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RESEARCH

Screening of Lactic Acid Bacteria Strains to Improve the Properties of Non-fat Set Yogurt by *in situ* EPS Production

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Abstract

Some lactic acid bacteria (LAB) are capable of producing exopolysaccharides (EPS), which can be used in the dairy industry to reduce syneresis and improve the viscosity and texture of fermented products. The aim of the present study was to screen the EPS-producing capacity of 123 LAB strains isolated from fermented foods to search for those able to produce EPS with the optimal technological aptitude to be applied in non-fat set yogurt manufacture. They were grown on MRS-sucrose and assigned to ropy or mucoid phenotype depending on the appearance of the colonies. Twenty-five of them were selected and assayed for production of both cell-bound EPS (EPS-b) and EPS released to the medium (EPS-r). Those resulting in the most EPS producers (*Levilactobacillus brevis* UCLM-Lb47, *Leuconostoc mesenteroides* subsp. *mesenteroides* 6F6-12 and *Leuconostoc mesenteroides* subsp. *mesenteroides* 2F6-9) were used to manufacture non-fat set yogurts. These yogurts were analyzed for microbiological and physicochemical properties (pH, titratable acidity, total solids), water-holding capacity, apparent viscosity, and sensory characteristics during a 28-day cold storage period. The yogurts made with the selected strains showed higher values of water-holding capacity, EPS concentration, and viscosity in the mouth than the control yogurt, which presented a more fluid texture. The results obtained suggest that the three selected strains could be used to replace hydrocolloids in non-fat set yogurt formulation, obtaining a clean-label product that would improve consumer acceptance.

Keywords Exopolysaccharides · Fermented milk product · LAB strains · *Leuconostoc* · Lactobacilli

Introduction

LAB have a long history of use in the food industry, both as starter cultures and as producers of compounds of interest like short-chain fatty acids, amines, bacteriocins, gamma aminobutyric acid, vitamins, and exopolysaccharides (EPS), among others (Wang et al., 2021).

The ability of LAB to produce EPS and the amount of EPS are largely species- and strain-specific (Prete et al., 2021), and it is therefore necessary to carry out a thorough screening in order to select the strains with the highest potential for the intended use. Ruas-Madiedo and de los Reyes-Gavilán (2005) reported that the nomenclature used to describe the different EPS-producing phenotypes of LAB was confusing, and they described the mucoid colonies as those having a glistening and slimy appearance on agar plates and being unable to produce strands when extended with an inoculation loop, whereas the ropy colonies form a long filament by this method. Another possible phenotype is that of the ropy/mucoid colonies, as reported by Bachtarzi et al. (2019). Some authors (Milanović et al., 2020; Zarour et al., 2018) have reported that EPS production has an inducible character, being affected by the pH and the composition of the growth medium (especially by the carbon and nitrogen sources added) and by the conditions (temperature and time) used for incubation. Palomba et al. (2012), comparing growth media supplemented with different carbohydrates, demonstrated that those supplemented with sucrose were the most suitable for screening EPS-producing LAB. EPS are classified as homoexopolysaccharides (HoPS) and heteroexopolysaccharides (HeEPS), depending on their chemical composition. Most of the EPS produced by LAB belongs to the HePS group.

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EPS produced by LAB have received special attention as safe and functional additives in the food industry (Peng et al., 2020; Sanalibaba & Çakmak, 2016; Tiwari et al., 2021). While HePS has been described as playing an important role in the rheology, texture, and "mouthfeel" of fermented milks and other fermented products, HoPS are mainly used for fermentation of non-dairy products (Notararigo et al., 2013). In addition to these technological benefits, EPS can also be considered prebiotics, and fermentation of foods with EPS-producing LAB strains that have probiotic and/or health-promoting properties (looking for a symbiotic effect), may provide additional benefits for consumers, such as antitumor, anti-ulcer, immunomodulatory, or cholesterol-lowering activities (Al-Dhaheri et al., 2017; Caggianiello et al., 2016; Mishra & Mishra, 2013; Saadat et al., 2019).

The use of potentially EPS-producing LAB strains for the manufacture of fermented products in order to improve their characteristics has been studied (Di Monaco et al., 2015; Mishra & Mishra, 2013). When added to foods, EPS act as pseudoplastics. They interact with milk proteins, enhancing those proteins' hydration levels while exerting a thickening effect by increasing the viscosity of the serum (Behare et al., 2009; Caggianiello et al., 2016). Therefore, they have been widely used in the dairy industry as thickeners, emulsifiers, and stabilizers to reduce the syneresis and to improve the viscosity, texture, and structure of the fermented products (Mende et al., 2016). Likewise, EPS improve fat- and water-retention capacity, providing smoother and creamier products without an undesirable mouthfeel or taste (Han et al., 2016). Their role in the manufacture of light dairy products, where they help to avoid texture and functionality defects that occur as a result of fat reduction, has also been revealed (Behare et al., 2009; Patel et al., 2012).

The pseudoplastic properties of EPS make them an attractive alternative to the addition of hydrocolloids or milk proteins both from the consumer's point of view, by allowing a "clean label", and from the producer's, by eliminating costly ingredients (Al-Dhaheri et al., 2017). In this respect, it is important to highlight that purified EPS are seldom used as direct food additives due to their low yield; instead, EPS produced *in situ* are often applied (Xu et al., 2019).

Therefore, the screening for new EPS-producing LAB strains from different origins is an interesting strategy to find those that can be best adapted for the fermented foods to be manufactured (Ale et al., 2016). In recent years, many efforts have been made to select LAB and bifidobacteria with high EPS yields to be applied in yogurt manufacture (Xu et al., 2019). However, in most of these studies, sucrose is added to milk for the production of EPS. The aim of the present study was to screen a collection of LAB isolates from fermented foods to search for those able to produce EPS with the optimal technological aptitude to be applied in non-fat set yogurt manufacture. It is noteworthy that a high number of strains from different origins, belonging to many genera and species of LAB, were screened as part of the study. This was one of the very few studies that used EPS-producer *Leuconostoc* strains for the manufacture of yogurt and, as far as we know, the first one that did not add sucrose to the milk for yogurt manufacture to promote *in situ* production of EPS.

Materials and Methods

LAB Strains and Growth Conditions

A total number of 123 LAB strains were assayed (Table 1). They had been isolated from spontaneously fermented foods (cheese, wine and Almagro eggplants), the air of a cellar, and beer, and identified in previous studies (Nieto-Arribas et al., 2009, 2010, 2011; Pérez-Martín et al., 2014; Ruiz et al., 2010, 2018; Seseña et al., 2004; Sánchez et al., 2005). They belonged to the species *Lactobacillus acidophilus* (3), *Lactobacillus delbrueckii* (1), *Lactiplantibacillus plantarum* (26), *Lactiplantibacillus paraplantarum* (1), *Levilactobacillus brevis* (10), *Lacticaseibacillus paracasei* (5), *Lactococcus* (*Lc.*) *lactis* subsp. *lactis* (21), *Lc. lactis* subsp. *cremoris* (4), *Leuconostoc* (*Ln.*) *mesenteroides* subsp. *dextranicum* (19), *Ln. mesenteroides* subsp. *mesenteroides* (29), *Ln. lactis* (1), and *Weissella* (*W.*) *paramesenteroides* (3).

Strains were kept frozen (−80 ºC) with 20% (v/v) glycerol, and prior to use, they were activated by cultivation in MRS broth (Pronadisa, Madrid, Spain) and incubated aerobically at 30 ºC.

Screening for EPS-producing Strains

First, an assay to determine the EPS-production capacity of the strains and their phenotype was carried out. Five microliters of an overnight culture of the LAB strains were streaked on MRS agar plates supplemented with 2% (w/v) sucrose (MRS-S) and incubated in the conditions mentioned above.

After the incubation period, the colonies were assigned to a ropy phenotype (R) if an unbreakable strand was observed when the streak was touched with a toothpick. Measurement using a rule of the length of the strand before breakage was used for classification of the strains: R^{+}: strand length between 1–10 mm; R^{2+}: 11–20 mm; R^{3+}: 21–30 mm; R^{4+}: 31–40 mm; R^{5+}: ≥ 40 mm. Strains were classified as mucoid (M) when the streak had a glistening, smooth and slimy appearance, without the occurrence of filament.

Following this assay, and in order to confirm the nature of the polymers produced, a modified version of

Table 1 LAB strains assayed and their origin

Species	Strain	Origin
Lactobacillus acidophilus	UCLM-Lb91; UCLM-Lb104; UCLM- Lb116	Almagro eggplants
Lactobacillus delbrueckii	UCLM- Lb32	Almagro eggplants
Lactiplantibacillus plantarum	UCLM-Lb33; UCLM-Lb34; UCLM-Lb36; UCLM-Lb37	Manchego cheese
	UCLM-Lb43; UCLM-Lb44	Wine
	UCLM-Lb53; UCLM-Lb54; UCLM-Lb56	Air from a cellar
	UCLM-Lb65; UCLM-Lb70; UCLM-Lb71; UCLM-Lb72; UCLM-Lb73; UCLM-Lb75; UCLM-Lb76; UCLM-Lb77; UCLM-Lb79; UCLM-Lb88; UCLM-Lb90; UCLM-Lb93; UCLM-Lb106; UCLM-Lb107; UCLM-Lb108; UCLM-Lb 112; UCLM-Lb114	Almagro eggplants
Lactiplantibacillus paraplantarum	UCLM-Lb74	Almagro eggplants
Levilactobacillus brevis	UCLM-Lb27; UCLM-Lb28; UCLM-Lb61; UCLM-Lb62; UCLM-Lb86; UCLM-Lb99; UCLM-Lb100; UCLM-Lb105; UCLM-Lb111	Almagro eggplants
	UCLM-Lb47	Wine
Lacticaseibacillus paracasei	UCLM-Lb5; UCLM-Lb24; UCLM-Lb29	Goat cheese
	UCLM-Lb38; UCLM-Lb41	Manchego cheese
Lactococcus lactis subsp. *lactis*	NB2MM3; NB4MM4; CB2SM2; NA1MM3; NB2SM1; CB2MM3; CB1MM4; CA0M5; NB4MM2; CA1MM5; CA2SM5; CB1SM3; CA0M4; NA2MM1; NA4MM4; NB2MM2; NB4MM3; NA4MM1; NA0M2; NB4MM1; NB1SM3	Manchego cheese
Lactococcus lactis subsp. *cremoris*	NA1SM2; CA0M1; NA4MM3; NB0M3	Manchego cheese
Leuconostoc mesenteroides subsp. *dextranicum*	C24W1; C16W5; C8W1; CM1; C4W1; C4W2; C4W3; C8W3; C8W4; C8W5; N2W3; N32W1; N0W3; N2W1; N2W4; N2W5; N24W2; N16W1; N16W2	Manchego cheese
Leuconostoc mesenteroides subsp. *mesenteroides*	1F7-18; 2F6-9; 3F7-14; 4F10-11; 5F7-13; 6F6-12; 7F2-11; 8F2-5; 9F2-3; 10F10-3; 11F2-13; 12F3-15; 13F4-15; 14F3-9; 15F4-9; 16F6-3; 17F4-5; 18F4-3; 19F4-12; 20F12-12; 21B1-18; 22BU-18; 23BU-9; 24B1-3; 25BU-814; 26F12-9; 27B1-12; 28B1-9; 29F8-13	Beer
Leuconostoc lactis	C0W2	Manchego cheese
Weissella paramesenteroides	C16W1; C16W3; N8W1	Manchego cheese

the procedure described by Sánchez (2005) was followed. Briefly, each strain was grown in quadruplicate in 10 mL of MRS-S broth and incubated at 30 ºC for 48 h. Cultures were centrifuged at 6,000 × *g* (in a fixed angle rotor 24 place, Universal 320/320 R Hettich centrifuge, Merck, Darmstadt, Germany) at 30 ºC for 10 min and the pellet washed with Ringer solution (¼) (Oxoid Ltd., Basingstoke, Hampshire, UK). Three of the pellets were resuspended in 5 mL of the following solutions: SDS (1% w/v) (Panreac, Barcelona, Spain), NaOH (0.05 M) (Panreac), and proteinase K (1.5 mg/mL) (Sigma-Aldrich, Madrid, Spain), respectively. Each of these suspensions was then incubated for 30 min at 45 ºC, 20 ºC and 50 ºC, respectively. The remaining pellet was resuspended in 5 mL of Ringer solution (¼) and used as a control. After incubation, the suspensions were again centrifuged (6,000 × *g*/30 ºC/10 min) and the appearance of the sediment was visually observed and compared with that of the control. All the assays were performed in duplicate.

Extraction, Purification, and Quantification of the EPS

Revitalized strains were inoculated (1%) in a semi-defined medium (SDM) (Vaningelgem et al., 2004) supplemented with 2% (w/v) sucrose, as reported by Sánchez (2005). The composition of the SDM was as follows: Tween 80 1 g/L; ammonium citrate 2 g/L; sodium acetate 5 g/L; $MgSO_4 \times 7H_2O$ 0.1 g/L; $MnSO_4$ 0.05 g/L; K_2HPO_4 2 g/L; yeast nitrogen base 5 g/L; tryptone 10 g/L. Cultures were incubated at 30 ºC for 48 h.

Both the EPS bound to the cells (EPS-b) and the EPS released to the medium (EPS-r) were extracted from these cultures, following the procedure described by Tallon et al. (2003) (Fig. 1), and dialyzed against distilled water for 48 h at 4 ºC, with 3 water changes per day. An acetate of cellulose membrane (D9277-100FT) (Sigma-Aldrich) (Molecular weight cut-off 12,000–14,000 Da), previously treated as recommended by the manufacturer, was used for the dialysis.

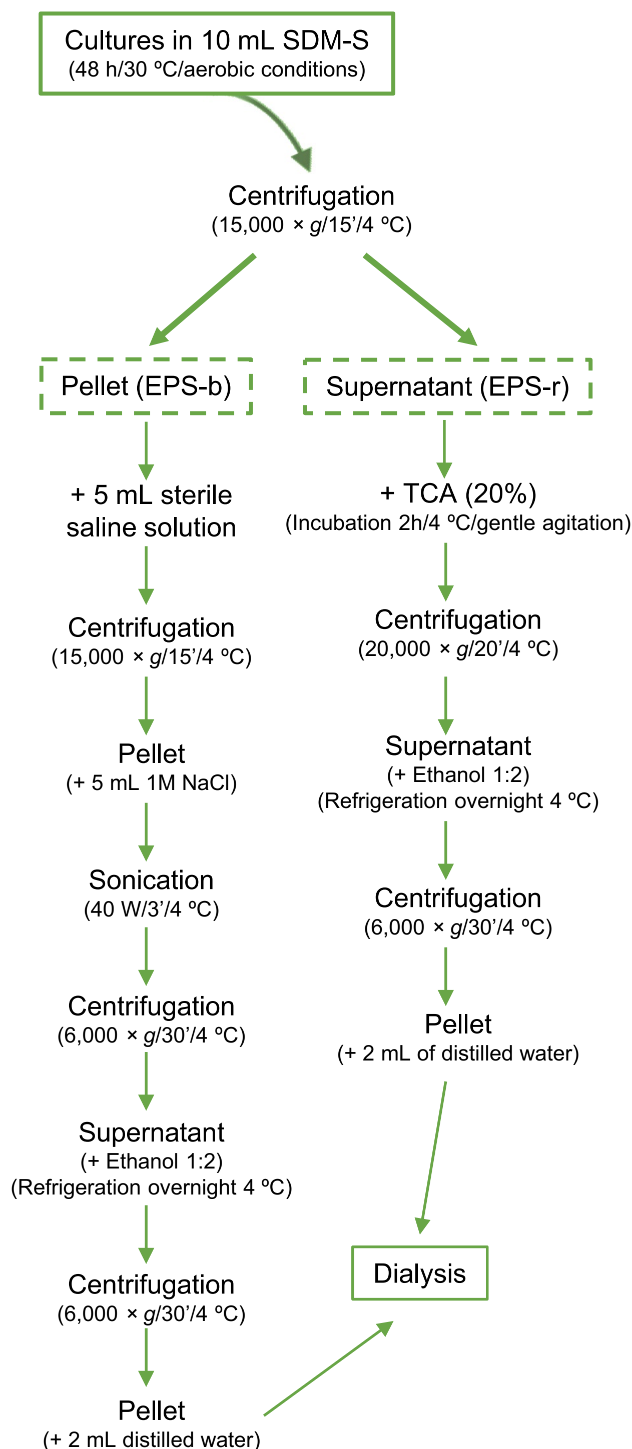

Fig. 1 Diagram of the procedure followed for the extraction of the EPS

After dialysis, samples inside the membranes were used for the EPS quantification assay, using the method of López-Legarda et al. (2017). For this purpose, 0.3 mL of each sample was mixed with 1 mL of sulphuric acid (Panreac) and the mixture was cooled by immersion in an ice bath for 2 min before the absorbance at 315 nm was read using a spectrophotometer (UV-1600PC, VWR, USA). Samples were assayed in duplicate. Results were expressed in mg equivalent of glucose per liter of growth medium. Total EPS concentration was calculated as the sum of the EPS-b and EPS-r concentrations.

Activation of the LAB Strains for Yogurt Manufacture

The strains *L. brevis* UCLM-Lb47, *Ln. mesenteroides* subsp. *mesenteroides* 6F6-12, and *Ln. mesenteroides* subsp. *mesenteroides* 2F6-9 were used for yogurt manufacture. For that purpose, they were grown twice in MRS broth inoculated at 10% (v/v), and the cultures incubated at 30 ºC for 24 h. Next, six passages in skimmed UHT milk (Hacendado, Spain) were carried out using the same conditions for the incubation. Cell population in the cultures reached at least 8 log CFU/mL.

In addition, a commercial starter culture (CS) (Ferlac yogurt Type I, Abiasa, S.L., Pontevedra, Spain) containing the species *Streptococcus (S.) thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* (1:1) was used. It was reconstituted in skimmed UHT milk (0.005%, w/v), following the manufacturer's instructions, and incubated for 16 h at 37 ºC to reach a cell population of at least 6 log CFU/mL.

Yogurt Manufacture

Four different yogurts (YC, Y1, Y2, and Y3) were manufactured using skimmed cow's milk (Hacendado) to which 2% skim milk powder was added to increase protein content and dry matter, as is usual in the industrial manufacture of yogurt. The milk composition (g/100 mL) was as follows: 5.2 g protein, 0.5 g fat, and 4.7 g lactose.

Prior to use, the milk was pasteurized by heating for 10 min at 90 ºC and then cooled to 42 ºC before inoculation. For each yogurt, 2 L of milk were inoculated with the corresponding culture (Table 2) and were dosed into sterile and transparent plastic 50 mL-cups (for microbiological, physicochemical, and sensory analyses) and 100-mL cups (for viscosity measurements) and incubated at 41.5 ± 0.5 ºC in a controlled temperature incubator. During incubation, the coagulation of milk was monitored for pH measurement, and when it reached a value of 4.50 ± 0.05, the yogurts were cooled and stored at 4.0 ± 0.5 ºC for 28 days. Yogurt manufacture was repeated one week later.

Physicochemical, microbiological, and sensory analyses of the yogurts were conducted at different times during the 28-day refrigerated storage period. All the analyses were performed in triplicate, except the sensory analysis, which was carried out in duplicate.

Table 2 Cultures and percentage of inoculum (%, v/v) used in the yogurt manufacture

Yogurt	Inoculated strains
YC	Commercial Starter Culture (CS) (1%)
Y1	*Levilactobacillus brevis* UCLM-Lb47 (2%) + CS (1%)
Y2	*Leuconostoc mesenteroides* subsp. *mesenteroides* 2F6-9 (2%) + CS (1%)
Y3	*Leuconostoc mesenteroides* subsp. *mesenteroides* 6F6-12 (2%) + CS (1%)

Fermentation Kinetics

During milk fermentation, the pH changes were initially monitored at 1-h intervals, and once the pH was close to pH 5.0 they were measured every 30 min, using a pH-meter (Crison, Barcelona, Spain). Maximum acidification rates (V_m), expressed in absolute values (m unit pH/min), were calculated from the pH-time curves using the following equation:

$$Vm = \left(\frac{dpH}{dt}\right)max$$

In addition, responses that characterized the kinetics of the process were calculated: T_m, the time at which the maximum rate of acidification was observed and T_e, the time at which pH 4.5 was reached.

Microbiological Analysis

For the determination of the cell population of *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus,* both from the CS, the pour plate method was used and serial dilutions of the samples were spread onto MRS agar (pH = 5.42, adjusted with HCl 0.1 M) or M17 agar (Scharlab, Barcelona, Spain) enriched in lactose (10%) (ISO, 2003), respectively. Both cultures were incubated for 72 h at 37 ºC (ISO, 2003).

To count the population of the *Levilactobacillus* and *Leuconostoc* strains added to the yogurts Y1, Y2 and Y3, serial dilutions of the samples were spread onto MRS agar or MSE agar (Condalab, Madrid, Spain), both with 1 mg/L vancomycin added (Fluka, Thermo Fisher Scientific, Madrid, Spain), respectively. Results in previous studies in our lab (Ramos et al., 2022; Rodríguez-Sánchez et al., 2021) had shown that these strains were vancomycin-resistant, and therefore addition of vancomycin to these media would avoid growth of the strains of the CS, which are vancomycin-sensitive (Coeuret et al., 2004). Plates were incubated at 30 ºC for 72 h. The results were provided as log CFU/mL.

Physicochemical Analysis

The pH was measured by using a digital pH-meter (Crison, Barcelona, Spain). Total solids (TS), determined gravimetrically after oven drying, and titratable acidity (TA) were determined according to the methods of the AOAC (2023).

Water-holding Capacity and Spontaneous Syneresis

The water-holding capacity (WHC) of yogurts was determined after centrifugation (in a JA-14 rotor, Avanti J-26 XP centrifuge, Beckman Coulter, Indianapolis, USA) of the samples at 5,000 × *g* at 4 ºC for 20 min (Chen et al., 2018). For this purpose, 20 g of yogurt sample (processed directly in a 50 mL centrifuge tube) were used. The WHC was calculated using the following equation:

$$WHC = \frac{W}{Wo} \times 100,$$

where W is the weight of the residue after centrifugation and W_o is the weight of the sample.

The extent of spontaneous syneresis was determined as the weight percentage of free whey in the total yogurt weight immediately after fermentation (Li et al., 2021).

Apparent Viscosity

A volume of 100 mL of yogurt, previously cooled to a temperature of 4 ºC by introducing it in a water/ice bath, was used. Samples were manually stirred clockwise for 60 s before taking the measurements (Mohamed Ahmed et al., 2021).

The apparent viscosity of the yogurts was measured as reported by Ramos and Poveda (2022), using a Brookfield digital rotational viscometer (model DV-II +, Brookfield Engineering Laboratories Inc., Middleboro, MA, USA) using a spindle 64 at a rotational speed of 30 rpm. The apparent viscosity reading in centipoises (cP) was taken at the 30th second, and torque was always maintained between 10 and 100%.

Analysis of the EPS Production in the Yogurts

The determination of the total EPS content in the yogurt samples was carried out according to the method proposed by Düven et al. (2021). Thirty grams of yogurt were centrifuged at 11,000 × *g* at 4 ºC for 4 min, and the supernatant was collected and mixed with ethanol (1:2). The mixture was maintained at 4 ºC overnight and then centrifuged at 2,000 × *g* at 4 ºC for 15 min. The supernatant was removed and a volume of 10 mL of distilled water was added to dissolve the precipitate. Then, 250 mL of trichloroacetic acid (80%) were added to precipitate the remaining proteins, and the mixture was maintained overnight at 4 ºC. The sample was again centrifuged (2,000 × *g*/4 ºC/15 min) and the supernatant collected. Finally, the EPS in the supernatant were collected using ethanol precipitation and cold storage, as described above, and the whole procedure for EPS purification, using water and trichloroacetic acid, was repeated once more. After that, the EPS were dried at 55 ºC in a rotary evaporator and weighed. The results were expressed as mg of crude EPS/kg of yogurt.

Sensory Analysis

Quantitative descriptive analysis (QDA) was run for the yogurts' appearance, odor, flavor, and texture attributes (ISO, 2016), following the procedure described in Ramos and Poveda (2022). A homogeneous panel of eight assessors aged between 24 and 60 years from the university staff was used. They had previously been trained following the ISO (2012), and all had experience in sensory analysis of dairy products and were familiar with the sensory attributes of yogurt. The evaluated attributes were selected by consensus from a list freely generated by the tasters after they had tasted several yogurts. The attributes selected were curd firmness and whey quantity (visual phase); yogurt odor, odor intensity, and odor quality (olfactive phase); acid flavor, yogurt flavor, flavor intensity, and flavor quality (taste phase), and viscosity in mouth. All samples were tasted in duplicate in two different sessions in a room equipped according to the requirements of the standard ISO (2007).

Statistical Analysis

The one-way analysis of variance (ANOVA) was applied using the Tukey test for comparison of the means ($P < 0.05$). Normality tests, heteroscedasticity, and the independence of samples had been previously checked. Principal component analysis (PCA) was applied to the yogurt results using the correlation matrix and Varimax rotation. These statistical analyses were performed using the IBM SPSS statistics package version 24.0 (SPSS Inc., Chicago, IL, USA).

Results and Discussion

EPS-producing Strains and Nature of the Polymers Produced

Results of growth on MRS-S displayed that 61.8% of the strains (76) were able to produce EPS, with 43.4% of them showing a ropy phenotype and the remaining ones a mucoid phenotype. Of those with ropy phenotype, 48.5% were R^{+}, 9.1% were R^{2+}, 15.1% were R^{3+}, 18.2% were R^{4+}, and the remaining were R^{5+}. These values are much higher than those obtained by Milanović et al. (2020), who reported that only 18% of the LAB strains assayed were able to produce EPS when screened cereal-sourced LAB, belonging to the genera *Lactobacillus*, *Weissella*, *Pediococcus*, *Leuconostoc*, and *Enterococcus.* Palomba et al. (2012) also reported lower values for LAB of the genera *Leuconostoc*, *Weissella*, *Lactobacillus*, *Lactococcus*, and *Enterococcus* used in sourdough for sweet products and pizza.

When the results for each of the genera were analyzed (Fig. 2), different percentages of EPS production were obtained, ranging between 25% for *Lactobacillus* genus and 100% for *Weissella* genus (data not shown). However, it is important to highlight that the number of assayed strains of each of the genera were also very different, e.g., 49 *Leuconostoc* strains versus 3 *Weissella* strains, and this fact may have had some influence on the results.

Similar percentages were obtained for *Leuconostoc* and *Lactococcus* strains, with 72% and 68%, respectively, being EPS-producing strains. However, while all those of the *Leuconostoc* genus showed a mucoid phenotype, only 4% of those of the *Lactococcus* genus did.

With respect to the *Leuconostoc* species (Fig. 2), it is remarkable that all the EPS non-producing *Ln. mesenteroides* subsp. *mesenteroides* strains were from beer. This fact would induce us to think that the origin of the strains has some influence on their capacity to produce EPS, and different opinions have been found in this respect in the literature. Zarour et al. (2018) also reported that *Ln. mesenteroides* strains from dairy products were EPS producers, while those from honey or oat were not. Contrarily, Montersino et al. (2008) reported strains of *Ln. mesenteroides* from musts and wines showing a mucoid phenotype.

Another fact to be highlighted is that the *Ln. mesenteroides* subsp. *mesenteroides* 28B1-9 strain, despite showing a mucoid phenotype, produced colonies with a much lower abundance of polymers than the remaining strains of this

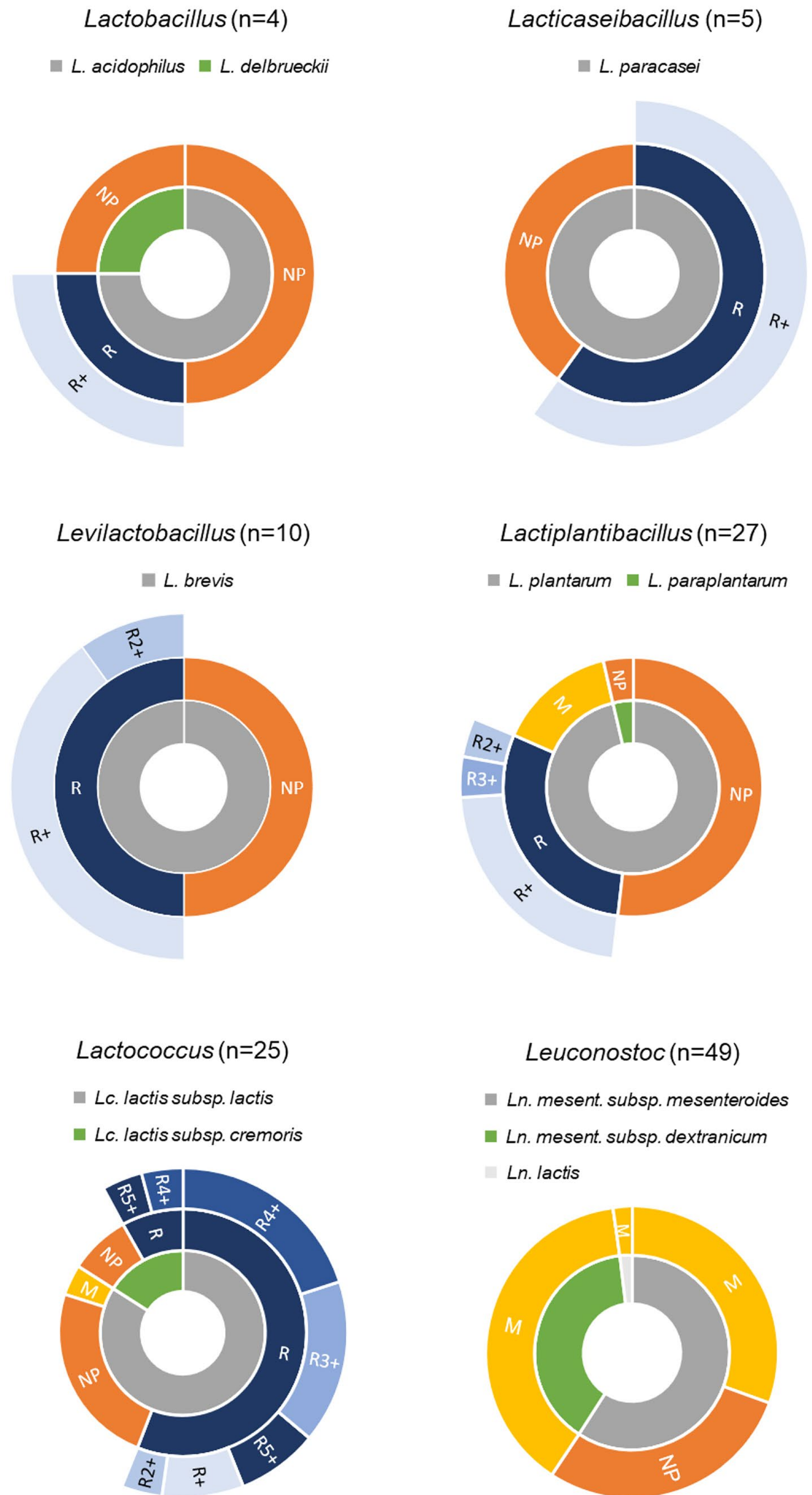

Fig. 2 Percentage of strains of each genus with the different phenotypes. R: Ropy; M: Mucoid; NP: Non-producer. R^{+}: 1–10 mm; R^{2+}: 11–20 mm; R^{3+}: 21- 30 mm; R^{4+}: 31- 40 mm; R^{5}+: ≥ 40 mm

species. Milanović et al. (2020) also reported differences in EPS production for strains of the same species and, therefore, it is possible to affirm that EPS production is a property with an intraspecific character.

Results for both subspecies of *Lc. lactis* were slightly different. As shown in Fig. 2, 50% of the *Lc. lactis* subsp. *cremoris* strains showed a ropy phenotype, a percentage somewhat lower than that for the *Lc. lactis* subsp. *lactis* strains (66.7%). In addition, 4.8% of the strains of *Lc. lactis* subsp. *lactis* showed a mucoid phenotype while none of the strains of *Lc. lactis* subsp. *cremoris* did. Contrary to these findings, Knoshaug et al. (2007) reported the isolation of *Lc. lactis* subsp. *cremoris* strains showing a mucoid phenotype. Percentages of strains of these subspecies with the different ropy phenotypes (R^{+}-R^{5+}) are shown in Fig. 2. It is remarkable that, of all those assayed in this study, the only strains assigned to the phenotypes R^{4+} and R^{5+} belonged to both subspecies of *Lc. lactis*. The strain *Lc. lactis* subsp. *lactis* NA4MM4 produced the longest strand (42.3 ± 2.5 mm) in this study, followed by *Lc. lactis* subsp. *lactis* NB2MM2 (40.3 ± 0.6 mm).

All the EPS-producing strains of the species *L. paracasei, L. brevis* and *L. plantarum* produced ropy colonies, except for 4 strains of *L. plantarum* assigned to the mucoid phenotype. Strains of these species were mainly assigned to the phenotype R^{+}, and only one *L. plantarum* was R^{3+}.

Two of three strains of *L. acidophilus* and all those of *L. paraplantarum* and *L. delbrueckii* were negative for EPS production, though the low number of strains of these species assayed did not allow us to come to any conclusive result. In fact, different authors (Burns et al., 2011; Nikolic et al., 2012) have reported the isolation of strains of these species with a ropy phenotype.

Finally, all three strains of *W. paramesenteroides* produced mucoid colonies (data not shown) as mentioned for the EPS-producing *Leuconostoc* strains. It is well known that *Weissella* genus is under the *Leuconostocaceae* family, and it has been reported that species of this genus yield much higher EPS concentrations than other LAB species (Kavitake et al., 2020).

In the light of these results, the 24 strains that had shown the longest strands or whose colonies had a more mucoid appearance were selected for the following assays. In addition, *Ln. mesenteroides subsp. mesenteroides* 28B1-9 was included because, as mentioned before, its growth on MRS-S agar plates had been clearly different than that of the remaining *Leuconostoc* strains. None of the *Weissella paramesenteroides* strains were selected for the following trials because of the limited use of the species of this genus for food fermentation. The lack of studies related with the safety traits of the *Weissella* species and a handful of reported cases of pathogenicity are the reasons for this fact (Ahmed et al., 2022).

Prior to the assay of EPS quantification, the nature of the polymers produced by the selected strains was analyzed. The pellets obtained after centrifugation of the cultures in MRS-S broth were treated with SDS (1% w/v), NaOH (0.05 M), and proteinase K (1.5 mg/mL). It was observed that only those treated with NaOH suffered changes and that, as reported by Forde and Fitzgerald (1999), the "fluffy" pellet was eliminated. This fact would be attributable to the breaking of the glycosidic linkages, confirming the polysaccharide nature of the polymers produced by all the selected strains.

EPS Quantification

Results from the assay to determine EPS-b, EPS-r and total EPS (sum of EPS-b and EPS-r) concentrations and those of the statistical analysis from the 25 selected strains are shown in Table 3. Total EPS concentrations ranged between 71.3 mg/L and 4940.6 mg/L; differences could be related to the type of polysaccharide produced. In this respect, Badel et al. (2011) and Hamet (2012) reported that homopolysaccharides (HoPS) are frequently produced in higher concentrations than heteropolysaccharides, reaching values of between 1 and 10 g/L. In accordance with this statement, Sanalibaba and Çakmak (2016) reported heteropolysaccharides (HePS) concentrations lower than 600 mg/L, which would be a major drawback if the EPS-producing strains are going to be used industrially.

EPS-r concentration was higher than EPS-b concentration for all the strains except for *L. plantarum* UCLM-Lb90, *L. brevis* UCLM-Lb28, *Ln. mesenteroides* subsp. *dextranicum* C8W4, and *Ln. mesenteroides* subsp. *mesenteroides* 28B1-9. To determine the impact of this fact on the final texture of fermented milk when LAB strains are used for *in situ* production of EPS is really complicated, as it is influenced by many factors, including the nature or the amount of the EPS produced, the acidity or the composition of the milk, the time of fermentation, etc. (Laws & Marshall, 2001). From the results in Table 3, it is important to highlight that *Ln. mesenteroides* subsp. *mesenteroides* 28B1-9 did not produce EPS-r, a fact that explained why colonies from this strain, although mucoid, were different, showing much lower quantities of polymers.

Analysis of the results for the strains of the different genera showed that EPS-b concentration for the strain *L. acidophilus* UCLM-Lb91 and those of *L. plantarum, L. brevis*, and *L. paracasei* species (the latter formerly also belonging to the *Lactobacillus* genus), ranged between 13.2 mg/L and 67.6 mg/L, with *L. plantarum* UCLM-Lb90 being the

Table 3 EPS production (mean ± SD; n = 3) by the selected LAB strains

Species	Strain	EPS production (mg/L)		
		[EPS-b]	[EPS-r]	[Total EPS]*
Lactobacillus acidophilus	UCLM-Lb91	$32.1^{k} \pm 1.2$	$114.2^{k,l} \pm 0.4$	$146.3^{j} \pm 1.6$
Lactiplantibacillus plantarum	UCLM-Lb70	$27.5^{k–m} \pm 0.6$	$72.1^{l–n} \pm 0.2$	$99.6^{j–l} \pm 0.8$
	UCLM-Lb90	$67.6^{h} \pm 0.2$	$57.7^{m,n} \pm 0.4$	$125.3^{j,k} \pm 0.2$
	UCLM-Lb108	$29.9^{k,l} \pm 0.1$	$52.5^{m,n} \pm 0.7$	$82.4^{k,l} \pm 0.8$
	UCLM-Lb112	$13.5^{n} \pm 0.1$	$109.2^{k,l} \pm 2.1$	$122.7^{j,k} \pm 2.2$
	UCLM-Lb114	$13.2^{n} \pm 0.1$	$79.7^{l,m} \pm 0.4$	$92.8^{a,b} \pm 0.3$
Levilactobacillus brevis	UCLM-Lb28	$55.5^{i} \pm 6.4$	$35.7^{n} \pm 0.5$	$91.1^{k,l} \pm 5.9$
	UCLM-Lb47	$27.7^{k–m} \pm 0.7$	$4912.8^{a} \pm 41.7$	$4940.6^{a} \pm 40.0$
	UCLM-Lb61	$25.5^{k–n} \pm 2.1$	$87.6^{l,m} \pm 3.4$	$113.1^{j–l} \pm 5.4$
Lacticaseibacillus paracasei	UCLM-Lb24	$15.9^{m,n} \pm 1.4$	$185.9^{i} \pm 0.4$	$201.8^{i} \pm 1.0$
Lactococcus lactis subsp. *lactis*	NA4MM4	$67.5^{h} \pm 2.2$	$188.3^{i} \pm 2.3$	$255.9^{h} \pm 4.5$
	NB2MM2	$74.6^{h} \pm 0.8$	$146.7^{j,k} \pm 6.2$	$221.3^{h,i} \pm 4.4$
	NB4MM3	$46.7^{j} \pm 0.6$	$78.9^{l,m} \pm 0.8$	$125.6^{j,k} \pm 0.3$
Lactococcus lactis subsp. *cremoris*	NA4MM4	$17.2^{l–n} \pm 2.6$	$112.4^{k,l} \pm 1.5$	$129.6^{j,k} \pm 4.2$
	NB0M3	$24.9^{k–n} \pm 0.6$	$46.5^{m,n} \pm 0.4$	$71.3^{l} \pm 1.1$
Leuconostoc mesenteroides subsp. *dextranicum*	C24W1	$143.4^{g} \pm 1.5$	$262.0^{g} \pm 7.3$	$405.4^{f} \pm 8.7$
	C16W5	$66.1^{h} \pm 4.0$	$161.3^{h,i} \pm 6.1$	$227.4^{h,i} \pm 10.0$
	C8W1	$72.5^{j} \pm 1.4$	$227.2^{h} \pm 11.2$	$299.7^{g} \pm 12.5$
	C4W3	$42.7^{j} \pm 0.9$	$392.5^{f} \pm 5.0$	$435.2^{f} \pm 4.0$
	C8W4	$339.2^{a} \pm 8.7$	$85.3^{l,m} \pm 4.2$	$424.5^{f} \pm 12.8$
Leuconostoc mesenteroides subsp. *mesenteroides*	2F6-9	$215.0^{c} \pm 4.2$	$2704.7^{c} \pm 9.8$	$2919.7^{c} \pm 14.0$
	6F6-12	$176.3^{f} \pm 4.1$	$3064.8^{b} \pm 42.9$	$3241.1^{b} \pm 47.0$
	8F2-5	$242.2^{b} \pm 14.3$	$1981.7^{d} \pm 13.7$	$2223.9^{d} \pm 28.0$
	24B1-3	$185.0^{e} \pm 3.7$	$1109.2^{e} \pm 24.8$	$1294.3^{e} \pm 28.5$
	28B1-9	$201.1^{d} \pm 5.0$	$0.0^{ñ} \pm 0.0$	$201.1^{i} \pm 5.0$

[a–ñ]Means in the same column with different letters indicate significant statistical differences ($P < 0.05$) between strains

*[Total EPS] = [EPS-b] + [EPS-r]

highest-producing strain ($P < 0.05$). It is interesting to note that this strain had been selected, in a previous study at our lab, for its resistance to gastrointestinal conditions, a fact that could be related to the EPS production now revealed, as the EPS would exert a protective effect favoring survival of the strain when the conditions were not optimal for growing, as reported by Ciszek-Lenda (2011). In addition, EPS could be used as a carbon source for the bacteria, acting as a prebiotic (Hussein et al., 2015). EPS produced by *L. plantarum* have been reported to have antitumor, antioxidant and antibiofilm properties, and they are widely used by the food industry as emulsifiers, gelling agents, and stabilizers, as well as to improve the texture in fermented foods (Silva et al., 2019).

It is also remarkable that the strain *L. brevis* UCLM-Lb47 yielded the highest concentration of EPS-r (4.9 g/L), a value significantly different from those of the remaining strains of all the genera assayed. Badel et al. (2011) reported that this species is widely recognized as one of the major EPS producers. However, in our study, only one of the three strains of this species assayed produced such a high concentration of EPS.

In this study, EPS production was once again shown to be an intraspecies trait, in agreement with the results obtained by other authors (Ermis et al., 2020; Milanović et al., 2020; Tallon et al., 2003). Ermis et al. (2020) reported even higher EPS production (between 10 and 35 g/L) by *Lactobacillus brevis* E25 under optimal growth conditions.

For the strains of *Lc. lactis* subspecies, it was observed that the concentrations of EPS-b by *Lc. lactis* subsp. *lactis* were significantly higher than those of *Lc. lactis* subsp. *cremoris*, with values ranging between 46.7 mg/L and 74.6 mg/L. Although somewhat higher than those for EPS-b, EPS-r concentrations for strains of both subspecies were far

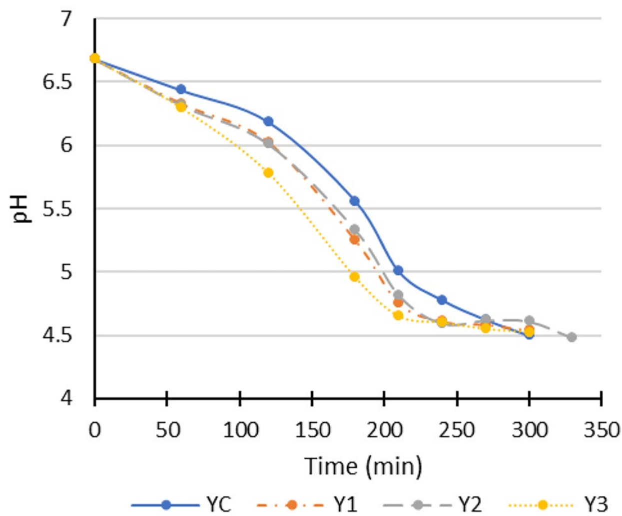

Fig. 3 Acidification profiles of the different yogurts produced

below the value for *L. brevis* UCLM-Lb47. Total EPS concentrations for *Lc. lactis* subsp. *lactis* strains were similar to those reported by Hamet (2012) for strains of the same subspecies, and those for *Lc. lactis* subsp. *cremoris* strains were double the ones reported by Behare et al. (2009).

As described for *Lc. lactis* subspecies, the results for both EPS-b and EPS-r concentrations by *Ln. mesenteroides* strains displayed differences between the subspecies. Thus, the *Ln. mesenteroides* subsp. *mesenteroides* strains yielded statistically higher ($P < 0.05$) concentrations of EPS-r, except for the 28B1-9 strain, which was the only one of the 25 strains assayed that did not produce EPS-r. The highest-producing strains were *Ln. mesenteroides* subsp. *mesenteroides* 6F6-12, followed by *Ln. mesenteroides* subsp. *mesenteroides* 2F6-9, with EPS-r concentrations of 3.06 g/L and 2.70 g/L, respectively. Ruas-Madiedo et al. (2010) reported EPS concentrations for *Ln. mesenteroides* strains of between 300 and 600 mg/L, indicating that EPS production depends on both the strain and the assay conditions.

Attending the results shown in Table 3, the strains *L. brevis* UCLM-Lb47 (isolated from wine), *Ln. mesenteroides* subsp. *mesenteroides* 6F6-12, and *Ln. mesenteroides* subsp. *mesenteroides* 2F6-9 (both from craft beer), which produced the highest concentrations of total EPS, were selected to be used in the yogurt manufacture.

Milk Fermentation Kinetics

The acidification kinetics of the four yogurt samples can be seen in Fig. 3 and in Table 4. A first stage can be observed during the first 60 min, in which the pH does not undergo major alterations and the microorganisms begin to adapt to the medium and the available substrates (Fig. 3). Then, until about two hours into fermentation, there is a slight drop in pH, possibly due to the fact that the lactic acid bacteria are starting to metabolize lactose faster. In the next stage, between 120 and 200 min, the pH decreases at a high rate, coinciding with the maximum performance of the bacteria. Finally, the pH continues to decrease slowly for another 100 min until all yogurts reach pH 4.5.

The experimental yogurts showed higher V_m values than the control yogurt, and among them, Y3 showed the highest value ($P < 0.05$) (Table 4). Y3 showed the lowest T_m value, followed by Y1 and Y2. On the other hand, YC showed the longest time to reach V_m ($P < 0.05$). In general, the acidification kinetics appeared to be affected by the composition of the strains present in the samples, which was in agreement with the results obtained by other authors (Lazaridou et al., 2014; Sözeri Atik et al., 2023). Sözeri Atik et al. (2023) reported V_m between 8.09 and 15.69 m unit pH/min for yogurts fermented with different cultures. These were similar values to those observed in this study, coinciding with a lower rate for the control yogurt. However, in general, T_m (172.50 and 217.50 min) and T_e (165.00 and 368.75 min) values were slightly lower.

On the other hand, the time needed to reach pH 4.5 (T_e) was similar ($P < 0.05$) for all the yogurts, except for Y2, which was higher. This may indicate that the addition of the EPS-producing strains to the experimental yogurts contributes to a faster acidification at the beginning of fermentation, but does not influence the total yogurt manufacturing time. This, in turn, is an advantage because yogurt gels produced with longer fermentation times have been reported to show

Table 4 Kinetic acidification parameters of the different yogurts

Parameters	YC	Y1	Y2	Y3
V_m (m unit pH/min)	$9.5^c \pm 0.1$	$10.4^b \pm 0.0$	$10.1^c \pm 0.0$	$11.3^a \pm 0.0$
T_m (min)	$269.5^a \pm 2.1$	$241.5^b \pm 0.71$	$239.4^b \pm 3.3$	$211.8^c \pm 1.1$
T_e (min)	$301.2^b \pm 1.6$	$306.3^b \pm 0.8$	$321.6^a \pm 0.0$	$303.7^b \pm 4.7$

V_m maximum acidification rate, T_m time to reach V_m, T_e time to reach the end of fermentation (pH 4.5)

$^{a-c}$Means in the same rows with different letters indicate statistically significant differences between the different yogurts ($P < 0.05$)

Table 5 Viable counts (mean values; n = 3) (log CFU/mL) of LAB inoculated in the different yogurts during refrigerated storage at 4 ºC

Species	Yogurt	Storage (days)		
		1	14	28
Streptococcus thermophilus	YC	9.2^{aA}	9.2^{aA}	9.1^{aB}
	Y1	9.2^{aA}	9.2^{aA}	9.1^{aB}
	Y2	9.2^{aA}	9.2^{aA}	9.1^{aB}
	Y3	9.1^{bA}	9.1^{bA}	9.0^{bB}
Lactobacillus bulgaricus	YC	9.3^{aA}	9.2^{aB}	9.2^{aB}
	Y1	6.1^{cA}	6.1^{cA}	6.0^{cB}
	Y2	8.2^{b}	8.2^{b}	8.2^{b}
	Y3	9.2^{aA}	9.1^{aB}	9.1^{aB}
Levilactobacillus brevis UCLM-Lb47	Y1	9.1^{A}	9.0^{B}	9.0^{B}
Leuconostoc mesenteroides subsp. mesenteroides 2F6-9	Y2	9.2^{A}	9.2^{A}	9.1^{B}
Leuconostoc mesenteroides subsp. mesenteroides 6F6-12	Y3	9.2^{A}	$9.1^{A,B}$	9.0^{B}

The absence of a letter indicates that there are no significant differences

$^{a-c}$Means in the same column with different letters indicate statistically significant differences between the different yogurts for each storage time ($P < 0.05$)

$^{A-B}$Means in the same rows with different letters indicate statistically significant differences between the different storage times for each yogurt ($P < 0.05$)

better textural properties than those obtained with shorter times (Peng et al., 2009).

Microbiological Analysis in the Yogurts

Microbial counts using specific culture media were carried out throughout the storage period at 4 ºC to ensure both the presence of the CS strains and the selected strains inoculated, as well as to determine their viability in the experimental yogurts (Table 5).

Viable cell counts of *S. thermophilus* were not significantly different between the different yogurts, except for Y3, which were slightly lower ($P < 0.05$) than the rest. Values for these counts were around 9 log CFU/mL. However, the cell counts for *L. delbrueckii* subsp. *bulgaricus* showed significant differences for the yogurts Y1 and Y2, which were respectively 3 and 1 log units lower ($P < 0.05$) than the rest (Table 5). The results show that the presence of *L. brevis* UCLM-Lb47 and *Ln. mesenteroides* subsp. *mesenteroides* 2F6-9 in these yogurts inhibited the growth of *L. delbrueckii* subsp. *bulgaricus,* with *L. brevis* UCLM-Lb47 strain showing the greatest inhibitory effect. This fact could be attributable to the production of organic acids (propionate, acetate or others), or even bacteriocins, all of them being intraspecific properties, as has been reported (Gu et al., 2021; Rodríguez-Sánchez et al., 2021; Sah et al., 2014). Gu et al. (2021) reported that the autochthonous strain *Lb. paracasei* IMC502 inhibited the growth of *Lb. bulgaricus* in yogurts made with different starter cultures. The counts obtained for the CS strains in the present study were consistent with the minimum microbial population required by legal regulations for yogurt during its shelf life (Codex Alimentarius, 2003).

On the other hand, the cell populations of the strains *L. brevis* UCLM-Lb47, *Ln. mesenteroides* subsp. *mesenteroides* 2F6-9, and *Ln. mesenteroides* subsp. *mesenteroides* 6F6-12 in yogurts Y1, Y2, and Y3, respectively, were similar between them and comparable to those of *S. thermophilus*. These values ensured the presence at high levels of the added selected LAB strains throughout the whole storage period of the yogurts. Counts for all the strains inoculated in the different yogurts decreased slightly ($P < 0.05$) during storage, due to the low temperature (4 ºC), and the decrease in nutrients and pH, conditions that are unfavorable for the LAB growth. These results are in agreement with those obtained by Abd El-Fattah et al. (2018) and Kariyawasam et al. (2021) in fermented cow's milk.

pH and Titratable Acidity

Figure 4 shows the pH and TA values of the different yogurts during the storage period. The pH and TA values of the Y1, Y2, and Y3 yogurts did not differ significantly ($P < 0.05$) from those of the control. On the other hand, the pH values decreased during the storage time, while those of TA increased significantly ($P < 0.05$) for all the yogurts.

These changes in pH and TA could be a consequence of the residual metabolic activity of viable LAB strains, with a continued production of organic acids, especially lactic acid, during storage, although the temperature was not the optimal for the bacterial growth. This behavior is widely

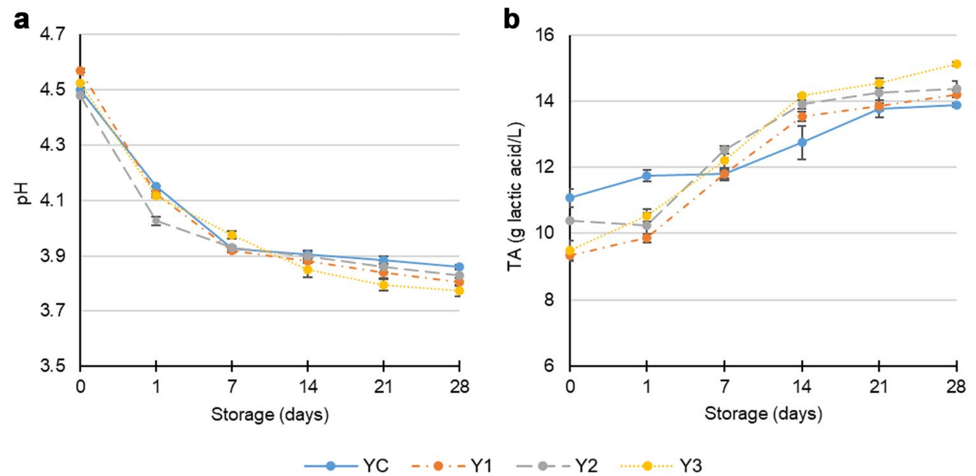

Fig. 4 **a** pH and **b** titratable acidity (TA) values (mean values ± SD, n = 3) for the yogurts during refrigerated storage at 4 ℃

reported in yogurt and has been the subject of recent study (Deshwal et al., 2021). In accordance with legal regulations, yogurt must have a minimum value of TA of 6 g lactic acid/L (Codex Alimentarius, 2003), a value reached by all the yogurts in this study, thus complying with this requirement.

Total Solids, Water-Holding Capacity, and Viscosity

As shown in Table 6, no significant differences ($P < 0.05$) were observed in the total solids content between the different yogurts or between the different storage times. This agrees with results from a recent study referring to the production of yogurt with ovine milk (Ramos & Poveda, 2022). The values of TS in the present study ranged between 10.2 and 10.9%, which comply with the regulation for non-fat set yogurt (Codex Alimentarius, 2003).

WHC refers to the water-holding capacity of the protein gel network in yogurt, which designates the firmness and compactness of the yogurt curd (Cui et al., 2021). Changes in this property may be due to modifications in the protein network, which affect the appearance of the yogurt and may limit its shelf-life and sensory acceptability (Huang et al., 2022). Yogurts Y1, Y2, and Y3 showed higher ($P < 0.05$) WHC than the YC for most of the storage times (Table 6). The Y3 yogurt presented the highest values for WHC, both at the beginning and at the end of the storage, while values for yogurts Y1 and Y2 were lower and similar to each other. It was observed that the WHC values for the YC yogurt were

Table 6 Values (mean ± SD; n = 3) for total solids (TS), water-holding capacity (WHC), and apparent viscosity for the different yogurts during refrigerated storage at 4 °C

	Yogurt	Storage (days)				
		1	7	14	21	28
TS (%)	YC	10.8 ± 0.2	10.7 ± 0.1	10.7 ± 0.1	10.7 ± 0.1	10.8 ± 0.1
	Y1	10.6 ± 0.1	10.6 ± 0.0	10.6 ± 0.1	10.5 ± 0.0	10.2 ± 0.2
	Y2	10.6 ± 0.1	10.8 ± 0.1	10.8 ± 0.2	10.5 ± 0.1	10.2 ± 0.2
	Y3	10.8 ± 0.1	10.9 ± 0.0	10.6 ± 0.1	10.5 ± 0.0	10.7 ± 0.2
WHC (%)	YC	35.5^{c} ± 0.1	37.1 ± 1.7	37.2^{b} ± 0.5	36.8^{b} ± 0.5	38.8^{b} ± 1.1
	Y1	36.5^{bC} ± 0.3	39.6^{B} ± 0.9	40.3^{aB} ± 0.1	43.1^{aA} ± 1.1	$41.3^{aA,B}$ ± 0.9
	Y2	34.8^{cC} ± 0.6	36.3^{B} ± 0.6	39.2^{aA} ± 0.2	41.2^{aA} ± 1.7	39.2^{aA} ± 0.2
	Y3	38.7^{aB} ± 0.1	38.6^{B} ± 1.2	42.3^{aA} ± 0.5	42.8^{aA} ± 0.6	42.9^{aA} ± 0.5
Apparent viscosity (cP)	YC	3890 ± 693	4290 ± 212	nd	4800 ± 1386	4490 ± 99
	Y1	3720 ± 170	4286 ± 320	nd	4930 ± 1174	4500 ± 509
	Y2	3900 ± 651	4370 ± 410	nd	4840 ± 1273	5081 ± 30
	Y3	3990 ± 806	4280 ± 594	nd	4640 ± 1047	5066 ± 65

The absence of a letter indicates that there are no significant differences

nd not determined

$^{a-c}$Means in the same column with different letters indicate statistically significant differences between the different yogurts for each storage time ($P < 0.05$)

$^{A-C}$Means in the same rows with different letters indicate statistically significant differences between the different storage times for each yogurt ($P < 0.05$)

not significantly different throughout the storage period. On the contrary, values for the experimental yogurts increased up to 14 days and remained constant until the end of storage ($P < 0.05$). This could favor the retention of water inside the yogurt gel over time, thus avoiding syneresis. In consonance with these results, when the presence of whey on the surface of the yogurts was evaluated, no syneresis was observed in any of them during storage, which would indicate that the protein gel formed in the yogurts can retain the whey inside during the whole storage period.

It has been reported that polysaccharide molecules have a large number of hydroxyl groups, which can interact with water molecules via hydrogen bonds, resulting in increased water-holding capacity. This increase in WHC can be attributed to the hydrophobicity of the polysaccharide molecules, which improves the rigidity of the protein gel network when absorbing water (Gyawali & Ibrahim, 2016). This could be related to the EPS production by LAB strains, which is discussed below. In the studies on yogurt production with different strains, similar results were obtained by Ramos and Poveda (2022), while Wang et al. (2022) found a clear reduction in WHC during storage.

The results obtained regarding viscosity did not show significant differences between the different yogurts, although yogurts Y2 and Y3 showed higher viscosity than YC and Y1 at the end of the storage period. In this regard, it should be noted that rheological parameters, such as viscosity, are closely influenced by the starter culture used for yogurt production, as reported by Sodini et al. (2002). No significant differences were observed either between the different storage times. However, a trend to increase in viscosity was observed over the storage time, in concordance with Abd El-Fattah et al. (2018) in a work on the manufacture of non-fat set yogurts. This trend of increasing viscosity with storage time was also observed by Ge et al. (2022) in set yogurts with *in situ* EPS production by a strain of *Lactobacillus helveticus*.

EPS Production in Yogurts

Figure 5 shows the concentrations of EPS in the four yogurts. The experimental yogurts showed significantly higher amounts ($P < 0.05$) than the control, with values between 2 and 4.5 times higher.

The presence of higher concentrations of EPS in the experimental yogurts was related to the higher values of WHC and the absence of syneresis in these samples, in concordance with Zhang et al. (2016), who reported an increase in WHC in non-fat yogurts made with an EPS-producing strain of *Lactobacillus bulgaricus*. Of the three experimental yogurts, Y3 showed the highest production of EPS, also coinciding with the highest WHC values in general during storage. This could be explained by the fact that EPS can act as a bridging link in the protein network, and in this way, higher amounts of water can be attached to larger aggregates made up of denatured whey proteins and EPS (Tiwari et al., 2021). Ale et al. (2016) reported that concentrations of 300 and 600 mg/L of EPS produced *in situ* by the strain *Lactobacillus fermentum* Lf2 in milk and yogurt, respectively, were able to produce changes in rheology and sensory characteristics in yogurts, improving consistency and defects compared to the control yogurts. If this is the case, the concentrations of EPS determined in the Y1, Y2 and Y3 yogurts of our study (up to 470 mg/kg) could be sufficient to modify their texture characteristics. Similarly, Khanal and Lucey (2018) reported that the presence of EPS in yogurts could have a thickening effect, playing an important role in increasing yogurt firmness.

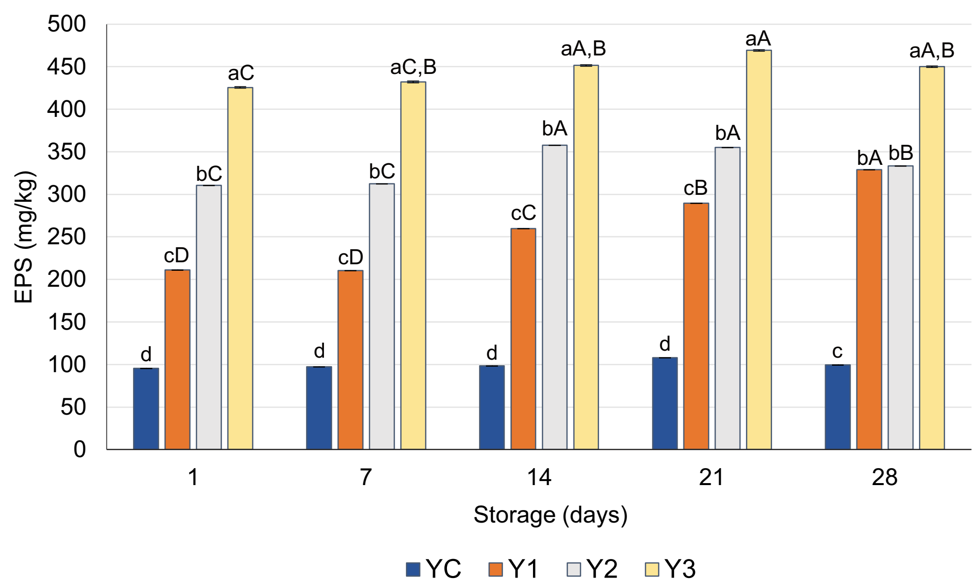

Fig. 5 EPS production (mean values ± SD, n = 3) in the different yogurts during refrigerated storage at 4 °C. a-d: columns with different letters indicate statistically significant differences between the different yogurts for each storage time ($P < 0.05$). A-D: columns with different letters indicate statistically significant differences between the different storage times for each yogurt ($P < 0.05$). The absence of a letter indicates that there are no significant differences

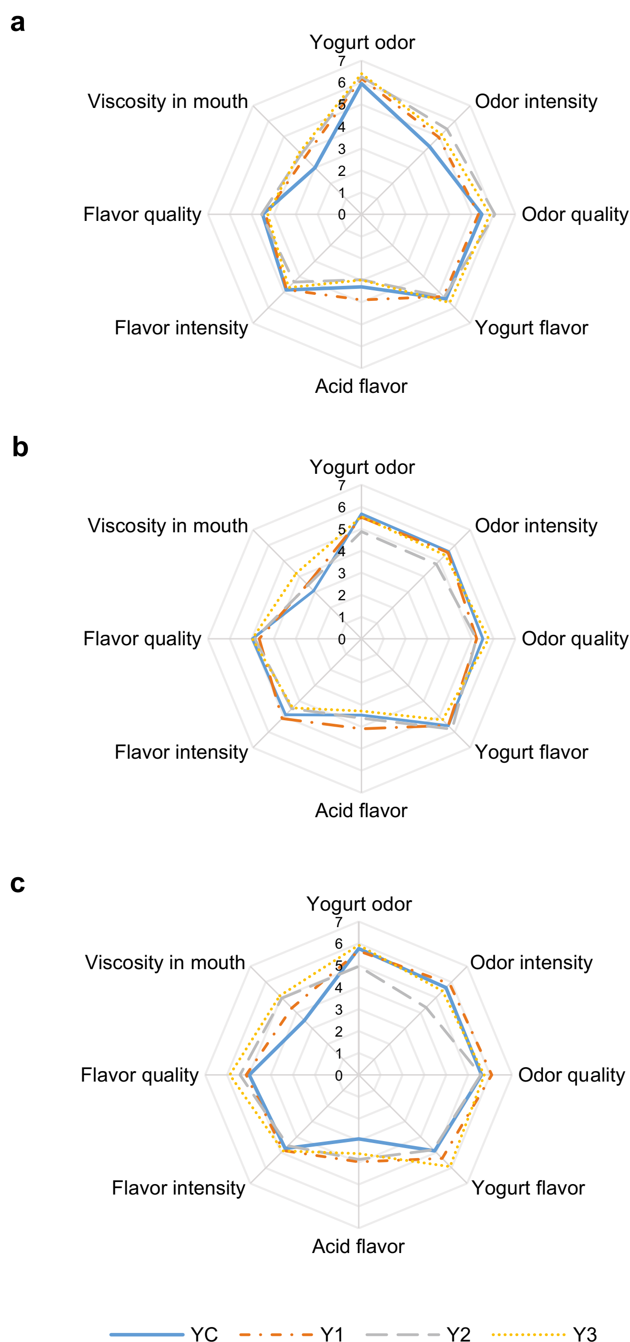

Fig. 6 Sensory spider plot of the olfactive and gustative analysis scores of the different yogurts during refrigerated storage at 4 °C. **a** 1 day; **b** 14 days; **c** 21 days. Data are the mean values of two different sessions

A significant increase in the EPS content of the experimental yogurts was observed during refrigeration storage ($P < 0.05$), while it remained constant in the case of the control. This shows the continuous production of EPS during the storage period by the LAB strains added to the Y1–Y3 yogurts.

Sensory Analysis of Yogurts

The yogurts manufactured with the selected LAB strains showed high odor intensity and quality and high yogurt odor values (Fig. 6). They also presented a moderate flavor intensity and quality, standing out for a high typical yogurt flavor, and they were moderately acid. The assessors hardly detected any differences in any of these odor and taste attributes between the experimental samples and the control yogurt, except for the flavor quality, which was slightly higher ($P < 0.05$) for the Y1, Y2 and Y3 yogurts after 21 days of storage, and for the odor intensity, which was the lowest for the Y2 yogurt after 21 days. The acid taste increased with the storage time in all the yogurts, and was related to the increase in their acidity observed during the cold storage.

On the other hand, differences were observed for the texture attribute viscosity in mouth, for which the Y1, Y2, and Y3 yogurts presented higher values than the control during the storage period. These values were significant at 21 days ($P < 0.05$). This observation could be related to the higher EPS concentrations and the higher values of WHC found in the experimental yogurts. Among them, the Y2 and Y3 yogurts received the highest scores for this attribute, coinciding with the highest EPS production. These results agreed with those described by Ale et al. (2016) in the development of yogurts with an EPS-producing *Lactobacillus* strain. EPS produced by microorganisms in yogurts can significantly affect the texture, stability, and sensory properties of these products, due to their capacity to bind water and its interactions with the protein network, leading to increased viscosity and creaminess of the products (Mende et al., 2016).

Principal Component Analysis

Results from the analyses in the yogurts (pH, TA, TS, WHC, apparent viscosity, EPS concentration and sensory attributes) were subjected to principal component analysis (PCA). Five principal components (PC) were obtained, which explained 90.8% of the total variance (TV). Among them, the first three PCs accounted for 67.5% of the TV. Figure 7 shows the projection of the samples on the plane defined by a: PC1 and PC2, and b: PC1 and PC3.

PC2 was highly correlated with viscosity in mouth, EPS concentration, and WHC, and separated the control samples (located at the bottom of the figure) from the rest of samples. On the other hand, the experimental samples were grouped according to the storage time along PC1, which was highly correlated with TA, pH, viscosity, flavor quality, and yogurt odor, with the exception of sample Y3 at 14 days. PC3 was highly correlated with flavor intensity and acidic flavor, and

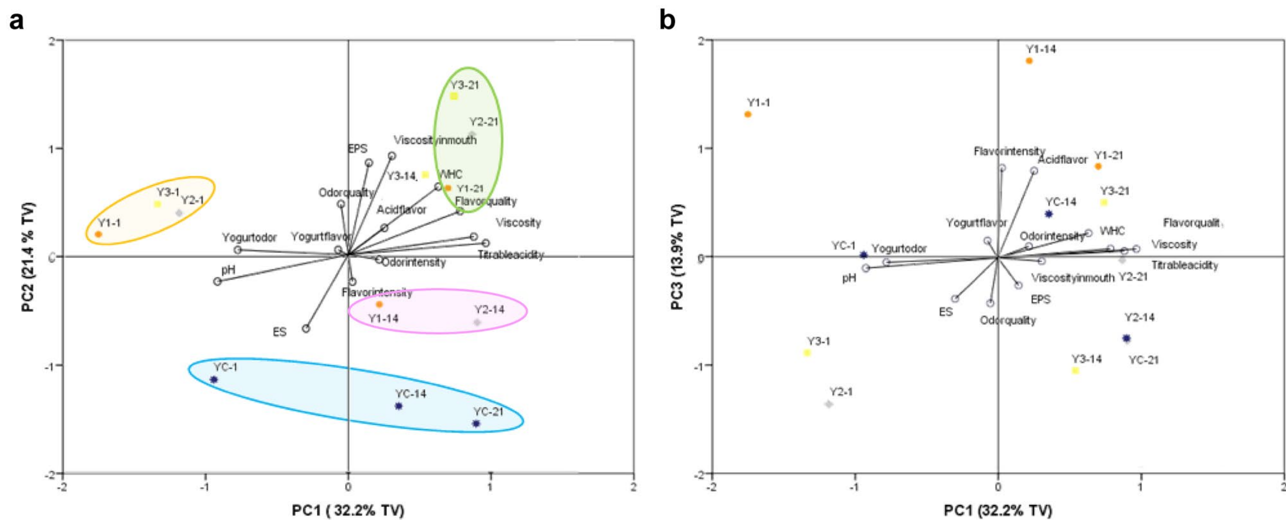

Fig. 7 Principal component analysis of the physicochemical and sensory properties of the different yogurts at 1, 14, and 21 days of refrigerated storage at 4 ºC. Biplot of the samples on the plane defined by a: PC1 and PC2, and b: PC1 and PC3

separated the samples of yogurt Y1, located in the upper side of this axis, from the rest.

These statistical results corroborate that the yogurts made with the selected LAB strains differed from the control, especially in mouth viscosity and EPS content, and thus, that PCA is an effective tool to classify the samples according to the starter type and storage time, as has been reported previously (Di Monaco et al., 2015).

Conclusions

The results of this study demonstrated that a high percentage of the LAB strains assayed were able to produce EPS when they were grown on MRS-S, and it has been confirmed that this is an intraspecific property of LAB. Three of the strains assayed, *L. brevis* UCLM-Lb47, *Ln. mesenteroides* subsp. *mesenteroides* 6F6-12, and *Ln. mesenteroides* subsp. *mesenteroides* 2F6-9, were selected for yogurt manufacture, as they produced the highest concentration of total EPS. Yogurts made with these strains generally presented higher values of water-holding capacity, EPS concentration, and viscosity in the mouth than the yogurt made only with commercial starter culture, which presented a more fluid texture. This would serve to avoid the addition of hydrocolloids to improve the texture of non-fat set yogurt, thus providing a more natural product without additives, which would have more acceptability among consumers, since they would identify it with a clean-label product. At the same time, it would have a lower cost for producers. Therefore, the 3 selected LAB strains are postulated as candidates to be added together with the commercial starter for the industrial manufacture of non-fat set yogurt. Nevertheless, further work on the textural properties of the yogurts is warranted.

Author Contribution Inés María Ramos: Data curation; formal analysis; resources; writing – original draft. Susana Seseña: Writing – review & editing. Justa María Poveda: Conceptualization; data curation; funding acquisition; investigation; methodology; formal analysis; visualization; writing – original draft; writing – review & editing. Maria Llanos Palop: Conceptualization; data curation; funding acquisition; investigation; project administration; supervision; visualization; writing – original draft; writing – review & editing.

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Data Availability All data generated or analyzed during this study are included in this manuscript.

Declarations

Competing Interests The authors declare no competing interests.

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