^{ab}

The data are the means of three independent experiments ± standard deviations (n = 3). $^{a-e}$*Values in the same row, with different superscript letters, differ significantly (P < 0.05).*

to the release of bound molecules and pouring compounds trapped in the cellular compartments to the matrix, becoming available to the LAB catabolic activity during the following step of fermentation.

Since *in vitro* assays are only considered as predictive tools for the antioxidant activity *in vivo* and testing a substance directly on animals or human is not an easy approach, different methods comprising cellular models were recently developed (Verni et al., 2019). In this study, a well-known human keratinocyte cell line was subjected to oxidative-induced stress with peroxide hydroxide and subjected to the MTT assay, which allows the cell survival estimation after stress exposure. The test was performed in presence of the extracts obtained from treated and untreated BSG, aiming at evaluating their protective effect against oxidative stress. Compared to the untreated BSG, bioprocessing conferred to the matrix a relevant protective activity in all the tested conditions, especially when *L. plantarum* PU1 was used as starter. Different BSG phenolic acids (e.g., ferulic, *p*-coumaric, sinapic, and caffeic acids) were previously correlated to protection against DNA oxidative damage (McCarthy et al., 2012). Moreover, previous studies already correlated the presence of bioactive peptides and phenolic compounds generated during LAB fermentation with the improved cell survival to the oxidative stress (Verni et al., 2019). Based on these considerations, phenol, and peptide profiles of bioprocessed BSG were characterized.

Bound phenolic profile of both raw and bioprocessed BSG was mainly characterized by phenolic acids and their derivatives. Several ferulic acid derivatives were found, and their concentration was higher in bioprocessed BSG, in which the extensive degradation of the cell walls caused the increase of the total phenols extractability. Catechin, epicatechin, quercetin, and safrole, this latter previously identified in hop (Yan et al., 2018), were found in both free and bound phenols fraction. During the industrial brewing process, the trub separated after boiling the wort is also added to BSG, therefore, it is possible to find hop components.

A relevant amount of the bound phenolics in untreated BSG, mainly represented by ferulic acid derivatives, was liberated as the consequence of xylanase treatment. Being xylanase the main enzymatic activity found in Depol 761P, it is hypothesized that the increase of phenolic compounds can be ascribed to direct and indirect effect of the fibers hydrolysis, as confirmed by the microstructure analysis reported above.

The free phenolics profile was characterized by the products of microbial metabolism of phenolic acids and their derivatives especially in eBSG and fermented BSG. Dihydroferulic, dihydrocaffeic, and phloretic acids are, in fact, the products of ferulic, caffeic, and coumaric acid reduction, respectively (Filannino et al., 2014). Overall, the highest metabolites concentration was found in eBSG fH46.

Results of this study revealed the complete absence of ferulic acid and its derivatives in untreated BSG, and their high content in eBSG. When fermentation followed enzymatic treatment, a decrease of the ferulic acid and its derivatives was observed as consequence of the conversion in dihydroferulic acid. Feruloyl esterases release ferulic acid and other cinnamic acids and many LAB, especially of the genus *Lactobacillus*, have been reported to possess such activity. Hydroxybenzoic and hydroxycinnamic acids, whose high concentrations negatively affect the microbial physiological functions (Gänzle, 2014), may be also decarboxylated by LAB to the corresponding phenol or vinyl derivatives or hydrogenated by phenolic acid reductases, and their products can exert higher biological activities than the precursors (Gänzle, 2014).

To complete the phenolics characterization, free proanthocyanidins were selectively extracted and quantified. Although their total content was lower than that found in a recent study on BSG (Martín-García et al., 2019), the amount of monomers resulted markedly higher than that previously found, most likely because this BSG contained maize, which was reported to have a high content of these forms (up to 43 g/kg) (Chen et al., 2017). Combined bioprocessing caused a relevant degradation of the polymeric forms of proanthocyanidins, although monomeric forms did not increase, suggesting a rapid degradation after their release. Lactobacilli and bifidobacteria were indeed previously shown to cleave the heterocyclic ring of catechin and epicatechin (Saìnchez-Pataìn et al., 2012).

In addition to phenolic compounds, BSG has been recently reported as a potential source of antioxidant peptides, released through enzymatic hydrolysis with proteases (Cermeño et al., 2019). In this study, since WSE was characterized by an increase of antioxidant activity and high peptide concentration, a role of the LAB in the release of active sequences through proteolysis was hypothesized. In particular, the activity was not affected by enzymatic digestion and heating, thus confirming the stability of the active sequences. Indeed, to be effective, antioxidant peptides should have overcome hydrolysis and modifications at the intestinal level and reach their targets (Sarmadi and Ismail, 2010).

Mixtures of small peptides, not reported before as antioxidant sequences, were identified in the active and purified WSE fractions obtained from eBSG fPU1. Overall, it was hypothesized that the strongest antioxidant activity was ascribed to the synergic effect rather than to the individual activity of the single peptides (Coda et al., 2012). All the sequences resulted encrypted in native barley and maize native proteins. As previously reported as common feature of the antioxidant sequences (Zou et al., 2016), the five identified peptides contained from 8 to 10 amino acid residues. Total hydrophobicity ratio ranged from 50 to 70%. This is an important feature, since hydrophobic amino acids enhance the solubility of peptides in lipids, thus facilitating access to hydrophobic radical species and to hydrophobic PUFAs (polyunsaturated fatty acids) (Sarmadi and Ismail, 2010; Zou et al., 2016). Identified peptides were rich in hydrophobic amino acids which are frequently included in the antioxidant peptides structure (Zou et al., 2016). Moreover, all identified sequences also contained one or more aromatic amino acids, capable to donate protons to electron-deficient radicals, therefore enhancing radical-scavenging activity of the molecule (Sarmadi and Ismail, 2010).

Despite being rich in fiber, proteins and phenolic compounds, BSG is still underutilized for human nutrition, mostly due to its characteristics (i.e., high fiber content and instability) which make BSG a challenging material for food applications. Different studies already described the positive role of pre-treatments with xylanases or fermentation on sensory, technological, and rheological properties of BSG-based foods (Waters et al., 2012; Ktenioudaki et al., 2015). Thus, new biotechnological approaches to improve BSG functionality and utilization are needed to reintroduce it in the food chain.

This study showed how tailored bioprocessing, combining a xylanase treatment and a sequential fermentation with selected LAB, could enhance BSG antioxidant potential and showed protective effect against oxidative stress in human keratinocytes. All the bioactive compounds, to exert their functionality, must be absorbed in the gastro-intestinal tract. In this perspective, fermentation was already recognized as an effective way to increase the bioaccessibility of polyphenols in cereal food matrices, promoting their bioavailability at gut level (Ribas-Agustí et al., 2018). Moreover, the absorption of small-molecular weight compound such as phenolic acids is easier compared to larger polyphenols such as proanthocyanidins, which need to be degraded into monomer or dimer units before being absorbed (Carbonell-Capella et al., 2014).

In our work, the bioprocessing with xylanase and LAB fermentation allowed the release of phenolic compounds otherwise bound to the BSG matrix and unavailable for physiological functions. In particular, the results of this investigation highlighted the fundamental role of the starters in releasing specific phenolic compounds and bioactive peptides, thus maximizing the antioxidant effect. However, more studies are required to confirm this functionality *in vivo*.

Brewers' spent grain with antioxidant properties can be used as novel ingredient for the production of cereal-based, daily-consumed food (i.e., baked goods, pasta), thus helping to increase the intake of antioxidant compounds. In conclusion, the innovative bioprocessing protocol of this study highlights the potential of simple processes as technological option to convert underutilized side streams like BSG into added-value, potential ingredient for innovative food applications.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

MV optimized the bioprocessing protocol, performed the *in vitro* experiments, analyzed the data, and drafted the original manuscript. EP performed the peptide characterization and related data elaboration. AK and SJ performed the microstructure analysis. DP and FR were involved in the *ex vivo*

experiments. VV and ED-C oversaw MV in the phenolic profile characterization. RC and CR conceived and designed the experimental plan. CR oversaw the writing process. All authors read and approved the final 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.2020.01831/full#supplementary-material

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frontiers in Microbiology

REVIEW
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Understanding the Mechanisms of Positive Microbial Interactions That Benefit Lactic Acid Bacteria Co-cultures

*Fanny Canon[1], Thibault Nidelet[2], Eric Guédon[1], Anne Thierry[1] and Valérie Gagnaire[1]**

[1] STLO, INRAE, Institut Agro, Rennes, France, [2] SPO, INRAE, Montpellier SupAgro, Université de Montpellier, Montpellier, France

Microorganisms grow in concert, both in natural communities and in artificial or synthetic co-cultures. Positive interactions between associated microbes are paramount to achieve improved substrate conversion and process performance in biotransformation and fermented food production. The mechanisms underlying such positive interactions have been the focus of numerous studies in recent decades and are now starting to be well characterized. Lactic acid bacteria (LAB) contribute to the final organoleptic, nutritional, and health properties of fermented food products. However, interactions in LAB co-cultures have been little studied, apart from the well-characterized LAB co-culture used for yogurt manufacture. LAB are, however, multifunctional microorganisms that display considerable potential to create positive interactions between them. This review describes why LAB co-cultures are of such interest, particularly in foods, and how their extensive nutritional requirements can be used to favor positive interactions. In that respect, our review highlights the benefits of co-cultures in different areas of application, details the mechanisms underlying positive interactions and aims to show how mechanisms based on nutritional interactions can be exploited to create efficient LAB co-cultures.

Keywords: positive interactions, co-culture, metabolic dependencies, lactic acid bacteria, cross-feeding, public goods, microbial community

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Reviewed by:
Giuseppe Spano, University of Foggia, Italy
Pasquale Filannino, University of Bari Aldo Moro, Italy

***Correspondence:**
Valérie Gagnaire
valerie.gagnaire@inrae.fr

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INTRODUCTION

It is widely acknowledged that microorganisms have colonized most natural ecosystems and that no single strain grows in isolation, so that microbes are intertwined and constantly interacting. Scientists are now trying to understand the mechanisms underlying these interactions in order to better control microbial communities and exploit them in a wide variety of bioprocesses.

Before detailing how microorganisms interact together, it is crucial to define the numerous terms used in the literature which refer to their association, such as microbial community or consortium, and mixed or co-cultures. As illustrated in **Figure 1**, we have chosen to use the term natural community when referring to self-assembled communities of environmental microbes in various ecosystems (Rodríguez Amor and Dal Bello, 2019), and co-culture when referring to

man-made associations of microorganisms (Zhang and Wang, 2016). We also use 'assembly' as an umbrella term which encompasses both self-assembled communities and assembled co-cultures. Biotechnological processes are reliant on three types of assemblies: enriched natural communities, artificial co-cultures and synthetic co-cultures (**Figure 1**). Here, artificial co-cultures refer to cultures composed of microorganisms that are generally not found together in nature, whereas synthetic co-cultures concern associations of microorganisms in which at least one of the strains is a genetically modified organism (GMO). Depending on the type of microbial assembly, the aim is (1) to increase or decrease concentrations in the targeted molecules, described as overyielding (Rapaport et al., 2020), by using the division of labor (DOL), or/and (2) to multiply the functions expressed compared to monocultures. Regardless of the objectives, in each case the outcomes are reliant on positive interactions between the microorganisms that also enhance their fitness. For this reason, negative interactions, e.g., competition, cheating or parasitism, will not be covered in this review.

Major efforts have been made to understand the mechanisms affecting the association of microorganisms in order to improve process outputs. The need for direct contact (Zengler and Palsson, 2012), use of quorum sensing (QS) (Rul and Monnet, 2015), environmental adjustments (Cheirsilp et al., 2003), the sharing of public goods (Cavaliere et al., 2017) and cross-feeding (D'Souza et al., 2018) have all been described during recent decades as possible ways to drive interactions among microbial communities.

The value of co-cultivation has been exploited in many fields, from wastewater treatments based on enriched microbial communities (Cydzik-Kwiatkowska and Zielińska, 2016) to the production of molecules of interest such as vitamin C using artificial or/and synthetic co-cultures (Zou et al., 2013; Wang et al., 2016) or a huge number of fermented foods produced worldwide. Increasing microorganism fitness, product functionalities and the production of specific molecules is crucial in the area of food applications. For example, it has been shown that co-cultures can enhance levels of peptides and amino acids (Gobbetti et al., 1994), organic acids (Settachaimongkon et al., 2014) and volatile compounds (Álvarez-Martín et al., 2008) in fermented foods, and contribute to more rapid microbial growth when compared to monocultures (Stadie et al., 2013).

Lactic acid bacteria (LAB) are ubiquitous in food fermentation and they have positive effects on the organoleptic, health, nutritional and sanitary properties of products. However, co-cultures of LAB seem to have been somewhat neglected because studies on their interactions are scarce in the literature. As reviewed by García et al. (2019), LAB are often found to be self-assembled with yeasts in fermented foods, or to a lesser extent with propionic acid bacteria (PAB) (e.g., in cheeses) or with acetic acid bacteria (e.g., in kombucha). Consequently, LAB-yeast interactions are the most widely described in the literature, such as in sourdough (Gobbetti, 1998) or kefir (Stadie et al., 2013), and have also been studied in chemically defined media (CDM) (Ponomarova et al., 2017; Carbonetto et al., 2020). The exception is the co-culture of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* added in yogurt, which is well characterized (Sieuwerts et al., 2008) and offers a perfect example which asserts that LAB co-cultures are of value.

The aim of this review is to highlight the optimum ways available at present that could be used to create positive interactions between LAB. We start with an overview of the different types of positive interactions in various existing microbial assemblies. We then address the added value of co-cultivation and explain which mechanisms govern the positive interactions encountered in microbial communities and co-cultures. Thirdly, we focus specifically on the possible strategies that could be used to assemble LAB. And finally, we highlight the particular role of nutritional interactions in LAB insofar as we consider their nutritional requirements to be the best lever to create positive interactions and new LAB co-cultures.

POSITIVE INTERACTIONS IN CO-CULTURES: POTENTIAL USEFULNESS, TYPES AND MECHANISMS

Microbial Communities and Co-cultures: Purposes and Benefits

As the saying goes, there is strength in numbers, whether this concerns microbial communities or man-made co-cultures. It is clear that natural transformations, for example the mineralization of organic matter in soils, can occur because microorganisms are able to grow in concert. Moreover, as stated by Ghosh et al. (2016), "*the application of co-culture in different bioprocesses can be more advantageous than monocultures from the perspective of broader array of substrate utilization, coupled metabolic performance and higher combined yield*". Thus, co-cultivation can be used for two purposes: to enable substrate conversion and improve process performance (**Figure 1**).

We can distinguish two types of microbial assembly: self-assembled communities and assembled co-cultures. The first gathers natural communities, enriched or not, which are spontaneous associations of microorganisms within a specific biotope. They present the highest complexity in terms of the microbial species present and hence of the resulting interactions. The dynamics of natural communities are governed by natural selection. Evolution occurs in such a way that there is a constant back and forth between the converted substrates available and the improvement in fitness of established microorganisms. Microbial interactions play a key part in improving the fitness of members in the population.

Natural microbial communities are exploited in numerous bioprocesses. It is possible to drive the conversion process by selecting certain microbial species under specific conditions. The change in microbial diversity implies that there are qualitatively fewer opportunities for substrate conversion and fewer secondary metabolites are produced overall when compared to natural communities. Enriched natural communities are oriented toward targeted results, meaning that only a few of the transformations possible are favored. This is the case in traditional fermented foods such as cheese or kimchi, as well as in composting

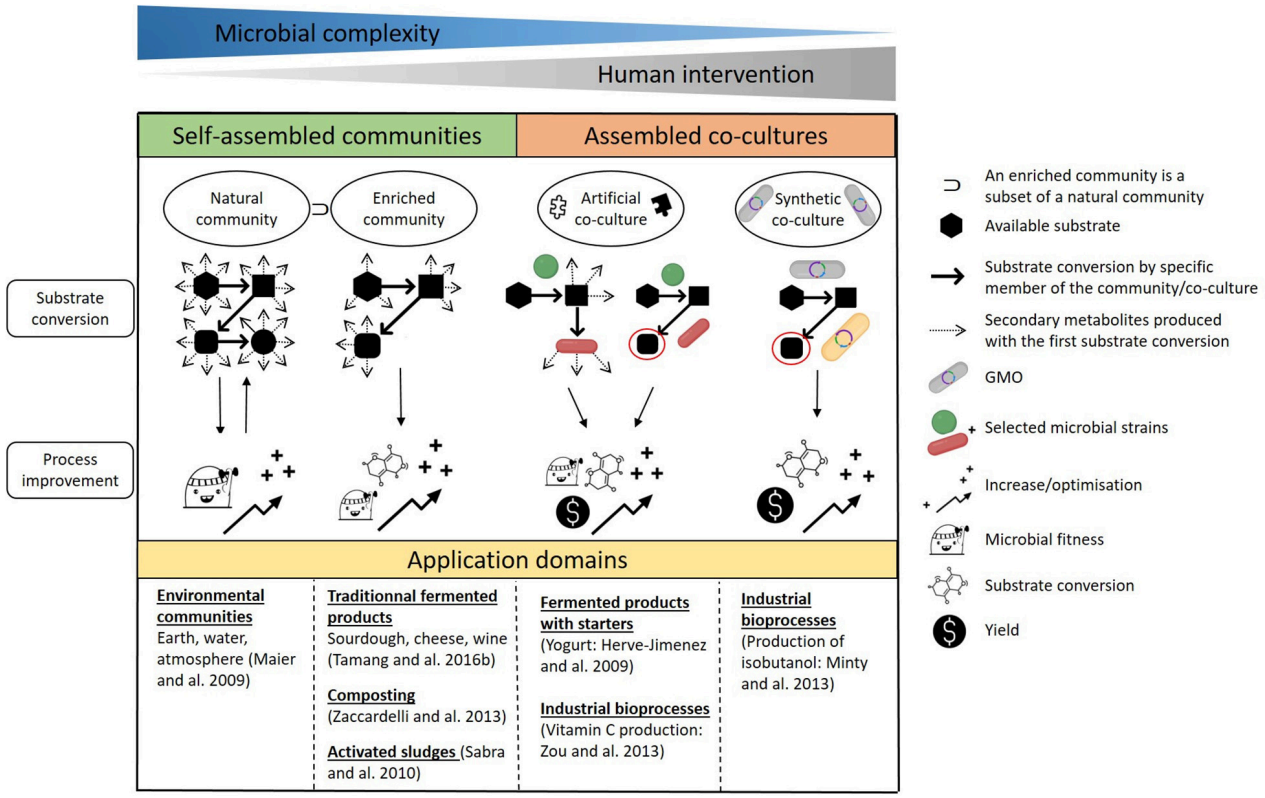

FIGURE 1 | Types of microbial communities and co-cultures, their use, benefits and some examples of their areas of application. Natural community: a self-assembled association of environmental microbes found in various ecosystems. Enriched natural community: a selection of specific microbes belonging to a natural community. Artificial co-culture: an association of microorganisms that may not necessarily be found in nature. Synthetic co-culture: an association of microorganisms in which at least one of the strains is a genetically modified organism.

or waste treatment. For example, methane can be produced from sludge under anaerobic conditions. The transformation of organic waste into biogas is considered to occur in four stages. During the first stage (hydrolysis), biological macromolecules are broken down into oligo- or monomers that are transformed during the second stage (acidogenesis) into volatile organic acids, alcohols, aldehydes, ketones, CO_2 and H_2. During the third stage (acetogenesis), the molecules produced in stage 2 are metabolized into acetic acid as well as some CO_2 and H_2. And during the last stage (methanogenesis), CH_4 is formed via the decarboxylation of acetate and the methanization of CO_2 and H_2 (Sabra et al., 2010).

The second type of microbial assembly gathers both types of artificial and synthetic co-cultures, which require human intervention and display less microbial complexity than natural communities. Jagmann and Philipp (2014) stated that "*it is a feasible approach in biotechnology to compose microbial communities* (here referred to as co-cultures) *that either consist of wild type strains that would not necessarily co-exist in nature or of one or more genetically engineered strains.*" Microorganisms are associated in order to reduce their metabolic burden by creating a division of labor. The energy-costly pathways that require cellular building blocks and ATP are divided between multiple strains (Roell et al., 2019; Wang et al., 2020).

Artificial co-cultures are assembled for two purposes. The first is to exploit the functions expressed by each strain. This is of particular interest when developing fermented food products in which the combined activity of microbial co-cultures is responsible for their characteristic flavor and texture (Garrigues et al., 2013; Bachmann et al., 2015). For example, the mutualistic behavior of *S. thermophilus* and *L. delbrueckii subsp. bulgaricus* during the manufacture of yogurt results in improved quality and stability of the final product when compared to monocultures (Herve-Jimenez et al., 2009). The second purpose for artificial co-culture assembly is to seek the production of a specific end-product, an approach that is relevant in fields such as the production of bio-energies and bio-chemicals (Ghosh et al., 2016). One example is the production of vitamin C, where *Ketogulonicigenium vulgare* converts L-sorbose into 2-keto-L-gulonic acid (2-KLG), the precursor of vitamin C, while *Bacillus megaterium* supplies growth factors to enable the growth of *K. vulgare* and the production of 2-KLG (Zou et al., 2013). In both cases, positive interactions between microorganisms are paramount to ensure optimized substrate conversion, production of the secondary metabolites anticipated, a higher yield and/or improved microbial fitness.

Synthetic co-cultures are defined as an association of at least one GMO with other microorganisms or several GMOs.

Their application is recent and mostly concerns the production of single end-products. Synthetic co-cultures are designed to increase yields and optimize substrate conversion. These goals are reliant on the positive interactions that occur between the microorganisms, which are ensured by creating metabolic dependency between them. In recent years, such metabolic dependencies have been shown to be one of the most promising ways to produce bio-energies. For example, to when producing isobutanol from cellulosic biomass, the biological functions necessary are divided between two specialists: a fungal cellulolytic specialist, *Trichoderma reesei*, which secretes cellulase enzymes to hydrolyze lignocellulosic biomass into soluble saccharides, and a fermentation specialist, a GMO *Escherichia coli* strain which metabolizes soluble saccharides into the desired product (Minty et al., 2013). It is important to mention that this use of GMOs is not conceivable for food applications, especially in Europe.

One fundamental difference between food applications and other bioprocesses is worth mentioning. In fermented food production, the composition of the initial medium is relatively simple, and the objective is to attain a complex balance between a large number of molecules such as organic acids, volatile and bioactive compounds, leading to improved shelf life, sensory qualities and health benefits of the food products. Other biotechnological processes tend to do the opposite: the initial medium is complex and the aim is to simplify this by producing a single end-product such as methane.

Positive Interactions That Occur in the Microbial World

Microbial interactions are crucial to the outcomes of bioprocesses. They are found in both natural communities and man-made co-cultures, where they are deliberately favored. The different types of interactions have been thoroughly detailed in some reviews (Großkopf and Soyer, 2014; García et al., 2019; Rodríguez Amor and Dal Bello, 2019). However, the terminology for the different types of positive interactions may slightly differ between authors. We therefore decided to specify what we consider to be positive interactions occurring between microorganisms. In all cases, positive interactions are defined as an improvement in the fitness of at least one partner involved in the interaction. Because interactions are often studied between two microorganisms, we will also present them in pairs in the sections below (**Figure 2**).

First, we should consider commensalism, which refers to an increase in the fitness of one partner in the interaction with no cost or benefit for the other. This is the only unidirectional positive interaction between two microorganisms. A notable example of this mode of interaction is that seen in Swiss-type cheese. Indeed, LAB strains contribute to the growth of PAB strains via two mechanisms: LAB produce lactic acid, which is further metabolized into propionic acid, acetic acid, and carbon dioxide by PAB (Smid and Lacroix, 2013) and they hydrolyze cheese proteins, thus supplying PAB with peptides and free amino acids (Gagnaire et al., 2001).

The other type of positive interaction is bidirectional. Three types of bidirectional positive interactions can be found in the literature, it can be difficult to distinguish them from each other: cooperation, mutualism and syntrophy, the most common being cooperation and mutualism. Cooperation implies the increased fitness of nearby cells that share a given genotype. This means that the interacting partners produce and use the same common goods, i.e., homotypic cooperation (Rodríguez Amor and Dal Bello, 2019). For instance, *Saccharomyces cerevisiae* cells are able to flocculate when they are challenged by environmental stress. This cooperative trait relies on the production of a cell-wall protein (FLO1) which enables all cells expressing FLO1 to adhere together (Smukalla et al., 2008). Unlike cooperation, mutualism refers to an increased fitness of both interacting partners that do not produce or use the same common goods, i.e., heterotypic cooperation (Rodríguez Amor and Dal Bello, 2019). Mutualism can be observed in traditional fermented food products such as kefir where yeasts supply essential amino acids and vitamins to LAB, which in turn lower the pH for yeasts (Cheirsilp et al., 2003; Stadie et al., 2013). Syntrophy is another mutual interaction that cannot be overcome by simply adding a co-substrate or any type of nutrient (Morris et al., 2013). In a co-culture of two microorganisms, syntrophy occurs when one strain benefits from the goods produced by the other strain, which in turn benefits from the consumption of these goods. The best known example is the H_2-mediated syntrophic interaction between secondary degraders and methanogens (Schink, 1997).

Division of labor (DOL) benefits from special status in the literature: it is described in natural communities and reproduced in co-cultures. Also referred to as functional specialization, the DOL is an association of multiple strains in order to perform complex tasks. In a recent article, Giri et al. (2019) suggested four criteria to determine whether an interaction does indeed constitute a DOL. The first is functional complementarity, meaning that every partner in the interaction carries out a function more efficiently that the others. Second, the interaction needs to involve a synergistic advantage and thus needs to be bidirectional. The third criterion is negative frequency-dependent selection, meaning that the interaction can be sustained for a long period. Finally, a positive assortment is necessary, which will be favored by natural selection. For example, during the nitrification process, ammonia is first of all oxidized to nitrite by ammonia-oxidizing microorganisms (AOM) and then to nitrate by nitrite-oxidizing bacteria (NOB). This sequential substrate conversion maximizes ATP production and hence growth rates. The interaction between AOM and NOB is complementary, synergistic, ecologically stable, and displays signs of positive assortment, thus suggesting that nitrification fulfils all the criteria to classify as a DOL. A DOL is often artificially or synthetically reproduced in bioprocesses strategies, but in this context only the first two criteria need to be fulfilled to qualify the interaction as a DOL.

The dynamic aspects of microbial interactions are rarely mentioned when describing their different types. Over a long timescale, microbial communities display a relative stability while displaying an alternance of dominant species or even strains at a reduced timescale of several days. In this way, the interactions between microorganisms are not fixed in time and may vary

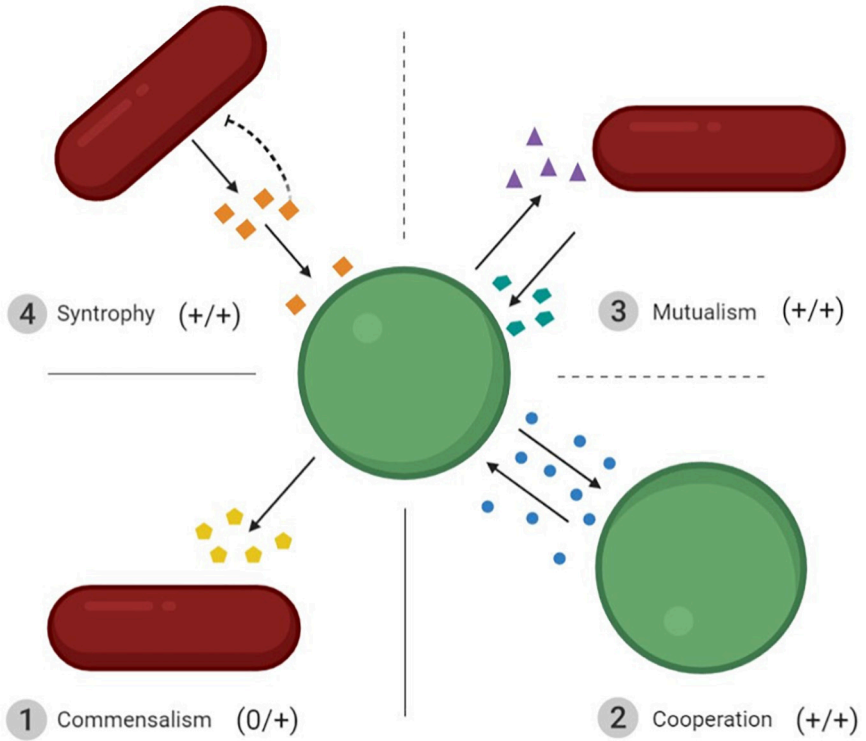

FIGURE 2 | Schematic representation of the four types of interactions resulting in positive outcomes for the microorganisms involved. ① Commensalism: increased fitness of one interacting partner without affecting the second. ② Cooperation: the two interacting partners share the same phenotype and improve each other's fitness. ③ Mutualism: the increased fitness of two interacting partners that do not benefit from the same molecules. ④ Syntrophy: one cell benefits from the metabolites produced by the other, and meanwhile removes the inhibition induced by these metabolites for the producer. The dotted lines between mutualism and syntrophy, and mutualism and cooperation, mean that they are both particular forms of mutualism. Interactions ② to ④ are bidirectional.

depending on environmental conditions or the occurrence of disturbances. In co-cultures, stability is less of a focus because co-cultures are essentially achieved using batch cultures that only last for a few days or months. However, the population dynamic may also be important in the case of sequential interactions that can result in commensalism.

Mechanisms Underlying Positive Interactions

General Mechanisms for Positive Microbial Interactions

As the principal factor influencing process outcomes, microbial interactions need to be characterized accurately if they are to be properly applied (Li et al., 2013). The decision tree presented in **Figure 3** illustrates how to describe these positive interactions. Positive interactions can require physical contact between the microorganisms. This contact may involve the flagellum, nanotubes, membrane, or vesicle chains. It is important to note that the molecules shared by direct contact are not detailed in **Figure 3** but may include some of those shared through diffusion in the medium. The pool of molecules available in the medium is referred as public goods; they can be costly to produce and are equally available to all microorganisms in the medium (Cavaliere et al., 2017; Giri et al., 2019). A wide variety of molecules can be shared: siderophores, enzymes, biosurfactants, biofilm matrix components and QS molecules, as reviewed by West et al. (2007). If they are not used by the interacting partner, these molecules do not qualify as public goods but may modify the physicochemical properties of the medium so that they better suit growth conditions for the partner (i.e., CO_2 production, increase or decrease of pH/redox). A distinction is made regarding the nature of the public goods thus produced: they may be nutritional or non-nutritional for the interacting partner; in other words, they can be used directly for growth or to improve its activity. Non-nutritional goods may include QS or biofilm components that can influence the spatial structure of the co-culture (Monnet et al., 2014). If nutritional goods such as amino acids or sugars are secreted, then the microorganisms interact via cross-feeding. Another nutritional interaction involves extracellular enzymes that hydrolyze proteins or complex polysaccharides directly in the medium.

The Interaction Gets Physical: The Case of Direct Transfers

As explained by Zengler and Palsson (2012), microbial interactions may require direct physical contact between interacting microorganisms. The best known way to create physical contact is to use a flagellum-mediated system which

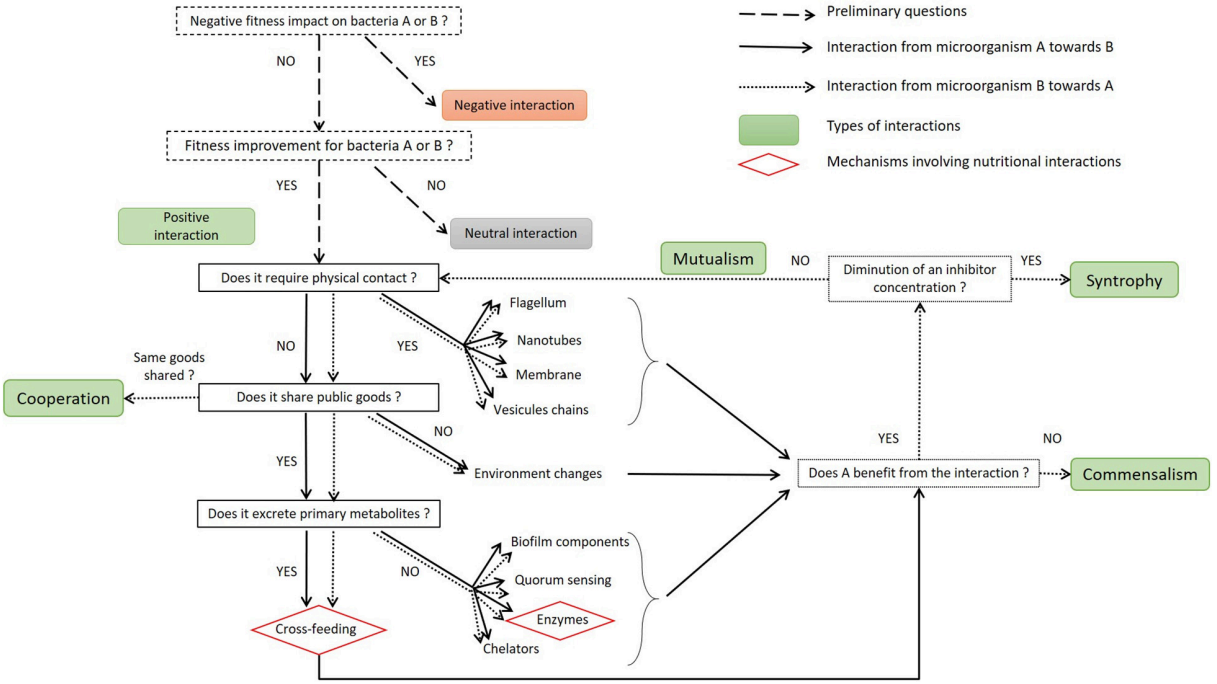

FIGURE 3 | Decision tree to characterize the mechanism underlying positive interactions between two microorganisms. The dotted squares contain preliminary questions to establish whether this is a case of positive interaction or not, or supplementary questions to establish its type. In the case of bidirectional interactions, the decision tree can be read twice: the first reading aims to characterize the effect of microorganism A on B (plain arrows), and the second aims to characterize the effect of B on A (dotted arrows). The sequential questions are designed to establish whether the interaction:

–is direct or indirect,

–engages a sharing of public goods or is due to physicochemical changes in the medium,

–is reliant on secreted primary metabolites or not.

enables the transmission of a signal to a specific partner by recognizing cell-surface proteins (Shimoyama et al., 2009). In the case of the syntrophic interaction between methanogens and secondary degraders, a specific inter-species cell-to-cell recognition system is necessary to ensure efficient hydrogen transfer. Indeed, random cell-to-cell associations with other microbial species may cause a deterioration of methanogenic metabolism. The role of a flagellum is therefore not just motility but also includes adhesion and environmental sensing (Kouzuma et al., 2015).

The use of nanotubes has also been described as being necessary to establish positive interactions between microorganisms. Pande et al. (2015) showed that in a well-mixed environment, *E. coli* can connect with other bacterial cells via membrane-derived nanotubes and then use them to exchange cytoplasmic constituents.

The direct delivery of molecules can also be mediated by membrane-membrane contact. This was demonstrated by Benomar et al. (2015) regarding the formation of an artificial co-culture of *Clostridium acetobutylicum* and *Desulfovibrio vulgaris* in which a cell–cell interaction was associated with an exchange of molecules. This induced changes to metabolic pathways, and enabled the growth of *D. vulgaris* despite a shortage of nutrients. Remis et al. (2014) revealed the presence of a fourth direct communication system, showing that *Myxococcus xanthus* was capable of producing a network of outer membrane extensions in the form of vesicles and vesicle chains that can interconnect cells.

The requirement for physical contact between positively interacting microorganisms can be assessed using different methods. If interactions occur in sequential cultures (Ponomarova et al., 2017), using co-culture devices (Paul et al., 2013) or inactivated microorganisms (Cheirsilp et al., 2003), this means that no physical contact is required. However, identifying the nature of a physical contact is complicated because this requires the use of electron and fluorescence microscopy and identification of the flagellum, nanotubes or vesicle constituents.

Influence of Spatial Structure and Quorum Sensing

Spatiality plays a key role in microbial interactions, even those of an indirect type. Indeed, spatially structured media favor the stabilization of mutualistic cross-feeding (Pande et al., 2016). It is known that spatial assortment strengthens local interactions, prevents cheaters from taking advantage (MacLean and Gudelj, 2006) and improves resilience in the face of environmental stresses (Lee et al., 2014). Kim et al. (2008) revealed the importance of spatial structure to bacterial community interactions using a mathematical model to show how it influences or stabilizes both negative and positive

interactions. One undeniable piece of evidence of the importance of spatial structure when considering bacterial interactions is biofilms. For more details in this field, the reader is referred to the interesting review by Nadell et al. (2016), which explain how positive or negative interactions (i.e., competition) are influenced by the spatial arrangement of different strains in biofilms.

The molecules involved in QS can modulate the spatiality between interacting microorganisms. In fact, two types of signaling molecules can modulate the expression of specific genes responsible for the synthesis of biofilm components, as well as bacteriocins, the conjugal transfer of plasmids, and a stress response (Di Cagno et al., 2007; Fontaine et al., 2007). These molecules are small signaling hormone-like autoinducers that accumulate and trigger cascade events when a quorum is reached (threshold concentration). The autoinducing peptide (AIP), also called peptide pheromone, is a species-specific communication signal found exclusively in Gram-positive bacteria. Autoinducer-2 (AI-2) furanones are universal signaling molecules that are also used in QS. QS manipulations are strategies that are sometimes implemented to increase yields in bioprocesses (Shong et al., 2012). It is in fact possible to modify synthesis of the signal molecule, sensitivity to the signal and the specificity of the response. Molecules involved in QS are important to bacterial dynamics in communities and co-cultures and can also affect the sensory quality and safety of foods as they may contribute to the elimination of undesirable microorganisms (spoilers or pathogens) and favor the development of those being targeted (Rul and Monnet, 2015).

In the case of unstructured media, the exchange of public goods, and particularly those of a nutritional type, may be trickier. In fact, this requires a subtle equilibrium between the metabolites produced, used and exchanged to obtain positive interactions between microorganisms (Bachmann et al., 2011). Interacting microorganisms need to produce more of the essential nutrients that they themselves require to sustain the growth of both partners, while avoiding cheating phenomena. In the case of proteolytic strains, a peptide concentration gradient surrounds the cells, so there is a specific location for nitrogen sources in the extracellular medium. This explains why proteolytic strains do not disappear in a co-culture that involves both proteolytic and non-proteolytic strains: the substrates are firstly used by the proteolytic cells and then diffused toward non-proteolytic cells (Bachmann et al., 2011).

Nutritional Dependencies Make Strong Allies

The sharing of primary metabolites is crucial in many positive interactions, whether they occur in natural communities, artificial or synthetic co-cultures. It is possible to distinguish two ways by which microorganisms supply these primary metabolites (**Figure 3**). The first is the sharing of hydrolytic extracellular enzymes such as invertases, lipases and proteases, which transform the substrates directly available in the public pool of molecules (Cavaliere et al., 2017). The second concerns cross-feeding, i.e., the phenomenon by which one microorganism (referred to as the donor) takes in a primary substrate and converts it into a product excreted as a public good, which is subsequently used by an interacting partner, referred to as the receiver. Cross-feeding has been extensively reviewed by D'Souza et al. (2018).

The evolution of natural communities has been described as the Black Queen Hypothesis, which implies that positive interactions are formed in complex habitats and strengthened through gene loss (Morris et al., 2012), thus creating dependencies between interacting microorganisms (Sachs and Hollowell, 2012). Over time, complex interactions develop between strains belonging to the same biotope, to the point where isolated strains can no longer sustain themselves. This theory highlights the fact that dependencies – particularly those of a nutritional type – between microorganisms not only improve their fitness but also strengthen their association against competitors, cheaters and environmental stress. Computational analyses have shown that metabolic dependencies are major drivers of species co-occurrence in nature (Zelezniak et al., 2015). Metabolism overflow is in fact frequent in microorganisms and can serve other community members (Paczia et al., 2012).

In artificial co-cultures, many positive interactions can be explained by extracellular enzyme sharing and cross-feeding that form the basis for the occurrence of positive interactions (Cavaliere et al., 2017), as exemplified below. Zuroff et al. (2013) used the cellulosic activity of *Clostridium phytofermentans* to supply fermentable simple carbohydrates to *Candida molischiana* to enable the production of ethanol. In milk fermentation, the sharing of extracellular protease, especially in LAB species such as *Lactococcus lactis*, has been shown to be paramount to ensuring microbial interactions in cheese and fermented milks (Smid and Lacroix, 2013). Nutritional interactions also occur as a result of cross-feeding. Ponomarova et al. (2017) showed that *S. cerevisiae* can sustain the growth of LAB strains by secreting essential amino acids and vitamins. The same observation was made regarding the fermentation of kefir water made from various fruit juices in which the growth of *Liquorilactobacillus hordei* and *Liquorilactobacillus nagelii* was supported by the release of amino acids and vitamin B6 by *Zygotorulaspora florentina* (Stadie et al., 2013). The cross-feeding of small carbohydrates from the hydrolysis of lactose or maltose can also occur between LAB and yeasts (Gobbetti, 1998; Ponomarova et al., 2017).

To create synthetic co-cultures, the most relevant interactions to be encouraged are commensalism and mutualism based on cross-feeding (Jagmann and Philipp, 2014). This means that co-cultures should be based on the excretion of nutritional compounds to favor positive interactions between microorganisms. Numerous examples have shown that this strategy is efficient in partitioning metabolic roles and engineering a DOL (Zhou et al., 2015; Ding et al., 2016; Johns et al., 2016). For example, Bernstein et al. (2012) genetically modified two strains of *E. coli* so that one converted glucose to acetate and the other, which was glucose-negative, used the metabolic by-product to increase total biomass production. In synthetic co-cultures, metabolic pathways have also been modified using specific mutations so that strains can only grow under strict cross-feeding. These pathways often involved amino acids, for which the strains are modified to become auxotrophs or overproducers (Wintermute and Silver, 2010; Mee et al., 2014). However, according to our definitions, it appears that the

sharing of public goods, and particularly extracellular enzymes, is also used in synthetic co-cultures to engineer a DOL. Bayer et al. (2009) managed to modify a strain of *S. cerevisiae* so that it produced methyl halides with the support of a cellulolytic strain of *Actinotalea fermentans*. For the conversion of xylan to ethanol, Shin et al. (2010) modified an *E. coli* strain to produce xylanase, thus providing the necessary substrate for a second strain of *E. coli* that had been modified to convert xylo-oligosaccharides into ethanol.

Cross-feeding also includes co-factors. For example, heme and quinones have been reported to switch the metabolism of LAB from fermentation to respiration (Seth and Taga, 2014). Respiring LAB display faster growth rates, improved long-term survival and changes to their metabolism (Pedersen et al., 2012). Corrinoids, which notably include vitamin B12, are also involved in nutritional cross-feeding, as they are essential for bacterial growth. However, a high proportion of bacteria and almost 70% of LAB lack the ability to produce corrinoids *de novo*. This means that bacteria need to procure them from their environment, either directly via the medium or through the activity of other microorganisms (Seth and Taga, 2014).

LAB CO-CULTURES TO MULTIPLY FUNCTIONALITIES

Although co-cultures have proved their worth in numerous applications, LAB-LAB co-cultures seem to have been somewhat neglected. In fact, yogurt is the only example of a LAB co-culture that is very widely exploited and whose interactions have been well characterized. LAB are endowed with a broad range of functions that could be multiplied with artificial co-cultures, particularly in food applications. Further, LAB have particular metabolic traits that favor the artificial establishment of nutritional dependencies.

LAB: Functional Microorganisms in Food Applications

LAB are Gram-positive, non-sporulating, facultative anaerobic and acid-tolerant bacteria. LAB species can be homofermentative, meaning that they mainly produce lactic acid; or heterofermentative, meaning that they also produce acetic acid, ethanol, CO_2 and formic acid (Wright and Axelsson, 2019). They are found in a variety of nutrient-rich food environments, and especially in dairy products, vegetables, cereals and meat, as autochthonous flora or added starter cultures. They also form part of the natural microbiota of animal hosts, where they are found in the gastrointestinal tract, the urogenital tract, oral cavity and skin. LAB are used as functional cultures because they contribute to the final organoleptic, nutritional, health and sanitary properties of food products. Zheng et al. (2020) suggested a new classification for the large group that represent LAB. They are now divided into five families: *Aerococcaceae*, *Carnobacteriaceae*, *Enterococcaceae*, *Streptococcaceae*, gathering 7, 16, 7, and 3 genera, respectively, and *Lactobacillaceae* now gathering 31 genera, while the former *Lactobacillus* genus has been expanded to 25 different genera. The most frequently isolated LAB species belong to the genera *Lactococcus*, *Lactobacillus*, *Lactiplantibacillus*, *Streptoccocus*, *Oenococcus*, *Leuconostoc,* and *Enterococcus*. New LAB species are constantly being described, and the total number of LAB species is evaluated at more than 200 (Holzapfel and Wood, 2014).

Organoleptic Changes Induced by LAB

LAB play an important role in organoleptic changes to food products. They actively contribute to both flavor and texture changes in fermented foods, and particularly dairy products. For example, in fermented dairy products, the proteolytic activity of LAB strains is crucial to both transformations. Indeed, the sapid peptides and amino acids produced by LAB participate in overall flavor perception and free amino acids are aroma precursors. LAB also produce different aroma compounds such as diacetyl and acetaldehyde, which are easily identifiable in the overall aroma note of yogurt (Zourari et al., 1992; Routray and Mishra, 2011), and a wide variety of aroma compounds derived from amino acid catabolism (Smid and Kleerebezem, 2014).

LAB activity is also essential to textural properties. Like many other bacteria, they can produce several types of polysaccharides or glycans that are natural biopolymers of carbohydrates and display enormous structural diversity (Ruas-Madiedo et al., 2002; Bernal and Llamas, 2012). Bacterial exopolysaccharides (EPSs) are either loosely bound to the cell surface or released into the surrounding environment, and they can change the texture of food products (Rahbar Saadat et al., 2019), whether through the production of peptides or their proteolytic activity (Lacou et al., 2016).

Nutritional Quality Improvements Induced by LAB

LAB also enhance the nutritional quality of foods; they may either improve nutritional intake and digestibility or reduce the presence of anti-nutritional compounds. LAB can produce vitamins (LeBlanc et al., 2011); for example, *Leuconostoc mesenteroides* and *Latilactobacillus sakei* produce riboflavin and folic acid in kimchi (Jung et al., 2011) and *L. lactis* produces K2 vitamin (menaquinones) (Morishita et al., 1999).

The EFSA recognizes that the LAB species found in yogurt are able to alleviate the symptoms of lactose malabsorption (Efsa Panel on Dietetic Products Nutrition and Allergies, 2010). This condition is due to a lack of human lactase in the small intestine. LAB diminish the lactose content during fermentation and contain the β-D-galactosidase enzyme that may further be active in the gastrointestinal tract (Shah, 2015). They may also reduce intestinal discomfort due to the non-digestible oligosaccharides present in many plants (such as stachyose and raffinose in legumes) by hydrolyzing these compounds in fermented plant-derived products. Improved protein digestibility has also been observed *in vitro* in sourdough (Bartkiene et al., 2012). The proteolytic activity of LAB may also decrease the protein allergenicity of food products such as soybean (Song et al., 2008).

Finally, LAB can also reduce the quantities of anti-nutritional compounds such as phytates that are present in some plant-based products (García-Mantrana et al., 2015), either by lowering the pH of the medium, which reactivates endogenous plant

phytase(s), and/or by producing bacterial phytase(s) that can release the inositol moieties of phytates.

Health Benefits

LAB exert beneficial effects on hosts as they can produce bioactive molecules, either in fermented products or directly in the gastrointestinal tract. According to the FAO and WHO, probiotics are "live microorganisms which, when administered in adequate amounts, confer a health benefit to the host". The most commonly used probiotic bacteria belong to LAB, and especially those in the former genera *Lactobacillus* and *Enterococcus* (Tamang et al., 2016a). Bioactive peptide production from LAB is often related to health benefits. Bioactive peptides are released from proteins by microbial or non-microbial enzymatic hydrolysis (Fitzgerald and Meisel, 2003). Because of their proteolytic system, LAB fermentation process can release these peptides in a protein-based medium (Venegas-Ortega et al., 2019). Bioactive peptides can act as immune modulators (Qian et al., 2011), and are antihypertensive by inhibiting angiotensin-converting enzyme (ACE) (Phelan et al., 2014), as in yogurts (Papadimitriou et al., 2007) and antioxidants as in sourdough (Coda et al., 2012) and yogurt (Sabeena Farvin et al., 2010). Antioxidant peptides have been found in soybean milk fermented by LAB and these can effectively eliminate free radicals (Liu et al., 2017). Antioxidant properties may also be the consequence of EPS production (Rahbar Saadat et al., 2019).

Other health benefits are related to the consumption of products fermented using probiotic LAB strains. Indeed, yogurt consumption produced some interesting results versus type 2 diabetes in a meta-analysis of dairy intake (Gijsbers et al., 2016). LAB are also involved in anti-cancer activities. For example, in milk, *Lactobacillus acidophilus* produces conjugated linoleic acid, an anti-carcinogenic agent (Macouzet et al., 2009). LAB also produce antimicrobials compounds whose properties can also contribute to the establishment of probiotic strains in the host (Kommineni et al., 2016) and thus counteract pathogenic bacteria in the gastrointestinal tract.

Food Preservation and the Safety of LAB

Another function of LAB is food preservation, generally considered to be the principal reason why they have been used empirically for centuries in traditional fermented foods. LAB inhibit the growth of numerous pathogens and spoilage microorganisms, by both lowering the pH as a result of lactic acid production and by producing a wide variety of antimicrobial compounds. Lactic acid induces ionic disruption (Warnecke and Gill, 2005), changes to the fatty acid composition of cell membranes (Cotter and Hill, 2003), and changes in transcriptional responses (Kirkpatrick et al., 2001) or oxidative stress within *Bacillus cereus* cells (Mols and Abee, 2011). In the presence of oxygen, some LAB are able to produce hydrogen peroxide which causes DNA damage, protein oxidation and membrane disruption in *Pseudomonas*. The ethanol produced by some LAB species, in association with other molecules, induces damage to cell membranes and denatures proteins (Ross et al., 2002). Diacetyl, involved in the butter-like aroma expected in some fermented dairy products, inhibits some Gram-negative bacteria (Kang and Fung, 1999). The overall antimicrobial impact of LAB therefore lies in the synergistic effect of a range of metabolites they produce. Organic acids from LAB also inhibit pathogenic and spoilage fungi (Crowley et al., 2013). The ability of LAB to inhibit microorganisms is supplemented by the production of bacteriocins, which are multifunctional peptides produced in the ribosome and display antimicrobial activity at particular concentrations (Chikindas et al., 2018). Bacteriocins are clustered in different classes depending on their structure, genetics and mode of action. They have been shown to inhibit a wide range of food spoilage bacteria and fungi (Zacharof and Lovitt, 2012; Leyva Salas et al., 2017). The addition of bacteriocins to food can indeed lower pathogen levels in a variety of products (O'Sullivan et al., 2002). Nisin, a bacteriocin produced by *L. lactis,* is the most widely exploited bacteriocin. It creates pores in the bacterial membrane that create leakage in Gram-positive bacteria (Bierbaum and Sahl, 2009). The use of LAB producing antibacterial and antifungal agents is a natural alternative to the addition of chemical preservatives. They can also improve the flavor of certain fermented foods (Younes et al., 2017).

Despite the wide and documented use of LAB in food production, safety concerns also need to be considered when they are added deliberately to foods. Two statuses are used to qualify LAB safety. A list of 48 LAB species benefit from a Qualified Presumption of Safety (QPS) status defined by the European Food Safety Authority (EFSA). *Enterococcus spp*., known for its ability to develop antibiotic resistance, is not included on the QPS list. However, it is important to note that some other species currently used as starters in foods do not (as yet) figure on the QPS list. QPS status offers species a safety qualification but does not preclude an evaluation of the risk involved in any (LAB) strain before it is used as a starter. There are two main risks, which depend on the LAB species or strains employed: the transfer of determinants of antibiotic resistance and the production of toxic or deleterious compounds such as biogenic amines and D-lactic acid. The selection of strains for use in foods must therefore be very thorough. The second status, Generally Regarded As Safe (GRAS) and determined by the United States Food and Drug Administration (FDA) qualifies LAB at the strain level.

Promoting LAB Positive Interactions in Co-cultures

Quorum Sensing and Its Technological Implications

QS is probably the most widely studied bacterial communication system. It regulates bacterial gene expression as a function of cell density and environmental conditions. It offers an adaptive advantage to specific bacterial populations in complex communities as well as to those which are enriched, such as in traditional fermented foods. In that regard, QS plays an important role in many of the microbial successions that occur during food fermentation: it can enhance the growth of specific species or strains while inhibiting others (Johansen and Jespersen, 2017). However, any evidence of positive interactions mediated by AIP between LAB in co-cultures is rare or even contrary. For example, it was observed that the growth and viability of *Lactiplantibacillus plantarum*

DC400 was enhanced when cultured with *Fructilactobacillus sanfranciscensis* and *Furfurilactobacillus rossiae* in sourdough, when QS-related genes responsible for stress response were activated (Di Cagno et al., 2009). Also, the growth and survival of the AIP-producing starter *L. plantarum* from Spanish-style olives increased when co-cultivated with AIP-inducing *Enterococcus faecium* and *Pediococcus pentosaceus* strains (Ruiz-Barba et al., 2010). In contrast, the survival of *L. plantarum* was not enhanced when co-cultivated with an AIP-inducing *L. lactis* strain (Caballero-Guerrero et al., 2013). Further, AIP production was reduced in both *L. plantarum* and *E. faecium* isolated from fermented vegetables during co-cultivation (Domínguez-Manzano and Jiménez-Díaz, 2013).

Although QS systems offer an important colonization advantage to LAB in complex communities, evidence of positive interactions in LAB co-cultures has yet to be produced. It was recently shown that exogenous synthetic AI-2 exerted positive effects on the growth of *E. faecium* and *Limosilactobacillus fermentum*, and on the cell density of *E. faecium* under acidic and alkaline stresses (Gu et al., 2020). Therefore, associating AI-2 producing LAB strains might lead to positive interactions. However, QS requires a certain cell density to regulate gene expression, thus implying that at least one partner in the interaction is able to grow independently. In addition, this phenomenon is only observed on solid or semi-solid structured matrices.

Nevertheless, QS is of considerable interest in food technologies as it can enhance food safety and quality through the production of antimicrobial compounds (Ruiz-Barba et al., 2010; Kareb and Aïder, 2020). In the near future, QS could be used to control microbial behavior and thus improve the quality of foods and beverages, for example by favoring the growth of LAB that enhance flavor and texture or by inhibiting the growth of spoilage or pathogenic microorganisms. QS also has a significant role to play in the probiotic functionalities of LAB strains, such as resistance to harsh environments; e.g., gastric acidity (Johansen and Jespersen, 2017).

Manufacturing Nutritional Dependencies Between LAB

LAB are found in in the majority of naturally fermented products ranging from milk to meat, as well as in various plants, which mostly offer nutrient-rich media (Tamang et al., 2016b). Their widespread occurrence can be explained by the fact that LAB display a huge diversity of phenotypes that vary within and between species. LAB can utilize many different substrates, notably carbohydrates and proteins (Duar et al., 2017). Some LAB species have also adapted insofar as they have developed auxotrophy for many nutrients (Teusink and Molenaar, 2017). These two characteristics make them ideal partners to create LAB-LAB positive interactions if account is taken of the composition of the medium, the possibility that one or other of the partners involved in the interactions can supply essential nutrients and by limiting potential substrate competition.

The auxotrophies present in LAB are markedly dependent on the biotopes from which they originate. They are therefore species- and strain- dependent. Many LAB strains have been shown to lack the capacity to produce the precursors of RNA and DNA: they are auxotrophic for nucleosides and nucleic acids (Kilstrup et al., 2005). LAB also grow poorly or even not at all in environments where vitamins (particularly from the B group), peptides and amino acids are not available. The development of chemically defined media (CDM) has offered crucial clues to identifying the nutritional requirements of LAB strains. For example, *L. lactis* strains are auxotrophic for 14 of 20 amino acids (Cocaign-Bousquet et al., 1995), *L. plantarum* and *L. mesenteroides* require between 3 and 11 amino acids (Teusink et al., 2005; Kim, 2012), while *Lactobacillus johnsonii* is unable to synthetize any of them (Hammes and Hertel, 2015). Requirements in leucine, isoleucine, valine, methionine, and glutamate are the most common. Most LAB strains also need exogenous sources of vitamins for growth, mainly pantothenate, pyridoxine, riboflavin, niacin and biotin (Zhang et al., 2009; Wegkamp et al., 2010; Aller et al., 2014), whereas some LAB strains are able to produce vitamins (LeBlanc et al., 2011; Capozzi et al., 2012). Finally, LAB are also known to be heme-auxotrophic (Gruss et al., 2012).

LAB also differ in their ability to utilize external resources. LAB can procure amino acids from their environment by using the peptide intracellular transport system. They can import peptides via an oligopeptide transport system (Opp) as well as di- and tri-peptides (DtpT and Dpp). Intracellular peptidases then hydrolyze these peptides into assimilable amino acids. In addition, some LAB strains possess a cell-envelope proteinase (CEP) that hydrolyzes the proteins present in the medium (Kunji et al., 1996). Five different types of such enzymes have been identified in LAB, including PrtP in *L. lactis* and homologs to PrtP in *Lacticaseibacillus paracasei*, PrtH in *Lactobacillus helveticus*, PrtR in *Lacticaseibacillus rhamnosus*, PrtS in *S. thermophilus*, and PrtB in *L. delbrueckii* subsp. *bulgaricus* (Savijoki et al., 2006). Depending on the LAB strain, the proteolytic equipment may differ in terms of the nature of peptidases and transport systems. In some species, the presence of CEP (Liu et al., 2010) and their number differ depending on the strain: *L. helveticus* strains can contain up to four different CEPs, in contrast with other LAB species (Sadat-Mekmene et al., 2011). LAB also display considerable differences in their abilities to ferment carbohydrates. While glucose is commonly preferred by many LAB strains, they present disparities in the use of other mono- and oligo-saccharides (Hayek and Ibrahim, 2013). Sugar intake depends on permeases, ATP-driven transporters or phosphoenolpyruvate: sugar phosphotransferase systems (PEP:PTS) present in the LAB genome (Poolman, 1993). For example, lactose intake is mediated by a PEP:PTS system in *L. lactis* whereas it is mediated by a permease in *L. delbrueckii* subsp. *bulgaricus* (Leong-Morgenthaler et al., 1991). Oligosaccharide intake requires initial enzymatic cleavage (with β-galactosidase, α-galactosidase or sucrase) in order to form monosaccharides which can enter the functional fermentation pathways. Sugars that are not metabolized are excreted into the medium (Wright and Axelsson, 2019). This is the case for *S. thermophilus,* which excretes the galactose moiety of lactose, which is further used via the Leloir pathway by *L. bulgaricus* in yogurt, for example (Zourari et al., 1992).

Nutritional dependencies are the key to creating artificial LAB co-cultures of metabolically dependent partners that positively interact through different mechanisms: the cross-feeding of vitamins or sugars or the sharing of public goods such as the CEP, which supplies peptides and free amino acids. A study by Settachaimongkon et al. (2014) investigated the interactions between proteolytic and non-proteolytic strains of *S. thermophilus* in co-culture with *L. delbrueckii* subsp. *bulgaricus* and showed that dependency between two LAB strains is essential for the interactions to be successful. The mutualistic interaction observed in yogurt does not occur when *S. thermophilus* has its own CEP. Another strategy might be to use strains which can hydrolyze complex sugars in order to furnish simple sugars to a LAB strain that does not possess the necessary enzymes. The fact that LAB are able to utilize a rich diversity of carbon sources for energy is an advantage as it limits resource overlaps and hence competition.

PERSPECTIVES FOR LAB – LAB CO-CULTURES IN FOOD FERMENTATION

In this review we have shown that co-cultures can be used for substrate conversion, multiplication of the functional traits expressed and the optimization of bioprocesses. We have also showed that the stronger the microbial interactions, the greater are the outputs. The positive combination of microbial strains seems mainly due to metabolic dependencies between the interacting partners. Our aim was to demonstrate that this is particularly relevant for the co-culture of LAB, which both (1) offers a broad range of functionalities to food products, such as organoleptic, sanitary, nutritional and health properties, and (2) displays a high level of metabolic diversity that enables the construction of nutritional dependencies between strains and thus the creation of positive interactions within co-cultures.

Artificial LAB co-cultures could be used to ferment new matrices containing different resource types that have more or less been exploited until now. In a context of developing more sustainable and healthy food products, animal-sourced proteins need to be consumed less, with a greater preference for plant-based proteins because of environmental impacts and the increasing need for proteins in general. It is therefore relevant to look for new ways to valorize plant resources, and particularly plants with a high protein content. Fermentation by LAB has already proved crucial in various traditional plant-derived products (Tamang et al., 2016b). By means of appropriate strain selection and associations to promote positive interactions, LAB could therefore make an important contribution to alleviating unpleasant sensory characteristics as well as nutritional and digestive disadvantages. They can in fact modify aroma profiles, and lower the concentrations of phytates and oligosaccharides responsible for intestinal discomfort. Kimoto-Nira et al. (2012) introduced the idea that artificial LAB co-cultures could also be used to ferment mixes of milk and plant-based substrates. They created a co-culture of *L. lactis* and *L. raffinolactis* that better acidified milk when the two strains grew together. The non-proteolytic *L. raffinolactis* strain was able to degrade melibiose, raffinose and stachyose which cause intestinal discomfort, while *L. lactic* was used for its production of a desirable flavor. It is important to note that when describing the interactions from an "end-product" point of view, positive interactions can involve either positive microbial interactions or no interactions. In fact, in a medium where both LAB grow without influencing one another, the product will gather more functions than if produced in a monoculture. It might therefore be possible to use a "pilot" strain responsible for the basic fermentation process (i.e., carbohydrate utilization) and complementary strains to add functionalities to the final product (i.e., production of aroma compounds or bioactive peptides).

Ultimately, a clearer understanding of positive interactions between LAB strains may lead to more efficient starters, as well as improvements to their production. Indeed, the mechanisms underlying interactions offer insights into possible LAB associations that might be more appropriate, depending on the results targeted. It may also enable the improved control of fermentation processes, leading to improved reproducibility. Further, starters are usually produced separately because of the disturbing effects on microbial viability of concentration, freeze-drying and storage steps. It is, however, possible to imagine co-cultures of LAB strains that would enable higher survival rates during these stressful processes, notably through an increased production of EPS, known to be a cryoprotectant. Finally, a clearer understanding of LAB interactions and their mechanisms could also facilitate the preservation of complex microbial assemblies, such as those found in cheese, and the cultivability of strains from complex environments.

The production of extracellular vesicles (EVs) (also referred as membrane vesicles) by LAB is most definitely a vector for interactions that should be considered in future studies. EVs are lipid bilayer-enclosed spherical structures which range in size from 20 to 300 nm and are released by cells from all living kingdoms (Kim et al., 2015; van Niel et al., 2018; Gill et al., 2019). They play a pivotal role in cell-to-cell communication through their ability to transport bioactive molecules (proteins, nucleic acids, lipids, metabolites, signaling molecules) from donor to recipient cells. In Gram-positive bacteria, most studies have been conducted on EVs generated by pathogenic bacteria (Brown et al., 2015; Liu et al., 2018b). They are implicated notably in the delivery of virulence factors, host cell modulation, antibiotic resistance, survival and microbial competition and cooperation. In LAB, EV production has been demonstrated in *L. plantarum*, *Enterococcus faecalis, L. rhamnosus*, *Limosilactobacillus reuteri,* and *Lacticaseibacillus casei* (Behzadi et al., 2017; Domínguez Rubio et al., 2017; Grande et al., 2017; Li et al., 2017) and to the best of our knowledge, their biological role was mainly considered with respect to LAB-host interactions. Considering the cargo molecules that Gram-negative and Gram-positive bacterial EVs can carry, as well as their ecological functions, LAB-derived EVs could also play crucial roles in microbial interactions, whether they are positive or negative. Indeed, they may be involved in nutritional interactions through the

cross-feeding of nutritional compounds, and in the delivery of antimicrobial compounds (Liu et al., 2018a). The participation of EVs generated by LAB in the structure and function of microbial communities/co-cultures is a promising research area of considerable interest to the field of food biotechnology that still needs to be explored.

The co-culture of LAB offers new opportunities for food fermentation in terms of controllable outputs, product functionalization and resource utilization. The construction of such artificial co-cultures needs to be well-reasoned as their outcomes depend on numerous criteria: the targeted functionalities of the final product or end-product, the composition of the medium, abiotic conditions, the metabolic complementary of strains and of course, their safety. There is therefore a crucial need for a dedicated database gathering information on LAB genomes, known interaction networks, phenotypic screening results and isolated biotopes. Existing databases such as FoodMicrobionet[1] can already provide valuable data and insights on the composition and relative abundances of the microorganisms in a food sample at a specific time (Parente et al., 2016), and Florilege[2] gathers knowledge on microorganism biotopes and phenotypes from the literature using text mining tools (Falentin et al., 2017). These databases could be further exploited, supplemented and hopefully become interoperable in the future.

With a clearer understanding of LAB interactions, the development of databases, the use of mathematical modeling and meta-omics approaches, it may be possible to develop more sophisticated LAB co-cultures. Indeed, this review focuses on the example of a two-strain co-culture as a starting point and we believe that increased bacterial diversity will only offer more benefits to fermented food products and strengthen the bonds between associated strains.

[1] http://www.foodmicrobionet.org

[2] http://migale.jouy.inra.fr/Florilege/#&welcome

AUTHOR CONTRIBUTIONS

FC collected the literature, wrote the manuscript, and the figures with the extensive contribution of VG and AT, who reviewed and edited each version. TN and EG reviewed the manuscript and the figures. All authors read and approved the final version of the manuscript.

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The Ability of Riboflavin-Overproducing *Lactiplantibacillus plantarum* Strains to Survive Under Gastrointestinal Conditions

*Annel M. Hernández-Alcántara[1], Sandra Pardo[1], Mari Luz Mohedano[1], Graciela M. Vignolo[2], Alejandra de Moreno de LeBlanc[2], Jean Guy LeBlanc[2], Rosa Aznar[3,4] and Paloma López[1]**

[1] Department of Microorganisms and Plant Biotechnology, Margarita Salas Center for Biological Research (CIB)-Consejo Superior de Investigaciones Científicas (CSIC), Madrid, Spain, [2] Reference Center for Lactobacilli (CERELA)-Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), San Miguel de Tucumán, Argentina, [3] Department of Preservation and Food Safety Technologies, Institute of Agrochemistry and Food Technology (IATA)-Consejo Superior de Investigaciones Científicas (CSIC), Paterna, Spain, [4] Department of Microbiology and Ecology, University of Valencia, Valencia, Spain

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University of Eastern Finland, Finland

***Correspondence:**
Paloma López
plg@cib.csic.es

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Riboflavin, vitamin B2, is essential for humans and has to be obtained from the diet. Some lactic acid bacteria (LAB) produce this vitamin, and they can be used for *in-situ* fortification of foods. This could be an alternative to supplementation with chemically synthesized vitamin, to palliate riboflavin deficiencies in specific groups of people. Moreover, if the producing LAB could survive in the gastrointestinal stress (GIT) they could be added as probiotics in this environment. In the present study we tested two riboflavin-overproducing *Lactiplantibacillus plantarum* strains (M5MA1-B2 and M9MG6-B2), spontaneous mutants of LAB isolated from chicha, a traditional Andean beverage. These two LAB, and also their isogenic strains M5MA1-B2[pRCR12] and M9MG6-B2[pRCR12], expressing the mCherry protein from the pRCR12 plasmid, were evaluated *in vitro* under simulated GIT conditions. Among other, specifically developed protein fluorescence assays were used. The four LAB showed similar levels of adhesion (>6.0%) to Caco-2 cells, higher than that of the probiotic *Lacticaseibacillus rhamnosus* GG strain (4.51%). Thus, LAB biofilm formation was assessed in the labeled cells by intracellular mCherry fluorescence and in the unlabeled parental strains by crystal violet staining. Both methods detected the formation of consistent biofilms by the *L. plantarum* strains. The quantification of mCherry fluorescence was also used to analyze LAB auto-aggregation properties. High levels of auto-aggregation were detected for both M5MA1-B2[pRCR12] and M9MG6-B2[pRCR12]. Survival of LAB included in a commercial cereal-based food matrix (Incaparina) under GIT conditions was also evaluated. The four LAB were resistant *in vitro* to the stomach and intestinal stresses, and proliferated in this environment, indicating a protective and nutritional effect of the Incaparina on the bacteria. Also, M9MG6-B2 survival in the presence or absence of Incaparina was evaluated *in vivo* in a BALB/c mouse model. The administration of the M9MG6-B2 strain alone or together with Incaparina had no

adverse effect on the health, growth and/or well-being of the rodents. In addition, an increment in the villus length/crypt depth ratio was observed. The overall results obtained indicate that the LAB studied have probiotic characteristics of interest for the development of functional foods.

Keywords: lactic acid bacteria (LAB), riboflavin, protein mCherry, *Lactiplantibacillus plantarum*, tolerance to gastrointestinal stress of *Lactiplantibacillus*

INTRODUCTION

Riboflavin (vitamin B2) is essential for life, but unlike many plants, fungi, and bacteria, humans are unable to synthesize it. Humans must therefore rely on exogenous sources of riboflavin provided in their diet, and also by its production by the microbiota of the large intestine (Hill, 1997; Powers, 2003). Important food sources of riboflavin included milk and dairy products, yeast, cereals, meats, oily fish, and green leafy vegetables. Generally grain products provide low amounts of riboflavin as much of the vitamin is lost due to processing; nevertheless, vitamin fortification practices make certain breads and other grain-based products good sources of riboflavin (Powers, 2003).

Riboflavin is very important for energy production, since it is the precursor of the two flavoprotein coenzymes, flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN). These coenzymes are crucial in many cellular process as they are cofactors in oxidation-reduction reactions; conversion and recycling of niacin, folate and vitamin B6, the synthesis of all heme proteins including hemoglobin, nitric oxide synthases, P450 enzymes, and in electron transfer and oxygen transport and storage (Pinto and Rivlin, 2013). These flavoproteins are also co-factors in the metabolism of essential fatty acids in brain lipids, the absorption and utilization of iron, and the regulation of thyroid hormones. Therefore, the interruption of any of these processes due to a riboflavin deficiency would have negative consequences for many body functions including brain functions (Kennedy, 2016). Riboflavin derivatives also have antioxidant properties and increase endogenous antioxidant status as essential cofactors in the glutathione redox cycle (Ashoori and Saedisomeolia, 2014).

The World Health Organization (WHO) has proposed riboflavin, as one of the six major indicators for assessing human growth, development, and nutritional status, and it was reported that riboflavin deficiency is endemic in populations whose diets lack dairy products and meats (Rohner et al., 2007). The European Food Information Council (EFSA, 2017), has established that the recommended daily allowance (RDA) of riboflavin is 1.7 mg/day; however, this can only be obtained by consuming balanced diet. The increased incidence of malnutrition, in addition to certain pathologies and drugs that affect absorption, is why sub-clinical deficiencies of riboflavin are very frequent throughout the world. For this reason, mandatory fortification of staple foods is now recommended as a general health policy in many countries. Currently, industrial production of riboflavin is mainly via microbial biosynthesis, and among the producer strains are two yeast-like fungi *Eremothecium ashbyii* and *Ashbya gossypii*, as well as the bacterium *Bacillus subtilis* (Alizadeh Behbahani et al., 2019).

Some lactic acid bacteria (LAB) are also able to synthesize B-group vitamins and have potential for their *in situ* production in fermented foods (Capozzi et al., 2011; Juarez del Valle et al., 2014). In this way, the use of riboflavin producing LAB to produce novel bio-enriched foods, and that also provide other health benefits, represents a more natural and consumer-acceptable alternative to using chemically synthesized vitamins (Gu and Li, 2016; Rodrigo-Torres et al., 2019). The levels of the vitamin that would be produced in such foods presumably would not have any negative health implications for humans. No upper limit of intake has been set for riboflavin in humans since excess intakes are excreted in urine (Flynn et al., 2003). In this regard, Institute of Medicine Food Nutrition Board (1998) and the European Food Safety Authority (2000) have both stated that intakes of up to 400 mg of riboflavina per day, almost 400 times the recommended intake, did not cause adverse side effects and concluded that this value could be used as the Tolerable Upper Intake Level.

Roseoflavin is a toxic riboflavin analog, and can be used to select mutations in the riboswitch regulatory region of the riboflavin (*rib*) operon, which results in constitutive expression of the riboflavin operon. This in turn leads to over-production of vitamin B2 (Kukanova et al., 1982). Hence, roseoflavin treatment is a widely used methods to obtain LAB strains that overproduce B2 vitamin (Capozzi et al., 2011; Juarez del Valle et al., 2014). This method has been successfully employed for *Limosilactobacillus fermentum, Lactiplantibacillus plantarum*, *Lactococcus lactis*, *Leuconostoc mesenteroides,* and *Propionibacterium freudenreichii* (Burgess et al., 2004, 2006; Arena et al., 2014), among others. In particular, a dairy product fermented with *P. freudenreichii* was shown to counteract the deficiency of riboflavin in an animal model (LeBlanc et al., 2006). An important characteristic to highlight, is that these roseoflavin resistant strains, are spontaneous, non-genetically modified, organisms and therefore could be exploited in the production of foods enriched with vitamin B2.

The concept of *in situ* production of riboflavin using selected LAB opens the possibility of developing novel food products designed for specific groups of people, such as the elderly, children, pregnant women, sportsmen, vegetarians, and adolescents (Ge et al., 2020), and even to use the food to deliver probiotic riboflavin-overproducing LAB for *in situ* vitamin B2 synthesis in the digestive tract. The chemical structure of riboflavin produced by LAB is the same as that used to

TABLE 1 | Lactic acid bacteria used in this study.

Strain	N° in type collections	Plasmid	Antibiotic resistance	Characteristics	References
Lactobacillus plantarum M9MG6-B2. Renamed *Lactiplantibacillus plantarum* M9MG6-B2	CECT 9435	–	–	Vitamin B2-overproducing strain	Yépez et al., 2019
Lactobacillus plantarum M5MA1-B2. Renamed by Zheng et al. (2020) *Lactiplantibacillus*	CECT 9434	–	–	Vitamin B2-overproducing strain	Yépez et al., 2019
Lactiplantibacillus plantarum M9MG6-B2 [pRCR12]	–	[pRCR12]	Cm^R	M9MG6-B2 strain fluorescently labeled with mCherry	This study
Lactobacillus plantarum M5MA1-B2 [pRCR12]. Renamed by Zheng et al. (2020) *Lactiplantibacillus*	CECT 9402	[pRCR12]	Cm^R	M5MA1-B2 strain fluorescently labeled with mCherry	Mohedano et al., 2019
Lactococcus lactis MG1363[pRCR12]	–	[pRCR12]	Cm^R	Source of plasmid pRCR12	Garay-Novillo et al., 2019
Lactobacillus rhamosus GG. Renamed by Zheng et al. (2020) *Lacticaseibacillus*	ATCC 53103	–	–	probiotic strain	Capurso, 2019

CECT, Colección Española de Cultivos Tipo; ATCC, American Type Culture Collection.

supplement foods, but its production costs are much lower (Liu et al., 2020). In addition, probiotic strains could provide the host not only with specific health promoting properties, but could also increase *in situ* production of riboflavin (Thakur et al., 2016). It has even been shown that riboflavin producing probiotic strains can provide anti-inflammatory and anti-cancer effects in animal models (LeBlanc et al., 2020), but the safety of using live vitamin-producing probiotics needs to be examined further before being administered to immuno-compromised patients.

In this context, *L. plantarum* strains isolated from chicha (a traditional beverage from Andean regions), were exposed to roseoflavin, and riboflavin-overproducing strains were isolated and tested for the production of functional cereal-based foods, with the objective that the technological properties they could provide in this type of food could palliate the deficiency of this vitamin in humans (Yépez et al., 2019).

These vitamin B2-overproducing LAB have also been used to standardize fluorescent detection of riboflavin production in real time during growth (Mohedano et al., 2019) and also showed the potential of strains *L. plantarum* M5MA1-B2 and M9MG6-B2, as producing high levels of riboflavin and possessing good technological properties. Both strains carry a different punctual mutation in the same position (G19A and G19C in the M5MA1-B2 and M9MG6-B2 strains, respectively) located in the regulatory region of the *rib* operon (Mohedano et al., 2019). Furthermore, the comparative analysis of the genomes of five of the parental riboflavin producing strains, as well as assessing some criteria to determine their food safety, revealed a great similarity between the strains studied. The M5MA1 strain was also shown to possess some unique genes in its genome (Rodrigo-Torres et al., 2019).

Therefore, the objective of this work was to evaluate some probiotic properties of the *L. plantarum* M5MA1-B2 and M9MG6-B2 strains using *in vitro* comparative analysis of behavior and survival in the human digestive tract followed by an *in vivo* evaluation of M9MG6-B2 in a cereal-based food matrix using a murine model.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

The LAB used in this work are presented in **Table 1**. The LAB strains were routinely grown in MRS broth (de Man et al., 1960) (Pronadisa, Spain) at 37°C. When evaluating the riboflavin production, the strains were grown in the chemical defined medium (CDM) (Sánchez et al., 2008) lacking riboflavin (CDM-Rib). LAB carrying the plasmid pRCR12 were growth in media supplemented with chloramphenicol (Cm) at 10 μg mL^{-1}, besides in the experiments performed in microtitre plates, in which the antibiotic was omitted. Solid media were prepared by addition of agar at a concentration of 1.5% (w/v) (Pronadisa, Spain).

Fluorescent Labeling of *L. plantarum* M9MG6-B2

The pRCR12 plasmid was obtained from *L. lactis* MG1363[pRCR12], grown until the beginning of stationary phase, using the JetStar 2.0 Plasmid Purification kit (Genomed, Löhne, Germany). Plasmid isolation was performed following the protocol supplied with the kit. For the insertion of plasmid pRCR12 into *L. plantarum* M9MG6-B2, 0.5 μg of plasmid were used for electroporation (25 μF, 1.3 kV and 200 Ω in 0.1 cm cuvettes) as previously described (Berthier et al., 1996). Transformants were selected on MRS-agar supplemented with Cm at 10 μg mL^{-1} at 37°C.

Simultaneous Detection of Bacteria Growth and Riboflavin or mCherry Production

To follow the growth and fluorescence of *L. plantarum* strains in real time, the method described by Mohedano et al. (2019) was used. Simultaneous spectrophotometric monitoring of growth at 37°C and of the fluorescence of the bacterial cultures was performed using the Varioskan Flask System (Thermo Fisher Scientific, USA) and sterile 96-well optical white w/lid cell culture polystyrene plates (Thermo Fisher Scientific, USA). The experiments were performed in triplicate by incubating at 37°C and measuring the optical density (OD) and fluorescence every 30 min during 14 h.

Growth and fluorescence due to mCherry protein were determined in cultures grown in MRS that were sedimented by centrifugation at 9,300 × *g*, for 10 min at room temperature and resuspended in fresh medium to an $OD_{600\,nm}$ of 0.1. Aliquots of 200 μL of each culture were analyzed in triplicate in the microtiter plate reader incubated at 37°C, using MRS as control. The $OD_{600\,nm}$ of the cultures was measured and the emission of the mCherry fluorescence at a wavelength of 610 nm upon excitation at a wavelength of 587 nm.

Growth and fluorescence due to riboflavin production were determined from cultures growth in MRS that were sedimented as above and resuspended in CDM-Rib to an initial $OD_{480\,nm}$ of 0.1. Aliquots of 200 μL of each culture were analyzed in triplicate as indicated above. Growth was monitored at an $OD_{480\,nm}$ and the riboflavin fluorescence upon excitation at a wavelength of 440 nm and detection of emission at a wavelength of 520 nm.

The growth of the LAB in liquid medium allowed the determination of the kinetic parameters, doubling time or generation time (dt) and growth rate (μ) as described by Maier (2009) and Widdel (2010).

Quantification of Riboflavin Production by Fluorescence

To quantify the riboflavin concentration, a calibration curve was constructed to correlate the fluorescence emitted by solutions with increasing concentrations of riboflavin at 520 nm (**Supplementary Figure 1**). To this end, serial dilutions of a riboflavin solution prepared in CDM-Rib medium at a concentration of 10 mg mL^{-1} were analyzed by placing aliquots of 200 μL of each dilution in a black, non-sterile, polypropylene 96-well plate (Nunc™, Thermo Fisher, USA), at an excitation wavelength of 440 nm and an emission wavelength of 520 nm in the Varioskan plate reader.

Quantification of riboflavin levels produced by the LAB strains was performed by measuring fluorescence in culture supernatants grown in CDM-Rib medium using 14 h cultures described in section Simultaneous Detection of Bacteria Growth and Riboflavin or mCherry Production that were recovered from each well after bacteria were removed by centrifugation. Aliquots of 200 μL of the supernatants were used to measure fluorescence in the plate reader. Riboflavin concentration was estimated by interpolating fluorescence values on the calibration curve. The determinations were made in triplicate.

Analysis of Bacterial Cultures by Fluorescence Microscopy

Exponential cultures of the *L. plantarum* strains were sedimented by centrifugation and resuspended in saline solution (0.85% w/v NaCl) to obtain a 10-fold concentrated suspension. Subsequently, a 5 μL aliquot of the suspensions was directly analyzed without fixation by phase contrast or fluorescence microscopy. A Leica DM1000 model microscope (Leica Microsystems, Mannheim, Germany) with a light source EL6000 and the filter system TX2 ET was used for detection of the mCherry fluorescence. The microscope was connected to a DFC3000G camera (Leica Microsystems) with a CCD sensor. Image analysis was performed with the Leica Application Suite X software (Leica Microsystems).

Caco-2 Cell Culture and Adhesion Assay

The human enterocyte cell line, obtained from the cell bank at the Centro de Investigaciones Biológicas Margarita Salas (Madrid, Spain), was seeded in 96-well tissue culture plates (Falcon Microtest™, USA) at a final concentration of 1.25 × 10^5 cells mL^{-1} and grown as monolayers of differentiated and polarized cells for 14 days as previously described (Nácher-Vázquez et al., 2017). For the adhesion assays, exponential-phase LAB cultures grown in MRS were sedimented by centrifugation (12,000 × *g*, 10 min, 4°C), and resuspended in the appropriate volume of Dulbecco's Modified Eagle medium (DMEM, Invitrogen) to give a final concentration of 1.25 × 10^6 colony forming units (cfu) mL^{-1}. Each bacterial suspension (0.1 mL) was added to a well (ratio 10:1, bacteria:Caco-2 cells) and the plates were incubated for 2 h at 37°C. The non-adhered bacteria were then removed and the cell-associated bacteria processed and quantified by plate counting on MRS-agar plates, as previously described (Nácher-Vázquez et al., 2017). All adhesion assays were conducted in triplicate. The percentage of adhesion to Caco-2 cells was calculated as:

$$Adhesion\ (\%) = \frac{\frac{cfu}{mL}\ attached\ to\ Caco-2\ cells}{\frac{cfu}{mL}\ added\ to\ Caco-2\ cells} \times 100$$

Detection of Biofilm Production on Polystyrene Plates as Abiotic Surface

Crystal Violet Staining Method

Assessment of biofilm formation on polystyrene plate was conducted as previously described by Stepanovic et al. (2007). LAB were grown in MRS medium until a concentration of 1.5 × 10^8 cfu mL^{-1} was reached and then diluted 1:100 in the same medium. Then, 200 μL of these suspensions (1.0 × 10^6 cfu mL^{-1}) were used to inoculate sterile 96-well polystyrene microtiter plates (BD, New Jersey, USA). MRS medium, was used as negative control (Ctr). After incubation for 48 h at 37°C the supernatant from each well was carefully removed to eliminate the planktonic cells, then wells were gently washed 3 times with sterile PBS (Phosphate-Buffered Saline) and dried prior the addition of 150 μL of methanol, as fixing agent, and incubation for 20 min. Afterwards, methanol was removed from the wells and biofilms subsequently stained with 150 μL of crystal violet solution (2.0%

w/v) (Fluka, USA) for 15 min at 21°C. The staining solution was then removed from the wells followed by two washes with distilled water to eliminate the excess of dye and dried. Finally, the dye was resolubilized with 150 μL of ethanol (96% v/v) for 30 min and the stained bacteria were quantified by measuring the $OD_{570\,nm}$ of the sample (OD_S) in comparison with the $OD_{570\,nm}$ of the control sample (OD_{Ctr}) in the Varioskan plate reader. Bacteria were classified for their ability to form biofilms in four categories according to Hashem et al. (2017): (i) non-adherent or negative biofilm, when sample $OD_{570\,nm}$ (OD_S) ≤ negative control $OD_{570\,nm}$ (OD_{Ctr}); (ii) weakly adherent, $OD_{Ctr} < OD_S \leq (2 \times OD_{Ctr})$; (iii) moderately adherent, $(2 \times OD_{Ctr}) < OD_S \leq (4 \times OD_{Ctr})$ and (iv) highly adherent, $(4 \times OD_{Ctr}) < OD_S$. Three cultures of each bacterium were analyzed, and experiments were performed in triplicate.

Fluorometric Method by mCherry Fluorescence Measurement

A new direct quantitative method, developed in this work, and based on the fluorescence emitted by the mCherry protein encoded by pRCR12 was used. The LAB biofilm formation was carried out as described in section Crystal Violet Staining Method. After 48 h of incubation, the biofilms were carefully washed three times with 200 μL of PBS. Subsequently, each well was resuspended in 200 μL of saline solution and the fluorescence determined as the emission of the mCherry fluorescence at a wavelength of 610 nm upon excitation at a wavelength of 587 nm was measured. Results were expressed as arbitrary units (au) of fluorescence emitted by each *L. plantarum* strain.

In vitro Auto-Aggregation Test

A method based on the detection of the mCherry fluorescence and developed in this work was used. A calibration curve for each *L. plantarum* strain was constructed to correlate the fluorescence emitted by the suspensions with the concentration of bacteria (**Supplementary Figure 2**). For this purpose, serial dilutions of a bacterial suspension prepared in saline solution at a concentration of 2.0×10^8 cfu mL^{-1} were analyzed by placing aliquots of 200 μL of each dilution in a 96-well polystyrene plate to measure fluorescence due to mCherry at an excitation wavelength of 578 nm and an emission wavelength of 610 nm in the Varioskan plate reader. The results obtained showed a linearity range between 2.5×10^7 cfu mL^{-1} and 2×10^8 cfu mL^{-1}.

To determine the auto-aggregation ability of *L. plantarum* strains, exponential cultures grown in MRS were sedimented and resuspended to a final concentration of 1.0×10^8 cfu mL^{-1} in saline solution. Subsequently, 200 μL aliquots of the bacterial suspensions were dispensed into a 96-well polystyrene plate and fluorescence was measured for detection of mCherry in the Varioskan equipment, before (initial fluorescence) and after (final fluorescence) incubation at 37°C without shaking for 15 h. Non-aggregated bacteria were carefully removed and the aggregated cells at the bottom of each well were resuspended in 200 μL of saline solution prior to determine the final fluorescence. The auto-aggregation was calculated as percentage, being 100% the initial fluorescence:

$$\%\ Auto{-}aggregation = \frac{Initial\ fluorescence - Final\ fluorescence}{Initial\ fluorescence} \times 100$$

Resistance to Simulated Gastrointestinal (GIT) Transit in a Food Matrix

The food matrix used in the trials was the cereal-based beverage Incaparina (Central de Alimentos, S.A., Guatemala City, Guatemala and **Supplementary Table 1**), which is enriched in protein, vitamins and other compounds provided to children in Guatemala kindergartens and approved by the Institute of Nutrition of Central America and Panama (INCAP). To perform the tests, the protocol described by Haffner et al. (2017) was adapted to this study (see details in **Figure 1**). The food matrix was dissolved in water and heated in a microwave oven following the directions of the product. Then, 10^8 cfu mL^{-1} of each LAB were resuspended independently in saline solution (1 mL), and each one used to inoculate 30 mL of the matrix. These bacterial suspensions were then subjected to the following consecutive exposures to stresses at 37°C with shaking (100 rpm):

(i) gastric phase, the pH of samples inoculated were gradually decreased from 6.5 (initial pH of the food matrix) to 2.0 with 2.5 M HCl, with intervals of 0.5–1.0 units and with 15 min incubation at each pH. Aliquots of 200 μL were withdrawn prior to each pH decrease;

(ii) small intestine phase, first the pH was increased with 2.5 M NaOH from 2.0 to 4.0. then, 10 mL of pancreatic and bile juice solution [0.6% w/v of bile salts (Sigma-Aldrich), 1.25% w/v of $NaHCO_3$ and 0.09% w/v of pancreatin (Sigma-Aldrich)] were added to the gastric phase, generating a pH increase to 7.0, and the mixture was incubated for 3 h. Aliquots of 200 μL were withdrawn after each hour.

Finally, 100 μL of the aliquots subjected to the treatments in adequate dilutions were streaked on MRS plates, incubated at 37°C for 48 h, and the number of cfu was calculated for each condition evaluated by plate counts. The results were expressed as percentage of surviving cells (initial bacterial concentration = 100%).

In vivo Animal Trials

Conventional adult BALB/c mice (male, 4 weeks old, weighing 12 ± 2 g) were obtained from the animal facility of the Centro de Referencia para Lactobacilos (CERELA-CONICET, San Miguel de Tucumán, Argentina) and were maintained in ventilated cages under controlled conditions (18–20°C and a 12 h light/dark cycle). The animal protocol was approved by the Animal Protection Committee of CERELA (protocol no. CRL-BIOT-LT-20142/A), and all experiments complied with the current laws of Argentina for the use of experimental animals.

Animals were divided into 6 experimental groups consisting of at least 6 mice each (see protocol in **Figure 2**). The Control group did not received any special treatment, and were fed with balanced conventional rodent food and diluted milk (1:30 low fat milk/water, v:v) *ad libitum*. The bacterial supplemented

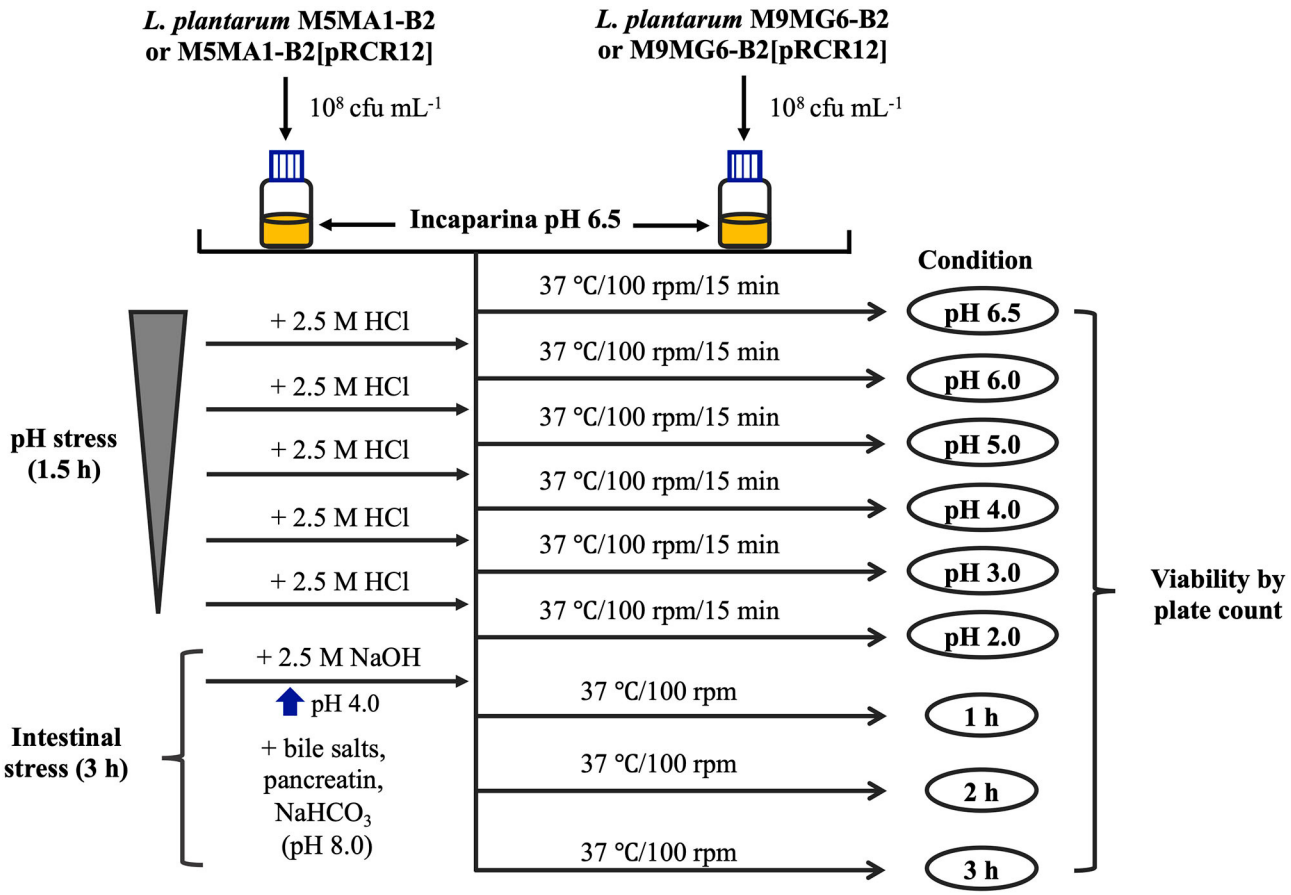

FIGURE 1 | Experimental *in vitro* protocol for GIT stress. Aliquots of 30 mL of the food matrix, Incaparina (pH 6.5), prepared according to the instructions of the product, were inoculated with either M5MA1-B2, M5MA1-B2[pRCR12], M9MG6-B2, or M9MG6-B2[pRCR12] strains. Subsequently, suspensions were subjected to sequential increasing acidic stress, by gradually decreasing the pH from 6.5 to 2.0. After 15 min of incubation at each pH, aliquots were withdrawn to determine cell viability. After gastric treatment, pancreatin and bile salt juices were added to the gastric phase to simulate intestinal stress. The whole mixture was incubated for 3 h and after every hour cell viability was measured.

groups received M9MG6-B2 or M9MG6-B2[pRCR12] strains in diluted milk at a concentration of 1×10^9 cfu mL^{-1}. To this end, strains were previously grown in MRS medium for 16 h at 37°C, sedimented (5,000 × *g*, 10 min, 4°C) washed with saline solution, resuspended in the original volume with low fat milk and diluted in water. The animals that were fed with Incaparina received the product as a replacement of their drinking water. In the groups that received both Incaparina and bacterial supplementation [Incaparina + M9MG6-B2 and Incaparina + M9MG6-B2[pRCR12] groups], the bacterial strains were prepared as stated above and resuspended in Incaparina at a concentration of 1×10^9 cfu mL^{-1}, and this mixture was given as a replacement of water. These supplements/water replacements were provided to the animals for 28 days (each drinking bottle was replaced twice daily); food and water/Incaparina consumption was measured daily. Animal live weights and behavior were determined on a bi-daily basis. Feces were collected every 5 days from the animals that consumed M9MG6-B2[pRCR12] in the presence or absence of Incaparina. These samples were homogenized and diluted in sterile saline solution and plated on MRS-agar plates containing Cm. Pink colonies, consisting of the strain that expressed mCherry were counted. At the end of the experimental period (28 days), animals were euthanized with an intraperitoneal injection of ketamine (Holliday, Scott S. A.) and xylazine (Rompun, Bayer S. A.) to obtain a final concentration of 100 and 5 μg/kg live body weight, respectively. Blood samples were taken by cardiac puncture and transferred into a tube containing EDTA as anticoagulant (1.5 mg mL^{-1} of blood) for hematological studies. The intestines of the animals were extracted, and the content removed with saline solution, cut into three parts and transferred separately to tubes containing 10% formaldehyde for histological analysis. Spleen, kidneys, and liver were excised, washed, air dried and weighed.

Histological Analysis of the Small Intestines

Fixed intestines were embedded in paraffin, sectioned (4 μm) and colored with hematoxylin and eosin. Measurements of small intestine villi length and crypts depth were performed using an optical microscope (Carl Zeiss-Axio Scope-A1,

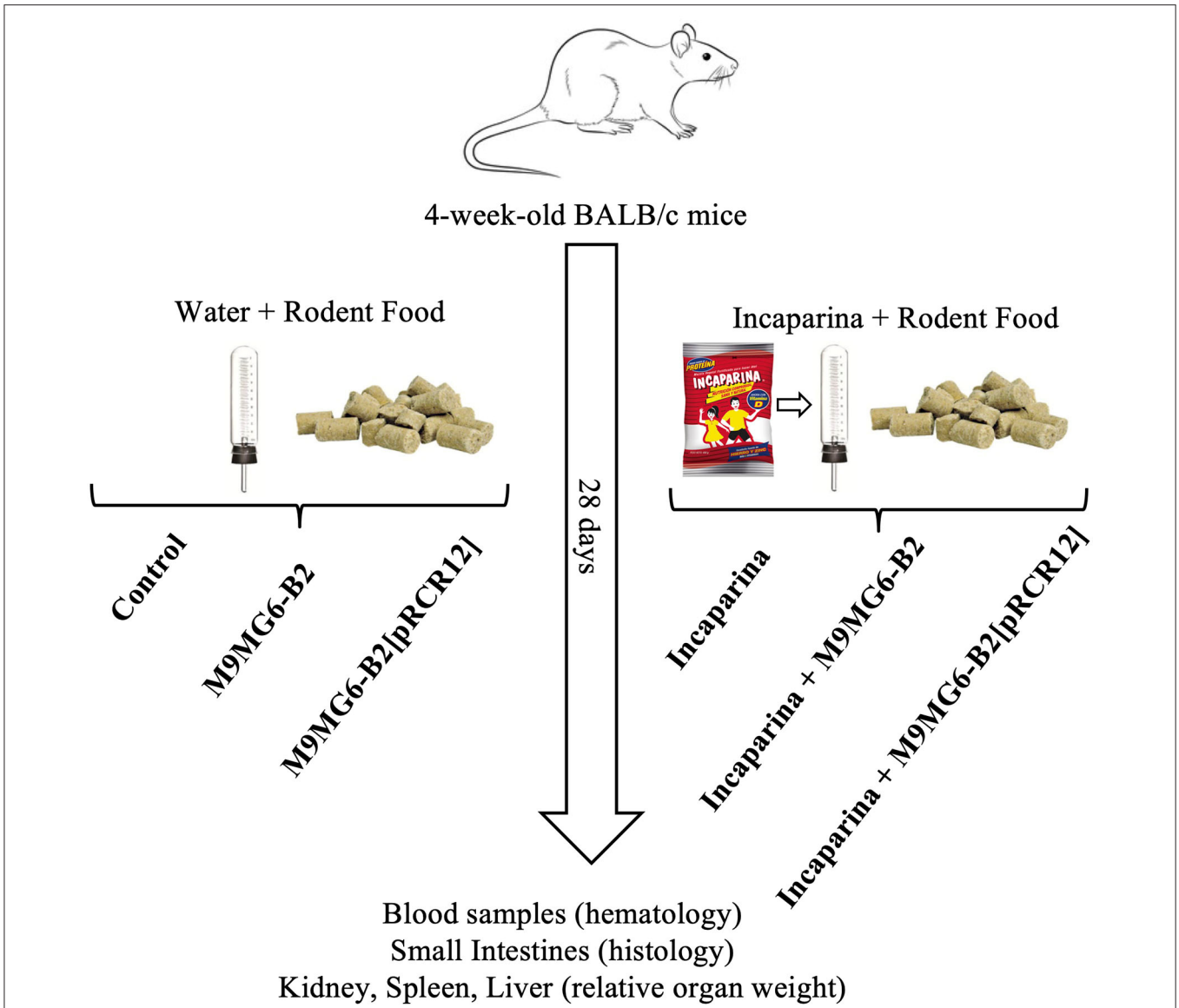

FIGURE 2 | Experimental animal protocol. Balb/c mice were divided into groups that received water and rodent food without bacterial supplementation (control group), that were supplemented with *L. plantarum* M9MG6-B2 (M9MG6-B2 group) or *L. plantarum* M9MG6-B2[pRCR12] [M9MG6-B2[pRCR12] group] or that received Incaparina and rodent food without bacterial supplementation (Incaparina group), supplemented with *L. plantarum* M9MG6-B2 (Incaparina + M9MG6-B2 group) or *L. plantarum* M9MG6-B2[pRCR12] [Incaparina + M9MG6-B2[pRCR12] group] during 28 days. Then, the animals were sacrificed and blood samples taken for hematological studies, small intestines used for histological evaluations and organs (kidney, spleen, and liver) used to determine relative organ weights.

Germany), with a 100 × magnification lens. The images were analyzed using AxioVision Release 4.8 software, calculating the villus length/crypt depth ratio (5 measurements per animal).

Statistical Analysis

All experiments were performed in triplicate. The results are expressed as the mean and the corresponding standard deviation. For the adhesion, auto-aggregation, and biofilm formation assays, in order to establish differences between strains, the data were subjected to one-way analysis of variance (ANOVA). Mean pairwise comparisons were computed with a Tukey's test with a $p \leq 0.05$. For analysis of the LAB survival cells (%) after the gastric and intestinal stresses, in the cases where strain and condition interaction was significant, *t*-tests were performed to determine if strains were significantly different at each condition level with a $p \leq 0.05$. All analyses were performed with the R software version 4.0.0 (R Core Team., 2020).

For the *in vivo* animal trials, data were assessed using the ANOVA general linear model, with the level of significance at $P \leq 0.05$. The results are presented as mean ± standard deviation (SD). Significant differences between groups were determined by

TABLE 2 | Riboflavin production, mCherry expression and kinetic growth parameters of *L. plantarum* riboflavin-overproducing strains.

Strain	MRS medium			CDM medium						
	μ (h^{-1})	dt (h)	mCherry fluorescence (au)	μ (h^{-1})	dt (h)	Fluorescence (au)	Riboflavin concentration (mg L^{-1})	$OD_{480\,nm}$	Specific concentration of riboflavin[a]	Mutation in the RFN region
M5MA1-B2	0.56 ± 0.02	1.24 ± 0.03	1.01 ± 0.07	0.52 ± 0.01	1.34 ± 0.01	25.34 ± 2.84	3.19 ± 0.42	4.0	0.80	G19A
M9M6G-B2	0.56 ± 0.01	1.24 ± 0.03	1.08 ± 0.10	0.55 ± 0.01	1.25 ± 0.03	21.23 ± 1.74	2.78 ± 0.22	3.7	0.75	G19C
M5MA1-B2 [pRCR12]	0.48 ± 0.01	1.45 ± 0.04	94.63 ± 3.8	0.34 ± 0.01	2.06 ± 0.07	25.54 ± 6.68	2.59 ± 0.27	3.2	0.81	G19A
M9MG6-B2 [pRCR12]	0.46 ± 0.01	1.52 ± 0.04	101.80 ± 3.17	0.40 ± 0.01	1.72 ± 0.05	24.37 ± 6.74	3.12 ± 0.87	4.6	0.68	G19C

All values are expressed as means ± standard deviation of three independent experiments. Values referred to riboflavin, mCherry and $D_{480\,nm}$ correspond to cultures in the stationary phase after 14 h of growth in the indicated medium. The growth rate (μ) and doubling time of these cultures during the exponential phase is also depicted.
[a] *Specific concentration of riboflavin = Riboflavin concentration/$OD_{480\,nm}$.*

Tukey's *post-hoc* test. Statistical analyses were performed using MINITAB 15 software (Minitab, State College, PA, USA).

RESULTS AND DISCUSSION

Analysis of the Effect of mCherry Labeling on the Growth of *L. plantarum* M5MA1-B2 and M9MG6-B2 and on Riboflavin Production

Plasmid pRCR12 encodes the mCherry protein, and previous construction and analysis of *L. plantarum* M5MA1-B2[pRCR12] revealed that the fluorescence conferred by the plasmid allows discrimination of the bacterium in a digestive tract microbiota context and also quantitative assessment of bacterial adhesion to Caco-2 cells (Mohedano et al., 2019). Therefore, in this current work we aimed to standardize new fluorescence-based quantification techniques to evaluate probiotic characteristics of *L. plantarum* strains not previously analyzed. To this end, after the construction of *L. plantarum* M9MG6-B2[pRCR12] by plasmid transfer, we compared the performance of the riboflavin-overproducing M5MA1-B2 and M9MG6-B2 strains and their isogenic labeled strains. As expected, M5MA1-B2 and M9MG6-B2 grown in MRS-agar medium developed white colonies, while M5MA1-B2[pRCR12] and M9MG6-B2[pRCR12] showed the characteristic pink color that indicates the functional expression of the mCherry protein (**Supplementary Figure 3A**). Likewise, red fluorescence was detected by fluorescence microscopy in M5MA1-B2[pRCR12] and M9MG6-B2[pRCR12] cultures grown in MRS broth, but not observed in cultures of the isogenic unlabelled parental strains (**Supplementary Figure 3B**).

Also, in cultures grown in MRS broth the bacterial growth and the mCherry fluorescence were monitored simultaneously during 14 h (results not shown) and from these data the growth rate (μ) and doubling time (dt) of the four LAB during the exponential phase of growth were calculated, as were the levels of the fluorescence at the end of the assays (**Table 2**). For M5MA1-B2 and M9MG6-B2 strains a μ value of 0.56 ± 0.02 h^{-1} was observed with a corresponding dt of 1.24 ± 0.04 h. A lower μ was observed for the labeled strains, 0.48 ± 0.01 h^{-1} for M5MA1-B2[pRCR12] vs. 0.46 ± 0.01 h^{-1} for M9MG6-B2[pRCR12], with a concomitant increment in the doubling time, 1.45 ± 0.04 h vs. 1.52 ± 0.04 h, respectively. Expression of the mCherry in cultures of the two strains carrying pRCR12 plasmid, was confirmed since 100 au of fluorescence were recorded, while in those of the isogenic M5MA1-B2 and M9MG6-B2 only about 1 au of background fluorescence was detected.

We have previously validated quantification of riboflavin levels by determination of vitamin B2 fluorescence during the *L. plantarum* growth in the non-fluorescent CDM-Rib medium deprived of the vitamin (Mohedano et al., 2019). Thus, this method was used to determine production of riboflavin by monitoring simultaneously the growth and the vitamin fluorescence in cultures grown in CDM-Rib medium. The substitution of MRS by CDM-Rib as growth medium had little effect on the M5MA1-B2 and M9MG6-B2 μ and dt parameters (**Table 2**). However, a clear negative effect was observed in the case of the pRCR12 plasmid-carrier strains, with a decrease in μ values from 0.48 to 0.34 h^{-1} for M5MA1-B2[pRCR12] and from 0.46 to 0.40 h^{-1} for M9MG6-B2[pRCR12], accompanied by an increase in the doubling time from 1.45 to 2.06 h and from 1.52 to 1.72 h, respectively. For all four strains, vitamin synthesis was directly associated with the exponential phase of growth, reaching its maximum during this phase and being practically constant throughout the stationary phase (results not shown). After the 14 h incubation period, 25 au of fluorescence were recorded for the M5MA1-B2 and M5MA1-B2[pRCR12] strains, whereas for the M9MG6-B2 and M9MG6-B2[pRCR12] the results were 20 and 24 au, respectively (**Table 2**). When fluorescences were expressed as riboflavin concentration, they ranged from 2.6 mg L^{-1} for M5MA1-B2[pRCR12] to 3.19 mg L^{-1} for M5MA1-B2 (**Table 2**), high values which were similar to those obtained in previous results (Mohedano et al., 2019). Also, when the specific concentration of vitamin B2 was estimated (considering the optical density of the cultures as reference of the cellular biomass), the values for M5MA1 and M5MA1-B2[pRCR12] were almost identical 0.80 and 0.81 and for M9MG6-B2 and M9MG6-B2[pRCR12], only slightly lower 0.75 and 0.68, respectively (**Table 2**).

Thus, the results obtained showed that the labeling with the plasmid pRCR12 did not significantly affected the production of riboflavin. In addition, the burden of carrying the plasmid only

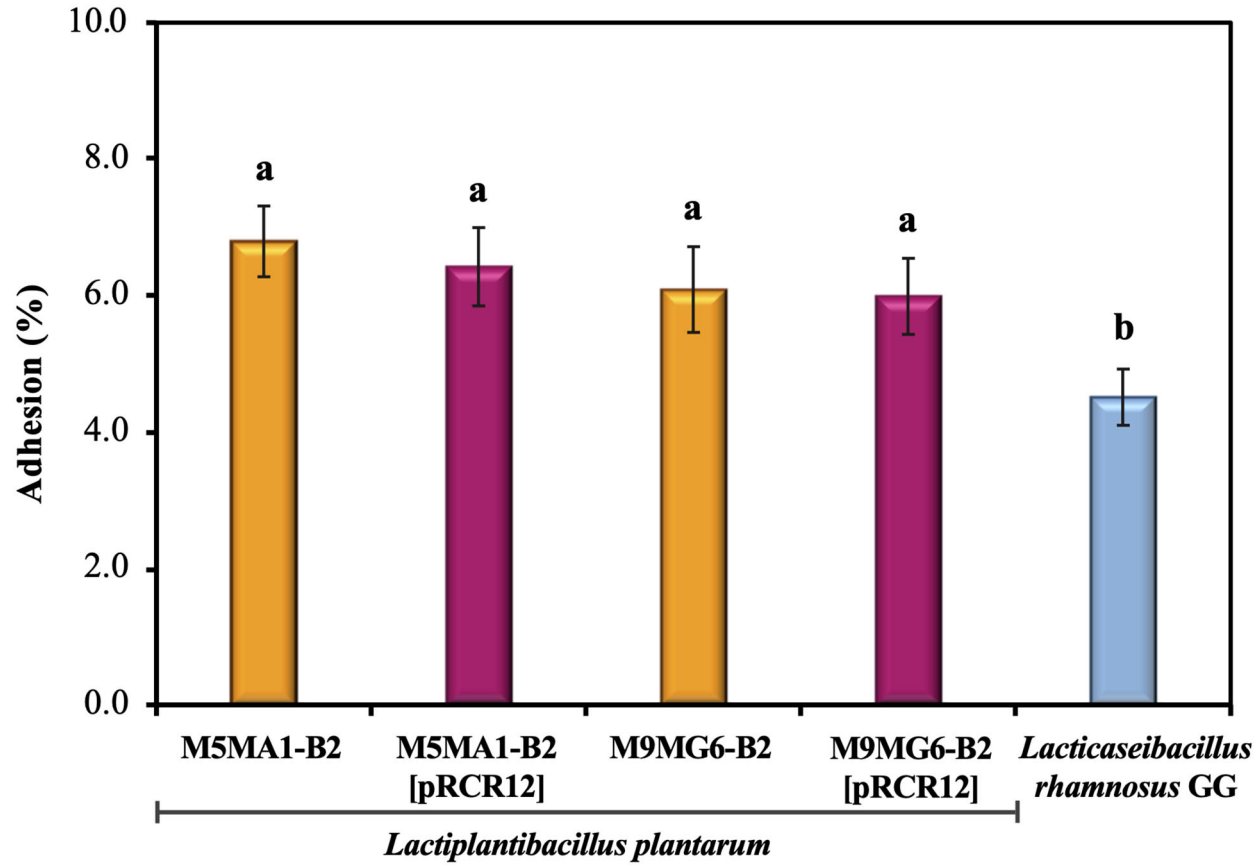

FIGURE 3 | Adhesion of the LAB strains to the biotic Caco-2 human cell line. The assays were performed during 2 h. Adhesion levels are expressed as the percentage of cfu bound. Hundred percentage corresponds to the number of bacteria added to the Caco-2 cells. The ANOVA statistical analysis of the results is depicted. A $p \leq 0.05$ was considered significant. The Tukey's test was employed ($\alpha = 0.05$) to test the statistically significant differences between samples. Means with the same letter were not significantly different.

moderately affected the growth of the strains, corroborating our previous observations with *L. plantarum* 90, B2 and M5MA1 strains (Russo et al., 2015; Mohedano et al., 2019) or *L. sakei* MN1 (Nácher-Vázquez et al., 2017) carrying the pRCR12 plasmid; thus, supporting the suitability of this vector in LAB for future studies with fluorescent labeling.

Interactions of Labeled and Unlabeled *L. plantarum* M5MA1-B2 and M9MG6-B2 Strains With Human Enterocytes

After ingestion of probiotic bacteria, their survival and persistence in the gut depend on host–bacteria interactions. Successful adhesion of probiotic bacteria to the gut epithelium has been reported to help establish colonization in the gut (Gueimonde and Salminen, 2006). As a first approach to test bacterial binding to enterocytes, human colon cell lines e.g., Caco-2 are used, due to the difficulty of examining the process *in vivo* (Kim et al., 2015). This cell line differentiates spontaneous to enterocyte-like cells after reaching confluence and expresses characteristics of mature enterocytes, including polarization, brush border and apical intestinal hydrolases (Pinto et al., 1983). Thus, the adhesion to Caco-2 cells of the four *L. plantarum* strains in comparison with that of the probiotic *L. rhamnosus* GG was investigated (**Figure 3**). The assays were performed with 1.0×10^5 bacteria exposed to 1.0×10^4 Caco-2 cells, with the aim to not saturate the binding capacity of the enterocytes in order to unmask differences between the strains. The levels of adhesion were very similar for the four *L. plantarum* strains analyzed, ranging from 6.0 to 6.81%. These results showed that both riboflavin-overproducing strains showed the same behavior and that the presence of the pRCR12 did not affect negatively the capability of any of the four bacteria to interact with the biotic surface. Moreover, *L. rhamanosus* GG displayed a 4.51% $\pm$ 0.41 adhesion level, slightly lower than that of the four *L. plantarum* under study. These adhesion levels highlight the probiotic potential of the M5MA1-B2 and M9MG6-B2 strains. In addition, the detected adhesion levels of M5MA1-B2 and M5MA1-B2[pRCR12] (6.81% $\pm$ 0.52 and 6.42% $\pm$ 0.57) were ~16-fold higher than that obtained previously (0.48% $\pm$ 0.07 and 0.34% $\pm$ 0.07 for the unlabelled and labeled strains, respectively) (Mohedano et al., 2019). This discrepancy was expected, since

TABLE 3 | *In vitro* adhesive properties of riboflavin-overproducing strains on an abiotic surface.

Strain	Biofilm determination		Auto-aggregation
	Methods		
	Crystal violet staining ($OD_{570\,nm}$)	mCherry fluorescence (au)	mCherry fluorescence method (%)
Ctr[1]	0.09 ± 0.17^{c}	1.25 ± 0.35^{b}	100^{a}
M5MA1-B2	3.84 ± 0.31^{a}	ND	ND
M9MG6-B2	3.52 ± 0.17^{b}	ND	ND
M5MA1-B2[pRCR12]	ND	102.6 ± 6.3^{a}	29.13 ± 4.0^{b}
M9MG6-B2[pRCR12]	ND	99.94 ± 7.8^{a}	34.68 ± 3.9^{a}

Values are expressed as means ± standard deviation for n = 3 independent experimental assays.

[a,b,c]Means with different letter differed significantly ($p \leq 0.05$).

ND, not determined.

[1]Ctr corresponds to either: (i) the values of $OD_{570\,nm}$ or mCherry fluorescence obtained for the biofilms formed with only medium instead of bacteria or (ii) the initial fluorescence (100%) of the 2×10^7 cfu used in the auto-aggregations assays.

the levels of binding were expressed as percentage of the added bacteria, and in our previous work the adhesion evaluation was performed using an over-saturation proportion of 5×10^9 bacteria to 1×10^5 Caco-2 cells. Supporting the results obtained here, studies performed with other *L. plantarum* strains isolated from both fermented foods and beverages showed adherence values similar to those obtained in the present study. Under similar experimental conditions to the ones performed here, an adhesion value to Caco-2 cells of 12.2%, 10.2% and 8% were reported for *L. plantarum* L15 (Alizadeh Behbahani et al., 2019), Lp91 (Duary et al., 2011) and 423 (Botes et al., 2008) strains isolated from cereal-dairy product, human fecal sample and sorghum beer, respectively.

Biofilms Formed by Labeled and Unlabeled *L. plantarum* M5MA1-B2 and M9MG6-B2 Strains on an Abiotic Surface

In addition to having the ability to bind to intestinal cells, probiotics should be able to form biofilms, a property that might provide the bacteria with protection against adverse environmental conditions, and consequently to promote their survival and persistence in the intestinal environment. Thus, to determine the capability of M5MA1-B2 and M9MG6-B2 to form biofilm, the crystal violet staining method was used (see Materials and Methods section Crystal Violet Staining Method) with polystyrene multi-well plates as the abiotic support (**Table 3**). The measurement of the $OD_{570\,nm}$ of the stained biofilms of M5MA1-B2 and M9MG6-B2 gave values of 3.84 ± 0.31 and 3.52 ± 0.17, the former being slightly but significantly higher than the latter ($p \leq 0.05$). In addition, these values were 39- and 44-fold higher than the value of the control sample (0.09 ± 0.17). Therefore, these results revealed that, according to the classification established by Hashem et al. (2017), the two *L. plantarum* strains, are very superior to the category of "strongly adherent or biofilm-forming" that only requires $OD_{570\,nm}$ of the samples 4-fold higher than that of the control (see details of categories in Material and Methods section Crystal Violet Staining Method). Detection of biofilm formation by the crystal violet staining method has been widely used for various bacterial species because the dye binds non-specifically to cells and matrix components, making it a high throughput method to quantify the total biomass that forms the biofilm (Merritt et al., 2005). However, parameters such as medium composition, temperature, and time of incubation may affect the biofilm quantification and interfere with the validity of this method. Thus, a study carried out with *L. plantarum* WCFS1 and other strains showed that the $OD_{570\,nm}$ of the biofilm was higher at 30 or 37°C vs. 20 or 25°C, but caused a reduction in the number of living cells (Fernández Ramírez et al., 2015). Similarly, in the same study a prolonged incubation time (24–48 h) caused an increase in the number of cells that make up the biofilm, but after 72 h, although higher values of $OD_{570\,nm}$ were detected, the viable cells decreased. These results were interpreted by the authors as indicating that the crystal violet may also bind to dead cells, extracellular DNA (eDNA), proteins, exopolysaccharides, and other intracellular components, which could interfere in biofilm quantification, thereby overestimating the number of viable cells that constitute the biofilm matrix. Therefore, in this work, we have standardized a fluorometric method to quantify biofilm formation on polystyrene multi-well plates as abiotic support by means of the intracellular fluorescence emitted by the mCherry protein (see details in Materials and Methods section Fluorometric Method by mCherry Fluorescence Measurement). The cultures used to generate the biofilms (1.0×10^6 cfu) showed an initial fluorescence of 1–2 au, while the biofilms of M5MA1-B2[pRCR12] and M9MG6-B2[pRCR12] formed after incubation for 48 h exhibited similar levels of fluorescence (102.60 ± 6.20 au vs. 99.94 ± 7.8) (**Table 3**). Moreover, these levels corresponded to $\sim 1.8 \times 10^8$ cfu and 1.4×10^8 cfu mL, respectively, values estimated using the calibration curve of each strain presented in **Supplementary Figure 2**. Thus, the increment of more than two $\log_{10}$ units in the cfu mL^{-1} demonstrated an efficient biofilm formation capability of the M5MA1-B2[pRCR12] and M9MG6-B2[pRCR12] strains, correlating with the qualitative estimation of "strongly adherent" obtained by the violet crystal staining method for their parental unlabeled strains, leading us to consider the method implemented in this work as valid for future quantifications.

Auto-aggregation Capability of Labeled *L. plantarum* M5MA1-B2 and M9MG6-B2 Strains

The ability of probiotic bacteria to form cellular aggregates via self-aggregation (auto-aggregation) or between genetically distinct cells (co-aggregation) is a desirable characteristic. Auto-aggregates can adhere to surface mucosa increasing probiotic persistence in the intestine where they can potentially inhibit the adherence of pathogenic bacteria, and prevent their colonization (Monteagudo-Mera et al., 2019). According to Abdulla et al.

(2014), this property also determines the ability of a probiotic to adhere to other surfaces such as the oral cavity, and the gastrointestinal and urogenital tracts. Since the usage of mCherry fluorescence measurement seemed to be a useful tool to assess the probiotic properties of *L. plantarum* (Mohedano et al., 2019) and above, we decided to expand the usage of the protein in this work to a method that allows the determination of the degree of bacterial auto-aggregation by measuring the fluorescence emitted by bacterial cultures (see details in Materials and Methods section 1.8). The method was used to establish that both labeled riboflavin-overproducing strains possess auto-aggregative profiles (**Table 3**). After 15 h of incubation at 37°C, an auto-aggregation value of 34.68 ± 3.9% for M9MG6-B2[pRCR12] strain was obtained, a value slightly, but statistically significantly higher than the 29.13 ± 4.0% detected for the M5MA1-B2[pRCR12] strain. The methodology used here to assess auto-aggregation has not been previously described, thus impairing our ability to make comparisons with the observations of other researchers. However, by means of a spectrophotometric technique, the aggregative profile of various LAB strains has been characterized. In particular, high percentages of auto-aggregation have been reported for *Lactiplantibacillus* strains: 28.27 ± 9.7% for *L. plantarum* 46a (Raveschot et al., 2020); around a 40 % for *L. plantarum* L15 (Alizadeh Behbahani et al., 2019), and within a range of 29.47 ± 2.63% to 33.57 ± 2.21% in other *L. plantarum* strains. Therefore, our results seem to be those expected for *L. plantarum* strains and indicate a good auto-aggregation capability of M5MA1-B2 and M9MG6-B2, which could have a certain relationship with their good adhesion to the biotic Caco-2 cells surface and the high capacity to form biofilms on abiotic surfaces, characteristics that would give them certain advantages to colonize the intestine. Similar observations have been described by several authors who have established that the ability of bacteria to aggregate seems to have an important effect on biofilm formation, and therefore this ability is related to the cell adherence properties, and the ability to survive and persist in the GIT (Vlková et al., 2008; Ferreira et al., 2011). In correlation with the above stated, some lactobacilli have been described to have an aggregation phenotype. However, the mechanisms of cellular aggregation have not been fully elucidated and may be species specific and environment dependent (García-Cayuela et al., 2014). Certain authors have proposed a correlation between auto-aggregation and hydrophobicity, so that high auto-aggregation values showed also high hydrophobicity (Nikolic et al., 2010). However, other authors did not observe such a correlation, which indicates that interaction of probiotics with intestinal cells involves more complex mechanisms (García-Cayuela et al., 2014). Similarly, Ramiah et al. (2008) reported that factors other than surface-bound proteins play an important role in adhesion. The glycoproteins, teichoic and lipoteichoic acids on the cell wall surface of bacteria contribute to the adhesion, auto-aggregation abilities, and hydrophobicity of the strains (Darilmaz et al., 2012; Haddaji et al., 2015). Several proteins involved in bacterial aggregation or biofilm formation have been described such as Esp protein (∼206 kDa) in *Enterococcus faecalis* that promotes adhesion and biofilm formation (Toledo-Arana et al., 2001) or Bap protein (∼250 kDa) in *Staphylococcus aureus* involved in biofilm formation (Cucarella et al., 2001). Hence, it is possible that the M5MA1-B2 and M9MG6-B2 riboflavin-overproducing strains may interact with epithelial cells of the host with a temporary colonization on it. In this way they might exert some beneficial effects, due to the considerable high adhesive profile probed in this study. According to Monteagudo-Mera et al. (2019) a temporary colonization facilitates the local action of metabolites produced by probiotics, such as short-chain fatty acids, as well as immunomodulatory effects.

Resistance to GIT Stresses of Labeled and Unlabeled *L. plantarum* M5MA1-B2 and M9MG6-B2 Strains Included in a Food Matrix

The acidic environments found in some foods, and in the stomach provide a survival challenge for probiotic microorganisms. Predominantly, the delivery vehicles for probiotic cultures have been yogurts and fermented milks, which themselves present an acid environment. Then, the probiotics must survive the highly acidic gastric juice in order to reach the small intestine in a viable state (Henriksson et al., 1999). Hence, gastric acid stress is a key test for their survival (Ranadheera et al., 2014). Additionally, a probiotic strain must pass through stress challenges in the GIT including tolerance to the bile salts in the upper parts of the small intestine (Begley et al., 2005). Thus, we have evaluated the capacity of the *L. plantarum* strains to resist simulated stress conditions following the protocol depicted in **Figure 1**. Previous work revealed that the parental strains isolated from chicha have good technological properties in cereal-based food matrices (Yépez et al., 2019). Thus, the LAB were suspended in a non-acidic food matrix, Incaparina, which is a cereal-based beverage administrated in children's day care services in Guatemala, and cell viability was determined after each stress (**Figure 4**). The initial bacterial concentration in the *in vitro* assay was considered as 100% and corresponded to 1.0×10^8 cfu mL^{-1}. The four *L. plantarum* strains were subjected to a decreasing pH gradient from 6.5 to 2.0 by sequential incubations at different pHs. These conditions were chosen, because humans secrete, on average, 2.5 L of gastric juice per day, resulting in a fasting gastric pH value of 1.5, which increases to between pH 3.0 and 5.0 during feeding (Hill, 2002). Also, many studies have reported that a value of pH < 2.0 could destroy bacteria; however, this pH rarely occurs in the stomach (Alizadeh Behbahani et al., 2019). The results obtained here show that the two parental strains M5MA1-B2 (**Figure 4A**) and M9MG6-B2 (**Figure 4B**) did not lose any viability during the exposure to the gastric stress. In addition, the number of cfu mL^{-1} increased after exposure to pH 4 to a maximum value of 142 or 139%, with 136 or 121% still remaining at pH 2 for strains M5MA1-B2 or M9MG6-B2, respectively. In the case of M5MA1-B2[pRCR12] (**Figure 4A**) and M9MG6-B2[pRCR12] (**Figure 4B**) an initial increase of viability was also observed for both strains with maximum values at pH 4 of 137 and 122%, respectively. In addition, the viability of M5MA1-B2[pRCR12] significantly decreased to 102% at pH 3 and to 79% at pH 2, whereas this loss of survival was less for M9MG6-B2[pRCR12],

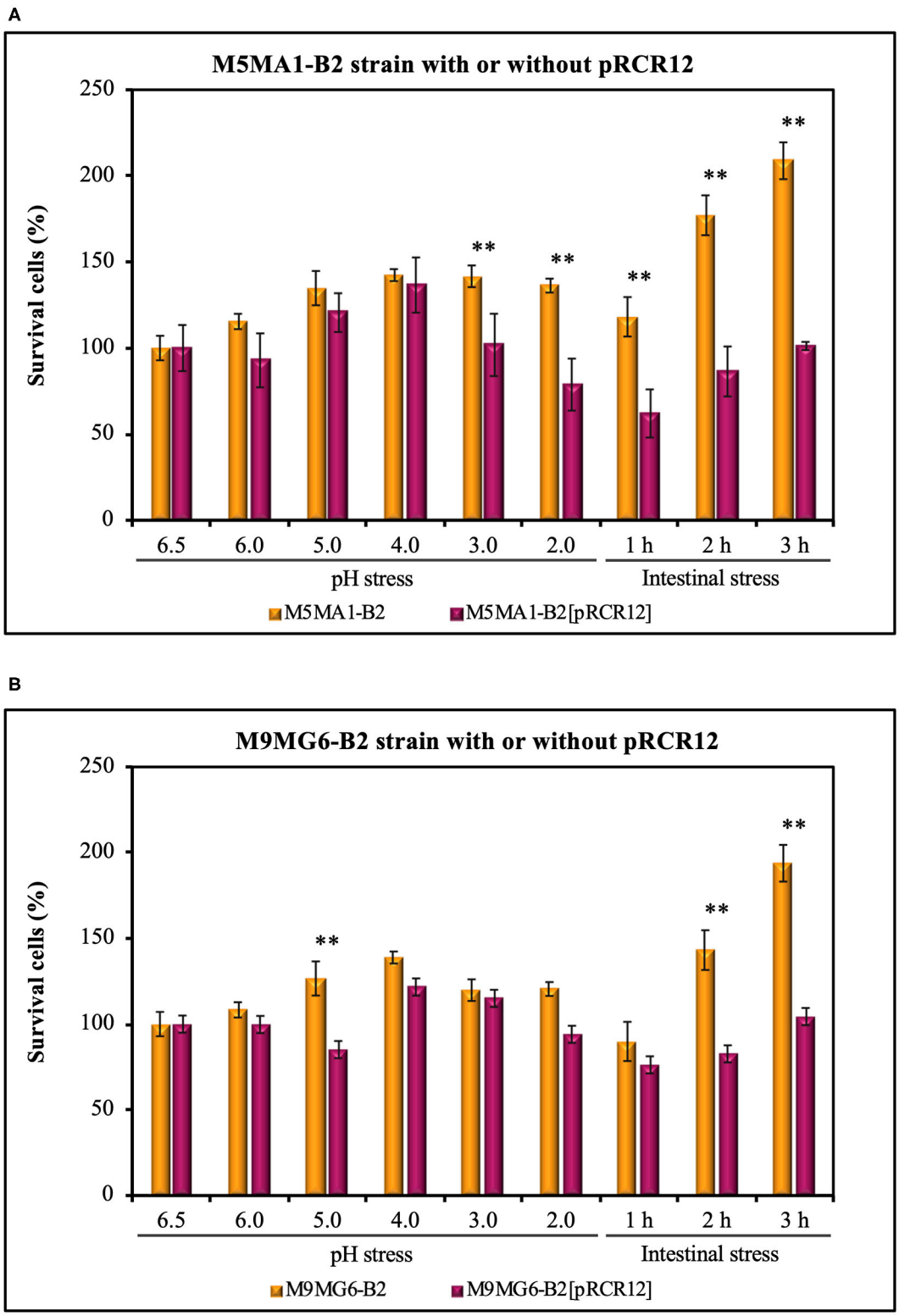

FIGURE 4 | Analysis of resistance to gut stresses of the LAB strains: M5MA1-B2 and M5MA1-B2[pRCR12] **(A)** as well as M9MG6-B2 and M9MG6-B2 **(B)** in a food matrix. The bacteria were subjected to acidic (pH) or intestinal stresses as described in **Figure 1**. Cell survival was determined by plate counting and expressed as percentage. The values are the mean of three independent experiments and are expressed as a total percentage of surviving cells compared to the initial bacterial population. A statistical *t*-test analysis of the results is depicted. A $p \leq 0.05$ was considered significant. Strains with **are significantly different at each condition tested.

TABLE 4 | Influence of *L. plantarum* M9MG6-B2 or M9MG6-B2[pRCR12] strains supplementation with or without Incaparina on BALB/c mice and their organs weights.

Group	Initial weight[a] (g)	Weight increase[b] (g)	Kidney/animal weight[c] (%)	Spleen/animal weight[c] (%)	Liver/animal weight[c] (%)
Control	13 ± 1	17 ± 2	1.5 ± 0.1	0.3 ± 0.1	5.0 ± 0.4
Incaparina	13 ± 2	16 ± 3	1.5 ± 0.2	0.3 ± 0.1	5.4 ± 0.4
M9MG6-B2	13 ± 2	17 ± 2	1.5 ± 0.1	0.3 ± 0.1	5.4 ± 0.3
M9MG6-B2[pRCR12]	12 ± 1	18 ± 2	1.5 ± 0.2	0.3 ± 0.1	5.3 ± 0.5
Incaparina + M9MG6-B2	13 ± 1	16 ± 2	1.5 ± 0.1	0.3 ± 0.1	5.0 ± 0.3
Incaparina + M9MG6-B2 [pRCR12]	12 ± 2	17 ± 3	1.4 ± 0.1	0.4 ± 0.1	5.1 ± 0.5

[a] *Initial weights of animals measured previous to starting the experiment.*
[b] *Weight increase was calculated by determining the difference between the animals' weights before and after the experiment (day 0 vs. day 28).*
[c] *Kidney, spleen and liver weights were divided by animal live weight at day 28.*
Data are expressed as means ± standard deviation for n = 6 animals in each group. A total N = 36 animals were used in the experiment).

with values of 115 and 94% at pH 3.0 and 2.0, respectively. Thus, the results show a high capacity of the four strains to survive in the stomach environment. This acid resistance could be intrinsic, as has been previously detected in other lactobacilli (de Angelis and Gobbetti, 2004; Wall et al., 2007; Broadbent et al., 2010). In Gram-positive microorganisms, including LAB, F_0F1-ATPase is one of the main mechanisms studied for protection against acidic conditions. F_0F_1-ATPase is induced at low pH and can increase the intracellular pH under acidic environmental conditions (Cotter and Hill, 2003). In addition, other mechanisms such as decarboxylation and deamination, cell membrane modification and macromolecule protection and repair have been proposed as alternative processes to mediate LAB acidic response (Guan and Liu, 2020).

The results obtained in this work also indicated an important role of the food matrix in the bacterial performance. The viability of the bacteria did not decrease, since quantification of bacteria by plate count revealed an increase of the cfu mL^{-1}, even during incubation at pH 4.0, indicating that the components of the food matrix were metabolized by the LAB and supported their growth. Furthermore, under any of the acid stress conditions, the viability of any of the LAB tested was higher than 21%, which could be due to a protective effect of the evaluated food matrices. Since the matrix has high viscosity, it might promote cellular aggregation that could be the first step of biofilm formation. The high auto-aggregation and biofilm formation ability of the strains detected here (**Table 3**) support this hypothesis. Likewise, it has been proposed for other LAB that formation of biofilms protects microbial cells against acidic shock. Thus, cell density, related to the formation of biofilms, is another factor affecting the acid resistance of microorganisms (Liu et al., 2015) and could be involved in the acid resistance detected for the M5MA1-B2 and M9MG6-B2 strains.

After feeding, bile is released into the duodenum and acts in the emulsification and solubilization of lipids in the small intestine. It can also destroy the phospholipids and proteins of bacterial cell membranes. Thus, a highly desirable quality of a probiotic strain is its ability to survive in the presence of bile salts in the upper parts of the small intestine (Begley et al., 2005). Thus, after, exposure to stomach stress, the *L. plantarum* strains were subjected to intestinal stress (**Figure 4**). The LAB were treated at pH 7.0 with bile salt and pancreatic juice containing protease, lipase and amylase. The exposure to this environment generated some deleterious effects on the viability of the four *L. plantarum* strains during the first hour of treatment, with recovery of cfu during the subsequent 2 h of incubation (**Figure 4**). A clear and significant 2-fold difference in bacterial survival was observed between, M5MA1-B2 and M5MA1-B2[pRCR12] (**Figure 4**). After 1 h of intestinal stress challenge, viability of the former remained at 119% of the initial values, whereas the latter showed only 62% survival (**Figure 4A**). This difference was also observed after 2 and 3 h of treatment, when M5MA1-B2 showed a viability of 177 and 209% vs. 86% and 101% for M5MA1-B2[pRCR12] (**Figure 4A**). This lower tolerance of M5MA1-B2[pRCR12] was also observed after challenge at pH 3.0 and 2.0 with 1.4- and 1.7-fold greater decrease of viability than M5MA1-B2 (**Figure 4A**). The presence of pRCR12 in M9MG6-B2 under intestinal stress for 1 h had no significant effect (**Figure 4B**), as was detected after acidic stress at pH 3.0 and pH 2.0, where, under the latter condition M9MG6-B2[pRCR12] was only slightly (1.2-fold) more sensitive than M9MG6-B2. However, the recovery after 2 and 3 h of intestinal treatment was higher for the unlabeled parental (143 and 194%) than the labeled (83 and 109%) strain (**Figure 4B**).

In vivo Animal Trials

The *in vitro* results presented above support the good probiotic potential of the riboflavin-overproducing M5MA1-B2 and M9MG6-B2 strains, and to Incaparina as a suitable food matrix to protect these LAB against the GIT stresses, as well as to promote their delivery to the intestine and therefore it supports their proliferation. However, there was not a clear-cut difference between the performances of these two strains. Therefore, M9MG6-B2 and M9MG6-B2[pRCR12] in the presence or absence of Incaparina were further tested in an *in vivo* murine model using BALB/c mice, evaluating growth parameters of the animals as well as possible changes in particular organs following the scheme depicted in **Figure 2**. All the animals started with similar initial weights. After the experimental period (28 days), no differences were observed in the animal weight increase, or in the relative weight of the kidneys, spleens, and liver between all the experimental groups (**Table 4**). These

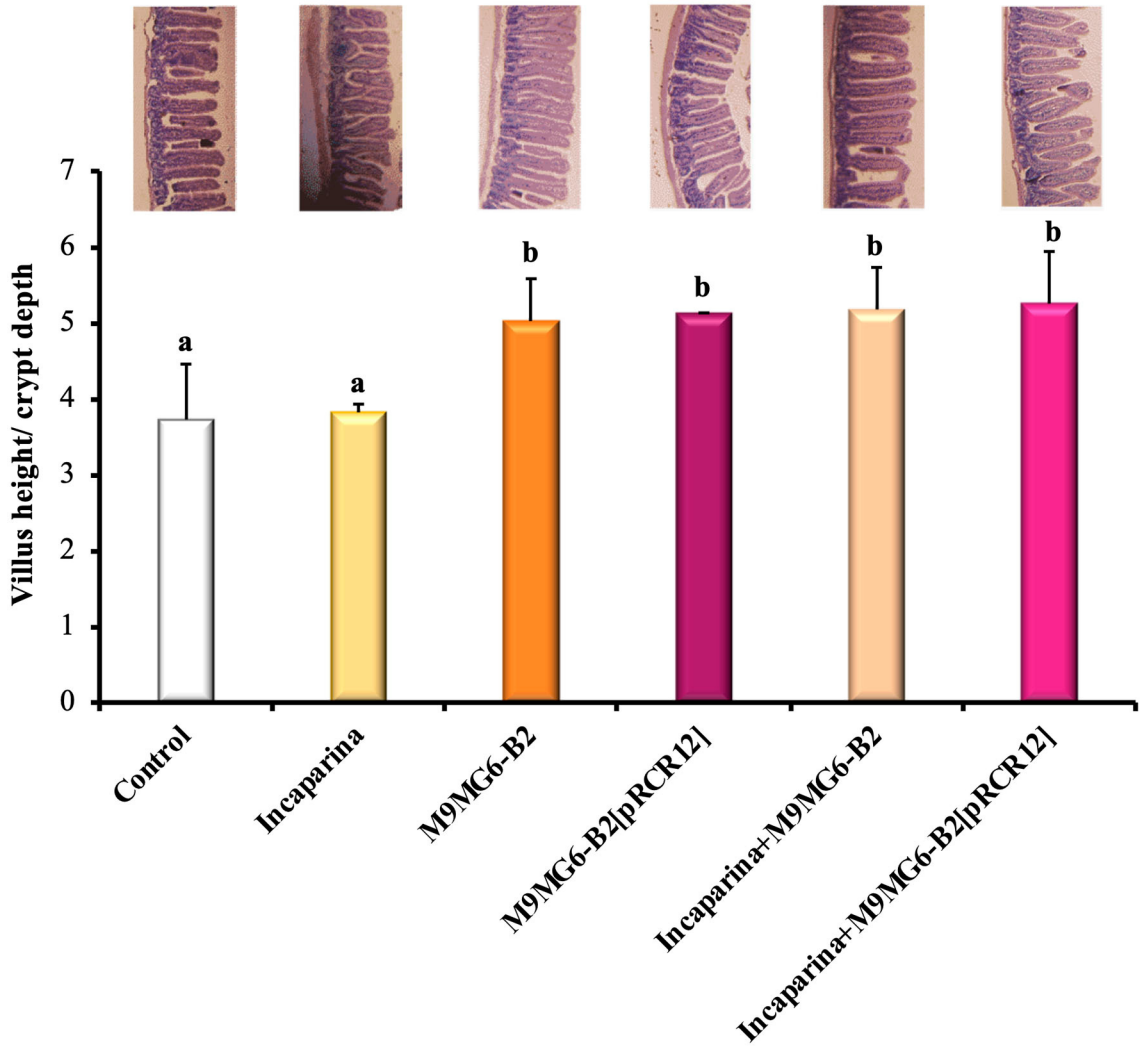

FIGURE 5 | Histological evaluation of the influence of *L. plantarum* M9MG6-B2 supplementation on the morphology of BALB/c mice small intestines. The organs of mice from the Control group, and those who received bacterial supplementation with either *L. plantarum* M9MG6-B2 or M9MG6-B2[pRCR12] strains with or without Incaparina were evaluated The ratio of the villus height/crypt depth is shown. [a,b]Columns with different letters are significantly different between each other with a $p \leq 0.05$. On top of each column is a representative photograph of each experimental group.

results demonstrate that bacterial supplementation by itself or included in the Incaparina matrix did not significantly affect animal growth. Similarly, hematological analysis did not show significant differences between the groups. White and red blood cells' counts, relative differential leukocytes, hemoglobin were maintained between the reference values for these animals (data not shown).

The maintenance of intestinal mucosa integrity requires coordination of the cell proliferation and differentiation processes (Parker et al., 2017). The analysis of intestine revealed that the administration of Incaparina did not alter its morphology; however, the administration of either the M9MG6-B2 or the M9MG6-B2[pRCR12] strain, with or without Incaparina, significantly increased the ratio of the villus height/crypt depth (**Figure 5**). These results revealed that the bacterial supplementation increased the total surface area of the intestinal villi and would thus be beneficial to nutrient absorption. This hypothesis is in line with recent results showing that the supplementation with folate-producing LAB resulted in an increase of the intestinal absorption surface (Cucick et al., 2020). Apart from the increased ratio of the villus height/crypt depth by the bacterial supplementation, no other changes were observed in the muscle layer or in the mucous layer of the small intestine.

In the fecal samples of the animals that consumed M9MG6-B2[pRCR12] in the presence or absence of Incaparina, the strains were recovered at a concentration of 4×10^4 CFU mL^{-1} as determined by counting the pink colonies in MRS-agar containing CM. This result is similar to those previously obtained with strain M5MA1-B2[pRCR12], where the mCherry labeling

was shown to be effective in following the survival of the strain in conventional mice (Mohedano et al., 2019). It was not possible to evaluate M9MG6-B2 recovery since this strain did not produce the mCherry protein and could not be differentiated from the normal intestinal microbiota of the mice. In addition, the presence of pRCR12 in M9MG6-B2 resulted *in vitro* in a slightly decreased growth rate (**Table 4**) and in an increased sensitivity of the bacterium to intestinal stress (**Figure 4**). Therefore, it is feasible that the *in vivo* colonization capability of M9MG6-B2 could be higher than that of M9MG6-B2[pRCR12].

Nevertheless, the overall results support a potential probiotic effect of *L. plantarum* M9MG6-B2 and its usage for the preparation of cereal-based functional food.

CONCLUSIONS

Currently, functional foods with potential health benefits are attracting increasing interest in which food-preserving microorganisms, like probiotics, play significant roles. According to Liu et al. (2017), probiotics not only provide high levels of nutraceuticals to the food, they also participate in health regulation of humans by production of functional molecules *in situ* in the GIT. In this context, our current results revealed that both *L. plantarum* M5MA1-B2 and M9MG6-B2 are able to tolerate the GIT stresses and to grow after adaptation to these conditions. Incaparina, as a food matrix, seems to provide the studied bacteria with the adequate nutrients to allow their proliferation, therefore, the possibility of carrying them in such a matrix would assure the production of commercial preparations with a high viability of probiotic LAB with additional functionalities, for the development of new functional foods. Moreover, both the M5MA1-B2 and M9MG6-B2 strains showed *in vitro* high adhesion capacity to Caco-2 epithelial cells as well as an aggregative phenotype and capability to form consistent and high biomass biofilms, which indicate a potential capability for colonization of the intestines. Furthermore, in a murine *in vivo* model, administration of M9MG6-B2 or M9MG6-B2[pRCR12] resulted in an increment in the surface area of the intestinal epithelium (without affecting the health of the animals) that could contribute to better absorption of the nutrients.

DATA AVAILABILITY STATEMENT

All datasets generated in this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Protection Committee of Centro de Referencia de Lactobacillus, Tucuman, Argentina.

AUTHOR CONTRIBUTIONS

AH-A and SP performed all the *in vitro* analysis of the LAB studied. MM planed the riboflavin production experiments, analysis of the results, and corrected the manuscript. RA provided the background for handling the riboflavin-producing LAB and corrected the manuscript. JL, GV, and AM performed the LAB analysis in the animal model, the description of the results, and corrected the manuscript. PL masterminded the direction and conceptualization of the study, the interpretation of the data obtained, and the final version of the manuscript. All authors listed have read and approved the final version of 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.2020.591945/full#supplementary-material

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Use of Autochthonous *Lactiplantibacillus plantarum* Strains to Produce Fermented Fish Products

Barbara Speranza, Angela Racioppo, Daniela Campaniello, Clelia Altieri, Milena Sinigaglia, Maria Rosaria Corbo and Antonio Bevilacqua**

Department of Agriculture, Food, Natural Resources and Engineering (DAFNE), University of Foggia, Foggia, Italy

The present research was aimed to the optimization of the production of a fish fermented *salami-like* product using autochthonous *Lactiplantibacillus plantarum* starters. The activity was performed through two phases: (1) Optimization of fermented fish product composition by using a 2^{k-p} Fractional Factorial Design: the variables tested were nitrites (0–150 ppm), salt (2.5–7.5%), sucrose (0–4%), white pepper (0–0.10%), and fermentation temperature (10–30°C); (2) Product realization and evaluation of its microbiological profile [aerobic microbiota (APC), *Pseudomonadaceae* (PSE), *Enterobacteriaceae* (E), and lactic acid bacteria (LAB) populations], chemico-physical parameters (pH and a_w), and sensorial quality (odor, texture, color, and overall acceptability) during its storage at 4°C for 21 days. In the first step, the fish pulp was mixed with the appropriate amounts of ingredients, according to the experimental design; each batch was individually inoculated with the studied starter (*L. plantarum* 11, *L. plantarum* 69, and *L. plantarum* DSM1055) at 10^7 cfu/g and incubated at 10, 20, or 30°C for 7 days. The lowest fermentation time (time to reach pH 4.4) was obtained with 4% sucrose, 100 ppm nitrite and a process temperature of 30°C. In the second step, *salami-like* were produced according to the individuated formulation and inoculated with the studied starters (10^7 cfu/g); the fish mixture was stuffed into a natural casing and left to ferment at 30°C for 7 days. The use of the selected strains not only assured a correct fermentation but reduced the process time at only 2 days; during refrigerated storage, a good microbiological, chemico-physical and sensorial quality of the final product was recorded for at least 21 days.

Keywords: starter, fermentation, fish, DoE, autochthonous bacteria

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Raffaele Coppola, University of Molise, Italy

****Correspondence:***
Maria Rosaria Corbo
mariarosaria.corbo@unifg.it
Antonio Bevilacqua
antonio.bevilacqua@unifg.it

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INTRODUCTION

The use of starters in food industry is a precious mean to improve both process efficiency and product quality (Corbo et al., 2016). In the last decade, the use of autochthonous microbial strains as specific starter cultures has been suggested: namely, the most robust strains are isolated from a food and studied as potential starter for the same matrix (Corbo et al., 2016; Speranza et al., 2017).

As mainly observed for meat and dairy fermented products, lactic acid bacteria (LAB) seem to give the best performances also as fish starters by significantly improving the products' quality, accelerating the acidification, and controlling the growth of spoilage bacteria and pathogens. However, while the studies about starter cultures for dairy products, wineries, sausages, and vegetables are very broad in literature, the starter to be used for fish fermentation are still rare and the suggested technologies are few: consequently, today there are no specific starters for fermented fish products.

Once, fermented fish products were mainly diffuse in east and southeast Asia, but more recently, with globalization and market opening, some products are being produced elsewhere or exported from Oriental countries to Europe, North America, and Africa (Adams, 2009); some examples are *Hákarl*, *Surströmming*, and *Rakfisk* diffuse in northern Europe (Jensen et al., 2012; Osimani et al., 2019) or *lanhouin* and *momoni* in West Africa (Anihouvi et al., 2007). Other well-known fermented fish products are *Suan yu* and fish sauces in China, *Plaa-som* and *Nam-pla* in Thailand, *fish nukazuke* and *narezushi* in Japan (Kanno et al., 2012). Due to the not homogeneous and complex nature of fish, the high diversity of fish species fermented, various additives used, and different processes applied (Kose and Hall, 2011), the use of allochthonous commercial starters is not advisable, while the use of autochthonous starters is still under-explored.

A number of researchers have proposed strains of the genera *Tetragenococcus*, *Pediococcus*, *Lactobacillus* and related genera, and *Staphylococcus* for several types of fish fermented products (Yongsawatdigul et al., 2007; Udomsil et al., 2010; Natteewan et al., 2011; Speranza et al., 2015, 2017; Zeng et al., 2016; Liao et al., 2018). Some studies highlighted that the addition of starter cultures shortened fermentation time (FT): for example, Yongsawatdigul et al. (2007) found that *Virgibacillus* sp. used for Thai fish sauce fermentation was able to shorten process time to 4 months. Other researchers found an improvement in the quality of traditional Chinese fermented fish using both *Lactiplantibacillus plantarum* and *Saccharomyces cerevisiae* (Liao et al., 2018) or *Tetragenococcus halophilus* (Udomsil et al., 2010).

In 2017, Speranza et al. (2017) proposed two autochthonous strains of *L. plantarum* as potentially multifunctional starters for fish fermented sausage production, since they gave promising results in preliminary assays by both reducing the FT and guaranteeing a good quality of the fish product. In addition, they also showed potential probiotic properties, such as a good low pH tolerance and promising cell adherence properties.

Therefore, the aim of this research was the optimization of the production of a fish fermented *salami-like* product using autochthonous *L. plantarum* starters. The activity was performed through two main phases:

1. Optimization of fermented fish product composition by using a 2^{k-p} Fractional Factorial Design: the variables tested were nitrites (0–150 ppm), salt (2.5–7.5%), sucrose (0–4%), white pepper (0–0.10%), and fermentation temperature (10–30°C).

2. Product realization and evaluation of its microbiological profile [aerobic microflora, *Pseudomonadaceae* (PSE), *Enterobacteriaceae* (E), and LAB populations], chemico-physical parameters (pH and a_w), and sensorial quality (odor, texture, color, and overall acceptability) during its storage at 4°C for 3 weeks.

MATERIALS AND METHODS

Optimization of Fermented Fish Product Composition

Strains

Three strains were used as potential starters:

Two autochthonous strains labeled with a numeric code (11–69), isolated from intestinal microbiota of sea bream (*Sparus aurata*) and identified at species level as *L. plantarum* (Speranza et al., 2017).

One allochthonous commercial strain, *L. plantarum* DSM 1055, isolated from bread dough and purchased from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germania).

The strains were stored at −20°C in de Man Rogosa and Sharpe broth (MRS, Oxoid, Milan, Italy) added with sterile glycerol (J.T. Baker, Milan, Italy); before each assay, they were revitalized in MRS broth incubated at 37°C for 24 h.

Design

The optimization of fermented fish product composition was obtained by using a 2^{k-p} Fractional Factorial Design. The variables tested were: concentrations of nitrites, salt, sucrose, white pepper, and fermentation temperature. **Table 1** shows the coded levels of each independent variable jointly to the eight combinations tested during the experiment. A control sample, i.e., a further combination in which the variables tested were set to level 0, was also reported. Three factorial designs were set up, one for each starter tested.

Samples Preparation

The optimization was performed by using specimens of croaker (*Argyrosomus regius*; Lepore Mare, Fasano, Brindisi, Italy). The fish was gutted, eviscerated, deboned, filleted, and washed in running tap water; then, the drained fillets were passed through a strainer (Meat Strainer Mod. MMG22, Electrolux, Rimini, Italy). Aliquots of fish pulp (20 g) were mixed with the appropriate amount of NaCl, $NaNO_2$, sucrose, and white pepper according to the Fractional Design reported in **Table 1**. Then, inocula were performed by adding each selected strain (*L. plantarum* 11, 69, or 1055) at a final level of 10^7 cfu/g. Not inoculated samples were used as negative controls. All samples were incubated at the temperature planned by the experimental design for 7 days, during which the pH decrease was monitored by a pH electrode 50*50T CRISON (Crison Instruments, Barcelona, Spain). The experiments were performed over three independent batches, i.e., for each combination three independent samples were analyzed.

Product Realization

Fish Sausages Preparation

Croaker pulp was obtained as described above. Then, it was mixed with (in g/kg): NaCl, 40; $NaNO_2$, 0.10; sucrose, 40; white pepper, 10. Strains (11, 69, and 1,055) were inoculated at 10^7 cfu/g. A control batch was also similarly prepared and not inoculated. After mixing, samples were mechanically stuffed into collagen casings (28 mm diameter) and incubated at 30°C for 7 days. During fermentation, the pH was evaluated by immersing

TABLE 1 | Coded levels and combinations used in the 2^{k-p} Fractional Factorial Design.

Coded level	Nitrites (ppm)	NaCl (%, w/w)	Temperature (°C)	Sucrose (%, w/w)	White pepper (%, w/w)
−1	0	2.5	10	0	0
0	75	5	20	2	0.05
1	150	7.5	30	4	0.10
Combinations					
A	0	7.5	30	0	0
B	150	7.5	30	4	0.1
C	150	2.5	30	0	0.1
D	150	2.5	10	0	0
E	150	7.5	10	4	0
F	0	7.5	10	0	0.1
G	0	2.5	30	4	0
H	0	2.5	10	4	0.1
Control	75	5	20	2	0.05

the probe approximately 1–2 cm below the casings. After 7 days, the samples were stored 4°C for 21 days, during which the changes in pH, a_w, aerobic microbiota (APC), PSE, E, and LAB populations were measured, as described in Speranza et al. (2017).

During the storage, sensorial quality was also assessed by 15 panelists aging between 25 and 50 years [students and researchers of the Department of Agriculture, Food, Natural resources and Engineering (DAFNE), University of Foggia]. Using a scale ranging from 0 to 5 (where 0 was very poor and 5 was excellent; the acceptability threshold was set to 2), the sensorial overall quality of the fermented sausages was evaluated by estimating color, odor, and texture attributes (Speranza et al., 2017). The experiments were performed in triplicate as reported above.

Statistics

The pH data obtained in the first phase were modeled through the Weibull equation, as modified by Mafart et al. (2002):

$$\mathrm{pH} = \mathrm{pH}_0 - \left(\mathrm{t}/\Delta\right)^{\mathrm{p}}$$

where, pH and pH_0 are the pH throughout the time and the initial pH, respectively; Δ, the first reduction time (days), i.e., the time to attain a reduction of 1 unit in cell count; *p*, the shape parameter ($p < 1$ upward curve; $p > 1$ downward curve).

Using the fitting parameters of the Weibull equation, the FT (defined as the time in days to attain a pH of 4.4) was calculated:

$$\mathrm{FT} = \delta\left(\mathrm{N}_0 - \mathrm{Lc}\right)1/\mathrm{p}$$

Where L_c is the critical limit (4.4) and it could be considered a "safe pH" (Riebroy et al., 2008).

Fermentation time was used as dependent variable for a multiple regression analysis; concentrations of nitrites, salt, sucrose, white pepper, and fermentation temperature were the independent variables. The modeling was performed through the software Statistica for Windows version 7.0 (Statsoft, Tulsa, Okhla). Significant differences amongst combinations for each fitting parameter were pointed out through one-way ANOVA and Tukey's test ($p < 0.05$).

Sensory scores were analyzed through the Krustal-Wallis test ($p < 0.05$).

RESULTS

The first phase was performed to individuate not only the optimal formulation of the fermented product, in terms of salt, sucrose, nitrates and spices concentrations, but also the fermentation temperature, in order to ensure a rapid acidification and guarantee the success of the transformation.

The optimization was achieved through the use of three different 2^{k-p} factorial designs, one for each of the tested targets: two autochthonous strains (*L. plantarum* 11–69) isolated from fish intestine (Speranza et al., 2017) and one allochthonous commercial strain (*L. plantarum* 1055).

For each combination studied, the pH data were fitted through the Weibull model (primary model): **Table 2** shows the fitting parameters obtained, i.e., *Δ* (time to reduce the pH by 1 unit) and *p* (the shape parameter), for each kinetics of acidification monitored. The initial pH was 6.20–6.50 and there was generally a gradual acidification over time, more or less vigorous depending on the combination considered. For example, for *L. plantarum* 69, the time to reduce pH by 1 ranged from a minimum of 0.52 days (±0.31) of the combination G (2.5% salt, 4% sucrose, without nitrites and pepper, fermentation temperature of 30°C) to a maximum of 23.05 days (±6.08) of the combination D (2.5% salt, without sugar and pepper, 150 ppm nitrites, temperature fermentation of 10°C). The other combinations showed not significant differences ($p > 0.05$). In the combinations E and F (with the highest salt concentration and the lowest fermentation temperature), no acidification was recorded.

Similar results were also observed for strain 11 with *Δ* values of 0.33 (±0.47) and 13.21 (±8.41) days, in the combinations G and D, respectively. For the reference strain (DSM 1055), the Δ values ranged from a minimum value of 1.02 (±0.83; combination G) to a maximum value of 9.07 (±2.84) days (combination A, 7.5% salt, without sugar, pepper and nitrites, fermentation temperature of 30°C).

Although not having a biological meaning, the "*p*" parameter (shape parameter) represents a useful tool to describe the

TABLE 2 | Fermentation time (FT, day) and fitting parameters of Weibull equation (Δ, time to reduce the pH by 1, day; *p*, shape parameter) for the kinetics of acidification.

	Δ	*p*	FT
Strain 69			
A	9.27 ± 3.00B,C	0.98 ± 0.46B	16.67 ± 5.12B
B	4.14 ± 1.18B	0.93 ± 0.32B	7.97 ± 2.23A
C	7.00 ± 4.84B	0.70 ± 0.54A,B	21.75 ± 5.87B
D	23.05 ± 6.08C	0.46 ± 0.18A	74.75 ± 3.45C
E	-*	-	-
F	-*	-	-
G	0.52 ± 0.31A	0.34 ± 0.07A	4.93 ± 1.55A
H	6.48 ± 0.43B	1.45 ± 0.38B	10.80 ± 0.56A
Control	5.00 ± 1.29B	1.06 ± 0.50B	9.29 ± 2.33A
Strain 11			
A	8.37 ± 1.88B,C	1.32 ± 0.76A,B	12.80 ± 1.80B,C
B	4.89 ± 0.78B	1.00 ± 0.25A,B	8.76 ± 0.87B
C	5.59 ± 2.81B	0.71 ± 0.45A	16.28 ± 4.56C
D	13.21 ± 8.41D	0.73 ± 0.44A	28.18 ± 7.86D
E	-*	-	-
F	-*	-	-
G	0.33 ± 0.47A	0.31 ± 0.13A	3.88 ± 0.34A
H	6.73 ± 0.32B	1.43 ± 0.25B	11.40 ± 1.12B,C
Control	4.94 ± 1.62B	1.24 ± 0.86A,B	8.42 ± 0.14B
Strain DSM 1055			
A	9.07 ± 2.84C	0.89 ± 0.39A,B	17.67 ± 5.67C,D
B	5.27 ± 0.62B	0.98 ± 0.19B	9.72 ± 0.54B,C
C	6.93 ± 4.69C	0.75 ± 0.61A,B	19.87 ± 5.78D
D	7.52 ± 0.76C	3.08 ± 1.48C	8.91 ± 0.57B
E	-	-	-
F	-	-	-
G	1.02 ± 0.83A	0.51 ± 0.17D	5.19 ± 1.01A
H	7.59 ± 0.58C	1.67 ± 0.58C	11.89 ± 0.35C
Control	5.32 ± 0.66B	1.35 ± 0.45C	8.64 ± 0.89B

*For each strain and parameter, letters indicate significant differences (one-way ANOVA and Tukey's test, p < 0.05). *no acidification.*

data trend. In fact, when $p > 1$, the curve has a downward concavity, with an initial phase in which the pH decreases very slowly or does not at all; after this kind of shoulder phase (Geeraerd et al., 2000), a rapid pH decrease is generally observed. On the other hand, when $p < 1$, the curve has an upward concavity, with a rapid initial pH decrease followed by a phase in which the parameter decreases very slowly or does not at all (tail effect). Regardless the strain inoculated, the lowest values of p were recorded for the combination G: in this formulation the kinetic always showed an upward concavity, with *p* values of 0.34 ± 0.07 for strain 69, 0.31 ± 0.13 for strain 11, and 0.51 ± 0.17 for the strain DSM 1055 (**Table 2**).

Using the fitting parameters of the Weibull equation, the FT (defined as the time in days to attain a pH of 4.4) was also calculated: this pH value is suggested as an essential requirement for food safety, especially to inhibit pathogenic and spoilage bacteria (Riebroy et al., 2008). The FT values are also reported in **Table 2**. The combination G was confirmed as the best formulation to guarantee the fastest acidification, with the lowest FT recorded, i.e., 4.93, 3.88, and 5.19 days for the kinetics by *L. plantarum* 69, *L. plantarum* 11, and *L. plantarum* 1055, respectively.

In order to evaluate the effects of the different studied variables [concentrations of sugar (Z), salt (S), nitrites (N), white pepper (P), and temperature (T)] on the FT, the FT values were used as input values for a multiple regression analysis: **Table 3** shows the standardized effects recovered.

TABLE 3 | Standardized effects of temperature, sugar, salt, pepper, and nitrite on the fermentation time.

	69	11	DSM 1055
Temperature	−6.36	−4.72	-
Sugar	−4.13	-	-
Salt	-	-	-
Nitrites	3.32	-	-
Pepper	-	-	-
Curvature	−4.00	−3.05	-
R^2_{ad}	0.896	0.797	-
MS	58.63	25.45	

MS, mean square residual.

The FT of strain 69 was strongly influenced by temperature and sucrose concentration, while the effects of pepper and salt were not significant ($p > 0.05$); the adjusted regression coefficient was 0.896, while the mean square residual was 58.63, thus suggesting an adequate fit of the model. In addition, the fitting estimates a curvature response with possible slight interactive terms; however, the design used in the present study could be used to estimate both interactive and quadratic response otherwise confounding effects and artifacts could be pointed out. A surface plot can be advantageously used to highlight these effects: in particular, these graphs can be obtained by plotting the FT as a function of two variables. As an example, **Figures 1A–D** show the surface plots for the effects of the interactions [T] / [N] (part **A**), [T] / [Z] (part **B**), [T] / [S] (part **C**), and [Z] / [N] (part **D**) on the FT values calculated for *L. plantarum* 69; as expected, the FT decreased as the process temperature and the sugar concentration increased, recording a minimum value at 30°C and 4% of sugar. At 30°C, the effect of salt was not significant (**Figure 1C**). For *L. plantarum* 11 (**Table 3**; **Figure 2**), only the temperature was significant. Finally, the FT of *L. plantarum* 1055 was not affected by any variable studied (**Table 3**).

Based on these results, the optimal composition was identified in (g/kg): NaCl, 40; $NaNO_2$, 0.10; sucrose, 40; white pepper, 10. The process temperature was set at 30°C. This last parameter, together with the sugar and nitrite concentrations, was chosen on the basis of the results obtained, while the concentrations of salt and white pepper were chosen on the basis of literature data (Adams, 2009).

In the second step, *salami-like* were produced according to the individuated formulation, inoculated with the studied starters (10^7 cfu/g), stuffed into a natural casing and fermented at 30°C for 7 days. The initial pH of fish mixture was 6.50: as the fermentation advanced, all inoculated samples exhibited lower pH than the control. In sausages inoculated with starter strains, the acidification started immediately after the inoculum and the minimum pH (4.3) was found after only 2 days, against the 6 days required to attain the same acidification level in control sample. During 21 days-storage, aerobic microflora, PSE, E, and LAB populations were assessed. In the control, the initial LAB count was low (3.0 log cfu/g), whereas LAB counts of 6.3–7.2 log cfu/g were recovered in all inoculated samples.

After 5 days, LAB attained a level of ca. 8.5 log cfu/g. Aerobic bacteria trends were similar to those observed for LAB.

Pseudomonadaceae and *Enterobacteriaceae* were ca. 3 log cfu/g in all samples for the entire storage, without significant differences between inoculated samples and the control (data not shown). pH and a_w values did not undergo significant changes, recording values about 4.25–4.30 and 0.96–0.97, respectively (data not shown).

Color and odor were the limiting factors for the overall acceptability (data not shown). **Figure 3** shows the box-whisker plot for this parameter during the storage: after 21 days, one-way ANOVA revealed that sensory scores of the sausages inoculated with *L. plantarum* 69 were significantly higher than other samples that attained a value nearby the non-acceptability limit (2).

DISCUSSION

The fermentation of fish for human consumptions has several benefits: first, it is a low cost convenient preservation method,

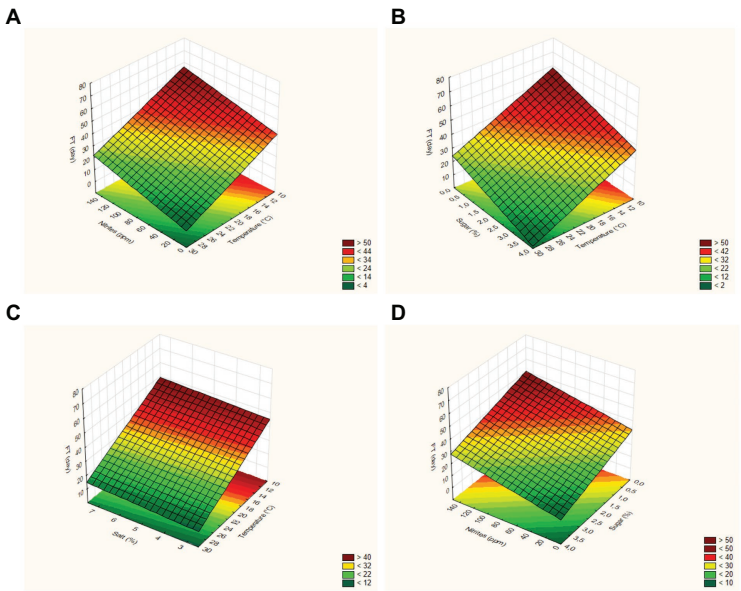

FIGURE 1 | Surface plots for the effects of the interactions [Temperature] / [Nitrites] (part **A**), [Temperature] / [Sugar] (part **B**), [Temperature] / [Salt] (part **C**), and [Sugar] / [Nitrites] (part **D**) on the FT (Fermentation time) values calculated for *Lactiplantibacillus plantarum* 69.

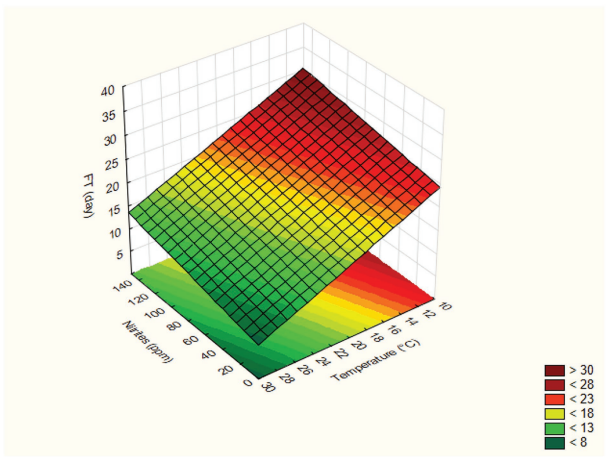

FIGURE 2 | Surface plot for the effects of the interactions [Temperature] / [Nitrites] on the FT (Fermentation time) values calculated for *L. plantarum* 11.

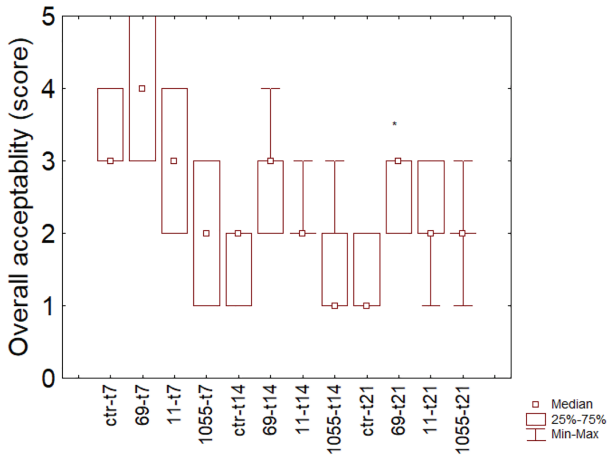

FIGURE 3 | Box-whisker for the sensory scores of fermented sausages during the refrigerated storage. 11, 69, 1055, products inoculated; ctr, control. The numbers after the isolate indicate the time of analysis (7, 14, or 21 days).

and then it improves the nutritional value and the digestibility or the raw material. Most of fermented fish products are still produced according to local tradition and preferences, leaving the fermentation process to indigenous microbes from the environmental or raw material (Adams, 2009). If referred to small-scale processing units using traditional techniques, even if a high level of product variability is observed, spontaneous fermentation could be still accepted. However, considering globalization and market opening, these products are now widespread also in Europe and North America, and inoculation with starter cultures has become increasingly necessary to improve and stabilize the quality of fermented fish (Nie et al., 2014). Fermented fish products are generally prepared by mixing the fish substrate with salt and other ingredients, such as carbohydrates, spices, or other additives; the product is fermented at various temperatures (from 10 to 45°C) for variable periods (1 week or different months). As for other fermented food, the primary role of starter cultures is to decrease the pH (<4.5), in order to create the conditions which, together with other variables (namely salt and spices) could inhibit the growth undesirable bacteria and extend shelf-life (Adams, 2009). Consequently, the first step of this study was aimed to individuate both the optimal formulation of the fermented product, in terms of salt, sucrose, nitrates and spices concentrations, and the fermentation temperature, since a faster acidification remains the main factor able to guarantee the success of the transformation. For each of the studied strains, the results obtained highlighted that the lowest fermentation time, i.e., time to attain a safe pH of 4.4 (Riebroy et al., 2008), was attained for a formulation containing 4% of sucrose and 100 ppm of nitrite, while the effects of pepper and salt were not significant. The acidification was also strongly influenced by the temperature, with an optimum at 30°C. If this last effect is not surprising, since the temperature is one of the critical parameters affecting the growth of starters and the production of enzymes during fermentation (Thomas et al., 2013), on the other hand, the not significant effect of salt was not expected. Salt concentration in fermented products often ranges from 1 to 10% (w/w); high salt concentrations generally were recommended to better inhibit the growth of spoilage microorganisms, but it should also be considered that a high salt concentration could reduce protease activity (Kim et al., 2003; Klomklao et al., 2006), resulting in a longer fermentation. The optimal formulation was chosen by considering the results obtained by the design for sugar (40 g/kg) and nitrite (0.10 g/kg) concentrations, whereas the concentrations of salt was chosen (40 g/kg) by considering that a too high concentration could cause great harm to human health (Alderman, 2000) and a delayed process (Klomklao et al., 2006). The pepper concentration (10 g/kg) was chosen because of literature data (Adams, 2009); in fact, the role of pepper is controversial. Some authors (Ono et al., 2015) reported a promoting effect of black and red pepper on the fermentation of *nukadoko* (a Japanese fermented rice), while other authors (Kang et al., 2015) underlined a negative effect in the fermentation of *kimchi*, a fermented cabbage.

In general, an optimization step is an overly complex phase, since researchers should focus on many variables; in the present study, this problem was solved using the approach of the Design of the Experiments (DoE). This technique allows to designing and planning significant experiments by minimizing the number of the samples to be analyzed (Bevilacqua et al., 2010).

Once individuated the formulation of ingredients and the process temperature (30°C), a validation in real conditions was performed. A process optimization usually involves a model building *in vitro* by laboratory assays followed by a validation in real system, but in this study, both the DoE and the confirmatory assays were conducted *in situ* (in fish).

The results obtained in the second phase highlighted that the starter strains used assured the correct course of fermentation, reduced the fermentation time and ensured a good microbiological, chemico-physical, and sensorial quality of the final product. In particular, the use of autochthonous starters appeared as a precious tool for the production of fermented fish products as also reported by Zeng et al. (2017), Han et al. (2020), Kusmarwati et al. (2020), and Zang et al. (2020). The latters, for example, used two strains of *L. plantarum* and one strain of *Lactococcus lactis* isolated from spontaneously fermented *Yucha*, a traditional Chinese home-made fermented fish product, as starter cultures for the same product and observed a faster acidification and an improvement of flavor and safety. Similarly, a better quality of *peda*, a traditional salted fermented fish product of mackerel fish of Indonesia, was observed during the use of *Leuconostoc mesenteroides* ssp. *cremonis* BN12 as starter culture (Kusmarwati et al., 2020).

CONCLUSION

The production of fermented-fish products with the appearance of salami is a complex process; natural fermentation could not assure the basic requirements of such products: low pH and rapid acidification. Thus, the use of starter cultures is advisable, mainly wild strains, as shown by the performances of *L. plantarum* 11 and 69.

Ingredients and temperature could play a significant role and the results of the first step highlighted the statistical weight of temperature, and nitrites; the use of sugar could increase the acidification rate.

On the other hand, salt and pepper did not exert a significant effect.

The production of a salami-like product through a guided fermentation with selected strains of *L. plantarum* assured a good microbiological quality and sensory scores for at least 21 days of refrigerated storage.

In conclusion, the results obtained in this study suggest that fermented food industries could formulate their own starters to standardize the process without affecting the sensorial characteristics of their traditional products.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

BS, AB, and MC: conceptualization. CA and AB: methodology. AB: software. BS, AR, and DC: investigation. MS and MC: resources. BS and AB: data curation and writing – original draft preparation. All authors contributed to writing – review and editing. MC and AB: supervision. MC: funding acquisition. All authors contributed to the article and approved the submitted version.

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Effect of Lactic Acid Bacteria Addition on the Microbiological Safety of Pasta-Filata Types of Cheeses

*Alžbeta Medved'ová, Martina Koňuchová, Karolína Kvočiková, Ivana Hatalová and L'ubomír Valík**

Department of Nutrition and Food Quality Assessment, Faculty of Chemical and Food Technology, Slovak University of Technology in Bratislava, Bratislava, Slovakia

In this work, the effects of different combinations of lactic acid bacteria (LAB) on the growth of coagulase-positive staphylococci (CPS) and *Escherichia coli* were evaluated during ripening of 23 curd cheeses, and their subsequent behavior during the manufacture and storage of pasta-filata cheeses was characterized. Three groups of cheeses were prepared in total: first, control cheeses from raw milk without LAB addition; further pasteurized milk cheeses with LAB, CPS and *E. coli* intentional inoculation; and finally, raw milk cheeses with LAB added. The aim was to compare the effect of LAB from starter culture, and also in combination with native LAB, and to evaluate the LAB effect as a group, and further to suggest the culture with the best inhibitory potential. Based on the results, counts of CPS increased over 24 h in control curd cheese by 1.76 ± 0.56 log CFU/g. On the other hand, in raw milk cheeses with the addition of starter culture, the increase in CPS counts by 0.76 ± 0.87 log CFU/g was noticed. Counts of *E. coli* increased during the first 24 h of curd manufacture by 3.56 ± 0.41 log CFU/g in cheeses without LAB addition. Contrary to this, using of LAB cultures resulted in an increase in *E. coli* counts by only 1.40 ± 1.07 log CFU/g. After steaming at 63.6 ± 1.9°C for 7.2 ± 2.1 min (temperature of heated acidified curd was 54.9 ± 1.7°C), CPS decreased by 0.58 ± 1.12 log CFU/g, and *E. coli* decreased by 1.23 ± 0.97 log CFU/g in all cheeses, regardless of LAB addition. Finally, during storage of cheeses at 6 ± 0.5°C for 28 days, the levels of *E. coli* in control cheeses and in raw milk LAB-enriched cheeses reached levels of 2.07 ± 2.28 log CFU/g and 1.20 ± 0.85 log CFU/g, respectively. In addition, the counts of CPS at the end of storage met the criteria of EU Commission Regulation (EC) No. 1441/2007 (2007) (less than 4 log CFU/g) in all manufactured cheeses with added LAB culture, while in the control raw milk cheeses, a level of 3.80 ± 1.22 log CFU/g was observed. Regarding the culture used, the best microbiological inhibitory effect in 28-day-old cheeses was reached by the combination of Fresco culture with *Lacticaseibacillus rhamnosus* GG, and the best sensory properties were judged to be those for cheeses manufactured with Culture A. A moderate negative

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***Correspondence:**
L'ubomír Valík
lubomir.valik@stuba.sk

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effect of storage on overall sensory acceptance was noted, according to the final evaluation of overall acceptability of pasta-filata cheeses. The most satisfactory overall acceptability after 28 days of storage at 6°C was reached for cheese with the addition of culture A.

Keywords: raw milk cheeses, *Staphylococcus aureus*, *Escherichia coli*, steaming process, sensory properties, predictive microbiology

INTRODUCTION

The pasta-filata cheeses include a wide range of cheeses originating primarily in the northern Mediterranean region: e.g., Italy, Greece, the Balkans, and Turkey. They have become global favorites as ingredients in a variety of foods, especially pizza (Albenzio et al., 2013). Some of these types of cheeses are artisanal variants that are also produced in Central and Southeast Europe. In Slovakia, the manufacture of pasta-filata type cheeses: e.g., Parenica, Oštiepok, Zázrivské vojky, Zázrivský korbáčik, and Oravský korbáčik, is of great importance to preserve national gastronomic heritage, and they are designated for the Protections of Geographical Indications (PGI) (2005) (EC No: SK/PGI/005/0485/19.07.2005). Traditionally, they are produced from lump cheese, which is also manufactured from raw milk in Slovakian upland cottages immediately after milking. The lump cheese is curdled with rennet, fermented by native lactic acid bacteria (LAB), and briefly ripened for 24 h. Then, the lumps are steamed at 60–70°C in hot water for 5–10 min, cooled (usually in salty water) and shaped (Licitra et al., 2018).

Artisanal pasta-filata cheeses are produced by a stretching process occurring after dipping a curd in hot water. During this process, the amorphous protein structure of the curd is transformed into an oriented structure composed of parallel protein fibers. The ongoing changes are influenced by an appropriate combination of *pH* level and calcium content in the curd during the heating-stretching step that is controlled by the activity of the starter cultures or by a direct acidification of milk with organic acids during the production of the curd (Zimanová et al., 2016). The resulting plastic paste is easily molded into various forms and shapes (Albenzio et al., 2013).

From the microbiological point of view, microorganisms, including LAB, live in clusters incorporated into the casein network and remain mostly in the curd, while some microorganisms leave the curd with whey (McSweeney, 2004). The development of curd fermentation relies on the spontaneous development of LAB, which produce lactic acid, helping the milk coagulate (Wouters et al., 2002). Stretching the cheese curd in hot water substantially affects the subsequent distribution and viability of the microbiota present; however, it does not destroy it completely (Gernigon et al., 2010; Hui et al., 2012). This is why residual lactose is further metabolized by bacterial survivors during pasta-filata manufacture and early stages of shelf life. Caseins that were hydrolyzed initially through residual coagulant activity by plasmin and other indigenous proteolytic enzymes to a range of large and intermediate peptides are further hydrolyzed by LAB proteinases and peptidases to shorter peptides and amino acids. As a consequence of hydrolysis and changes in water-binding ability, including changes in *pH* that in turn may cause other changes such as the migration and precipitation of calcium phosphate, pasta-filata cheese texture may soften (McSweeney, 2004). Thus, the flavor of pasta-filata cheese is very mild; other flavor notes are readily detected at low intensities and are generally considered defects (Kindstedt et al., 2010).

Despite a short fermentation of lump cheese, the metabolic activity of LAB leads to the desired degradation of saccharides, lipids, proteins and other milk components (e.g., citrates, inorganic compounds), forming a wide range of metabolites that improve the technological and sensory properties of the final product (Marth and Steele, 2001; Ljungh and Wadström, 2009; Lahtinen et al., 2011; Mančušková et al., 2018). In addition to organic acids (lactic, acetic, benzoic, formic, pyroglutamic, phenyllactic), other substances (e.g., hydroperoxide, diacetyl, acetoin, exopolysaccharides, etc.) that contribute to the flavor, aroma and textural properties of dairy products are produced by the action of LAB. The metabolism of proteins results in the production of various amino acids that are precursors for aldehydes, alcohols, carboxylic acids, hydroxyacids, dimethyl sulfide, dimethyl disulfide or dimethyl trisulfide and methanethiol (Østlie et al., 2003; McSweeney, 2004; Sádecká et al., 2014; Kowalczyk et al., 2016). Through the activity of lipases and esterases various free fatty acids, methyl ketones, lactones, thioesters, and ketoacids are formed (Pot and Tsakalidou, 2009). These metabolic products serve as a tool for controlling the growth and multiplication of spoilage and pathogenic microorganisms synergistically with a lowering of the *pH* (Adams, 2001; Wouters et al., 2002; Charlier et al., 2008). Moreover, in addition to the inhibitory effect of LAB, they are widely used because of their positive effects on the sensory properties of final products. However, it should be kept in mind that thermal treatment influences not only the microbiological, biochemical, physicochemical, and functional characteristics of the cheese but also, in turn, markedly affects the sensory properties of these products (Kindstedt et al., 2010). Therefore, the choice of an appropriate active starter culture for controlled fermentation processes and production of pasta-filata cheeses with thermal treatment is of utmost importance.

Despite all the benefits of cheese consumption and even though cheese is considered one of the safest foods, pathogenic bacteria can be transmitted by dairy products since their presence in raw milk cannot be completely avoided. Moreover, microbial contamination can impart a considerable load of microorganisms during the steaming process and ultimately determines the microbiological quality and safety of the final pasta-filata cheeses. Originating from raw milk or from manufacturing environments, salmonellae and *Listeria monocytogenes* are rare, and *Escherichia*

coli and *Staphylococcus aureus* are more frequent microbial contaminants of lump cheeses (Little et al., 2008; Valík, 2013). The prevalence of *Listeria monocytogenes*, *E. coli* and coagulase-positive staphylococci in raw milk Slovak cheeses was found to range from 0.96 – 5.26%, 45.21 – 86.96%, and 9.96 – 12.28%, respectively from 2015–2018 (Ministry of Agricultural and Rural Development of the Slovak Republic, 2020).

Generally, it is known that efficient inhibition of microbial contaminants requires fast growth and high metabolic activity of LAB. As steaming is the only process responsible for inactivating undesirable microbiota, the following questions associated with the roles of LAB remain to be answered in this study. First, to what extent can LAB control the growth of accompanying microorganisms before steaming? The question of what proportions of high LAB numbers survive the steaming process naturally follows. On the other hand, it is also necessary to know the heat resistance of relevant representative microbiota, including LAB. Additionally, can LAB survivors control the behavior of other survivors? If yes, for what a period of time they do not affect sensorial properties of final pasta-filata cheeses? To answer these questions, we performed quantitative experiments aimed at the addition of various LAB cultures to increase the overall quality of specific traditional pasta-filata cheeses.

MATERIALS AND METHODS

Microorganisms and Preparation of Microbial Suspensions

During cheese manufacture, several microbial cultures were used:

- Fresco DVS 1010 (Christian Hansen, Hørsholm, Denmark) consisting of *Lactococcus* (*L.*) *lactis* ssp. *lactis*, *L. lactis* ssp. *cremoris,* and *Streptococcus salivarius* ssp. *thermophilus*;
- Culture A (Rajo, Inc., Bratislava, Slovakia) consisting of *L. lactis* ssp. *lactis*, *L. lactis* ssp. *cremoris*, *Leuconostoc mesenteroides* ssp. *cremoris,* and *Lactobacillus* (*Lb.*) *acidophilus*;
- *Lb. acidophilus* LA145 (Christian Hansen, Hørsholm, Denmark);
- *Lacticaseibacillus* (*Lcb.*) *rhamnosus* VT1 isolated from tartar sauce by Assoc. Prof. Plocková at the University of Chemistry and Technology in Prague (Czech Republic);
- *Lcb. rhamnosus* GG provided by Dr. Salminen (University of Turku, Turku, Finland)

A few grains of the frozen Fresco culture were inoculated into 100 ml of sterile milk and incubated at 30 ± 0.5°C for 5 h until the stationary phase was reached. After that, 40 ml of this culture (in the case of PM7 cheese 150 ml of culture was used) was then inoculated into milk to gain a density of 5 log counts. Culture A was inoculated into the milk used for cheese manufacture, directly from commercial packaging, to reach a cell counts of 5 log CFU/g. *Lcb. rhamnosus* GG, VT1 and *Lb. acidophilus* LA 145 were inoculated into 10 ml of sterile MRS broth (Biokar Diagnostics, Beauvais, France), and incubated anaerobically (5% CO_2) at 37 ± 0.5°C for 48 h. After that, 30 ml of each culture was inoculated into milk to obtain densities of 6 log counts.

For the cheeses manufactured from pasteurized milk, *Staphylococcus aureus* and *Escherichia coli* strains and isolates were used to analyze the effect of LAB on their growth ability or survival during cheese manufacture. The strains used were:

- *S. aureus* CCM 3953 and *E. coli* CCM 3988 from the Czech Collection of Microorganisms.

In addition, certain *S. aureus* and *E. coli* isolates were used. All these isolates are maintained in a collection of the Department of Nutrition and Food Quality Assessment, Slovak University of Technology in Bratislava, where we positively identified them by standard microbiological, biochemical and molecular methods and by MALDI-TOF spectroscopy.

- *S. aureus* 16 isolated from pasta-filata cheese "Parenica,"
- *S. aureus* 14733 isolated from a vending machine drain valve, producer of SED,
- *S. aureus* 2064 isolated from ewes' lump cheese,
- *S. aureus* 9V1 isolated from a laboratory-produced pasta-filata cheese from raw cows' milk,
- *E. coli* BR isolated from ewes' cheese "Bryndza,"
- *E. coli* KF isolated from raw cows' milk,
- *E. coli* PSII isolated from laboratory-produced pasta-filata cheese from raw cows' milk,
- *E. coli* ZV isolated from ewes' lump cheese.

A standard suspension of each isolate was prepared from a 24-h-old culture grown in BHI broth (Sigma-Aldrich, St. Louis, Missouri, United States) at 37 ± 0.5°C. These cultures were then diluted in saline-peptone solution and from 10^{-3} dilutions; 0.6 ml of each culture was inoculated into milk to obtain densities of 3 log counts.

Cheese Manufacture and Microbiological Analysis

The raw cows' milk was obtained between November 2018 and March 2020 from a milk vending machine situated in Bratislava, Slovakia, and immediately transported into the laboratory in cooled boxes at 6 ± 0.5°C. Manufacture of the pasta-filata "Nite" cheeses followed the scheme depicted in **Figure 1** and **Supplementary Material**. The rennet used for cheese manufacture was Fromase (220 IMCU/ml; DSM, Heerlen, the Netherlands).

The raw milk, whey, fresh curd, lump cheese (at 3, 6, and 24 h), fresh pasta-filata cheese, pasta-filata cheese after 7, 10, 14, 21, and 28 days of storage, salty water and hot water were analyzed to enumerate LAB (presumptive counts of lactococci and presumptive counts of lactobacilli, hereafter denoted only as counts of lactococci or lactobacilli), total coagulase-positive staphylococci (CPS) and *E. coli*.

The actual counts of lactococci were determined on M17 agar (Biolife, Milan, Italy) according to EN ISO 15214 (2002), with aerobic incubation at 30 ± 0.5°C for 48 h. Numbers of lactobacilli were determined according to EN ISO 15214 (2002) on MRS agar (Biokar Diagnostics, Beauvais, France), with anaerobic (5%

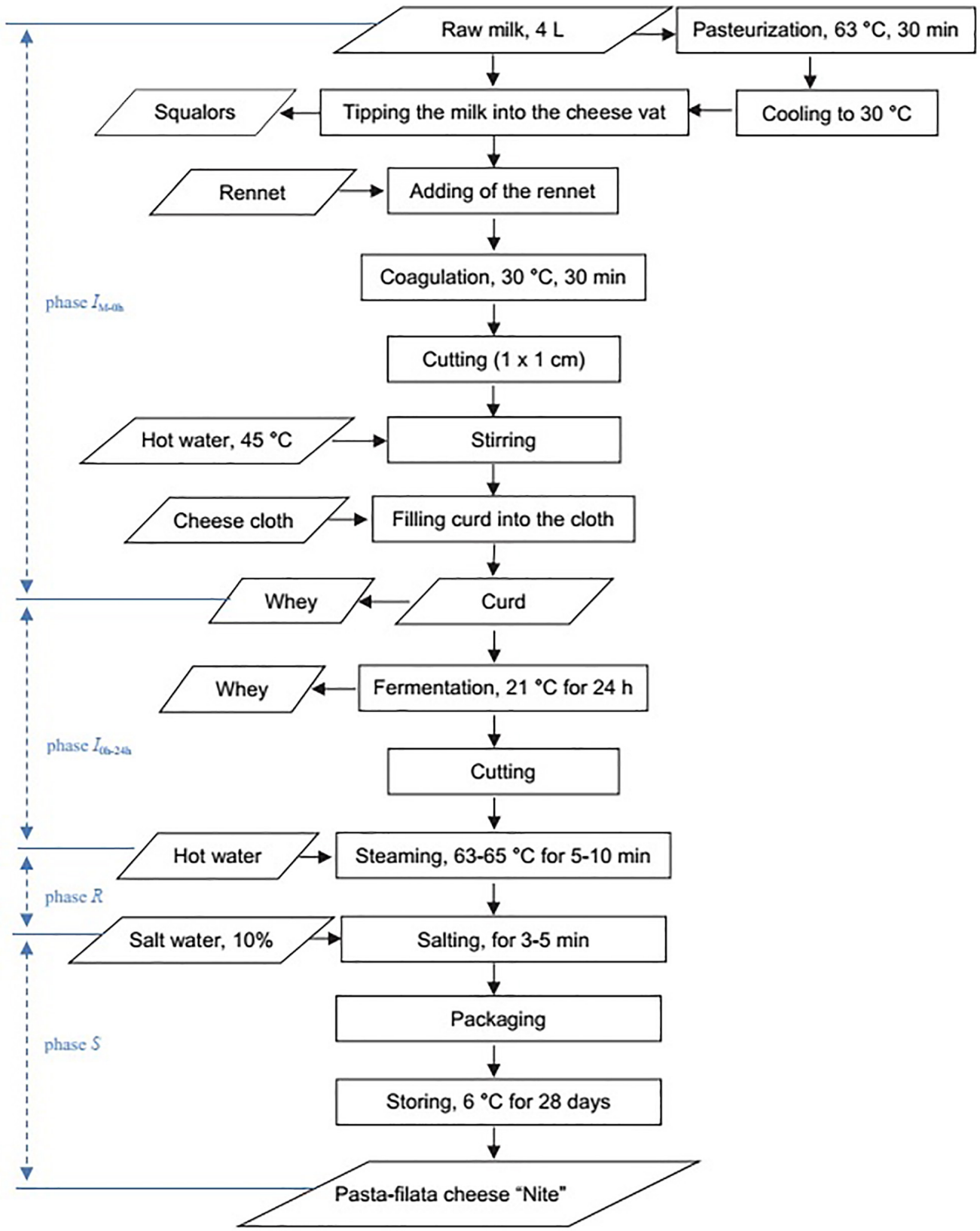

FIGURE 1 | Flow diagram of production of pasta-filata "Nite" cheeses.

CO_2) incubation at 37 ± 0.5°C for 72 h. Counts of *E. coli* were determined according to National Standard Method F23 (2005), with incubation at 37 ± 0.5°C for 24 h. Presumptive counts of CPS were determined on Baird-Parker medium (Merck KGaA, Darmstadt, Germany) with aerobic incubation at 37 ± 0.5°C for 48 h, according to EN ISO 6888-1 (2001).

The actual temperatures and duration of each steaming process were recorded by a temperature data logger (Ebro EBI 100-T100 with internal sensor, −40 to + 150°C; Xylem Analytics Germany Sales GmbH & Co., KG Ebro, Ingolstadt, Germany) connected to Winlog.pro 2.66 software, 2014 (Xylem Analytics Germany Sales GmbH & Co., KG Ebro, Ingolstadt, Germany). Moreover, for the same samples, water activity (a_w) measured by a LabMaster-aw (Novasina, Lachen, Switzerland) and *pH* values measured with a 100L pH meter (VWR International GmbH, Wien, Austria) equipped by glass electrode Sen Tix 81 (WTW GmbH, Weilheim, Germany) were also determined.

Sensorial Analysis of Cheese

Sensory Scoring of Cheeses

Quantitative descriptive analysis (QDA), based on a procedure described by Stone et al. (2012), was used to quantify the sensory attributes of the produced pasta-filata cheeses. The detailed sensory profile procedure was performed according to ISO 13299 (2016) in the Laboratory of Sensory Analysis of the Slovak University of Technology in Bratislava by a panel composed of 10 trained assessors (3 male, 7 female, aged 20–60, ISO 8586, 2012). The sensory evaluation was performed using a sensory profile of fresh and stored (6 ± 0.5°C for 10, 14, and 28 days) pasta-filata cheeses. Six samples were submitted for this evaluation (CC1, RM1, RM 4, RM6, RM8, and RM9). None of PM cheeses was evaluated due to the presence of enterotoxinogenic isolate *S. aureus* 14733. Approximately 20 g of each cheese sample was placed at ambient temperature (approximately 20°C) on a plastic plate 30 min before sampling and coded with a random 3-digit number. Overall sensory quality using a 5-point hedonic scale for each attribute was evaluated (1 and 5 representing "absence of sensation" and "highest intensity of observable sensory parameter," respectively). Each cheese sample was evaluated for appearance (color intensity, color homogeneity/uniformity and visual suitability), texture (chewiness, springiness/elasticity, stickiness), aroma (milky, acidic, intense, rancid, moldy, cowshed, grassy, spicy, fruity) and taste (bitter, rotten, oxidized/metallic, cowshed, grassy, spicy, fruity, taste of fresh cheese, milky, acidic, acidic taste suitability). After tasting, each panelist rated the overall acceptability of the product. Assessors used plain crackers and water to clean their palates in between tasting of samples.

Statistical Analysis

Statistical analysis of the results obtained was performed using Microsoft Excel 365 (Microsoft, Redmond, United States). The statistical significance of differences in the mean counts of microorganisms and in sensory parameters in different cheeses was evaluated by using ANOVA model. When differences were significant ($p < 0.05$), the means were compared with Tukey's *post-hoc* test.

RESULTS

Production of Lump Cheeses

"Nite" cheeses are typical Slovak pasta-filata cheeses of thick (2–10 mm) string shape. They are very popular in Slovakia, being sold in supermarkets, shops with traditional food products, markets and even in refrigerated vending machines. These products are usually consumed as snacks (Tomáška et al., 2019). To study the effect of LAB presence on microbiological and sensory quality of pasta-filata cheeses, we prepared 23 cheeses divided into 3 groups as specified in **Table 1**: 5 cheeses from raw milk without any added LAB culture (denoted CC group – control cheeses), 8 cheeses made from pasteurized milk with intentional inoculation of *S. aureus* and/or *E. coli* and LAB culture (denoted PM group) and finally, 10 cheeses from raw milk with addition of different LAB cultures (denoted RM group).

Applying the rationale of the Food Safety Objective concept (International Commission for the Microbiological Specification of Foods (ICMSF), 2002), the initial counts H_0 and the steps associated with the increase (I) or reduction (R) of microbial counts were identified to assess their total increase ΣI or decrease ΣR. As all the procedures were performed in the laboratory and mostly using close-to-sterile utensils, the increase in microbial numbers from possible recontamination was not taken into consideration. The average initial microbial numbers (H_0) among all milk samples varied as follows: 4.55 ± 0.98 log CFU/ml for lactococci, 3.82 ± 0.80 log CFU/ml for lactobacilli, 1.86 ± 1.03 log CFU/ml for *E. coli* and 2.94 ± 0.51 log CFU/ml for CPS.

Milk Coagulation

Naturally, during the manufacture of cheeses from raw milk (M) until the fresh curd (0h) preparation (phase I_{M-0h}, **Figures 2A–D**, **3A–C**), the most statistically significant ($p < 0.05$) increases in lactobacilli ($I_{G1,LB} = 2.73 \pm 1.00$ log CFU/g) and lactococci ($I_{M-0h,LC} = 2.20 \pm 1.11$ log CFU/g) were observed in curds with LAB culture addition, in both the PM and RM groups. However, in CC curds $I_{M-0h,LB}$ was only 0.46 ± 0.65 log CFU/g and $I_{M-0h,LC} = 0.88 \pm 1.34$ log CFU/g. As a result of intentional inoculation of *E. coli* its growth was more intensive in PM group ($I_{M-0h,EC} = 1.69 \pm 1.43$ log CFU/g) compared to both the CC and RM groups, with $I_{M-0h,EC} = 0.94 \pm 1.10$ log CFU/g. In the case of CPS, their growth during curd preparation was only minimal, and the highest increment of $I_{M-0h,EC} = 0.35 \pm 0.57$ log CFU/g was noticed in the CC group.

Distribution of Microorganisms Between Curds and Whey

As a consequence of syneresis, microbial partitioning between the curds and whey occurred. As one shown in **Figure 4**, where the partitioning $\{\%W/L = (N_{whey}/N_{L\ 0h}) * 100\%\}$ during syneresis is depicted, the presence of all microbial groups in whey was lower for LAB-enriched cheeses. This was especially true in the case of *E. coli*; its ratios in whey from cheeses with LAB culture addition (group PM and RM) were only 2.1 ± 3.6% and 0.4 ± 0.8%, respectively. Additionally, most CPS remained

TABLE 1 | Characterization of manufactured pasta-filata cheeses, depending on the addition and intended inoculum concentration of starter, additional cultures of LAB, or intentional inoculation of *S. aureus* and *E. coli* mixture.

Group	Cheese	Starter culture with intended final concentration	Additional culture with intended final concentration	Inoculated undesirable MO with intended final concentration
CC	CC1 – CC5	None	None	None
PM	PM1	Fresco 10^6 CFU/ml	None	*E. coli* mixture 10^3 CFU/ml
	PM2	Fresco 10^6 CFU/ml	None	*S. aureus* mixture 10^3 CFU/ml
	PM3	Fresco 10^6 CFU/ml	None	*E. coli* mixture + *S. aureus* mixture twice, 10^3 CFU/ml
	PM4	Culture A 10^6 CFU/ml	None	*E. coli* mixture 10^3 CFU/ml
	PM5	Culture A 10^6 CFU/ml	None	*S. aureus* mixture 10^3 CFU/ml
	PM6	Culture A 10^6 CFU/ml	None	*E. coli* mixture + *S. aureus* mixture 10^3 CFU/ml
	PM7	Fresco 10^7 CFU/ml	None	*E. coli* mixture + *S. aureus* mixture 10^3 CFU/ml
	PM8	Culture A 10^7 CFU/ml	None	*E. coli* mixture + *S. aureus* mixture 10^3 CFU/ml
RM	RM1 - RM3	Fresco 10^6 CFU/ml	None	None
	RM4, RM5	culture A 10^6 CFU/ml	None	None
	RM6	Fresco 10^6 CFU/ml	LA145 10^6 CFU/ml	None
	RM7	Fresco + culture A 10^6 CFU/ml	None	None
	RM8	Fresco 10^6 CFU/ml	VT1 10^6 CFU/ml	None
	RM9	Fresco 10^6 CFU/ml	LGG 10^6 CFU/ml	None
	RM10	Fresco (ewes' milk) 10^6 CFU/ml	LGG 10^6 CFU/ml	None

CC – control cheeses without the addition of LAB, PM – cheeses from pasteurized milk with the addition of culture A or Fresco and intentional inoculation with a mixture of 5 isolates of S. aureus and/or E. coli, RM – raw milk cheeses with the addition of different LAB cultures, LA145 – Lb. acidophilus LA145, VT1 – Lcb. rhamnosus VT1, GG – Lcb. rhamnosus GG.

in curd (94.3 ± 9.8% and 80.7 ± 20.0%) in both PM cheeses and RM cheeses, respectively, thus indicating the need for their rapid inhibition during further cheese fermentation. On the other hand, as the initial counts of LAB in milk were higher (as a result of their addition), and they grew faster, inhibitory activity against *E. coli* and CPS was achieved earlier.

Fermentation of Curds

During the next 24-h lasting fermentation step (phase I_{0h-24h}, **Figures 3A–D**), a further increase in microbial counts (I_{0h-24h}) was naturally observed. In all microbial groups, the largest I_{0h-24h} was noted for CC curds, with a declining tendency occurring gradually in PM and RM curds. At the same time and in all the curds, values of $I_{0h-24h,EC} > I_{0h-24h,CPS}$, and, specifically $I_{0h-24h,EC}$ was 3.56 ± 0.41 log CFU/g, 2.18 ± 1.66 log CFU/g and 1.40 ± 1.07 log CFU/g in CC, PM and RM curds, respectively. For CPS in the same order of curds, values of $I_{0h-24h,CPS}$ were only 1.76 ± 0.56 log CFU/g, 0.94 ± 1.71 log CFU/g and 0.76 ± 0.87 log CFU/g, respectively. Finally, similar declining trends of $I_{0h-24h,LC}$ and $I_{0h-24h,LB}$ were found for CC, PM and RM curds, respectively.

Despite the growth of CPS and *E. coli* during fermentation (**Figures 3A–D**), the addition of LAB cultures finally led to their statistically significant ($p < 0.05$) suppression in PM and RM compared to CC curds. Moreover, there was also a higher total increase ($I_{M-0h} + I_{0h-24h}$ phases) of LAB in cheeses with LAB addition. As a result of LAB activity, the counts of CPS and *E. coli* after 24 h of fermentation were lower than 4 log CFU/g in all 18 LAB-enriched cheeses. In contrast, the average counts of CPS and *E. coli* reached values of 5.10 ± 0.87 log CFU/g and 5.97 ± 0.55 log CFU/g in the CC group, respectively.

Steaming Process

The initial *pH* value of the fresh curds was 6.4 ± 0.2. Curds suitable for steaming should have a *pH* of approximately 5.0–5.3 (Fuentes et al., 2015; Pappa et al., 2019), as a lower *pH* results in a soft curd that collapses during stretching (Niro et al., 2014). In laboratory-manufactured cheeses, after 24 h of fermentation, the *pH* values of curds in the CC group decreased to $pH = 6.0 \pm 0.5$. In PM and RM cheeses, significantly lower ($p < 0.05$) *pH* values were noticed after 24 h of fermentation, specifically, $pH = 5.2 \pm 0.3$ in the PM cheeses group and $pH = 5.1 \pm 0.1$ in the RM cheeses group. All the curds were cooked for 7.2 ± 2.1 min in hot water at 63.6 ± 1.9°C, resulting in a curd temperature of 54.9 ± 1.7°C recorded by the Ebro EBI 100-T100 temperature data logger.

The observed reduction (R) of all microbial groups as a result of the steaming process is depicted in **Figures 2A–C** and in **Figures 3A–D**. Comparing the viability of lactococci and lactobacilli, the former ones exhibited higher tolerance to the heating process. The values of R_{LB} ranged from 2.50 ± 1.02 log CFU/g in the PM cheese group to 3.03 ± 0.89 log CFU/g in the CC cheese group. The R_{LC} values were lower; 0.75 ± 1.88 log CFU/g, 1.60 ± 0.84 log CFU/g and 2.09 ± 0.98 log CFU/g in CC, PM and RM, respectively. While lactococci survivors in fresh pasta-filata cheeses remained above 6.5 log (6.58 ± 0.79 log CFU/g, 7.25 ± 0.63 log CFU/g and 6.58 ± 0.95 log CFU/g), counts of lactobacilli only exceeded 4.5 log counts (4.89 ± 0.88

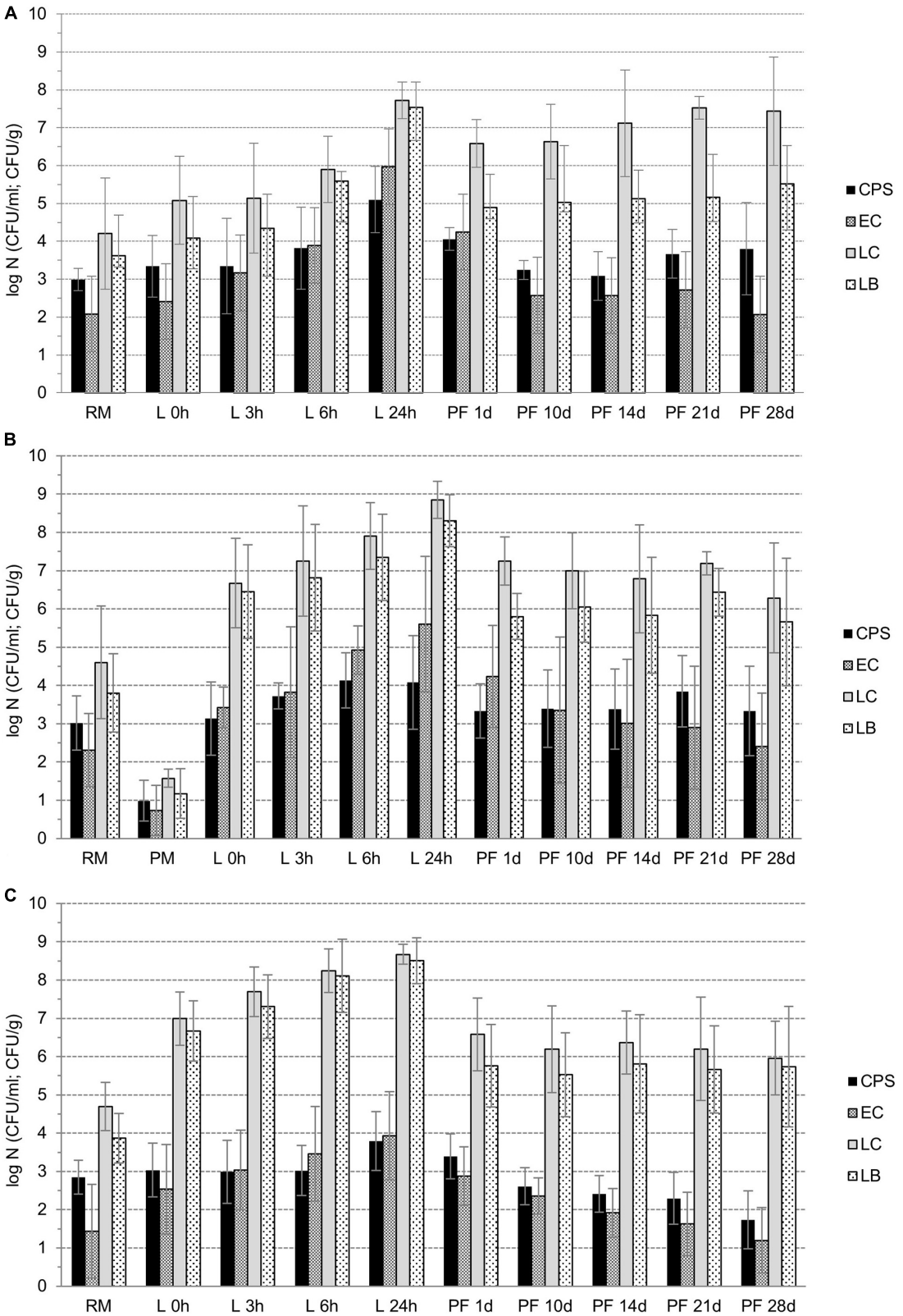

FIGURE 2 | A–C: The counts of selected microbial groups (CPS – coagulase-positive staphylococci, EC – *E. coli*, P-LC – presumptive counts of lactococci, P-LB – presumptive counts of lactobacilli) during manufacture of pasta-filata cheeses from raw milk (RM) to curd (L 0h), during fermentation of curd (L 3 h, L 6 h, and L 24 h), and during manufacture and storage of pasta-filata cheeses (PF1d – PF28d) at 6 ± 0.5°C for 28 days dependent of various cheese groups (**A** – CC **B** – PM, **C** – RM).

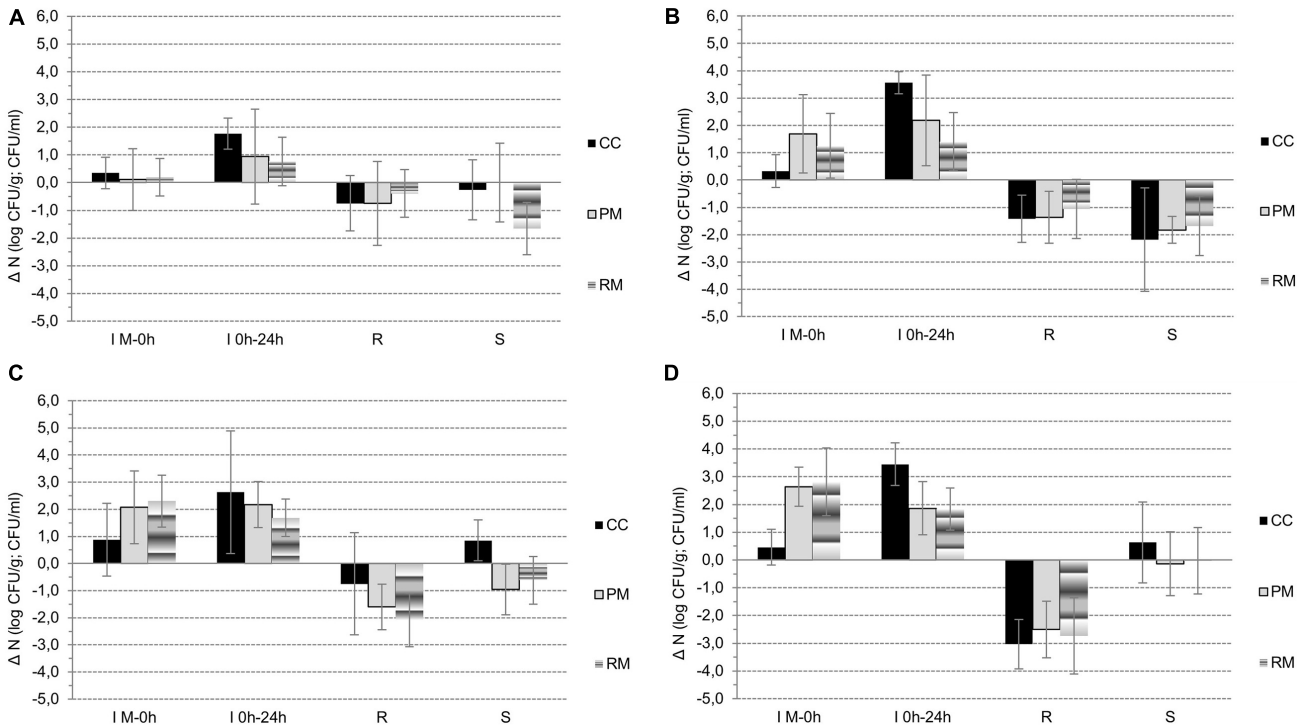

FIGURE 3 | A–D: The changes in microbial counts (**A** – CPS, **B** – *E. coli*, **C** – lactococci, **D** - lactobacilli) during the manufacture of curds from raw milk to curd (phase G1), during fermentation of curd (phase G2), and steaming (phase D) and storage (phase S) of CC, PM and RM pasta-filata cheeses.

log CFU/g, 5.80 ± 0.60 log CFU/g and 5.79 ± 1.07 log CFU/g) in CC, PM and RM cheeses, respectively.

Although the reduction of undesirable microbiota was lower in LAB-enriched cheeses, it seems that this may have resulted from their previous slower growth during phase G. The decrements of *E. coli* counts (R_{EC}) were calculated to be 1.42 ± 0.86 log CFU/g, 1.37 ± 0.95 log CFU/g and 1.06 ± 1.08 log CFU/g in CC, PM and RM, respectively. CPS proved to be more thermotolerant, as their R_{CPS} values were only 0.75 ± 1.00 log CFU/g, 0.75 ± 1.51 log CFU/g and 0.40 ± 0.86 log CFU/g, respectively, for the same order of cheese groups. However, it is also important to note that both *E. coli* and CPS surviving populations in the CC group of cheeses without LAB addition were higher than 4 log counts, while the counts for CPS in PM and RM cheeses were 3.33 ± 0.71 log CFU/g and 3.40 ± 0.59 log CFU/g, respectively. Numbers of *E. coli* lower than 3 log CFU/g after steaming were surprisingly observed only in fresh RM pasta-filata cheeses with LAB added (2.88 ± 0.76 log CFU/g).

Refrigerated Storage

To determine further quantitative changes in selected microbial populations, the fresh pasta-filata cheeses were stored at 6 ± 0.5°C for 28 days and analyzed regularly after 10, 14, 21, and 28 days. The results are depicted in **Figures 2A–C** and in **Figures 3A–D** (storage phase S, during that increase in microbial counts – labeled as *I*s; or decrease of their counts – labeled as R_S was observed). Only in control raw milk pasta-filata cheeses did lactococci and lactobacilli grow during storage, represented by $Is_{,LC} = 0.85 \pm 0.75$ log CFU/g and $Is_{,LB} = 0.63 \pm 1.45$ log CFU/g. In other cheeses with LAB addition, the lactobacilli remained at the same level after steaming, lactococci numbers decreased on average less than 1 log CFU/g, $R_{S,LC} = 0.96 \pm 0.93$ CFU/g in the PM group and $R_{S,LC} = 0.63 \pm 0.88$ CFU/g in the RM group. At the end of storage the counts of lactococci in the CC, PM and RM groups of cheeses were 7.43 ± 0.43 log CFU/g, 6.28 ± 1.43 log CFU/g and 5.96 ± 0.96 log CFU/g, respectively, significantly ($p < 0.05$) lower than those in the CC group. On the other hand, the counts of lactobacilli in all cheese groups on day 28 were higher than 5.5 log CFU/g, with no statistically significant ($p < 0.05$) differences between groups.

Higher levels of LAB together with their inhibitory metabolites acted as the hurdle for other microbial contaminants. This was confirmed in all groups of cheeses, as further reductions (R_S) were observed in both CPS and *E. coli* during storage of pasta-filata cheeses despite the fact that differences between the counts in each group of cheeses were not statistically significant ($p < 0.05$). For example, for *E. coli*, the final reductions $R_{S,EC}$ were approximately 2 log CFU/g in 28 days of storage. The lowest final *E. coli* counts of 1.20 ± 0.85 log CFU/g were reached in RM pasta-filata cheeses, while in the CC and PM groups, the *E. coli* counts exceeded 2 log CFU/g. For CPS, there was a statistically significant decrease ($p < 0.05$) during storage of RM pasta-filata cheeses ($R_{S,CPS} = 1.5$ log CFU/g) and a difference in final counts of CPS between PM and RM cheeses. The most important results is the observation that the final average counts of CPS were lower than 4 log CFU/g in all groups of cheeses, specifically 3.80 ± 1.22

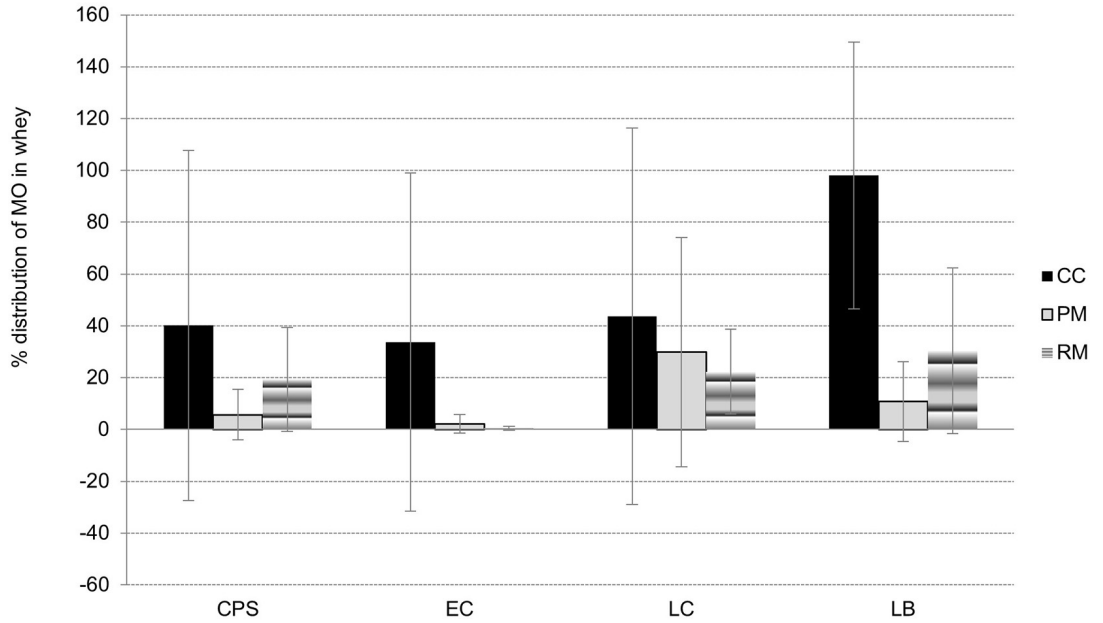

FIGURE 4 | The distribution of microbial groups (CPS – coagulase-positive staphylococci, EC – *E. coli*, LC – presumptive counts of lactococci, LB – presumptive counts of lactobacilli) between whey and curd during manufacture of lump cheeses for CC, PM, and RM, later used as raw materials for pasta-filata cheeses.

log CFU/g, 3.33 ± 1.17 log CFU/g and 1.73 ± 0.76 log CFU/g in RM, PM and RM pasta-filata cheeses, respectively.

Effect of LAB on the Sensory Quality of Pasta-filata Cheeses

The panelists did not determine significant differences ($p < 0.05$) in appearance between the LAB-enriched cheeses and the CC cheeses and described them as being white to creamy white, with the overall uniformity in color that is a basic requirement of pasta-filata cheeses (Council Regulation (EC) No 510/2006, 2006; Zimanová et al., 2016). However, color intensity and uniformity tended to decrease between the 1st and 28th days (4.6 ± 0.6 to 4.2 ± 0.8 and 4.9 ± 0.3 to 4.5 ± 0.7, respectively). The most stable scoring of both parameters was noticed for RM9 cheese, and its rating did not decrease below 4.5 points even at the end of the consumption period. Fresh pasta-filata cheeses exhibited visual suitability ranging from 4.1 to 4.9 points (on average 4.6 ± 0.3), which indicates a very acceptable appearance for consumers. The storage influenced the sensory evaluation of this parameter, which it decreased approximately 0.8 points after 28 days of storage.

Mechanical Texture

Mechanical texture attributes such as chewiness, elasticity and stickiness did not significantly ($p < 0.05$) change among samples during the study. The mean values of those characteristics were 4.0 ± 0.4, 3.8 ± 0.6, and 4.5 ± 0.3 points, respectively, on the 5-point scale used. In general, not too much force was required to masticate to a state ready for swallowing, and cheeses were able to regain their initial thickness rapidly after compression and deformation. Nevertheless, the panelists perceived better textural properties in RM cheeses in comparison with products without the LAB addition, especially in the case of chewiness (4.2 ± 0.8 vs. 3.1 ± 0.9). However, the sensory profiles reported by the assessors' panel showed no noteworthy differences.

Aroma and Taste

The indicators of aroma and taste were classified by the descriptors that could have positive or negative effects on the overall sensory value of pasta-filata cheeses. In final products, the main purpose was preservation of primary descriptors - delicious milky, slightly sour and mild in flavor. On the other hand, the evaluation of possible negative effects (rancid, moldy, cowshed or other foreign) on the overall flavor was an important step in the assessment.

The panelists perceived higher intensities of milky aroma ($p < 0.05$) in RM cheeses (3.6 ± 1.0) than in CC cheeses (2.7 ± 0.8). Furthermore, the addition of selected LAB adjuncts positively controlled the aroma intensity. RM cheeses received hedonic scores near the neutral point (3.4 ± 0.8), indicating appropriate mild aroma of the prepared products. Control sample CC1 featured a higher aroma intensity (4.4 ± 0.7). The intensity of acidic aroma is a descriptor that can increase the sensory acceptance and quality of pasta-filata cheeses. Scores for acidic aroma above 3.0 could be considered unpleasant and disturbing. The results of this attribute were not affected by the adjunct cultures used. The panelists rated the acidic aroma of all the cheese samples as weak to moderate (from 2.0 to 3.0), which corresponds to an average value of 2.4 ± 0.3. The storage period did not significantly ($p < 0.05$) affect the evaluation of this aroma, which was almost identical to that of fresh pasta-filata cheeses. Overall, evaluation in terms of unacceptable aroma of

LAB-enriched samples was positive and samples did not have any off-flavor marks. The sensory scoring of grassy, spicy and fruity aroma reported by the assessors' panel was very low and did not change during the study ($p < 0.05$; 1.2 ± 0.4, 1.1 ± 0.3, and 1.2 ± 0.4, respectively). Aroma defects such as rancid, moldy and cowshed odor were observed mostly in cheese without the addition of LAB cultures, and significant differences ($p < 0.05$) between the RM and the CC were observed.

The addition of LAB during cheese manufacture substantially enhanced the taste of the pasta-filata cheeses. The values of desirable milky taste attribute of fresh cheeses were significantly ($p < 0.05$) higher in RM (4.2 ± 0.3) that in CC cheeses (3.0 ± 0.5). After 28 days of storage, a gradual decrease in milky taste was noticed for RM and CC cheeses (2.8 ± 0.4, and 2.2 ± 0.6, respectively). Descriptors associated with the taste of fresh cheese prevailed in LAB-enriched fresh cheeses (4.2 ± 0.7) in comparison with fresh CC (3.0 ± 0.8). Sensory evaluation of this attribute after the storage period significantly ($p < 0.05$) decreased for both the RM (2.8 ± 0.8) and CC (1.6 ± 0.7) groups of cheeses. Acidic taste is associated with the basic taste sensation for pasta-filata cheeses. The results of this descriptor ranged from 1.8 to 3.2, indicating a weak to moderate acidic taste. However, cheeses without LAB addition exhibited significantly ($p < 0.05$) higher scores (3.1 ± 0.9) for acidic taste than did RM cheeses (2.2 ± 0.8). Similarly, the perception of acidic taste suitability was evaluated and showed acceptability ranging from satisfactory to almost pleasant (3.9 ± 0.9). Furthermore, taste defects such as rotten, metallic, grassy, spicy and fruity defects were generally rated as unnoticeable (1.1 ± 0.4, 1.1 ± 0.4, 1.1 ± 0.3, 1.2 ± 0.5 and 1.1 ± 0.3, respectively). Several assessors also identified cowshed taste, but it was generally rated as unnoticeable (1.5 ± 0.6) in the RM group of cheeses in comparison with the CC group of cheeses (2.5 ± 0.9). Bitter taste was not affected by the adjunct culture used, but scores of the stored cheeses in the sensory acceptance test showed a tendency to increase approximately 0.4 points after 28 days of storage (1.4 ± 0.6 to 1.8 ± 0.8).

Overall Acceptability

All of the abovementioned sensory aspects were assessed and expressed as the overall level of acceptability for the pasta-filata cheeses (**Figure 5**). The final evaluation of the overall quality has a key role in the total sensory consumer acceptance of prepared products. The mean values and standard deviations (SD) of the overall acceptability used in the quantitative descriptive analysis

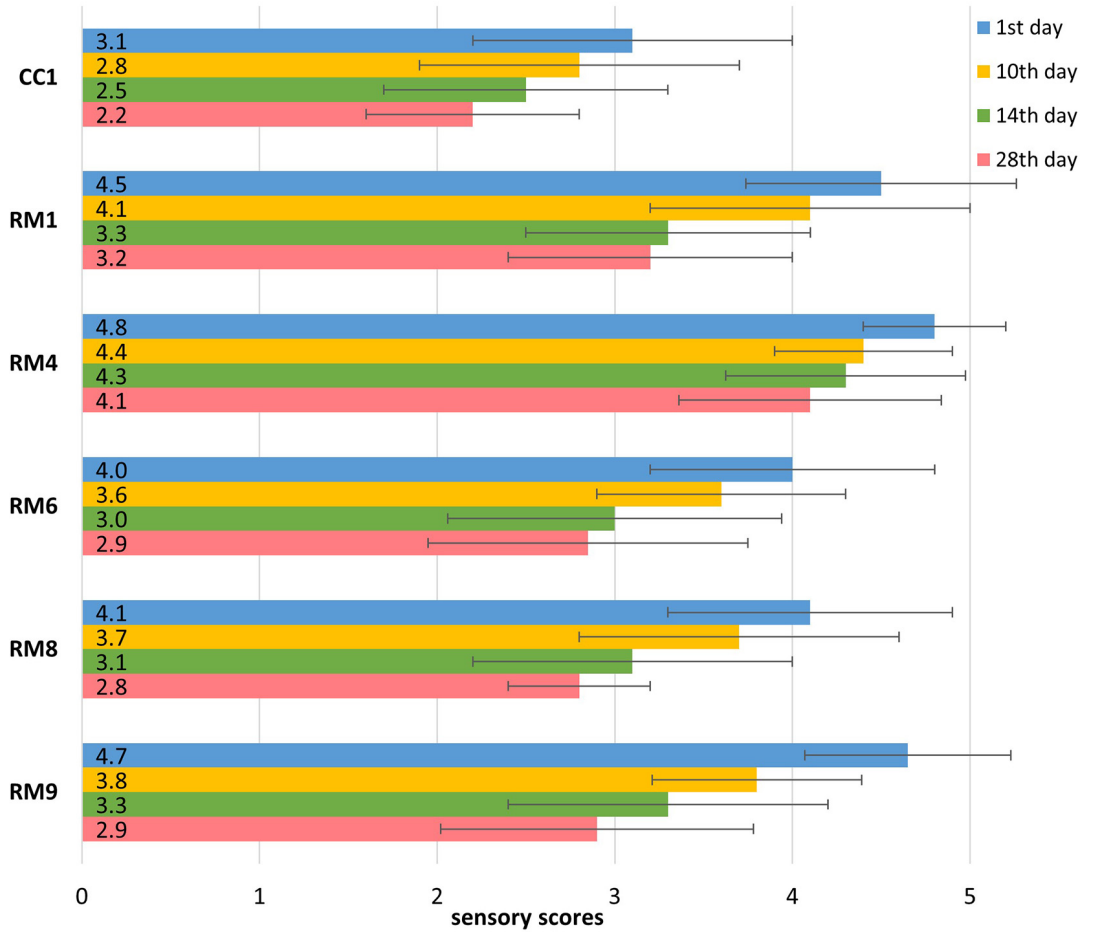

FIGURE 5 | The qualitative sensory evaluation of overall acceptability of chosen pasta-filata cheese.

TABLE 2 | Sensory scores of the overall acceptability of selected pasta-filata cheeses during storage period.

Cheese	Inoculated cultures with intended final concentration	1st day	10th day	14th day	28th day
CC1	none	$3.1 \pm 0.9^{a,y}$	$2.8 \pm 0.9^{a,y}$	$2.5 \pm 0.8^{a,y}$	$2.2 \pm 0.6^{a,z}$
RM1	Fresco 10^6 CFU/ml	$4.5 \pm 0.8^{a,x}$	$4.1 \pm 0.9^{a,x}$	$3.3 \pm 0.8^{b,y}$	$3.2 \pm 0.8^{b,y}$
RM4	culture A 10^6 CFU/ml	$4.8 \pm 0.4^{a,x}$	$4.4 \pm 0.5^{a,x}$	$4.3 \pm 0.7^{a,x}$	$4.1 \pm 0.7^{a,x}$
RM6	Fresco 10^6 CFU/ml LA145 10^6 CFU/ml	$4.0 \pm 0.8^{a,x}$	$3.6 \pm 0.7^{a,x}$	$3.0 \pm 0.9^{a,y}$	$2.9 \pm 0.9^{a,y}$
RM8	Fresco 10^6 CFU/ml VT1 10^6 CFU/ml	$4.1 \pm 0.8^{a,x}$	$3.7 \pm 0.9^{a,x}$	$3.1 \pm 0.9^{b,y}$	$2.8 \pm 0.4^{b,y}$
RM9	Fresco 10^6 CFU/ml LGG 10^6 CFU/ml	$4.7 \pm 0.6^{a,x}$	$3.8 \pm 0.6^{b,x}$	$3.3 \pm 0.9^{b,y}$	$2.9 \pm 0.9^{b,y}$

1 – worst possible value, 5 – best possible value (results are mean values ± standard deviation of 10 evaluations).
^{a,b} – within a row, letters with a differing superscript are significantly different ($p < 0.05$).
^{x,y,z} – within a column, letter with differing superscript are significantly different ($p < 0.05$).
LA145 – Lb. acidophilus LA145, VT1 – Lcb. rhamnosus VT1, GG – Lcb. rhamnosus GG.

(QDA) for cheeses (1, 10, 14, and 28 days) are presented in **Table 2**. According to the overall preference ratings, CC1 tended to be the least preferred sample in terms of the overall quality regardless of storage period. Samples enriched with LAB were, on average, 37% more acceptable products than CC1. The highest average scores were obtained by RM4 and RM9 cheeses on the day of production (4.8 ± 0.4, and 4.7 ± 0.6, respectively). The storage period decreased the overall acceptability of the final products. However, 28-day-old RM cheeses were rated from 2.8 to 4.1, showing acceptability ranging from satisfactory to almost pleasant.

DISCUSSION

Traditional raw milk cheeses are of great importance to maintain national heritage and tend to display greater variability, and strong and unique sensory properties compared with cheeses from pasteurized milk (Pappa et al., 2019); however, the usage of raw milk carries a potential health risk (Verraes et al., 2015). Therefore, every effort should be made to minimize this risk, with the addition of LAB being one of the oldest and simplest options. This was also the aim of our study, to evaluate the effects of adjunct cultures not only on microbiological quality and safety but also on the sensory properties of final products.

Autochthonous LAB are necessary to transform curds into cheese (Settanni and Moschetti, 2010) and enhance the stability and sensory properties of the final products (Guarrasi et al., 2017; Todaro et al., 2018). The growth and fermentative metabolism of LAB, as a permanent component of raw milk microbiota, can act as a natural inhibitory barrier in a wide variety of fermented dairy products. The most effective inhibiting activity against pathogenic and spoilage microorganisms is achieved by the production of organic acids with a subsequent *pH* decrease. It is relevant not only during the curd production phase, in which release of soluble Ca and change in casein structure occurs, but also in later stages when lower *pH* results in lower hardness (Fuentes et al., 2015). In addition, the potential presence of bacteriocins, H_2O_2 and aromatic compounds limits the growth of undesirable microbiota, and LAB, as a strong competitor, reveal the competition for nutritional factors (nicotinamide, biotin or niacin, etc.) needed for the growth of the bacteria present (Marth and Steele, 2001; Østlie et al., 2003; McSweeney, 2004; Charlier et al., 2008; Lahtinen et al., 2011). In the case of small amounts or insufficient acid production, no or minimal inhibitory effects are observed, as we have previously shown for *E. coli* and *S. aureus* (Le Marc et al., 2009; Ačai et al., 2016; Valík et al., 2018). Therefore, the attempts of scientists and technologists are naturally focused on the best possible cultures that will have appropriate metabolic activity and, in addition, will contribute to the improvement of technological and sensory properties of products. In addition, the selected cultures have to maintain their viability to a sufficient degree during the entire production process, especially the heating steps and storage periods.

Guidone et al. (2015) and Reale et al. (2019) reported that the *Lactobacillus casei* group is the most frequently used adjunct in several cheese types. The *Lactobacillus casei* group is acid-resistant, tolerates stress factors encountered in food processing and in the gastrointestinal tract, has a low frequency of antibiotic resistant phenotypes and the absence of the most frequently acquired antibiotic resistance genes, is better adapted to the cheese environment and survives resolutely at high counts in different mature cheeses.

Regarding viability, the study by Cuffia et al. (2017) proved the viability of *Lcb. rhamnosus* GG at levels higher than 7.5 log CFU/g during the cheese-making process and during the whole storage period. The loss of viability at 62.5 ± 0.5°C for 10 min was only 0.44 ± 0.12 log CFU/g. Moreover, counts of total LAB were higher than 9.1 log CFU/g during 15 days of storage at 4°C regardless of the day of storage or *Lcb. rhamnosus* GG presence. Additionally, *Lb. acidophilus* LA5 survived the heating process in pasta-filata cheese production, as its counts decreased after 1 day of storage by only 0.5 log CFU/g, and during ripening, the counts remained stable above 8 log CFU/g (Cuffia et al., 2017). Additionally, in the study of Cuffia et al. (2019), the counts of *Lcb. rhamnosus* GG and *Lb. acidophilus* LA5 were higher than 8 log CFU/g, and the counts of *S. thermophilus* STI-14 were higher than 9 log CFU/g during 15 days of storage. Pappa et al. (2019) reported that *S. thermophilus*, as a thermotolerant bacterium, exhibits better viability during the process of heating acidified curds than do mixed mesophilic *L. lactis* starter strains. The heating process at 72°C for 2 min destroyed 91% (*S. thermophilus*, *Lc. lactis*, *Lb. casei*; actually less than 2 logs decrease) and 85% (*S. thermophilus*, *Lb. delbrueckii* ssp. *bulgaricus*) of selected starter LAB cultures; however, during 180 days of ripening and storage,

the counts of LAB were above 7 log CFU/g. Moreover, they also reported that microbial recovery was much more pronounced in raw milk cheeses than in pasteurized milk cheeses. Additionally, in the study of Reale et al. (2019), the counts of the primary starter *S. thermophilus* were close to 9 log CFU/g, and during the steaming process they decreased by approximately 2 log CFU/g.

In this context, we used *Lcb. rhamnosus* GG, *Lcb. rhamnosus* VT1, and *Lb. acidophilus* and, as a starter culture, Fresco culture (consisting of *L. lactis* ssp. *lactis*, *L. lactis* ssp. *Cremoris,* and *S. salivarius* ssp. *thermophilus*) and culture A (consisting of *L. lactis* ssp. *lactis*, *L. lactis* ssp. *cremoris*, *Leuconostoc mesenteroides* ssp. *Cremoris,* and *Lb. acidophilus*) in pasta-filata cheese manufacture. During the steaming process, their counts also decreased to a similar degree as described above.

To minimize the risk and increase the technological and sensory properties of final products, it is also necessary for LAB to be the dominant microbes in pasta-filata cheeses at the end of the refrigerated storage period. This was the case for 224 pasta-filata cheeses prepared by Todaro et al. (2017), in which LAB dominated the microbial communities during the entire 180 days of storage at levels between 7-8 log CFU/g. In 192 cheeses presented in the work of Todaro et al. (2018), the counts of mesophilic cocci and bacilli were higher than 7.3 log CFU/g. Additionally, Minervini et al. (2012) observed that counts of mesophilic lactobacilli remained above 7.4 log CFU/g after 14 days of Fior di Latte (chemically acidified Italian pasta-filata) cheeses. In our case, in all 23 pasta-filata cheeses, after 28 days at 6 ± 0.5°C, the average counts of lactococci were 6.3 ± 1.2 log CFU/g, and the average counts of lactobacilli were 5.7 ± 1.6 log CFU/g. It might be a reason of either different composition of raw milk microbiota in regard to their metabolic activity, or development of non-cultivable non-starter LAB and their activity, and inhibitory effect of salting on starter culture LAB (in our case, the dominating bacteria in starter cultures were lactococci). Also the higher redox potential on the surface of "Nite" cheeses (their diameter is in average 0.5 cm) may not favor some LAB growth. In addition, in studies by Todaro et al. (2017, 2018) the cheeses were packed in 70% of N_2 and 30% of CO_2 atmosphere preferring the growth of anaerobic or facultative anaerobic LAB. Additionally, in the study by Šipošová et al. (2020) of 39 Slovak raw milk pasta-filata cheeses, the average counts of lactococci were 6.7 ± 1.1 log CFU/g; however, lower average counts of lactobacilli (4.4 ± 1.0 log CFU/g) were reported. Similarly, in the study by Tomáška et al. (2019), the levels of lactococci and lactobacilli were high, 6-8 log CFU/g in "Nite" cheeses from raw milk.

In addition, LAB do not always originate solely from starter culture, and their origin is also attributed to raw milk; therefore, in raw milk cheeses, their counts are naturally higher (Niro et al., 2014). This was also observed in our investigation, with lowest average counts of LAB in CC curds, followed by PM. The highest counts of LAB were observed in RM curds as a result of autochthonous LAB presence and addition of starter cultures. Fuentes et al. (2015) also reported that the addition of adjuncts significantly increased the counts of LAB after 30 days of ripening, in the case of lactobacilli by approximately 1–1.2 log CFU/g and in the case of lactococci by approximately 0.4–0.8 log CFU/g, depending on the adjuncts used, and their counts in 18 Oaxaca (Mexican non-aged pasta-filata) cheeses remained higher than 8.1 log CFU/g and 6.3 log CFU/g, respectively, for 24 days of storage at 8°C. Additionally, as reported by Niro et al. (2014), mesophilic bacteria showed an increasing trend during ripening and reached levels of approximately 7 log CFU/g from the initial 5-6 log CFU/g. In the case of total counts of LAB in 16 commercial Hispanic pasta-filata cheeses, their average value (6.8 log CFU/g) was similar to the abovementioned values; however, in 4 cheeses, there was less than 2.7 log CFU/g of total LAB (Jimenez-Maroto et al., 2016).

As Cuffia et al. (2019) showed, the addition of *Lcb. rhamnosus* GG or *Lb. acidophilus* LA5 during pasta-filata soft cheese manufacture did not influence their gross composition (moisture, fat, protein) or *pH* value, which remained stable during ripening. In our case, we were not able to determine the *pH* values of "Nite" cheeses, because the diameter of cheeses was less than that of the pH-meter sensor. In Slovak pasta-filata cheeses included in the study of Šipošová et al. (2020), the average *pH* of commercial samples was 5.36 ± 0.12 (data not published in the study), and the *pH* value of curds in this study (before steaming) averaged 5.32 ± 0.48. Moreover, based on studies by Minervini et al. (2012); Guidone et al. (2015), Cuffia et al. (2017; 2019) and Pappa et al. (2019), *pH* values of samples remained stable during the entire storage period and so the adjuncts did not contribute to the undesirable decrease of acids. Lower *pH* is frequently observed in cheeses produced with NSLAB adjuncts as a result of acetic acid production (Guidone et al., 2015).

Additionally, the final average a_w value (0.965 ± 0.006) of our 23 pasta-filata cheeses after 28 days of storage was consistent with values for cheeses in the study of Šipošová et al. (2020), where the average a_w value of 39 cheeses was 0.956 ± 0.012 (data not published in the study). In contrast, Todaro et al. (2017) reported higher a_w value (0.984) after 30 days and an a_w value of 0.971 after 180 days of storage at 4°C. Lower a_w values at the end of storage prevent the development of undesirable microbial populations (Robertson, 1993).

Considering the LAB inhibitory properties against the growth of CPS and *E. coli*, the most favorable effect was observed in cheeses with LAB as the adjunct. In the case of 24-h-old curds, the lowest counts of CPS and *E. coli* (2.4 log CFU/g for both) were observed in cheeses manufactured with culture A (in the case of CPS) or Fresco + *Lcb. rhamnosus* VT1. In contrast, CPS and *E. coli* counts in CC curds ranged from 3.60 – 5.82 log CFU/g and 5.30 – 6.72 log CFU/g, respectively. The steaming process naturally reduced microbial numbers; counts of CPS and *E. coli* in the CC group ranged from 3.88 – 4.40 log CFU/g and 3.92-4.78 log CFU/g, respectively. Fresh RM pasta-filata cheese with the lowest value of CPS (2.78 log CFU/g) was manufactured using the combination of Fresco culture and culture A, while the best fresh pasta-filata cheese with the lowest *E. coli* level (1.23 log CFU/g) was manufactured with the addition of Fresco culture and *Lb. acidophilus* LA145. At the end of storage at 6 ± 0.5°C, neither *E. coli* or CPS were detected in cheeses manufactured with the addition of Fresco culture combined with *Lcb. rhamnosus* GG, and *E. coli* was also not detected in cheese enriched with Fresco and A culture combined. Our results are consistent with

the findings of Jimenez-Maroto et al. (2016); of 16 commercial Hispanic pasta-filata cheeses, none were found to be positive for the presence of CPS, and the counts of coliforms in 44% of the cheeses were lower than 10 CFU/g, and in 37% the counts were lower than 2 log CFU/g. In 3 cheeses, the counts were 3.2–4.1 log CFU/g. Additionally, in other studies, *E. coli* and CPS were either below the detection limit during the entire storage period (Todaro et al., 2017; 2018), were found at very low levels (Niro et al., 2014; Fuentes et al., 2015), or in Kashkaval cheeses, *E. coli* counts were below 10 CFU/g since day 12 (Pappa et al., 2019). Conversely, the level of CPS remained high in Kashkaval cheeses during the entire period, and in 30-day-old cheeses, it reached the highest value, 5.5 ± 0.4 log CFU/g. Similar results were observed in other Slovak raw milk commercial pasta-filata cheeses, where the average counts of *E. coli* and CPS were 1.85 ± 0.99 log CFU/g and 3.94 ± 0.99 log CFU/g, respectively (Šipošová et al., 2020). In raw milk "Nite" cheeses, the level of CPS was on the order of 2-3 log CFU/g. Higher counts of CPS may be related to post-steaming contamination, especially in manually prepared cheeses (Tomáška et al., 2019).

For the incorporation of LAB into food products, cultures used should be technologically suitable, such that they establish viability and efficacy throughout the storage. In addition, during refrigeration, adjunct strains should not lead to undesirable changes in texture, flavor, or aroma characteristics. The sensory attributes of cheeses, such as appearance and texture, are clearly visible, and thus are a prerequisite for consumer acceptance (Karimi et al., 2012).

The sensory profiles showed no noteworthy differences in appearance attributes between the LAB-enriched and control pasta-filata cheeses. Similarly, Cuffia et al. (2017), examining the acceptability of soft pasta-filata cheese (Fior di Latte type) enriched with *Lcb. rhamnosus* GG, found no differences in color from that of conventional commercial cheese ($p < 0.05$). Dinakar and Mistry (1994) also reported that some bifidobacteria had no significant effect on the appearance of cheddar cheese through 24 weeks of storage. Gomes et al. (2011) reported that cheese with LAB addition received lower scores for appearance. In contrast, Albenzio et al. (2013) and Braghieri et al. (2015) reported that incorporation of LAB enhanced the color uniformity of Scamorza cheese. The negative effect of storage period on the appearance of Slovak pasta-filata "Parenica" was also noted by Semjon et al. (2019). Fresh unsmoked samples showed a descriptor of appearance approximately 25% higher than for cheeses after a 14-day storage period. In our case, this attribute decreased approximately 12 – 36% (on average 22%) after 28 days of storage.

The type of adjunct culture in our study did not result in significant sensory differences in textural parameters. However, due to LAB incorporation, a higher appreciation of our sample texture was observed. Assessors evaluated that chewiness in LAB-enriched samples was enhanced. Cuffia et al. (2017; 2019) reported that the chewiness of probiotic pasta-filata cheese was comparable to that of the control cheese (non-probiotic). Conversely, Sandoval-Copado et al. (2016) showed that both Oaxaca cheese made with mesophilic (*L. lactis* ssp. *lactis* and *L. lactis* ssp. *cremoris*) and thermophilic (*S. salivarius* ssp. *thermophilus*) LAB cultures were less chewable than were cheeses from naturally acidified raw milk. In terms of elasticity, we observed positive changes and improvement of this mechanical textural attribute in cheeses with LAB. Addition of *Lb. acidophilus* LA5 and *Lcb. rhamnosus* GG has been previously reported to improve the elasticity of pasta-filata soft cheese (Cuffia et al., 2017, 2019). Nevertheless, Braghieri et al. (2015) reported that LAB incorporation into Scamorza induced lower elasticity. In our study, no significant differences were detected in elasticity after storage of pasta-filata cheeses; however, after storage, there was less tendency for the cheeses to return to its initial shape after being compressed. This trend was consistent with the findings of Kindstedt (1993), Fuentes et al. (2015) and Semjon et al. (2019). These authors have associated the modification of the elasticity of cheese due to LAB incorporation with proteolysis. Similarly, Reale et al. (2019) recorded the ranking for elasticity of Scamorza with LAB around the neutral point of the hedonic scale (5 points on a scale from 1 to 9) after 30 days of storage. In our study, we observed mean scoring just above the middle category (3.4 ± 0.6) at the end of the storage period. As was also observed in our study, Cuffia et al. (2019) reported no uniform effect of LAB addition into pasta-filata on mechanical attributes such as stickiness. Nevertheless, Albenzio et al. (2013) determined higher stickiness in LAB-enriched pasta-filata.

Many investigations have indicates that the addition of LAB has an impact on the aroma and taste of innovatively produced cheeses (Ryhänen et al., 2001; Ong et al., 2007; de Souza et al., 2008; Hoorde et al., 2010; Gomes et al., 2011; Guidone et al., 2015; Hammam et al., 2018). In our study, LAB-enriched pasta-filata cheeses received a better score for aroma intensity compared to the CC group. In contrast, some studies have concluded that scores for aroma intensity of pasta-filata cheese increased with adjunct culture (Minervini et al., 2012; Cuffia et al., 2017, 2019; Reale et al., 2019). Some of the aroma and taste descriptors described in our study are consistent with results obtained in previous artisanal pasta-filata cheese studies. Braghieri et al. (2015) and Albenzio et al. (2013) found that milk aroma in LAB-enriched cheeses was higher than in control samples. Sandoval-Copado et al. (2016) incorporated lactococci and streptococci into Oaxaca cheese and found that LAB-enriched cheeses featured lower intensity of undesirable cowshed aroma. Several studies focusing on the incorporation of LAB into pasta-filata have reported a higher level of sensory perception of bitterness (Sandoval-Copado et al., 2016; Cuffia et al., 2019; Reale et al., 2019) in comparison with conventional (non-LAB) cheeses. As also observed by Albenzio et al. (2013) and Sandoval-Copado et al. (2016), the scoring of acid taste was comparable with that of the control sample, but pasta-filata cheeses with the addition of LAB received slightly lower scores regarding intensity of acid taste. In contrast, Braghieri et al. (2015), Cuffia et al. (2017; 2019) and Reale et al. (2019) determined that the LAB addition in cheeses resulted in rejection by consumers with respect to higher acid taste in comparison to control cheese.

The sensory profile of pasta-filata cheeses showed that the incorporation of LAB enhanced the overall acceptability, and the most satisfactory overall acceptability after 28 days of storage at 6 ± 0.5°C was reached for cheese with the addition of culture A (RM4, **Figure 5**). Having better acceptability of LAB-enriched

cheeses is consistent with findings of Minervini et al. (2012) and Braghieri et al. (2015), who reported that pasta-filata cheeses produced using adjunct LAB cultures induced higher values of an acceptability score than did with control cheeses. Cuffia et al. (2019), who inoculated pasta-filata with *Lb. acidophilus* LA5 and *Lcb. rhamnosus* GG, received similar or higher sensory scores for LAB-enriched samples compared to control (non-LAB-inoculated) samples. In the study performed by Reale et al. (2019), the sensory profiles of the control sample and LAB-enriched Scamorza cheese were comparable. All these results showed that LAB mostly improved the sensory properties of pasta-filata cheeses, as was also observed in our study, or at least did not reduce in consumer acceptance in comparison to the original product.

As expected, the overall acceptance of RM cheeses was negatively affected by storage time. Deterioration of cheeses was mainly attributed to the bitter taste and slight softness. Based on overall acceptability, RM products stored for 14 days received scores from the judges approximately 29% lower, and the appreciation of 28-day refrigerated cheeses decreased by 38% in comparison with fresh samples. Semjon et al. (2019) also noted a negative effect of 14-day storage period on the overall acceptability of unsmoked "Parenica" from 8.1 to 4.9. Similarly, Fuentes et al. (2015) noted that the acceptability of cheese for consumption decreased by approximately 36% after 16 days of storage in comparison with products immediately after preparation.

These results suggest that the addition of LAB can improve the sensory profile of pasta-filata cheese. The differences in perceived textural characteristics, aroma and taste depend on the metabolic activity of the strains used in the cheese-making process. Contribution to sensory properties is attributed to the higher accumulation of microbial metabolites in LAB-enriched cheeses. Generally, proteolysis plays a key role in the development of the typical sensory characteristics of a cheese (de Souza et al., 2008; Gomes et al., 2011; Karimi et al., 2012; Niro et al., 2014; Braghieri et al., 2015; Guidone et al., 2015; Hammam and Ahmed, 2019; Reale et al., 2019) with the production of different amino acids that are precursors for specific sensory active metabolites (see Introduction).

CONCLUSION

In conclusion, beyond the fermentation activity of natural LAB microbiota, the use of starter cultures is strongly recommended in artisanal lump cheeses that use raw milk for pasta-filata cheese production. This is the only approach capable of assuring the initial dominance of LAB and supporting the growth of the natural LAB present in raw milk in competition with other undesirable bacteria. Moreover, the addition of dairy culture will also enhance the sensory acceptance of final products. At this point, we would like to emphasize the great importance of LAB, including their dairy cultures, in preserving the national gastronomic heritages that are registered and protected in the EU.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

KK and IH carried out the experimental work. MK and AM analyzed the dataset. AM and ĹV set up the experimental design. AM, MK, and ĹV wrote the manuscript. All authors contributed to the article and approved the submitted version.

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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.2020.612528/full#supplementary-material

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Immunomodulatory Properties of a γ-Aminobutyric Acid-Enriched Strawberry Juice Produced by *Levilactobacillus brevis* CRL 2013

Pablo G. Cataldo†, Julio Villena†, Mariano Elean, Graciela Savoy de Giori, Lucila Saavedra and Elvira M. Hebert**

Centro de Referencia para Lactobacilos (CERELA-CONICET), San Miguel de Tucumán, Argentina

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***Correspondence:**
Lucila Saavedra
lucila@cerela.org.ar
Elvira M. Hebert
ehebert@cerela.org.ar;
elviramaria.hebert@gmail.com
orcid.org/0000-0001-8288-9701

†These authors have contributed equally to this work

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Gamma-aminobutyric acid (GABA) plays a key role in mammals as the major inhibitory neurotransmitter of the central nervous system. Although GABA may not be able to cross the human blood-brain barrier, it was approved as a food ingredient because of its benefits to the host after oral administration including anti-hypertensive, anti-depressant and anti-inflammatory activities. Considering the current trend toward the development of new functional and natural products and that microbial fermentation is one of the most promising methods to produce this non-protein amino acid, the *in situ* production of GABA through fermentation of strawberry and blueberry juices by the efficient GABA producer strain, *Levilactobacillus brevis* (formerly known as *Lactobacillus brevis*) CRL 2013, was evaluated. A high GABA production (262 mM GABA) was obtained after fermenting strawberry juice supplemented with yeast extract for 168 h, being GABA yield significantly higher in strawberry juices than in the blueberry ones. Thus, GABA-enriched fermented strawberry juice (FSJ) was selected to carry out *in vivo* and *in vitro* studies. The *in vitro* functional analysis of the GABA-enriched FSJ demonstrated its ability to significantly decrease the expression of *cox-2* gene in LPS stimulated RAW 264.7 macrophages. In addition, *in vivo* studies in mice demonstrated that both, *L. brevis* CRL 2013 and the GABA-enriched FSJ were capable of reducing the levels of peritoneal, intestinal and serum TNF-α, IL-6, and CXCL1, and increasing IL-10 and IFN-γ in mice exposed to an intraperitoneal challenge of LPS. Of note, the GABA-enriched FSJ was more efficient than the CRL 2013 strain to reduce the pro-inflammatory factors and enhance IL-10 production. These results indicated that the CRL 2013 strain exerts anti-inflammatory effects in the context of LPS stimulation and that this effect is potentiated by fermentation. Our results support the potential use of *L. brevis* CRL 2013 as an immunomodulatory starter culture and strawberry juice as a remarkable vegetable matrix for the manufacture of GABA-enriched fermented functional foods capable of differentially modulating the inflammatory response triggered by TLR4 activation.

Keywords: GABA, lactic acid bacteria, *Levilactobacillus brevis*, strawberry fermented juice, anti-inflammatory properties, TLR4 activation

INTRODUCTION

Gamma-aminobutyric acid (GABA) is an ubiquitous non-protein amino acid widely distributed among microorganisms, plants and animals, having diverse physiological functions and great potential health benefits (Ramos-Ruiz et al., 2018). GABA exerts some positive effects on mammalian physiology; such as hypotensive, relaxation, antidiabetic and immunity enhancement effects (Adeghate and Ponery, 2002; Abdou et al., 2006; Yang et al., 2012). Due to its relevance and health benefits, GABA is becoming recognized as an essential nutrient for a healthy and balanced diet. GABA has been authenticated as new resource food by China Food and Drug Administration in 2009 and it is listed in the Chinese Pharmacopeia (Ramos-Ruiz et al., 2018). Additionally, a healthy diet following the WHO food-based dietary guidelines (FBDG) and/or the Healthy Eating Plate (Harvard) will provide a considerable amount of GABA as a natural nutrient. Nevertheless, GABA content in natural animal- and plant-based food products is low (Quílez and Diana, 2017). Therefore, efforts are being devoted to the development of new technological processes for GABA enrichment in traditional foods.

Lactic acid bacteria (LAB) are microorganisms that inhabit nutrient-rich environments associated with food, seeds, plants, animals, and humans. Due to their Qualified Presumption of Safety (QPS) status and their use in food, biotechnology, and therapeutic applications, LAB are highly industrially relevant microorganisms and represent a multi-billion Euros business worldwide (Johansen, 2017). Thus, LAB strains, mainly belonging to *Levilactobacillus brevis* (formerly known as *Lactobacillus brevis*) species, constitute the most competitive and technologically relevant group of microorganisms used to synthesize GABA since they are able to produce high levels of this compound within a variety of food matrices (Park and Oh, 2007; Kim et al., 2009; Wu et al., 2015; Li et al., 2016; Villegas et al., 2016; Bao et al., 2020; Cui et al., 2020). In this regard, we have recently demonstrated that *L. brevis* CRL 2013 is an efficient microorganism for the conversion of monosodium glutamate (MSG) to GABA in hexose-supplemented complex media with conversion ratios about 99% (Cataldo et al., 2020). This GABA yield was one of the highest values observed among lactobacilli grown in batch culture (Villegas et al., 2016; Cataldo et al., 2020; Cui et al., 2020). Then, our results support the potential use of *L. brevis* CRL 2013 as a starter culture for the manufacture of GABA-enriched functional foods.

In dietary guidelines worldwide, an increased consumption of fruits and vegetables is recommended. The intake of the so-called "superfruits" like berries that are rich in nutrients, phytochemicals and constitute important dietary reservoirs of bioactive compounds can prevent various diseases and disorders (Nile and Park, 2014). Thus, berries have potent antioxidant, anticancer, antimutagenic, antimicrobial, anti-inflammatory, and anti-neurodegenerative properties, both *in vitro* and *in vivo* (Nile and Park, 2014). Among berries, blueberry (*Vaccinium corymbosum*) and strawberry (*Fragaria x ananassa*) are widely cultivated in Argentina, which is the second largest strawberry producer in The Common Market of the Southern Cone (MERCOSUR), being Canada, United States, and the European Union the main export destinations of Argentinian strawberries. These fruits are not only available fresh but also generally consumed frozen and processed into juices, yogurts, beverages, jams, and jellies. In general, berries have a low GABA content, ranging from 0.016 mg/g for strawberries to 0.079 mg/g for blueberries (Ramos-Ruiz et al., 2018). Fortifying beverages with GABA is being intensively studied due to its potential health benefits (Kim et al., 2009; Quílez and Diana, 2017). However, artificially produced GABA cannot be added to food manufacture since GABA is not a legal additive in several countries; including countries of the European Union and Argentina [Efsa Panel on Dietetic Products, and Nutrition, and Allergies [NDA], 2009; Kim et al., 2009]. Then, the use of efficient GABA producer starter cultures such as *L. brevis* CRL 2013 to increase GABA concentrations in berry-juices would be an alternative to bioenrich these beverages with this bioactive compound. Therefore, the aims of this work were: (a) to obtain a GABA-enriched berry juice fermented by the high GABA producer *L. brevis* CRL 2013, combining the health benefits of both, GABA and berries; and (b) to evaluate the potential anti- inflammatory properties of this GABA-enriched juice by using *in vitro* and *in vivo* approaches in the context of Toll-like receptor (TLR)-4 mediated inflammation.

MATERIALS AND METHODS

Microorganism and Growth Conditions

Levilactobacillus brevis CRL 2013 was isolated from Andean Real Hornillos quinoa sourdough (Cataldo et al., 2020) and belongs to the CERELA culture collection (CERELA-CONICET, Argentina). The strain was routinely propagated and cultivated in a modified MRS broth (pH 6.5) containing 1% glucose and 1% fructose instead of 2% glucose. When assessing GABA production, cells were statically grown in capped test tubes containing 267 mM of monosodium glutamate (MSG) at 30°C. For the juice fermentation assays, eighteen-hour cultures were harvested by centrifugation (9,000 × *g* for 10 min), washed twice with sterile 0.8% (w/v) NaCl and used as inoculums to reach an initial optical density at 600 nm (OD_{600}) of about 0.1 (approximately 5×10^7 CFU/ml).

Determination of GABA Concentration

Gamma-aminobutyric acid was tittered using a modified version of the GABase method previously described by Tsukatani et al. (2005). Briefly, 86 mM Tris–HCl buffer (pH 9), 5 mM α-ketoglutarate, 3.3 mM 2-mercaptoethanol, 1.2 mM $NADP^+$, and 0.03 U of GABase were added to each well of a 96-well microtiter plate. The mixture was warmed at 25°C, and then the standard or sample solution (fermented juice supernatants) was added (Cataldo et al., 2020). The NADPH formation was measured at 340 nm every 1 min for 10 min at 25°C in a Biotek Synergy HT microplate reader (Winooski, VT, United States). GABA concentration in each sample was calculated from the calibration curve of the standard solutions (0.10, 0.25, 0.50, and 1 mM GABA).

Development of GABA-Enriched Fermented Juices

Blueberries (*Vaccinium corymbosum*) and strawberries (*Fragaria x ananassa*) harvested in 2018 were purchased from production fields in Lules (Tucumán, Argentina). For the preparation of blueberry (BJ) and strawberry (SJ) juices, frozen fruits were thawed at room temperature for 4 h and then crushed on a Russell Hobbs JM550SRH Juice extractor (China). Juices were then centrifuged three times at 14,000 × *g* for 10 min to get rid of the remaining pulp and then sterilized at 115°C for 15 min. When needed, pulp-free juices were supplemented with 267 mM MSG, 1% (w/v) yeast extract (YE) or tryptein (T). The initial pH was either adjusted to approximately 6.5 with 0.1 M $NaHCO_3$ or left unmodified. All juices were inoculated with *L. brevis* CRL 2013 to an initial cell density of 5×10^7 CFU/ml. Cell growth (OD_{600}), pH and GABA production were determined at different time intervals for 7 days. To determine cell growth, bacterial culture was washed twice with phosphate buffered solution (PBS) and resuspended to the original volume using the same solution. Non-inoculated berry juices processed in the same way as the sample were used as controls. Juices fermentations were followed up until the conversion rate from MSG to GABA reached its maximum values for this strain (around 98%).

Cell Culture

Mouse RAW 264.7 macrophages were obtained from IMBICE, CONICET (Argentina). Cells were routinely kept in a RPMI Medium (Genbiotech, Argentina), supplemented with 10% (w/v) fetal bovine serum (FBS, NATOCOR, Argentina), penicillin G (100 U/ml, Gibco, ThermoFisher, Argentina), streptomycin (100 μg/ml, Gibco) and amphotericin B (25 μg/ml, Gibco) at 37°C in a humidified 5% CO_2-95% air incubator (standard conditions).

MTT Cell Viability Assay

The influence of GABA-enriched berry juices on cell proliferation was analyzed by the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay (Marcial et al., 2014). Briefly, RAW 264.7 cells were seeded at 5.0×10^4 cells per well with RPMI containing 10% FBS in 96-well plates and cultured for 24–48 h at 37°C in a humidified environment until 85% of confluence was reached. Then, the medium was removed, cells were washed twice with PBS and adherent cells were incubated at 37°C for 24 h in the presence of GABA (0.1 and 1.0 mM) (Sigma-Aldrich Co., MO, United States), filtrated dilutions of fermented strawberry juice (FSJ) supernatants to obtain a final GABA concentration of 1 mM or the same dilution of the non-fermented strawberry juice (NFSJ). After incubation, the medium was discarded, cells were washed with PBS and 50 μl of MTT solution (2.6 mg/ml in PBS) were added in each well and incubated for 4 h at 37°C in a 5% CO_2 incubator. Living cells convert MTT into a purple colored formazan product. MTT was removed from the plates and 50 μl DMSO/well were added to dissolve the formazan crystals. After 5 min the absorbance at 570 nm, which is directly proportional to the cellular metabolism, was measured in a Biotek Synergy HT microplate reader (Winooski, VT, United States) and cell viability was estimated as the percentage absorbance of sample relative to the positive control.

RT-qPCR

RAW 264.7 cells were inoculated in 6-well plates (0.5×10^6 cells/ml) and incubated as described above for 24–48 h. At 85% confluence, the medium was replaced with fresh media containing GABA (0.1 or 1 mM), filtrated dilutions of FSJ supernatants to obtain a final GABA concentration of 1 mM or the same dilution of the NFSJ, and cells were pre-incubated for 1 h at 37°C. Then, cells were treated with 1 μg/ml of lipopolysaccharide (LPS) for 4 h to induce the inflammatory state. Adherent cells were harvested after the LPS stimulation and total RNA was isolated using the TRIzol reagent following the manufacturer's instructions (Life Technologies, Buenos Aires, Argentina). Briefly, cells were lysed in 400 μl of TRIzol; and 200 μl of chloroform were added to each tube. Finally, the suspensions were centrifuged for 15 min at 1,2000 × *g* and 4°C. RNA was isolated from the upper hydrophilic phase by adding 100 μl of isopropyl alcohol and allowing it to precipitate for 2 h at −20°C. Samples were centrifuged again, the supernatants were discarded and pellets were washed twice with 70% cold ethanol, resuspended in 15 μl of RNase-free water and stored at −80°C. Total RNA was quantified using the Qubit RNA HS assay kit (Life technologies). The reverse transcription was carried out employing the SuperScript III First Strand Kit (Life technologies) following the supplier's instructions.

The specific oligonucleotides sequences for cyclooxygenase (*cox-2*) gene and β-actin used in this study were previously described by Chang et al. (2016). Real Time qPCR was performed on an iQ5 Real-Time PCR Detection System (BioRad) with the IQTM SYBR®supermix (Bio-Rad) in 96-well plates. PCR was performed with 1 μl of cDNA (100 ng) or water in the non-template controls, 4 μl of primer mix (0.3 μM of each primer), 5 μl of RNase-free water and 10 μl of IQTM SYBR®supermix as described by Brown et al. (2017). The PCR cycles consisted in: 95°C for 4 min, 40 cycles of 95°C for 15 seg, 52°C for 30 seg, 72°C for 30 seg and 95°C for one-minute. A non-template control was included within each PCR reaction. Amplification efficiencies were validated and normalized against the β-actin gene. A melting curve analysis was performed immediately at the end of each experiment at a linear temperature transition rate of 0.1°C/s from 55 to 95°C to determine the specificity of the amplification. The relative mRNA expression (as fold change) was determined using β-actin as normalizing housekeeping gene by the $2^{-\Delta\Delta CT}$ or Livak method (Livak and Schmittgen, 2001).

Animals and Feeding Procedures

Male 4-week-old Balb/c mice were obtained from the closed colony kept at CERELA (Tucuman, Argentina). They were housed in plastic cages with controlled room temperature (22 ± 2°C temperature, 55 ± 2% humidity) and were fed *ad libitum* conventional balanced diet. This study was carried out in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the Guidelines for Animal Experimentation of CERELA. The CERELA Institutional

Animal Care and Use Committee prospectively approved this research under the protocol BIOT-CRL-17.

Mice were housed individually during the experiments and the assays for each parameter studied were performed in five mice per group. *L. brevis* CRL 2013 was administered to mice for 3 consecutive days at a dose of 2×10^8 cells/mouse/day in the drinking water (4 ml per mice per day). Different groups of mice were also treated with GABA-enriched fermented strawberry juice (FSJ) containing ∼140 mM GABA or diluted (1:2) GABA-enriched FSJ containing ∼70 mM GABA [FSJ + CRL2013 and FSJ + CRL2013 (d), respectively] during 3 consecutive days *ad libitum*. The GABA-enriched fermented strawberry juice (FSJ + CRL2013) contains about 2.7×10^7 CFU/ml. Groups of mice treated with non-fermented strawberry juice (NFSJ) or NFSJ supplemented with yeast extract (NFSJ + YE) were used as controls. One day after the end of the treatments, mice were challenged with LPS to induce inflammation as described previously (Garcia-Castillo et al., 2019). Mice received 8 mg/kg of LPS from *Escherichia coli* O55:B5 by intraperitoneal injection.

Cytokine Concentrations

The concentration of cytokines was determined in peritoneal fluid, blood and intestinal samples. Peritoneal fluid was collected from mice as described previously (Garcia-Castillo et al., 2019). Blood samples were obtained through cardiac puncture at the end of each treatment and collected in heparinized tubes. Intestinal fluid samples were obtained by separating the small intestine and flushing it with 5 ml of PBS. The fluid was centrifuged (10,000 × *g*, 4°C for 10 min) to separate particulate material. The supernatant was kept frozen until use. Blood and tissue samples were obtained from mice after the intraperitoneal injection of ketamine (80 mg/kg) and xylazine (10 mg/kg) according to the recommendations of the CERELA Institutional Animal Care and Use Committee.

Tumor necrosis factor α (TNF-α), interferon γ (IFN-γ), interleukin (IL)-10, IL-6, and the IL-8 mouse homolog chemokine KC or chemokine (C-X-C motif) ligand 1 (CXCL1) concentrations were measured with commercially available enzyme-linked immunosorbent assay (ELISA) kits following the manufacturer's recommendations (R&D Systems, MN, United States). TNF-α (Mouse TNF-α ELISA Kit, sensitivity: 1.5 pg/ml), IFN-γ (Mouse IFN-gamma Quantikine ELISA Kit, sensitivity: 2 pg/ml), IL-6 (Mouse IL-6 Quantikine ELISA Kit, sensitivity: 1.8 pg/ml), IL-10 (Mouse IL-10 Quantikine ELISA Kit, sensitivity: 5.2 pg/ml), and CXC1 (Mouse CXCL1/KC DuoSet ELISA, sensitivity 2.3 pg/ml) kits were used.

Intestinal Tissue Injury

Intestinal tissue injury was evaluated as described previously (Elean et al., 2020). Briefly, small intestine samples from all experimental groups were excised and washed out with PBS. Then, tissues were immersed in 4% (v/v) formalin saline solution. Once fixed, samples were dehydrated and embedded in Histowax (Leica Microsystems Nussloch GmbH, Nussloch, Germany) at 56°C. Finally, intestines were cut into 4 μm serial sections and stained with hematoxylin-eosin for light microscopy examination. All slides were coded and evaluated blindly. A semiquantitative scoring index was used to evaluate alterations in the intestine. The presence/absence and intensity of edema, epithelial injury, degranulation of Paneth cells and inflammation were considered. Each parameter was rated on a point damage scale from 1 to 4 (1, absence; 2, slight; 3, moderate; 4, severe alteration) and the final score results were expressed as the sum of the individual scores given to each parameter (Torres et al., 2017).

Statistical Analyses

Statistical analyses were performed with the software package Minitab 17 (Minitab Inc.) using ANOVA general linear models followed by Tukey's *post hoc* test where $p < 0.05$ was considered significant. Unless otherwise specified, all reported values were the means of three independent trials ± standard deviation. No significant differences were observed between individual replicates.

RESULTS

Formulation of GABA-Enriched Fermented Berry Juices

In order to develop GABA-enriched fermented berry juices, firstly, we studied the growth of *L. brevis* CRL 2013 in blueberry and strawberry pulp-free juices supplemented with 267 mM MSG. As control, the strain was grown in the same juices without extra supplementation. GABA production was not observed in the fermented berry juices supplemented only with MSG (data not shown). Thus, growth parameters and GABA production were evaluated in the pulp-free juices supplemented with MSG and YE or tryptein. Initial pH was either adjusted to *ca*. 6.5 using 0.1 M $NaHCO_3$ or left unmodified and the fermentation was monitored for 7 days at 30°C. No significant differences in cell density and pH time course were observed between juices with adjusted and unadjusted initial pH (data not shown). In both YE and tryptein-supplemented media, *L. brevis* CRL 2013 was able to reach a sustained growth (**Figure 1**). YE-supplemented juices presented higher cell densities than those observed for the tryptein-supplemented ones (**Figure 1**). In the first 24 h of fermentation, the cell growth was accompanied by a decrease in the pH of the media independently of the initial pH. Thereby, pH values for strawberry and blueberry juices sharply decreased during the first 24 h and 48 h, reaching values of ∼4.86 and ∼4.75 for the YE supplemented strawberry (**Figure 1A**) and blueberry juices (**Figure 1B**), respectively.

Although juices supplemented with tryptein allowed a sustained growth of *L. brevis* CRL 2013, no increase in the pH of the medium was observed and GABA was not detected in the culture supernatants. Contrariwise, in strawberry juice supplemented with YE (**Figure 1A**), GABA synthesis began after 24 h of fermentation whereas in blueberry juice (**Figure 1B**), GABA became detectable only after 48 h. In both cases, GABA production was accompanied by a concomitant increase of the pH of the extracellular milieu (**Figure 1**). In YE-supplemented juices GABA production progressed up to 168 h. Thus, the highest productivity (262 mM GABA) was obtained after

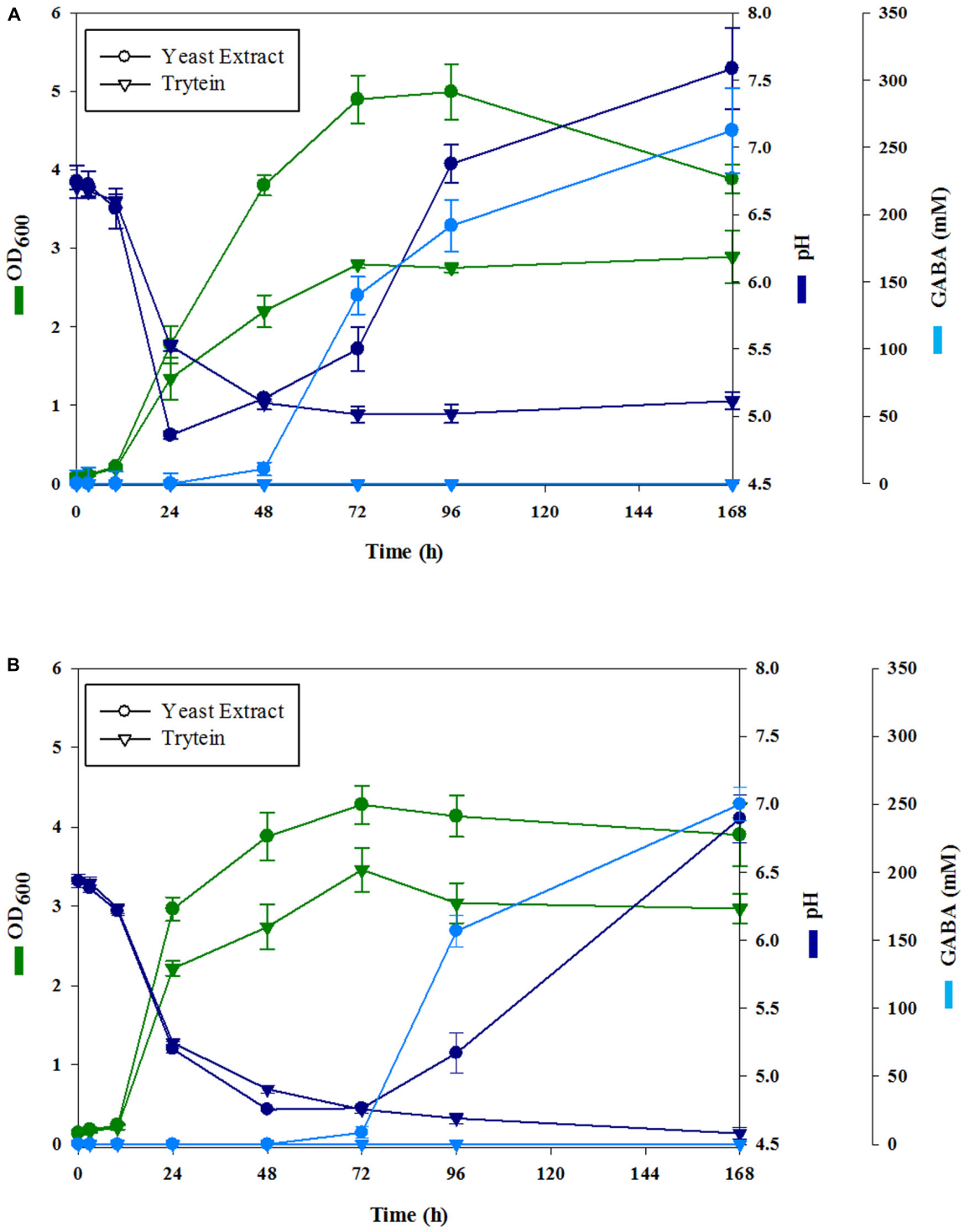

FIGURE 1 | Cell growth (green lines), pH (blue lines), and GABA production (cyan lines) by *L. brevis* CRL 2013 in MSG strawberry **(A)** and blueberry **(B)** juices supplemented with yeast extract (●) or tryptein (▼) at an initial pH of 6.5. Cell growth is expressed as ln x/x0, where x0 is initial biomass, and x is biomass at the indicated time. All values are means ± standard deviations from at least three separate experiments.

fermenting strawberry juice supplemented with YE for 168 h, being GABA yield significantly higher in strawberry juices than in the blueberry ones. No interference by other compounds was found during GABA determinations and GABA was not detected in any of the intact juices without MSG supplementation. Based on these results, we selected the GABA-enriched fermented

strawberry juice (FSJ) for 72 h as a model for further functional studies. This juice contains about 2.7×10^7 CFU/ml and a GABA concentration of around 140 mM, which would be compatible with the doses reported by Taranukhin et al. (2017) to carry out functional assays.

Anti-inflammatory Effect of GABA-Enriched FSJ on Mouse Macrophages

We aimed to evaluate *in vitro* whether the GABA-enriched FSJ was able to modulate the response of macrophages to TRL4 activation. For this purpose, we first studied whether the GABA-enriched FSJ was able to exert any detrimental effect on RAW 264.7 macrophages by evaluating their viability (**Supplementary Figure 1**). Then, mouse macrophages were stimulated with GABA-enriched FSJ that was diluted until reaching a GABA concentration equal to 1 mM. This final amount of GABA was selected considering that this concentration was the optimal to modulate the expression of inflammatory factors in RAW 264.7 cells (Han et al., 2007). In addition, NFSJ as well as stimulations with 0.1 and 1.0 mM GABA were used for comparisons. All the treatments yielded survival percentages around 82% (**Supplementary Figure 1**), which allowed the use of these samples for pro-inflammatory marker expression studies. No significant differences were observed in the viability of macrophages after their treatment with both fermented and non-fermented juices or GABA alone.

The potential anti-inflammatory properties of the GABA-enriched FSJ were assessed through the evaluation of the relative expression of a key factor that is up-regulated in response of TLR4 activation: COX-2 (Lee et al., 2003). As expected, control macrophages stimulated with LPS significantly increased the expression levels of *cox-2* (**Figure 2**). The treatment of macrophages with 0.1 mM GABA did not induce remarkable effects in the response of these cells to the activation of TLR4, however, when GABA was used at a concentration of 1 mM, a significant reduction of *cox-2* transcripts was observed compared to LPS-challenged control cells (**Figure 2**). Similarly, mouse macrophages treated with GABA-enriched FSJ had significantly lower levels of *cox-2* than LPS-challenged controls. Moreover, the expression of *cox-2* in macrophages treated with GABA-enriched FSJ was not different from that found in cells stimulated with 1 mM GABA. No significant differences in the expression of *cox-2* were observed when control macrophages and cells treated with NFSJ were compared (**Figure 2**). These results indicated that the GABA-enriched FSJ obtained after fermentation with the *L. brevis* CRL 2013 strain has the potential to beneficially modulate the TLR4-mediated inflammation.

Effect of GABA-Enriched FSJ in Mice Cytokine's Profile

We next aimed to evaluate *in vivo* the effect of the GABA-enriched FSJ. For this purpose, mice were treated with the GABA-enriched FSJ or the diluted GABA-enriched FSJ (1:2) during 3 days. In addition, to assess the potential intrinsic immunomodulatory effect of the bacterial strain, a group of animals treated only with *L. brevis* CRL 2013 was included. Animals treated with NFSJ or NFSJ supplemented with YE were also used for comparisons. When the levels of the pro-inflammatory factors TNF-α, IL-6 and CXCL1 were evaluated in the peritoneal cavity after treatments with GABA-enriched FSJ, diluted GABA-enriched FSJ or *L. brevis* CRL 2013 no significant differences were found between these groups or the untreated control mice (**Figure 3**). Similarly, no differences in the levels of intestinal and serum TNF-α were detected when GABA-enriched FSJ- or CRL 2013-treated mice were compared to controls (**Figure 4**). Of note, mice receiving GABA-enriched FSJ or *L. brevis* CRL 2013 had higher levels of peritoneal IFN-γ and IL-10 than control animals, an effect that was not observed in mice receiving the diluted GABA-enriched FSJ (**Figure 3**). In addition, mice treated with GABA-enriched FSJ or *L. brevis* CRL 2013 had higher levels of intestinal IFN-γ and IL-10 than control animals (**Figure 4**). The CRL 2013 strain was more efficient than the GABA-enriched FSJ and the diluted FSJ to increase the intestinal IFN-γ. Interestingly, the GABA-enriched FSJ was more efficient than *L. brevis* CRL 2013 and the diluted FSJ to increase the intestinal IL-10. Only *L. brevis* CRL 2013 was able to increase the levels of serum IFN-γ when compared to controls (**Figure 4**). In addition, although the treatments with CRL 2013 and GABA containing juices increased the levels of serum IL-10, the treatment with non-diluted GABA-enriched FSJ was more efficient to induce this effect (**Figure 4**). Animals treated with NFSJ or NFSJ supplemented with YE had values of peritoneal (**Figure 3**), intestinal and serum cytokines (**Figure 4**) that were not different from untreated control mice.

Anti-inflammatory Effect of GABA-Enriched FSJ in Mice

Finally, we aimed to confirm our *in vitro* findings by evaluating *in vivo* the anti-inflammatory effects of GABA-enriched FSJ in the context of TRL4 activation. Then, animals received the five treatments described before and on the day 4 they were challenged with an intraperitoneal injection of LPS. The activation of TLR4 significantly increased levels of the pro-inflammatory factors TNF-α, IL-6 and CXCL1 as well as IFN-γ and IL-10 in the peritoneal cavity (**Figure 5**). Mice receiving the GABA-enriched FSJ showed the most remarkable differences in the peritoneal cytokines 'profile. This group of mice had significantly lower levels of TNF-α, IL-6 and CXCL1 and higher concentrations of IFN-γ and IL-10 when compared to LPS-challenged controls. The treatment with diluted GABA-enriched FSJ was also capable of reducing the levels of inflammatory factors and slightly increases IL-10; however, it did not achieve the effect induced by the non-diluted GABA-enriched FSJ (**Figure 5**). Of note, mice treated with *L. brevis* CRL 2013 had reduced levels of TNF-α and CXCL1 that were similar to those found in the diluted GABA-enriched FSJ group. The CRL 2013 strain was not able to modify the levels of IL-6 when compared to controls. In addition, mice treated with *L. brevis* CRL 2013 had increased levels peritoneal IFN-γ and IL-10. Whereas the levels of IFN-γ were similar to those found in the GABA-enriched FSJ group, the levels of IL-10 were significantly lower (**Figure 5**).

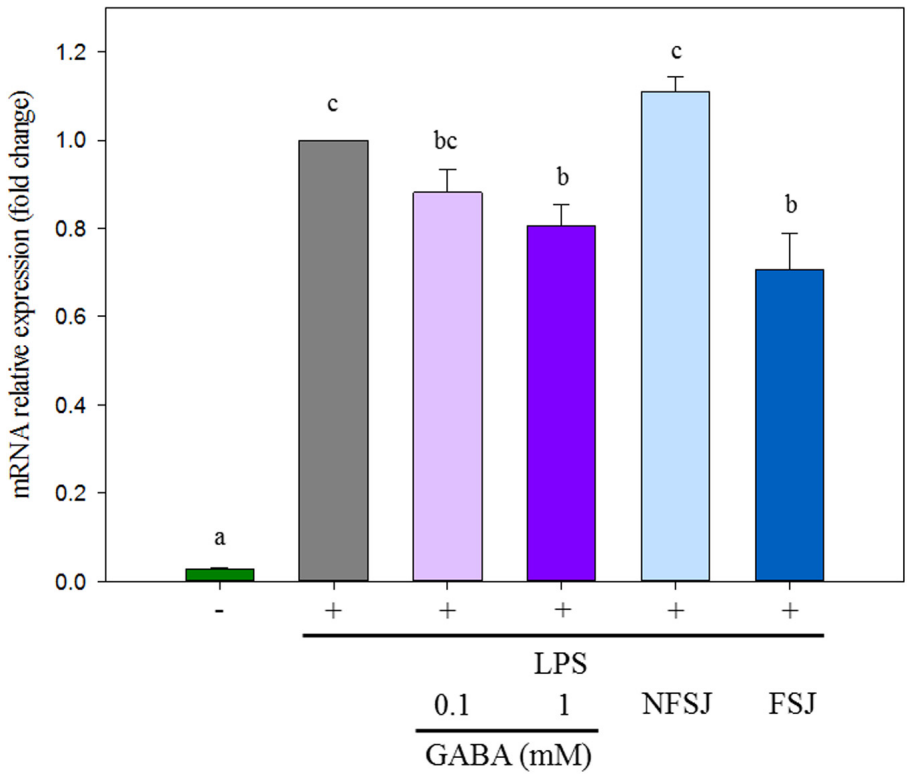

FIGURE 2 | Effects of GABA, non-fermented strawberry juice (NFSJ) and GABA-enriched fermented strawberry juice (FSJ) on *cox-2* expression in RAW 264.7 cells stimulated by LPS. After treatment with the indicated compounds for 1 h, cells were stimulated for 4 h with LPS (1 μg/ml). Total RNA was isolated, and then RT-qPCR was used to measure the mRNA levels of *cox-2* with β-*actin* expression as an internal control. Data represent means ± standard error of three independent experiments. Means for each bar without a common letter differ significantly. Significance with Tukey's HSD *post hoc* test following a one-way ANOVA is indicated as $p < 0.05$.

In addition, the GABA-enriched FSJ was able to induce a significant reduction of intestinal and serum TNF-α, and to increase IFN-γ and IL-10 when compared to LPS-challenged controls (**Figure 6**). The treatments with *L. brevis* CRL 2013 and diluted GABA-enriched FSJ were also capable of increasing and reducing the levels of IL-10 and TNF-α, respectively. However, the concentrations of both cytokines in those groups did not reach the values found in the non-diluted FSJ group (**Figure 6**). On the other hand, the treatment of mice with the CRL 2013 strain induced a similar increase of intestinal and serum IFN-γ than the observed in the GABA-enriched FSJ (**Figure 6**). Animals treated with NFSJ or NFSJ supplemented with YE had values of peritoneal (**Figure 5**), intestinal and serum cytokines (**Figure 6**) that were not different from control mice in the context of TLR4-induced inflammation (**Figure 5**).

The histopathological examination of the intestinal tissue revealed that LPS administration was capable of increasing the damage score from 4 (normal score) up to ∼14 (**Figure 7**). Indeed, the LPS challenge induced intestinal edema, epithelial injury, infiltration of inflammatory cells and a moderate degranulation of Paneth cells. The treatments with non-diluted and diluted GABA-enriched FSJ as well as with *L. brevis* CRL 2013 significantly reduced the scores of intestinal injury. Of note, although there were not statistical significant differences between the score of these three groups, mice receiving the non-diluted GABA-enriched FSJ had a tendency to lower infiltration of inflammatory cells in the intestinal mucosa. Animals treated with NFSJ or NFSJ supplemented with YE had values of intestinal injury scores that were not different from control mice (**Figure 7**).

DISCUSSION

Gamma-aminobutyric acid is a non-protein amino acid that has been extensively described as a health-promoting functional compound (Diana et al., 2014; Quílez and Diana, 2017). Considering that berries contain powerful antioxidants and other functional compounds, biotechnological approaches are being currently used to increase the content of specific health-related compounds in these fruits and its derivatives (Battino et al., 2009). Since LAB are currently the most interesting group of microorganisms capable of producing GABA at high yields, there is an opportunity to isolate and identify GABA producing strains to be used as starter cultures in the design and development of novel functional fermented foods or as probiotics. In this regard, based on previous screenings performed in our laboratory (Cataldo et al., 2020), *L. brevis* CRL 2013 was selected as the

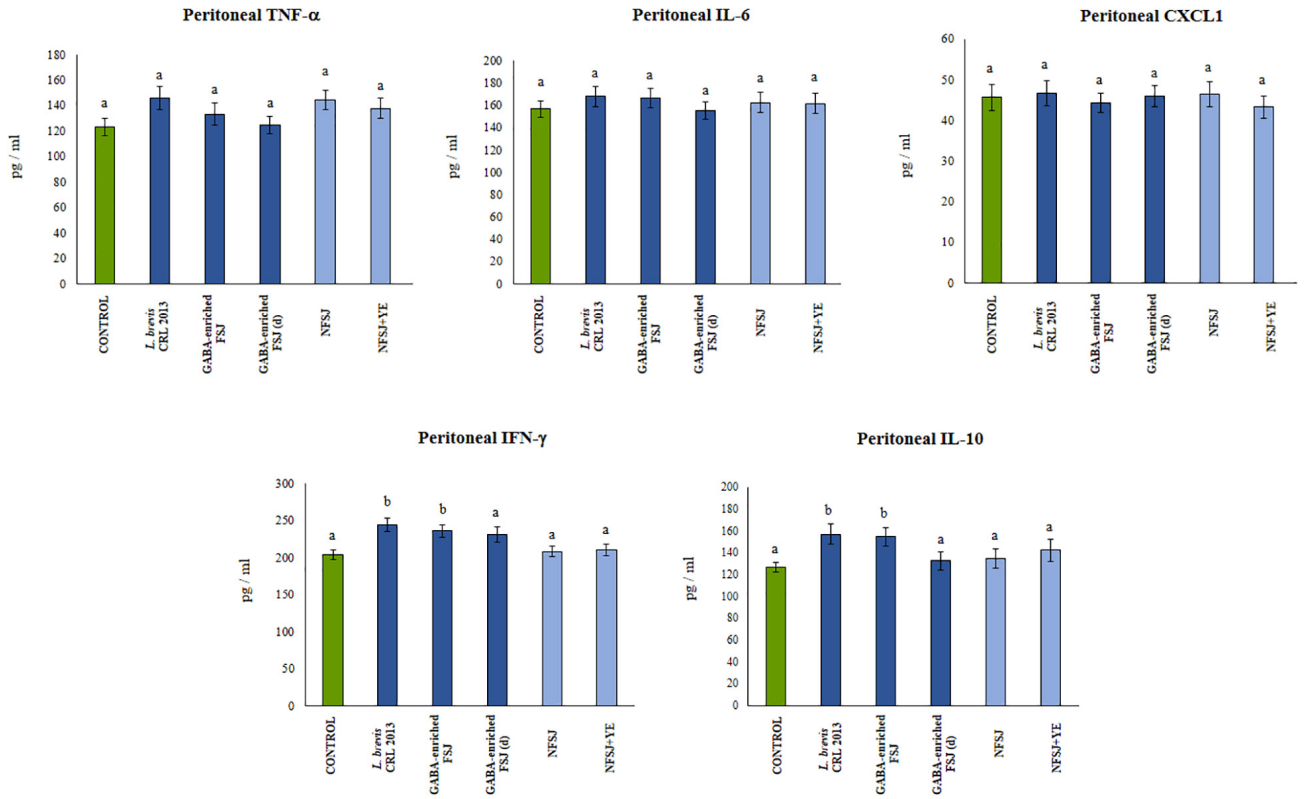

FIGURE 3 | Effect of *L. brevis* CRL 2013, GABA-enriched strawberry fermented juice (FSJ) and diluted (d) GABA-enriched FSJ on peritoneal cytokines and chemokines in Balb/c mice. Animals treated with NFSJ or NFSJ supplemented with YE were used for comparisons. Results are expressed as mean ± SD. Means for each bar without a common letter differ significantly. Significance with Tukey's HSD *post hoc* test following a one-way ANOVA is indicated as $p < 0.05$.

most appropriate GABA-producing LAB for the manufacture of a functional fermented beverage.

Since lactic acid fermentation has a remarkable role in improving the intrinsic functional and nutritional properties of many vegetable food matrixes (Di Cagno et al., 2016) and due to its well-known positive effects in traditional fermented foods, in this work both blueberry and strawberry juices were subjected to a process of lactic fermentation by *L. brevis* CRL 2013. The acidification rate during both strawberry and blueberry fermentation was similar to that observed by Kim et al. (2009) when fermenting a black raspberry juice with *L. brevis* GABA 100. In order to set the vegetable matrix for the fermentation, additional sources of nitrogen were added to the juices, an alternative also used by Di Cagno et al. (2010) when fermenting a grape must. A survey of the genome of CRL 2013 strain (Accession number: NZ_MZMW00000000.1) confirms that *L. brevis* CRL 2013 lacks the gene encoding cell envelope associated proteinase. Since *L. brevis* CRL 2013 is a non-proteolytic strain, the growth recovery was probably due to the presence of several free amino acids and peptides in both YE and tryptein, which are essential for bacterial growth (Hebert et al., 2001). Although GABA synthesis began earlier in the strawberry juice, it continued to progress up to 168 h in both juices, reaching a maximum GABA level of 262 mM GABA. In this sense, GABA in raspberry juices fermented by *L. brevis* GABA 100 at 30°C reached the maximum levels on the 12th day (Kim et al., 2009). To our knowledge, this is the first report regarding the GABA-enrichment of blueberry and strawberry juices by lactic acid fermentation.

The functional *in vitro* and *in vivo* studies performed in this work allow us to reach three important conclusions: (a) the GABA-enriched FSJ produced with *L. brevis* CRL 2013 is able to differentially modulate the inflammatory response induced by the activation of TLR4; (b) the immunoregulatory effect induced by the GABA-enriched FSJ depends on its concentration, and (c) the intrinsic immunomodulatory properties of the CRL 2013 strain may contribute to the modulatory effect of the GABA-enriched FSJ.

In our hands, the GABA-enriched FSJ was capable of reducing the expression of the inflammatory marker *cox-2* in RAW macrophages activated by TLR4 stimulation. In line with our results, it was reported previously that GABA is capable of inhibiting immune cells activation by modulating NF-kB pathway, thus diminishing the production of inflammatory mediators such as TNF-α, COX-2 and iNOS (Jin et al., 2013; Prud'homme et al., 2015). In addition, the ability of GABA to inhibit the *in vitro* release of IL-6 and IL-12 by LPS-stimulated peritoneal macrophages was also reported by Reyes-García et al. (2007). Besides, it was shown that a GABA-enriched pepino extract obtained after fermentation by *L. brevis* BCRC 12310 was

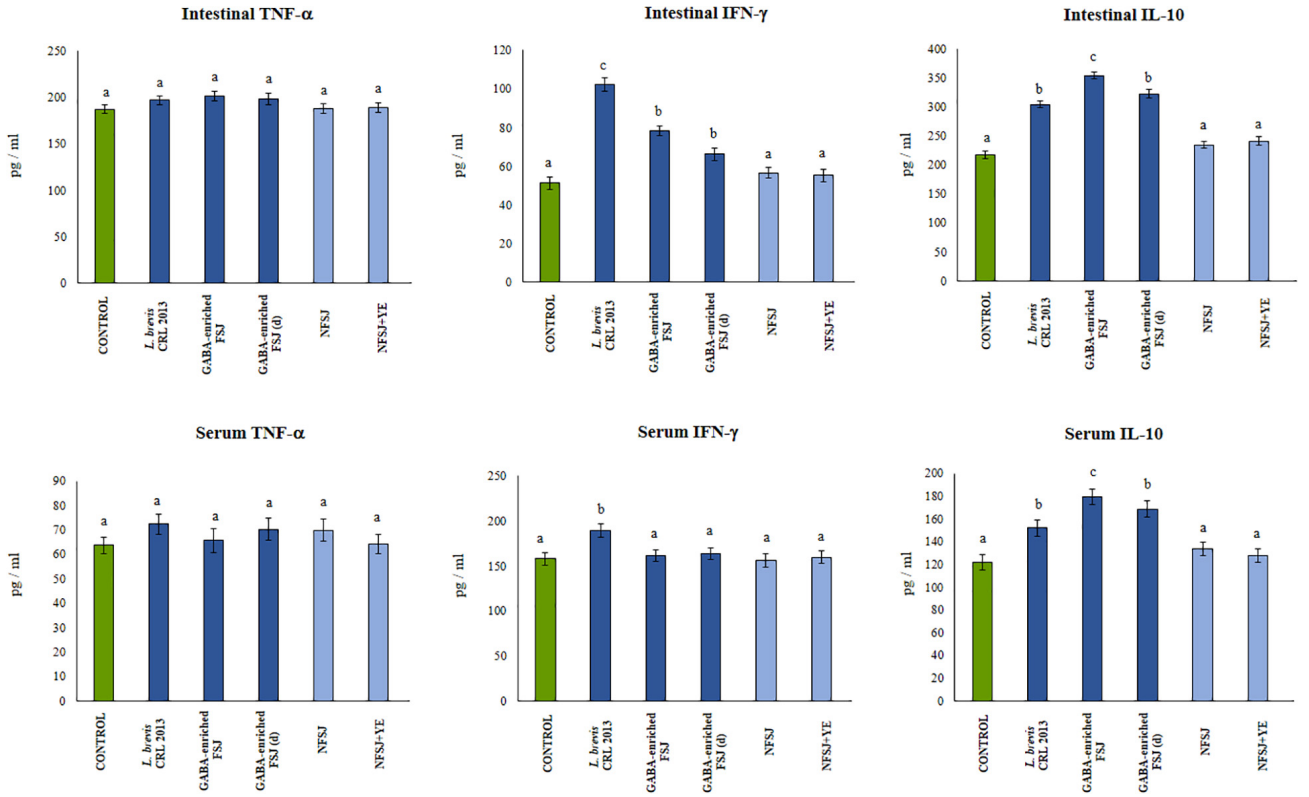

FIGURE 4 | Effect of *L. brevis* CRL 2013, GABA-enriched strawberry fermented juice (FSJ) and diluted (d) GABA-enriched FSJ on intestinal and serum cytokines and chemokines in mice. Animals treated with NFSJ or NFSJ supplemented with YE were used for comparisons. Results are expressed as mean ± SD. Means for each bar without a common letter differ significantly. Significance with Tukey's HSD *post hoc* test following a one-way ANOVA is indicated as $p < 0.05$.

able to inhibit the expression of TNF-α in LPS-induced RAW 264.7 macrophages (Chang et al., 2016).

To the best of our knowledge, the immunoregulatory effects of GABA-enriched products after fermentation with lactobacilli have not been studied in depth within *in vivo* models. For this reason, we also aimed in this work to demonstrate *in vivo* the potential immunomodulatory effect of the GABA-enriched FSJ obtained after the fermentation with the CRL 2013 strain. We were able to demonstrate that the oral treatment with the GABA-enriched FSJ was capable of modulating the inflammatory response triggered by the activation of TRL4 at the intestinal mucosa, in the peritoneal cavity and at the systemic level. The GABA-enriched FSJ reduced the production of TNF-α, IL-6 and CXCL1 as well as improved the levels of IL-10 in the three body compartments analyzed (**Figure 8**). Activation of TLR in macrophages after the binding of its ligand induce a signaling cascade that culminates in the expression and secretion of various cytokines, chemokines, and other inflammatory factors which signal and prime neighboring immune cells. Although necessary to confer protection against invading pathogens, the TLR-mediated inflammation needs to be tightly controlled at mucosal surfaces such as the intestinal tissue to avoid exaggerated inflammatory responses and damage. Then, we speculated that the differential cytokine profile induced by the GABA-enriched FSJ in the intestinal mucosa after TLR4 activation would be capable of protecting against the inflammatory damage. The histological studies performed here, demonstrated that this statement was correct since a significantly lower intestinal damage score, mainly related to a lower infiltration of inflammatory cells, was observed in mice treated with the GABA-enriched FSJ when compared to controls.

Interestingly, it has been shown that GABA-enriched products obtained by fermentation with lactobacilli can modulate not only the immune response mediated by macrophages but also by other immune cells such as lymphocytes. In this regard, it was recently reported that live bacteria-free supernatant collected from the GABA-producing *L. brevis* BGZLS10-17 is able to differentially modulate the proliferation, the MHCII and CD80 expression and the production of IFN-γ and IL-17 in concanavalin A-stimulated mesenteric lymph node lymphocytes (Bajić et al., 2020). Furthermore, GABA-containing supernatants were able to increase the expression of immunoregulatory molecules Foxp3, IL-10, and TGF-β in immune cells. These *in vitro* findings correlated with the *in vivo* ability of the GABA producing BGZLS10-17 strain to protect against inflammatory-induced destruction of intestinal barrier in an experimental autoimmune encephalomyelitis model (Sokovic Bajic et al., 2019). These results indicate that it would be of great importance to study the immunomodulatory effect of the CRL 2013-derived GABA-enriched FSJ in the context of other inflammatory challenges, to

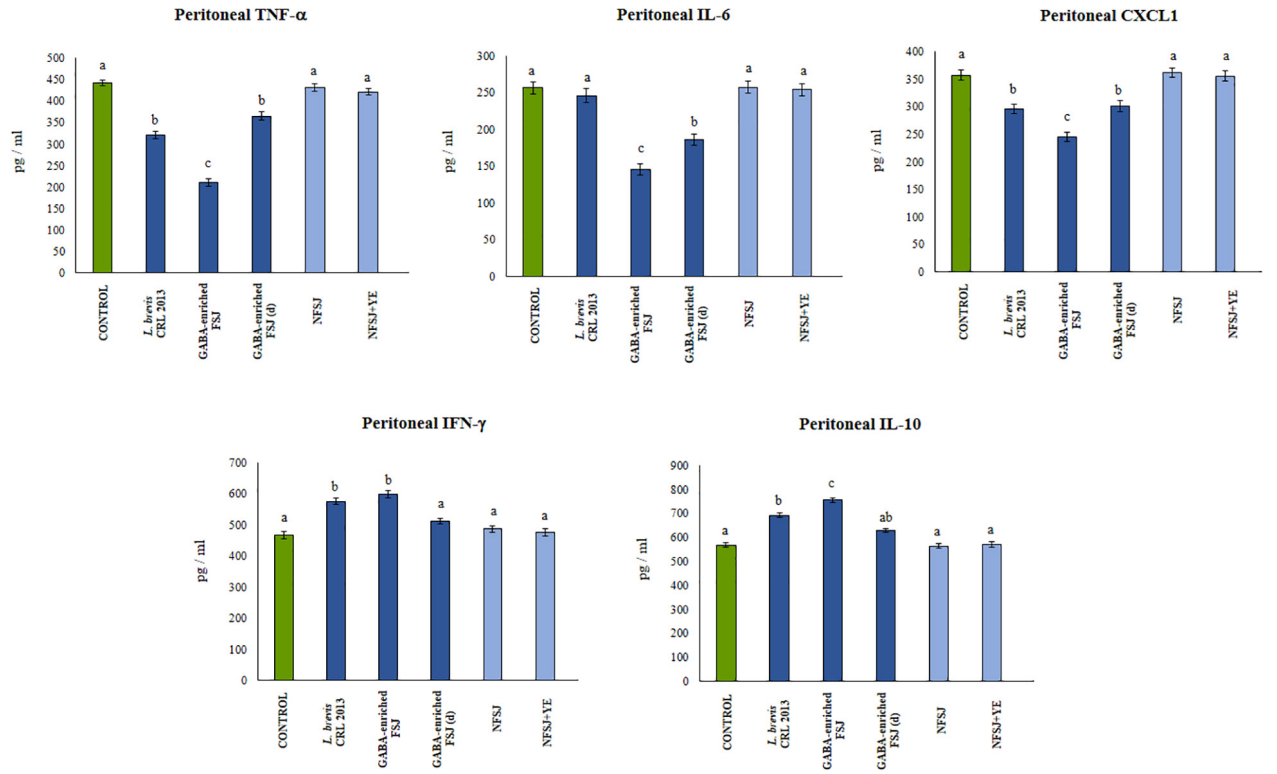

FIGURE 5 | Effect of *L. brevis* CRL 2013, GABA-enriched strawberry fermented juice (FSJ) and diluted (d) GABA-enriched FSJ on peritoneal cytokines and chemokines in mice challenged with LPS. Animals treated with NFSJ or NFSJ supplemented with YE were used for comparisons. Results are expressed as mean ± SD. Means for each bar without a common letter differ significantly. Significance with Tukey's HSD *post hoc* test following a one-way ANOVA is indicated as $p < 0.05$.

know its potential to be applied in the prevention or treatment of autoimmune and inflammatory diseases.

In the present study, we also observed a correlation between GABA concentration and the degree of repression of *cox-2* in murine macrophages activated by TLR4 stimulation; 1 mM GABA pretreatment presented inhibitory influence whereas 0.1 mM GABA was not enough concentration to achieve a significant inhibition. This dose-dependent inhibition is in agreement with a previous result in which 1 mM GABA was the optimal concentration to reach the highest inhibition on the expression of TNF-α and iNOS in LPS-induced RAW 264.7 cells (Han et al., 2007). Furthermore, these *in vitro* results are in line with the *in vivo* studies performed here that demonstrated the reduced ability of the diluted GABA-enriched FSJ to modulate the inflammatory response triggered by TLR4 activation in mice when compared to non-diluted GABA-enriched FSJ. It should be noted that another possibility for the lower ability of the diluted GABA-enriched FSJ to modulate the innate immune response could be related to the dilution of the lactobacilli dose. It would be interesting to study in the future the effect of different doses of *L. brevis* CRL 2013 and concentrations of GABA-enriched FSJ to modulate the TLR4-triggered inflammation to find the optimal doses that exert their beneficial effects.

Interestingly, the oral administration of *L. brevis* CRL 2013 to mice was able to increase the intestinal, peritoneal and serum levels of IFN-γ and IL-10. Several studies have reported the beneficial effects of immunomodulatory lactobacilli and have highlighted that the most remarkable effect of lactobacilli on the intestinal cytokine profile is the increase of TNF-α, IFN-γ, and IL-10 (Salva et al., 2010; Marranzino et al., 2012; Villena et al., 2012). Moreover, it is considered that through the release of intestinal TNF-α and IFN-γ, immune enhancing lactobacilli are capable of stimulating the activities of peritoneal macrophages. In this regard, we have shown that the oral administration of the highly immune enhancing strains *Lacticaseibacillus casei* CRL431 (formerly known as *Lactobacillus casei* CRL431), *Lactiplantibacillus plantarum* CRL1506 (formerly known as *Lactobacillus plantarum* CRL1506), and *Lacticaseibacillus rhamnosus* CRL1505 (formerly known as *Lactobacillus rhamnosus* CRL1505) increase the production of TNF-α, IFN-γ, and IL-10 in the intestine after their oral administration (Salva et al., 2010; Marranzino et al., 2012; Villena et al., 2012). More recently, we observed that immunomodulatory strains such as *Limosilactobacillus fermentum* UCO-979C (formerly known as *Lactobacillu fermentum* UCO-979C) significantly improved the production of intestinal IFN-γ, and IL-10 but not of TNF-α (Garcia-Castillo et al., 2019). This probiotic strain is not a highly stimulating strain of the immune system, but rather behaves like an immunoregulatory strain, moderately stimulating effector responses and inducing anti-inflammatory responses.

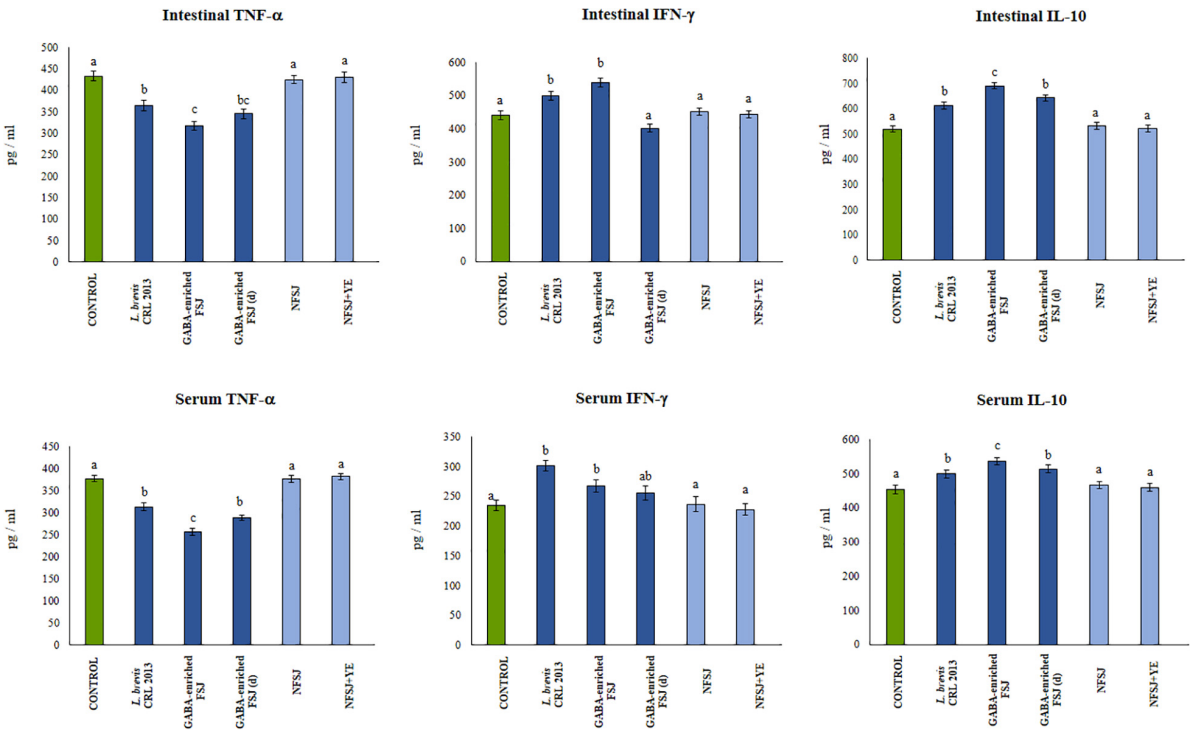

FIGURE 6 | Effect of *L. brevis* CRL 2013, GABA-enriched strawberry fermented juice (FSJ) and diluted (d) GABA-enriched FSJ on intestinal and serum cytokines and chemokines in mice challenged with LPS. Animals treated with NFSJ or NFSJ supplemented with YE were used for comparisons. Results are expressed as mean ± SD. Means for each bar without a common letter differ significantly. Significance with Tukey's HSD *post hoc* test following a one-way ANOVA is indicated as $p < 0.05$.

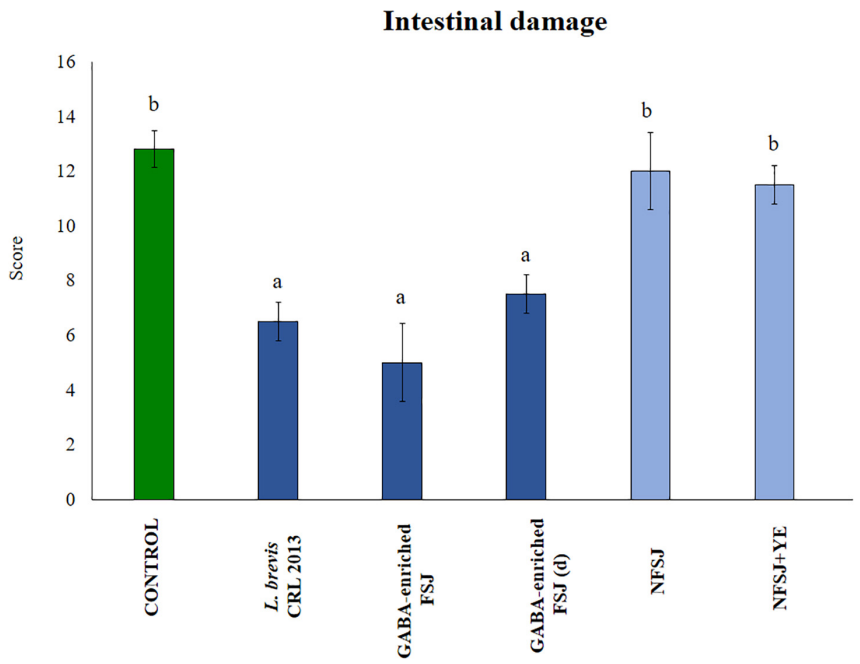

FIGURE 7 | Effect of *L. brevis* CRL 2013, GABA-enriched strawberry fermented juice (FSJ) and diluted (d) GABA-enriched FSJ on intestinal damage in mice challenged with LPS. Animals treated with NFSJ or NFSJ supplemented with YE were used for comparisons. Score values were calculated considering the presence/absence and intensity of edema, epithelial injury, degranulation of Paneth cells and inflammation. Results are expressed as mean ± SD. Means for each bar without a common letter differ significantly. Significance with Tukey's HSD *post hoc* test following a one-way ANOVA is indicated as $p < 0.05$.

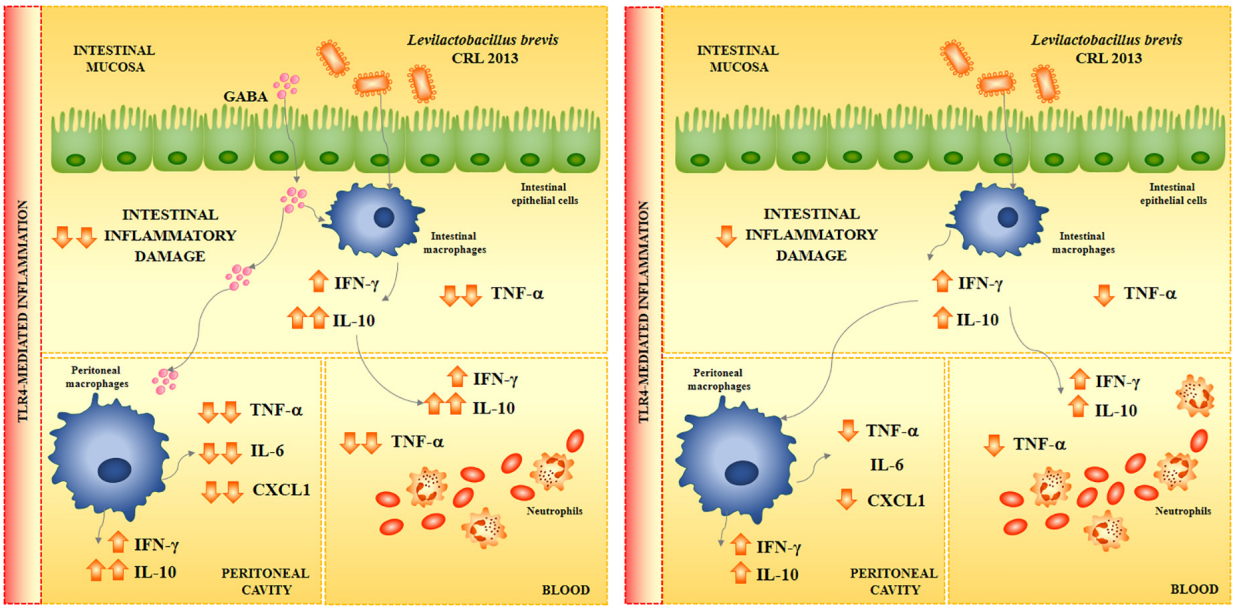

FIGURE 8 | Proposed mechanism for the immunomodulatory effect of *Levilactobacillus brevis* CRL 2013 and GABA in the immune response triggered by the activation of TLR4.

The results obtained in this work, allow concluding that the CRL 2013 strain belongs to this group of lactic acid bacteria. In fact, *L. brevis* CRL 2013-treated mice were able to differentially regulate the response to TLR4 by reducing the production of TNF-α and CXCL1, and further enhancing IL-10 (**Figure 8**).

Some studies have described immune enhancing *L. brevis* strains. The treatment of macrophages with *L. brevis* KCTC 12777BP increased their phagocytic activity and the production of TNF-α, IL-6, and nitric oxide (Jeong et al., 2020). *L. brevis* ZLB004 was capable of increasing IFN-γ concentration enhancing the immune health status of weaned pigs (Liu et al., 2015) while *L. brevis* ATCC 8287 reduced TGF-β1 and increased IL-6 expressions in the small intestine of pigs (Lähteinen et al., 2014). However, no strains of *L. brevis* have been described to possess a mixed stimulatory/anti-inflammatory profile. Then, our results raise the question of whether *L. brevis* CRL 2013 could be used by itself as an immunomodulatory probiotic strain to exert beneficial effects in other immune-related diseases. It should be noted that the intrinsic anti-inflammatory activity of the CRL 2013 strain was increased when it was administered together with GABA. However, it is possible to rule out the effect of GABA in the bacteria administered alone, since the concentrations of GABA that this bacterium can produce *in situ* in the intestinal mucosa are far below those necessary to obtain the immunomodulatory effect, as demonstrated by our comparative studies using non-diluted and diluted GABA-enriched FSJ. Then, it would also be of great value to investigate which bacterial molecule(s) are responsible for the intrinsic immunomodulatory effect observed in the CRL 2013 strain.

In conclusion, *L. brevis* CRL 2013, a major GABA producer among the strains evaluated in our laboratory, was used for the formulation of a GABA-enriched fermented berry juice. Considering that a significantly higher GABA yield was observed in fermented strawberry than in fermented blueberry juice, the former one was selected for further studies. The highest GABA production was obtained by fermenting the YE-supplemented strawberry juice with *L. brevis* CRL 2013 as starter culture. The GABA-enriched strawberry juice modulated the expression of *cox-2* in LPS stimulated RAW 264.7 macrophages and exerted a remarkable anti-inflammatory effect *in vivo* in the context of TLR4 activation. Our observations strikingly support the potential of GABA and GABA-enriched strawberry fermented juices as promising functional foods to help ameliorate the exacerbated inflammatory response of chronic inflammatory diseases in addition to the other well-known GABA positive properties such as diuretic, antihypertensive, hypoglycemic and antidepressant compound. To our knowledge, this is the first report of a bio-enriched fermented strawberry juice capable of positively modulating the TLR4-mediated inflammatory response.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by The CERELA-CONICET Institutional Animal Care and Use Committee

prospectively approved this research under the protocol BIOT-CRL-17.

AUTHOR CONTRIBUTIONS

PC conducted most of the experiments, analyzed the results, and wrote the manuscript. EH conceived the idea for the project. LS coordinated and contributed in cell culture and RT-PCR assays. JV, LS, and EH coordinated the study and wrote the manuscript. GS and ME contributed to the discussion of the manuscript. All authors read and approved the final 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.2020.610016/full#supplementary-material

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REVIEW
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Lactic Acid Bacteria in Wine: Technological Advances and Evaluation of Their Functional Role

*Carla Virdis[1], Krista Sumby[1,2], Eveline Bartowsky[2,3] and Vladimir Jiranek[1,2]**

[1] Department of Wine Science, University of Adelaide, Urrbrae, SA, Australia, [2] Australian Research Council Training Centre for Innovative Wine Production, Urrbrae, SA, Australia, [3] Lallemand Australia, Edwardstown, SA, Australia

Currently, the main role of Lactic Acid Bacteria (LAB) in wine is to conduct the malolactic fermentation (MLF). This process can increase wine aroma and mouthfeel, improve microbial stability and reduce the acidity of wine. A growing number of studies support the appreciation that LAB can also significantly, positively and negatively, contribute to the sensorial profile of wine through many different enzymatic pathways. This is achieved either through the synthesis of compounds such as diacetyl and esters or by liberating bound aroma compounds such as glycoside-bound primary aromas and volatile thiols which are odorless in their bound form. LAB can also liberate hydroxycinnamic acids from their tartaric esters and have the potential to break down anthocyanin glucosides, thus impacting wine color. LAB can also produce enzymes with the potential to help in the winemaking process and contribute to stabilizing the final product. For example, LAB exhibit peptidolytic and proteolytic activity that could break down the proteins causing wine haze, potentially reducing the need for bentonite addition. Other potential contributions include pectinolytic activity, which could aid juice clarification and the ability to break down acetaldehyde, even when bound to SO_2, reducing the need for SO_2 additions during winemaking. Considering all these findings, this review summarizes the novel enzymatic activities of LAB that positively or negatively affect the quality of wine. Inoculation strategies, LAB improvement strategies, their potential to be used as targeted additions, and technological advances involving their use in wine are highlighted along with suggestions for future research.

Keywords: MLF, LAB, wine aroma, quality, enzymes, management

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***Correspondence:**
Vladimir Jiranek
vladimir.jiranek@adelaide.edu.au

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INTRODUCTION

The fruit of the grapevine, *Vitis vinifera*, was first transformed into wine sometime between 8500BC and 4000BC (Varriano, 2010). However, wine, as we know it nowadays, is a complex beverage in which many key elements shape the final product. These key elements include the quality of the grapes and their varietal and clonal genotype, the yeasts and bacteria conducting the alcoholic fermentation (AF) and the malolactic fermentation (MLF), respectively, the aging vessels and the winemaking techniques (Styger et al., 2011).

The main role of lactic acid bacteria (LAB) in wine has traditionally been to perform the conversion of malic acid to lactic acid. In the last decades, various papers have shown that LAB metabolism also involves a large array of secondary enzymatic activities capable of generating many

volatile secondary compounds (Matthews et al., 2004; Sumby et al., 2010; Bartowsky and Borneman, 2011; Michlmayr et al., 2012; Cappello et al., 2017; Takase et al., 2018).

Although it is evident that wines originating from a specific grape variety display particular characters that distinguish them from other varieties, in many cases these active-flavor compounds are not detectable at pre-fermentative stages (Swiegers et al., 2005). Often, they are instead the product of the metabolism of yeast and bacteria and are modified and released in wine during the fermentation processes. Enzymes from LAB that can exert their activity in wine include glycosidases, esterases, proteases and others (Liu, 2002). The activity of these enzymes can significantly add to the appearance, flavor, texture and aroma of wine, ultimately, helping to define its structure (Swiegers et al., 2005).

In this review we examine the current literature with regards to the functional role of LAB in winemaking (**Figure 1**) including; inoculation strategies, modification of wine aroma, impact on wine color, potential novel uses to aid winemaking processes, and their overall effect on the wholesomeness of wine. Finally, we discuss current limitations and future prospects.

THE ROLE OF LACTIC ACID BACTERIA IN WINEMAKING

The Malolactic Fermentation

Once the primary fermentation has finished, or simultaneously in some cases, most red, some white and sparkling wines undergo a secondary microbial fermentation. During MLF, LAB convert the dicarboxylic malic acid into the monocarboxylic lactic acid and carbon dioxide (**Figure 1**) with no free intermediary products (Ribéreau-Gayon et al., 2006a). MLF typically achieves a more palatable wine by reducing the tart taste of malic acid. Additionally, MLF reduces the amount of residual nutrients able to support microbial growth making the wine more stable prior to being bottled (Lonvaud-Funel, 1999).

Several factors affect the performance of LAB in wine, their growth, their ability to complete MLF and their metabolic properties (Sumby et al., 2019). Some known limiting factors are high ethanol content, low pH, sub-optimal temperatures and high SO_2, however, their inhibiting effect can be potentiated by their synergistic action (Lerm et al., 2010).

Inoculation Strategies

Malolactic fermentation can be facilitated by autochthonous LAB or can be induced by inoculating the wine with selected bacterial starters (**Table 1**). Before the use of MLF starters became a common winemaking practice, a secondary fermentation in wine was often enabled by the microbial populations that originated in the vineyard or winery and were brought to the wine via the grape skin or the winery vessels (Bokulich et al., 2016). Although not common, uninoculated MLF performed by autochthonous LAB is still used in winemaking (**Table 1**), mainly with the aim of producing wines with a regional character or a particular style (Bokulich et al., 2016). However, when MLF is uninoculated and carried out by autochthonous bacteria, the conversion of malic acid into lactic acid can be slow or incomplete, or undesired volatile compounds and potentially hazardous compounds can be produced (Lonvaud-Funel, 1999). The use of bacterial starters can help minimize these risks (Lonvaud-Funel, 1999). Considerable research has been dedicated to identifying robust starters with desirable metabolic activity (Lerm et al., 2011). More recently, efforts have been directed to exploring the biodiversity of wine associated geographic areas, with the aim of finding new LAB starters with a high degree of adaptation to the specific niches (Miranda-Castilleja et al., 2016; López-Seijas et al., 2020). For example, two potential new autochthonous MLF starters with interesting β-glucosidase activity, *Lacticaseibacillus paracasei* (formerly known as *Lactobacillus paracasei*) UVI-2 and *Lentilactobacillus hilgardii* (formerly known as *L. hilgardii*) UVI-23, have been identified from Albariño grapes in Val do Salnés, Spain (López-Seijas et al., 2020). In another study, the isolation of numerous *Lactiplantibacillus plantarum* (formerly known as *Lactobacillus plantarum)* highlighted a great intra-species genetic variability in North Patagonia, Argentina, and suggested also their potential use as MLF starter cultures for regional wines (Bravo-Ferrada et al., 2013). This is especially interesting considering that the regional identity of wines, or terroir, can be an important factor in increasing the value of the final product (Bartowsky et al., 2015).

When selected bacterial starters are used (**Table 1**), the winemaker can opt for a sequential inoculation with yeast, where *Oenococcus oeni* is inoculated at the end of AF, or for a coinoculation strategy, where LAB starters are inoculated simultaneously or shortly (24–48 h) after the beginning of AF (Lucio et al., 2017; Bartle et al., 2019; Sumby et al., 2019). The main advantages of the coinoculation technique, compared to the more traditional sequential inoculation, are the potentially positive contribution to wine composition and the reduction of microbial spoilage risk (Sumby et al., 2019). Reducing the overall AF and MLF time allows the winemaker to protect the wine with SO_2 additions earlier in the winemaking process, thus avoiding the production of volatile phenols from spoilage microorganisms such as *Brettanomyces bruxellensis* (Sumby et al., 2019). During coinoculation, the interactions between yeasts and bacteria may affect the efficiency of MLF and the sensory properties of the final wine (Bartle et al., 2019; Du Plessis et al., 2019). For more information regarding yeast and bacteria interactions, refer to the recent review from Bartle et al. (2019). Lastly, bacteria can be inoculated before yeast. Although not common this technique has proven to be very efficient in promoting MLF, especially when *Liquorilactobacillus mali* (formerly known as *Lactobacillus mali*), *L. paracasei, Liquorilactobacillus satsumensis* (formerly known as *Lactobacillus satsumensis*) *and L. plantarum* strains were used as starters (Lucio et al., 2017). Amongst the above mentioned genera, the best results in terms of growth in juice and malic consumption efficiency were obtained with *L. plantarum* (Lucio et al., 2017).

In recent years, mixed inoculation strategies have also been trialed (**Table 1**). The use of blended cultures of *L. plantarum* and *O. oeni* as MLF starters, can facilitate a rapid consumption of malic acid, whilst contributing significantly to the volatile profile of wine (Brizuela et al., 2018). Preparations comprising mixtures

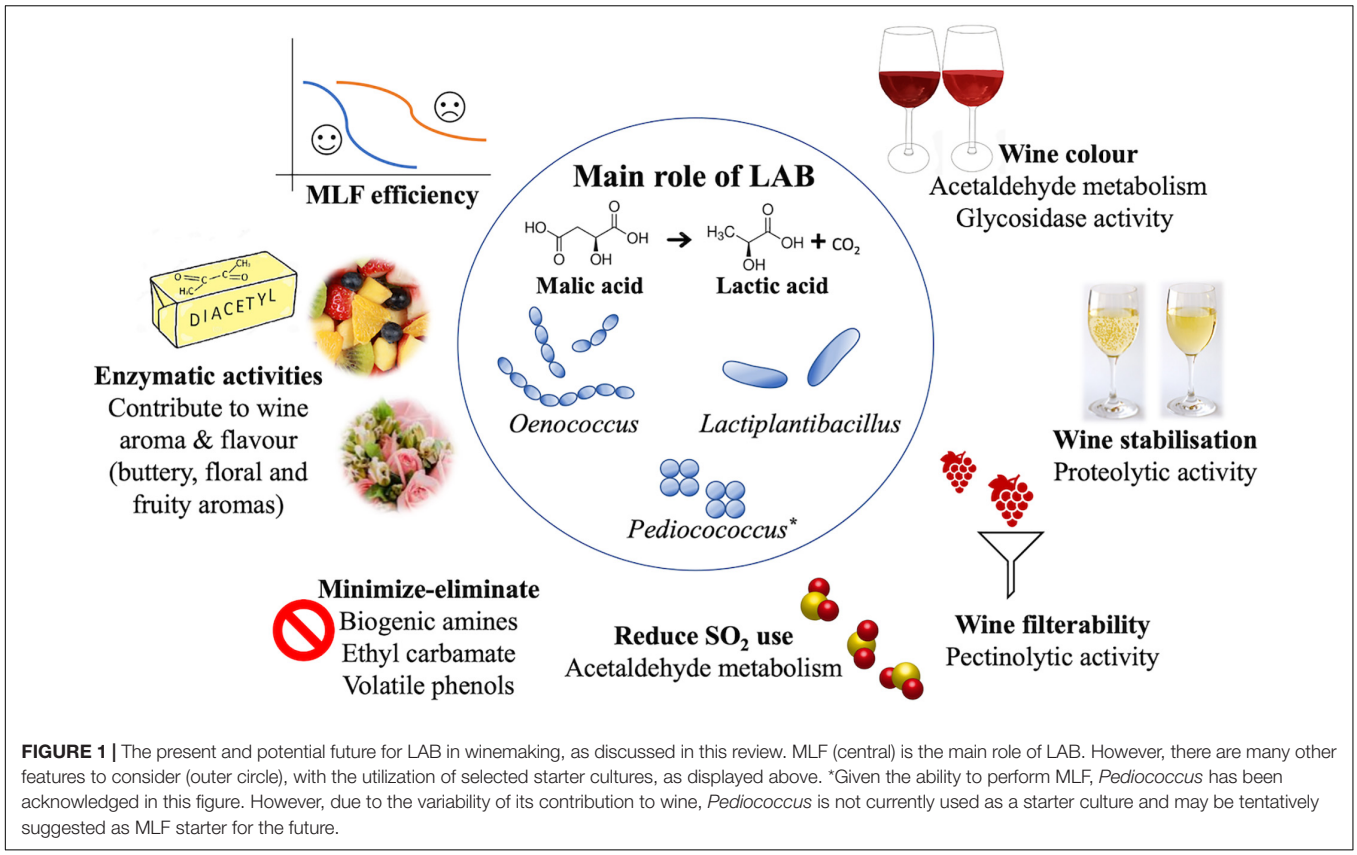

FIGURE 1 | The present and potential future for LAB in winemaking, as discussed in this review. MLF (central) is the main role of LAB. However, there are many other features to consider (outer circle), with the utilization of selected starter cultures, as displayed above. *Given the ability to perform MLF, *Pediococcus* has been acknowledged in this figure. However, due to the variability of its contribution to wine, *Pediococcus* is not currently used as a starter culture and may be tentatively suggested as MLF starter for the future.

TABLE 1 | Overview of the main LAB of enological interest, and their main roles in wine; past, present, and future.

	Strains used	When	Major roles	Other roles	References
Past	• Autochthonous LAB	Not controllable	Softer and more aromatic wine		Bokulich et al., 2016
Present	• Single bacterial starter cultures: *O. oeni* *L. plantarum* • Mixed bacterial starter cultures	During and after AF	Softer mouthfeel, Higher microbiological stability Lower acidity	Production of diacetyl, esters, aromatic thiols and other aromatic compounds, Liberation of glycoside-bound aromatic compounds	Bartowsky and Borneman, 2011; Krieger-Weber et al., 2020
	• Autochthonous LAB	Not controllable	Softer and more aromatic wine	Development of regional character	Bokulich et al., 2016
Future	• Single bacterial starter cultures: *O. oeni* *L. plantarum* *Pediococcus spp.* Other LAB? • Mixed bacterial starter cultures • Autochthonous, LAB starters	Before, during and after AF	Softer mouthfeel, Higher microbiological stability, Lower acidity, Production of diacetyl, esters, aromatic thiols and other aromatic compounds, Liberation of glycoside-bound aromatic compounds	Reduction of Acetaldehyde content in wine, Reduction of biogenic amine content in wine, Protein stability, Juice clarification, Reduction of SO_2 required in winemaking, More properties?	Wade et al., 2018; Iorizzo et al., 2016; Wells and Osborne, 2011; López-Seijas et al., 2020

of *L. plantarum* and *O. oeni*, recommended for coinoculation use, are commercially available (for example Anchor Oenology).

The timing of inoculation leads to different aroma compounds being released in wine, qualitatively and quantitatively modifying wine profiles (Lasik-Kurdyś et al., 2018). However, independently from the inoculation time, the key of the success of MLF seems to be the correct management of the inoculation technique (Lombardi et al., 2020). For example, pre-adapting the bacterial starters to sub-optimal pH (5.0) can improve the consumption of malic acid (Lombardi et al., 2020).

Lactic Acid Bacteria Used in Winemaking

Lactic Acid Bacteria are Gram-positive bacteria, grouped in the phylum Firmicutes, class Bacilli, order Lactobacillales (Holzapfel

et al., 2014). Several genera of the family *Lactobacillaceae* are used in the food industry and are involved in the production of numerous fermented foods, such as yogurt, cheese and sauerkraut (Szutowska, 2020). *Oenococcus, Leuconostoc*, and *Pediococcus*, within the formerly known Leuconostocaceae family, and the formerly known genus *Lactobacillus*, within the formerly known Lactobacillaceae family, are the only genera associated to wine (Holzapfel et al., 2014; Zheng et al., 2020).

Recently the description of LAB has been amended, following a modern multifactorial approach used to re-evaluate the taxonomy of these microorganisms (Zheng et al., 2020). Core genome phylogeny, physiological and metabolic criteria, and the ecology of the organisms are some of the parameters used for this evaluation (Zheng et al., 2020). The formerly known genus *Lactobacillus* has been restructured into 25 new genera and the former *Lactobacillaceae* and *Leuconostocaceae* families have been fused into a new larger *Lactobacillaceae* family (Zheng et al., 2020).

Lactic acid bacteria can be both detrimental and beneficial to the quality of wine (Bartowsky, 2009). Their performance in wine is related to the specific species and strain genetics but also to many other factors including environmental conditions and microbial interactions (Cappello et al., 2017; Devi and Ka, 2019). At present, *O. oeni* is one of the three, and the most known, species in the *Oenococcus* genus (Lorentzen and Lucas, 2019). Due to its high tolerance for low pH, high ethanol concentrations and scarcity of nutrients, *O. oeni* is the main LAB of choice in winemaking (Bartowsky, 2005). However, with increasing temperatures during growth and harvest, and a consequent rising pH trend for many wines, other LAB have the potential to become a valid alternative to *Oenococcus*, playing an important role in the modifications of wine aroma (Du Toit et al., 2010; Mira and de Orduña, 2010; Berbegal et al., 2019; Krieger-Weber et al., 2020; López-Seijas et al., 2020; Shao-Yang et al., 2020; Sun et al., 2020). Above all, *Lactiplantibacillus* strains, with their fast consumption of malic acid (up to 3 g/L in 2–4 days) and the suppression of the activity of other spontaneous LAB populations, are an ideal starter choice for the winemaker (Du Toit et al., 2010; Krieger-Weber et al., 2020). Furthermore, as *L. plantarum* is homofermentative for hexoses it does not produce volatile acidity (VA) through sugar metabolism. Currently, only a few freeze-dried starter cultures of *L. plantarum* are commercially available and their use is especially recommended for coinoculation strategies in wines with high pH (>3.4) and a high risk of autochthonous LAB contamination[1,2].

Lastly, the genus *Pediococcus* is generally considered a spoilage microorganism in wine (Wade et al., 2018). *P. damnosus, P. inopinatus, P. parvulus*, and *P. pentosaceus* have been reported to produce excessive diacetyl, exopolysaccharides, biogenic amines, acrolein and more generally off-odors, flavors and textures, thus contributing detrimentally to wine quality (Wade et al., 2018). However, recent findings have shown that the presence of *Pediococcus* species in wine does not always lead to spoilage and that some species and strains within this genus may contribute positively to wine aroma and can inhibit the formation of 4-ethylphenol from the spoilage yeast, *B. bruxellensis* (Strickland et al., 2016; Wade et al., 2018). Given the variability of the contribution to wine from *Pediococcus* strains, further studies on this genus are of crucial importance. Understanding the differences between strains, and the interactions of this genus with other microorganisms, could open up the possibility of using selected *Pediococcus* starter cultures, potentially reconsidering its role in winemaking.

[1]www.lallemandwine.com

[2]www.chr-hansen.com

HOW CAN LAB IMPROVE WINE AROMA?

Citrate Metabolism

Oenologically, one of the major aroma compounds associated with LAB is diacetyl, which originates from citrate fermentation (Bartowsky and Borneman, 2011). At low concentrations (1–4 mg/L) diacetyl confers the typical buttery character to wine, while at high concentrations (>5–7 g/L) it is associated with undesirable aromas (Bartowsky and Henschke, 2004). A 2002 survey showed that the concentration of diacetyl in wine varies widely, ranging from 0.3 to 0.6 mg/L in Chardonnay wines and from 0.3 to 2.5 mg/L in red wines (Bartowsky et al., 2002). The sensory threshold can vary greatly across different types of wines and is greatly affected by the presence in wine of other compounds such as sulfur dioxide (Bartowsky et al., 2002).

Referring to their ability to degrade citrate, LAB can be classified in cit^+ and cit^-. Cit^+ strains have all the genes that encode for the necessary permease and the lyase subunits needed to degrade citrate, leading to the production of pyruvate. Conversely, cit^- strains may lack one or more genes in this metabolic pathway and cannot degrade citrate, however, they can still produce diacetyl from the pyruvate that originates in the glycogenesis pathway (Lerm et al., 2011; Mink et al., 2015). Cit^+ LAB strains typically produce more D-lactate, acetate, diacetyl and acetoin from pyruvate (Pretorius et al., 2019). The content of diacetyl found in wine is therefore dependent on the LAB species used as a starter for MLF (Lonvaud-Funel, 1999; Bartowsky and Henschke, 2004).

Glycosidase Activity

Odorless, sugar-bound aromas represent a reservoir of wine aroma that can be released by cleaving the bonds between the glycosidic molecule and the volatile, aromatic aglycone (**Figure 2**), such as terpenes, C13 norisoprenoids, volatile phenols, C6 compounds and others (Baumes, 2009). A group of enzymes called glycosidases are responsible for the enzymatic hydrolysis of these compounds. The type of aroma precursor determines which specific glycosidase is needed to break the bonds. The aromatic volatiles can be conjugated to glucose (β-D-glucopyranoside) or to a disaccharide. In the latter case, the inner molecule is a glucose unit and the outer one is a second sugar unit (e.g., α-L-arabinofuranose, α-L-rhamnopyranose, β-D-xylopyranose, or β-D-apiofuranose) (Williams et al., 1982; Gunata et al., 1988). When the aromatic compound is linked

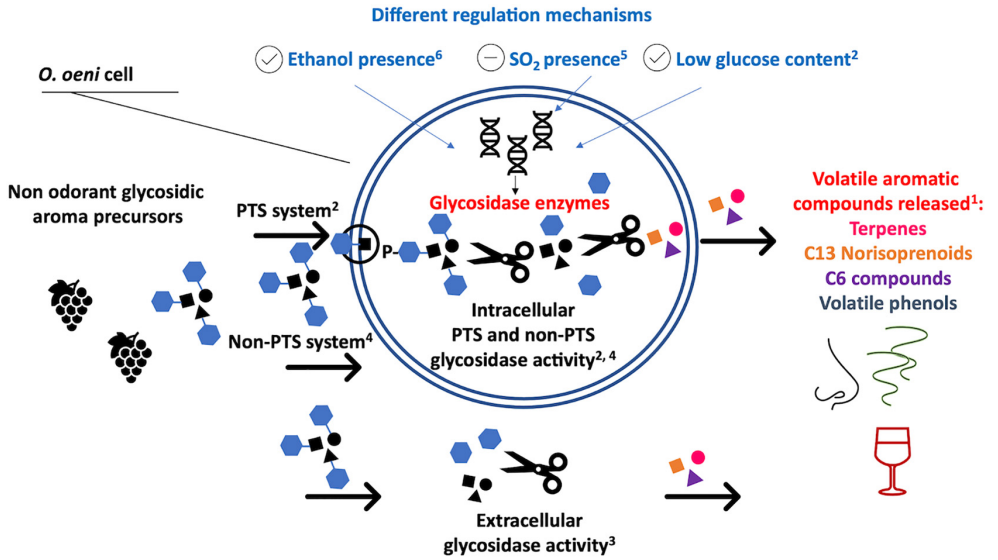

FIGURE 2 | Reported glycosidase activity in *O. oeni*. [1]Baumes, 2009; [2]Capaldo, 2012; [3]Grimaldi et al., 2000; [4]Michlmayr et al., 2010a,b; [5]Spano et al., 2005; [6]Grimaldi et al., 2000.

to a glucose, a β-glucosidase is required to hydrolyze the bond. When the aromatic compound is linked to a disaccharide two enzymes are needed to break the bond: an exo-glycosidase e.g., α-L-arabinofuranosidase, α-L-rhamnopyranosidase, β-D-xylopyranosidase, or β-D-apiofuranosidase, cleaves the second sugar unit and subsequently a β-glucosidase removes the remaining glucose (Gunata et al., 1988).

Glucosidases can originate from different sources, including grapes (Aryan et al., 1987), yeasts (Swiegers et al., 2005) and bacteria (Bartowsky and Borneman, 2011; Capaldo, 2012; Cappello et al., 2017). The addition of enzymatic preparations with hydrolytic activity to musts and wines can increase the content of free aromatic compounds such as terpenes and norisoprenoids (Pretorius and Rensburg, 2000; Codresi et al., 2012). However, purified enzymes can be expensive and may have low specificity and efficiency (Tavernini et al., 2020). To overcome these issues, polymeric chitosan beads have been successfully trialed as matrix for the immobilization of β-D-glucosidases and α-L-arabinofuranosidases sourced from a commercial, *Aspergillus niger*-derived preparation and previously organized in a cross-linked matrix (Tavernini et al., 2020). There is also a high potential for the use of glycosidases from bacteria, especially LAB, because these enzymes could perform better under stressful conditions such as high concentrations of alcohol and acidity (Bartowsky and Borneman, 2011).

Several authors have shown that LAB are able to enzymatically hydrolyze glycosides and that this activity is widespread amongst different *O. oeni* strains (Grimaldi et al., 2000, 2005a; Barbagallo et al., 2004; D'Incecco et al., 2004; Spano et al., 2005; Hernandez-Orte et al., 2009; Fia et al., 2018) and other LAB genera (Grimaldi et al., 2005b; Spano et al., 2005; Hernandez-Orte et al., 2009). *Lactiplantibacillus* strains show a varied array of enzymes that can contribute to wine aroma, and particularly, a great ability to release volatile aromas from glycosidic precursors (Lerm et al., 2011; Iorizzo et al., 2016).

Although considerable efforts have been directed toward understanding the genetics and the expression mechanisms, the glycosidase activity of LAB is not yet clear. The carbohydrate phosphotransferase system (PTS) seems to play a key role in *O. oeni*, by phosphorylating and transporting the glycosides into the cell (**Figure 2**) through a membrane-associated enzymatic system (Capaldo, 2012). The activated glycosides can then be hydrolyzed by intracellular phosphoglycosidases (Capaldo, 2012). Some phosphoglycosidases have been characterized and their hydrolytic activity against *p*-nitrophenol-β-D-glucopyranoside-6-phosphate confirmed (Capaldo, 2012). Intracellular non-PTS glycosidase activity has been reported by a number of authors (**Figure 2**) and some of these glucosidases have also been characterized (Michlmayr et al., 2010a,b; Capaldo, 2012). However, other authors reported extra-cellular and parietal glycosidase activity, suggesting that the glycosidase activity in *O. oeni* is the consequence of a complex enzymatic system, involving many different genes, enzymes and regulation mechanisms (Grimaldi et al., 2000; Barbagallo et al., 2004). Little is known regarding the molecular mechanisms involved in the glycosidase activity of other LAB, such as *Lactiplantibacillus* and *Pediococcus*. It is known that abiotic stresses have a key role in upregulating the expression of these enzymes, although data are controversial. Low levels of glucose and the presence of ethanol seem to lead to an enhancement of the glycosidase activity, while low pH values and sulfite content seem to repress the activity (D'Incecco et al., 2004; Grimaldi et al., 2005a,b; Spano et al., 2005; Capaldo, 2012). See **Figure 2** for an overview of the glycosidase activity of LAB in wine.

Finally, it is important to note that glycosidase activities in wine can also have a negative effect on the final product. Similar

to yeast, the bacterial starters, with their ability to hydrolyse the glycoconjugate compounds, can also heavily affect the evolution of volatile phenols associated with smoke taint, influencing the intensity of smoke taint-associated aromas (Kennison et al., 2008; Jiranek, 2011). The aroma compounds associated with the smoky and earthy notes that characterize smoke tainted wines, such as guaiacol, 4-methylguaiacol, 4-ethylguaiacol and 4-ethylphenol, typically peak in finished wine (388 μg/L for guaiacol and 93 μg/L 4-for methylguaiacol) (Kennison et al., 2007). This can be explained by the presence of conjugated precursor compounds, particularly disaccharide conjugates, which can be liberated via enzymatic hydrolysis (Kennison et al., 2008; Hayasaka et al., 2010; Dungey et al., 2011). For the impact that LAB glycosidase activity can have on wine color see the dedicated paragraph: "how can LAB impact the wine colour?".

Release of Volatile Thiols From Precursor Compounds

Volatile thiols, such as 3-sulfanylhexan-1-ol and 3-sulfanylhexyl acetate, first identified in Sauvignon Blanc, are important aromatic compounds that contribute varietal aromas like grapefruit and passionfruit (Takase et al., 2018). Their non-odorant precursors, such as glutathione S-conjugate, cysteinyl-glycine S-conjugate and cysteine S-conjugate, are found in grapes and are enzymatically released by yeast during AF (Thibon et al., 2008). A 2019 study has shown that *L. plantarum* is able to enzymatically convert the cysteine S-conjugates and cysteinyl-glycine S-conjugates, with a noticeable preference for the latter compound under experimental conditions (Takase et al., 2018). The enzymes responsible for this transformation and the underlying regulatory mechanisms have not yet been characterized. However, this represents an opportunity to improve the varietal aroma of wines, and further research is warranted to provide a better understanding of this metabolic pathway.

Esterase Activity

Esters are secondary or tertiary aroma compounds that contribute significantly to wine aroma. In wine, esters can either be formed in a process called esterification, or broken down via ester hydrolysis (Waterhouse et al., 2016). They can be formed during the primary or secondary fermentation by yeast or bacteria, and their concentration and composition is slowly changed during wine aging (Liu, 2002; Sumby et al., 2010, 2013; Antalick et al., 2012). The contribution of LAB to the ester composition of wine has been highlighted with several wine volatile profiling studies conducted after MLF (Ugliano and Moio, 2005; Antalick et al., 2012; Costello et al., 2012; Sumby et al., 2012; Lytra et al., 2020). In wine, LAB can both increase and decrease the content of esters (Sumby et al., 2013). The degree of LAB contribution to the ester profile of wine is strain-specific (Sumby et al., 2013; Gammacurta et al., 2018) and MLF inoculation strategy can affect the quantity and quality of esters released by the bacteria (Lasik-Kurdyś et al., 2018). It seems that the use of a coinoculation technique increases the release of ethyl esters, particularly ethyl lactate, diethyl succinate and ethyl acetate, thus (depending on their concentration) enriching the wine with floral and fruity notes (Lasik-Kurdyś et al., 2018).

Activities That Can Affect the Content of Volatile Phenols in Wine

Ethyl phenols are crucial aromatic compounds associated with unpleasant odors in wine such as horse sweat, leather and stable. Ethyl phenols are produced by some yeasts within the genus *Brettanomyces*/*Dekkera* from hydroxycinnamic acids (HCAs) precursors (Chatonnet et al., 1992). *Brettanomyces* yeasts can decarboxylase the free HCAs, converting them into vinylphenols first, via an hydroxycinnamate decarboxylase enzyme (HCDC), and then into the unpleasant ethyl phenol, via a vinylphenol reductase enzyme (VPhR) (Chatonnet et al., 1992). HCAs can be found in the must as free acids but, most commonly, as their tartrate esters, which are hydrolyzed slowly throughout the winemaking process (Waterhouse et al., 2016; Lima et al., 2018). This leads to small amounts of ethyl phenol precursors being continuously released in wine and made available to *Brettanomyces* metabolism.

It has been reported that the use of *O. oeni* strains with cinnamoyl esterase activity can lead to higher amounts of 4-ethylphenol and 4-ethylguaiacol in Pinot Noir after MLF, compared to wine that did not undergo MLF, or underwent MLF with bacterial starters with no cinnamoyl esterase activity (Chescheir et al., 2015). Cinnamoyl esterase activity is strain-specific in *O. oeni* and may be constitutively expressed (Chescheir et al., 2015; Collombel et al., 2019). Thus, to avoid the faults that arise from *Brettanomyces* spoilage, it can be beneficial to use bacterial starters with low cinnamoyl esterase activity (Chescheir et al., 2015).

LAB have also been reported to be directly responsible for the production of 4-vinylphenol (Silva et al., 2011). In *L. plantarum*, *Secundilactobacillus collinoides* (formerly known as *Lactobacillus collinoides)* and *Pediococcus pentosaceus*, this activity is enhanced by the presence of hydroxycinnamic acids, especially caffeic acid, in the growth medium (Silva et al., 2011). Conversely, a tannin content of 1 g/L (the average range for red wines is 1–4 g/L) can inhibit the release of volatile phenols in wine by *L. plantarum* (Silva et al., 2011). However, the literature regarding this matter is still controversial. It has also been suggested that the addition to the must of cinnamoyl esterase enzymes can help reduce the formation of ethyl phenols (Morata et al., 2013). The intermediate vinylphenols are also able to react with anthocyanins, producing very stable vinylphenolic pyranoanthocyanins (Rentzsch et al., 2007). When vinylphenols are present in their bound form, they can help reduce the number of precursors for the formation of ethyl phenols and also stabilize the color of wine (Morata et al., 2013). In support of this, wines fermented with *Saccharomyces* yeast with an increased hydroxycinnamate decarboxylase activity (HCDC+) have an increased content of vinylphenolic pyranoanthocyanins and a reduced content of ethyl phenols (Morata et al., 2013). The addition to the must of cinnamoyl esterase enzymes, to help release more quickly the free HCAs paired with the use of HCDC+ *Saccharomyces cerevisiae* strains, could be a way

to reduce the formation of ethylphenols (Morata et al., 2013). Similarly, the use of LAB with enhanced cinnamoyl esterase activity could be beneficial for the quality of wine, helping to prevent the formation of unwanted off flavors and favoring the development of stable color compounds.

Furthermore, the levels of volatile phenols in the finished wine seem to depend more on differences between *Brettanomyces* strains rather than on the cinnamoyl esterase activity of the LAB employed for MLF (Madsen et al., 2017). Two different *O. oeni* strains, with and without cinnamoyl esterase activity, were tested on Cabernet Sauvignon and, although there were differences in the degradation rate of tartaric esters of HCAs into free HCAs, this did not affect the final content of volatile phenols (Madsen et al., 2017).

Further studies are needed to characterize the cinnamoyl esterase activity in LAB. Understanding the basis for the differential cinnamoyl esterase activity of LAB could enable the winemaker to make appropriate decisions in regards the choice of bacteria to employ.

Acetaldehyde Metabolism

Acetaldehyde is an important compound in wine, affecting aroma, color, stability and microbiological properties (Liu and Pilone, 2000). Wine typically contains 20-100 mg/L of acetaldehyde, and, at low concentrations, it can enhance the fruity character of wine (Waterhouse et al., 2016). However, at higher concentrations it is associated with an unpleasant rotten apple aroma (Waterhouse et al., 2016). Acetaldehyde is an intermediate compound produced by yeast during AF and also plays a vital role in the stabilization of wine color (see next section "how can LAB impact the wine color?") (Waterhouse et al., 2016; Forino et al., 2020). During AF acetaldehyde is readily reduced to ethanol, thus at this stage, its content in wine is typically low (25–40 mg/L) depending on yeast strain and the concentration of SO_2 in the must (Waterhouse et al., 2016). However, in later stages, the oxidation of alcohol can lead to an increase in acetaldehyde content (Waterhouse et al., 2016).

Excessive accumulation of acetaldehyde is not desirable because it strongly binds to sulfur dioxide making it less active (see section "reducing the SO_2 required in winemaking") and requiring the winemaker to add additional SO_2 to protect the wine (Wells and Osborne, 2011). Additionally, as acetaldehyde has potential toxic and carcinogenic effects, high amounts are undesirable in beverages for human consumption (Lachenmeier and Sohnius, 2008).

Acetaldehyde can exert a stimulating or inhibiting effect on LAB growth in wine (Liu and Pilone, 2000). Both heterofermentative and homofermentative LAB strains can degrade free and SO_2-bound acetaldehyde into small amounts of ethanol and acetic acid (Osborne et al., 2000). However, this could also lead to higher VA in wine (Waterhouse et al., 2016). The efficiency of this activity seems to be strain-specific (Mira and de Orduña, 2010). The ability of probiotics, including LAB, to break down acetaldehyde, is therefore of interest to the wine industry and could be used to reduce acetaldehyde levels in wine. Indeed, a patent describing "a composition of probiotics including LAB, able to degrade alcohol and acetaldehyde," to help prevent and treat alcohol-related diseases, has been filed (Chung, 2018).

HOW CAN LAB IMPACT THE WINE COLOR?

Wine color is an important sensory attribute (**Figure 1**) that is relevant to red wines, largely related to the grape variety and the vintage, and to a minor extent to the winemaking practices (González-Neves et al., 2013). Wine color can also be affected by the activity of yeast and LAB (Morata et al., 2005; Benito et al., 2011; Burns and Osborne, 2013, 2015; Devi and Ka, 2019; Devi et al., 2019). Color loss is common in wines that have undergone MLF (Abrahamse and Bartowsky, 2012; Burns and Osborne, 2013, 2015). Independently from pH, wines post-MLF have lower levels of polymeric pigments, lower Visitin A and B content and a higher content of monomeric anthocyanins than their respective controls that did not undergo MLF (Burns and Osborne, 2013). An explanation for the color loss in wine post-MLF is the LAB metabolism of acetaldehyde (see acetaldehyde metabolism above) (Burns and Osborne, 2015). Acetaldehyde is crucial for the formation and stabilization of wine color because it mediates the formation of stable ethylene-linked pigments, which are more stable than their respective monomeric anthocyanins and show better colorimetric properties (Forino et al., 2020). Furthermore, pyruvic acid and acetaldehyde can react with pigments such as malvidin-3-glucoside, generating the relatively stable pyranoanthocyanins Visitin A and Visitin B (Waterhouse et al., 2016).

Recent studies have shown that *O. oeni* and *L. plantarum* strains can also absorb anthocyanin glucosides such as delphinidin-3-glucoside, malvidin-3-glucoside and peonidin-3-glucoside through the cell wall (Devi et al., 2019). These LAB can produce β-glycosidase enzymes that cleave the anthocyanin glucoside glycosidic bonds and can further degrade the aglycons into phloroglucinol aldehyde and corresponding phenolic acids (Devi et al., 2019). The absorption rate of anthocyanin glucosides, the β-glycosidase activity and the degradation rate of anthocyanins are dependent on the species and strains of LAB (Devi et al., 2019). Different inoculation regimes, such as coinoculation or sequential inoculation of yeast and bacteria, can determine different wine color outcomes, seemingly due to microbial interactions, rather than to absorption mechanisms (Devi and Ka, 2019). A higher color loss occurs when MLF is performed with sequential inoculation regimes, rather than with coinoculation regimes (Abrahamse and Bartowsky, 2012).

Little has been reported regarding the long-term effects of MLF on wine color. A 2016 study reported that, nine months after MLF completion, up to 9% color intensity loss and lower acylated and non-acylated anthocyanins levels were detected in wines (Izquierdo-Cañas et al., 2016). Interestingly an increase of the pyranoanthocyanin concentration was also observed (Izquierdo-Cañas et al., 2016).

Further research is warranted to understand the specific yeast-bacteria interactions during the secondary fermentation, and how they subsequently affect wine color. Considering the multitude

of factors involved, understanding the basis of wine color is not simple and far from fully achieved. However, this knowledge would greatly benefit the industry by enabling the winemaker to plan MLF in order to obtain the desired color outcome even in wines naturally low in pigments.

POSSIBLE CONTRIBUTIONS OF LAB TO WINEMAKING

Reducing the Need for Bentonite

Residual grape proteins can represent a problem for the quality of wine. Thaumatin-like proteins, chitinases and, to a lesser extent, β-glucanases, are the main classes of proteins responsible for wine protein instability (Van Sluyter et al., 2015; Cosme et al., 2020). Protein aggregation, particularly during wine storage, can lead to the formation of an unwanted sediment or haze (**Figure 1**) once the wine is bottled (Ferreira et al., 2001). Haze formation in white wines is mainly an esthetic issue, but it can economically depreciate the final product (Van Sluyter et al., 2015).

To alleviate this problem, the main treatment currently applied during winemaking is the addition of bentonite. However, this is a costly treatment and it involves a loss of wine volume, wine aroma and assimilable nitrogen resources (Muhlack et al., 2006). It has been shown that proteolytic and peptidolytic activities are common amongst LAB (Savijoki et al., 2006; Moslehishad et al., 2013; Atanasova et al., 2014; García-Cano et al., 2019). LAB proteolytic activities could be exploited in winemaking and have the potential to replace or reduce the use of fining agents such as bentonite. However, more studies are needed as most of the literature refers to work done in the dairy industry, at pH values of 6.5 and with considerably different substrates (Moslehishad et al., 2013; Atanasova et al., 2014; García-Cano et al., 2019).

Aiding Clarification and Filtration of Juice

Grape juice is naturally rich in polysaccharides, such as pectin, cellulose, hemicellulose, and other substances, originating mainly from grape cell walls and the middle lamellae. In particular, the high content of pectins leads to the formation of a colloid structure, which can make processing of juice difficult (Sandri et al., 2011). The addition of fungal pectinase enzymes can help with the clarification and filtration of the juice, increasing must yield, and favoring the extraction of polyphenols, pigments and aromas (Merín et al., 2011). Some yeast strains are capable of degrading polysaccharides (Merín et al., 2014; Belda et al., 2016). Similarly, LAB may also possess pectinolytic activity, however, little research has been done in this field (Ruiz Rodríguez et al., 2019).

Reducing the SO_2 Required in Winemaking

Due to its antimicrobial and antioxidant properties, SO_2 is one of the most common agents added to wine (Lisanti et al., 2019). Despite the benefits that come from SO_2, the sensitivity of some consumers to this compound, have raised concerns regarding its safety in the food and beverage industries (Benito, 2019). Thus, the addition of potassium metabisulfite to wine is strictly regulated, with maximum limits varying depending on local regulations. As a general guide, the international code of enological practices recommends a residual limit of 150 mg/L total sulfur dioxide for red wines and 200 mg/L for white and rosé wines with up to 4 g/L of reducing substances[3].

As mentioned already, acetaldehyde strongly binds free SO_2, making it less effective in its antiseptic, antioxidant and antioxidasic roles (Ribéreau-Gayon et al., 2006b). Thus, reducing the content of acetaldehyde in wine could help minimize the amount of SO_2 required in winemaking (**Figure 1**) and, at the same time, could improve the aroma of wine (Lisanti et al., 2019). The use of LAB starters that are able to degrade SO_2-bound acetaldehyde (see acetaldehyde metabolism above) could represent a winemaking strategy to minimize SO_2 additions to wine by converting the bound SO_2 into the more effective free SO_2. Importantly, acetaldehyde also plays a crucial role in the development of wine color. Thus, its content in wine has to be optimized to determine the balance between optimizing color, flavors and aromas, and health associated risks.

THE IMPACT OF LAB ON THE WHOLESOMENESS OF THE WINE

LAB can positively and negatively affect the impact that wine may have on human health (**Figure 3**). LAB can produce numerous metabolites, including organic acids (mainly lactic and acetic), phenyllactic acid, diacetyl, cyclic dipeptides, and bacteriocins that can inhibit the growth of spoilage or pathogenic bacteria (Corsetti et al., 2015; Szutowska, 2020). They have also been reported to have antifungal activity, although the mechanisms of action are not clear (Bianchini, 2015). LAB can exert a high detoxifying action toward mycotoxins in various foods including wine (Muhialdin and Saari, 2020). This is likely to be due to the proteolytic activity of LAB, however, other detoxification mechanisms have been suggested, including the binding of mycotoxins to LAB metabolites such as acids, phenolic compounds and small bioactive peptides, and the absorption of mycotoxins by the bacterial cell walls (Muhialdin and Saari, 2020). Recent studies show that wines that underwent MLF have higher contents of melatonin and other tryptophan related compounds, which are associated with several human health benefits (Fracassetti et al., 2020). LAB could also have a role in the bio-absorption of copper, which, at high concentrations, is associated with health risks and negative effects in wine too (Harald, 2020).

Biogenic Amines

Biogenic amines (BA) are potentially hazardous compounds that are commonly found in wine (**Figure 1**) and other fermented foods (Benito, 2019). Although BA can be produced in the grapes (Del Prete et al., 2009) and by yeast (Tristezza et al., 2013), their

[3]http://www.oiv.int

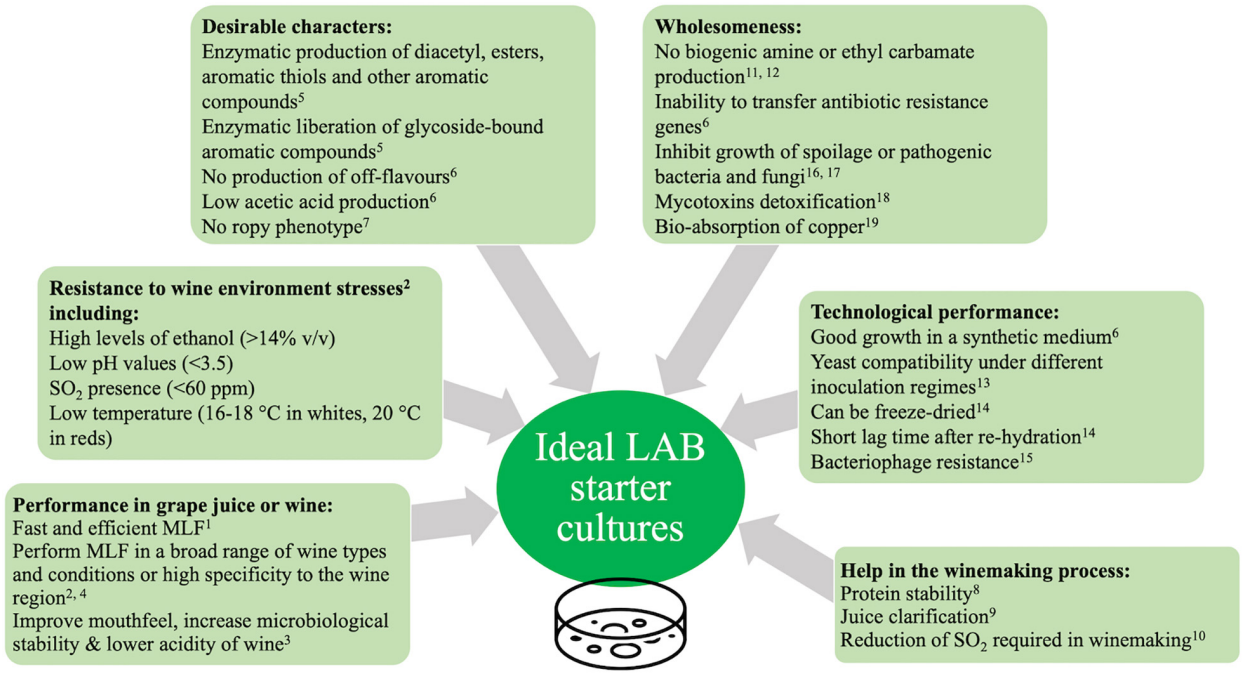

FIGURE 3 | Guidelines for the selection of MLF starters for use in winemaking. Adapted from Henick-Kling, 1995 and Torriani et al., 2011. [1]Henick-Kling, 1995; [2]Costello et al., 2015; [3]Lonvaud-Funel, 1999; [4]Bartowsky et al., 2015; [5]Matthews et al., 2004; [6]Torriani et al., 2011; [7]Wade et al., 2018; [8]García-Cano et al., 2019; [9]Ruiz Rodríguez et al., 2019; [10]Lisanti et al., 2019; [11]Callejón et al., 2014; [12]Araque et al., 2009; [13]Bartle et al., 2019; [14]Fonseca et al., 2015; [15]Mozzi et al., 2015; [16]Szutowska, 2020; [17]Bianchini, 2015; [18]Muhialdin and Saari, 2020; [19]Harald, 2020.

presence in wine is primarily due to LAB metabolism (Costantini et al., 2019). The main BAs produced during MLF are histamine, putrescine and tyramine (Landete et al., 2007).

These BAs are formed through the activity of the LAB enzymes, histidine decarboxylase (*hdc*), ornithine decarboxylase (*odc*), and tyrosine decarboxylase (*tdc*), which decarboxylate the precursor compounds histidine, ornithine and tyramine, with both the enzymatic activity and presence of the genes encoding these proteins being strain dependent (Coton et al., 2010; Costantini et al., 2019). In addition to this, several LAB genera can produce putrescine via the agmatine deiminase, from its precursor agmatine (*agdi*) (Lucas et al., 2007).

Some LAB can degrade BA in culture media, and to different degrees in wine, through the action of multicopper oxidases or amine oxidases (García-Ruiz et al., 2011; Callejón et al., 2014). Many species, including *Levilactobacillus brevis* (formerly *Lactobacillus brevis*), *L. delbrueckii*, *L. hilgardii*, *L. paracasei*, *Lactiplantibacillus pentosus* (formerly *Lactobacillus pentosus*), *L. plantarum*, *P. parvulus*, and *P. pentosaceus* have been biochemically tested and showed degrading activity against BA (García-Ruiz et al., 2011; Callejón et al., 2014). Seemingly this property of LAB is strain dependent for most species tested, except in the case of *L. plantarum*, in which a high level of activity was widespread amongst all strains (Callejón et al., 2014). Similar results were obtained in a previous study which aimed to investigate the BA degrading activity of two *L. plantarum* strains (NDT 09 and NDT16) (Capozzi et al., 2012). Interestingly, the degrading activity of *L. plantarum* NDT09 and *L. plantarum* NDT16 toward putrescine and tyramine, in synthetic media, was higher when the two strains were used in conjunction (Capozzi et al., 2012). Recently, a *L. plantarum* with good MLF and stress tolerance properties, that is also able to decrease the histamine, tyramine and cadaverine content by over 57%, has been obtained, through successive screenings (Sun et al., 2020).

Currently, the options available to control the BA content in wine are the reduction of precursor compounds, the inhibition of spoilage bacteria growth and the use of selected non biogenic amine-producer starters (Callejón et al., 2014). Given the recent findings on the ability of LAB, particularly *L. plantarum* strains, to degrade BA, the use of MLF starters that are able to reduce BA in wine would represent an innovative and alternative option for the winemaker.

Ethyl Carbamate

Ethyl carbamate (**Figure 1**) is a carcinogenic compound found in many fermented foods and beverages (Benito, 2019). The reaction of ethanol with N-carbamyl compounds, such as urea (produced by yeast) and citrulline (produced by some LAB), is responsible for the main formation of ethyl carbamate in wine (Liu et al., 1994).

Some species of LAB can degrade L-arginine, through the arginine deaminase pathway, producing ATP, CO_2 and ammonium as final products (Araque et al., 2009). The intermediate steps in this pathway yield potential ethyl carbamate precursors like citrulline and carbamyl phosphate (Liu et al., 1994; Araque et al., 2009). The genes *arcA*, *arcB*, and *arcC*,

which encode for the enzymes arginine deiminase, ornithine transcarbamylase and carbamate kinase, respectively, have been characterized in many genera (Araque et al., 2009). The activity of these genes, and the production of ethyl carbamate precursors, has been reported for all wine-related genera (Liu et al., 1994, 1995; Araque et al., 2009).

The presence of the *arc* genes is generally a good predictor of the capability of LAB to degrading arginine (Liu et al., 1995; Araque et al., 2009); however, it is not indicative of the expression of the genes under particular circumstances. Both genotypic and phenotypic screenings are recommended to correctly characterize the capability of LAB to produce ethyl carbamate precursors in wine. On the other hand, LAB can produce urea-degrading enzymes that can selectively hydrolyze urea, contributing to reduction of ethyl carbamate in wine (Fang et al., 2016).

IMPROVEMENT OF LAB AND TECHNOLOGICAL ADVANCES

Strain Selection

The selection of new LAB starters requires the assessment of many properties related to the new strain including resistance to biotic and abiotic stresses, technological performances and safety aspects (Henick-Kling, 1995; Torriani et al., 2011; Costello et al., 2015). See **Figure 3** for a summary of the properties of interest. Traditionally a phenotypic approach has been used for the purpose of selecting new starters (Torriani et al., 2011). In recent years, phenotypic and genotypic approaches are used in tandem to rapidly characterise new potential starter candidates (Torriani et al., 2011).

Microbial Integrity: Phage Resistance

One of the major issues that can arise during fermentation processes is the contamination of the LAB starter cultures with bacteriophages (Mozzi et al., 2015). Most of the knowledge regarding phage infections in LAB comes from the dairy industry, with *Lactococcus lactis* and *Streptococcus thermophilus* being the most susceptible and studied species (Mozzi et al., 2015). However, since *O. oeni*, *Lacticaseibacillus casei* (formerly known as *Lactobacillus casei), L. delbrueckii* and other species have become more prominent in the food industry, more research has been directed toward investigating their phages, disclosing multiple phage infections within these genera (Mozzi et al., 2015). In a 2017 study, 15 *O. oeni* phages have been studied, containing more than one type of integrase genes each and varying in genome size (Costantini et al., 2017). In the same study, it was shown that the presence of ethanol and low pH values inhibited the phages from attacking the cells, suggesting that inoculation time of the bacterial starters could play a significant role in preventing bacteriophage infections (Costantini et al., 2017). The first sequence of an *O. oeni* phage, E33PA, has been published in 2018 by Jaomanjaka and co-workers (Jaomanjaka et al., 2018). Interestingly, although most of phage isolated from the *Oenococcus* genus are temperate, E33PA was found to have a predominately lytic lifestyle (Jaomanjaka et al., 2018).

The adaptive immune system of bacteria is a potential mechanism for defense against phage, which has been highlighted by recent studies (Barrangou and Marraffini, 2014). For example, it is now known that Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated genes, are essential elements in phage defense (Barrangou and Marraffini, 2014). CRISPR-Cas systems can confer phage resistance to the bacterial cell through three steps: during the adaptation, foreign genomic sequences can be integrated in the CRISPR arrays as new spacers, bordered by the palindromic repeats. During the expression the CRISPR locus is transcribed and processed, generating multiple copies of small CRISPR RNAs, able to direct the Cas proteins to foreign, complimentary DNA. Lastly, during the interference, foreign nucleic acid is targeted and cleaved (Crawley et al., 2018).

The CRISPR-mediated immunity mechanism causes phages to mutate at high rates, to maintain their ability to infect the hosts; furthermore, it's likely that phages and CRISPR systems are continuously co-evolving (Sorek et al., 2008). Interestingly, there is a rich variety of type II CRISPR-Cas systems within LAB (Sun et al., 2015); in a 2015 study comprising 213 different LAB genera, type II systems were found in 36% of them, representing a wealth of tools for LAB genetic engineering (Sun et al., 2015).

Conversely, CRISPR-Cas systems are either uncommon or absent in *O. oeni*. We searched six different *O. oeni* whole genome sequences from NCBI on the CRISPR finder database (version 4.2.20) (Couvin et al., 2018) showing that there are no CRISPR arrays or Cas clusters.

However, two closely related *Oenococcus* species contained both Cas clusters and CRISPR arrays at a high-quality score (quality score of 4). These were *O. kitaharae* DSM 17330 (CAS clusters = 1, CRISPR arrays = 1) and *O. sicerae* UCMA 15228 (CAS clusters = 2, CRISPR arrays = 2).

This inconsistent distribution of CRISPR-Cas immune systems within and between bacterial species is not uncommon and it may be explained by the fact that bacterial cells that have lost the CRISPR-Cas system from their genome avoid damage caused by autoimmune targeting (Rollie et al., 2020). Additionally, the efficacy of CRISPR-Cas is dependent on viral mutation rate and the frequency of spacer incorporation. *O. oeni* has a slow growth rate (∼7 days to reach OD_{600} of 1.0) and may not be able to mount a response fast enough or acquire the spacers quickly enough for the system to be effective (Sorek et al., 2008).

Strategies to Harness LAB Enzymatic Activity

Directed evolution of microorganisms is a widely used technique for improving desired properties of bacterial strains (Bachmann et al., 2017). It is based on the concept that, during evolution, the environment selects the fittest variants. Growth limiting conditions, such as scarcity of nutrients, and environmental stresses induce a stress response and can lead to the production of genetic mutations (Rosenberg and Hastings, 2003). To generate an array of genetic and phenotypic variants treatments of UV and ethylmethanesulfonate (EMS) can also be applied to the culture (Dragosits and Mattanovich, 2013; Li et al., 2015).

As part of a broader study involving metabolic networks, a strain of *L. plantarum* has been successfully adapted, through serial dilutions, to grow well in a medium containing glycerol as main carbon source (Teusink et al., 2009). Directed evolution has been used to successfully generate acid-resistant mutants of *L. casei* and *Leuconostoc mesenteroides* (Zhang et al., 2012; Ju et al., 2016). An ethanol tolerant *O. oeni* strain, A90, has been generated after exposure to increasing ethanol concentrations, in approximately 330 generations (Betteridge et al., 2018). This strain was further evolved to withstand the multiple stresses typical of wine environment, obtaining after approximately 350 generations, an alcohol tolerant, acid tolerant (3.35 pH) and SO_2 tolerant (26 mg/L) strain (Jiang et al., 2018).

Directed evolution, as a strategy to harness LAB enzymatic activity, offers several advantages (Bennett, 2002). It does not involve the use of recombinant technology, posing less problems in the public acceptance of the products. It does not require a specific knowledge of the underlying genetics behind the specific phenotypic traits that are object of interest. Finally, whereas bioengineering approaches often target one gene at the time, directed evolution can result in multiple beneficial mutations, broadening the possible outcomes (Bloom and Arnold, 2009). However, the specific application of directed evolution to the LAB species associated with wine, is still a relatively new but promising technique. By using this approach, it could be possible to produce LAB strains that exhibit a higher desired enzymatic activity and/or are more tolerant to the harsh wine environment.

Another way to enhance the enzymatic activity of bacteria is the immobilization of the organisms onto supports such as alginate beads or apple pieces, corn cobs, delignified cellulosic material, grape skins and grape stems (Genisheva et al., 2013; Bleve et al., 2016; Simó et al., 2017; Nikolaou et al., 2020). Immobilized bacteria can perform MLF twice as fast, compared to free cells, and are also more resistant to ethanol content, SO_2 content and elevated temperatures (Genisheva et al., 2013).

Currently, it may not be practical to apply this methodology in a traditional winemaking system, considering the large amounts of required supporting material (up to 30 g/L). Nonetheless this is a promising technique, with a great potential to improve MLF in wine. Furthermore, it would be interesting to know if secondary enzymatic activities of LAB would also be affected by the use of immobilized cells.

Genome Editing of LAB

In addition to strain selection and non-GMO strategies to harness enzymatic activity of LAB, genome editing is a promising strategy to develop high performing bacterial starters. Transformation with plasmids, transduction and conjugative transposons systems are the main recombinant methods used to genetically manipulate LAB (Sumby et al., 2014). The transformation of *Lactiplantibacillus* and *Lacticaseibacillus* strains has been successfully performed by many authors (Spath et al., 2012; Xin et al., 2018). However, the rate of success of this method depends on the plasmid vector, on the bacterial strain and the possible presence of a enzymatic restriction system in the host cell (Teresa Alegre et al., 2004). Currently, not many expression vectors are available for *O. oeni* (Sumby et al., 2014). A plasmid, pGID052, was developed in 2004 and successfully mobilized from *L. lactis* to *O. oeni* (Beltramo et al., 2004). The same plasmid, pGID052, encoding a truncated form of the ClpL2 protein was later introduced in *O. oeni* ATCC BAA-1163, trialing an optimized electroporation method (Assad-García et al., 2008). Although promising, there has been no follow up research to these findings, perhaps due to the low copy level of this plasmid (Sumby et al., 2014).

The most promising technology is currently based on CRISPR-Cas systems. This strategy exploits the functions of CRISPR and CRISPR-associated genes (Doudna and Charpentier, 2014; van Pijkeren and Barrangou, 2017). Desired edits can be obtained with different methods, e.g., utilizing a plasmid-encoded recombineering sequence or an oligonucleotide sequence with an inducible DNA recombinase (Leenay et al., 2019). The efficiency of the selected method varies dramatically across different strains of *Lactoplantibacillus plantarum* (Leenay et al., 2019).

Bacterial Starter Implementation

Freeze drying is the method of choice for long term storage of bacteria and continuous research has been done toward the optimization of the process (Fonseca et al., 2015, 2019; Polo et al., 2017). Freeze dried bacterial starters have a good rate of cell viability over long periods and are easy to use, store and transport (Fonseca et al., 2015). To protect the integrity of the cells membranes and the structural conformation of proteins and DNA during the dehydration process, lyoprotectans such as trehalose, sucrose and monosodium glutamate, are typically added (Wang et al., 2019). More recently, soluble extracellular polymeric substances (sEPS) from *O. oeni* have been successfully tested as lyoprotectans. Notably, a mixture of 5% sEPS, 15% sucrose, 15% trehalose, and 0.5% MSG, increased the cell viability to 92.83% (Wang et al., 2019). The positive effects of ethanol acclimatization on cell survival rate of lyophilized *O. oeni* have also been reported (Yang et al., 2020). Due to the length and costs associated with this process, other techniques have been investigated, including vacuum drying, spray drying, drum drying, fluidized bed drying and air drying (Santivarangkna et al., 2007). Currently these alternative drying processes do not perform as well as freeze drying, especially regarding the cell viability and the lag time required to restore the metabolic activity (Santivarangkna et al., 2007). However, they could represent a cheaper, fast and efficient way to preserve commercial bacterial starters. Promising results have also been obtained by freeze drying bacterial cultures previously immobilized onto natural supports (Nikolaou et al., 2020).

LIMITATIONS AND FUTURE PROSPECTS

Currently the main enological role of LAB is performing MLF. Thus, the use of LAB, with the intention of increasing wine aroma and facilitating the winemaking process, is an unexplored and promising field. In this new broader

role, LAB could replace, or partially substitute, the use of purified enzymatic preparations. Furthermore, using whole cells, rather than purified enzymes, provides a series of advantages (De Carvalho and da Fonseca, 2007).

Firstly, whole cells are a natural environment for enzymes, preventing loss of activity in non-conventional media. Secondly, they are able to efficiently regenerate co-factors. However, more studies are needed in order to improve the knowledge of LAB's secondary enzymatic activities and their potential usability in the production of wine.

To benefit from the LAB's secondary metabolism a system to control and enhance the expression of these activities is needed.

Currently, disregarding studies on recombinant technology, little research has been done on this point (Renault, 2002). Directed evolution has been successfully used to produce highly performing LAB strains in the dairy industry, but seldomly in the wine industry (Betteridge et al., 2015). However, without resorting to genetic engineering techniques, no other methods are currently available to enhance a chosen secondary enzymatic activity in a LAB strain.

Overall, the benefits of harnessing the enzymatic activities of LAB will increase the options open to winemakers to sculpt the wine of their choice. The industry could benefit greatly from the advent of these extra tools.

AUTHOR CONTRIBUTIONS

CV and KS prepared the first draft. All authors reviewed and finalized the manuscript. All authors contributed to the development of the outline and scope of the review.

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Zooming Into the Microbiota of Home-Made and Industrial Kefir Produced in Greece Using Classical Microbiological and Amplicon-Based Metagenomics Analyses

Maria Kazou†, Andriana Grafakou†, Effie Tsakalidou and Marina Georgalaki*

Laboratory of Dairy Research, Department of Food Science and Human Nutrition, Agricultural University of Athens, Athens, Greece

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***Correspondence:**
Maria Kazou
kmaria@aua.gr

†These authors have contributed equally to this work

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Kefir is a high nutritional fermented dairy beverage associated with a wide range of health benefits. It constitutes a unique symbiotic association, comprising mainly lactic acid bacteria, yeasts, and occasionally acetic acid bacteria, which is strongly influenced by the geographical origin of the grains, the type of milk used, and the manufacture technology applied. Until recently, kefir microbiota has been almost exclusively studied by culture-dependent techniques. However, high-throughput sequencing, alongside omics approaches, has revolutionized the study of food microbial communities. In the present study, the bacterial, and yeast/fungal microbiota of four home-made samples (both grains and drinks), deriving from well spread geographical regions of Greece, and four industrial beverages, was elucidated by culture-dependent and -independent analyses. In all samples, classical microbiological analysis revealed varying populations of LAB and yeasts, ranging from 5.32 to 9.60 log CFU mL^{-1} or g^{-1}, and 2.49 to 7.80 log CFU mL^{-1} or g^{-1}, respectively, while in two industrial samples no yeasts were detected. *Listeria monocytogenes*, *Salmonella* spp. and *Staphylococcus* spp. were absent from all the samples analyzed, whereas *Enterobacteriaceae* were detected in one of them. From a total of 123 isolates, including 91 bacteria and 32 yeasts, *Lentilactobacillus kefiri*, *Leuconostoc mesenteroides,* and *Lactococcus lactis* as well as *Kluyveromyces marxianus* and *Saccharomyces cerevisiae* were the mostly identified bacterial and yeast species, respectively, in the home-made samples. On the contrary, *Streptococcus thermophilus, Lactobacillus delbrueckii* subsp. *bulgaricus,* and *Lacticaseibacillus rhamnosus* along with *Debaryomyces hansenii* and *K. marxianus* were the main bacterial and yeast species, respectively, isolated from the industrial beverages. In agreement with the identification results obtained from the culture-dependent approaches, amplicon-based metagenomics analysis revealed that the most abundant bacterial genera in almost all home-made samples (both grains and drinks) were *Lactobacillus* and *Lactococcus,* while *Saccharomyces, Kazachstania,* and *Kluyveromyces* were the predominant yeasts/fungi. On the other hand, *Streptococcus, Lactobacillus,* and *Lactococcus* as well as *Kluyveromyces* and

Debaryomyces dominated the bacterial and yeast/fungal microbiota, respectively, in the industrial beverages. This is the first report on the microbiota of kefir produced in Greece by a holistic approach combining classical microbiological, molecular, and amplicon-based metagenomics analyses.

Keywords: kefir, grains, microbiological analysis, microbiota, 16S metagenomics analysis, ITS metagenomics analysis, high-throughput sequencing

INTRODUCTION

At both research and industrial level, dairy fermented foods are the protagonists among functional foods, i.e., foods having a positive impact on human health beyond the known nutritional value, such as benefits concerning gastrointestinal health, hypertension, cholesterol reduction, immune system regulation, interaction between gut and brain, etc. (Shiby and Mishra, 2013). Fermented milks, with yogurt standing as the main representative, are probably the most prominent among them, offering a wide range of appealing sensory characteristics and well-being benefits (Robinson and Itsaranuwat, 2006). Other fermented milks, such as kefir, koumiss, chigo, viili, nunu, amabere, amaruranu, and suusac (Ishii et al., 1997; Lore et al., 2005; Kahala et al., 2008; Akabanda et al., 2013; Nyambane et al., 2014; Yao et al., 2017) are known as alcoholic milk beverages, since milk is fermented by both lactic acid bacteria (LAB) and yeasts (yeast-lactic fermentation) (Wszolek et al., 2006).

Kefir, a name most likely deriving from the Turkish and Caucasian word keyif meaning pleasure (Kurman et al., 1992; Kabak and Dobson, 2011), is one of the most cherished functional dairy products. It is believed to have its origins in the Caucasian, Tibetan and Mongolian mountains, but is also manufactured artisanally for centuries in several countries, such as the former Union of Soviet Socialist Republics (USSR), Bulgaria, Slovakia, Hungary, Portugal, Turkey, and France (Farnworth, 2005; Wszolek et al., 2006). In recent years, kefir consumption has increased worldwide, and by 2023, the global kefir market is expected to reach 1.85 billion $US (Shahbandeh, 2019). This can be attributed not only to its unique sensory characteristics, such as flavor, aroma, freshness, and viscosity, but also to a plethora of bioactive compounds, such as peptides and vitamins (Farnworth, 2005; Kabak and Dobson, 2011; Leite et al., 2012, 2013; Diosma et al., 2014). Consumption of kefir has been associated with numerous health benefits including anticarcinogenic and anti-inflammatory effects (Rodrigues et al., 2005; de Moreno de Leblanc et al., 2007; Lee et al., 2007; Kim et al., 2016), alleviation of lactose intolerance symptoms and cholesterol reduction (Hertzler and Clancy, 2003; Liu et al., 2006).

This viscous, slightly carbonated dairy beverage comprises a complex microbial association of mainly LAB, acetic acid bacteria (AAB) and yeasts. When LAB and yeasts are used as starters for the industrial production of kefir beverage, the respective counts in the final product should be at least 7 and 4 log CFU mL^{-1}, until the "date of minimum durability" (Codex Alimentarius Commission, 2003). In kefir symbiotic ecosystem, LAB ferment lactose and decrease the pH, while yeasts stimulate LAB growth by producing B-group vitamins and hydrolyzing milk proteins (Farnworth, 2005; Álvarez-Martín et al., 2008). This symbiosis results in an elastic, white to yellow and slimy granules with irregular, cauliflower-like structure of different size with folded or uneven surface, called kefir grains, which also comprise coagulated milk proteins and mucous polysaccharides known as kefiran (Farnworth, 2005; Wszolek et al., 2006; Dobson et al., 2011; Kabak and Dobson, 2011; Leite et al., 2013). However, kefir beverage can be also produced artisanally by the "Russian method," i.e., by fermenting milk with kefir grains. At the end of the fermentation the grains are removed and stored for subsequent use (Kabak and Dobson, 2011; Prado et al., 2015). Nevertheless, at the end of the fermentation, kefir beverage has a typical pH value of 4.5–4.6 (Guzel-Seydim et al., 2005).

The microbial composition of kefir grains and beverages has been well documented by culture-dependent and -independent fingerprinting methods, mainly PCR-denaturing gradient gel electrophoresis (PCR-DGGE) (Pintado et al., 1996; Witthuhn et al., 2005; Chen et al., 2008; Miguel et al., 2010; Kesmen and Kacmaz, 2011; Leite et al., 2013; Diosma et al., 2014). However, the development and application of high-throughput sequencing (HTS), alongside omics approaches, fundamentally altered the way of studying food microbial consortia, highlighting also the impact of various factors, such as the type of milk, the geographical origin and the manufacture method on kefir microbiota (Dobson et al., 2011; Leite et al., 2012; Gao et al., 2013; Marsh et al., 2013; Nalbantoglu et al., 2014; Garofalo et al., 2015; Korsak et al., 2015; Bourrie et al., 2016; Zamberi et al., 2016; Dertli and Con, 2017). Nowadays, amplicon-based metagenomics analysis targeting either the 16S rRNA gene or the internal transcribed spacers (ITS) DNA region is the most widely used technique to analyze the bacterial and yeasts/fungal communities, respectively, in a food matrix (De Filippis et al., 2017; Ferrocino and Cocolin, 2017).

The aim of the present study was to elucidate and compare the bacterial and yeast/fungal microbiota of home-made and industrial kefir samples produced in Greece, deriving from well-spread geographical origin and types of milk, using a dual approach that includes both classical microbiological and amplicon-based metagenomics analyses.

MATERIALS AND METHODS

Samples

Four home-made kefir grains (G) and the respective drinks (D) deriving from well spread geographical regions of Greece, as well

TABLE 1 | Kefir samples examined.

Sample*	Production	Geographical origin	Milk type	pH
1G	Home-made	Athens, Central Greece	Cow and Goat (1:1)	3.43
1D	Home-made	Athens, Central Greece	Cow and Goat (1:1)	4.17
2	Industrial	Kilkis, Northern Greece	Cow	4.40
3	Industrial	Pella, Northern Greece	Goat	3.72
4	Industrial	Sindos, Northern Greece	Buffalo	4.49
5	Industrial	Volos, Central Greece	Goat	4.24
6G	Home-made	Ioannina, North-western Greece	Cow	4.54
6D	Home-made	Ioannina, North-western Greece	Cow	4.37
7G	Home-made	Gythio, Southern Greece	Cow	5.18
7D	Home-made	Gythio, Southern Greece	Cow	5.95
8G	Home-made	Karditsa, Central Greece	Cow	3.90
8D	Home-made	Karditsa, Central Greece	Cow	3.80

**G and D denote grain and drink kefir samples, respectively.*

as four industrially produced kefir beverages (no grains present) were analyzed in this study (**Table 1**). Home-made samples were prepared from commercial pasteurized milk fermented with home-maintained grains (kept in milk or milk and tap water for 7–20 days at 4°C) for 18–24 h, at ambient temperature (25–30°C), while industrial samples were prepared from pasteurized milk fermented with commercial kefir starters. pH was measured using a digital pH meter (model 827 pH lab; Metrohm, Herisau, Switzerland).

Microbiological Analysis

The following groups of microorganisms were enumerated in both kefir grains and drinks: (1) thermophilic LAB in MRS agar (pH 6.2; Biokar Diagnostics, Beauvais, France) at 37°C for 3 days; (2) mesophilic LAB in MRS agar at 30°C for 3 days; (3) non-starter LAB (NSLAB) on Rogosa agar (Biokar) at 30°C for 5 days, anaerobically (double agar layer); (4) enterococci on kanamycin aesculin azide (KAA) agar (Merck, Darmstadt, Germany) at 37°C for 1 day; (5) AAB on medium called GYP, containing 1% w/v glucose (PanReac AppliChem, Darmstadt, Germany), 0.8% w/v yeast extract (Biokar), 1.5% w/v bacteriological peptone (Sigma-Aldrich, St. Louis, US) and 1.5% w/v agar (Condalab, Madrid, Spain) at 30°C for 2 days; (6) yeasts and molds on yeast extract glucose chloramphenicol (YGC) agar (Merck) at 25°C for 3–4 days; (7) Enterobacteriaceae on violet red bile glucose (VRBG) agar (Biokar) at 37°C for 1 day, anaerobically (double agar layer); (8) *Listeria monocytogenes* on Harlequin Listeria Chromogenic Agar (LabM) at 30°C for 1 day; (9) *Salmonella* spp. on xylose lysine deoxycholate (XLD) agar (LabM, Heywood, United Kingdom) at 37°C for 1 day; and (10) micrococci-staphylococci on mannitol salt agar (MSA) (Biokar) at 37°C for 1 day. Cycloheximide (Merck) was added (150 $\mu g\ mL^{-1}$) to media 1, 2, 3, and 5, in order to eliminate growth of yeasts (Camu et al., 2008). Results were expressed as log CFU g^{-1} (grains) or mL^{-1} (beverages).

Based on morphology (shape, color, and size), colonies were collected from MRS (37 and 30°C), Rogosa, GYP, and YGC agar plates, purified by repetitive streaking, and identified at the species level.

DNA Extraction and Rep-PCR Fingerprinting

Genomic DNA of bacterial isolates was extracted from a 2 mL overnight culture as described previously (Georgalaki et al., 2017). In addition, DNA extraction from yeast cells grown overnight in medium containing 5% w/v yeast extract and 20% w/v glucose was performed according to the protocol of Kopsahelis et al. (2009), with some modifications in two steps. First, cell pellet was washed twice with dd H_2O and incubated at 65°C for 10 min before lysis to decrease the content of PCR inhibitors, and second (last protocol steps), DNA pellet was washed with iced-cold 70% v/v ethanol and re-suspended in a small volume (30–50 μL) of Tris-EDTA (TE) buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8). DNA concentration of both bacteria and yeast isolates was measured using a Quawell Q5000 Read First photometer (Quawell Technology Inc., San Jose, CA, United States).

Repetitive extragenic palindromic elements-PCR (rep-PCR) analysis of bacteria isolates was performed according to Georgalaki et al. (2017). For yeasts, a slightly modified protocol of da Silva-Filho et al. (2005) was employed. In details, amplification was performed in 25 μl PCR reaction volume containing 50 ng DNA, 0.3 mM $(GTG)_5$ primer (5′-GTG GTG GTG GTG GTG-3′; VBC Biotech, Vienna, Austria) and 12.5 μl OneTaq-Quick Load 2× Master Mix (New England Biolabs, MA, United States). PCR was performed using a SimpliAmp Thermal Cycler (Thermo Fisher Scientific, Waltham, MA, United States) as follows: initial denaturation at 94°C for 5 min, 35 cycles with denaturation at 94°C for 15 s, primer annealing at 55°C for 45 s and primer extension at 72°C for 90 s, followed by a final extension at 72°C for 15 min.

Bacteria and yeast rep-PCR products were electrophoretically separated and the BioNumerics version 6.0 (Applied Maths, Ghent, Belgium) was used for rep-PCR fingerprints clustering (Georgalaki et al., 2017).

Bacteria and Yeasts Identification

Representative bacterial and yeast isolates based on the clustering of the rep-PCR analysis were identified at the species level by

sequencing the 16S rRNA gene and the ITS DNA region using the primers 16SF (5′-GGA GAG TTA GAT CTT GGC TCA G-3′)/16SR (5′-AGA AAG GAG GTG ATC CAG CC-3′) (Ntougias et al., 2006) and ITS1 (5′-TCC GTA GGT GAA CCT TGC GG-3′)/ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) (Korabecna, 2007), respectively. After electrophoresis, PCR products were purified using the NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel, Duren, Germany).

Amplicon-Based Metagenomics Analysis

Total DNA Extraction and Amplicon Sequencing

Microbial DNA from the kefir grains and beverages (home-made and industrial ones) was extracted using the DNeasyPowerSoil Kit (Qiagen, Valencia, CA, United States) and the DNeasyPowerFood Microbial Kit (Qiagen), respectively, according to the manufacturer's instructions. DNA was eluted in 30 μL of preheated (70°C) DNA-free PCR grade water and stored at −20°C until amplicon sequencing. DNA concentration and quality were determined using a Quawell Q5000 Read First photometer (Quawell Technology Inc.).

Amplicon sequencing (bTEFAP) was performed on the Illumina MiSeq sequencing platform at Molecular Research DNA (MR DNA, Shallowater, Texas). Bacterial diversity was evaluated through the amplification of the V1–V3 hypervariable region of the 16S rRNA gene using primers 27F (5′-AGR GTT TGA TCM TGG CTC AG-3′) and 519R (5′-GTN TTA CNG CGG CKG CTG-3′). On the other hand, primers ITS1F (5′-CTT GGT CAT TTA GAG GAA GTA A-3′) and ITS2R (5′-GCT GCG TTC TTC ATC GAT GC-3′) were used to amplify part of the ITS DNA region of yeasts/fungi, namely ITS1-ITS2. The PCR conditions and purification of amplicon products were performed according to Papademas et al. (2019). Operational taxonomic units (OTUs) were defined after removal of singleton sequences, clustered at 97% similarity and taxonomically assigned using the Nucleotide Basic Local Alignment Search Tool (BLASTn) against a curated National Center for Biotechnology Information (NCBI) deriving database (Dowd et al., 2008). Raw sequencing data are deposited at the European Nucleotide Archive (ENA) under the study PRJEB37688.

Alpha-and Beta-Diversity Analysis

Bacterial and yeast/fungal microbiota was analyzed in R version 3.6.3 using the R packages phyloseq and ggplot2, as well as several custom packages (Poirier et al., 2018). Therefore, one bacterial and one yeast/fungal phyloseq object was created, each containing three files, namely otu_table, in which the OTU abundances were normalized using the median sequencing depth of all samples analyzed, taxa_table and sample_data. These two datasets were used for all downstream analyses.

Alpha-diversity analysis was calculated according to the Observed species and the Shannon and inverse Simpson indexes for species diversity according to the abundance and uniformity of OTUs (Thukral, 2017). Analysis of variance (ANOVA) was performed to determine whether the differences among the samples were statistically significant, using a threshold value of 0.05. Moreover, clustered OTUs were used to construct the rarefaction curves in order to assess species richness and estimate the sequencing depth.

In addition, beta-diversity analysis was conducted to examine similarities and dissimilarities among the different food samples based on the bacterial and yeast/fungal microbiota. Thus, a MultiDimensional Scaling (MDS) Principle Coordinates Analysis (PCoA) based on Bray-Curtis distance metrics and a hierarchical clustering of Bray-Curtis using ward.d2 were performed on the bacterial and yeast/fungal communities taxonomically assigned at the family level (Whittaker, 1972).

RESULTS AND DISCUSSION

Classical Microbiological Analysis

Results obtained through the classical microbiological analysis are summarized in **Table 2**. In general, high populations of presumptive mesophilic and thermophilic LAB were counted in all kefir samples, no matter home-made, industrial, geographical origin, type of milk or pH of the product. More specifically, counts ranged from 6.50 to 9.60 log CFU mL^{-1} or g^{-1} (mesophilic LAB, home-made samples), from 6.60 to 9.20 log CFU mL^{-1} or g^{-1} (thermophilic LAB, home-made samples), from 6.38 to 9.15 log CFU mL^{-1} (mesophilic LAB, industrial samples) and from 5.32 to 8.60 log CFU mL^{-1} (thermophilic LAB, industrial, samples). Concerning the home-made samples, no differences were observed between grains and drinks in samples 6 and 8, while in samples 1 and 7, counts in grains were by 1–2 log higher than those in the respective drinks. This can be probably attributed to microbial aggregation and/or biofilm formation in the kefir grains resulting in a variability of biodiversity between grains and beverages, as well as to the variable microbial communities within the grain layers (Dobson et al., 2011; Wang et al., 2012). Accordingly, similar or even higher LAB populations than those of the present study have been reported in previous studies for kefir grain and their respective drink samples (Pintado et al., 1996; Guzel-Seydim et al., 2005; Kesmen and Kacmaz, 2011; Garofalo et al., 2015; Korsak et al., 2015). However, no significant differences were observed between grains and drinks (Guzel-Seydim et al., 2005; Kesmen and Kacmaz, 2011). Interestingly, the lowest LAB values were obtained with the industrial samples 4 and 5, namely 6.38 and 6.62 log CFU mL^{-1} at 30°C, and 5.32 and 6.02 log CFU mL^{-1} at 37°C, respectively. These results showed that only the industrial samples 2 and 3 complied with the 2003 Codex Alimentarius standard (7 log CFU mL^{-1}).

Furthermore, high populations of NSLAB were also counted in all kefir samples, ranging from 6.11 to 8.56 log CFU mL^{-1} (home-made kefir drinks), from 7.60 to 8.74 log CFU g^{-1} (home-made kefir grains) and from 6.41 to 8.33 log CFU mL^{-1} (industrial samples). As in the case of mesophilic and thermophilic LAB, no differences between grains and drinks were observed in samples 6 and 8, while, again, in samples 1 and 7 counts in grains were higher than those in the respective drinks. Similarly, the lowest NSLAB counts were obtained in the industrial samples 4 and 5. There are no reports on the population of NSLAB in kefir using Rogosa agar, although enumeration of

TABLE 2 | Microbial counts (log CFU g^{-1} or mL^{-1} ± *SD*) in the kefir samples examined.

	Home-made samples*			
	1G	**1D**	**6G**	**6D**
Thermophilic LAB (MRS agar, 37°C)	8.43 ± 0.02	7.29 ± 0.08	8.67 ± 0.02	8.60 ± 0.02
Mesophilic LAB (MRS agar, 30°C)	8.47 ± 0.13	7.27 ± 0.06	8.75 ± 0.04	8.54 ± 0.08
NSLAB (Rogosa agar, 30°C)	8.42 ± 0.04	6.11 ± 0.02	8.74 ± 0.17	8.56 ± 0.04
Enterococci (KAA agar, 37°C)	nd	nd	6.60 ± 0.03	5.60 ± 0.21
AAB (GYP agar, 30°C)	7.40 ± 0.08	6.11 ± 0.19	8.67 ± 0.02	7.78 ± 0.04
Yeasts and molds (YGC agar, 30°C)	6.61 ± 0.18	6.11 ± 0.08	6.32 ± 0.09	5.67 ± 0.01
Enterobacteriaceae (VRBG agar, 37°C)	nd	nd	1.00 ± 0.08	1.48 ± 0.03
Listeria monocytogenes (HALO10 agar, 30°C)	nd	nd	nd	nd
Salmonella spp. (XLD agar, 30°C)	nd	nd	nd	nd
Staphylococcus spp. (MSA agar, 37°C)	nd	nd	nd	nd
	Home-made samples*			
	7G	**7D**	**8G**	**8D**
Thermophilic LAB (MRS agar, 37°C)	9.20 ± 0.04	7.80 ± 0.08	6.60 ± 0.09	6.60 ± 0.29
Mesophilic LAB (MRS agar, 30°C)	9.60 ± 0.13	7.60 ± 0.02	6.50 ± 0.09	6.50 ± 0.02
NSLAB (Rogosa agar, 30°C)	8.40 ± 0.05	7.00 ± 0.09	7.60 ± 0.08	7.60 ± 0.06
Enterococci (KAA agar, 37°C)	nd	3.48 ± 0.05	0.00	0.00
AAB (GYP agar, 30°C)	6.40 ± 0.09	8.50 ± 0.02	7.10 ± 0.02	7.10 ± 0.03
Yeasts and molds (YGC agar, 30°C)	7.80 ± 0.05	5.70 ± 0.12	6.10 ± 0.05	6.10 ± 0.03
Enterobacteriaceae (VRBG agar, 37°C)	3.50 ± 0.02	6.50 ± 0.07	nd	nd
Listeria monocytogenes (HALO10 agar, 30°C)	nd	nd	nd	nd
Salmonella spp. (XLD agar, 30°C)	nd	nd	nd	nd
Staphylococcus spp. (MSA agar, 37°C)	nd	nd	nd	nd
	Industrial samples			
	2	**3**	**4**	**5**
Thermophilic LAB (MRS agar, 37°C)	8.03 ± 0.02	8.60 ± 0.02	5.32 ± 0.02	6.02 ± 0.05
Mesophilic LAB (MRS agar, 30°C)	9.15 ± 0.08	8.60 ± 0.07	6.38 ± 0.03	6.62 ± 0.10
NSLAB (Rogosa agar, 30°C)	8.01 ± 0.12	8.33 ± 0.13	6.41 ± 0.09	6.76 ± 0.02
Enterococci (KAA agar, 37°C)	nd	nd	2.48 ± 0.02	0.81 ± 0.07
AAB (GYP agar, 30°C)	8.01 ± 0.02	8.60 ± 0.02	6.77 ± 0.05	8.24 ± 0.02
Yeasts and molds (YGC agar, 30°C)	nd	nd	2.49 ± 0.08	5.63 ± 0.09
Enterobacteriaceae (VRBG agar, 37°C)	nd	nd	0.60 ± 0.02	1.40 ± 0.12
Listeria monocytogenes (HALO10 agar, 30°C)	nd	nd	nd	nd
Salmonella spp. (XLD agar, 30°C)	nd	nd	nd	nd
Staphylococcus spp. (MSA agar, 37°C)	nd	nd	nd	nd

**G and D denote grain and drink kefir samples, respectively; nd, not detected; Values are the mean ± SD (n = 3).*

kefir mesophilic lactobacilli and cocci using MRS and M17 agar, respectively, resulted in similar counts (Guzel-Seydim et al., 2005; Kesmen and Kacmaz, 2011).

Enterococci were detected in two out of the four home-made samples, namely 6 and 7, in both grains and drinks for sample 6 (6.60 log CFU g^{-1} in 6G, and 5.60 log CFU mL^{-1} in 6D), but only in the drink of sample 7 (7D; 3.48 log CFU mL^{-1}). Moreover, they were detected in two out of the four industrial samples, namely 4 and 5 at rather low counts of 2.48 and 0.81 log CFU mL^{-1}, respectively. To the best of our knowledge, no enterococci enumeration in kefir using KAA agar has been reported so far. However, *Enterococcus faecalis* and *Enterococcus durans* have been isolated from Tibetan kefir beverages (Yang et al., 2007), *E. durans* has been isolated from kefir grains belonging to the Centro de Investigación y Desarrollo en Criotecnología de Alimentos (CIDCA) collection (Carasi et al., 2014), while *Enterococcus* spp. isolates have been identified in home-made or commercial kefir grains on M17 agar (Garofalo et al., 2015). Thus, the presence of enterococci in kefir is mostly ambiguous. It should be stressed that although enterococci comprise a significant part of many fermented foods, they are, at the same time, considered as indicators of poor hygienic processing conditions, with strains of some species exhibiting virulence factors (Foulquié Moreno et al., 2006).

Moreover, high microbial counts on GYP agar plates (dedicated for AAB) were observed, with values ranging between 6.40 and 8.67 log CFU g^{-1} for grain samples, and 6.11 and 8.60 log CFU mL^{-1} for drink samples, either home-made or industrial. However, isolates from the GYP agar plates were found to be either Gram-positive bacteria or yeasts. Problematic cultivation and enumeration of AAB from natural environments has been already reported (Camu et al., 2008; Gulitz et al., 2011). Some authors assign this to the viable but non-culturable state switch of AAB under not favorable growth media and conditions (Gomes et al., 2018). Thus, while Pintado et al. (1996) and Witthuhn et al. (2005) reported the absence of AAB in Portuguese and African kefir grains, respectively, Garofalo et al. (2015), using three different growth media (all containing cycloheximide at 400 $\mu g\ mL^{-1}$), reported AAB viable counts in different grain samples and of different origin, ranging from 3 to 6 log CFU g^{-1}.

Yeasts were detected in all home-made samples, both grains and drinks, with counts ranging from 6.10 to 7.80 log CFU g^{-1} (grain samples) and from 5.67 to 6.11 log CFU mL^{-1} (drink samples). On the other hand, they were only detected in two out of the four commercial samples, namely 4 and 5, at 2.49 and 5.63 log CFU mL^{-1}, respectively. Consequently, only the commercial sample 5 met the Codex Alimentarius standard for yeasts. Similar counts, ranging between 5.0 and 6.55 log CFU g^{-1} or mL^{-1}, were reported by Korsak et al. (2015) for grains, either home-made or found in the market, and by Guzel-Seydim et al. (2005) for grains and drinks produced at a Turkish University, whereas Garofalo et al. (2015) reported yeast populations at 7 log CFU g^{-1} for grain samples produced by various suppliers. Additionally, Kalamaki and Angelidis (2016) reported that in kefir samples produced in Greece, yeasts were counted at 7.7 log CFU g^{-1} in grains and from < 0.4 to 6.7 log CFU mL^{-1} in drinks. In all cases however, yeast populations were lower than the mesophilic and thermophilic LAB.

Enterobacteriaceae were detected in two of the four home-made samples, namely 6 and 7, in both grains (1.0 and 3.5 log CFU g^{-1}, respectively), and drinks (1.48 and 6.5 log CFU mL^{-1}, respectively). Interestingly, *Enterobacteriaceae* population of the home-made sample 7D was higher (3 log difference) than the respective grain sample 7G, which indicates the advantageous milk environment for the growth of this bacterial group. They were also detected in two of the four industrial samples, namely 4 and 5, at low levels of 0.6 and 1.4 log CFU mL^{-1}, respectively, though. Samples with > 0.7 log CFU mL^{-1} do not fulfill the above-mentioned Commission Regulation microbiological criteria for liquid dairy products, which requires presence of *Enterobacteriaceae* < 0.7 log CFU mL^{-1}. The detection of both enterococci and *Enterobacteriaceae* in the same industrial samples, namely 4 and 5, as well as home-made samples 6 and 7, could be probably attributed to deficient hygienic handling and storage conditions. Detection of *Enterobacteriaceae* has been reported by Chen et al. (2008) who isolated an *Escherichia coli* strain from Taiwanese kefir grains and attributed its occurrence to possible environmental contamination.

Finally, no growth was observed on the media used for *L. monocytogenes, Salmonella* spp., and *Staphylococcus* spp., and in any of the samples examined; thus all samples fulfilled the microbiological criteria of the Commission Regulation (2005) No 2073/2005.

Strain Fingerprinting and Identification of Microbial Isolates

Based on colony morphology, a total of 123 isolates, including 91 bacteria and 32 yeasts, were selected from the MRS (37 and 30°C), Rogosa, GYP, and YGC agar plates. Rep-PCR analysis clustered bacteria in 27 and yeasts in 10 groups. Representative isolates of all groups were selected and subjected to 16S rRNA gene and ITS DNA region sequencing, respectively.

According to the sequencing results (**Supplementary Table S1A** and **Supplementary Figure S1**), among the 91 bacterial isolates, 31 isolates were identified as *Lentilactobacillus kefiri* (basonym *Lactobacillus kefiri*), 16 as *Leuconostoc mesenteroides,* 12 as *Lacticaseibacillus rhamnosus* (basonym *Lactobacillus rhamnosus*), eight as *Streptococcus thermophilus*, seven as *Lactococcus lactis*, seven as *Leuconostoc mesenteroides/pseudomesenteroides,* four as *Enterobacter clocae/ludwigii/kobei*, two as *Staphylococcus warneri*, one as *L. delbrueckii* subsp. *bulgaricus,* one as *Lactococcus raffinolactis,* one as *Streptococcus parauberis,* and one as *Klebsiella oxytoca*. Interestingly, all bacterial species used as starters for the production of industrial sample 4 were isolated and identified (*L. lactis*, *S.*thermophilus, and *Leuconostoc* sp.), while information on the starters used for the rest of the samples was not available. Additionally, *L. kefiri* was found exclusively in home-made samples where it is considered the dominant species (62.0%) along with *L. mesenteroides/pseudomesenteroides* (30.0%), whereas *S.* thermophilus was only found in the industrial ones. The dominant species identified in the industrial samples were *L. rhamnosus* (29.3%), *S. thermophilus* (22.0%), and *L. mesenteroides/pseudomesenteroides* (19.5%) depending on the sample analyzed. Interestingly, the eight isolates belonged to species, which are considered (opportunistic) pathogens, i.e., *S. warneri, E. clocae/ludwigii/kobei, S. parauberis,* and *K. oxytoca* were all isolated from the home-made sample 7 (the *S. warneri* from the grains and the other from the drink), which indicates poor hygiene practices in the production of sample 7. The presence of the bacterial species identified has been routinely reported in kefir (Pintado et al., 1996; Chen et al., 2008; Miguel et al., 2010; Kesmen and Kacmaz, 2011). However, to the best of our knowledge, this is the first time that the above mentioned (opportunistic) pathogens are detected in kefir samples.

Among the 32 yeast isolates (**Supplementary Table S1B** and **Supplementary Figure S2**), 14 were identified as *Kluvyeromyces marxianus*, nine as *Saccharomyces cerevisiae,* three as *Kazachstania turicensis,* two as *Geotrichum candidum/Galactomyces candidum,* two as *Yarrowia lipolytica,* one as *Pichia kudriavzevii,* and one as *Debaryomyces hansenii. K. marxianus* and *S. cerevisiae* were the dominant species in the home-made samples, while four out of the six isolates of the industrial samples belonged to *K. marxianus* and *Geotrichum candidum/Galactomyces candidum*. Interestingly, no yeasts were obtained from the industrial samples 2 and 3, while *D. hansenii,* which was the yeast species used as starter for the production of

industrial sample 4 was isolated and identified. *K. marxianus* and *S. cerevisiae* are considered as the predominant species in kefir (43.8 and 28.1%, respectively). *K. marxianus* and *K. turicensis* play an important role in kefir grain formation (Wang et al., 2012), *Geotrichum candidum* can be found at the early stages of kefir production and it covers the grain surface (Witthuhn et al., 2005), while *Y. lipolytica* and some *Pichia* species are of significant importance in the production of fermented milks, such as kefir and koumiss (Fleet, 2006). Similar results have been reported in kefir samples from Argentine (Diosma et al., 2014), Italy (Garofalo et al., 2015) and Africa (Witthuhn et al., 2005).

Amplicon-Based Metagenomics Analysis

Sequencing Data of Microbial Communities in Kefir Grains and Beverages

A total of 1,061,686 bacterial raw sequences were obtained from the 12 samples analyzed, i.e., four kefir grains (samples 1G, 6G, 7G, and 8G) and the respective home-made drinks (samples 1D, 6D, 7D, and 8D) as well as four industrial beverages (samples 2, 3, 4, and 5). After the quality control of the 16S reads, 666,437 sequences were used for taxonomic classification, with an average of 55,536 ± 9,374 sequences per sample. In total, 160 bacterial OTUs were assigned among the samples. Interestingly, the average number of OTUs was similar among the three food sample groups, i.e., 73 ± 22, 75 ± 12, and 68 ± 17 OTUs per kefir grain, home-made and industrial drinks, respectively. On the contrary, the number of yeast/fungal raw sequences obtained from the 12 samples was higher compared to that of bacterial sequences, i.e., 1,413,675, as well as the number of sequences that passed the quality control, i.e., 938,653, with an average of 78,221 ± 52,180 sequences per sample. Among the 12 samples analyzed, 463 yeast/fungal OTUs were identified, ranging from 113 (sample 6D) to 301 (sample 2) OTUs, with an average of 148 ± 12, 149 ± 25, and 250 ± 58 OTUs per kefir grain, home-made, and industrial drinks, respectively.

Alpha-and Beta-Diversity Analysis

The rarefaction curve analysis was performed to evaluate the sufficiently recovered OTUs by the Illumina MiSeq sequencing. Rarefaction curves of both 16S and ITS data of the majority of the samples analyzed attained the saturation plateau, indicating that the sequencing depth was sufficient (**Figure 1**). It should be noted though, that rarefaction curves of the ITS data for kefir grains 1G, 7G, and 8G, home-made drink 1D and industrial drinks 2 and 5, did not tend to approach the saturation plateau, indicating that the yeast/fungal richness in these samples was probably underestimated (**Figure 1B**).

The microbial complexity (richness and evenness) was estimated on the basis of alpha-diversity indices, namely Observed, Shannon, and inverse Simpson. The richness estimation according to Observed species, indicated that the yeast/fungal microbiota of industrial beverages was significantly higher ($P < 0.05$) compared to that of home-made drinks and kefir grains, while the species richness of bacterial communities did not differ significantly among the samples (**Figure 2**). The microbial richness based on Observed species was strongly supported also by the rarefaction curves analysis, as mentioned above (**Figure 1**). On the other hand, a significant difference ($P < 0.05$) was observed in Shannon and inverse Simpson indices of diversity, in both bacterial and yeast/fungal communities among the samples (**Figure 2**). In details, bacterial microbiota of home-made beverages was found to be the most abundant, followed by that of industrial drinks and kefir grains (**Figure 2A**). However, this was not the case for yeast/fungal microbiota, since the abundance of industrial beverages was higher than that of home-made drinks and kefir grains (**Figure 2B**).

To further explore the degree of diversity among the samples, an MDS/PCoA plot and a hierarchical clustering were generated based on OTUs that were taxonomically assigned at the family level. As shown in **Figure 3A**, two major clusters were observed in MDS/PCoA plot based on bacterial microbiota; the first, included the kefir grains 1G, 7G, and 8G, and the home-made drink 1D, and the second one contained the industrial beverages 2, 4, and 5. The specific grouping pattern of samples was also evident by the hierarchical clustering (**Figure 3C**). On the other hand, MDS/PCoA and hierarchical clustering based on the yeast/fungal communities grouped together the majority of the samples, i.e., all kefir grains (samples 1G, 6G, 7G, and 8G), home-made drinks 1D, 7D, and 8D and industrial drink 5, indicating that the majority of the samples analyzed shared a yeast/fungal microbiota at the family level (**Figures 3B,D**).

Phylogenetic Composition of the Bacterial Microbiota

To further analyze the microbial community structure of the samples, bacterial, and yeast/fungal OTUs were used to calculate relative abundances of taxa. Although V1–V3 region of the 16S rRNA gene offers the discriminatory power for bacterial identification at the species level (Johnson et al., 2019), due to the high-level similarity between closely related taxa, both bacterial and yeast/fungal microbiota of the samples were evaluated up to the genus level for a more accurate identification.

The bacterial microbiota of the 12 kefir grains and drinks analyzed, was covered by seven phyla, in which *Firmicutes* was the dominant with relative abundances ranging from 33.17 to 99.18%, followed by *Proteobacteria*, which was identified mainly in the home-made drinks 6D (61.35%), 7D (66.80%), and 8D (53.22%), and *Actinobacteria* found mostly in the industrial beverage 2 (8.57%) (**Figure 4A** and **Supplementary Table S2A**). Among *Firmicutes* and *Proteobacteria*, families *Lactobacillaceae and Streptococcaceae,* as well as *Pseudomonadaceae, Enterobacteriaceae,* and *Moraxellaceae,* respectively, were the most abundant (**Figure 4B** and **Supplementary Table S2B**). In details, the family *Lactobacillaceae* was the predominant in kefir grains 1G, 7G, and 8G, accounting for approximately 96% of the bacterial sequences. However, a more diverse microbiota was identified in grain sample 6G, which consisted of bacteria belonging to three main families, namely *Lactobacillaceae* (45.10%), *Streptococcaceae* (44.66%), and *Moraxellaceae* (7.87%). Although influenced by the microbial communities of kefir grains, the bacterial microbiota in respective home-made drinks was more diverse, apart from sample 1, in which the family *Lactobacillaceae* was predominant in both home-made kefir grains (1G: 97.30%) and drink (1D: 91.19%). Specifically, in the home-made drink 6D, bacterial family *Pseudomonadaceae*

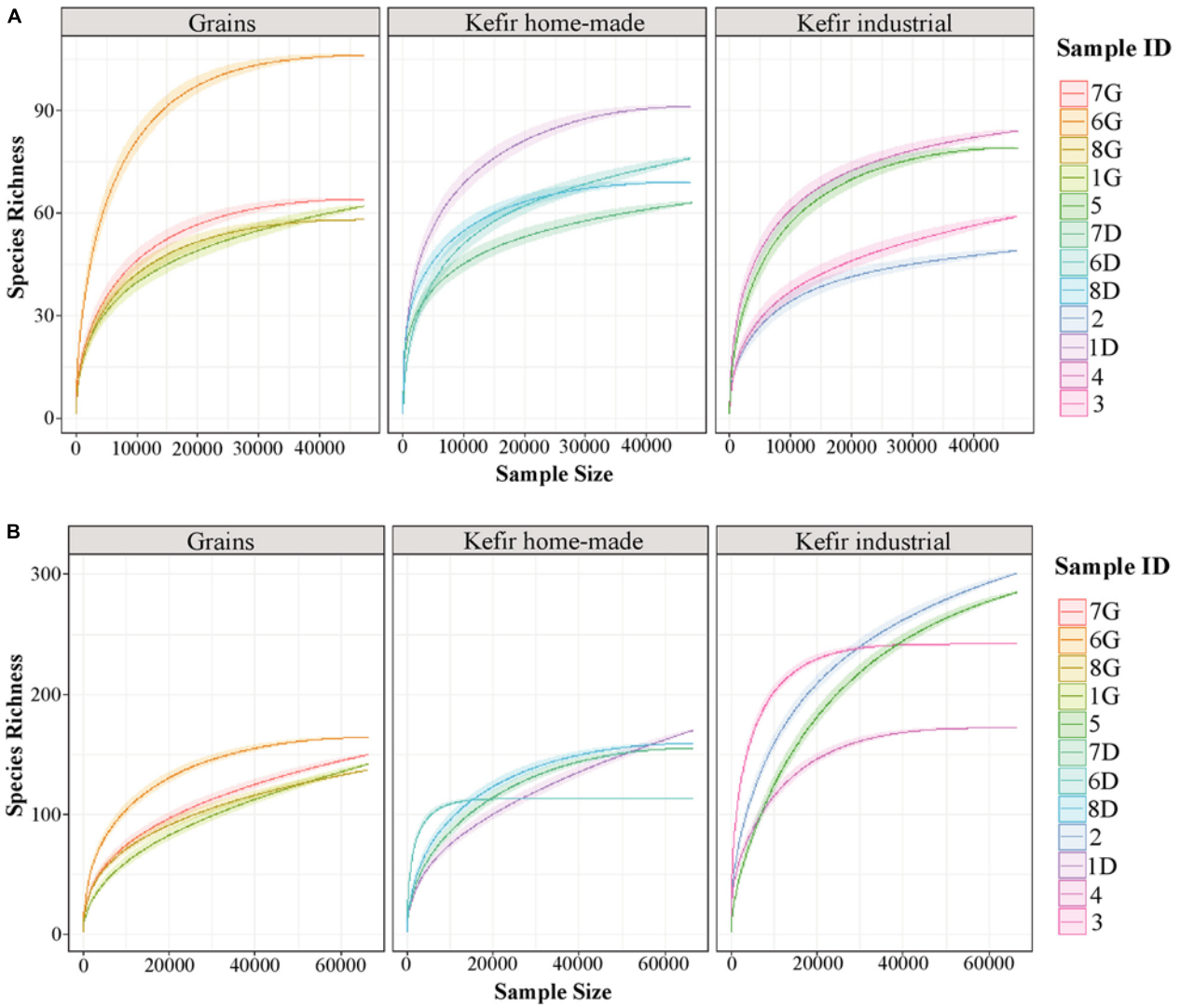

FIGURE 1 | Rarefaction curves of species richness showing the sequencing depth of 16S **(A)** and ITS **(B)** data obtained from kefir grains (samples 1G, 6G, 7G, and 8G), home-made (samples 1D, 6D, 7D, and 8D) and industrial (samples 2, 3, 4, and 5) drinks. The rarefaction curves for each sample were displayed by different colors. The *x*-axis represents the sequencing depth in number of reads and the *y*-axis the estimation of the OTU richness detected at species level.

(60.31%) was found to be the most abundant, followed by *Lactobacillaceae* (19.20%) and *Streptococcaceae* (18.34%). In contrast, the family *Enterobacteriaceae* was the dominant (64.98%) in home-made drink 7D, followed by *Streptococcaceae* (26.57%) and *Lactobacillaceae* (6.60%). The dominance of *Enterobacteriaceae* family in sample 7D, together with either the absence or the relatively low abundances of this family in the other kefir grains and drink samples ($<$ 4.05%), was in accordance with the results of the classical microbiological analysis, since *Enterobacteriaceae* counts were higher in sample 7D (6.5 log CFU mL^{-1}) compared to the other samples ($\leq$ 3.50 log CFU mL^{-1} or CFU g^{-1}; **Table 2**). In addition, the microbial community structure of home-made drink 8D mainly consisted of bacteria belonging to the families *Lactobacillaceae* (45.68%), *Moraxellaceae* (31.20%), *Pseudomonadaceae* (15.62%), and *Shewanellaceae* (6.11%). As it was expected, bacterial microbiota of industrial beverages was less diverse compared to that of the home-made drinks, as also revealed by the alpha-diversity indices (**Figure 2A**). This is not surprising, as dairy industries use commercial starter cultures for a well-controlled fermentation that results in a standardized and safe final product. Bacterial communities identified in the industrial drinks (samples 2, 3, 4, and 5) belonged to three main families, namely *Streptococcaceae, Lactobacillaceae,* and *Bifidobacteriaceae,* with varying abundances among the samples. A closer look at the bacterial community structure classified at the genus level, revealed a similar distribution pattern to that observed at the family level, as one or two genera corresponded to all reads assigned to that family (**Figure 4C** and **Supplementary Table S2C**). Therefore, the genus *Lactobacillus*

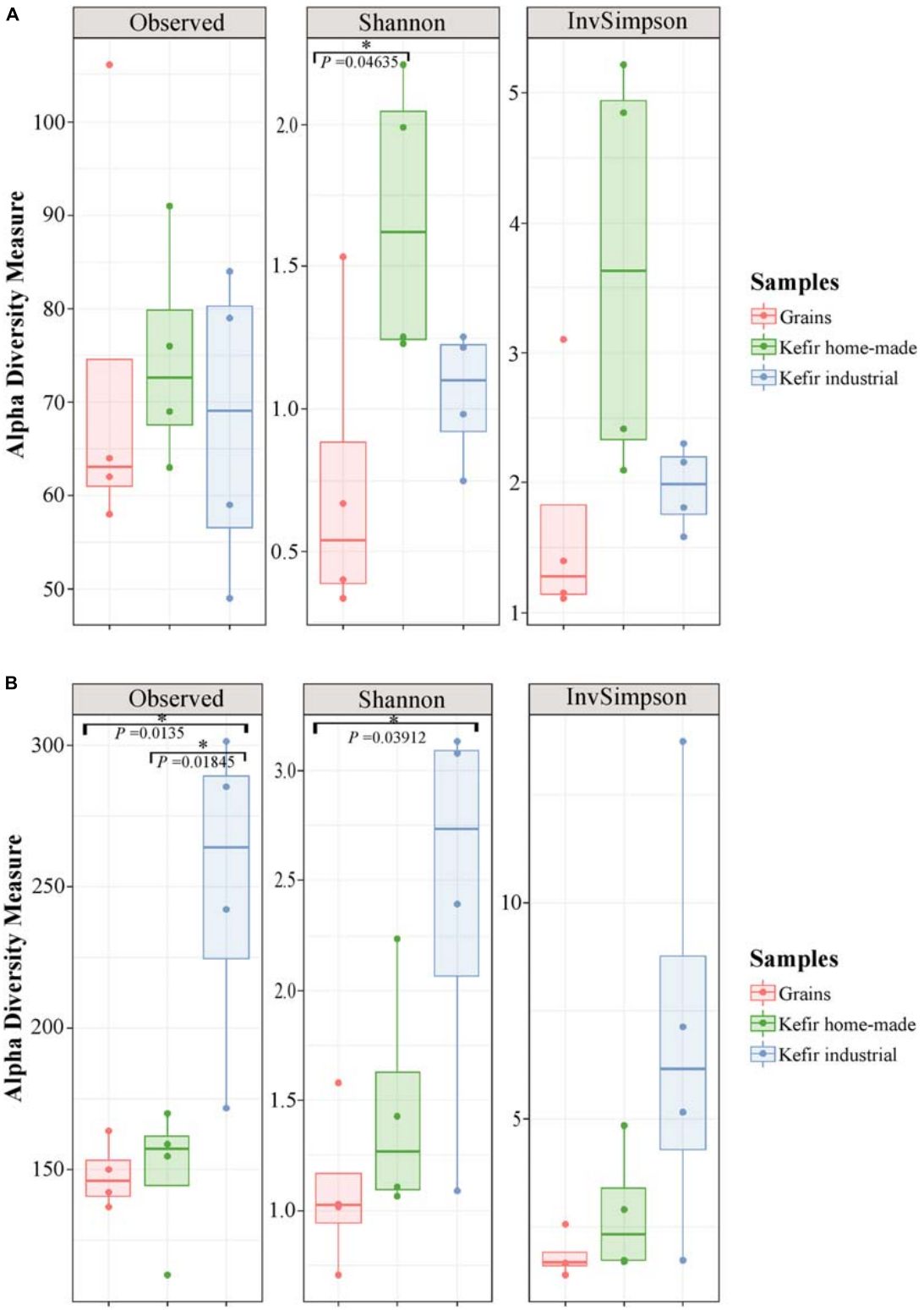

FIGURE 2 | Boxplots of alpha-diversity indices, namely Observed, Shannon, and inverse Simpson for bacterial **(A)**, and yeast/fungal **(B)** communities identified in kefir grains, home-made, and industrial drinks. Samples are colored according to the different food groups, i.e., red for kefir grains, green for home-made drinks and blue for industrial beverages. An asterisk denoted $P < 0.05$.

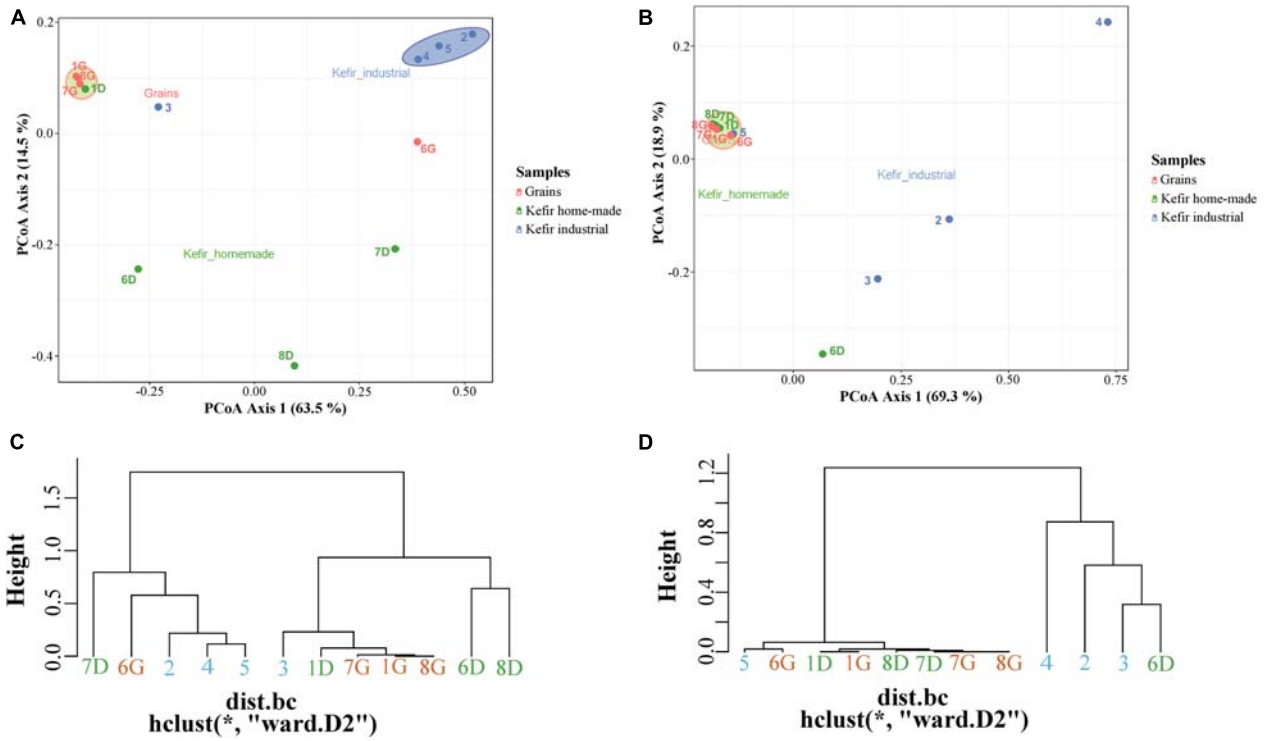

FIGURE 3 | Principal coordinates analysis (PCoA) among bacterial **(A)** and yeasts/fungal **(B)** communities found in kefir grains (samples 1G, 6G, 7G, and 8G), home-made (samples 1D, 6D, 7D, and 8D) and industrial (samples 2, 3, 4, and 5) drinks. Hierarchical clustering of bacterial **(C)** and yeasts/fungal **(D)** communities identified in kefir grains, home-made, and industrial drinks. All OTUs in both PCoA and hierarchical clustering within the communities were taxonomically assigned at the family level. Samples are colored according to the different food groups, i.e., red for kefir grains, green for home-made drinks and blue for industrial beverages.

was the most abundant in kefir grains 1G (93.70%), 7G (84.48%), and 8G (95.51%), as well as in home-made drink 1D (65.18%). Furthermore, the genus *Lentilactobacillus* was also identified in relatively high abundances in samples 1G (3.37%), 7G (11.63%), 8G (1.51%), 1D (25.75%), 7D (2.65%), and 8D (6.51%). This was in consistent with the identification results obtained from the culture-dependent analysis, since *L. kefiri* was found to be the dominant species in these samples (**Supplementary Table S1A**). It is interesting though, that 7G was the only sample analyzed in which classical microbiological analysis identified two out of seven bacterial isolates as *S. warneri,* as mentioned above (**Supplementary Table S1A**). However, at the same time, none of the 16S sequences obtained from the metagenomics analysis for sample 7G was taxonomically assigned at the genus *Staphylococcus* (**Supplementary Table S2C**). Therefore, as *S. warneri* is a skin commensal of humans and animals (Kloos and Schleifer, 1975), the presence of the species in sample 7G could potentially be attributed to contamination during classical microbiological analysis. Furthermore, according to the results of the 16S metagenomics analysis, bacterial microbiota of kefir grains 6G was dominated by the genera *Lactococcus* (44.17%), *Leuconostoc* (37.15%), *Acinetobacter* (7.86%), and *Lactobacillus* (6.24%), while the respective home-made drink (sample 6D) by the *Pseudomonas* (60.31%), *Lactobacillus* (14.73%), and *Lactococcus* (17.96%) genera. On the other hand, classical microbiological analysis identified *L. mesenteroides* and *L. lactis* as the dominant species in samples 6G and 6D, respectively (**Supplementary Table S1A**). Moreover, *Lactobacillus* (39.02%), *Acinetobacter* (31.20%), *Pseudomonas* (15.62%), *Lentilactobacillus* (6.51%), and *Shewanella* (6.11%) were the most abundant genera found in home-made sample 8D. On the other hand, though, *Leclercia* (31.46%), *Klebsiella* (22.74%), *Lactococcus* (22.73%), *Lentilactobacillus* (2.65%), and *Lactobacillus* (2.22%) were the main genera of the home-made sample 7D bacterial microbiota, whereas *Enterobacter* species were mostly identified during microbiological analysis (∼56% of the bacterial isolates). However, the abundance of the genus *Enterobacter* was relatively low, i.e., 1.34% (**Supplementary Table S2C**). It should be noted, that the relatively high abundances of genera, such as *Pseudomonas, Leclercia, Klebsiella, Acinetobacter,* and *Shewanella,* in most of the home-made drinks analyzed, probably reflect the home-made production of these drinks.

In contrast, the bacterial communities of industrial beverages, namely 2, 3, 4, and 5, was dominated by common genera used as starter or adjunct cultures in dairy industry. More specifically, the genera *Streptococcus* (1.25–72.58%), *Lactococcus* (0.68–90.50%), *Lactobacillus* (1.06–78.52%), *Lacticaseibacillus* (0.03–14.06%), and *Bifidobacterium* (0.02–8.57%), were found to be the dominant ones with varying abundances among

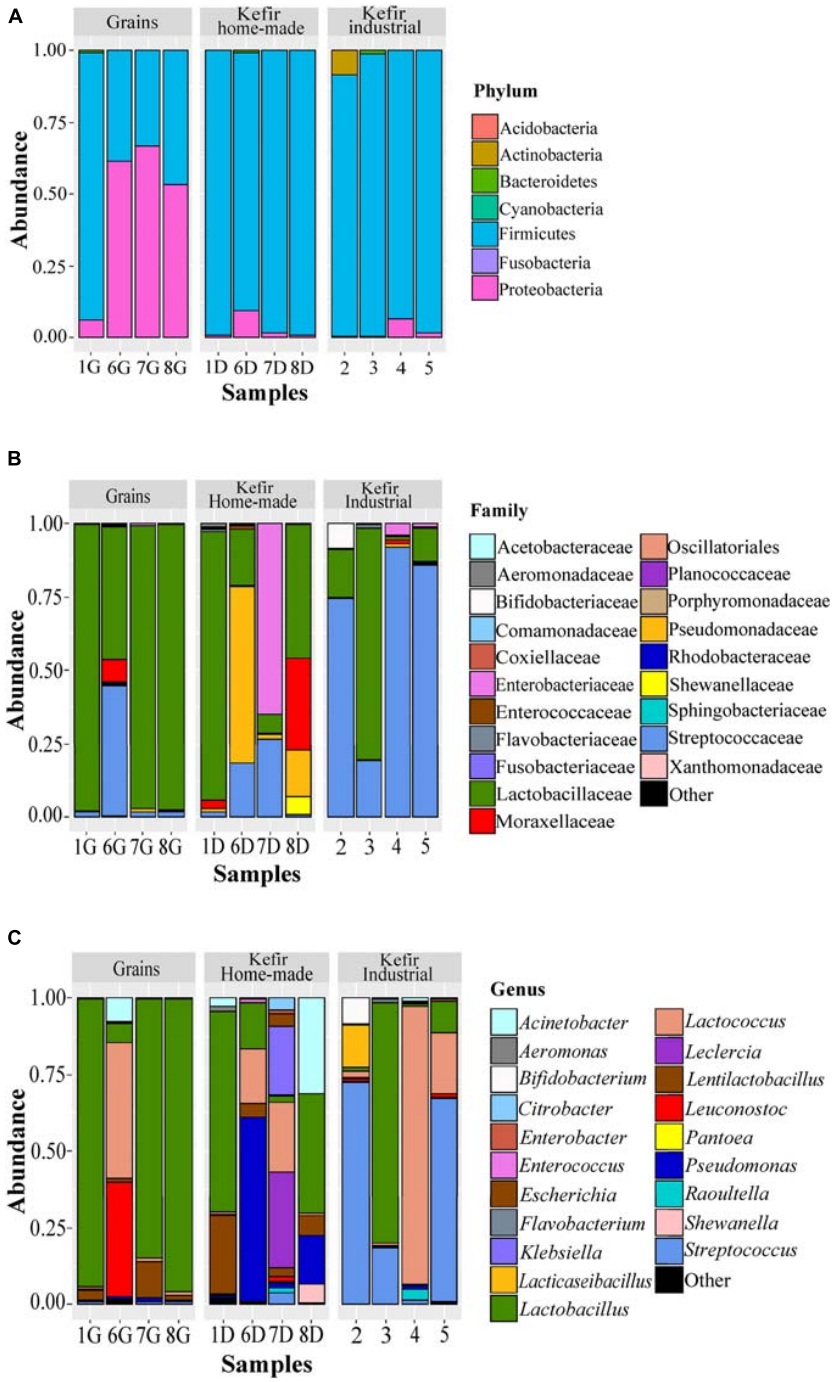

FIGURE 4 | Composition plots of the relative abundances of the 20 most abundant bacterial OTUs taxonomically assigned at the phylum **(A)**, family **(B)**, and genus **(C)** level in kefir grains (samples 1G, 6G, 7G, and 8G), home-made (samples 1D, 6D, 7D, and 8D) and industrial (samples 2, 3, 4, and 5) drinks. Samples from each food group, i.e., kefir grains, home-made, and industrial drinks, are presented together in subpanels.

samples (**Figure 4C** and **Supplementary Table S2C**). As it was expected, the results of the classical microbiological analysis were consistent with those of the 16S metagenomics analysis. All the bacterial isolates from the industrial samples were identified as *S. thermophilus, L. delbrueckii* subsp. *bulgaricus, L. lactis, L. mesenteroides,* and *L. rhamnosus* (**Supplementary Table S1A**). Furthermore, as also mentioned above, even though high microbial counts were observed in GYP medium for all samples analyzed (6.11–8.67 log CFU mL^{-1} or CFU g^{-1}; **Table 2**), Gram staining revealed that the majority of the isolates

corresponded to either Gram-positive bacteria or yeasts. The almost absence of AAB using classical microbiological analysis was consistent with the results of the metagenomics analysis, since the family *Acetobacteraceae* was found only in two samples, namely 6G and 6D in trace levels, i.e., 0.09 and 0.25%, respectively (**Supplementary Table S2B**). However, several HTS studies have demonstrated that the genus *Acetobacter* is commonly found in both kefir grains and beverages as part of the subdominant bacterial microbiota (Leite et al., 2012; Gao et al., 2013; Marsh et al., 2013; Garofalo et al., 2015; Korsak et al., 2015; Walsh et al., 2016). Moreover, relatively high counts were also observed in KAA medium for samples 6G, 6D, 7D, 4, and 5 (**Table 2**). However, the genus *Enterococcus* was found in trace amounts (< 0.32%) in all samples except of sample 5 (1.07%; **Supplementary Table S2C**). Although not frequently, the genus *Enterococcus* has been identified in kefir grains and beverages as part of the subdominant microbiota (Dobson et al., 2011; Marsh et al., 2013; Nalbantoglu et al., 2014; Dertli and Con, 2017). In addition, the absence of *Listeria monocytogenes* and *Salmonella* spp. in all samples analyzed was confirmed by both classical microbiological and amplicon based-metagenomics analyses (**Table 2** and **Supplementary Tables S1A**, **S2C**).

The identification of *Lactobacillaceae*/*Lactobacillus* as the dominant bacterial taxa in kefir grains and beverages has already been established by previous HTS studies. However, due to the new taxonomy of LAB, which was recently proposed by Zheng et al. (2020), the genus *Lentilactobacillus* should also be considered as part of the dominant bacteria microbiota of kefir grains and beverages since it includes, among others, the species *L. kefiri*. Apart from *Lactobacillus*, 16S rRNA metagenomics analysis of six Italian kefir grains, i.e., four home-made, one from the University of Perugia and one from a biotechnology company, revealed that although of different origins, the subdominant microbiota of all samples was in high concordance and consisted mainly of the genera *Acetobacter, Streptococcus,* and *Lactococcus* (Garofalo et al., 2015). Furthermore, HTS analysis of one kefir grain from the Ege University in Turkey and five home-made samples from different regions of the country revealed also highly similar communities dominated by *Lactobacillus* and several genera as part of the rest microbiota, such as *Pediococcus, Leuconostoc, Enterobacter,* and *Acinetobacter* (Nalbantoglu et al., 2014; Dertli and Con, 2017). However, there are also HTS studies that reported the identification of almost only *Lactobacillus* in kefir grains and beverages (Dobson et al., 2011; Leite et al., 2012; Zamberi et al., 2016). In addition, *Lactobacillus* and *Acetobacter* were found to be the most abundant genera identified in 28 kefir grains, either home-made or commercial, from eight distinct regions (Ireland, United Kingdom, United States, Spain, France, Italy, Canada, and Germany), whereas *Lactococcus*, *Acetobacter*, *Lactobacillus,* and *Leuconostoc* dominated the bacterial microbiota of the associated beverages (Marsh et al., 2013; Walsh et al., 2016). It should be noted though, that in a few studies, the genus *Lactobacillus* was not the predominant in kefir grains. According to Gao et al. (2013), HTS analysis of four Tibetan grains from different areas in China revealed that the genus *Lactococcus* was the most abundant (40.93–72.02%), while *Lactobacillus* was found to be among the subdominant microbiota along with *Acetobacter* and *Shewanella*. This was also the case for one commercial kefir grain sample from Belgium, in which the genus *Lactococcus* was the most abundant (93.7%), followed by *Leuconostoc* and *Lactobacillus* (Korsak et al., 2015). In the same study, four additional Belgian kefir grain samples were analyzed, i.e., two home-made and two obtained from the Ministry of Agriculture, and according to the 16S metagenomics analysis all samples were dominated by the genus *Lactobacillus* (> 85%), followed by *Gluconobacter*, *Lactococcus*, *Enterobacter,* and *Acetobacter*. Interestingly, although the respective kefir drinks comprised the same microbiota members, the relative abundances of the subdominant genera significantly increased in drinks compared to grains (Korsak et al., 2015). A similar trend was also observed in our samples, namely 7 and 8, since the bacterial microbiota of both 7G and 8G was dominated by the genus *Lactobacillus* (> 84%), while that of 7D and 8D was highly diverse including several genera, such as *Lactococcus*, *Klebsiella*, *Leclercia,* and *Acinetobacter* (**Figure 4C** and **Supplementary Table S2C**). To the best of our knowledge, this is the first study reporting the presence of *Acinetobacter* in kefir beverages. Although the genus is commonly present in soil and water (Baumann, 1968), *Acinetobacter* species have been also found in raw milk and cheeses, and contribute to the organoleptic characteristics of the final product due to their lipolytic and proteolytic activities (Fuka et al., 2010; Gurung et al., 2013; Pangallo et al., 2014). It should be noted however, that some *Acinetobacter* species, such as *Acinetobacter baumannii,* are considered as opportunistic pathogens mainly associated with hospital-acquired infections (de Amorim and Nascimento, 2017).

Phylogenetic Composition of the Yeasts/Fungal Microbiota

The yeast/fungal microbiota of kefir grains and beverages was characterized by a high level of *Ascomycota* phylum with relative abundances ranging from 79.18 to 99.93%. It should be noted however, that yeasts/fungi belonging to the phylum *Basidiomycota* were found to be present in relatively high abundances in industrial beverages 2 (9.66%), 3 (11.94%), and 4 (12.82%) (**Figure 5A** and **Supplementary Table S3A**). Compared to bacteria, yeasts/fungal microbiota classified at the family level was less diverse in kefir grains and home-made drinks, with *Saccharomycetaceae* being the dominant family (> 95.00%) in all samples but 6D, in which *Saccharomycetaceae* and *Dipodascaceae* were identified in similar abundances, i.e., 49.12 and 48.17%, respectively (**Figure 5B** and **Supplementary Table S3B**). The identification of *Dipodascaceae* family in drink sample 6D, could be explained by the proportion of the same family in kefir grain 6G (2.81%). However, based on the two proportions, i.e., 48.17 and 2.81% for samples 6D and 6G, respectively, we can assume that the milk environment favored the abundance of yeasts/fungi belonging to the family *Dipodascaceae*. Moreover, as also revealed by the alpha-diversity indices (**Figure 2B**), the diversity of industrial beverages was significantly higher than that of home-made ones (**Figure 5B**). It was interesting though, that this was not the case for the industrial drink sample 5, in which *Saccharomycetaceae* was the

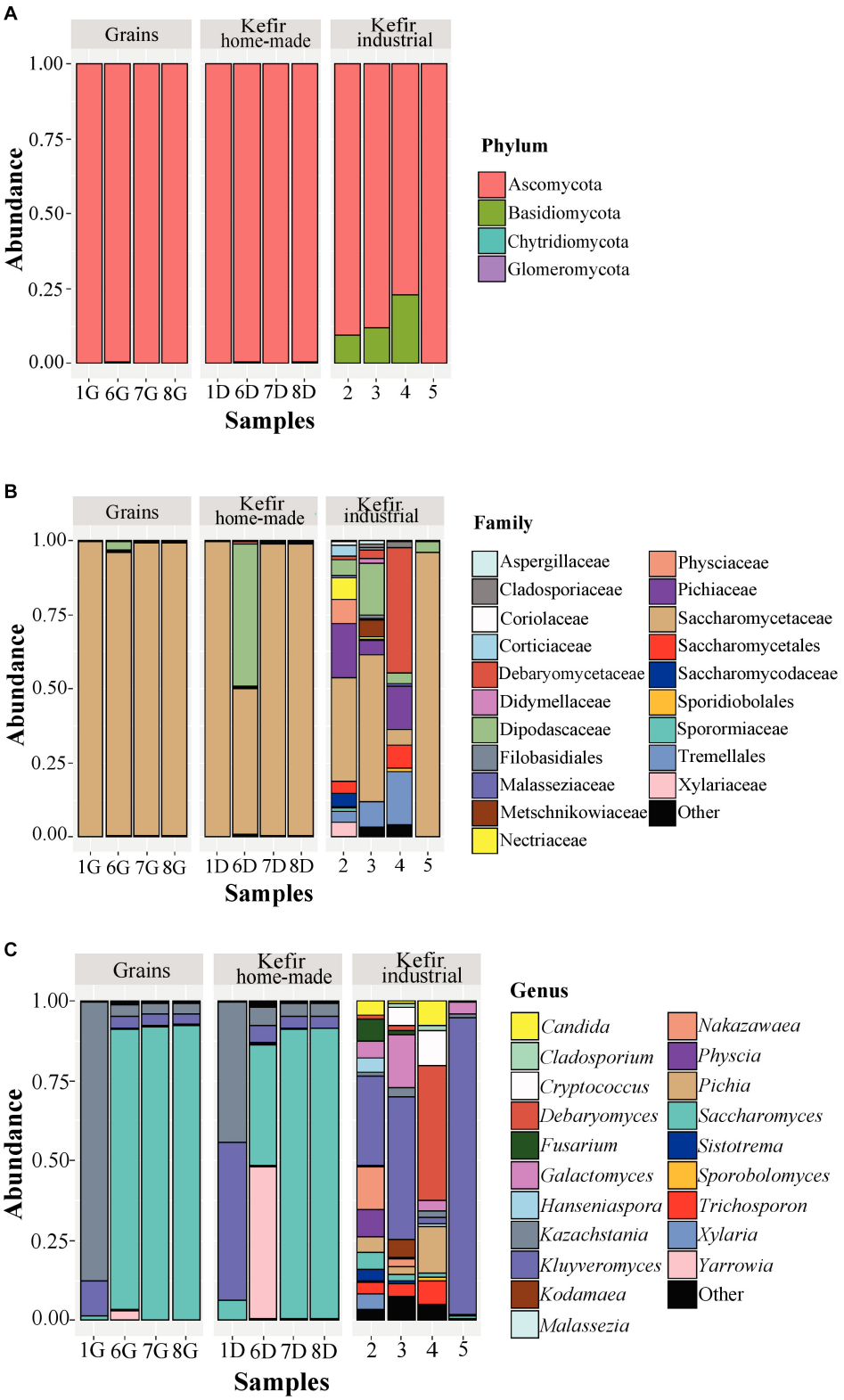

FIGURE 5 | Composition plots of the relative abundances of the 20 most abundant yeast/fungal OTUs taxonomically assigned at the phylum **(A)**, family **(B)**, and genus **(C)** level in kefir grains (samples 1G, 6G, 7G, and 8G), home-made (samples 1D, 6D, 7D, and 8D) and industrial (samples 2, 3, 4, and 5) drinks. Samples from each food group, i.e., kefir grains, home-made, and industrial drinks, are presented together in subpanels.

predominant family at 95.76% abundance. In the industrial drink sample 2, *Saccharomycetaceae* (34.78%), *Pichiaceae* (18.16%), *Physciaceae* (8.48%), and *Nectriaceae* (6.95%) were the main families identified, while yeast/fungal microbiota of the industrial sample 3 was dominated by the families *Saccharomycetaceae* (49.57%), *Dipodascaceae* (17.44%), and *Tremellales* (8.64%). The only sample that the family *Saccharomycetaceae* was not the dominant, was the industrial drink 4. In this sample, *Debaryomycetaceae* was the most abundant (42.47%), followed by *Tremellales* (18.11%), *Pichiaceae* (14.80%), *Saccharomycetales* (7.53%), and *Saccharomycetaceae* (5.34%). The dominance of the family *Debaryomycetaceae* in sample 4 could be explained by the use of eXact KEFIR1 culture for milk fermentation, which contains five bacterial species along with the yeast species *D. hansenii*. At the genus level, yeast/fungal communities of kefir grains 7G and 8G were almost identical to those of the respective home-made drinks, namely 7D and 8D, and dominated by the genus *Saccharomyces* (> 90%), followed by *Kazachstania* and *Kluyveromyces* with abundances for both genera below 4% (**Figure 5C** and **Supplementary Table S3C**). Similar were also the yeast/fungal microbiota profiles between samples 1G and 1D, although with different abundances. In detail, *Kazachstania* (87.40%) and *Kluyveromyces* (11.12%) were found to be the most abundant genera in grain sample 1G, while in home-made drink 1D, *Kluyveromyces* (49.58%) was the dominant genus, followed by *Kazachstania* (43.74%) and *Saccharomyces* (6.08%). The yeast/fungal microbiota of kefir grain 6G and home-made drink 6D had an analogous to the bacterial microbiota trend. Although the genus *Yarrowia* was found in trace levels in sample 6G, the abundance of this genus in sample 6D was significantly higher, i.e., 47.67%, and along with *Saccharomyces* (38.03%), were the most abundant genera sample 6D. In contrast, the yeast/fungal microbiota of sample 6G was dominated by the genus *Saccharomyces* (87.99%). The trend obtained by the ITS metagenomics analysis, i.e., similar yeast/fungal microbiota profiles between each grain-drink pair was also revealed by the classical microbiological analysis. *K. marxianus* and *K. turicensis* were the only species identified in both samples 1G and 1D, *S. cerevisiae* and *Y. lipolytica* in samples 6G and 6D and *S. cerevisiae* in samples 7G and 7D as well as 8G and 8D (**Supplementary Table S1B**). Concerning the industrial beverages, sample 5 was the least diverse, as also observed by the classification at the family level, and was dominated by the genus *Kluyveromyces* (93.57%). Although the industrial samples 2 and 3 were more diverse than sample 5, the genus *Kluyveromyces* was also found to be the most abundant, followed by several other genera, such as *Nakazawaea, Physcia, Galactomyces, Fusarium,* and *Saccharomyces*, with abundances varying between the two samples (**Supplementary Table S3C**). Similarly to the identification results at the family level, yeast/fungal community structure in industrial beverage 4 was dominated by the genus *Debaryomyces* (42,47%), followed by *Pichia* (14,68%) and *Cryptococcus* (10,89%). The results from the ITS metagenomics analysis were in accordance with those obtained by the classical microbiological analysis concerning samples 4 and 5. *P. kudriavzevii, D. hansenii,* and *Geotrichum candidum/Galactomyces candidum* as well as *K. marxianus* and *Geotrichum candidum/Galalactomyces candidum* were the yeast species identified in samples 4 and 5, respectively (**Supplementary Table S1B**). It is interesting however, that although high abundances of yeast/fungal genera revealed by the ITS metagenomics analysis for samples 2 and 3, yeasts were not detected during the microbiological analysis. The absence of yeast counts in samples 2 and 3 may imply that their abundances derived from the amplification of dead or compromised cells, which is a well-known disadvantage of DNA-based metagenomics techniques (De Filippis et al., 2017).

Our study is among the few elucidating the yeast/fungal microbiota of kefir grains and beverages using metagenomics analysis. Although yeasts/fungi also contribute to the organoleptic characteristics of several dairy products, including kefir, the number of HTS studies describing the bacterial communities is significantly higher than that of yeasts/fungi (De Filippis et al., 2017). The dominance of *Saccharomyces, Kazachstania,* and *Kluyveromyces* in our kefir grains (**Figure 5C**) was in accordance with the results of the other HTS studies. Specifically, the yeast/fungal microbiota of five kefir grains of different origins revealed a similar profile among the samples, with *Kazachstania* and *Kluyveromyces* being the most abundant genera identified (Korsak et al., 2015). In contrast, a different yeast/fungal microbial profile was found between home-made and academic/commercial samples. The latter were dominated almost exclusively by the genera *Kazachstania* and *Dekkera*, while the community structure of home-made samples was more diverse containing also the genera *Saccharomyces* and *Hanseniaspora* with varying abundances depending on the sample analyzed (Garofalo et al., 2015). An even more diverse microbiota was found in four home-made samples from different regions of Turkey, composed by members of the family *Dipodascaceae* and the genera *Saccharomyces, Kazachstania, Candida, Issatchenkia,* and *Rhodotorula* in lower abundances (Dertli and Con, 2017). The yeast/fungal communities of our home-made beverages analyzed were also dominated by the genera *Saccharomyces*, *Kazachstania,* and *Kluyveromyces*, as in the case of kefir grains. However, in sample 6D, *Yarrowia* was also identified (**Figure 5C**). On the other hand, the yeast/fungal microbiota of industrial samples was significantly more diverse than that of the home-made ones, as also revealed by the alpha-diversity indices (**Figure 2B**), and mainly composed by the genera *Kluyveromyces, Debaryomyces, Galactomyces, Cryptococcus, Pichia,* and *Nakazawaea* (**Figure 5C**). Interestingly, according to the study of Marsh et al. (2013), the identified yeast/fungal communities of 25 kefir grains (home-made and industrial) obtained from eight geographically distinct regions and the respective drinks were, in high concordance, dominated by the genera *Kazachstania*, *Naumovozyma,* and *Kluyveromyces*. This was also the case in the study of Walsh et al. (2016), in which *Saccharomyces* and *Kazachstania* were the most abundant genera found in three kefir grains from France, Ireland, and the United Kingdom and their drinks accounted for > 99% of the ITS sequences. Eventually, the relatively few reports regarding the yeast/fungal microbiota of both kefir grains and beverages, highlight the need of more HTS studies to unravel the microbial composition of these ecosystems.

CONCLUSION

The concurrent employment of a culture-dependent approach and amplicon-based metagenomics analysis unraveled, in a consistent way, the rich diversity of the microbiota of home-made and industrial kefir samples produced in Greece. Culturing methods enabled not only the estimation of the viable counts within various microbial groups but also the isolation of microorganisms for potential future applications in kefir production. Identification of isolated strains revealed that certain bacterial and yeast/fungal genera were mainly associated with either the home-made or the industrial samples. The presence of *Enterobacteriaceae* in both home-made and industrial samples along with the identification of (opportunistic) pathogens in one home-made sample, underlines the necessity of employing good hygiene practices in kefir production both at home and industrial level.

Interestingly, in the home-made samples the dominant genera of grains were different from those of the respective drinks. Overall, the in-depth study of kefir microbiota can help us recognize and possibly tune microbial activities to improve sensory characteristics and product quality and safety.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ebi.ac.uk/ena, PRJEB37688.

AUTHOR CONTRIBUTIONS

MK supervised the total DNA extraction for metagenomics analysis, performed the bioinformatics analysis of the amplicon-based metagenomics data and participated in the writing and review of the manuscript. AG performed the classical microbiological analysis and the total DNA extraction for the metagenomics analysis and participated in the writing of the manuscript. ET supervised the classical microbiological and metagenomics analyses and participated in the writing and review of the manuscript. MG conceived the project, supervised the classical microbiological analysis, and participated in the writing of the manuscript. All authors read and approved the final manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.621069/full#supplementary-material

Supplementary Figure 1 | Rep-PCR fingerprinting of bacterial isolates using the BOXAIR primer. Image analysis was performed using BioNumerics v. 6.0. Arrows denote isolates, which were selected for 16S rRNA gene sequencing.

Supplementary Figure 2 | Rep-PCR fingerprinting of yeast isolates using the $(GTG)_5$ primer. Image analysis was performed using BioNumerics v. 6.0. Arrows denote isolates, which were selected for ITS DNA region sequencing.

Supplementary Table 1 | Identification of bacterial isolates using classical microbiological analysis **(A)**. Identification of yeasts isolates using classical microbiological analysis **(B)**.

Supplementary Table 2 | Relative abundance of bacterial phyla identified in kefir grains, homemade, and industrial drinks **(A)**. Relative abundance of bacterial families identified in kefir grains, homemade, and industrial drinks **(B)**. Relative abundance of bacterial genera identified in kefir grains, homemade and industrial drinks **(C)**.

Supplementary Table 3 | Relative abundance of yeast/fungal phyla identified in kefir grains, homemade, and industrial drinks **(A)**. Relative abundance of yeast/fungal families identified in kefir grains, homemade, and industrial drinks **(B)**. Relative abundance of yeasts/fungal genera identified in kefir grains, homemade, and industrial drinks **(C)**.

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Therapeutic Effects of Probiotic Minas Frescal Cheese on the Attenuation of Ulcerative Colitis in a Murine Model

Bárbara F. Cordeiro[1], Juliana L. Alves[1], Giovanna A. Belo[1], Emiliano R. Oliveira[1], Marina P. Braga[1], Sara H. da Silva[1], Luisa Lemos[1,2], Jonas T. Guimarães[3], Ramon Silva[3,4], Ramon S. Rocha[3,4], Gwénaël Jan[5], Yves Le Loir[5], Marcia Cristina Silva[4], Mônica Q. Freitas[3], Erick A. Esmerino[3], Alfonso Gala-García[1], Enio Ferreira[1], Ana Maria C. Faria[1], Adriano G. Cruz[4], Vasco Azevedo[1†] and Fillipe L. R do Carmo[1,5†]*

[1]Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais (UFMG), Belo Horizonte, Brazil, [2]Department of Infectious Diseases, Harvard Medical School, Massachusetts General Hospital, Boston, MA, United States, [3]Faculdade de Medicina Veterinária, Universidade Federal Fluminense (UFF), Niterói, Brazil, [4]Departamento de Alimentos, Instituto Federal de Educação, Ciência e Tecnologia do Rio de Janeiro (IFRJ), Rio de Janeiro, Brazil, [5]INRAE, STLO, Institut Agro, Agrocampus Ouest, Rennes, France

Inflammatory bowel diseases (IBDs) constitute disturbances of gastrointestinal tract that cause irreversible changes in the structure and function of tissues. Ulcerative colitis (UC), the most frequent IBD in the population, is characterized by prominent inflammation of the human colon. Functional foods containing probiotic bacteria have been studied as adjuvants to the treatment or prevention of IBDs. The selected probiotic strain *Lactococcus lactis* NCDO 2118 (*L. lactis* NCDO 2118) exhibits immunomodulatory effects, with promising results in UC mouse model induced by dextran sodium sulfate (DSS). Additionally, cheese is a dairy food that presents high nutritional value, besides being a good delivery system that can be used to improve survival and enhance the therapeutic effects of probiotic bacteria in the host. Therefore, this work investigated the probiotic therapeutic effects of an experimental Minas Frescal cheese containing *L. lactis* NCDO 2118 in DSS-induced colitis in mice. During colitis induction, mice that consumed the probiotic cheese exhibited reduced in the severity of colitis, with attenuated weight loss, lower disease activity index, limited shortening of the colon length, and reduced histopathological score. Moreover, probiotic cheese administration increased gene expression of tight junctions' proteins *zo-1*, *zo-2*, *ocln*, and *cln-1* in the colon and increase IL-10 release in the spleen and lymph nodes. In this way, this work demonstrates that consumption of probiotic Minas Frescal cheese, containing *L. lactis* NCDO 2118, prevents the inflammatory process during DSS-induced colitis in mice, opening perspectives for the development of new probiotic functional foods for personalized nutrition in the context of IBD.

Keywords: functional food, probiotic, colitis, cheese, *Lactococcus lactis*, inflammatory bowel disease

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***Correspondence:**
Fillipe L. R do Carmo
fillipelrc@gmail.com

[†]These authors share senior authorship

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INTRODUCTION

Functional food products are defined as "natural or processed foods containing known or unknown biologically active compounds which provide a clinically proven and documented health benefit for the prevention, control or treatment of chronic diseases when in used in defined, effective and non-toxic amounts" (Rolim et al., 2020). Among them, functional foods containing probiotic bacteria have been proposed for being safe for consumption and have the ability to modulate the responses in the host by cellular components or metabolites produced (Rabah et al., 2017). In this context, there is a wide demand for new functional foods, in particular, foods enriched by the addition of probiotics, driving the food's industry to develop new products proven effective for health (Sperry et al., 2018).

Cheese is one of the most consumed dairy food and comprises, from the nutritional view, a source with good nutritional value, given high contents of protein, minerals, and vitamins (Matera et al., 2018). Moreover, cheese, especially soft cheese, is an excellent delivery system to introduce probiotics into the gastrointestinal tract (GIT), due to it is anaerobic conditions created by the protein-fat contents, which can form complex coacervates that microencapsulated the probiotic bacteria and also high pH and low acidity present in this kind of the cheese (Silva et al., 2018). These coacervates reduce the contact with a highly acidic gut environment and thereby promote probiotic bacteria survival (Castro et al., 2015). Noteworthy, the Minas Frescal cheese is one of the most consumed cheese in Brazil and constitutes one important activity of the dairy industries, due to the high yield and absence of maturation period, which allows a quick return on investment and, consequently, lowers costs to the consumer (Sperry et al., 2018; Rocha et al., 2020). These features show that Minas Frescal cheese is a good candidate for manufacturing a new probiotic function dairy food.

Lactococcus lactis strain is a Gram-positive lactic acid bacteria (LAB) that exhibit simple metabolism and rapid growth and, due to that, are widely used in food fermentation (Da Silva et al., 2019). More specifically, NCDO 2118, used in this work, is a strain of *L. lactis* subsp. *lactis* isolated from frozen peas (Oliveira et al., 2014) and was previously demonstrated anti-inflammatory and immunomodulatory activities in the treatment of diseases, especially, in inflammatory bowel diseases (IBDs; Luerce et al., 2014). Furthermore, the functional analysis of *L. lactis* NCDO 2118 genome reflected a physiological adaptation ability to environmental changes like industrial processes and transit through the human GIT (Da Silva et al., 2019). These characteristics make *L. lactis* NCDO 2118 an excellent candidate to be introduced in probiotic functional foods.

IBDs, which include ulcerative colitis (UC) and Crohn's disease (CD), are marked by periods of remission and relapse of an inflammation condition in the GIT and have a high prevalence in westernized countries, reaching about 0.5% of these populations (Silva et al., 2019). The etiology of IBD still not well understood, but scientific evidence suggests that that the genetic susceptibility, associated with intestinal microbiota alterations, causing an exacerbated immune response in the host is involved in IBD pathogenesis (Zhang and Li, 2014). UC is the most frequent condition of IBD in the population, affecting the large intestine, also called the colon. UC causes small irritation and ulcers in the colon, pain, diarrhea often with blood in the stool, and weight loss (Cordeiro et al., 2019). Nowadays, studies have shown that consumption of probiotic bacteria has therapeutic effects on UC, which is demonstrated to decrease the colon inflammation in a mouse model as well as in UC patients (Mañé et al., 2009; Luerce et al., 2014; Santos Rocha et al., 2014; Berlec et al., 2017; Jakubczyk et al., 2020; Rabah et al., 2020). Thus, this study hypothesizes that Minas Frescal cheese, made using *L. lactis* NCDO 2118, has a therapeutic effect in dextran sodium sulfate (DSS)-induced colitis mouse model.

MATERIALS AND METHODS

Cheese Processing

The cheese processing was performed in accordance with Grom et al. (2020). Fifty liters of raw milk with 3.2% w/w fat (Núcleo Avançado de Tecnologia de Alimentos) was pasteurized for 15 s at 72°C (Model pro110, Arpifrio, São Paulo, Brazil), cooled to 37°C, and equally divided into two portions of 25 l, each for processing of conventional and probiotic Minas Frescal cheese. Then, 0.2 g/l of calcium chloride (Labsynth, Sao Paulo, Brazil) and 3 g/l of coagulant powder (Halamix power, Chr. Hansen) were added into the milk and maintained in a double-jacketed tank for 40 min to coagulate. After, 0.1 g/l at probiotic culture *L. lactis* NCDO 2118 [7–8 log colony-forming unit (CFU)/g] was added to the probiotic cheese, while no addition of lactic bacteria was performed on conventional cheese. The curd was cut, the cheese whey was removed, and the grains were put in 250-g plastic molds. Dry salting was performed by direct addition of 0.8% w/v NaCl on the cheese surface. Cheeses were packed and stored at 5°C.

Physicochemical Analyses of Conventional and Probiotic Cheese

The proximate composition (moisture, protein, and fat) was evaluated according to the methodology previously described (BRASIL, 2006). To determine the moisture content of cheeses, we oven-dried 5 g of a sample at 100–105°C, for 24 h. Protein quantification and fat levels were determined by the Kjeldahl and Gerber methods, respectively (BRASIL, 2006). All results were expressed as g/100 g.

The content analysis of calcium and sodium levels in both kinds of cheeses were performed by inductively coupled plasma (ICP) optical emission spectrometry (Spectro Analytical Instruments, Kleve, Germany) previously described by Felicio et al. (2016). Sodium and calcium standards were used to obtain the calibration curves. Ten grams of samples was hydrolyzed, for 16 h, using 2 ml of nitric-perchloric acid solution (2:1), at 120 ± 2°C. Samples were heated in a digestion block (Technal, São Paulo, Brazil) to 100 ± 2°C for 1 h and

maintained for more than 2 h, at 170 ± 2°C. Then, after the samples reach room temperature, we added 2 ml of nitric-perchloric acid and heated them again for a further 4 h at 170 ± 2°C.

To obtain the pH levels of both cheese, we inserted a digital pH meter electrode (Micronal, B-375, Digimed, Piracicaba, São Paulo, Brazil) into the diluted cheese samples as previously described (Silva et al., 2017).

Bioactivity

To measure the bioactive peptides in cheese samples, we evaluate the angiotensin I-converting enzyme inhibitor (ACEI), antioxidant activity assay [2,2-diphenyl-1-picrylhydrazyl (DPPH)], and α-amylase and α-glucosidase inhibition.

The ACEI in probiotic and conventional cheese was determined by spectrophotometric assay, according to Konrad et al. (2014). The ACEI was calculated as follows: ACE inhibitory activity (%) = [(B − A)/(B − C)] × 100, where A is the absorbance in the presence of ACE and ACE components, B is the absorbance with ACE and without the ACE component, and C is the absorbance without the ACE or ACE component.

The DPPH radical-scavenging method previously described was used to determine the antioxidant activity capacity of cheeses (Lee et al., 2016). For that, 200 μl of 10% cheese sample was mixed with 1 ml of 100 μmol/l of DPPH solution. Besides that, as a positive control, butylated hydroxytoluene at 1 mg/ml concentration was used. After 15 min, the absorbance was measured at 517 nm using a spectrophotometer. The DPPH was calculated as follows: DPPH radical-scavenging activity (%) = [1 − (sample absorbance at 517 nm/control absorbance at 517 nm)] × 100.

The measurement of α-glucosidase and α-amylase inhibitory activities was determined according to Grom et al. (2020). The α-glucosidase inhibitory activity was determined dissolving 100 μl of α-glucosidase (0.2 units/ml) in 100 μl of phosphate buffer (pH 6.8), mixed with 150 μl of water-soluble extracts, and incubated for 20 min at 37°C. Then, 100 μl of 2.5 mM of p-nitrophenyl α-D-glucopyranoside was added to start the reaction. After incubation at 37°C for 20 min, the reaction was stopped, and 80 μl of sodium carbonate solution (0.2 mol/l) was added. The absorbance of *p*-nitrophenol was read at 405 nm using CMax Plus microplate reader (Promega, São Paulo, Brazil).

The α-amylase inhibitory activity was measured, and 100 μl of human salivary α-amylase (20 units/ml) with 100 μl of water-soluble extracts was added and incubated at 37°C for 20 min. Then, 250 μl of starch solution (10 g/l) in phosphate buffer (pH 6.8) was added, and the solution was incubated at 37°C for 5 min. To stop the reaction, 250 μl of dinitrosalicylic reagent (1% 3,5-dinitrosalicylic acid and 12% sodium potassium tartrate in 0.4 M of NaOH) was added and heated at 100°C for 10 min. After that, the sample was cooled at room temperature using a cold-water bath, and then 2.000 μl of distilled water was added to the mixture. The absorbance was performed at 540 nm using a spectrophotometer. The percentage (%) of α-glucosidase and α-amylase inhibition was calculated as described by Grom et al. (2020).

Evaluation of Therapeutic Effects of Minas Frescal Cheese Containing *L. lactis* NCDO 2118 in the Dextran Sodium Sulfate-Induced Colitis Model

Animals

Conventional female C57BL/6 mice of 8 weeks of age, obtained at Universidade Federal de Minas Gerais (UFMG, Belo Horizonte, Brazil), were used in this work. They were housed in plastic cages in a room with controlled temperature (18–23°C), light cycle of 14-h light/10-h dark, relative humidity (40–60%), and *ad libitum* access to food and water. All experimental procedures realized in this work were approved by the Ethics Committee on Animal Experimentation of the Universidade Federal de Minas Gerais (CEUA-UFMG, Brazil) by protocol no. 364/2018.

Experimental Design and Dextran Sodium Sulfate-Induced Colitis

Prior to intragastric gavage, cheese samples were daily prepared resuspending 250 mg of each cheese, separately in 250 ml of phosphate buffer (pH 7.4; 1:1), and homogenized for 2 min using an IKA T 10 Basic Ultra Turrax homogenizer. Bacterial viability in both cheese solutions was determined by CFU counts.

The mice were divided randomly into six main groups, each containing six animals per group (**Table 1**). Groups 1–3 represented the healthy control group (no DSS) that received drinking water from the same source and consisted of group 1 received only water content (Group Naive); group 2 received conventional Minas Frescal cheese (group conventional ch.); and group 3 was given probiotic Minas Frescal cheese containing *L. lactis* NCDO 2118 (group NCDO ch.). All mice from groups 4–6 (experimental groups) received DSS (36–50 kDa, MP Biomedicals, CAT 260110, LOT Q5756), as the only drinking source, prepared to a concentration of 1.7% in filtered drinking water and provided to the animals daily, for 7 days, according to acute colitis model previously described (Wirtz et al., 2017). Animals from group 4 received only drinking water with DSS (group DSS) and no treatment; mice from group 5 were treated with conventional cheese (group DSS + conventional ch.), and group 6 were treated with probiotic Minas Frescal cheese containing *L. lactis* NCDO 2118 (DSS + NCDO ch.). For this experimental procedure, all mice received 0.5 ml of the respective treatments, in a single daily dose, by intragastric gavage, concomitantly with DSS induction (for 7 days). Each animal received approximately 2.5×10^6 CFU/g of probiotic bacteria, per day, according to the results obtained by previous studies (Cordeiro et al., 2019; Rabah et al., 2020) and the adequate amount of bacteria for effect on the colon (Minelli and Benini, 2008). Mice were euthanized on the seventh day. All *in vivo* experiments were done in biological triplicate.

Assessment of Colitis Disease

Mouse body weight was individually measured during all experimental days. Water and food consumption were also recorded daily. The disease activity index (DAI) was determined

TABLE 1 | Experimental groups and the respective treatments.

Healthy control group (consumption of drinking water)		Inflamed groups [consumption of DSS (1.7%) in the drinking water]	
Group	**Treatment**	**Group**	**Treatment**
Naive	H_2O	DSS	H_2O
Conventional ch.	Conventional cheese	DSS + conventional ch.	Conventional cheese
NCDO ch.	Probiotic cheese containing *L. lactis* NCDO 2118	DSS + NCDO ch.	Probiotic cheese containing *L. lactis* NCDO 2118

All groups were gavaged daily, with 0.5 ml of the appropriate treatments, for 7 days.
DSS, dextran sodium sulfate.

on the last experimental day, as described by Cooper et al. (1993). This score measurement three major colitis clinical signs: weight loss, levels of diarrhea, and presence of rectal bleeding.

To access the intestine and colon for future assays, a longitudinal abdominal incision was performed in all mice. The colon length of each mouse was individually measured (from the cecum to rectum), and the values obtained were used to indicate the mean of each experimental group, in cm. Then, the distal portion of each colon was collected and washed with phosphate-buffered saline (PBS) for making colonic segment rolls for histomorphological analysis. These rolls were immersed in formaldehyde solution (4%, v/v) for tissue fixation, and after that, they were embedded in paraffin. A section (4 μm) was placed on a glass slide and stained with hematoxylin and eosin (H&E; Marchal Bressenot et al., 2015). Then, the sections were photographed (20× magnification objective) using a digital camera (Spot Insight Color) coupled to an optical microscope (Olympus, BX-41, Japan). The histological inflammation score was determined by a pathologist. To measure the level of histological inflammation in the colon tissue, the score previously described was used (Wirtz et al., 2017). This score considered the following features: tissue damage (0: none; 1: isolated focal epithelial damage; 2: mucosal erosions and ulcerations; 3: extensive damage deep into the bowel wall) and lamina propria inflammatory cell infiltration (0: infrequent; 1: increased, some neutrophils; 2: submucosal presence of inflammatory cell clusters; 3: transmural cell infiltrations). The total score ranging from 0 (no changes) to 6 (widespread cellular infiltrations and extensive tissue damage) was obtained by the sum of these two sub-scores (tissue damage and lamina propria inflammatory cell infiltration). Furthermore, to stain mucus-producing goblet cells, other cuts of the paraffinized colon samples were produced and stained by the Periodic acid-Schiff (PAS; Prisciandaro et al., 2011). Ten random field images from each sample were made using the 40× objective, and then with the use of ImageJ software (version 1.8.0), the intact goblet cells were counted. The total number of goblet cells was expressed as the number of cells per high-power field (hpf; 40×, 108.2 μm^2).

Measurement of Secretory Immunoglobulin A

Secretory immunoglobulin A (sIgA) was determined by linked immunosorbent assay (ELISA), according to Cordeiro et al. (2018). For that, 96-well plates (Nunc-Immuno Plates, MaxiSorp) were coated with anti-IgA antibodies (Southern Biotechnology, Birmingham, AL, United States) and incubated overnight. Plates were washed in salina-Tween (salina with 0.05% of Tween-20; SIGMA Chemical Co) and blocked with 200 μl of PBS-casein (0.05%) for 1 h at room temperature. Intestinal lavage contents were added, and the plate was serially diluted (1:100) and incubated at room temperature for 1 h. Plates were washed with salina-Tween, and then, biotin-conjugated anti-mouse IgA antibody (Southern Biotechnology; 1:10,000 in PBS-casein) was added. Plates were incubated for 1 h at 37°C, and then, biotinylated monoclonal antibody anti-IgA (BD Biosciences) was added and incubated for 1 h at room temperature. Subsequently, peroxidase-labeled streptavidin (Southern Biotechnology) was added. Plates were washed in salina-Tween and incubated again with 100 μl of *o*-phenylenediamine (OPD; Sigma, St. Louis, MO, United States) and H_2O_2 (0.04%) for 1 h at room temperature. For stop reaction, 20 μl/well of 2 N of H_2SO_4 was added. Reading was performed on Bio-Rad Model 450 Microplate Reader at 492-nm absorbance. The results of total sIgA were measured, according to the standard curve, in a concentration of sIgA (ng) per ml of intestinal fluid.

Measurement of the Activity of Myeloperoxidase

The levels of neutrophil infiltration in the colon tissue were assessed by measurement of myeloperoxidase (MPO) activity, as previously described by Porto et al. (2019). For MPO quantification, a piece of colon tissue (10 mg) was homogenized proportionally in 1.9 ml/100 mg of PBS and centrifuged at 12,000 *g* for 10 min. The supernatant was discarded, and the pellet formed was lysed and centrifuged again. The supernatant formed was discarded again, and the pellet was resuspended proportionally in 1.9 ml/100 mg of 0.5% hexadecyltrimethyl ammonium bromide (HTAB) diluted in PBS. Afterward, were subjected to a freeze-thaw cycle (3×) using liquid nitrogen and then centrifuged at 12,000 *g* at 4°C for 10 min. To realize the enzymatic assay, we added an equal amount of substrate (1.5 mM/l of OPD and 6.6 mM/L of H_2O_2 in 0.075 mM/L of Tris-HCl pH 8.0) to the supernatant. To stop the enzymatic reaction, 50 μl of 1 M of H_2SO_4 was added. The absorbance was read in a spectrophotometer (Spectramax M3, Molecular Devices, LLC, Sunnyvale, CA, United States) at 492 nm. The results were expressed as arbitrary units (AU/mg).

Gene Expression Analysis in the Colon

The quantitative gene expression in colon fragment was determined according to Do Carmo et al. (2019). For that, fragments of 1 cm of the colon were collected, and then, the total RNA was isolated using PureLink RNA Mini Kit (Thermo Fisher Scientific)

according to the manufacturer's protocol. Afterward, DNase I (Invitrogen, Waltham, MA, United States) was used to digest residual genomic DNA of samples, and then Turbo DNA-free Kit (Ambion, Austin, TX, United States) was used for DNA removal following the manufacturer's protocol. RNA quality was checked by agarose gel and NanoDrop® ND-1000 (260/230 ratio). To obtains the sample cDNA, the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, United States) was used. Quantitative PCR (qPCR) was determined using iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, United States) and gene-specific primers, according to Do Carmo et al. (2019), for zonula occludens 1 and 2 (*zo-1* and *zo-2*, respectively), occludin (*ocln*), claudin-1 (*cln-1*), mucin-2 (*MUC-2*), inducible nitric oxide synthase (*iNOS*), and cytokine genes for interleukin-10 (*IL-10*), *IL-17*, *IL-1β*, as well as housekeeping genes encoding β-actin (*actβ*) and GAPDH (*gapdh*). The amplification cycles were performed as follows: 95°C for 30 s and 40 cycles of 95°C for 15 s and 60°C for 30 s on ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). Results were expressed as a fold change of expression levels, using the mean and standard deviations of target expression ($2^{-\Delta\Delta CT}$).

Cell Preparation for Culture and Flow Cytometry

Cell suspension preparation for cytokine analysis and flow cytometry measurements were performed according to Canesso et al. (2018). Firstly, as UC affects the distal portions of the intestine, especially, the colon (Mizoguchi et al., 2020), we extracted the cecal lymph node (which drains the cecum) and the colonic lymph node (which drains the colon) for cell culture (Vieira et al., 2012; Esterházy et al., 2019). As the colon lymph node is very small, we did a pool mixing the two lymph nodes to reach enough cells for cell labeling. After that, the organs were macerated with sterile complete RPMI medium [containing 10% fetal bovine serum (FBS)] using a glass tissue macerator. Them, the organs were centrifuged and resuspended in a complete RPMI medium. For the spleen, cell-culture preparation was necessary to lysate the red blood cells, adding 9 ml of distilled water for 5 s. To stop this lysis process, 1 ml of PBS (10×) was added. The spleen capsule was removed to facilitate the presence of only immune cells. These cells were centrifuged and isolated from medium and then were incubated at 1×10^6 cells per well, for cytokine secretion analyses, and another 1×10^6 cells were incubated with antibodies for flow cytometry.

Cytokine Quantification by ELISA

Cells isolated from spleen and lymph node culture were cultured in 96-well plate (1×10^6 cells/well) in sterile supplemented RPMI 1640 and stimulated or not with 1 mg/ml of anti-CD3 and anti-CD28, according to Canesso et al. (2018). The cells were incubated in an atmosphere of 5% CO_2 for 48 h at 37°C, for measurement of IL-10, IL-17, and IL-1β cytokines, by ELISA, according to the manufacturer's instructions (R&D Systems).

Flow Cytometry Analyses

Isolated cells from the spleen and lymph nodes were washed with PBS and pre-incubated with purified rat anti-mouse CD16/CD32 (Fc Block, clone: 2.4G2, BD Biosciences Pharmingen) for 20 min at 4°C to block FcγRII/III receptors. For surface staining, cells were incubated at 4°C for 30 min with anti-CD45.2 (FITC; clone: 104, BD Biosciences Pharmingen) and anti-CD4 (Pacific Blue, clone: RM4-5, BD Biosciences Pharmingen) fluorochrome-conjugated monoclonal antibodies. For intracellular staining, cells were first permeabilized following the *eBioscience Foxp3 Kit*, according to the manufacturer's instructions, and later incubated with anti-FoxP3 (APC) [Alexa Fluor® 647, clone R16-715 (RUO), BD Biosciences Pharmingen], anti-LAP (PerCP-eFluor 710, clone: TW7-16B4, eBioscience), and anti-RORγt (PE; clone: Q31-378, BD Biosciences Pharmingen) fluorochrome-conjugated monoclonal antibodies for 30 min at 4°C. Individual controls (singles) were made containing only one labeled antibody in each tube, and also tubes with fluorescence minus one (FMO) were used. The gating strategy and the FMO controls are based on forward and side scatters, selecting splenocytes as a function of cell size and granularity ($n = 6$). Flow cytometry analysis was performed on a FACSCanto (BD Biosciences, San Jose, CA). The frequency (%) of positive cells and the mean fluorescence intensity were analyzed with the aid of the FlowJo program, version 10.0 (Tree Star, Ashland, OR, United States).

Statistical Analyses

Data were analyzed using one-way ANOVA followed by Tukey post-test and performed in GraphPad Prism version 7.00 for Windows (GraphPad Software, San Diego, CA, United States). The experimental assays were performed in triplicate, and the results were expressed as mean ± standard deviation. Asterisks demonstrated in all figures represent the significant differences between the experimental groups and were indicated as follows: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

RESULTS

Proximate Composition and Mineral Content of Conventional and Probiotic Minas Frescal Cheese

Proximate composition (moisture, protein, fat, and lactose), sodium, calcium contents, and pH values are presented in **Table 2**. Our results showed that the addition of *L. lactis*

TABLE 2 | Proximate composition and mineral contents of conventional and probiotic Minas Frescal cheese.

	Conventional cheese	Probiotic cheese
Moisture	67.2 ± 1.43	68.2 ± 1.55
Proteins	16.2 ± 1.56	17.5 ± 1.34
Fat	14.4 ± 1.11	14.7 ± 1.13
Lactose	2.2 ± 0.3	1.8 ± 0.1
Na	542 mg/kg	549 mg/kg
Ca	312 mg/kg	316 mg/kg
pH	5.52 ± 0.34	5.42 ± 0.21

Values are expressed as mean ± standard deviation. Moisture, protein, fat, and lactose values are expressed in g/100 g cheese, while sodium and potassium values are expressed in mg/kg. pH is dimensionless. Cheese analysis performed in triplicate.

TABLE 3 | Bioactive compounds from conventional and probiotic cheese.

	Conventional cheese	Probiotic cheese
DPPH	22.3 ± 0.3b	43.3 ± 0.65a
ACEI	13.3 ± 0.73b	32.4 ± 1.13a
α-Amylase	19.2 ± 1.45b	28.9 ± 0.99a
α-Glucosidase	10.3 ± 0.91b	16.7 ± 1.12a

Values are expressed in mean ± standard deviation. a–b Different letters in the same row indicate a significant difference ($p < 0.05$). ACEI, DPPH, α-amylase, and α-glucosidase are expressed as a percentage of inhibition (%). Cheese analysis performed in triplicate.
ACEI, angiotensin I-converting enzyme inhibitor; DPPH, 2,2-diphenyl-1-picrylhydrazyl.

NCDO 2118 did not affect significantly ($p > 0.05$) the proximate composition and mineral content of Minas Frescal cheese, compared with conventional cheese. Overall, probiotic cheese presented 68.2 ± 1.55, 17.5 ± 1.34, and 14.7 ± 1.13 (g/100 g) of moisture, protein, and fat, respectively, while conventional cheese presented 67.2 ± 1.43, 16.2 ± 1.56, and 14.4 ± 1.11, respectively. Regarding lactose amount, conventional cheese shows 2.2 ± 0.3, while probiotic cheese presented 1.8 ± 0.1 g/100 g. Regarding Na and Ca values, probiotic Minas Frescal cheese exhibited 549 and 316 mg/kg, respectively; meanwhile, conventional cheese presented similar values, 542 mg/kg of sodium and 312 mg/kg of calcium. pH values also did not present differences in both kinds of cheese, retaining a range of 5.

Lactococcus lactis NCDO 2118 on Cheese Enhances the Production of Bioactive Compounds

Table 3 shows the evaluation of bioactive compounds produced by conventional and probiotic cheese. Our results demonstrated that the antioxidant potential (DPPH), ACE inhibitory activity (ACEI), α-amylase, and α-glucosidase on the probiotic cheese, containing *L. lactis* NCDO 2118, presented increased values and were significantly different ($p < 0.05$) compared with conventional cheese. Regarding DPPH inhibition, we observed that values ranged from 22.3 ± 0.3% (convention cheese) to 43.3 ± 0.65% (probiotic cheese). Furthermore, probiotic cheese presented 32.4 ± 1.13% of ACEI, while the conventional cheese presents only 13.3 ± 0.73%. Likewise, probiotic cheese presented the highest values of α-amylase and α-glucosidase (28.9 ± 0.99% and 16.7 ± 1.12%, respectively), while conventional cheese presented 19.2 ± 1.45% and 10.3 ± 0.91%, respectively.

Treatment of Probiotic Cheese Did Not Alter the Liquid and Food Consumption or Caloric Intake of Mice

Figure 1 shows the liquid consumption (**Figure 1A**), the total food consumption (**Figure 1B**), and the caloric intake per mice (**Figure 1C**) during the experimental procedure. We observed a decrease in liquid intake on groups of mice that consumed water solution containing 1.7% of DSS over the experimental day, exhibiting the lowest consumption on the seventh day (3.3 ± 0.45 ml/animal). On the other hand, this consumption remains stable in groups receiving only drinking water (6.3 ± 0.441 ml/animal per day; $p < 0.001$). No differences were observed in liquid consumption in mice treated with conventional or probiotic cheese ($p > 0.05$) in health or unhealthy mice. Mice of all experimental groups consumed, on average, the same amount of food (3 g/animal per day), with no statistical difference between any experimental groups studied. Concerning caloric intake (**Figure 1C**), no difference between experimental groups has found by the intragastric gavage with conventional or probiotic cheese. Thus, both kinds of cheese did not alter the daily caloric content.

Probiotic Minas Frescal Cheese Reduced the Weight Loss in Dextran Sodium Sulfate-Induced Ulcerative Colitis Mice

The consumption of probiotic cheese, containing *L. lactis* NCDO 2118 strain, was challenged in the DSS-induced colitis model. Mouse weight loss, monitored during the DSS administration, showed that the animals receiving DSS exhibited body weight loss starting from the third day, after DSS consumption (**Figure 1D**). Otherwise, mice from all healthy control groups presented a significant weight gain throughout the experiment days ($p < 0.0001$). **Figure 1E** shows that treatment with probiotic cheese has a protective effect on colitis-induced body weight loss. Mice from the DSS group that did not receive any treatment showed a marked weight loss (−8.08 ± 2.09%); however, mice treated with the probiotic cheese showed significant improvement in body weight (+0.23 ± 0.80%, $p < 0.0001$). Body weight variation was also statistically different ($p < 0.05$) between DSS + conventional cheese (−4.5 ± 5.9%) and DSS + NCDO cheese (+0.23 ± 0.80%).

Probiotic Minas Frescal Cheese Alleviated Clinical and Macroscopic Signs of Colitis Disease

The shortening of colon length (**Figure 1F**) and the DAI (**Figure 1G**) were analyzed to verify major colitis macroscopic and clinical symptoms. Our results showed that the administration of DSS causes a pronounced shortening in the colon length (4.2 ± 1.12 cm) when compared with the naive group (7.0 ± 1.15 cm, $p < 0.0001$). However, the treatment with probiotic cheese, containing *L. lactis* NCDO 2118, prevents the shortening of the colon (6.2 ± 0.99 cm), being statistically different ($p < 0.0001$) when compared with the DSS group and results in a similar length to healthy animals ($p > 0.05$). Regarding DAI analyses, we observed that the administration of DSS was able to increase significantly (8.6 ± 2.1, $p < 0.0001$) the DAI score, compared with healthy groups (0.06 ± 0.2). Nevertheless, the intake of probiotic cheese was able to decrease significantly the DAI score (4.3 ± 1.8) compared with the DSS group ($p < 0.0001$) or with DSS + conventional cheese group (6.6 ± 3.3, $p < 0.05$).

Colon Mucosal Damages Were Reduced in Mice Treated With Probiotic Minas Frescal Cheese

Figures 1H, 2 reveal the impact of DSS administration and the effect of the probiotic treatment on the morphological

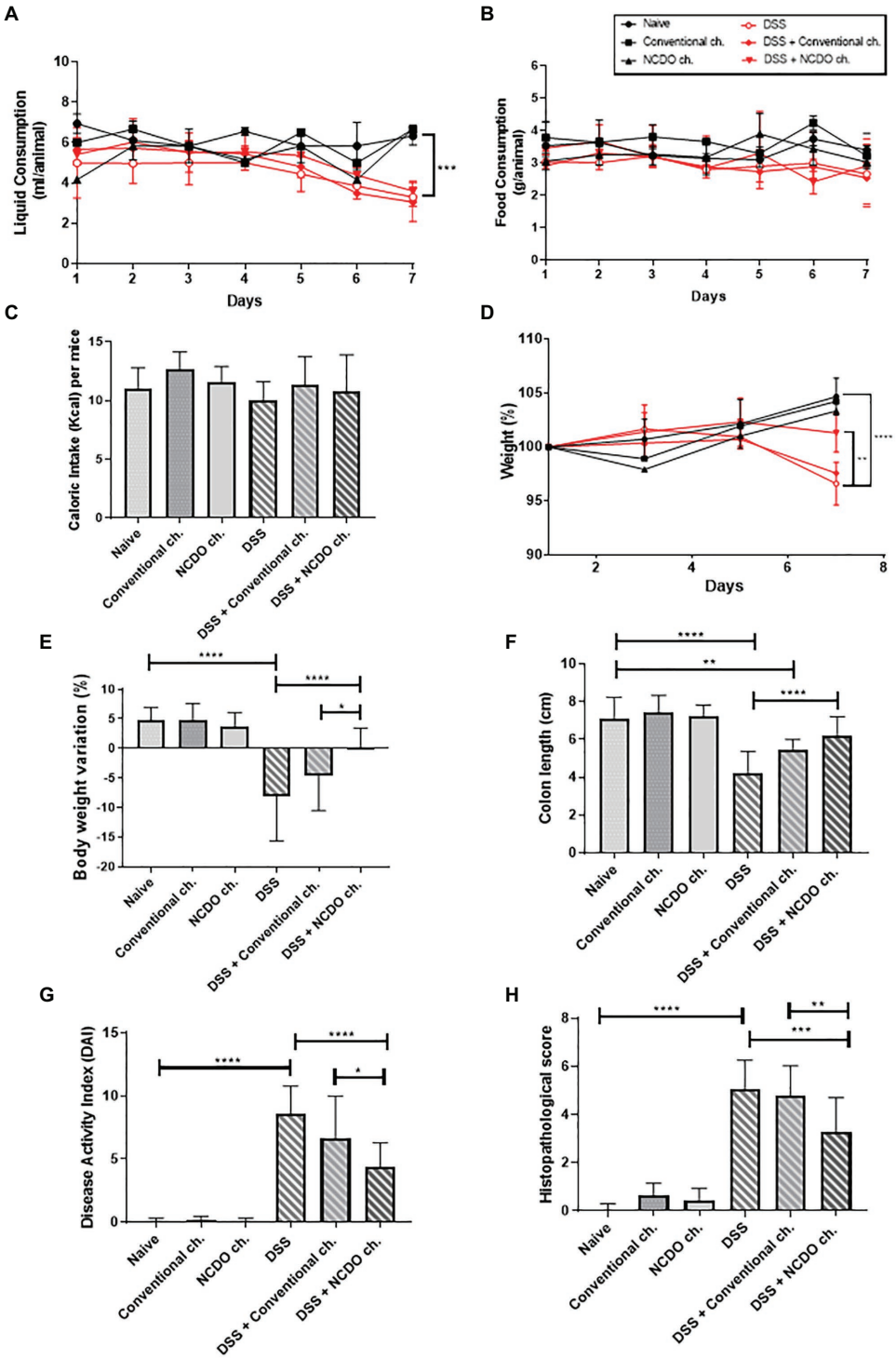

FIGURE 1 | Impact of treatment with probiotic cheese on mice. **(A)** Liquid intake, **(B)** food consumption, and **(C)** caloric intake of mice across the different experimental groups. **(D)** Time course of mouse body weight monitoring during the seven experimental days. **(E)** Body weight loss observed on the seventh day of dextran sodium sulfate (DSS) colitis induction, and differences across the groups. **(F)** Changes in mouse colon length. **(G)** Disease activity index (DAI), a composite measure of weight loss, stool consistency, and presence of blood in stool. **(H)** Histopathological score obtained in mice. Values indicate the mean ± standard deviation. The data represent the mean ± SD ($n = 6$). Asterisks represent statistically significant differences, as follows: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

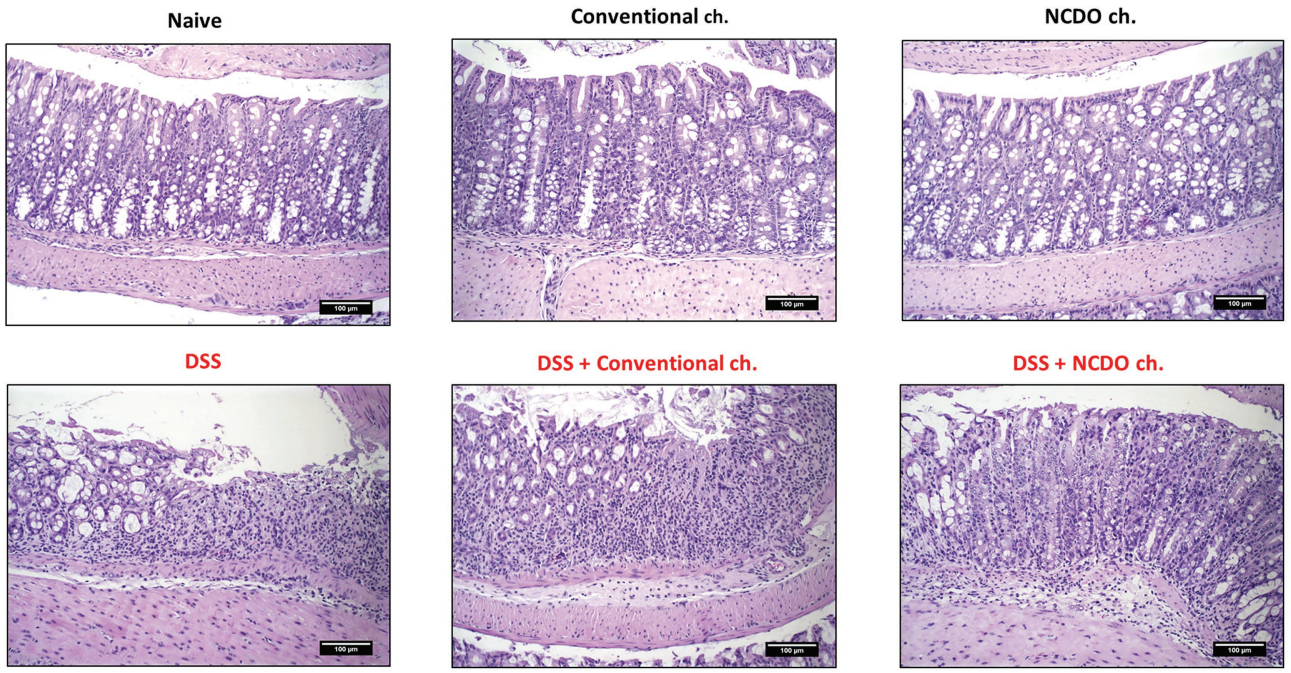

FIGURE 2 | Effect of probiotic cheese administration on the dextran sodium sulfate (DSS)-induced histopathological damages. Representative H&E staining of colon mucosa sections. Image acquisition was done with a 20× magnification objective. Scale bar = 100 μm (n = 6).

structure of the mouse colon. Histopathological score (**Figure 1H**) and histological slide analysis (H&E staining, **Figure 2**) show that mice subjected to DSS consumption presented alterations in the morphological architecture of the colon, with extensive damage deep into the tissue, erosions and ulcerations in the colon of some mice, and increased inflammatory cell infiltration. However, consumption of probiotic cheese in DSS colitis mice was able to ameliorate these mucosal damages. Mice from healthy control groups showed a null histological score, while the DSS group presented a score on average of 5.0 ± 1.1 ($p < 0.0001$). Consumption of probiotic cheese, in turn, decreases the score to 3.2 ± 1.4, being statistically different to the DSS group ($p < 0.001$) and DSS + conventional cheese group (4.8 ± 1.2, $p < 0.01$).

Treatment With Probiotic Minas Frescal Cheese Prevented Degeneration of Goblet Cells and Improved Secretory IgA Production

The administration of DSS provokes a substantial decrease in the number of goblet cells in the colon tissue (56.4 ± 25 goblet cell/hpf) when compared with the naive group (103 ± 27.5, $p < 0.01$, **Figures 3A,B**). Nonetheless, consumption of probiotic Minas Frescal cheese was still able to prevent this degeneration of goblet cells (101 ± 32.2 goblet cell/hpf), when compared with the DSS group ($p < 0.01$). Interestingly, the consumption of probiotic cheese provokes an improvement in the number of intact goblet cells, and also in the healthy control group (143.9 ± 16.3 goblet cell/hpf). **Figure 3C** shows levels of sIgA in the small intestine of mice. Our results showed that consumption of probiotic Minas Frescal cheese was able to increase the levels of sIgA (2,800.4 ng/ml) when compared with the naive group (1,750.3 ng/ml) and the DSS group (1,579.7 ng/ml).

Probiotic Cheese Reduced the Inflammatory Cell Infiltration

In this work, we assessed the presence of colon neutrophil infiltrates by detecting its specific MPO enzymes (**Figure 3D**). Our results showed that mice in the DSS group had an inflammatory infiltrate with a very high level of neutrophils (0.0248, 0.008, $p < 0.001$) when compared with the naive group (0.0109 ± 0.003). However, when mice were treated with probiotic cheese, we found a significant reduction of these cells (0.0137, ± 0.003, $p < 0.05$), showing MPO levels very similar to those found in healthy animals ($p > 0.05$). Interestingly, we observed that conventional Minas Frescal cheese also presented reduced values of MPO (0.0148), being statistically different from those of the DSS ($p < 0.05$) group and similar to those of the DSS + NCDO group.

Probiotic Minas Frescal Cheese Modulated Gene Expression in the Mice Colon

In this work, we sought to evaluable the colonic mRNA expression levels of epithelial barrier genes (*zo-1*, *zo-2*, *ocln*, and *cln-1*), production of mucin gene (*MUC-2*), colonic oxidative

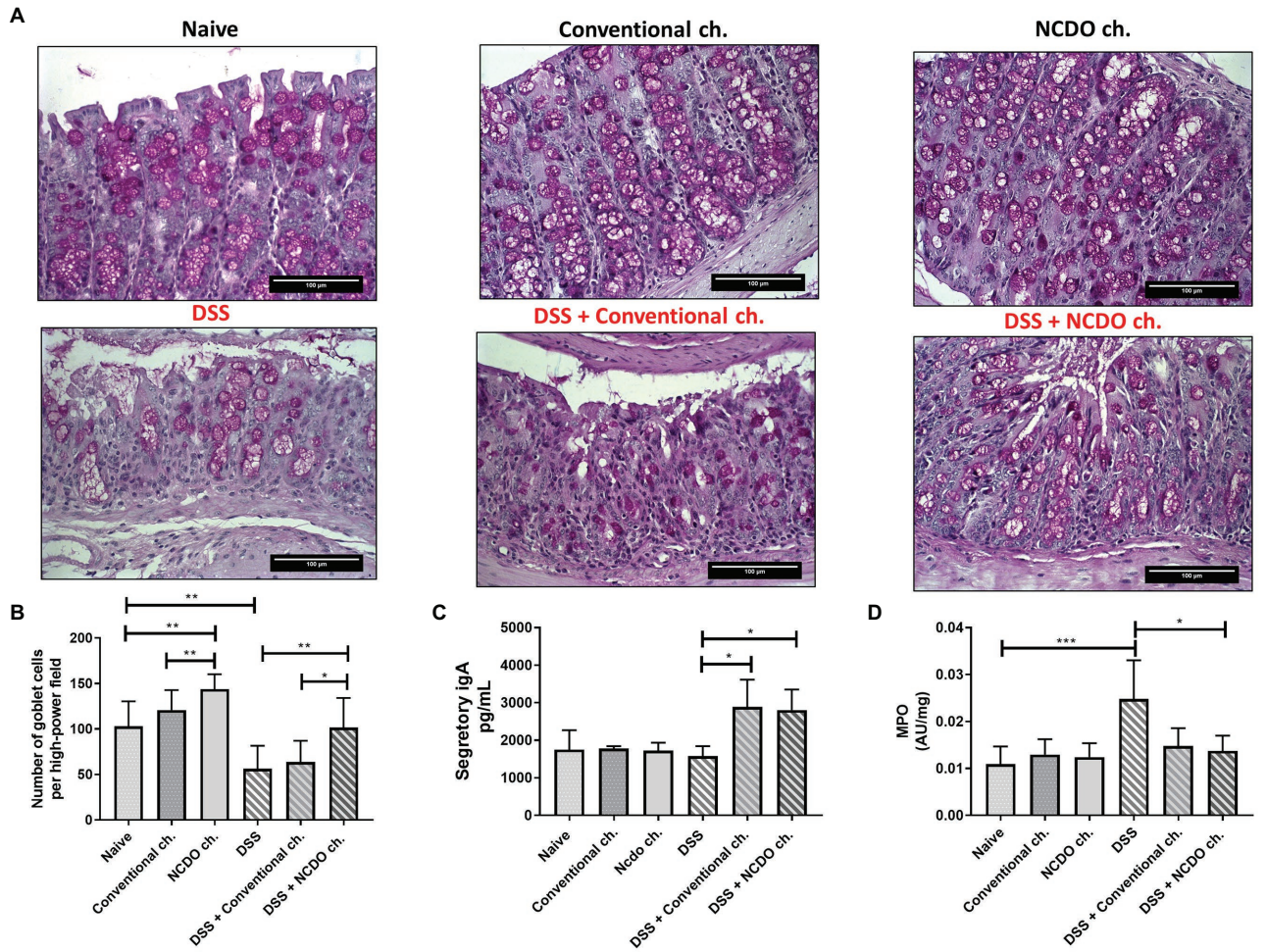

FIGURE 3 | Effect of probiotic cheese administration on goblet cells, secretory immunoglobulin A (sIgA) levels, and the enzyme activity of myeloperoxidase (MPO). **(A)** Representative Periodic acid-Schiff (PAS) staining of colon mucosa sections. **(B)** Number of goblet cells, given by counting intact cells in 10 random field images on mouse colon. **(C)** Quantification of sIgA in the small intestine. **(D)** Levels of MPO activity that indirectly determines the neutrophil concentration in 10 mg of colon tissue. Values indicate the mean ± standard deviation ($n = 6$). Image acquisition was done with a 20× magnification objective. Scale bar = 100 μm ($n = 6$). Asterisks represent statistically significant differences: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

stress (*iNOS*; **Figure 4**), and cytokine gene expression (*IL-10*, *IL-1β*, and *IL-17*; **Figures 5A–C**). Our results showed that the consumption of conventional or probiotic cheese in healthy control groups (naive, conventional ch., and NCDO ch. groups) was not able to alter the expression of the genes evaluated ($p > 0.05$). The intake of DSS in drinking water also did not alter the expression of *zo-1*, *zo-2*, *ocln*, *cln-1*, *MUC-2*, *IL-10*, and *IL-17* genes, when compared with the naive group. On the other hand, we observed an increase in the expression of *iNOS* and *IL-1β* genes, when compared with the DSS group and naive group ($p < 0.0001$). Interestingly, we observed that the consumption of probiotic cheese in unhealthy mice induced an increase in the expression of *zo-1*, *zo-2*, *ocln*, and *cln-1* genes of epithelial barrier, compared with the DSS and naive groups, while the expression of *iNOS* and *IL-1β* genes was decreased in animals treated with probiotic cheese. Also, we observed that *MUC-2* gene expression tended to increase in animals treated with probiotic cheese but was not statically different from DSS ($p > 0.05$).

Probiotic Cheese Modulated Cytokine Production in Mice

To clarify the potential mechanisms by which probiotic cheese exerts its beneficial effects, we evaluated the cytokine profiles in the spleen and lymph nodes of mice (**Figure 5**). Our data showed that oral administration of probiotic cheese increased the levels of the anti-inflammatory cytokine IL-10 in the spleen (329.4 pg/ml, **Figure 5D**) and in the lymph nodes (24.9 pg/ml, **Figure 5G**), when compared with the DSS group (233.6 and 4.24 pg/ml, respectively) and DSS + conventional ch. (161.4 and 3.73 pg/ml, respectively). The intake of DSS led to increased cytokine IL-1β in the spleen (243.7 pg./ml, **Figure 5E**) and IL-17 in the lymph nodes (163.9 pg./ml, **Figure 5I**), when compared with the naive group (123.8 and 44.4 pg./ml). On the other hand, consumption

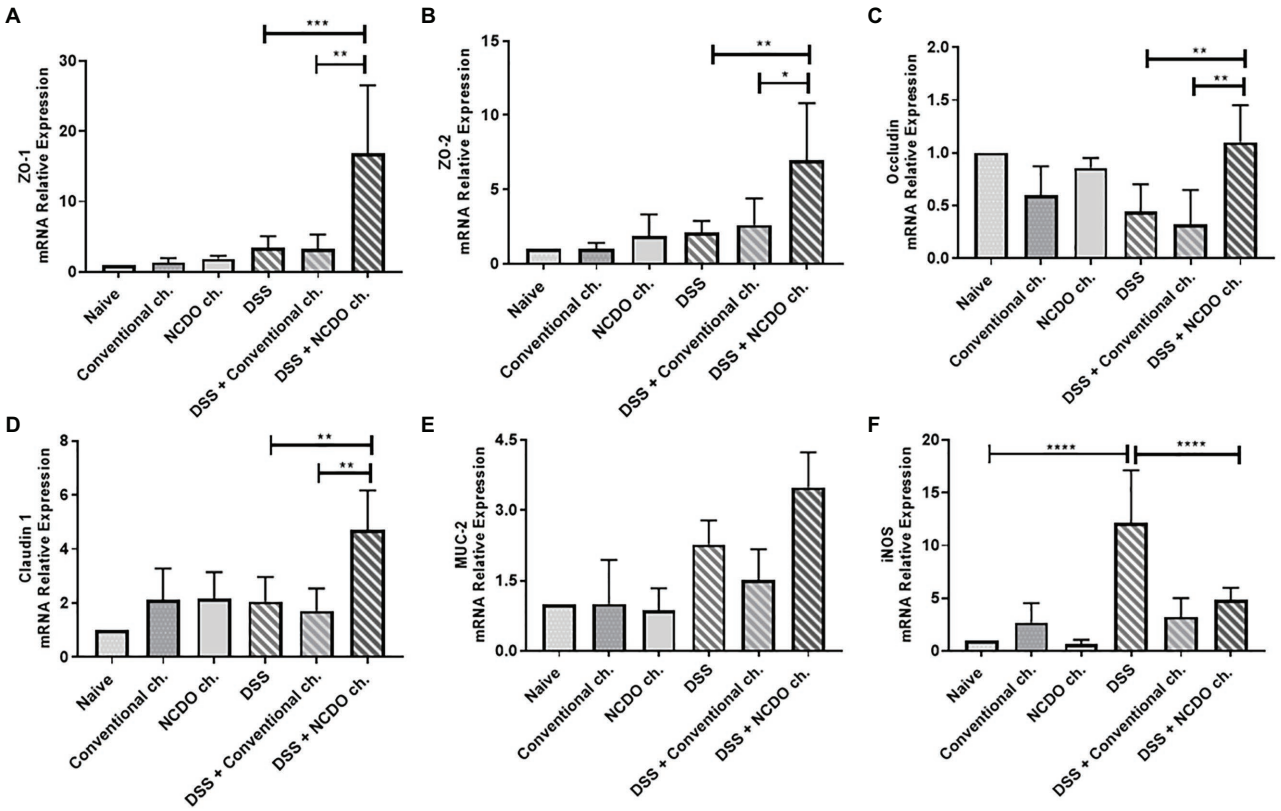

FIGURE 4 | Impact of probiotic cheese on colonic expression markers of cell barrier and oxidative stress. Colonic mRNA expression levels of **(A)** *zo-1*, **(B)** *zo-2*, **(C)** *ocln*, **(D)** *cln-1*, **(E)** *MUC-2*, and **(F)** *iNOS* genes analyzed by qRT-PCR. The data represent the mean ± SD ($n = 6$). Asterisks represent statistically significant differences: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

of probiotic cheese in the DSS mice group was able to maintain IL-1β and IL-17 levels of production similar to healthy animal levels (140.9 and 41.53 pg/ml, respectively).

Probiotic Bacteria, *L. lactis* NCDO 2118, Did Not Alter the Frequency of Tregs in the Spleen and Lymph Nodes

T-cell subpopulation (Treg $CD4^{+}Foxp3^{+}$, $CD4^{+}LAP^{+}$, and $CD4^{+}Ror\gamma T^{+}$) was evaluated in mice spleen and lymph nodes by using flow cytometry (**Figure 6**). The probiotic cheese, containing *L. lactis* NCDO 2118, did not change the percentage of Tregs on the spleen and lymph nodes in both healthy and inflamed mice. No statistical differences were found between the DSS and naive groups for all cells analyzed here.

DISCUSSION

The importance of diet in human health has been described by various scientific evidence; therefore, the development of new food products with health-giving additives and medical benefits is a pressing need (Domínguez Díaz et al., 2020). In this context, probiotic functional foods have been proposed, due to the proven therapeutic benefits of probiotic bacteria by the consumers (Carmo et al., 2017). In this work, we developed a new probiotic Minas Frescal cheese for the treatment of UC.

The addition of certain bacteria to cheese can contribute to altering glycolysis, proteolysis, and lipolysis processes that change the proximate composition and mineral contents of cheese and modify the organoleptic properties of the final product (Cárdenas et al., 2014). Thus, it was necessary to investigate whether the Minas Frescal cheese manufactured with *L. lactis* NCDO 2118 altered the centesimal composition and bioactive compounds of the cheese, as well as whether Minas Frescal cheese was a good matrix to maintain the viability of this probiotic strain. It is worth emphasizing that the beneficial effects of foods containing probiotics strains depend on the ability of these bacteria to survive to industrial process after passing through the GIT, which imposes unfavorable bacterial conditions and can affect probiotic potential (Cordeiro et al., 2019). In this sense, the regulatory agencies around the world recommended that for a probiotic product to be able to exercise its benefits, there must be a viable amount of probiotic bacteria of between 10^{6} and 10^{7} CFU/g (Castro et al., 2015). In this work, we observed that after manufacturing of Minas Frescal cheese, *L. lactis* NCDO 2118 presented 10^{7} CFU/g of viable cells counts, according to the recommendation, and this reinforces that Minas Frescal cheese

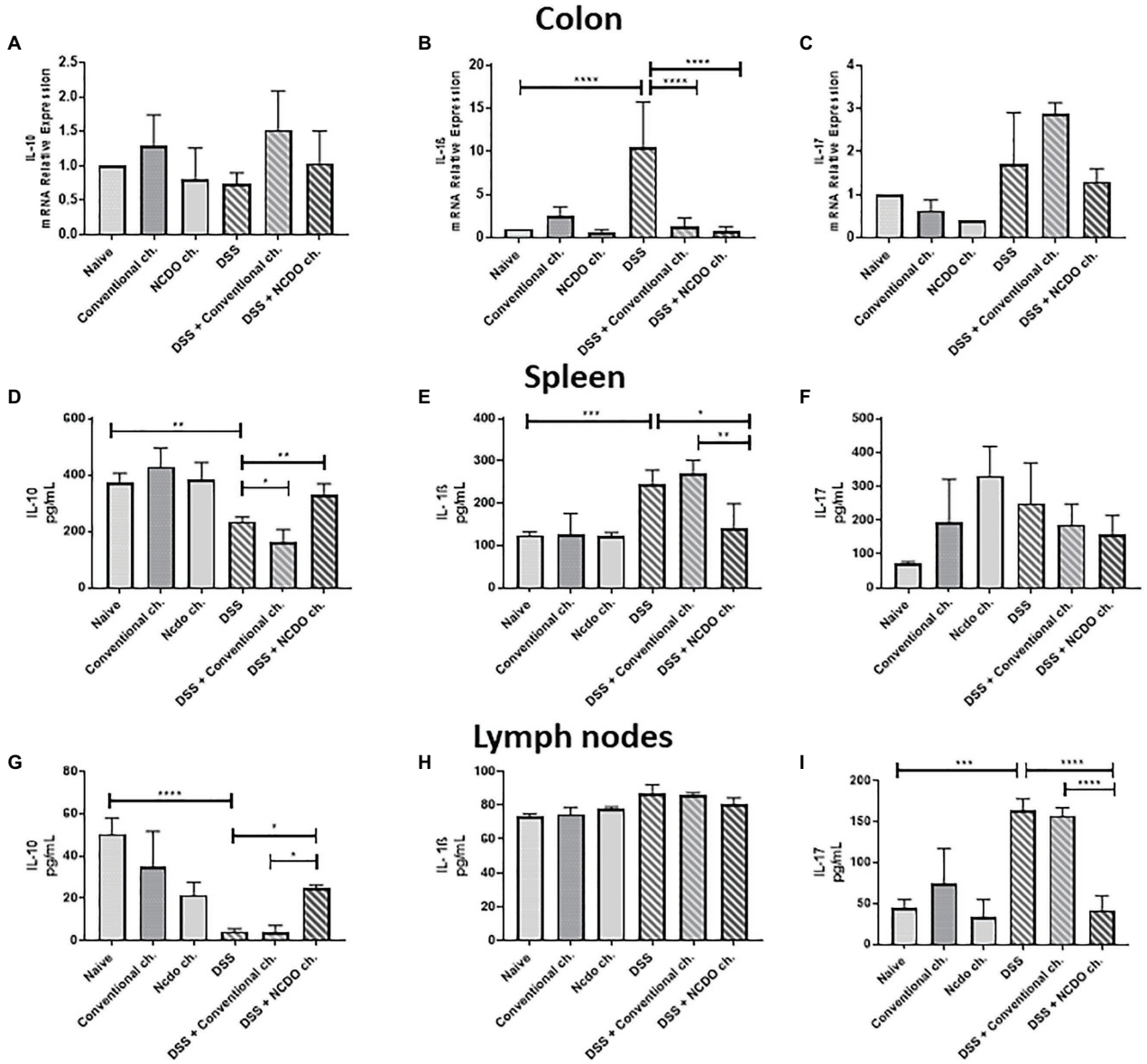

FIGURE 5 | Colonic expression of cytokines genes and immunomodulatory effect of probiotic cheese in dextran sodium sulfate (DSS) colitis mice. Colonic mRNA expression levels of **(A)** *IL-10*, **(B)** *IL-1β*, and **(C)** *IL-17* genes analyzed by qRT-PCR. Enzyme-linked immunosorbent assay (ELISA) of **(D,G)** IL-10, **(E,H)** IL-1β, and **(F,I)** IL-17 cytokines in spleen and lymph node cell culture supernatant, respectively. The data represent the mean ± SD ($n = 6$). Asterisks represent statistically significant differences: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

is a good delivery system to maintain the viability of this probiotic bacteria through manufacturing processes. Previous studies showed that soft cheeses, like Minas Frescal cheese, are a good protective matrix for bacteria (Hosoya et al., 2012; Lollo et al., 2012, 2015; Sperry et al., 2018). In addition, our work demonstrated that *L. lactis* NCDO 2118 added on Minas Frescal cheese did not alter the proximal composition parameters evaluated (moisture, protein, fat, and lactose), as well the sodium and calcium contents, and pH values of the cheese. The probiotic cheese developed in this work still maintained the specifications recommended by the Brazilian legislation law established for moisture (>55%) and fat (25–44.9%) in Minas Frescal cheese dry matter (FDM; Matera et al., 2018).

Cheeses are recognized not only for their high nutritional value but also for the production of bioactive peptides, from casein hydrolyzed by proteases and peptidases (López-Expósito et al., 2017). Some of these peptides can resist gastrointestinal digestion, responsible for biological activities such as antihypertensive, antioxidant, and antidiabetic activities (Livney, 2010). However, the introduction of some probiotic bacteria,

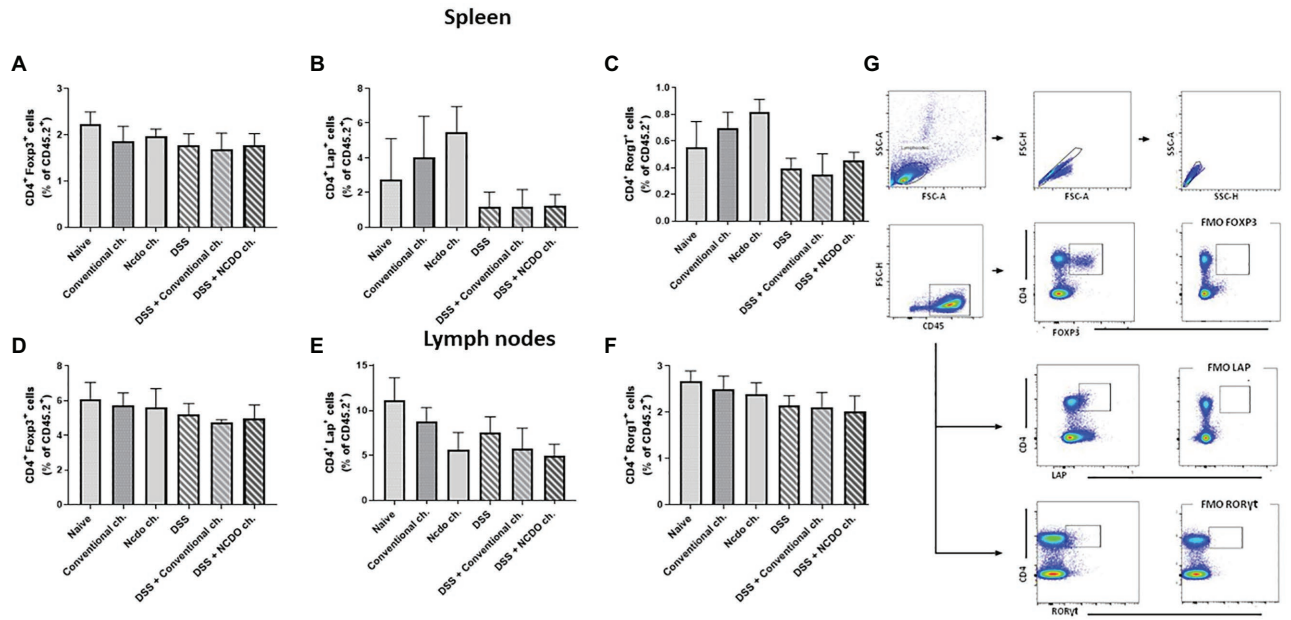

FIGURE 6 | Evaluation of T lymphocyte production in spleen and lymph nodes after induced colitis. T cells were isolated from mouse spleen and lymph node, and the frequencies of $CD4^+Foxp3^+$ **(A,D)**, $CD4^+RORγt+$ T cells **(B,E)**, and $CD4^+Lap+$ T **(C,F)** cells as frequency (%) of $CD4^+$ T cells were assessed by flow cytometry. **(G)** Plots with the gating strategy based on forward and side scatters, selecting splenocytes as a function of cell size and granularity ($n = 6$).

i.e., LAB, in this dairy product can increase the production of bioactive peptides (Smacchi and Gobbetti, 2000; Ayyash et al., 2018). Sperry et al. (2018) suggested that *Lactobacillus casei* 01 can generate high levels of antihypertensive (ACE-I) and antioxidant peptides (DPPH) in Minas Frescal cheese. In this sense, our probiotic Minas Frescal cheese, with *L. lactis* NCDO 2118, also induced an increased amount of antihypertensive (ACEI), antioxidant (DPPH), and antidiabetic activities (α-amylase and α-glucosidase) when compared with conventional Minas Frescal cheese. Interestingly, it is recognized that oxidative stress (OS) is one of the factors involved in the onset of IBD symptoms (Moura et al., 2015); therefore, we suggest that the increase in DPPH levels in probiotic Minas Frescal Cheese could help to ameliorate inflammation conditions in UC mice.

Considering the cheese properties presented, we decided to exploit the therapeutic effect of the consumption of the probiotic Minas Frescal cheese, containing *L. lactis* NCDO 2118, in the context of DSS-induced colitis in mice.

The UC disease symptoms include weight loss, tummy pain, recurring diarrhea with blood in the stool, and malaise (Zhang and Li, 2014). The inflammation reaches the mucosa and submucosa layers of the colon section, with the presence of edema, significant depletion of goblet cells, and changes in tissue architecture and ulcerations (Cordeiro et al., 2019). UC treatments are based on the control of the symptoms and administration of anti-inflammatories and antibiotics, immunosuppressive drugs, and surgeries in severe cases. However, none of these treatments are curative and instead provoke serious collateral effects in UC patients (Chibbar and Dieleman, 2015). In this context, functional probiotic foods have been suggested to be used alone or in combination with conventional drugs and act like adjuvant therapy to enhance remission in UC patients (Rabah et al., 2020). Regarding the mice weight loss triggered by DSS administration, we observed that the treatment with the probiotic Minas Frescal cheese, for seven experimental days, was able to prevent weight loss in mice. It is important to clarify that no differences in food consumption or caloric intake were observed in all groups analyzed, which suggested that this weight gain is linked to probiotic cheese administration. As in previous studies, consumption of probiotic bacteria in a dairy food was able to prevent weight loss in inflammatory disorder mouse models (Santos Rocha et al., 2014; Plé et al., 2016; Cordeiro et al., 2018).

In this work, we observed that unhealthy mice treated with probiotic Minas Frescal cheese exhibited attenuated clinical and macroscopic signs of colitis disease. This is mainly demonstrated by a decrease in DAI, hence, less diarrhea and rectal bleeding as well as the prevention of colon shortening triggered by DSS action. Similarly, Luerce et al. (2014) demonstrated that *L. lactis* NCDO 2118 improved the clinical signs of colitis by reducing the macroscopic inflammatory score of the disease, also seen by Rabah et. al (2020) who observed a reduction in the signs of UC induced by DSS by the consumption of a probiotic Emmental cheese.

Pathological assessment of UC is evidenced by extensive architectural damage of colon tissue, with erosions and ulcerations and depletion of the mucosal surface. Moreover, there was an increase in inflammatory cell infiltration in the *lamina propria*, i.e., neutrophil infiltrates, and also depletion of goblet cells

(Jeengar et al., 2017). The activity of MPO is an indicator of this extent of neutrophil infiltrates in the mucosa (Ivanovska et al., 2017). Nevertheless, our results showed that the administration of probiotic Minas Frescal cheese protects the colon mucosa from DSS injury, marked by a decrease in the histological score and also a decrease in MPO levels. Besides that, we observed that probiotic cheese was able to decrease the expression of *iNOS* gene. This gene encodes the enzyme responsible for the generation of cytotoxic and immunoregulatory free radical NO, which is related to several inflammatory processes (Sakthivel and Guruvayoorappan, 2013). These results together demonstrated that there is less inflammation in the colon tissue of DSS mice treated with probiotic cheese.

Probiotic Minas Frescal cheese, with *L. lactis* NCDO 2118, was also able to preserve the number of intact goblet cells in the colon mucosa. These cells are responsible for producing the mucus that covers the intestinal mucosa. Considering that this mucus contains high levels of sIgA, we suggested that the increased sIgA levels, observed in the intestinal content of mice, were driven by the maintenance of the number of goblet cells due to the consumption of probiotic cheese. Precisely, mucus production by goblet cells and increased levels of sIgA were reported to be some of the mechanisms of probiotic action in the host (Rogier et al., 2014). Interestingly, dairy milk can significantly induce the host response to pathogens, enhance the integrity of the mucus layer (Tong et al., 2020), and increase secretory IgA in the small and large intestines (Schofield and Palm, 2018). In our previous works, we verified that dairy milk matrices, including cheese matrix, can increase IgA secretion (Cordeiro et al., 2018 Rabah et al., 2020), while the milk matrix shows an increase in the number of goblet cells (Cordeiro et al., 2018). Thus, this would explain the increases in the levels of sIgA and goblet cells found in the group treated with our probiotic cheese. On the other hand, to uncover the exact mechanisms, the expression of intestinal immune-related gene and sIgA levels needs to be better explored. Besides that, it is recognized that the presence of the mucus in the gut prevents the adhesion of microorganisms to the mucosa and their translocation into the lumen (Grondin et al., 2020). Moreover, the mucus is important for the lubrication and protection of the intestinal epithelium from toxic substances coming from the external environment, such as DSS (Abrantes et al., 2020). Thus, in mouse colon inflammation caused by DSS intake, it is common to observe a decrease in goblet cell number, but it can be restored by the consumption of probiotic bacteria (Rodrigues et al., 2018; Zhang et al., 2018; Abrantes et al., 2020). It is important to highlight that MUC-2 is the major glycoprotein constituent of intestinal mucus and is secreted primarily by goblet cells (Perez-Vilar, 2007). Interestingly, we also observed an increase in *MUC-2* gene expression in mice treated with probiotic cheese, corroborating with the observed increased production of the mucus in these mouse groups. As seen in a previous study, probiotic strain can stimulate *MUC-2* expression in intestinal goblet cells and mitigate acute colitis in a mouse model (Ma et al., 2020). Moreover, our findings showed that probiotic cheese administration also increased the gene expression of *zo-1*, *zo-2*, *ocln*, and *cln-1*. These genes are responsible for the expression of tight junction proteins that maintaining the epithelial barrier and control cellular permeability (Landy et al., 2016).

The host's cytokine-mediated immune response plays a pivotal role during the development of acute colitis (Ko and Auyeung, 2014). Probiotic bacteria have a great ability to promote increased levels of anti-inflammatory cytokines and also to lead to a decrease in the production of pro-inflammatory cytokines (Carvalho et al., 2017). In IBD, IL-17 and IL-1β cytokines are related to the extensive lymphocyte, plasma cell, and macrophage infiltration into the tissue (Melgar et al., 2005; Shen and Durum, 2017). The decrease in the transcriptional colonic levels of IL-1β and the secretion of IL-1β and IL-17 (spleen and lymph node, respectively) by the consumption of probiotic cheese in the disease DSS group can be mediated by the action of IL-10 secretion (spleen and lymph node). IL-10 is the most important cytokine to control homeostasis in the intestinal mucosa (Sun et al., 2018), and probiotic bacteria, mainly LAB, are known to be able to increase IL-10 levels in the gut (Maldonado Galdeano et al., 2019). Interestingly, low transcriptional levels of IL-1β on the DSS groups treated with cheese (conventional or probiotic cheese) were observed. It is plausible to say that the downregulation of IL-1β can be associated with milk components in the cheese matrix, as noted by Kanwar et al. (2016). In addition, we can observe a systemic effect on the increase in IL-10 and decrease in IL-1β in the spleen of animals treated with probiotic cheese. In the local effects (lymph nodes), we see a decrease in IL-17 and an increase in IL-10, which corroborates with the low transcriptional colonic levels of IL-1β. This suggests that the effect of probiotic cheese may be associated with a decrease of pro-inflammatory Th1 and Th17 cytokines that can be linked to the enhanced production of IL-10 in the lymph nodes and spleen, as previously reported (Santos Rocha et al., 2014).

$Foxp3^+$ Tregs are the subgroup of $CD4^+CD25^+$ T cells that have the capacity to inhibit the reactive effects of T cells by producing cytokine transforming growth factor β1 (TGF-β1) and IL-10 (Maldonado Galdeano et al., 2019). $CD4^+$ T cells expressing $FOXP3^+$, LAP^+, and RORγt+ as analyzed in mouse spleens and lymph nodes (cecum and colon) show that treatment with probiotic cheese did not change regulatory T cell populations. Our work suggests that $Foxp3^+$ Tregs are not responsible directly for the therapeutic effects of probiotic bacteria, despite increased levels of IL-10 (lymph nodes and spleen). It is plausible that the therapeutic effects of probiotic Minas Frescal cheese did not act *via* the adaptive immunity. However, IL-10 staining in regulatory T cells populations can be conducted to elucidate this hypothesis. Precisely, to confirm these results, it is necessary for other experiments to be able to indicate if the release of IL-10 is by innate immune cells such as macrophages and dendritic cells.

CONCLUSION

We demonstrated that Minas Frescal cheese containing the well-characterized probiotic bacteria *L. lactis* NCDO 2118 was able to alleviate the severity of DSS-induced colitis in

a mice model, limiting histopathological damages, restoring intestinal barrier by increased expression of gene related to tight junction protein, and modulating the cytokine production in mice. Probiotic Minas Frescal cheese was also able to prevent the degeneration of goblet cells and to reduce the inflammatory cell infiltration in the colon mucosa. Moreover, experimental probiotic cheese investigated in this work was able to produce high levels of bioactive peptides with antihypertensive, antioxidant, and antidiabetic activities. These results, together, open new perspectives for the development of probiotic functional foods for use in combination with conventional drugs or for use as an adjuvant therapy to enhance remission in UC patients.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethics Committee on Animal Experimentation of the Universidade Federal de Minas Gerais (CEUA-UFMG, Brazil, protocol 364/2018).

AUTHOR CONTRIBUTIONS

BC, VA, and FC conceived and designed the experiments. JA, LL, and AF performed and analyzed immunomodulatory experiments. MB was a major contributor to animal experimentation. EF performed, analyzed, and interpreted the histological analysis from colon slides. BC, GB, AG-G, and FC wrote the original draft. GJ and YL gave scientific advice. JG, RS, RR, MS, MF, EE, and AG-G manufactured the cheeses and performed centesimal and mineral composition. All authors contributed to data interpretation, drafted the manuscript, critically revised the manuscript, and approved its final version.

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Comprehensive Substrate-Based Exploration of Probiotics From Undistilled Traditional Fermented Alcoholic Beverage '*Lugri*'

Neha Baliyan[1,2], *Kiran Dindhoria*[1,2], *Aman Kumar*[1,2], *Aman Thakur*[1,2] and *Rakshak Kumar*[1,2]*

[1] Biotechnology Division, CSIR-Institute of Himalayan Bioresource Technology, Palampur, India, [2] Academy of Scientific and Innovative Research (AcSIR), CSIR- Human Resource Development Centre, Ghaziabad, India

Cereal-based traditional fermented beverages (TFBs) are prevalent among India's ethnic community, and *lugri* is one such TFB popular among the tribal people of the Lahaul valley in North-Western Himalaya. Previous studies have reported that *lugri* harbors probiotics and contains amino acids and vitamins but comprehensive substrate-specific exploration of *lugri* for probiotic attributes is unexplored. The present study selected three substrate-based *lugri* (wheat, rice, and barley) to study their biochemical properties and explore potential probiotics. This study screened the best probiotic strains for antioxidant studies and the fermentative process. A biochemical analysis determined that rice-based *lugri* had a higher alcohol content, electric conductivity, crude protein, and lower pH than barley and wheat-based *lugri.* A total of 134 distinct morphotypes were screened, and 43 strains were selected based on their qualitatively superior acid and bile tolerance. Rice-based undistilled *lugri* harbored the most probiotics, with 22 out of 43 strains isolated. All 43 bacterial isolates exhibited properties like cell surface hydrophobicity, cell-auto aggregation, β-galactosidase, and exopolysaccharide production, supporting them as possible probiotics. Based on antibiotic susceptibility, hemolytic activity, and biofilm formation, all the bacterial strains were found to be non-pathogenic. Taxonomically, they ranged among eight distinct genera and 10 different species. Statistically, 12 isolates were found to be the most promising probiotic, and eight strains were isolated from rice-based undistilled *lugri*. Furthermore, the antioxidant activity of the promising isolates was tested, based on free-radical scavenging ability toward 2,2-diphenyl-1-picrylhydrazyl (4.39–16.41%) and 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (15.29–57.74%). The strain *Lacticaseibacillus paracasei* LUL:01 showed the best antioxidant activity and probiotic attributes, and hence was used for the production of fermented milk. The strain LUL:01 fermented the sterile milk within 18 h, and the viable count remained above the legal requirement of 6 $\log_{10}$ CFU/ml during 28 days storage at 4°C. The strain represents a suitable candidate for applying probiotic functional food formulation with several health benefits.

Keywords: traditional fermented beverages, North-Western Himalaya, probiotics, cereal based beverages, antioxidant activity

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***Correspondence:**
Rakshak Kumar
rakshak@ihbt.res.in

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INTRODUCTION

Alcoholic beverages are important components in different social cultures all over the globe. Since antiquity, people have been preparing traditional fermented beverages (TFBs) that are unique to their local cultural practices. In general, TFBs are prepared from cereals such as rice, wheat, corn, barley, and sorghum, etc. (Salmerón et al., 2015). These cereals are rich in nutrients like carbohydrates, proteins, antioxidants, vitamins, minerals, and dietary fibers and transmit these properties to cereal-based TFBs during their preparation, thus making them very nourishing (Blandino et al., 2003; Kreisz et al., 2008). In a report by the World Health Organization (WHO), TFB alcohol accounts for one quarter (25.5%) of all the alcohol consumed worldwide (World Health Organization [WHO], 2019). Despite being the largest dietary source for major parts of the population and having several health-promoting properties, very little attention has been given to the production of cereal-based fermented probiotic products in developing countries.

Lugri is a very popular TFB among the tribal people of Lahaul valley in the North-Western Himalayan region. It is indigenously prepared from cooked cereals like rice, wheat, barley, and a starter culture locally termed as '*phab*' (Thakur and Bhalla, 2004). The *phab* initiates the fermentation process in food and consists of different types of lactic acid bacteria, yeasts, and molds (Thakur et al., 2015), which get enriched in the later maturation phases of TFBs. The purified and the distilled form of *lugri* is known as '*Arak*,' which is a famous traditional drink, very common in local ceremonies and contains up to 5–7% of the alcohol content (Angmo and Bhalla, 2014). It has been found that these alcoholic beverages have many ethnomedicinal properties worthy of scientific attention (Ray et al., 2016). The preparation process and the bacterial diversity of this TFB is also documented in other literature (Sharma et al., 2013; Rai and Kumar, 2015; Thakur et al., 2015), but it has never been explored for its probiotic properties. Moreover, the literature still lacks a comprehensive study of *lugri*, especially concerning cereal-based substrates. The substrates like cereals harbor a number of Lactic acid bacteria (LAB), which produce substances like oligosaccharides, organic acids, and polyphenolic compounds during fermentation, with health benefits for consumers (Vinderola and Reinheimer, 2003; Leroy and De Vuyst, 2004; Enujiugha and Badejo, 2017). Moreover, in another study LAB have been found to play an important role in the fermentation of cereals, vegetables, meat, and dairy products, mainly due to their acidifying, proteolytic, and aromatic compound producing activity (Ashaolu and Reale, 2020). The current study isolated probiotics from undistilled *lugri* prepared from the three substrates rice, wheat, and barley. Our main aim was to identify the most suitable substrate-based *lugri* with regard to residential probiotic diversity. We also checked the probiotic attributes, functional analysis and safety evaluation along with their potential strain for functional food formation such as fermented milk in order to explore various health benefits provided by these strains.

MATERIALS AND METHODS

Collection and Biochemical Analysis of Undistilled *Lugri* Samples

The samples of undistilled *lugri* prepared from rice, wheat, and barley substrates were collected from Ghosal (32.54911°N–76.96941°E), Jundha (32.64105°N–76.84492°E), Kanjar (32.87251°N–76.85702°E), Upper Sumnam (32.55920°N–76.98329°E), and Urgosh (32.85852°N–76.79588°E) villages of the Lahaul valley of Himachal Pradesh (**Figure 1**). All the samples were collected in sterile containers and were stored at 4°C until further use.

To assess the biochemical analysis, pH, electric conductivity (EC), alcohol content, ash, moisture content, crude fat, and protein content of the collected samples were performed according to the method provided by AOAC (2016). The pH and EC (mS/cm) of various samples were recorded using the digital pH and EC meter (Eutech, India). For moisture content analysis, 10 g of the sample was weighed and placed at 110°C for 2 h in a hot air oven until the sample weight became stable. The sample was then brought to room temperature in a desiccator, and the weight of the sample was measured again. To determine the ash content, 5 g of the sample were weighed and placed in an electric muffle furnace at 550–600°C for at least 5 h. The light gray-colored ash obtained after incubation was then cooled down in a desiccator and again weighed to estimate ash content. The crude protein was determined by the Kjeldahl nitrogen method using 40% NaOH and 4% boric acid. For crude fat content, 5 g of the moisture-free sample was used to extract the fat content with petroleum ether in Soxhlet extraction apparatus for 18 h. The ether extract solvent was added to a pre-weighed beaker and again weighed after the complete evaporation of petroleum ether.

Isolation and Screening of Probiotic Bacteria

Bacterial isolation from various *lugri* samples was conducted using serial dilution and spread plate technique (Zommiti et al., 2018). 100 μl of the *lugri* samples were serially diluted from 10^{-1} to 10^{-7} in sterile normal saline (0.85% NaCl). Aliquots of 100 μl from serial dilutions were spread plate on de-Mann Rogosa Sharpe (MRS) agar (Hi-media Lab., Mumbai, India). The plates were incubated under aerobic conditions for 48 h at 37°C. Colonies with unique morphologies were further streaked on MRS agar to obtain the pure cultures. The glycerol stock (25% v/v) of each isolate was prepared and stored at −80°C for long term use.

The unique morphotypes were further estimated qualitatively for their growth at different pH (2–4) ranges and bile salt concentrations (0.3–3.0%) on the MRS agar plate at 37°C. Based on their qualitative assay, the primary identification of bacterial strains was carried out using gram staining and catalase test. For the catalase test, 3.0% of hydrogen peroxide was added to bacterial cultures. The observation of effervescence indicated the presence of a catalase enzyme. The reference type strain, *Lacticaseibacillus rhamnosus* (ATCC 53103) was used as a positive control for comparison in all the experiments.

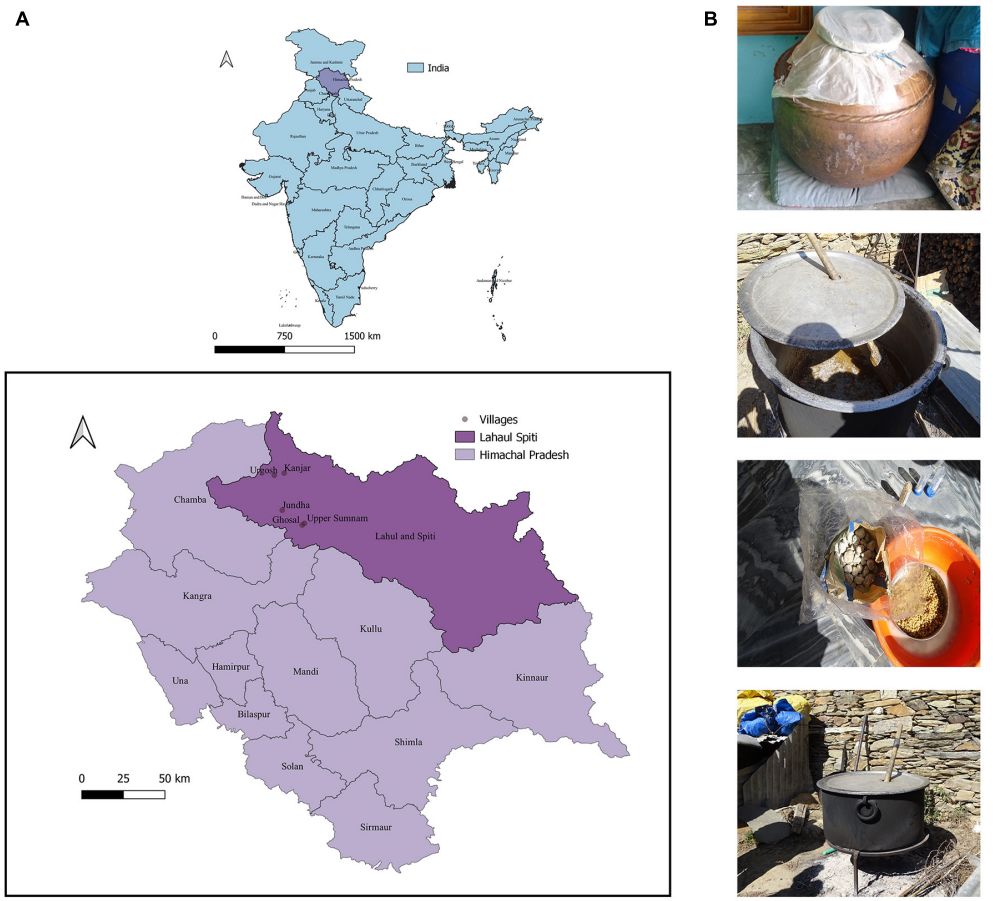

FIGURE 1 | Sampling site location and various undistilled *lugri* samples. **(A)** Illustration of the map depicting the geographical location of the Lahaul and Spiti district of Himachal Pradesh. The sites include the tribal villages of the Lahaul valley namely: Ghosal (32.54911°N–76.96941°E), Jundha (32.64105°N–76.84492°E), Kanjar (32.87251°N–76.85702°E), Upper Sumnam (32.55920°N–76.98329°E), and Urgosh (32.85852°N–76.79588°E), and samples were collected in sterile sample containers. The map was made in QGIS version 3.10.5 (URL: http://qgis.org). The villages from where the sample was collected are shown after zooming out. **(B)** The traditional method of preparation of *lugri*, the figure shows vessel and starter culture "*phab*" used to prepare *lugri*.

Physicochemical Characterization

The bacterial cultures were grown overnight and inoculated (1% v/v) in the MRS broth containing different NaCl concentrations (1, 2, 3, and 4%, w/v), temperature (4, 15, 37, and 45°C), and pH conditions (pH 4–9) at 37°C for 24 h.

Characterization of Probiotic Strains in *in vitro* Simulated Gastric Tract Conditions

Acid and Bile Tolerance

Acid tolerance assay was conducted using the method provided by Shehata et al. (2016) with slight modifications. The overnight cell culture was centrifuged at 5,000 × *g* for 15 min at 4°C, and the obtained pellet was washed three times with phosphate buffer saline (PBS, pH 7.2). The pellet was again resuspended in the same buffer. The simulated gastric juice was prepared with the addition of pepsin (3% v/v, Sigma-Aldrich) in normal saline (0.5%) with three different pH 2.0, 2.5, and 3.0 values. The cell suspension of 1 ml was mixed properly with 5 ml of simulated gastric juice and vortexed for 25 s, followed by incubation at 37°C. 100 μl aliquots were taken at 2 h and 12 h, respectively, and plated on MRS agar to check the viability count.

The isolates' ability to grow in the presence of bile salt was determined according to the method given by Hamon et al. (2011) with a few modifications. Each isolate was grown overnight and then inoculated (1% v/v) into MRS broth containing 1, 2, and 3% (w/v) ox-bile salt concentration (Hi-media, Mumbai, India). The culture broth was incubated at 37°C, and after 12 h of incubation, the absorbance was taken at $A_{560\ nm}$ using a spectrophotometer (Synergy LX multimode reader, BioTek). The results were expressed in optical density (O.D.) of media in the presence of bile salts compared to the control (without bile salts).

Determination of Cell Adhesion

Cell Auto-Aggregation

The auto-aggregation ability of all the cultures was determined following the method by Kumari et al. (2020). Each isolate was

grown for 18 h in MRS broth at 37°C and was centrifuged at 5,000 × *g* for 15 min at 4°C. The cell pellets were washed twice with phosphate buffer saline (PBS, pH 7.2) and resuspended in the same buffer to adjust the absorbance to 0.5 O.D. at 600 nm (A_0). The cell suspension was vortexed for 15 s and incubated for 24 h at 37°C. The upper layer of this suspension (A_t) was measured ($A_{600\ nm}$) using a spectrophotometer. The percentage of auto-aggregation was calculated as:

$$Cell\ auto-aggregation(\%) = [(A_0 - A_t)/A_0] \times 100$$

Cell Surface Hydrophobicity

Over-night grown bacterial isolates were centrifuged at 5,000 × *g* for 15 min. The pellet was washed twice with phosphate urea magnesium buffer (pH 7.1), and then the pellet was resuspended in the same buffer. The absorbance was adjusted to ∼0.7 O.D. at 600 nm (A_0), and then, 1 ml of n-hexadecane (Hi-media, Mumbai, India) was added in 3 ml of the cell suspension. The mixture was vortexed for 20 s and incubated at 37°C for 24 h. After incubation, the absorbance (A_t) of the aqueous phase was measured at 600 nm (Mallappa et al., 2019). The percentage of cell surface hydrophobicity (%) was calculated as follows:

$$Cell\ surface\ hydrophobicity(\%) = [(A_0 - A_t)/A_0] \times 100$$

Functional Attributes

Antimicrobial Activity

Antimicrobial activity of the isolates was assessed using well diffusion assay against Gram-positive [*Bacillus subtilis* (MTCC 121), *Micrococcus luteus* (MTCC 2470), and *Staphylococcus aureus* (MTCC 96)], and Gram-negative [*Escherichia coli* (MTCC 43), *Klebsiella pneumoniae* (MTCC 109), and *Pseudomonas aeruginosa* (MTCC 2453)] opportunistic pathogen type strains. The inhibition zone diameter was measured after the incubation for 24 h at 37°C (Yadav et al., 2016).

Exopolysaccharide (EPS) Production

The bacterial cultures were evaluated for the EPS production on MRS plates containing 5 and 10% concentrations of sucrose and lactose, respectively, as the carbon sources. The overnight grown cultures were streaked on the modified MRS plates and incubated at 37°C for 3 days (Kumari et al., 2020).

β-Galactosidase Activity

The bacterial isolates were spotted on MRS agar plates containing 60 μl *X*-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and 10 μl IPTG (isopropyl-thio-β-D-galactopyranoside) as an inducer, followed by incubation at 37°C for 2 days (Angmo et al., 2016).

Safety Evaluation of Selected Bacterial Isolates

Antibiotic Susceptibility Test

The antibiotic susceptibility of the bacterial isolates was assessed by the disk diffusion method (Kumari et al., 2020). For the assay, 100 μl of overnight grown bacterial cultures (0.5 McFarland standard) were spread plated on MRS agar, and antibiotics disk (Hi-media, Mumbai, India) containing azithromycin (11.5 mcg), kanamycin (30 mcg), tetracycline (30 mcg), ciprofloxacin (5 mcg), rifampicin (5 mcg), and vancomycin (30 mcg) were placed on it under sterile conditions. The results were determined as sensitive (S) and resistant (R), based on the measured inhibition zone diameter after the incubation for 24 h at 37°C.

Hemolysis Assay

The bacterial isolates were spot inoculated on blood agar (Hi-media, Mumbai, India) supplemented with 5% human blood. The plates were incubated for 48 h at 37°C to determine the non-pathogenic nature of the cultures (Argyri et al., 2013).

Biofilm Formation Assay

For safety evaluation, biofilm assay was performed using the method described by Borges et al. (2012), with some modifications. The cultures were grown overnight in MRS broth for 18 h at 37°C, and then, cells were harvested by centrifugation at 5,000 × *g* for 15 min. The cell pellet was washed three times with PBS (pH 7.2) and resuspended in the same buffer with O.D. adjusted equivalent to 0.5 McFarland standard. 10 μl of the cell suspension was inoculated in a sterile 96 well microtiter plate containing 190 μl tryptic soy broth (TSB). After 12 h incubation at 37°C, the microtiter plate wells were washed thrice with 200 μl PBS. The remaining attached cell culture was fixed with 99% methanol. The plate was air-dried for 15 min at room temperature, followed by staining with 200 μl of 2% crystal violet solution. After 5 min of incubation, the unbound dye was gently removed with running tap water, and then the plate was air-dried. 200 μl of absolute ethanol was added to each well to resolubilize the dye bound to adherent cells. The absorbance was measured spectrophotometrically at 595 nm. The un-inoculated TSB was taken as a negative control, whereas, *S. aureus* (MTCC 96), *B. subtilis* (MTCC 121), *E. coli* (MTCC 43), *M. luteus* (MTCC 2470) were used as positive controls.

The optical density value of negative control was taken as optical density cut off (OD_C). The results of isolates were described as non-biofilm, weak, moderate, and strong biofilm producers based on their OD values $OD \leq OD_C$, $OD_C \leq OD (2 \times OD_C)$, $2 \times OD_C < OD \leq (4 \times OD_C)$, and $(4 \times OD_C) < OD$ respectively Gómez et al. (2016).

Molecular and Physiological Characterization of Selected Bacterial Isolates

The universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′- TACGGTACCTTGTTACGACTT-3′) were used for 16S rRNA gene sequencing (Kumar et al., 2013). The generated sequences were used to perform Basic Local Alignment Search Tool (BLAST) analysis to determine the nearest neighbor against the available type strain database[1]. Molecular Evolutionary Genetics Analysis software (MEGA version X) was used for Phylogenetic analysis (Kumar et al., 2018). The

[1]http://www.eztaxon.org/

sequences were aligned using the ClustalW algorithm in built-in MEGA X. Neighbor-joining method was employed to construct the Phylogenetic tree with 1,000 bootstrap replications to assess the nodal support in the tree. Based on high sequence similarity percentage and clear phylogenetic clustering in the same branch, the isolates were assigned to a species described earlier (Kumar et al., 2013).

Nucleotide Sequence GenBank Accession Numbers

The 16S rRNA gene sequences of the characterized probiotic strains were submitted in the NCBI GenBank. The obtained accession numbers are shown in **Table 2**.

Antioxidant Activity

Preparation of Cell-Free Extract

The bacterial strains were grown overnight and centrifuged at 10,621 × *g* for 10 min and washed twice with PBS. The pellet was resuspended in PBS and adjusted to 1.0×10^{10} CFU/ml. The cells were disrupted by ultra- sonification (Sonic's vibra cells VCX 750) (10 and 5 s ON/OFF) at 4°C for 15 min. The cell fragments were separated by centrifugation at 6,000 × *g* for 15 min at 4°C. The cell-free extract (CFE) obtained was used for the investigation of the antioxidant property.

Free Radical Scavenging Activity Toward DPPH

The free radical scavenging ability of the isolates toward 2,2-diphenyl-2 picrylhydrazyl (DPPH) was estimated using Tang et al. (2017). The CFE and DPPH radical solution (0.2 mM in ethanol) were taken in equal amounts (A_{sample}). The mixture was placed in the dark at room temperature for 30 min and centrifuged at 6,000 × *g* for 10 min. The scavenging capacity of isolates was analyzed by measuring absorbance at 517 nm. The control contained an equal amount of water instead of the sample ($A_{control}$), and the blank was prepared using an equal amount of ethanol in the place of DPPH (A_{blank}). Free radical scavenging activity toward DPPH (%) was determined using the formula:

$$= [1 - (A_{sample} - A_{blank})/A_{control}] \times 100$$

ABTS Radical Cation Scavenging Assay

This assay measured the isolates' capacity to scavenge ABTS radical cation (Afify et al., 2012). The stock solution of 2,2 -azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (7 mM) was added to potassium persulfate (2.45 mM) in equal amounts and left overnight until the reaction and absorbance became stable. After incubation for 24 h at room temperature, the absorbance was adjusted at 0.70 ($A_{734\ nm}$) by diluting it with sterile distilled water. The blank was set with distilled water and ABTS (A_{blank}), and the control contained distilled water and sample ($A_{control}$). The CFE (0.2 ml) and ABTS (0.8 ml) solution were mixed and incubated in the dark room for 5 min, and then absorbance was observed spectrophotometrically at 734 nm.

ABTS radical cation scavenging assay (%) was evaluated using the given formula:

$$= [1 - (A_{sample} - A_{blank})/A_{control}] \times 100$$

TABLE 1 | Biochemical analysis of the collected undistilled *lugri* samples from different villages of Lahaul valley in North Western Himalaya.

Sample name	Substrate	Area[a]	GPS[b]	Elev.[c]	pH	E.C.[d]	(%)				Microbial count (CFU/g)
							Moist.[e]	Ash	Crude fat	Crude protein	Alc.[f]	
LUL	Barley	Ghosal	32.54911°N 76.96941°E	2925	3.4 ± 0.01	3.72 ± 0.43	86.84 ± 0.81	23.54 ± 0.33	2.75 ± 0.04	3.41 ± 0.30	0.30 ± 0.01	$5.6 \pm 0.80 \times 10^8$
LUM	Barley	Ghosal	32.54911°N 76.96941°E	2925	3.6 ± 0.01	3.69 ± 0.23	85.97 ± 0.45	24.46 ± 0.30	2.56 ± 0.06	2.90 ± 0.02	0.27 ± 0.01	$6.1 \pm 1.21 \times 10^6$
LUR	Wheat	Upper sumnam	32.55920°N 76.98329°E	3201	3.9 ± 0.02	3.97 ± 0.11	83.25 ± 0.43	38.54 ± 0.40	4.65 ± 0.06	2.5 ± 0.13	0.29 ± 0.01	$3.7 \pm 3.01 \times 10^8$
LWK	Wheat	Kanjar (Myar valley)	32.87251°N 76.85702°E	3360	4.2 ± 0.01	3.89 ± 0.22	84.84 ± 0.15	27.75 ± 0.37	5.32 ± 0.17	2.1 ± 0.02	0.37 ± 0.01	$1.5 \pm 1.18 \times 10^7$
LUP	Rice	Urgosh	32.85852°N 76.79588°E	3247	3.3 ± 0.05	4.41 ± 0.32	77.05 ± 0.36	27.34 ± 0.44	2.50 ± 0.15	4.20 ± 0.21	0.59 ± 0.02	$1.9 \pm 2.31 \times 10^5$
LRJ	Rice	Jhundha	32.64105°N 76.84493°E	2866	3.2 ± 0.02	4.27 ± 0.02	72.11 ± 0.62	19.94 ± 0.70	2.05 ± 0.12	4.32 ± 0.04	0.36 ± 0.02	$9.4 \pm 2.34 \times 10^6$

Area[a], different location of Lahaul valley of North Western Himalaya; GPS[b], latitude and longitude; Elevation[c], elevation (values in meter), and Biochemical characterization of collected lugri sample (Traditional fermented beverage) from Lahaul valley of North Western Himalayan: pH; EC[d], electric conductivity (mS/cm); Moist[e], moisture, ash, crude fat, crude protein; and Alc.[f], alcohol content.

Preparation of Fermented Milk

The fermented milk was prepared according to the method explained by Angmo et al. (2016). The sterile skim milk medium (4% w/v) was inoculated with 1% (v/v) of bacterial culture (∼0.8 O.D.) and incubated at 37°C for 18 h. After fermentation, it was stored for 28 days at 4°C, and samples were withdrawn every week for determining the bacterial viability and pH changes in the fermented milk. To study the existence of coliform and enterobacteria, every week, samples were withdrawn and spread plated on eosin methylene blue (EMB) and violet red bile glucose agar (VRBG) agar plates.

Statistical Analysis

The statistical analysis of the data was done using IBM SPSS Statistics version 26. The principal component analysis (PCA) plot was constructed with XLSTAT software v2020.3.1. The data of acid, bile, cell auto-aggregation, and cell surface hydrophobicity were used as input values in the PCA plot.

RESULTS

Biochemical Analysis of Undistilled *Lugri* Samples

The biochemical analysis of undistilled *lugri* prepared from barley, wheat, and rice was evaluated for alcohol, pH, EC, moisture content, ash, crude fat, and protein content (**Table 1**). The rice-based *lugri* has higher alcohol, crude protein content, EC, and low pH as compared to barley and wheat-based *lugri* (**Table 1**). Likewise, the moisture content, ash, and crude fat content of the wheat-based *lugri* were slightly higher than the other two substrates of *lugri*. The bacterial load of all three substrate-based *lugri* falls in the range of $1.9 \pm 2.31 \times 10^5$ to $5.6 \pm 0.80 \times 10^8$ CFU/g (**Table 1**).

Screening and Physicochemical Characterization of Isolates

The substrate-based undistilled *lugri* was screened for different probiotic strains. The initial screening revealed 348 bacterial isolates from six different samples of undistilled *lugri*. Out of these isolates, 134 unique morphotypes showing distinct appearance on MRS medium were randomly selected. The qualitative estimation for acid and bile tolerance was performed for the unique morphotypes, and among these 43 bacterial strains showed the best tolerance in an acidic environment (2.0, 2.5, 3.0, 3.5, and 4.0) and different bile salt concentrations (0.3, 0.5, 1.0, 2.0, and 3.0%) (**Supplementary Tables 1A,B**). Out of 43 selected strains, the highest 22 isolates were found from rice-based *lugri*, whereas barley and wheat harbored the remaining 12 and 9 strains, respectively (**Table 2**). The selected bacterial strains were also assessed for their physicochemical characterization (**Table 2**). All the strains were found to be Gram-positive, catalase-negative and the physicochemical characterization of the selected strains showed optimum growth at 37°C, pH 5, and 4% NaCl (**Table 2**). The selected bacterial strains were further characterized for probiotic and functional attributes.

Characterization of Isolates for Probiotic Attributes

Acid and Bile Tolerance

The bacterial strains were grown in simulated *in vitro* gastric juice of pH 2.0, pH 2.5, and pH 3.0 for the time interval of 2 and 12 h, respectively. Among the bacterial stains, 12 isolates retained a similar viability level, and LRJ15:13 and LRJ15:14:01 showed the maximum survivability when exposed to three pH ranges for 2 h (**Table 3**). Similarly, when the bacterial isolates were exposed to 12 h in three pH ranges, 29 isolates showed similar survivability, and LRJ15:14:01 exhibited the highest viability level (**Table 3**).

All the bile tolerance capability of the bacterial isolates was determined in different bile concentrations (1, 2, and 3%) with incubation at 37°C for 12 h. At 1% bile concentration, 21 strains showed a similar level of bile tolerance; whereas, at 2%, only 11 isolates exhibited a good survivability range (**Table 3**). On 3% bile salt, the reduction in viability was indicated as only six strains (LUR:07, LUL:01, LUL:07, LRJ15:14:01, LRJ15:04, and LRJ15:05) were able to survive in the high concentration (**Table 3**).

Cell Auto-Aggregation and Cell Surface Hydrophobicity

A high range of variation was observed in the cell auto-aggregation ability of the bacterial isolates (36.40 ± 2.30 to $90.70 \pm 0.70\%$) after 24 h of incubation (**Figure 2A** and **Supplementary Table 2**). The highest auto-aggregation was observed in the strain LUL:07 ($90.70 \pm 0.70\%$), while 14 strains exhibited higher auto-aggregation activity (≥80%). On the other hand, 24 bacterial strains were found in the moderate range (≤80%), and five isolates showed the least activity (≤60%) of auto-aggregation.

The hydrophobicity of all the bacterial strains was performed using n- hexadecane as the hydrocarbon to assess their adhesion abilities. The bacterial strains exhibited highly variable adhesion capabilities (>38–99.90%) (**Figure 2B** and **Supplementary Table 2**). Out of all the bacterial isolates, 32 strains showed a higher percent of hydrophobicity (≥90%), and the maximum hydrophobicity was observed of the strain LWK:07 ($99.90 \pm 0.14\%$). The results indicated that some isolates have a high relative hydrophobicity due to their adherence to hydrocarbons.

Antimicrobial Activity

The antimicrobial activity of bacterial isolates showed different degrees of inhibition against opportunistic pathogenic type strains using the well diffusion method. Out of 43 bacterial isolates, 14 strains were able to inhibit the growth of at least four pathogenic strains, but no bacterial strain inhibited all the pathogens (**Supplementary Table 3**). The strain LWK:03 and LUP:03 showed the maximum zone of inhibition (>5 mm) against *K. pneumonia* (MTCC 109), *M. luteus* (MTCC 2470), *E. coli* (MTCC 43), and *S. aureus* (MTCC 96). However, most isolates exhibited weak inhibition against *B. subtilis* (MTCC 121) and *E. coli* (MTCC 43). In addition, the maximum

TABLE 2 | Identification of probiotic strains isolated from traditional fermented beverage *lugri* of North Western Himalaya.

Substrate type	Closest Match (type strain)	Sequenced strains	Accession number	% sim[a]	nt[b]	T[c]	pH[d]
Rice	*Limosilactobacillus reuteri* JCM1112^T	**LUP:03**	MT337545	99.63	1290	15–37	4–8
		LUP:07	MT337546	99.69	1290	15–37	4–7
	Pediococcus acidilactici DSM20284^T	LUP:09	MT337547	99.92	1332	15–37	4–8
		LRJ1:01	MT329719	100	1222	15–37	4–9
		LRJ1:06:01	MT329720	99.92	1288	15–37	4–9
	Limosilactobacillus fermentum CECT562^T	LRJ1:03	MT32971	99.93	1340	15–37	4–7
		LRJ1:04	MT329718	99.86	1404	15–37	4–7
		LRJ15:08	MT329731	99.92	1319	15–37	4–7
	Lactiplantibacillus pentosus DSM20314^T	LRJ1:11	MT355098	99.92	1430	15–37	4–7
		LRJ15:12	MT329725	100	1225	15–37	4–7
	Lactiplantibacillus paraplantarum DSM 10667^T	LUP:01	MT337544	100	1283	15–37	4–8
		LRJ1:08	MT329721	100	1309	15–37	4–7
		LRJ1:09	MT329722	100	1252	15–37	4–7
		LRJ1:12	MT329723	100	1283	15–37	4–7
		LRJ15:07	MT329730	100	1312	15–37	4–7
		LRJ15:10	MT329732	100	1281	15–37	4–7
		LRJ15:13	MT329726	100	1223	15–37	4–7
		LRJ15:14:01	MT329733	100	1319	15–37	4–7
	Pediococcus acidilactici DSM20284^T	LJR15:03	MT329727	100	1291	15–37	4–7
		LRJ15:04	MT329728	99.92	1318	15–37	4–9
		LRJ15:05	MT329729	99.92	1322	15–37	4–9
	Lactobacillus argentoratensis	LRJ15:11	MT329724	99.92	1219	15–37	4–7
Barley	*Lactiplantibacillus paraplantarum* DSM 10667^T	LUL:02	MT337540	100	1358	15–37	4–7
		LUL:03	MT337541	100	1319	15–37	4–7
		LUL:08	MT355100	100	1324	15–37	4–8
		LUL:18	MT337539	100	1264	15–37	4–8
		LUM:03	MT337584	100	1278	15–28	4–9
	Lacticaseibacillus paracasei subsp. *tolerans* JCM1171^T	LUM:04	MT337585	100	1363	15–37	4–8
		LUM:06	MT337586	100	1317	15–37	4–7
		LUL:01	MT355099	99.77	1334	15–37	4–7
		LUL:04	MT337542	100	1370	15–37	4–7
	Lactiplantibacillus pentosus DSM20314^T	LUM:09	MT337588	100	1236	15–37	4–8
		LUL:07	MT337543	100	1370	15–37	4–7
	Pediococcus acidilactici DSM20284^T	LUM:11	MT337583	99.92	1272	15–37	4–8
Wheat	*Bacillus licheniformis* ATCC14580^T	LUR:01	MT355101	99.85	1355	15–45	4–7
	Lacticaseibacillus paracasei subsp. *tolerans* JCM1171^T	LUR:04	MT337577	100	1252	15–37	4–7
		LWK:04	MT337573	100	1279	15–37	4–7
	Levilactobacillus brevis ATCC14869^T	**LUR:05**	MT337578	99.85	1323	15–37	4–8
		LUR:07	MT337579	99.84	1263	15–37	4–7
		LWK:07	MT337575	100	1288	15–37	4–8
	Companilactobacillus crustorum LMG23699^T	LWK:03	MT337572	100	1239	15–37	4–9
		LWK:06	MT337574	100	1275	15–37	4–9
	Lactiplantibacillus paraplantarum DSM 10667^T	LWK:10	MT337576	100	1275	15–37	4–8

[a]*Percentage sequence similarity of isolated strains with type strains of validly published prokaryotic names (available online http://eztaxon-e.ezbiocloud.net/); nt[b]: Length of 16S rRNA gene sequence;* [c]*Temperature;* [d]*pH.*
The most promising 12 strains selected after PCA showing in bold font.

pathogenic inhibition by different bacterial isolates was seen against *M. luteus* (MTCC 2470).

Exopolysaccharide (EPS) and β-Galactosidase Activity

The production of EPS in the selected strains was confirmed by the formation of mucoid colonies (**Supplementary Table 4**). All the bacterial exhibited mucoid colonies on modified MRS media containing different concentrations (5 and 10%) of sucrose and lactose as carbon sources.

The β-galactosidase activity was observed by the formation of blue color colonies on the modified MRS agar plates. All the bacterial isolates except LUM:03, LUR:07, LWK:03,

TABLE 3 | Survival of bacterial strains isolated from different undistilled substrate-based *lugri* (rice, barley, and wheat) under *in vitro* gastric phase containing pepsin and different bile concentration.

Substrate type	Bacterial isolates	Acid tolerance						Bile tolerance		
		2 h			12 h			12 h		
		pH 2	pH 2.5	pH 3	pH 2	pH 2.5	pH 3	1%	2%	3%
Rice	LUP:03	5.84 ± 0.01 m	6.14 ± 0.55 a	6.89 ± 0.00 c	4.81 ± 0.04 bc	5.82 ± 0.01 gh	5.88 ± 0.00 ghi	0.54 ± 0.01hijk	0.43 ± 0.00ijk	0.41 ± 0.01cdef
	LUP:07	5.87 ± 0.02 m	6.08 ± 0.60 a	5.54 ± 0.02 k	5.64 ± 0.01 abc	4.74 ± 0.05 p	5.91 ± 0.01 fghi	0.23 ± 0.00qrst	0.17 ± 0.00opqrs	0.14 ± 0.00ef
	LUP:09	6.53 ± 0.00 hi	5.87 ± 0.30 a	6.64 ± 0.00 ef	5.09 ± 0.02 abc	5.82 ± 0.00 gh	5.64 ± 0.01 jkl	0.56 ± 0.00 hij	0.38 ± 0.03jkl	0.24 ± 0.01def
	LRJ1:01	5.23 ± 0.07 q	5.60 ± 0.06 a	4.69 ± 0.12 n	4.39 ± 0.12 bc	5.36 ± 0.05 1	4.15 ± 0.21 p	0.22 ± 0.01rst	0.18 ± 0.00nopqrs	0.13 ± 0.01ef
	LRJ1:06:01	5.88 ± 0.00 m	5.86 ± 0.37 a	5.81 ± 0.02 ij	4.54 ± 0.08 bc	5.88 ± 0.00 g	5.81 ± 0.02 hij	0.44 ± 0.05hijk	0.40 ± 0.00cdef	0.42 ± 0.04hijk
	LRJ1:03	6.52 ± 0.02 hi	5.91 ± 0.78 a	5.91 ± 0.00 i	5.64 ± 0.01 abc	5.66 ± 0.02 i	5.36 ± 0.05 m	0.52 ± 0.02fghi	0.48 ±0.03 cdef	0.40 ± 0.03 jklm
	LRJ1:04	6.56 ± 0.02 fgh	5.65 ± 0.67 a	5.81 ± 0.02 ij	5.52 ± 0.01 abc	6.46 ± 0.00 d	6.45 ± 0.01 c	0.61 ± 0.03defg	0.52 ± 0.01cdef	0.43 ±0.02efgh
	LRJ15:08	6.11 ± 0.02 1	5.97 ± 0.45 a	5.59 ± 0.07 k	5.83 ± 0.00 ab	5.76 ± 0.01 h	5.62 ± 0.03 jkl	0.67 ± 0.04bcdef	0.64 ± 0.03 def	0.41 ± 0.01 jklm
	LRJ1:11	5.83 ± 0.02 m	6.03 ± 0.70 a	5.39 ± 0.021	4.65 ± 0.07 bc	5.07 ± 0.04 n	5.74 ± 0.02 ijk	0.21 ± 0.00 lmnpq	0.29 ± 0.021mno	0.36 ± 0.00mnopq
	LRJ15:12	6.73 ± 0.00 cd	6.35 ± 0.00 a	7.03 ± 0.00 b	.000 ± 0.00 e	.000 ± 0.00 s	6.88 ± 0.00 b	0.32 ± 0.01cdef	0.31 ± 0.0lopqrst	0.26 ± 0.00lmnopqr
	LUP:01	5.65 ± 0.00 n	5.49 ± 0.18 a	5.38 ± 0.03 1	5.88 ± 0.00 ab	5.50 ± 0.01 k	5.11 ± 0.04 n	0.58 ± 0.01 ghij	0.35 ± 0.00klm	0.21 ±0.00def
	LRJ1:08	5.65 ± 0.00 n	6.41 ± 0.13 a	6.73 ± 0.00 de	4.87 ± 0.03 bc	5.81 ± 0.02 gh	5.73 ± 0.02 ijk	0.21 ± 0.011mnop	0.13 ± 0.00st	0.11 ± 0.01rst
	LRJ1:09	6.53 ± 0.00 hi	6.02 ± 0.51 a	6.65 ± 0.00 ef	4.30 ± 0.00 bc	6.57 ± 0.01 c	6.35 ± 0.02 cd	0.37 ± 0.01cdef	0.24 ± 0.02nopqrs	0.19 ± 0.02pqrst
	LRJ1:12	6.46 ± 0.00 ij	6.30 ± 0.07 a	5.54 ± 0.02 k	5.73 ± 0.02 abc	6.13 ± 0.00 f	5.40 ± 0.06 m	0.32 ± 0.02nopqrst	0.26 ± 0.00lmnopqr	0.23 ± 0.00 def
	LRJ15:07	6.42 ± 0.02 jk	6.27 ± 0.87 a	5.58 ± 0.07 k	5.82 ± 0.01ab	5.88 ± 0.01 g	5.67 ± 0.03 jkl	052 ± 0.08bcdef	0.49 ± 0.02defg	0.46 ± 0.02 d
	LRJ15:10	6.55 ± 0.00 gh	5.65 ± 0.13 a	5.79 ± 0.05 ij	5.38 ± 0.03 abc	6.56 ± 0.00 c	6.07 ± 0.01 efg	0.63 ± 0.01bcde	0.46 ± 0.05 cdef	0.43 ± 0.01 d
	LRJ15:13	6.95 ± 0.00 a	6.25 ± 1.10 a	7.15 ± 0.00 a	4.15 ± 0.21 bc	6.58 ± 0.01 c	6.98 ± 0.00 ab	0.50 ± 0.02ghij	0.51 ± 0.0lghij	0.48 ±0.02hijk
	LRJ15:14:01	6.79 ± 0.00 bc	6.61 ± 0.74 a	7.10 ± 0.00 ab	5.89 ± 0.00 e	6.79 ± 0.00 a	7.09 ± 0.02 a	0.63 ± 0.05bcdef	0.56 ± 0.02efgh	0.50 ± 0.04ijkl
	LJR15:03	6.16 ± 0.001	5.87 ± 0.72 a	6.52 ± 0.00 fg	5.53 ± 0.00 abc	6.65 ± 0.01 bc	6.20 ± 0.02 de	0.80 ± 0.61bcd	0.65 ± 0.07efgh	0.43 ± 0.01 defg
	LRJ15:04	5.63 ± 0.02 no	6.00 ± 0.84 a	7.01 ± 0.00 b	.000 ± 0.00 e	.000 ± 0.00 s	6.11 ± 0.03 ef	0.799 ± 0.02 bedef	0.66 ± 0.08 defg	0.61 ± 0.19 bedef
	LRJ15:05	6.54 ± 0.04 hi	6.26 ± 0.72 a	6.35 ± 0.01 h	2.00 ± 2.83 d	4.54 ± 0.08 q	6.83 ± 0.00 b	0.76 ± 0.06bcdef	0.61 ± 0.01defg	0.58 ± 0.01 de
	LRJ15:11	5.54 ± 0.02 p	6.43 ± 0.30 a	6.49 ± 0.00 g	5.75 ± 0.00 abc	6.29 ± 0.01 e	5.78 ± 0.04 hij	0.19 ± 0.01 t	0.12 ± 0.02st	0.10 ± 0.00 f
Barley	LUL:02	5.66 ± 0.02 n	6.45 ± 0.12 a	5.56 ± 0.04 k	5.34 ± 0.02 abc	5.35 ± 0.011	5.41 ± 0.07 m	0.45 ± 0.10 jklmn	0.41 ± 0.03 cdef	0.37 ± 0.02fd
	LUL:03	6.64 ± 0.01 ef	6.57 ± 0.09 a	5.64 ± 0.02 k	5.29 ± 0.01 abc	5.58 ± 0.02 ijk	5.16 ± 0.02 n	0.45 ± 0.15hijk	0.35 ± 0.0lmnopqr	0.31 ± 0.01cdef
	LUL:08	6.38 ± 0.02 jk	5.86 ± 0.30 a	5.81 ± 0.02 ij	5.16 ± 0.02 abc	5.84 ± 0.01 gh	5.96 ± 0.02 fgh	0.65 ± 0.21bcdef	0.59 ± 0.01 d	0.48 ±0.00defg
	LUL:18	5.52 ± 0.01 p	6.08 ± 1.01 a	5.13 ± 0.07 m	5.09 ± 0.02 abc	5.61 ± 0.01 ij	4.54 ± 0.08 o	0.55 ± 0.00bcdef	0.42 ± 0.03ijk	0.41 ± 0.00klmno
	LUM:03	5.90 ± 0.01 m	5.46 ± 0.44 a	5.38 ± 0.03 1	4.54 ± 0.08 bc	5.20 ± 0.03 m	4.69 ± 0.12 o	0.61 ± 0.33fghi	0.41 ± 0.00cdeij	0.36 ± 0.01cdef
	LUM:04	6.83 ± 0.00 b	5.45 ± 0.42 a	6.53 ± 0.00 fg	4.48 ± 0.00 bc	5.89 ± 0.01 g	6.21 ± 0.01 de	0.28 ± 0.19cdef	0.25 ± 0.00pqrstt	0.15 ± 0.00qrst

(Continued)

TABLE 3 | Continued

Substrate type	Bacterial isolates	Acid tolerance						Bile tolerance		
		2 h			12 h			12 h		
		pH 2	pH 2.5	pH 3	pH 2	pH 2.5	pH 3	1%	2%	3%
	LUM:06	6.53 ± 0.00 hi	6.12 ± 1.00 a	6.65 ± 0.00 ef	4.15 ± 0.21 bc	5.66 ± 0.01 i	6.06 ± 0.02 efg	0.32 ± 0.02opgrs	0.30 ± 0.01cdef	0.27 ± 0.021mnopqt
	LUL:01	6.37 ± 0.02 k	6.28 ± 0.31 a	6.50 ± 0.00 g	4.39 ± 0.12 bc	5.92 ± 0.02 g	5.66 ± 0.01 jkl	0.75 ± 0.02cdef	0.61 ± 0.00defg	0.50 ± 0.01fghi
	LUL:04	6.87 ± 0.00 ab	5.93 ± 0.60 a	6.65 ± 0.00 ef	4.00 ± 0.00 c	5.86 ± 0.02 gh	6.05 ± 0.01 efg	0.52 ± 0.49 cdef	0.23 ± 0.04mnopqrs	0.34 ± 0.00mnopqrs
	LUM:09	5.84 ± 0.01 m	5.70 ± 0.07 a	5.78 ± 0.03 j	5.33 ± 0.04 abc	5.34 ± 0.02 1	5.50 ± 0.02 1m	0.20 ± 0. 0llmnop	0.13 ± 0.02rst	0.11 ± 0.00ef
	LUL:07	5.56 ± 0.04 op	5.98 ± 0.53 a	5.41 ± 0.07 1	5.82 ± 0.01 ab	5.40 ± 0.06 1	5.14 ± 0.08 n	0.97 ± 0.06c	0.87 ± 0.00 c	0.61 ± 0.03defg
	LUM:11	5.68 ± 0.04 n	5.83 ± 0.00 a	5.76 ± 0.02 j	4.39 ± 0.12 bc	4.65 ± 0.07 p	4.69 ± 0.12 o	0.14 ± 0.00ef	0.30 ± 0.03st	0.23 ± 0.00pqrst
Wheat	LUR:01	5.71 ± 0.03 n	6.58 ± 0.06 a	6.65 ± 0.00 ef	4.15 ± 0.21 bc	4.00 ± 0.00 r	6.10 ± 0.01 ef	0.62 ± 0.04bcdef	0.48 ± 0.04efgh	0.41 ± 0.06fghi
	LUR:04	5.14 ± 0.04 r	6.45 ± 0.12 a	6.75 ± 0.00 de	4.15 ± 0.21 bc	5.54 ± 0.02 jk	6.18 ± 0.00 de	0.30 ± 0.30 lmn	0.15 ± 0.01pqrst	0.05 ± 0.00t
	LWK:04	6.83 ± 0.00 b	5.71 ± 0.13 a	6.53 ± 0.00 fg	4.81 ± 0.04 bc	5.54 ± 0.02 jk	5.11 ± 0.09 n	0.30 ± 0.00cdef	0.28 ± 0.0llmno	0.19 ± 0.02 t
	LUR:05	6.66 ± 0.00 de	6.52 ± 0.43 a	6.80 ± 0.00 cd	4.30 ± 0.00 bc	4.87 ± 0.03 o	6.21 ± 0.01 de	0.28 ± 0.02cdef	0.04 ± 0.00 t	0.29 ± 0.02opqrst
	LUR:07	6.64 ± 0.00 e	6.50 ± 0.20 a	6.65 ± 0.01 ef	5.37 ± 0.04 abc	5.38 ± 0.03 1	5.54 ± 0.02 klm	0.56 ± 0.03bcdef	0.51 ± 0.00bcdef	0.50 ± 0.05jklm
	LWK:07	6.65 ± 0.01 e	6.64 ± 0.16 a	6.51 ± 0.00 g	5.52 ± 0.01 abc	6.37 ± 0.00 de	5.38 ± 0.02 m	0.31 ± 0.0lopqrst	0.21 ± 0.07nopqrs	0.17 ± 0.00ef
	LWK:03	5.56 ± 0.03 op	5.58 ± 0.09 a	5.57 ± 0.04 k	5.11 ± 0.04 abc	5.37 ± 0.01 1	5.40 ± 0.06 m	0.44 ± 0.02cdef	0.20 ±0.00st	0.15 ± 0.07pqrst
	LWK:06	6.54 ± 0.01 hi	6.43 ± 0.10 a	5.64 ± 0.02 k	5.38 ± 0.02 abc	6.73 ± 0.00 ab	5.06 ± 0.02 n	0.52 ± 0.03cdef	0.39 ± 0.00ljkl	0.38 ± 0.00jkl
	LWK:10	5.67 ± 0.03 n	5.93 ± 0.40 a	5.54 ± 0.02 k	5.54 ± 0.02 abc	5.38 ± 0.02 1	5.39 ± 0.04 m	0.38 ± 0.00lmnop	0.28 ± 0.031mnop	0.20 ± 0.01 cdef
	Control	6.63 ± 0.02 efg	6.73 ± 0.02 a	6.69 ± 0.03 de	6.65 ± 0.02 a	6.37 ± 0.04 de	6.80 ± 0.00 b	0.92 ± 0.01 a	0.82 ± 0.02 a	0.79 ± 0.02 a

Values represented as mean ± SD; for each row, different subscripts upper case letters indicate significantly different at $p < 0.05$, as measured by 2-sided Tukey's HSD between pH 2, pH 2.5, and pH 3 Control and bile salt% 1, 2, and 3. Acid tolerance (Values expressed in log10CFU/ml in 2 and 12 h) whereas; For bile tolerance (Values represented in OD at the absorbance A560nm). Lacticaseibacillus rhamnosus (ATCC 53103) are reference control.

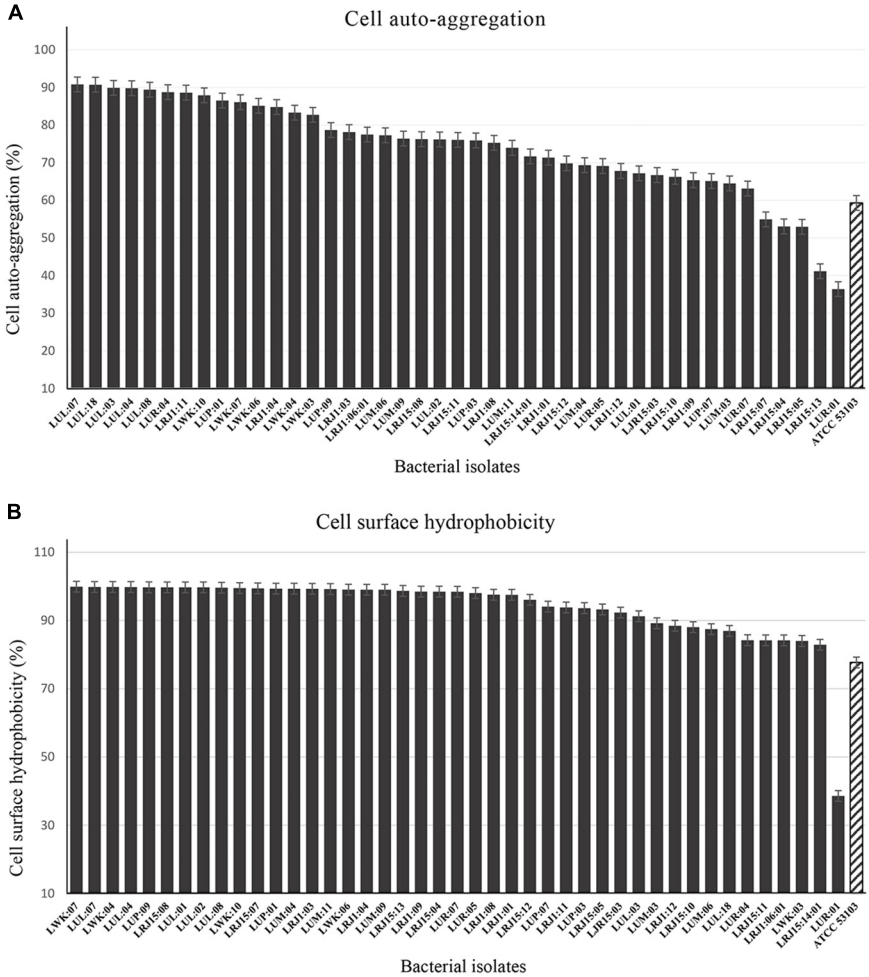

FIGURE 2 | Cell auto-aggregation and cell surface hydrophobicity activity of bacterial isolates after 24 h. **(A)** The adhesion characteristics with cell auto-aggregation test (%) of bacterial isolates after 24 h with reference control *Lacticaseibacillus rhamnosus* (ATCC 53103). **(B)** Adhesion properties characterized with cell surface hydrophobicity test (%) of bacterial strains against n-hexadecane with reference control *L. rhamnosus* (ATCC 53103). Error bars and standard deviations showed with respect to the mean ± S.D. values of triplicate analyses.

LWK:07, LUR:01, and LRJ15:11 were positive for the β-galactosidase production after 48 h of incubation at 37°C (**Supplementary Table 4**).

Antibiotic Susceptibility

The bacterial isolates were tested for their antibiotic susceptibility, and all were found sensitive to azithromycin and tetracycline (**Supplementary Table 5**). However, all the strains showed resistance to vancomycin, and only 11 strains were resistant to ciprofloxacin. Likewise, six isolates exhibited resistance to kanamycin, and five isolates were found to be resistant to rifampicin.

Biofilm Formation and Hemolysis Assay

Biofilm formation for all the isolates was assessed in MRS broth, and based on their O.D., the maximum number of strains were found to be biofilm producers (**Supplementary Table 6**). The highest biofilm formation was observed in LUM:04 and LUL:01 strains that showed ≥2.5 O.D. However, six strains exhibited moderate (O.D. ≤ 0.78) biofilm formation and three strains showed weak biofilm formation (O.D. ≤ 0.37).

The isolates were further screened for the hemolytic activity that indicates the strain's non-pathogenic nature. All the isolates gave negative results for hemolytic activity.

16S rRNA Gene Sequencing and Phylogenetic Analysis

16S rRNA gene sequencing and phylogenetic analysis of 43 bacterial isolates were characterized based on probiotic attributes, and safety assessment was performed (**Table 2**). All the sequences of representative bacterial strains showed >99 to 100% similarity within the GenBank sequences. Based on 16S rRNA gene sequencing, all the 43 bacterial strains were affiliated to eight different genera and ten different species (**Table 2**). To classify each bacterial strain at the species level, the phylogenetic tree was constructed from 16S rDNA sequences from evolutionary

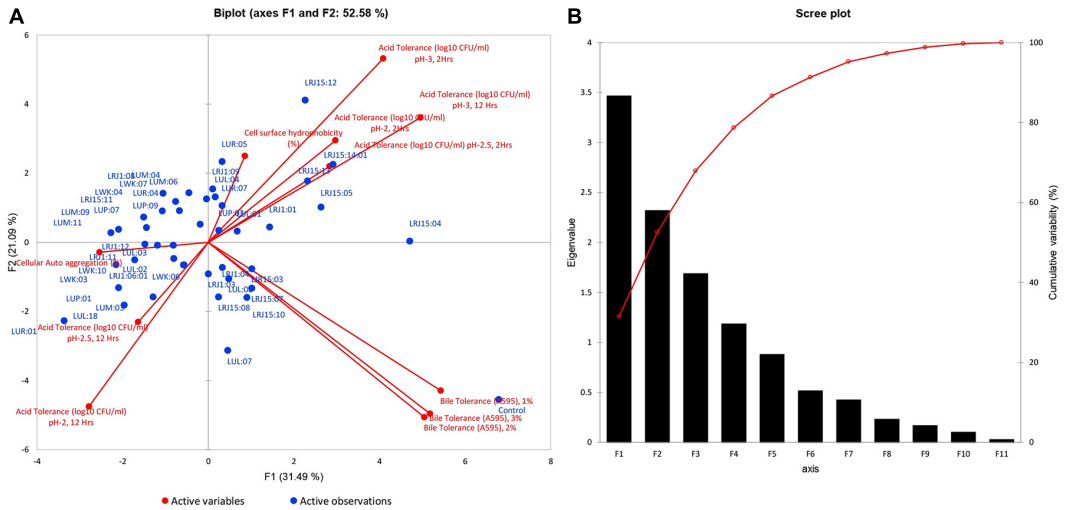

FIGURE 3 | Principal component analysis of the probiotic attributes (acid and bile tolerance at different pH and bile concentration, cell auto-aggregation, cell surface hydrophobicity) of 43 bacterial isolates. **(A)** Principal component analysis (PCA) biplot projection based on probiotic attributes for the selection of most promising probiotic strain isolated from Traditional fermented beverages *lugri*. The percentage of variance is explained by the first two factors F1 and F2, reported after each axis. **(B)** Scree biplot (eigenvalue) of principal components (F1–F11) for the probiotic potential of different isolates from traditional fermented beverage *lugri*.

distances by the neighbor-joining method (**Supplementary Figures 1A–C**). The 16S rRNA gene sequence of the strains was submitted to the GenBank database, and the accession number are given in **Table 2**.

Principal Component Analysis (PCA)

The selection of the most promising strains was conducted through PCA and considered for further experimental evaluation. The PCA revealed 52.58% of the total variation in two principal components, and the variable homogenous distribution on the principal plane component showed F1 and F2 with 31.49 and 21.09% variation, respectively (**Figure 3A**). The maximum bacterial isolates were correlated to F1 and F2 components and suggested that these variables contribute to selecting potential strains (**Supplementary Table 7**). PCA revealed that 12 isolates (LUL:01, LUL:04, LUP:03, LUR:05, LUR:07, LRJ15:04, LRJ15:05, LRJ1:01, LRJ15:13, LRJ15:14:01, LRJ1:09, and LRJ15:12) present in the quadrant I showed the maximum correlation with respect to the variables. Out of 12 promising probiotics, eight isolates were selected from rice-based *lugri* and two each from barley and wheat-based *lugri*. LUL: 01 and LRJ15:14:01 showed the highest probiotic attributes belonging to barley and rice-based *lugri*, respectively.

Antioxidant Activity

The cell-free extracts of 12 potential probiotic strains were assessed for their free-radical scavenging ability toward the DPPH and ABTS inhibition (**Table 4**). In the DPPH assay, all the isolates showed antioxidant activity in the range of 4.39 ± 2.14 to 16.41 ± 2.13%, and for ABTS inhibition, the range of antioxidant activity was between 15.29 ± 0.50 to 57.74 ± 1.63%. The strain *L. paracasei* LUL: 01 exhibited the highest antioxidant activity for the inhibition of DPPH (16.41 ± 2.13%) and ABTS free radical (57.74 ± 1.63%), respectively.

Fermented Milk

The most potential strain *L. paracasei* LUL: 01 was used to prepare a dairy-based fermented drink (**Figure 4**). The isolate LUL: 01 was able to grow in sterile milk, and the viable count reached 8.6 $\log_{10}$ CFU/ml within 18 h at 37°C. After finishing fermentation time (18 h), LUL: 01 was able to lower the pH value (4.11 ± 0.01) of the fermented milk. A change in the viability count and pH of the fermented milk was recorded weekly during the storage at 4°C for 28 days (**Figure 4**). The viability count of the LUL: 01 strain was found to be 8.6 $\log_{10}$ CFU/ml in the

TABLE 4 | Characterization of antioxidant activity (ABTS and DPPH) of the most promising probiotic isolates from traditional fermented beverages.

S. no.	Strain name	Free radicals scavenging activity (%)	
		ABTS	DPPH
(1)	LUL:01	57.74 ± 1.63a	16.41 ± 2.13b
(2)	LUL:04	37.90 ± 1.77b	15.44 ± 0.59b
(3)	LUP:03	24.53 ± 1.77c	4.67 ± 0.97ef
(4)	LUR:05	54.73 ± 2.28a	4.39 ± 2.14f
(5)	LUR:07	28.87 ± 2.82c	6.39 ± 1.88def
(6)	LRJ15:14:01	15.74 ± 1.71d	5.82 ± 1.04def
(7)	LRJ1:01	22.43 ± 1.38cd	8.78 ± 2.14def
(8)	LRJ1:09	21.98 ± 8.61cd	9.25 ± 1.27de
(9)	LRJ15:04	27.27 ± 3.59c	5.93 ± 2.50def
(10)	LRJ15:05	15.29 ± 0.50d	8.26 ± 0.60def
(11)	LRJ15:12	26.76 ± 1.16c	10.33 ± 1.94cd
(12)	LRJ15:13	27.09 ± 0.73c	13.58 ± 2.05bc
(13)	Control	59.01 ± 1.09a	21.70 ± 0.94a

Values are represented as mean ± SD of triplicate analysis. Means in the column with the same superscripts lowercase letters (a–f) are not significantly different as measured by 2-sided Tukey's–HSD test between replications ($p < 0.05$).

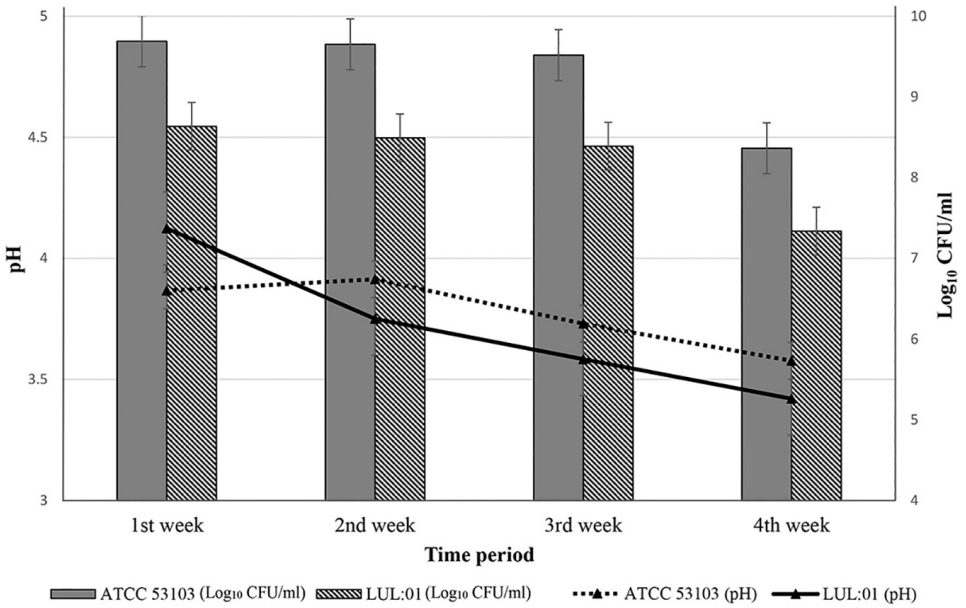

FIGURE 4 | Preparation of fermented milk using the most potential strain *Lacticaseibacillus paracasei* LUL:01, compared with the reference type strain *L. rhamnosus* (ATCC 53103). The changes in the pH and viable count during the storage period of fermented milk (28 days at 4°C). The variation in the pH and viable count (log $_{10}$ CFU/ml) showing in the line and bar graph, respectively. Error bars and standard deviations showed with respect to the mean ± S.D. values of triplicate analyses.

first week of study, but a slight reduction (7.33 $\log_{10}$ CFU/ml in the 4th week) was observed during the storage time (**Figure 4**). However, a continued decrease in the pH value (3.39 ± 0.02) of fermented milk was observed (**Figure 4**). The fermented milk was also assessed every week for the presence of any coliform and enterobacteria. The plate assays showed no growth of pathogenic bacteria (**Supplementary Table 8**).

DISCUSSION

Lugri is a mildly alcoholic beverage prepared by fermentation of rice, barley, and wheat using a traditional starter culture called '*phab*' (Angmo and Bhalla, 2014; Thakur et al., 2015). The substrate-specific biochemical characterization of undistilled *lugri* revealed rice has relatively higher alcohol content (0.36 to 0.59 ± 0.02%) but lower pH than the barley and wheat-based *lugri*. The variation in the acidic nature of *lugri* samples was probably due to the production of organic acids during the fermentation process (Sharma et al., 2013). However, the distilled form of *lugri* known as '*Arak*' has displayed higher alcohol content (5–7%) (Angmo and Bhalla, 2014). Furthermore, rice-based *lugri* have maximum EC and crude protein content, suggesting their higher mineral content and proteinous metabolites (Bhatt and Maheshwari, 2020). Accordingly, among the three-substrate-based *lugri*, rice had lower moisture, microbial load, crude fat, and ash content (**Table 1**). These results are in accordance with the fact that the lower the moisture content, the shelf life increases, and the microbial load decreases, leading to prolonged storage (Bhatt and Maheshwari, 2020). A few previous reports on TFB's such as *Grawa*, *borde*, *tej*, and *kodo ko jannr* have shown similar moisture, pH, and crude protein content to the three substrate-based *lugri* (Thapa and Tamang, 2004; Nemo and Bacha, 2020).

Cereal-based fermented beverages are a major source of probiotics and have significant applications in industries (food, beverages, and pharmaceuticals) (Rezac et al., 2018; Ashaolu and Reale, 2020). The three substrate-based *lugri* were observed to be dominated by probiotic strains during the fermentation process. The identification of selected 43 probiotics revealed diverse taxonomic affiliations ranging from eight distinct genera and 10 species (**Supplementary Figure 1** and **Table 2**). In the present study, we explored the separate substrate-specific *lugri* and observed the highest diversity in rice-based *lugri*, where six species belonged to five distinct genera (**Supplementary Table 9**). Secondly, wheat-based *lugri* revealed five species among five genera, and in barley-based *lugri* we observed three species belonging to two genera (**Supplementary Table 9**).

Two separate earlier studies identified three bacterial genera (*Pediococcus*, *Lactobacillus*, and *Bacillus*) and three species (Thakur et al., 2015). In another report, three genera (*Lactobacillus*, *Serratia,* and *Bacillus*) and four species were reported from *lugri* (**Supplementary Table 9**). The current study is the first to explore substrate-specific *lugri* comprehensively; hence we observed additional five genera (*Limosilactobacillus, Lactiplantibacillus, Levilactobacillus, Companilactobacillus,* and *Lacticaseibacillus*) apart from the previous reports by Sharma et al. (2013) and Thakur et al. (2015) (**Supplementary Table 9**). Although these previous studies on *lugri* identified bacterial populations, they lacked any exploration of their probiotic attributes, functional analysis, and safety evaluation (Sharma et al., 2013; Thakur et al., 2015).

The selected 43 strains in the current study qualified all the required probiotic attributes, as prescribed under the FAO/WHO Guidelines (FAO/WHO, 2002). The basic criteria for the microorganisms relevant to probiotics are the ability to survive and colonize in the human gastrointestinal (GI) tract (Reale et al., 2015; Gómez et al., 2016). Selected bacterial strains survived at varied pH and bile salt concentrations showing their tolerance level in the human GI tract (**Table 3**). The isolates were explored for their cell adhesion properties (cell auto-aggregation and hydrophobicity) that enable bacterial attachment to the GI epithelial and mucus surface (Wan et al., 2016; Mays et al., 2020) (**Figure 2** and **Supplementary Table 2**). The bacterial strains were also observed for EPS that are extracellular biopolymers produced by bacteria for their protection in the adverse conditions present in the GI tract (Ryan et al., 2015; Kumari et al., 2016) (**Supplementary Table 4**). The β-galactosidase assay is another attribute observed in the strains for the production of β-galactosidase that helps to hydrolyze intra-intestinal lactose or modulate the colonic microbiota (Zárate and Chaia, 2012) (**Supplementary Table 4**). Based on probiotic attributes, 12 isolates out of 43 were statistically found to be the most promising strains (**Figure 3** and **Supplementary Table 7**). Out of the selected superior probiotics, eight belonged to rice-based *lugri*, while two each were isolated from barley and wheat-based *lugri*, respectively. The abundance of probiotics in rice-based *lugri* may suggest rice as the favored substrate for the preparation of *lugri*.

The antioxidant activity of the 12 strains was assessed for their role in protection from free radicals and to overcome the oxidative stress in the GI tract (Bhattacharyya et al., 2014) (**Table 4**). All the isolates showed free radical-scavenging abilities, and *L. paracasei* LUL:01 exhibited the best antioxidant activity (**Table 4**). Similar results of *L. paracasei* demonstrating antioxidant activity were also previously reported (Zhang et al., 2017). Due to the best antioxidant results displayed by LUL: 01, the strain was used for the production of fermented milk. The LUL: 01 strain was able to ferment the sterile milk in 18 h, and the viable count was found to be 7.33 $\log_{10}$ CFU/ml after the fourth week of the study, only one log decrease lower than the type strain *L. rhamnosus* (ATCC 53103) (8.3 $\log_{10}$ CFU/ml) (**Supplementary Table 8**). However, the microbial count was higher than six $\log_{10}$ CFU/ml, the recommended microbial count for functional food development (Angmo et al., 2016) (**Figure 4** and **Supplementary Table 8**). Our results were in agreement with previous studies, where the strains showed similar variation in pH and microbial count during the fermentation of milk (Angmo et al., 2016; Nami et al., 2018).

All 12 strains are suitable for their application in functional food formulation, as the strains demonstrated remarkable probiotic attributes and antioxidant activity. Out of the 12 strains, eight were selected from rice-based *lugri*, which suggests the best substrate choice for *lugri* preparation. *L. paracasei* LUL:01 was selected based on the best antioxidant activity for its application in functional food formulation. The LUL: 01 strain was able to ferment sterile milk and survived in acceptable numbers during the storage time. Hence, the characterized probiotics promise to be suitable candidates for the production of probiotic functional foods.

CONCLUSION

Lugri is a cereal-based TFB prevalent among the ethnic community of the Lahaul valley. The substrate-specific exploration of *lugri* (rice, wheat, and barley) was conducted for the first time to study the biochemical properties, isolate potential bacterial strains and explore their probiotic attributes, functional analysis, and safety evaluation. The biochemical analysis determined that rice-based *lugri* had a higher alcohol content, EC, crude protein, and low pH, ash, and moisture content as compared to barley and wheat-based *lugri*. The substrate-based *lugri* was explored for potential probiotics, and a total of 134 distinct morphotypes were isolated. Based on acid and bile tolerance, 43 potential strains were selected and identified among eight genera and 10 species. The rice-based lugri harbored the maximum diversity, where six species belonged to five distinct genera. All the 43 strains were tested for their probiotic attributes, and statistically, 12 strains were found to be the most promising probiotic candidates. Among the selected superior probiotic strains, eight were isolated from rice-based *lugri*, and two each belonged to barley and wheat-based lugri, respectively.

The 12 strains were further tested for their free-radical scavenging activity, and all the isolates demonstrated remarkable antioxidant activity. Among the 12 strains, *L. paracasei* LUL:01 exhibited the best results for free-radical scavenging activity and hence was selected for its application in functional food formulation. Strain LUL: 01 was able to ferment sterile milk in 18 h, and the viable count remained above the legal requirement of 6 $\log_{10}$ CFU/ml during 28 days storage at 4°C. Lacticaseibacillus paracasei LUL: 01 has shown its suitability for use in the production of milk-based probiotic products. All the 12 strains demonstrated prominent probiotic attributes and antioxidant activity, exhibiting health benefits and suitability for applications in functional food formulation. Based on the current findings, rice-based *lugri* exhibited the maximum number of probiotic diversity and maybe hypothesized as the best substrate for the preparation of lugri. TFBs and potentially, other fermented foods of Himalaya are a rich source of potential probiotics and provide future opportunities for their investigation. The characterized probiotic strains will also be further processed for the development of functional food.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in online repositories. The names of the repository/repositories can be found in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

NB: methodology, validation, data curation, and writing-original draft. KD and AK: data curation and writing-original draft preparation. AT: writing, revision, and data curation. RK: conceptualization, writing – review and editing,

supervision, and funding acquisition. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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Regulatory Mechanisms of L-Lactic Acid and Taste Substances in Chinese Acid Rice Soup (Rice-Acid) Fermented With a *Lacticaseibacillus paracasei* and *Kluyveromyces marxianus*

Na Liu[1,2], Likang Qin[1,3] and Song Miao[2]**

[1] Key Laboratory of Plant Resource Conservation and Germplasm Innovation in Mountainous Region (Ministry of Education), Collaborative Innovation Center for Mountain Ecology & Agro-Bioengineering (CICMEAB), College of Life Sciences/Institute of Agro-Bioengineering, Guizhou University, Guiyang, China, [2] Teagasc Food Research Centre, Moorepark, Fermoy, Ireland, [3] School of Liquor and Food Engineering, Guizhou University, Guiyang, China

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Paola Lavermicocca, Institute of Sciences of Food Production, Italian National Research Council, Italy

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Massimo Ferrara, Institute of Sciences of Food Production, Italian National Research Council, Italy
Maria De Angelis, University of Bari Aldo Moro, Italy

***Correspondence:**
Likang Qin
lkqin@gzu.edu.cn
Song Miao
song.miao@teagasc.ie

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Rice-acid has abundant taste substances and health protection function due to the various bioactive compounds it contains, including organic acids. L-lactic acid is the most abundant organic acid in rice-acid, but the regulatory mechanisms of L-lactic acid accumulation in rice-acid are obscure. In this study, we analyzed the dynamic changes in organic acids and taste substances in rice-acid in various fermentation phases and different inoculation methods. We identified the key genes involved in taste substance biosynthesis by RNA-Seq analysis and compared the data of four experimental groups. We found that the interaction of the differences in key functional genes (L-lactate dehydrogenase and D-lactate dehydrogenase) and key metabolism pathways (glycolysis, pyruvate metabolism, TCA cycle, amino acid biosynthesis, and metabolism) might interpret the accumulation of L-lactic acid, other organic acids, and taste substances in rice-acid fermented with *Lacticaseibacillus paracasei*. The experimental data provided the basis for exploring regulatory mechanisms of taste substance accumulation in rice-acid.

Keywords: rice-acid, L-lactic acid, organic acid, taste substances, transcriptomics, regulatory mechanism

INTRODUCTION

As a traditional sour food of the Miao and Dong Nationalities, sour soup is popular in China because of its unique taste and flavor. Sour soup mainly includes "acid rice soup" (rice-acid) prepared with rice and water and "acid tomato soup" prepared with tomato or red pepper. Rice-based fermented cereal products are widely loved by consumers in the world, such as fermented rice vinegar, rice wine, and rice noodle. Rice-acid, as another rice-based fermented food, has unique taste substances and various physiologically active substances. Furthermore, it is believed that rice-acid has anti-fatigue, anti-aging, and immunity regulation properties alongside probiotic microbiota and can be used to adjust the human intestinal micro-ecological balance and prevent digestive diseases (Yuan, 2010).

The flavor quality of fermented foods can be improved by optimizing the microbiota structure and regulating the substance metabolism (Zang et al., 2020). Lactic acid is the main flavor component and the key nutritional component in lactic acid foods fermented with various lactic acid bacteria (LAB). However, the human body has only L-lactic acid dehydrogenase, which can only catalyze L-lactic acid. The excessive intake of D-lactic acid or DL-lactic acid may cause metabolic disorders and adverse reactions such as acidosis in the human body (Singh et al., 2019). It is, therefore, necessary to explore the metabolism pathway of L-lactic acid. Yeasts are widely used to ferment foods due to their contributions to the flavors of beverage and fermented cereal foods. LAB and yeasts in fermented foods interact with each other mostly in the metabolism of carbohydrates and nitrogen via stimulatory or inhibitory compounds (Minervini et al., 2014). In recent years, the composite fermentation systems of LAB and yeasts have been used to adjust the characteristics of acid, taste, and aroma. Du et al. (2018) explored the formation of ethyl carbamate in Chinese Moutai-flavor Liquor inoculated with LAB and nonconventional yeasts. Romanens et al. (2019) screened and selected the LAB and yeast strains in cocoa bean fermentation with a suitable flavor and quality. Both LAB and yeasts affect each other through the production of some metabolic products. The regulation mechanisms of organic acid metabolism or taste substance metabolism in these LAB-yeast cooperative fermentation systems, however, have not been reported. The mechanisms underlying L-lactic acid biosynthesis in rice-acid fermentation remain unclear.

The combination of RNA sequencing technologies, metabolic modeling, and physiological indexes offers a powerful tool to study the global response of cells to environmental changes and identify the regulatory mechanisms of targeted metabolites (Wang et al., 2019; Yuan et al., 2019). Actively expressed genes can be explored through transcriptome sequencing of the cDNA after RNA reverse transcription. The transcriptome sequencing can reveal actively expressed genes within a specific period and space, which can be associated with ongoing metabolomic changes as well as the formation of flavors and tastes in fermented foods (Chen et al., 2017). Importantly, the global response of LAB under yeast culture conditions should be studied and RNA sequencing can be used to obtain a global vision of the up- and down-regulated genes (Sun et al., 2019). Aiming at the key flavors (organic acids and taste substances), based on our previous metabolite data, we analyzed the biosynthesis pathways of organic acids and taste substances in the rice-acid fermentation process through the joint application of transcriptomics and metabolite data analysis.

In the study, we fermented rice-acid with *Lacticaseibacillus paracasei* (previously *L. paracasei*, Zheng et al., 2020) and *Kluyveromyces marxianus* (*K. marxianus*) in order to gain suitable organic acids and taste substances. Interestingly, *L. paracasei* has a good effect on the flavor and is usually found in the human intestinal tract or other natural habitats such as cereal products and plant materials. *L. paracasei* with some probiotic properties, including H4-11, has been used in the production of functional foods to improve flavors and extend the shelf life (Mantzourani et al., 2019). It was demonstrated that in fermentation foods, *K. marxianus* could metabolize rice into water, sucrose, glucose, fructose, gum, proteins, minerals, vitamins, beneficial compounds, etc. (Ortiz-Basurto et al., 2008). We attempted to investigate the biological regulation of *L. paracasei* during the maturation of rice-acid by using transcriptomics. Four pairwise comparisons were set in the experiment, including different fermentation time (1 and 3 days) and different inoculation methods (single inoculation of *L. paracasei* and mixed inoculation of *L. paracasei* and *K. marxianus*), in order to investigate the crucial genes of *L. paracasei* for the production of organic acids and taste substances in different rice-acid fermentation processes. The study aims to investigate the molecular mechanisms of formation and metabolic pathways of flavors in rice-acid fermented with *L. paracasei* under the conditions of different times and inoculation methods.

MATERIALS AND METHODS

Preparation Methods of Fermented Rice-Acid

Two strains of *L. paracasei* H4-11 (*L. paracasei* H4-11, CCTCC 2021074) and *K. marxianus* L1-1 (*K. marxianus* L1-1, CCTCC 2021073) were isolated from the traditional fermented rice-acid with high contents of acid and aroma compounds and used in the fermentation experiments (Liu et al., 2020a,b). First, *L. paracasei* H4-11 was cultivated at 37°C for 48 h in MRS medium. *K. marxianus* L1-1 was cultivated at 30°C for 72 h in PDA medium. Single colonies of *L. paracasei* H4-11 and *K. marxianus* L1-1 were selected. Selected colonies of *L. paracasei* H4-11 were cultivated into MRS broth medium at 37°C for 16 h. Selected colonies of *K. marxianus* L1-1 were cultivated into PDB broth medium at 30°C for 24 h. Then, the two broth media were centrifuged at 3000 rpm for 5 min. Cells were washed three times with saline water containing 0.85% (*w/v*) NaCl and finally resuspended in saline water. The resuspended cells were used to ferment rice-acid. The cell densities of *L. paracasei* H4-11 and *K. marxianus* L1-1 were 8.99 log CFU/mL and 7.69 log CFU/mL, respectively.

Rice-acid was fermented according to the following method. Firstly, the selenium rice (Danzhai, Guizhou, China) was broken with a high-speed pulverizer and sieved twice with an 80-mesh sieve to prepare rice flour. Then, water was added into rice flour according to the proportions of 8.0% rice flour and 92.0% water and boiled under stirring conditions to obtain rice soup. Then, boiled rice soup was gelatinized for 30 min in a water bath at 60°C under stirring conditions to prevent uneven gelatinization and local deterioration of the gelatinization solution. Then 1.0% α-amylase was added into gelatinized rice soup for 30-min liquefaction at 90 °C. Then, 0.02% saccharifying enzyme (glucoamylase) was added for 2-h saccharification at 60°C. The mixture was sterilized at 90°C for 20 min. After the rice soup was cooled to about 30°C, *K. marxianus* L1-1 and *L. paracasei* H4-11 were added according to the cell densities of 5.69 log CFU/mL and 7.89 log CFU/mL. Then, the prepared raw materials

were poured into the sterilized rice-acid fermenter and sealed immediately. Finally, the mixture was fermented in a constant-temperature incubator at 30°C for 3 day. We compared different fermentation stages (1 and 3 days) in two rice-acid samples inoculated with *L. paracasei* H4-11, namely, L1 and L3 days. The other two rice-acid samples were inoculated with the mixed culture (*L. paracasei* H4-11 and *K. marxianus* L1-1) for 1 and 3 days, namely, LY 1 and LY 3 days.

Determination of the Cell Densities of *L. paracasei* H4-11 and *K. marxianus* L1-1

Four samples (L1, L3, LY 1, and LY 3 days, 5 g) were mixed separately with 45 mL of saline water containing 0.85% (*w/v*) NaCl and shaken at 150 rpm for 30 min at room temperature. Then, the mixed rice-acid was serially diluted by 10^5 times with saline water containing 0.85% (*w/v*) NaCl and spread on MRS agar medium and PDA medium, respectively. *L. paracasei* H4-11 was cultured in MRS medium at 37°C for 48 h. *K. marxianus* L1-1 was cultured in PDA medium at 30°C for 72 h. The cell densities of *L. paracasei* H4-11 and *K. marxianus* L1-1 were determined in 1 and 3 days, respectively.

Determination of Organic Acids and Taste Substances

After settlement, the samples of rice-acid were filtered with double-layer filter paper. The obtained filtrate was filtered through a 0.22-μm microporous membrane and then passed through a ZORBAX SB-AQ solid-phase cartridge for HPLC analysis (equipped with G1329B autosampler, G1311C quaternary low-pressure ladder, G1316A column oven, and G1315D diode array UV–visible light detector) with ZORBAX SB-Aq column (4.6 × 250 mm, 5 μm, American Agilent Corporation) according to the following parameters: 0.02 mol/L NaH_2PO_4 solution as the mobile phase (pH 2.7), the injection volume (10 μL), flow rate (0.9 mL/min), column temperature (35°C), and detector wavenumber (UV 210 nm). The purity of L-lactic acid was determined according to the method of Moon et al. (2012). L-lactic acid concentration was measured with Amplite™ Colorimetric l-Lactate Assay Kit (AAT Bioquest Inc., United States). Firstly, 0.5, 1.0, 2.0, 5.0, and 10.0 mL of organic acid standards (oxalic acid, tartaric acid, malic acid, acetic acid, citric acid, and succinic) were taken, respectively, diluted to a volume of 25 mL with ultrapure water in a volumetric flask, and filtered through a 0.22-μm aqueous phase filter membrane in order to obtain organic acids with different concentrations. With acid standard solutions, a standard curve with peak area versus concentration was plotted to obtain the linear range regression equation and correlation coefficient and calculate the concentration of each organic acid in rice-acid. In this way, the concentrations of oxalic acid, tartaric acid, malic acid, acetic acid, citric acid, and succinic acid in rice-acid samples were determined.

Rice-acid filtrate (8 mL) was diluted to a volume of 80 mL and analyzed by an electronic tongue (Insent SA-402B, Atsugi-chi, Japan) to determine taste substances (sourness, bitterness, astringency, umami, richness, saltiness, aftertaste-A, and aftertaste-B) according to the method of Qiu et al. (2015). The electronic sensor was cleaned in a cleaning solution for 90 s. The first reference solution was added to clean the sensor for 120 s. The second reference solution was added to clean the sensor for 120 s. After cleaning, the sensor was zeroed to the equilibrium position for 30 s and then placed in the sample cup for 30 s. After the test, the sensor was sequentially washed in two reference solutions for 3 s. Each sample was tested four times. After excluding the first repeated result, the average of the remaining three results was used in the subsequent analysis.

Transcriptome Sequencing and Data Analysis

RNA Extraction and Library Construction for Transcriptome Analysis

The four samples (L1, L3, LY1, and LY3 days, and each sample had three biological repeats) were filtered to get the cells and then centrifuged at 10,000 g for 10 min at 4 °C to obtain the *L. paracasei* cells, which was put in liquid nitrogen quickly and then stored in a refrigerator at −80°C. The RNA extraction of *L. paracasei* H4-11 cells, library construction, RNA-seq, and primary data analysis was performed by PTM BIO Co., Ltd. (Hangzhou, China). The bacterial powder was suspended in 1 mL of Trizol reagent (Invitrogen, Carlsbad, CA, United States) and manipulated according to the manufacturer's instructions. The RNA samples were resuspended in RNase-free water (Thermo Fisher Scientific, Waltham, MA, United States). RNA degradation and contamination were monitored on 1% agarose gels. Then, RNA purity was checked with NanoPhotometer® spectrophotometer (IMPLEN, CA, United States). RNA concentration was measured with Qubit® RNA Assay Kit in Qubit® 2.0 Flurometer (Life Technologies, CA, United States). RNA integrity was assessed by using the RNA Nano 6,000 Assay Kit of the Agilent Bioanalyzer 2,100 system (Agilent Technologies, CA, United States).

A total of 3 μg RNA per sample was used as the input material for the RNA sample preparation. Firstly, ribosomal RNA was removed by Epicentre Ribo zeroTM rRNA Removal Kit (Epicentre, United States) and rRNA-free residue was cleaned by ethanol precipitation. Subsequently, sequencing libraries were generated with the rRNA-depleted RNA by NEBNext® UltraTM Directional RNA Library Prep Kit for Illumina® (NEB, United States) according to the manufacturer's recommendations. Briefly, fragmentation was carried out with divalent cations at an elevated temperature in NEBNext First Strand Synthesis Reaction Buffer (5X). First-strand cDNA was synthesized with random hexamer primers and M-MuLV Reverse Transcriptase (RNase H-). Second strand cDNA synthesis was subsequently performed with DNA Polymerase I and RNase H. In the reaction buffer, the dTTP of dNTPs were replaced by dUTP. The remaining overhangs were converted into blunt ends via exonuclease/polymerase activities. After adenylation of 3′ ends of DNA fragments, the NEBNext Adaptor with a hairpin loop structure was ligated to prepare for hybridization. In order to preferentially select cDNA fragments of 250∼300 bp in length,

the library fragments were purified with the AMPure XP system (Beckman Coulter, Beverly, United States). Then 3 μL of USER Enzyme (NEB, United States) was incubated with size-selected and adaptor-ligated cDNA at 37°C for 15 min, followed by 5-min incubation at 95°C before PCR. PCR was then performed using Phusion High-Fidelity DNA polymerase, Universal PCR primers, and the Index (X) Primer. At last, PCR products were purified (AMPure XP system) and the library quality was assessed on the Agilent Bioanalyzer 2,100 system.

The clustering analysis of the index-coded samples was performed on cBot Cluster Generation System with Novaseq 6000 PE Cluster Kit (Illumia) according to the manufacturer's instructions. After the generation of clusters, the library preparations were sequenced on an Illumina Novaseq 6000 platform and 150-bp paired-end reads were generated.

De novo Transcriptome Assembly and Annotation

Raw reads were generated by a sequencing machine and transformed by base calling. Clean data (clean reads) were obtained by removing reads containing adapter, reads containing ploy-N, and low-quality reads from raw data. Q20, Q30, and GC content of clean reads were calculated. To obtain the clean reads, those reads were filtered with the procedure developed by PTM BIO Co. Ltd. (Hangzhou, China). Next, clean reads were de novo assembled into contig with the Trinity platform (v2.0.12). We selected TopHat as the mapping tool since TopHat could generate a database of splice junctions based on the gene model annotation file and a better mapping result than other non-splice mapping tools. The reference genome (*L. paracasei* ATCC 334) and gene model annotation files can be directly downloaded from the genome website[1]. Cuffquant and cuffnorm (v2.2.1) were used to calculate FPKMs (reads per kilobase of exon region per million mappable reads) of genes in each sample. The transcriptome sequencing was performed according to the method of Huang et al. (2019). The differentially expressed genes (DEGs) were identified in the four pairwise comparisons. DEGs were defined as the value of | Fold Change (FC) | >1.5 and a false discovery rate (FDR) < 0.01. Both Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways with q-value ≤ 0.05 were significantly enriched in DEGs.

Statistical Analysis

All experiments were conducted in triplicate. Data were represented as mean ± standard deviation. Duncan's multiple range test and t-test were carried out to analyze significant differences in SPSS Version 20.0 (SPSS Inc., Chicago, IL, United States). $P < 0.05$ was considered to be statistically significant. In the analysis process of differential expression genes, the recognized Benjamini-Hochberg correction method was used to correct the significance p-value obtained from the original hypothesis test, and a FDR (FDR < 0.01) was used as the key indicator for screening differential expression genes. The correlations between organic acids (oxalic acid, tartaric acid, malic acid, acetic acid, citric acid, and succinic) and taste substances (sourness, bitterness, astringency, umami, richness, saltiness, aftertaste-A, and aftertaste-B) and the expressions of organic acid metabolism and transporter genes were measured with Pearson's correlation coefficients (Jawad et al., 2020).

[1]http://bacteria.ensembl.org/Lactobacillus_paracasei_atcc_334_gca_000014525/Info/Index

RESULTS

Variations of the Cell Densities of *L. paracasei* H4-11 and *K. marxianus* L1-1 in the Fermentation Process of Rice-Acid

The cell densities of *L. paracasei* and *K. marxianus* in rice-acid inoculated with single starter and mixed starters increased in the fermentation period from 1 to 3 days (**Figure 1A**) and the increasing rate of *K. marxianus* L1-1 cells was faster than that of *L. paracasei* H4-11 cells. After 3-day fermentation, the cell density of *L. paracasei* in the group of single inoculation was higher (8.19 log CFU/mL) than that in the mixture of a single inoculation (8.12 log CFU/mL) ($P > 0.05$). Likely, the cell density of *K. marxianus* in the group of mixed inoculation (7.43 log CFU/mL) was lower than that in the group of single inoculation (7.61 log CFU/mL) ($P > 0.05$).

Variations of Organic Acids in the Fermentation Process of Rice-Acid

The concentration of L-lactic acid in the group of mixed inoculation was 22.08 ± 2.42 g/kg after 3-dday fermentation (**Figure 1B**) and the optical purity of L-lactic acid was as high as 96%. The concentration of L-lactic acid in rice-acid inoculated with *K. marxianus* L1-1 was only 6.02 ± 1.67 g/kg. The other six organic acids showed different changing trends during the fermentation process (**Figure 2**). The values of malic acid and acetic acid increased in the fermentation process and the value of oxalic acid showed no significant variation. The values of three organic acids, including tartaric acid, citric acid, and succinic acid, slightly increased in the fermentation period from 1 to 3 days.

Variations of Taste Intensity in the Fermentation Process of Rice-Acid

The intensity variations of eight tastes measured by E-tongues within the three-day fermentation process decreased according to the following order: sourness > bitterness > umami > saltiness > astringency> richness > aftertaste-A > aftertaste-B (**Figure 3**). The group of mixed inoculation showed lower bitterness intensity than the group of single inoculation. The umami index showed a small difference between the group of single inoculation of *L. paracasei* H4-11 and the group of mixed inoculation. Interestingly, the mixed inoculation had smaller values of saltiness and astringency than single inoculation. Saltiness values of rice-acid showed slight differences between the groups of different inoculation methods due to the degradation of different proteins by different fermentation strains. The richness index in the group of mixed inoculation was higher than that in the group of single inoculation in the late stage. Aftertaste-A and aftertaste-B had a small influence on the flavor of rice-acid.

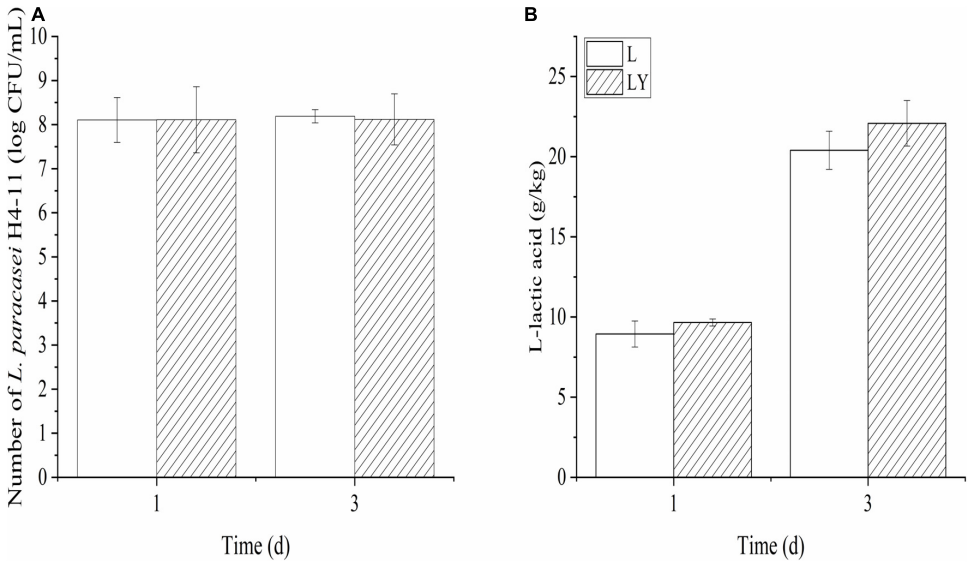

FIGURE 1 | The variations of the number of *L. paracasei* **(A)** and the concentration of L-lactic acid **(B)** in the fermentation period of 1 and 3 days of rice-acid inoculated with *L. paracasei* and mixed-culture with *L. paracasei* and *K. marxianus* (L means rice-acid inoculated with *L. paracasei*, LY means rice-acid inoculated with *L. paracasei* and *K. marxianus*).

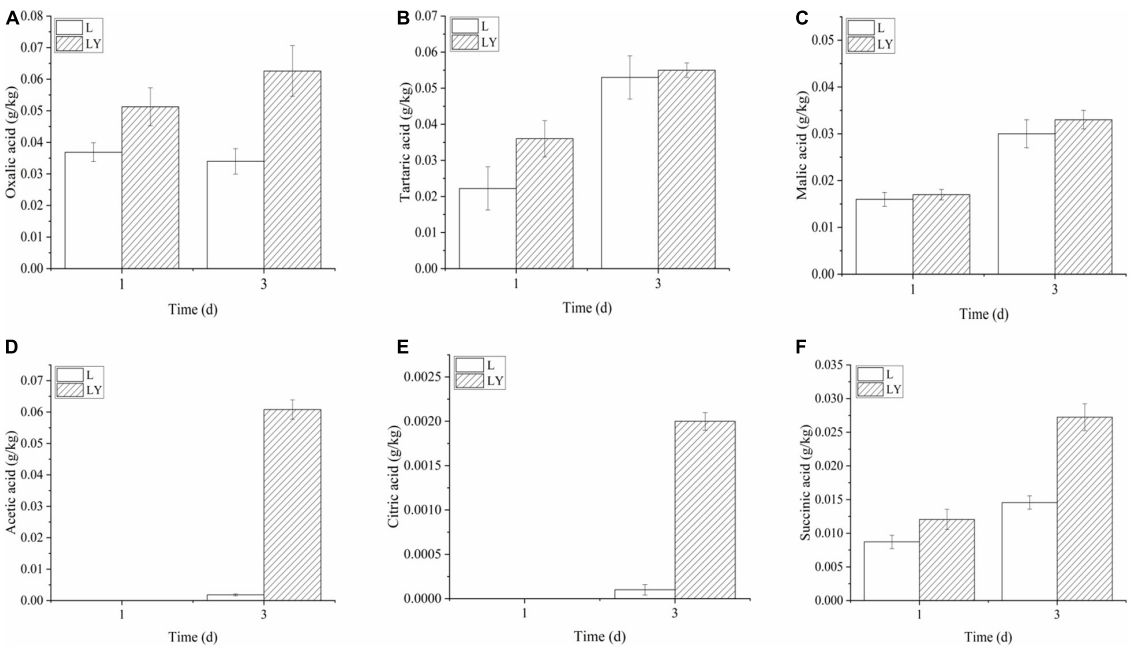

FIGURE 2 | The variations of organic acids in the fermentation period of 1 and 3 days of rice-acid inoculated with *L. paracasei* and mixed-culture with *L. paracasei* and *K. marxianus* [L means rice-acid inoculated with *L. paracasei*, LY means rice-acid inoculated with *L. paracasei* and *K. marxianus*. Organic acids include oxalic acid **(A)**, tartaric acid **(B)**, malic acid **(C)**, acetic acid **(D)**, citric acid **(E)**, and succinic acid **(F)**].

RNA Sequencing Results and Differential Expressions of Genes

The four independent cDNA libraries (L1, L3, LY1, and LY3 days) constructed for high-throughput sequencing produced 7,824,981∼10,228,721 pair-end reads and 2,347,494,300∼3,068,616,300 clean reads after strict quality check and data filtering (Q20 bases > 97.54%, Q30 bases > 92.66%, G + C approximately 45.25%∼50.29%) (**Supplementary Table 1**, Supporting Information). The filtered clean sequence data were compared with the reference genome (*L. paracasei* ATCC 334). The percentage of the sequences that could be localized to the genome exceeded 90.00%, which suggested that the throughput

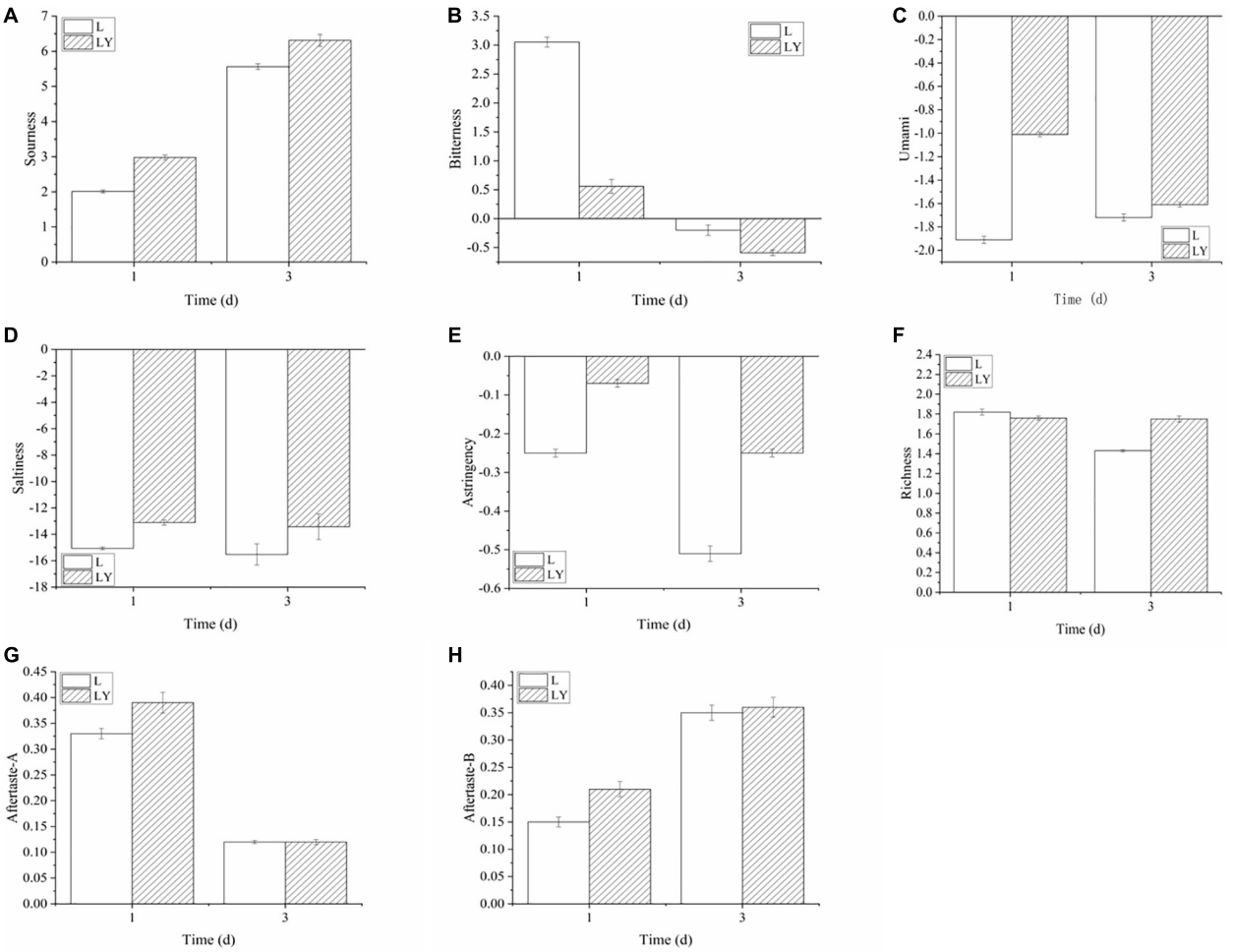

FIGURE 3 | The variations of taste signal value of taste substances in the fermentation period of 1 and 3 days of rice-acid inoculated with *L. paracasei* and mixed-culture with *L. paracasei* and *K. marxianus* [L means rice-acid inoculated with *L. paracasei*, LY means rice-acid inoculated with *L. paracasei* and *K. marxianus*. Taste substances include sourness **(A)**, bitterness **(B)**, umami **(C)**, saltiness **(D)**, astringency **(E)**, richness **(F)**, aftertaste-A **(G)**, and aftertaste-B **(H)**].

and sequencing quality were high enough for further analysis. Moreover, we used Pearson's correlation coefficient to analyze whether the differences depended on fermentation time and inoculation methods (mixed inoculation and single inoculation). R values in the three replicates of the same experiment were more than 0.989 (**Supplementary Figure 1**).

The comparison of different transcripts revealed that 1141 and 1094 genes were significantly differentially expressed in the period from L1 to L3 days and the period from LY1 to LY3 days, respectively. In addition, 601 significantly DEGs were found between the data of LY1 and L1 days, and 329 significantly DEGs were found between the data of LY3 and L3 days. The up-regulated and down-regulated genes accounted for approximately 50% (**Supplementary Table 2**).

GO Enrichment Analysis

To further elaborate on the function of DEGs, we firstly conducted GO enrichment analysis. GO analysis of the DEGs showed the enrichment of major biological processes, molecular function, and cellular components (**Supplementary Figure 2**). The groups of different fermentation methods (single inoculation and mixed inoculation) were compared. The DEGs were enriched in catalytic activity and binding in terms of molecular functions. In terms of cellular components, most of the DEGs were enriched in four categories including membrane, membrane part, cell, and cell part.

Among the up-regulated and down-regulated DEGs, related biological processes mainly included the lysine biosynthetic process, oxidation-reduction process, NAD biosynthetic process, and purine nucleobase biosynthetic process. Related cellular components mainly included the ribosome, cytoplasm, and plasma membrane. Molecular functions mainly included structural components of ribosome and rRNA binding. The above terms were commonly enriched GO terms in the four pairwise comparisons (**Supplementary Figure 3**). Interestingly, there were more molecular function-related enriched GO terms in the fourth pairwise comparison (LY3 vs L3 days).

Composite Analysis of KEGG Metabolism Pathways, Organic Acids, and Taste Substances

The top 20 significantly enriched pathways in the four pairwise comparisons were selected (**Supplementary Figure 4**). ABC transporters, ribosome, purine metabolism, amino sugar metabolism, and nucleotide sugar metabolism were enhanced in the four pairwise comparisons. These pathways were related to the general features of different transcriptomes of fermented rice-acid. The comparison results of different fermentation time (3 and 1 days) indicated that the enrichment of the differentially transcribed genes in the categories of beta-Lactam resistance, photosynthesis, nicotinate and nicotinamide metabolism, and oxidative phosphorylation were high in rice-acid only inoculated with *L. paracasei*, whereas the enrichment of the differentially transcribed genes in the categories of ribosomes, lysine biosynthesis, cysteine, and methionine metabolism, fatty acid biosynthesis and photosynthesis were high in rice-acid with mixed inoculation. The comparison results of different inoculation methods (single inoculation of *L. paracasei* and mixed inoculation) indicated that the enrichment of the differentially transcribed genes in the categories of alanine, aspartate and glutamate metabolism, aminoacyl-tRNA biosynthesis and TCA cycle were high in the first day, whereas the enriched differentially transcribed genes in the categories of ribosomes, histidine metabolism, purine metabolism, and ABC transporters were high in the third day.

Moreover, we calculated Pearson correlation coefficients with the expression data obtained at different fermentation times (**Figure 4**). The expression levels of several genes (LSEI_0166, LSEI_1784, LSEI_2104, LSEI_2191, and LSEI_2866) had significant and positive correlation with L-lactic acid concentration (r = 0.983*, 0.974*, 0.966*, 0.951*, and 0.992**) in rice-acid fermented with the single inoculation of *L. paracasei* and the mixed inoculation. The expression levels of several genes (LSEI_0594, LSEI_1126, LSEI_1310, LSEI_1365, LSEI_1787, LSEI_1808, LSEI_2410, and LSEI_2510) had the significant and negative correlation with L-lactic acid concentration (r = −0.981*, −0.997**, −0.998**, −0.956*, −0.999**, −0.955*, −0.961*, and −0.960*) in fermented rice-acid. The expression levels of the two genes of LSEI_0630 and LSEI_0949 had a significant and positive correlation with the concentrations of acetic acid, succinic acid, and citric acid. The expression levels of the gene LSEI_2477 had a significant and negative correlation with the concentrations of acetic acid, succinic acid, and citric acid. Similarly, the expression levels of the three genes (LSEI_0700, LSEI_2104, and LSEI_2866) had a significant and positive correlation with sourness intensity (r = 0.950*, 0.984*, and 0.961*) in fermented rice-acid. The expression levels of the gene LSEI_2549 had a positive correlation with L-lactic acid concentration and sourness intensity and LSEI_2549 had a significant and negative correlation with bitterness intensity (r = 0.953*). The expression levels of the two genes (LSEI_0401 and LSEI_2272) had a significant and negative correlation with astringency intensity (r = −0.964* and −0.974*). The expression levels of the three genes (LSEI_0322, LSEI_0775, and LSEI_1746) had a significant and positive correlation with umami intensity (r = 0.958*, 0.966*, and 0.965*) in fermented rice-acid.

Genes Involved in Ribosomes, ABC Transporters, and Purine Metabolism

The comparison results of different fermentation time (3 and 1 days, **Supplementary Table 3**) indicated that ribosomal transcription levels of the genes encoding large subunit ribosomal protein L32 (LSEI_1358) and large subunit ribosomal protein L7/L12 (LSEI_2272) were up-regulated in the group of the single inoculation with *L. paracasei*, whereas the genes encoding large subunit ribosomal protein L17 (LSEI_2476) and small subunit ribosomal protein S12 (LSEI_2510) were down-regulated in the group of the mixed inoculation. The comparison results of two inoculation methods (mixed inoculation and single

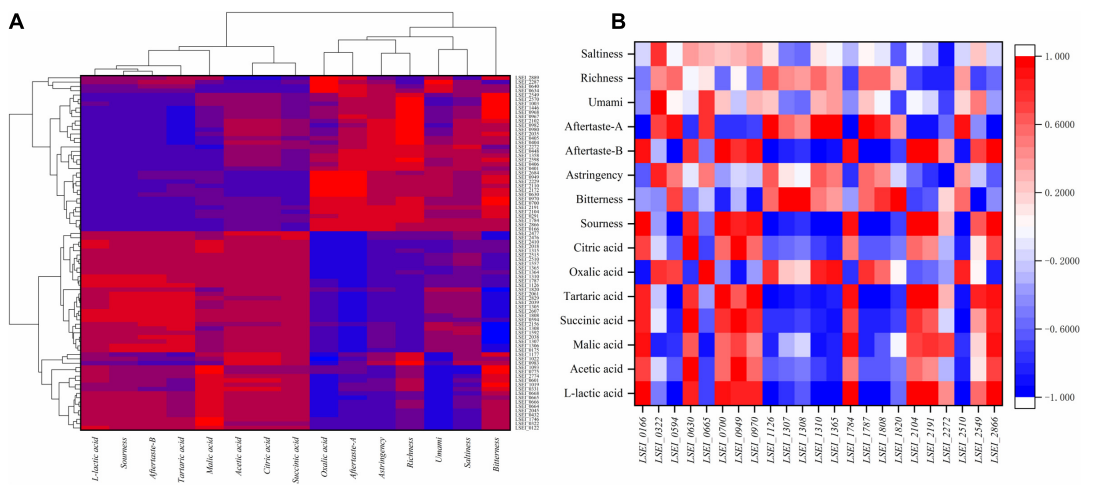

FIGURE 4 | Correlations among the characteristic flavor (organic acids and taste substances) and gene expression **(A)**, Correlations among the characteristic flavor (organic acids and taste substances) and the key 23 genes **(B)**.

inoculation of *L. paracasei*) indicated that the gene LSEI_2272 was down-regulated on the first day and the third day in the rice-acid fermentation process, whereas the genes LSEI_2476 and LSEI_2510 were down-regulated on the third day of rice-acid fermentation.

The comparison results of different fermentation time (3 and 1 days, **Supplementary Table 3**) indicated that ABC transporter transcription levels of the gene encoding L-cystine transport system substrate-binding protein (LSEI_0601) and the gene encoding D-methionine transport system substrate-binding protein (LSEI_1177) were up-regulated, whereas ABC transporter transcription levels of some genes encoding oligopeptide transport system substrate-binding protein (LSEI_0175), ATP-binding cassette, subfamily B, multidrug efflux pump (LSEI_1592), and oligopeptide transport system ATP-binding protein (LSEI_2061) were down-regulated in the group of the single inoculation of *L. paracasei*. The five genes except LSEI_1592 were down-regulated in the group of the mixed inoculation. The comparison results of different inoculation methods (mixed inoculation and single inoculation of *L. paracasei*) indicated that the changes of transcription levels of key genes in the first day of rice-acid fermentation were similar to those of the first pairwise comparison (L3 vs L1 days). The genes encoding L-cystine transport system substrate-binding protein (LSEI_0601) and D-methionine transport system substrate-binding protein (LSEI_1177) were up-regulated in the first and third pairwise comparisons.

The comparison results of different fermentation time (**Supplementary Table 3**) indicated that purine metabolism transcription levels of the genes encoding putative integral membrane protein with a TlyC-like hemolysin domain (LSEI_2229) was up-regulated, whereas the transcription level of the gene encoding adenylosuccinate synthase (LSEI_0122) was down-regulated in the group of the single inoculation of *L. paracasei*. The genes encoding phosphoglucomutase (LSEI_0949), ribonucleoside-triphosphate reductase (LSEI_2287), and the gene (LSEI_2229) were up-regulated and the genes encoding adenine phosphoribosyltransferase (LSEI_1557), phosphoribosylamine-glycine ligase (LSEI_1746), DNA-directed RNA polymerase subunit alpha (LSEI_2477) and DNA-directed RNA polymerase subunit beta′ (LSEI_2515) and the gene (LSEI_0122) were down-regulated in the group of the mixed inoculation. The comparison results of inoculation methods (single inoculation of *L. paracasei* and mixed inoculation) indicated that the genes LSEI_0122 and LSEI_1746 were up-regulated on the first day of rice-acid fermentation. On the third day of rice-acid fermentation, the genes of LSEI_0122, LSEI_0949, LSEI_1746, LSEI_2229, and LSEI_2287 were up-regulated and the genes of LSEI_1557, LSEI_2477, and LSEI_2515 were down-regulated.

Genes Involved in Amino Sugar and Nucleotide Sugar Metabolism and Starch and Sucrose Metabolism

The key DEGs involved in amino sugar and nucleotide sugar metabolism and starch and sucrose metabolism were related to energy production and conversion, carbohydrate transport and metabolism, cell wall/membrane/envelope biogenesis, and general function prediction. We further confirmed 21 DEGs at corresponding positions of amino sugar and nucleotide sugar metabolism (**Supplementary Table 4**). The comparison results of the different fermentation times confirmed 10 up-regulated genes and 6 down-regulated genes in the group of the single inoculation of *L. paracasei* and 5 up-regulated genes and 6 down-regulated genes in the group of the mixed inoculation. The comparison results of different inoculation methods confirmed six up-regulated genes and three down-regulated genes on the first day of fermentation and three up-regulated genes and three down-regulated genes on the third day of fermentation. The up-regulated genes exhibited positive effects on hexosaminidase (LSEI_0291), PTS system, mannose-specific components (LSEI_0401 and LSEI_0561), phosphoglucomutase (LSEI_0949), glutamine-fructose-6-phosphate transaminase (LSEI_1019), UTP–glucose-1-phosphate uridylyltransferase (LSEI_1093), and glucosamine-6-phosphate deaminase (LSEI_2889).

We further confirmed 17 key DEGs at corresponding positions of starch and sucrose metabolism. The comparison results of different fermentation times (3 and 1 days) confirmed 11 up-regulated genes and three down-regulated genes in the group of the single inoculation of *L. paracasei* and six up-regulated genes and two down-regulated genes in the group of mixed inoculation. The comparison results of different inoculation methods (single inoculation of *L. paracasei* and mixed inoculation) confirmed six up-regulated genes and two down-regulated genes on the first day of fermentation and three up-regulated genes and four down-regulated genes on the third day of fermentation (**Supplementary Table 4**). The up-regulated genes including oligo-1,6-glucosidase (LSEI_0406), trehalose-6-phosphate hydrolase (LSEI_0630), beta-glucosidase (LSEI_0700), and phosphoglucomutase (LSEI_0949) exhibited positive effects on the fermentation process in the groups of single inoculation of *L. paracasei* and mixed inoculation.

Genes Involved in Glycolysis

Different inoculation methods had different enhancement effects on rice-acid metabolism. We further confirmed 20 key DEGs at corresponding positions of glycolysis metabolism (**Table 1**). The up-regulated and down-regulated genes in glycolysis metabolism were related to carbohydrate transport and metabolism and energy production and conversion (**Figure 5**). The comparison results of different fermentation times (3 and 1 days) confirmed 10 up-regulated genes and 3 down-regulated genes in the group of single inoculation of *L. paracasei* and 4 up-regulated genes and 8 down-regulated genes in the group of mixed inoculation. The comparison results of inoculation methods (single inoculation of *L. paracasei* and mixed inoculation) confirmed 8 up-regulated genes on the first day of fermentation and 3 up-regulated genes and 2 down-regulated genes on the third day of fermentation. The key genes encoding L-lactate dehydrogenase (LSEI_2549, FC = 1.89), glyceraldehyde 3-phosphate dehydrogenase (LSEI_0967, FC = 1.71), phosphoglycerate kinase (LSEI_0968, FC = 2.22) and enolase (LSEI_0970, FC = 1.59) were up-regulated in the first pairwise comparison (L3 vs L1 days), indicating that these genes could affect the increase of L-lactic acid, sourness and richness in the fermentation process of rice-acid only inoculated

TABLE 1 | The key genes involved in glycolysis, pyruvate metabolism, and TCA cycle in single inoculation with *L. paracasei* and mix inoculation during rice-acid fermentation process among four pairwise comparisons.

Gene ID	EC ID	KEGG annotation	FC (L3 vs L1 days)	FC (LY3 vs LY1 days)	FC (LY1 vs L1 days)	FC (LY3 vs L3 days)
Glycolysis						
LSEI_0331	K15634	Integral membrane protein	4.82	−2.43	5.58	−2.04
LSEI_0432	[EC:4.1.2.13]	K01624 fructose-bisphosphate aldolase, class II	–	−1.97	2.71	–
LSEI_0448	[EC:3.2.1.86]	K01223 6-phospho-beta-glucosidase	2.25	–	–	–
LSEI_0634	[EC:1.1.1.27]	K00016 l-lactate dehydrogenase	–	1.99	–	1.53
LSEI_0668	[EC:5.1.3.3]	Aldose 1-epimerase; K01785 aldose 1-epimerase	–	−3.48	2.81	−1.68
LSEI_0775	[EC:1.1.1.1]	K04072 acetaldehyde dehydrogenase / alcohol dehydrogenase	2.07	–	4.35	1.99
LSEI_0949	[EC:5.4.2.2]	K01835 phosphoglucomutase	–	2.01	–	1.59
LSEI_0967	[EC:1.2.1.12]	K00134 glyceraldehyde 3-phosphate dehydrogenase	1.71	–	–	–
LSEI_0968	[EC:2.7.2.3]	K00927 phosphoglycerate kinase	2.22	–	1.66	–
LSEI_0970	[EC:4.2.1.11]	K01689 enolase	1.59	1.62	–	–
LSEI_1126	[EC:5.3.1.9]	K01810 glucose-6-phosphate isomerase	−1.56	–	–	–
LSEI_1310	[EC:1.1.1.27]	K00016 l-lactate dehydrogenase	−2.17	−2.64	–	–
LSEI_1364	[EC:2.7.1.11]	K00850 6-phosphofructokinase 1	–	−1.83	–	–
LSEI_1365	[EC:2.7.1.40]	K00873 pyruvate kinase	–	−2.00	–	–
LSEI_1446	[EC:1.8.1.4]	K00382 dihydrolipoamide dehydrogenase	2.46	–	1.88	–
LSEI_2045	[EC:3.1.3.11]	K04041 fructose-1,6-bisphosphatase III	–	−1.57	2.04	–
LSEI_2191	[EC:3.2.1.86]	K01223 6-phospho-beta-glucosidase	2.66	2.35	–	–
LSEI_2549	[EC:1.1.1.27]	K00016 l-lactate dehydrogenase	1.89	–	1.61	–
LSEI_2598	[EC:5.1.3.3]	K01785 aldose 1-epimerase	1.58	–	–	–
LSEI_2607	[EC:1.1.1.27]	K00016 l-lactate dehydrogenase	−3.34	1.95	–	–
Pyruvate metabolism						
LSEI_0166	[EC:2.7.2.1]	K00925 acetate kinase	–	1.72	–	–
LSEI_0322	[EC:1.3.5.4]	K00244 fumarate reductase flavoprotein subunit	–	–	2.17	–
LSEI_0594	[EC:1.3.5.4]	General function prediction only K00244 fumarate reductase flavoprotein subunit	−1.71	–	–	–
LSEI_0634	[EC:1.1.1.27]	K00016 l-lactate dehydrogenase	–	1.99	–	1.53
LSEI_0775	[EC:1.1.1.1]	Energy production and conversion K04072 acetaldehyde dehydrogenase / alcohol dehydrogenase	2.07	–	4.35	2.00
LSEI_1305	[EC:1.2.4.1]	K00161 pyruvate dehydrogenase E1 component alpha subunit	−1.82	–	–	–
LSEI_1306	[EC:1.2.4.1]	K00162 pyruvate dehydrogenase E1 component beta subunit	−1.83	–	−1.60	–
LSEI_1307	[EC:2.3.1.12]	K00627 pyruvate dehydrogenase E2 component (dihydrolipoamide acetyltransferase)	−1.95	–	−1.96	–
LSEI_1308	[EC:1.8.1.4]	K00382 dihydrolipoamide dehydrogenase	−1.54	–	−1.80	–
LSEI_1310	[EC:1.1.1.27]	K00016 l-lactate dehydrogenase	−2.17	−2.64	–	–
LSEI_1446	[EC:1.8.1.4]	K00382 dihydrolipoamide dehydrogenase	2.46	–	1.88	–
LSEI_1784	[EC:1.2.3.3]	K00158 pyruvate oxidase	3.58	6.73	−1.54	–
LSEI_1787	[EC:2.3.1.9]	Lipid transport and metabolism K00626 acetyl-CoA C-acetyltransferase	−2.30	−2.35	–	–
LSEI_2110	[EC:2.1.3.15]	K01963 acetyl-CoA carboxylase carboxyl transferase subunit beta	1.82	4.38	–	1.75
LSEI_2156	[EC:1.1.1.28]	K03778 d-lactate dehydrogenase	−4.99	–	−4.63	–
LSEI_2172	[EC:2.7.2.1]	K00925 acetate kinase	2.57	3.73	1.64	2.19
LSEI_2410	[EC:4.2.1.2]	K01679 fumarate hydratase, class II	−1.69	−2.00		–
LSEI_2549	[EC:1.1.1.27]	l-lactate dehydrogenase	1.89	–	1.61	–
LSEI_2607	[EC:1.1.1.27]	K00016 l-lactate dehydrogenase	−3.34	−1.95	–	–
LSEI_2866	[EC:1.1.1.38]	K00027 malate dehydrogenase (oxaloacetate-decarboxylating)	3.94	8.46	–	–
TCA cycle						
LSEI_0322	[EC:1.3.5.4]	K00244 fumarate reductase flavoprotein subunit	–	–	2.17	–
LSEI_0594	[EC:1.3.5.4]	General function prediction only/ K00244 fumarate reductase flavoprotein subunit	−1.71	–	–	–
LSEI_1305	[EC:1.2.4.1]	Energy production and conversion K00161 pyruvate dehydrogenase E1 component alpha subunit	−1.83	–	–	–
LSEI_1306	[EC:1.2.4.1]	K00162 pyruvate dehydrogenase E1 component beta subunit	−1.83	–	−1.60	–
LSEI_1307	[EC:2.3.1.12]	K00627 pyruvate dehydrogenase E2 component (dihydrolipoamide acetyltransferase)	−1.95	–	−1.96	–
LSEI_1308	[EC:1.8.1.4]	K00382 dihydrolipoamide dehydrogenase	−1.54	–	−1.80	–
LSEI_1315	[EC:6.4.1.1]	K01958 pyruvate carboxylase	–	−2.75	1.66	–
LSEI_1446	[EC:1.8.1.4]	K00382 dihydrolipoamide dehydrogenase	2.46	–	1.88	–
LSEI_1820	[EC:4.1.1.49]	K01610 phosphoenolpyruvate carboxykinase (ATP)	–	–	−1.83	–
LSEI_2410	[EC:4.2.1.2]	K01679 fumarate hydratase, class II	−1.69	−2.00	–	–

– means no significant difference.

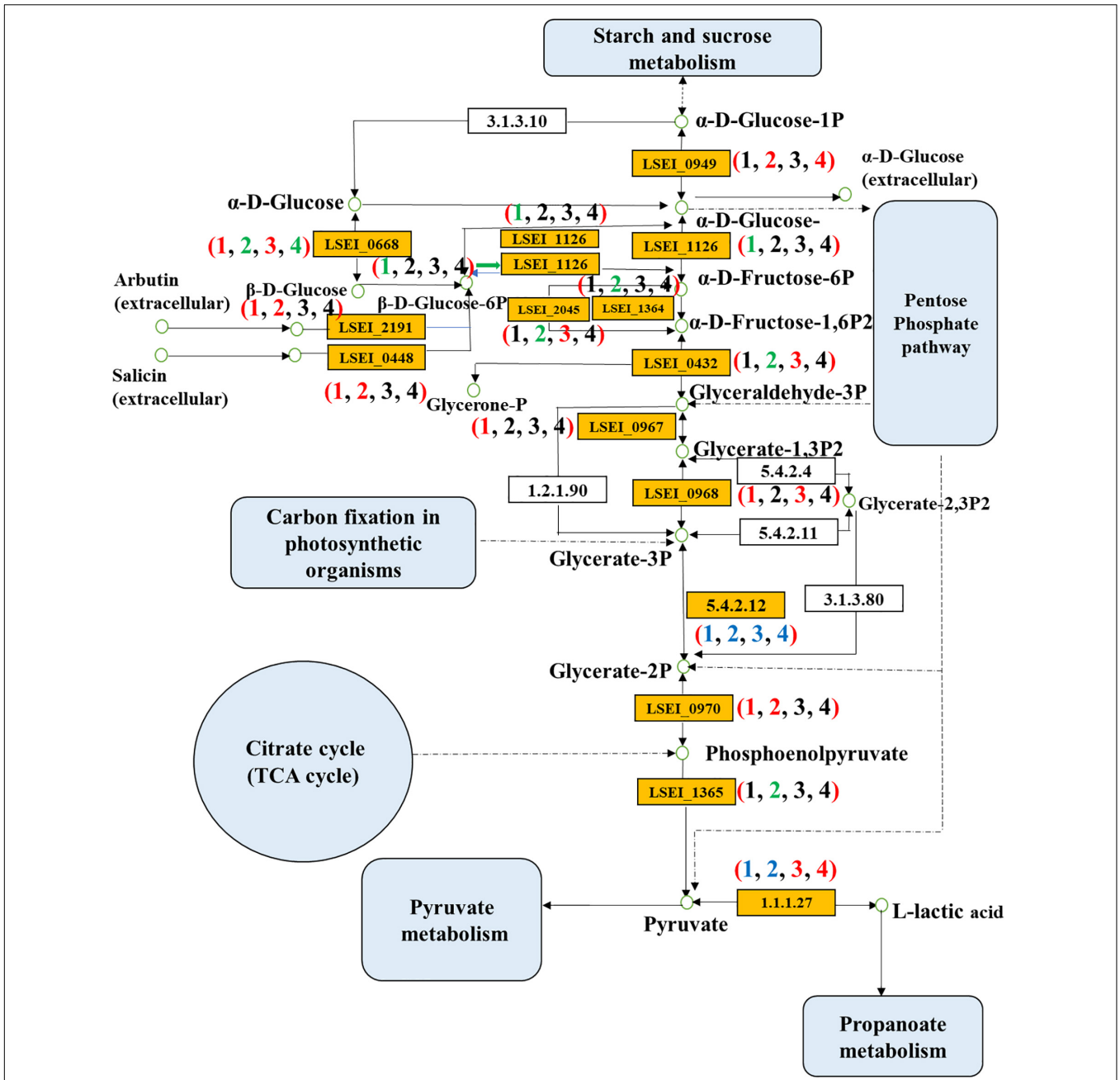

FIGURE 5 | Differentially expressed genes of glycolysis pathway in four pairwise comparisons (Numbers in brackets 1, 2, 3, and 4 refer to the L3 vs L1 days, LY3 vs LY1 days, LY1 vs L1 days, and LY3 vs L3 days, respectively. L1 day refers to single inoculation with *L. paracasei* on the first day, L3 day refers to single inoculation with *L. paracasei* on the third day, LY1 day refers to mix inoculation with *L. paracasei* and *K. marxianus* on the first day, LY3 day refers to mix inoculation with *L. paracasei* and *K. marxianus* on the third day. The colors red, green, blue, and black represent the up-regulated gene, down-regulated gene, both of up-regulated gene and down-regulated gene, and no change gene, respectively).

with *L. paracasei*. The key genes encoding 6-phosphofructokinase (LSEI_1364, FC = 1.83), fructose-1,6-bisphosphatase III (LSEI_2045, FC = 1.57), fructose-bisphosphate aldolase Class II (LSEI_0432, FC = 1.97), pyruvate kinase (LSEI_1365, FC = 2.00) and l-lactate dehydrogenase (LSEI_2607, FC = 1.95) were down-regulated in the third pairwise comparison (LY3 vs LY1 days), indicating that these genes could have a negative effect on the increase of L-lactic acid, sourness and richness in the fermentation process of rice-acid with mixed inoculation. The comparison results of different inoculation methods in the first day of fermentation indicated that the key genes of LSEI_2549 (FC = 1.61), LSEI_2045 (FC = 2.05), LSEI_0432 (FC = 2.71), and LSEI_0968 (FC = 1.66) exhibited positive effects on the formation of L-lactic acid, whereas the key genes showed no

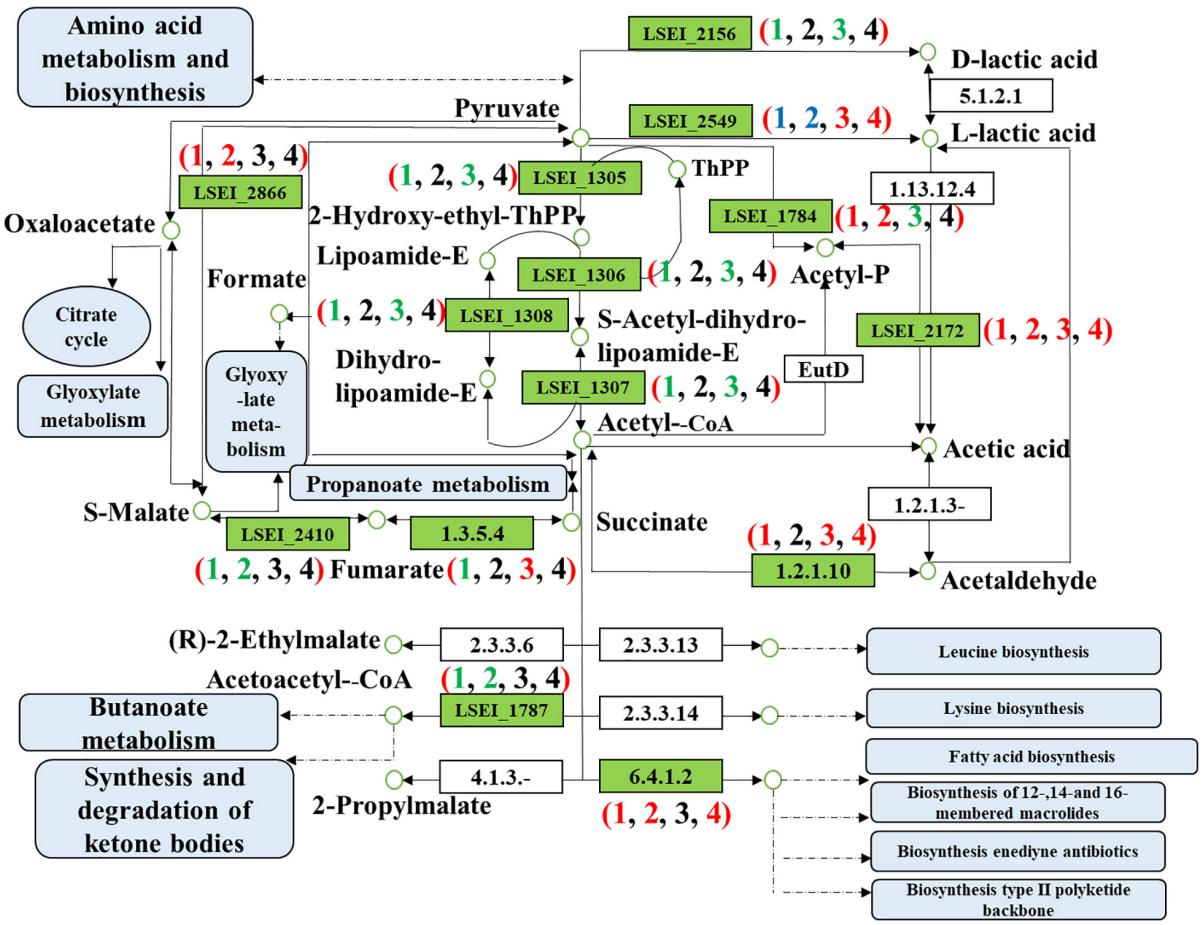

FIGURE 6 | Differentially expressed genes of pyruvate metabolism in four pairwise comparisons (Numbers in brackets 1, 2, 3, and 4 refer to the L3 vs L1 days, LY3 vs LY1 days, LY1 vs L1 days, and LY3 vs L3 days, respectively. L1 day refers to single inoculation with *L. paracasei* on the first day, L3 day refers to single inoculation with *L. paracasei* on the third day, LY1 day refers to mix inoculation with *L. paracasei* and *K. marxianus* on the first day, LY3 day refers to mix inoculation with *L. paracasei* and *K. marxianus* on the third day. The colors red, green, blue, and black represent the up-regulated gene, down-regulated gene, both of up-regulated gene and down-regulated gene, and no change gene, respectively).

negative effect on the formation of L-lactic acid in the third day of fermentation.

Genes Involved in Pyruvate Metabolism

The key DEGs involved in pyruvate metabolism were related to energy production and conversion, carbohydrate transport and metabolism, and lipid transport and metabolism. We further confirmed 20 key DEGs at corresponding positions of pyruvate metabolism (**Table 1** and **Figure 6**). The comparison results of different fermentation times (3 and 1 days) confirmed 7 up-regulated genes and 10 down-regulated genes in the group of single inoculation of *L. paracasei* and 6 up-regulated genes and 4 down-regulated genes in the group of mixed inoculation. The comparison results of different inoculation methods confirmed five up-regulated genes and five down-regulated genes on the first day of fermentation and four up-regulated genes on the third day of fermentation. The gene encoding d-lactate dehydrogenase (LSEI_2156, FC = 4.99) in the third day of fermentation showed the lower mRNA level than that on the first day in the group of the single inoculation of *L. paracasei*. Similarly, the gene encoding d-lactate dehydrogenase (LSEI_2156) in the first day of fermentation in the group of the mixed inoculation showed lower mRNA levels than that in the group of the single inoculation of *L. paracasei*. This result reasonably interpreted the phenomenon that the decrease of d-lactate dehydrogenase led to the increase in L-lactic acid. The gene encoding l-lactate dehydrogenase (LSEI_2549, FC = 1.89) in the third day of fermentation showed a higher mRNA level than that on the first day in the group of the single inoculation of *L. paracasei*. Similarly, the gene encoding l-lactate dehydrogenase (LSEI_2549) in the first day of fermentation in the group of the mixed inoculation showed a higher mRNA level than that in the group of the single inoculation of *L. paracasei*. Another gene encoding L-lactate dehydrogenase (LSEI_0634) showed a positive effect on L-lactic acid formation in the two pairwise comparisons (LY3 vs LY1 days; LY3 vs L3 days). These results reasonably demonstrated the increase in the L-lactic acid in the fermentation process in the study. The genes encoding

acetate kinase (LSEI_0166 and LSEI_2172) were up-regulated in the four pairwise comparisons and the gene LSEI_2172 had different FC values (2.57, 3.73, 1.64, and 2.19) in the four pairwise comparisons. These data proved that acetic acid showed different increasing trends in the group of mixed inoculation and the group of single inoculation in the rice-acid fermentation process. The up-regulated gene encoding malate dehydrogenase (LSEI_2866, FC = 3.94 and 8.46) reasonably interpreted the increase in malic acid in the fermentation process. The function of up-regulated genes was related to the sourness and bitterness of taste substances measured with E-tongues. The indication requires further experimental verification in the future.

Genes Involved in TCA Cycle

The key DEGs in TCA cycle were related to energy production and conversion and general function prediction. We further confirmed 10 DEGs at corresponding positions of TCA cycle (**Table 1** and **Supplementary Figure 5**). The comparison results of different fermentation times (3 and 1 days) confirmed one up-regulated gene and six down-regulated genes in the group with single inoculation of *L. paracasei* and two down-regulated genes in the group with mixed inoculation. The comparison results of different inoculation methods (mixed inoculation and single inoculation of *L. paracasei*) confirmed three up-regulated genes and four down-regulated genes on the first day of fermentation, but no up-regulated or down-regulated genes were confirmed on the third day of fermentation. The gene encoding dihydrolipoamide dehydrogenase (LSEI_1446, FC = 2.46) in the third day showed a higher mRNA level than that on the first day in the group with single inoculation of *L. paracasei*. Although six genes were down-regulated, their FC values were low. The comparison results of two inoculation methods indicated that the genes encoding fumarate reductase flavoprotein subunit (LSEI_0322, FC = 2.17), pyruvate carboxylase (LSEI_1315, FC = 1.66), and dihydrolipoamide dehydrogenase (LSEI_1446, FC = 1.88) showed higher mRNA levels on the first day of fermentation.

Genes Involved in Amino Acid Biosynthesis and Metabolism

In order to identify the differences in main amino acid metabolic pathways between single inoculation and mixed inoculation, we further confirmed DEGs and analyzed 10 amino acid metabolic pathways (**Supplementary Table 5**). The comparison results of different fermentation time (3 and 1 days) indicated that the genes of arginine and proline metabolism (P = 0.04), arginine biosynthesis (P = 0.05), and histidine metabolism (P = 0.02) showed the significant difference in the group with single inoculation of *L. paracasei*, whereas the genes of cysteine and methionine metabolism (P = 0.006) and lysine biosynthesis (P = 0.004) showed the extremely significant difference in the group with mixed inoculation. The comparison results of two inoculation methods indicated that the genes of alanine, aspartate, and glutamate metabolism (P = 0.003) showed the extremely significant difference in the first day of fermentation, whereas the genes of histidine metabolism (P = 0.0001) and tyrosine metabolism (P = 0.0379), respectively, showed the extremely significant difference and significant difference in the third day of fermentation.

DISCUSSION

Rice-acid as a functional fermented product is favored by Chinese consumers. Due to its rich probiotic flora, unique flavor, and nutrients, rice-acid is being developing into a beverage. Since the majority of fermentation processes rely on the conversion of sugars to lactic acid, LAB plays the most important role, followed by yeasts (Tofalo et al., 2019). Notably, during the production process, rice-acid undergoes a series of flavor changes. In order to investigate the molecular mechanisms of formation and metabolic pathways of flavors in rice-acid fermented with *L. paracasei* under the conditions of different fermentation time and inoculation methods. In this study, we investigated the transcriptome of *L. paracasei* during the maturation of rice-acid. Firstly, we measured the cell densities of *L. paracasei* and *K. marxianus* during the different fermentation times. Then, we obtained the key organic acids and taste substances of different fermented rice-acids. Finally, we compared the DEGs related to the KEGG pathways based on comparative transcriptomics.

The cell density of *K. marxianus* in the group of mixed inoculation was lower than that in the group of single inoculation because *K. marxianus* was more adapted to the rice-acid fermentation system and might compete with *L. paracasei* in the late fermentation stage (the data were not shown). *L. paracasei* and *K. marxianus* could cooperate or compete with each other and contribute to the production of some important flavors in rice-acid due to the increased flux in the acetyl-CoA synthetic pathway and TCA cycle of *K. marxianus* (Sakihama et al., 2019). The metabolism pathway of *L. paracasei* was discussed in the study. It is worth noting that *L. paracasei* has special probiotic functions. A recent report demonstrated that the pomegranate beverages with the better quality could be obtained by the inoculation with *L. paracasei* (Mantzourani et al., 2020). Interestingly, L-lactic acid was the most important organic acid in rice-acid because the inoculation with *L. paracasei* could produce L-lactic acid (Thakur et al., 2019). It could be inferred that *L. paracasei* H4-11 played an important role in the formation of L-lactic acid. In addition, L-lactic acid could form poly-L-lactic acid, which could be combined with functional additives to improve their mechanical and biological properties for cardiovascular implantation applications (Kang et al., 2019). Therefore, it is necessary to develop fermented rice-acid with the L-lactic acid metabolism ability. Among eight taste substances in the group of mixed inoculation, the sourness had the highest intensity due to the dynamics from saccharification to alcoholization and acidification in the fermentation process of rice-acid inoculated with lactobacillus and yeasts (Jiao et al., 2017). The result also demonstrated that mixed inoculation had a synergistic effect on the acid production capacity. The variation in sourness was consistent with the variation in L-lactic acid in this study.

R-values proved that our experimental results obtained with comparative transcriptomics had high biological repeatability.

In addition, different *R*-values in the pairwise comparisons proved the rationality of the experimental design. The four pairwise comparisons suggested different KEGG metabolism pathways, thus resulting in the different concentrations of L-lactic acid, other organic acids, and taste substances. The enrichment of the differentially transcribed genes in the categories of beta-Lactam resistance and photosynthesis was obtained in three pairwise comparisons except for the third pairwise comparison (LY1 vs L1 days). Importantly, the KEGG metabolism pathways of starch and sucrose metabolism, glycolysis/gluconeogenesis, pyruvate metabolism, TCA cycle, and amino acid biosynthesis and metabolism showed the differences in the four pairwise comparisons due to the difference in the sourness and taste characteristics of different rice-acid samples. It is worth noting that the enrichment of the differentially transcribed genes in glutathione metabolism in the first pairwise comparison was higher than that in the other three pairwise comparisons. Glutathione S-transferase (LSEI_1467) and glutathione peroxidase (LSEI_0893) found in *L. paracasei* were reported to play an important role in acid stress response in other LAB (Lee et al., 2010). Interestingly, we found that the differentially transcribed genes were enriched in the longevity regulation pathway, thus interpreting the longevity of local residents who often drank rice-acid. The indication requires to be further experimentally verified.

Most ribosomal genes were related to the functions of translation, ribosomal structure, and biogenesis in this study. The down-regulated genes were related to the ribosome, indicating that total ribosomes limited protein synthesis and growth, as reported by Zhang et al. (2020). In addition, the up-regulated genes indicated that the synthesis pathway of ribosomes provided more energy and resources for other cellular components. Most ABC transporters were related to the functions of amino acid transport and metabolism, energy production and conversion, inorganic ion transport and metabolism, and defense mechanisms. Most genes showed a lower mRNA level on the third day because the third day was the end of fermentation. The result also confirmed our proper choice of fermentation time. Purine metabolism refers to the metabolic pathways that synthesize and degrade purines in many organisms (Klotz et al., 2020). Most purine metabolism-related genes were related to the functions of nucleotide transport and metabolism, carbohydrate transport and metabolism, general function prediction, replication, recombination, repair, and transcription. The down-regulated genes prevented the purine nucleotide synthesis, thus leading to significant phosphate consumption in yeasts (Pinson et al., 2009). Up-regulated and down-regulated genes strongly confirmed the interactive symbiotic system of *L. paracasei* and *K. marxianus*.

Amino sugar and nucleotide sugar were the important precursors of flavor substances in the fermentation system of rice-acid. The abundance of amino sugar and nucleotide sugar could affect the glycan complexity and the final glycosylation profile (Naik et al., 2018). The comparison results of different inoculation methods indicated that the down-regulated genes were less than the up-regulated genes. This result suggested that these up-regulated genes promoted the fermentation process of rice-acid in the interaction between *L. paracasei* H4-11 and *K. marxianus* L1-1. In addition, a recent study demonstrated that amino sugar and nucleotide sugar metabolism played the important role in the interactions between *L. sanfranciscensis* and *S. cerevisiae* (Yang et al., 2020). The demonstration was consistent with the interactions between *L. paracasei* and *K. marxianus* in our study.

Starch and sucrose were also the important precursors of flavor substances in the fermentation system of rice-acid. Under the action of enzymes, starch and sucrose were hydrolyzed and converted into acids and other taste components (Li et al., 2020). Interestingly, the comparison results of different inoculation methods indicated that the gene encoding UTP–glucose-1-phosphate uridylyltransferase (LSEI_1093) was up-regulated. This gene (LSEI_1093) played a crucial role in the mixed inoculation. The genes encoding alpha-glucosidase (LSEI_0980 and LSEI_2102), maltose phosphorylase (LSEI_0982), and beta-phosphoglucomutase (LSEI_0983) in the group of the mixed inoculation showed lower mRNA levels than those in the group of the single inoculation, indicating that the three genes exhibited negative effects on the mixed inoculation in rice-acid. Those genes might be associated with the decrease in bitterness intensity and astringency intensity in the group of mixed inoculation compared to the group of single inoculation of *L. paracasei*.

Glycolysis is a central and important metabolic pathway and can provide energy, reducing power, and pyruvate to fuel the TCA cycle, and precursors for amino acid, fatty acid, and secondary metabolite biosynthesis (Plaxton, 1996). The key glycolysis genes discussed in the study included up-regulated and down-regulated genes during the fermentation period from the organic acid formation stage to the taste substance maturation and ripening stages. Different gene states resulted in different taste substances in rice-acid with single inoculation or mixed inoculation during the fermentation process. The up-regulation of glycolytic genes in the exponential phase could provide the energy for the rapid growth of strains (Qiao et al., 2019). Glycolysis is the important metabolic pathway, which is related to the formation of L-lactic acid. Up-regulation of L-lactate dehydrogenase indicated that L-lactic acid was mainly produced in the early fermentation phase for energy production. The down-regulation of the genes encoding pyruvate kinase (LSEI_1365) and 6-phosphofructokinase (LSEI_1364) indicated that L-lactic acid was stable in the late fermentation stage. The different results for LSEI_2549 could be caused by the post-transcriptional regulation. Consistently, the genes encoding l-lactate dehyrogenase (LSEI_2549) and 6-phosphofructokinase (LSEI_1364) had been identified to promote bacterial adhesion to mucin and epithelial cells (Izquierdo et al., 2009). We could infer that mixed inoculation with *L. paracasei* H4-11 and *K. marxianus* L1-1 had an interactive symbiotic relationship, as reported in the previous study (Ishii et al., 1999). Therefore, the key genes could affect the content of L-lactic acid, sourness, and richness in the fermentation process of rice-acid.

The pyruvate metabolism contributes to the production of α-keto acids, which is the prerequisite for the synthesis of more flavor substances in yeasts (Sun et al., 2019). Yeasts play an important role in rice-acid fermentation. Therefore,

L. paracasei H4-11 and *K. marxianus* L1-1 strains were inoculated in rice-acid. In our study, the genes encoding acetate kinase (LSEI_0166), pyruvate oxidase (LSEI_1784), L-lactate dehyrogenase (LSEI_2549), and malate dehydrogenase (LSEI_2866) showed the up-regulation trend. A recent report proved that the genes (L-lactate dehydrogenase and pyruvate kinase) had been identified to promote L-lactic acid formation (Yao et al., 2019), so the DEGs in pyruvate metabolism strongly proved that *L. paracasei* and *K. marxianus* had a symbiotic relationship. Thus, the metabolic pathways of pyruvate were also the decisive factor in the formation of flavor compounds in the group of mixed inoculation. Interestingly, the previous study proved that in addition to the transformation from EMP to PKP, more redox-related metabolic reactions were affected by the co-cultivation of LAB and yeasts which were involved in pyruvate metabolism (Xu et al., 2019). We will further explore the antioxidant mechanism of rice-acid with mixed inoculation.

TCA cycle is a series of chemical reactions for the oxidation of acetyl-CoA derived from carbohydrates, fats, and proteins to provide energy. TCA cycle-related metabolites (malic acid and other organic acids) act as energy storage materials during the fermentation process of rice-acid inoculated with *L. paracasei*. Functionally, carbohydrate metabolism influences a number of processes including glycolysis, TCA cycle, and hexose metabolism, and may be associated with the expansion of *L. paracasei* in rice-acid fermentation as well as the generation of energy (Mendes Ferreira and Mendes-Faia, 2020). Furthermore, dihydrolipoamide dehydrogenase (LSEI_1446) is a crucial enzyme in the fermentation process and may be related to the formation of taste substances. Notably, dihydrolipoamide dehydrogenase (LSEI_1446) is related to the antioxidant function and it is a redox enzyme involved in decarboxylation of pyruvate to form acetyl-CoA in the cascade of glucose metabolism and mitochondrial adenine triphosphate (ATP) production (Yang et al., 2019). The genes involved in the TCA cycle and related to amino acids (the precursor of taste substances) included LSEI_0322, LSEI_1307, LSEI_1308, and LSEI_1820 and showed different expression in the four comparisons. The result indicated that mixed inoculation had a significant effect on the TCA cycle in *L. paracasei*. The function of up-regulated genes might be responsible for the differences in taste substances, including the substances of sourness, and other taste characteristics measured with E-tongues.

The amino acid metabolism pathways showed the close relationship with taste substances in fermented foods (Yu et al., 2015; Zhao et al., 2016; Hu et al., 2020). Arginine and proline metabolism, arginine biosynthesis, and tyrosine metabolism had a positive effect on the formation of umami and bitterness. Histidine metabolism had a positive effect on the formation of saltiness. Cysteine and methionine metabolism had a positive effect on the formation of astringency and saltiness. Lysine biosynthesis had a positive effect on bitterness and richness in the fermentation process. Alanine, aspartate, and glutamate metabolism in the group of mixed inoculation led to a higher sourness value than that in the group of single inoculation. Alanine, aspartate, and glutamate metabolites might be the precursors of L-lactic acid. These pathways verified the results of taste substances measured with E-tongue. The different pathways contributed to taste substances generated in the rice-acid fermentation process in the group of single inoculation of *L. paracasei* and the group of mixed inoculation. Furthermore, most amino acids promoted the formation of bitterness. The structural requirements for ACE-inhibitory activity were related to the structural characteristics of bitter peptides and many bitter dipeptides showed the ACE-inhibitory activity (Pripp and Ardö, 2007). These results of amino acid metabolism reasonably demonstrated the positive effects of the symbiotic system composed of two strains (*L. paracasei* H4-11 and *K. marxianus* L1-1) on the formation of taste characteristics in rice-acid.

The genes related to the metabolism of some amino acids showed higher relative mRNA levels, including alanine, aspartate, glutamate, arginine, and proline metabolism, which had a crucial effect on the formation of taste substances. Especially, the genes encoding fumarate reductase flavoprotein subunit (LSEI_0322), alcohol dehydrogenase (LSEI_0775), and glycine ligase (LSEI_1746) showed a significant and positive correlation with the umami intensity. The genes encoding the PTS system, mannose-specific IIB component (LSEI_0401), and large subunit ribosomal protein L7/L12 (LSEI_2272) showed the significant and negative correlation with astringency intensity. The gene encoding L-lactate dehyrogenase (LSEI_2549) showed the significant and negative correlation with bitterness intensity, indicating the correlation with the formation of amino acids. Moreover, the produced amino acids were converted into corresponding α-keto acids by aminotransferases. The conversion of amino acids to α-keto acids is the first step in the formation of taste substances (Pangallo et al., 2019). Branched-chain amino acids are crucial precursors of protein synthesis. Leucine plays an important role in the regulation of intracellular signal transduction for the control of mRNA translation (Liu et al., 2014). Transamination of leucine is associated with the production of 3-methylbutanoic acid, and serine deamination may supply pyruvate, which can be subsequently converted into acetic acid, 2,3-butanedione, and acetoin (Wang et al., 2020). Amino acid metabolism is not only the starting compound for the synthesis of various metabolites such as glycine, cysteine, and serine phospholipids but also building blocks for protein synthesis (Shimizu et al., 2008). Moreover, most amino acids are crucial metabolites that play an important role in the growth and protein synthesis of *L. paracasei* in the rice-acid fermentation process.

CONCLUSION

In summary, the study provides a comprehensive overview of the global changes at the transcription level in the growth of *L. paracasei* in the rice-acid fermentation process. Most of the observed growth phase-dependent changes at the mRNA level appeared in the early stage and the mRNA levels of relevant genes became stable in the later stage. In particular, the genes related to amino sugar and nucleotide sugar metabolism and starch and sucrose metabolism affected the energy required for the growth of *L. paracasei* in the early stage. Many transcripts related to glycolysis, pyruvate metabolism, TCA cycle, and amino acid biosynthesis and metabolism were differentially expressed

in the growth of *L. paracasei* in the presence of *K. marxianus*. The differential expressions of the genes related to the synthesis of L-lactic acid and other organic acids and taste characteristics were observed in the whole growth period. The DEGs of various metabolic pathways revealed the formation mechanism of L-lactic acid. The study provides the theoretical basis for the industrial applications of the symbiotic system of LAB and yeasts.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: BioProject accession PRJNA658256.

AUTHOR CONTRIBUTIONS

NL performed the experiments, analyzed the data, and prepared the manuscript. LQ and SM contributed to the experimental design, manuscript revision, and overall support of this study. All authors read and approved the final 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.2021.594631/full#supplementary-material

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