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Technological, rheological and sensory characterizations of a yogurt containing an exopolysaccharide extract from *Lactobacillus fermentum* Lf2, a new food additive

Elisa C. Ale [a], Marcos J. Perezlindo [a], Yanina Pavón [b], Guillermo H. Peralta [a], Silvia Costa [b], Nora Sabbag [b], Carina Bergamini [a], Jorge A. Reinheimer [a], Ana G. Binetti [a,*]

[a] Instituto de Lactología Industrial (UNL – CONICET), Facultad de Ingeniería Química (UNL), Santiago del Estero 2829, 3000 Santa Fe, Argentina
[b] Instituto de Tecnología de Alimentos (UNL), Facultad de Ingeniería Química (UNL), Santiago del Estero 2829, 3000 Santa Fe, Argentina

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ABSTRACT

Lactobacillus fermentum Lf2, an autochthonous strain isolated as a non starter culture in Cremoso cheese, produces high EPS levels (~1 g/L) in optimized conditions (SDM broth, pH 6.0, 30 °C, 72 h). Technological (texture profile and rheological analysis) and sensory properties of non-fat yogurts with 300 and 600 mg EPS/L were studied at 3 and 25 days after manufacture. Yogurts with different EPS concentrations showed higher hardness values than the control group at both periods of time, being the only significant difference that remained stable during time. The consistency index was also higher for the treated samples at both times evaluated, being significantly different for samples with 300 mg/L of EPS extract, while the flow behavior index was lower for EPS-added yogurts. The thixotropic index was lower ($P < 0.05$) for samples with the highest EPS extract concentration at the end of the storage time. Regarding the sensory analysis, those yogurts with 600 mg/L of EPS extract presented the highest values of consistency at 3 days of storage. No considerable differences for defects (milk powder, acid, bitter and cooked milk flavors) were perceived between treated and control samples at both times evaluated. Syneresis was also studied and samples with 600 mg/L of EPS extract presented the lowest syneresis values at 25 days of storage, which considerably decreased with the time of storage.

In conclusion, the EPS from L. *fermentum* Lf2, used as an additive, provided yogurt with creamy consistency and increased hardness, without the presence of unwanted defects and improving the water holding capacity of the product. All the analysis done showed the potential of this extract to be used as a technofunctional natural ingredient, and it should be considered its positive impact on health, according to previous studies.

1. Introduction

Many food-grade microorganisms including lactic acid bacteria (LAB) are known to synthesize exopolysaccharides (EPS) that are classified as homopolysaccharides (HoPS, which contain a single type of monosaccharide) and heteropolysaccharides (HePS, which comprise repeating units of different monosaccharides). EPS from LAB, mainly HePS, have been widely used in the dairy industry, mainly when they are produced *in situ* during fermentation to improve certain attributes such as emulsifying and thickening properties, syneresis reduction and firmness increase (Dabour, Kheadr, Benhamou, Fliss, & LaPointe, 2006; Zisu & Shah, 2005). Apart from the techno-functional relevance the EPS may have for the producing bacteria and their industrial applications, there is increasing evidence that EPS exert a positive impact on human health (Fanning et al., 2012; Salazar et al., 2011, 2014; Ale et al., 2016). Despite these beneficial aspects, the low yield of 80 to 600 mg/L or even less (Cerning, 1995; Ruas-Madiedo, Tuinier, Kanning, & Zoon, 2002) prevents their commercial use, in contrast to HoPS of another microbial origin such as dextran.

Techno-functional properties, such as firmness, syneresis and mouth feel are relevant in dairy products, as consumers expect a homogeneous texture. The increasing demand of novel dairy products, mainly reduced in sugar and fat contents, requires a better understanding of the effect of EPS on food matrix and, at the same time, the search for new EPS-producing strains with desirable properties (Ruas-Madiedo & de los Reyes-Gavilán, 2005). *Lactobacillus fermentum* Lf2 was isolated as a non-starter culture in Cremoso cheese manufacture and it belongs to the collection of the INLAIN (Instituto de Lactología Industrial, UNL-CONICET, Santa Fe, Argentina). The particular interest in it resides in the high levels (approximately 1 g/L of crude extract) of EPS that it produces when it is grown in well-defined conditions. This yield was found to be higher than hetero-EPS produced by other LAB and, in particular, *L. fermentum* strains (Behare, Singh, Nagpal, & Rao, 2013). The only strain

* Corresponding author.
E-mail address: anabinetti@fiq.unl.edu.ar (A.G. Binetti).

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of this bacterial species which was studied in relation to the EPS production is L. *fermentum* TDS030603 (Fukuda et al., 2010). Genomic information indicates that EPS biosynthetic pathway is controlled in housekeeping genes and a cluster of EPS-related genes, involving four functional regions (regulation of EPS production, chain length determination, biosynthesis of the repeating unit and polymerization and export; Dan et al., 2009). For the same strain and more recently, Shi, Aryantini, Uchida, Urashima, and Fukuda (2014) indicated that the optimal C/N ratio and/or microaerobic condition can alter the expression levels of several exopolysaccharide biosynthesis-related genes promoting the EPS production, achieving the best yield (199 mg/L) in a chemically defined medium supplemented with 5% glucose and 1% ammonium citrate without pH control, after 48 h cultivation and at 30 °C.

The high amount of EPS produced by L. *fermentum* Lf2, combined with the increasing request of new functional food ingredients, were the main factors to address the aims of this work: to isolate and characterize the EPS produced by this strain, regarding the technological, rheological and sensory aspects when added in yogurt, focusing on its application as a functional food additive.

2. Materials and methods

2.1. Organisms and growth conditions

L. fermentum Lf2 (INLAIN collection) was stored at −80 °C in MRS (Biokar, Beauvais, France) broth plus 15% (v/v) glycerol. It was routinely grown in MRS broth at 37 °C for 16 h. For yogurt production, two commercial strains, *Streptococcus thermophilus* SC42 and *Lactobacillus delbrueckii* subsp. *bulgaricus* 254 (both from Biochemical, Argentina), were selected based on their inability (visual test) to produce EPS in milk. They were routinely grown in 10% (w/v) reconstituted skimmed milk (RSM) at 43 °C and stored at −80 °C in the same medium.

2.2. Growth of L. fermentum Lf2

Cultivations were performed in a 2-L fermentor (Sartorius Biostat A plus®, Goettingen, Germany) in SDM (Semi-Defined Medium) broth (Kimmel & Roberts, 1998) with the aim to minimize interferences in EPS isolation by replacing yeast extract, beef extract and proteose peptone from MRS broth by yeast nitrogen base and Bacto Casitone (both from Difco, Becton, Dickinson and Company, Le Pont de Claix, France). *L. fermentum* Lf2 was inoculated from an overnight culture (0.1% v/v) and incubations were made at 30 and 37 °C for 72 h, with agitation (6 ×*g*) and sparging with CO_2 (0.2 L/min). Samples were aseptically withdrawn at different times to determine cell counts (MRS Agar, 48 h, 37 °C, aerobiosis), end products and EPS yield. The pH was kept automatically at 6.0 with sterile 8 M NaOH. Simultaneously, cultures without pH control were included in each experiment.

2.3. Determination of residual sugar and organic acids

The analysis of residual glucose and organic acids was carried out by HPLC, according to Peralta, Wolf, Perotti, Bergamini, and Hynes (2016). Chromatographic separation was performed isocratically at 65 °C with a mobile phase of 0.01 M H_2SO_4 at a flow rate of 0.6 mL/min on a Bio-Rad Aminex HPX-87H column (300 × 7.8 mm; Bio-Rad Laboratories, Hercules, CA, USA) equipped with a cation H + microguard cartridge (Bio-Rad Laboratories, USA), which allows the simultaneous quantification of sugars and organic acids using UV and IR detectors connected in series. HPLC equipment consisted of a quaternary pump, an online degasser, a column oven, a UV–visible detector (all Series 200) and a refractive index detector thermostated at 35 °C (Series Flexar) (Perkin Elmer, USA). The UV detector was set at 210 nm for the detection of organic acids, while the IR detector was used for the analysis of glucose. Data were collected and processed on a computer with Chromera® software (Perkin Elmer). Samples were diluted in 0.01 M H_2SO_4, filtered through 0.45 μm membranes (Millex, Millipore, Sao Paulo, Brazil) and injected into the chromatograph, using a loop of 60 μL. Analytical grade glucose and organic acids (Sigma Aldrich) were used as standards to obtain calibration curves. The results were expressed in mg/mL. Determinations were done for, at least, two independent cultivations.

2.4. Isolation and quantification of EPS

After incubation, bacteria were removed by centrifugation (19,630 ×*g*, 30 min, 5 °C) and EPS was extracted and precipitated at 4 °C for 48 h by adding 2 volumes of chilled absolute ethanol (Cicarelli, Buenos Aires, Argentina) according to Ruas-Madiedo, Gueimonde, Margolles, de los los Reyes-Gavilán, and Salminen (2006). The precipitate was collected by centrifugation (4050 ×*g*, 30 min, 5 °C), dissolved in ultrapure water and dialyzed against distillated water, using 12–14 kDa MWCO membranes (Sigma Aldrich) for 3 days, at 4 °C with daily change of water. Finally, the EPS solution was freeze-dried (Chris Alpha 1-4 LD Plus, Tokyo, Japan), weighed and expressed as mg crude EPS/L. A control of the SDM broth (without inoculum) was treated in the same way of the culture, with the aim of subtracting the contribution of substances that precipitate from the medium. Total protein concentration of the crude extract was determined with the Bio-Rad (Hercules, CA, USA) protein assay based on the Bradford method, in triplicate. Additionally, a purification of the EPS-crude fraction was performed with a treatment with DNAse I (5 μg/mL; Sigma Aldrich) at 37 °C for 12 h and Pronase E (50 μg/mL; Roche, Germany) at 37 °C for 18 h. Then, a precipitation step with TCA (12% w/v) with a posterior neutralization with NaOH was done. The suspension was dialyzed against distillated water and freeze-dried as indicated above to obtain the EPS-purified fraction (Lopez et al., 2012). At least two replicate experiments were carried out.

2.5. Monosaccharide composition of the polysaccharide

For monosaccharide analysis, EPS-purified fraction was hydrolyzed with 2 N trifluoroacetic acid at 121 °C for 2 h, then freeze-dried and resuspended in the mobile phase. Chromatographic separation was performed isocratically at 85 °C with distilled water (0.6 mL min^{-1}) on an Aminex HPX-87N column (300 × 7.8 mm) equipped with a cation N microguard cartridge (Bio-Rad), using a refractive index detector (Series Flexar, Perkin Elmer, Norwalk, CT, USA). Data were collected and processed with the software ChromeraR (Perkin Elmer). The analyzed standards were D(+)-glucose, D(+)-galactose, D(+)-manose, D(−)-arabinose, D(−)-ribose, D(−)-levulose, D-manitol and L-rhamnose (Sigma Aldrich).

2.6. Manufacture of yogurts

Yogurts were made with 10% (w/v) RSM inoculated with *S. thermophilus* SC42 and L. *delbrueckii* subsp. *bulgaricus* 254 (10^6 and 10^5 UFC/mL, respectively), with 0 (control), 300 (equivalent to 73 mg pure EPS/L) and 600 (equivalent to 146 mg pure EPS/L) mg/L of crude EPS added. The incubation was at 43 °C until a final pH value of 4.6 was reached. The concentrations of crude EPS were chosen taking into account the principle that, for "real commodities" such as yogurt, EPS content frequently ranges from 13 to 170 mg/L (as pure extract; Mende, Peter, Bartels, Rohm, & Jaros, 2013). Following fermentation, yogurts were immediately cooled-down and stored at 4 °C for 25 days.

2.7. Rheological analysis

A concentric cylinder viscometer Rotovisco Haake RV-2 (Haake Mess-Technik, Karlsruhe, Germany) with a cell of 50 N·cm and a MVII rotatory sensor system was used. Yogurt samples were gently stirred 10 times in clockwise direction with a medium size spoon and then

they were allowed to rest for 10 min inside the cylindrical cup before starting the analysis (Hassan, Ipsen, Janzen, & Qvist, 2003). The shear rate was increased from 0 to 200 s^{-1} and then reduced to 0 s^{-1} (Mende et al., 2013). Rheograms of shear stress *vs* shear rate were obtained and the area between the upward and downward shear stress curves (thixotropic index, TI) was calculated for each sample. The Power law model was applied in order to estimate the consistency index (K) and the flow behavior index (n). Measurements were made in duplicate, after 3 and 25 days of storage.

2.8. Texture profile analysis (TPA)

TPA was carried out according to Szczesniak (2002) using a Universal Testing Machine (Instron Bluehill®, MA, USA) equipped with a 10 N load cell. The following mechanical properties were determined in set yogurts: hardness, adhesiveness, cohesiveness, gumminess, elasticity and masticability. The setting parameters used in the tests were double penetration of 30 mm into the samples, speed penetration of 1 mm s^{-1}, penetrometer diameter of 12 mm and cylinder diameter of 36 mm at 10 °C (Pons & Fiszman, 1996; Santini et al., 2007). Analyses were performed in quadruplicate at 10 °C, after 3 and 25 days of storage.

2.9. Sensory analysis

The samples were also evaluated by a trained sensory panel of ten assessors (7 women and 3 men from 25 to 55 years old), who have been using quantitative descriptive analysis (International Organization for Standardization [ISO], 1993) on regular basis for the past 2 years. The panel was trained in the use of the chosen attributes in six training sessions. During these sessions, panelists discussed and agreed on the definitions and the way to qualify the attributes in a scale using commercial natural yogurts while following the recommendations of the International Dairy Federation (International Dairy Federation [IDF], 1997). Texture descriptors (consistency, graininess, creaminess and lumps presence) were evaluated on a 10 cm unstructured line scale anchored with appropriate terms on the left (1 = 'almost nothing') and right (9 = 'a lot') extremes. When defects were detected (strange, rancid, metallic and old tastes), their intensity was evaluated as described before. A value was then assigned to each descriptor, using the mean value for the statistical analysis. Flavor attributes (acid, sweet, bitter, milk powder and cooked flavors) were assessed using an equal interval scale (using 5 categories in this order: barely, little, moderately, very and extremely perceptible). For the flavor attributes, each category was assigned to a scalar number (1, 3, 5, 7 and 9, respectively) and a weighted average (considering the total assessors that chose one specific category) was calculated, together with perceived percentage. Test samples, identified by a three-digit code, were presented to the panelists in a randomized order and at 10 °C after 3 and 25 days of storage at 4 °C. All tests were conducted in duplicate and in a standardized room (ISO, 1988).

2.10. Syneresis of yogurts

The syneresis of yogurt samples was determined using the centrifugation method adapted by Amatayakul, Halmos, Sherkat, and Shah (2006). Measurements were made in triplicate, 3 and 25 days after yogurt manufacture.

2.11. Statistical analysis

Statistical analysis was performed with the SPSS software (SPSS Inc., Chicago, IL, USA) using one way ANOVA for the comparison of more than two groups, and Kruskal-Wallis when the ANOVA assumptions were not satisfied. These tests were applied to evaluate differences in the rheological, sensory and texture analysis, and to evaluate the syneresis of yogurts as well. Experiments were done in duplicate for the first two assays (for the sensory assay, each panelist repeated the determination twice), and in quadruplicate and triplicate for the texture and syneresis analysis, respectively. The differences between means were detected by the Tukey's Multiple Range Test or by Dunns when Kruskal-Wallis was applied. Two way ANOVA was applied to verify that there was no significant interaction between the time of yogurt manufacture and the EPS concentration. *t*-Test was applied to analyze the response variable between two groups (yogurt samples at two different manufacture times), and Mann Whitney was used for non-normal distributions. Data were considered significantly different when $P < 0.05$ for all the tests previously described.

3. Results and discussion

3.1. Growth of L. fermentum Lf2 and EPS production

During growth in SDM broth at pH 6.0, the strain reached its stationary phase at 16 h, with cell counts of 9.1 and 8.9 $\log_{10}$ cfu/mL at 30 and 37 °C, respectively (Fig. 1). All through the 72 h of growth, the highest counts were registered at 30 °C (Fig. 1, A). Our results showed that 30 and 37 °C were suitable for the growth of this strain, but 30 °C and pH 6.0 represented the best conditions for EPS production, for the evaluated variables. The highest EPS value (791 and 531 mg/L as crude extract, at 30 and 37 °C, respectively) was found after 72 h, corresponding to the late stationary phase of growth of L. *fermentum* Lf2, when glucose was almost consumed. The percentage of proteins was 0.9% and 2% for the crude EPS produced at 30 °C and 37 °C, respectively. Leo et al. (2007) reported, for L. *fermentum* TDS030603, EPS yields in MRS (30 °C, 72 h) of 568.6 mg/L and 151.2 mg/L, in crude and purified forms, respectively. Three isolates of *L. fermentum* from Burkina Faso fermented milk produced similar EPS yields (from 322 to 713 mg crude EPS/L in MRS broth in which glucose was replaced by lactose, 35 °C, 20 h; Savadogo et al., 2004). Considering that MRS broth contains a large amount of yeast extract, beef extract and proteose peptone that strongly interferes in EPS precipitation (Kimmel & Roberts, 1998), the production of EPS by L. *fermentum* Lf2 in SDM broth (in which these components were replaced to provide minimal interference) at 30 °C and pH 6.0 is, to the best of our knowledge, the highest production reported for this bacterial species. Due to the diversity of quantification methods employed, it is difficult to compare EPS yields from diverse LAB species; in the case of hetero EPS from *Lactobacillus* species, the reported yields range from 25 to 150 mg/L (Ruas-Madiedo & de los Reyes-Gavilán, 2005). Without pH control, similar growth curves were observed (Fig. 2) but the EPS production reached the highest value at 40 h at 30 °C (330.6 mg/L as crude extract; Fig. 2, A) and at the same time at 37 °C (395.5 mg/L as crude extract; Fig. 2, B), declining the EPS yield at 72 h for both temperatures. This could be attributed to the enzymatic degradation of EPS at pH values below 5 (Deegest, Mozzi, & De Vuyst, 2002; Fukuda et al., 2010; Pham, Dupont, Roy, Lapointe, & Cerning, 2000).

At controlled pH, the highest productions of lactic and acetic acids were accompanied with the highest levels of consumed glucose, which were verified at 40 h (at 30 °C) and 16 h (37 °C) of growth (Fig. 3). In cultivations at pH 6 and 30 °C, the concentration of lactic acid was significantly lower at 16 h than those obtained at 40 and 72 h of incubation, while no significant differences were observed for acetic acid. Neither concentrations of lactic and acetic acids between 16 and 72 h during fermentations at controlled pH and 37 °C presented significant differences. It is noticeable that the rate of glucose consumption was higher when the bacteria grew at controlled pH at 37 °C than when it grew at controlled pH and 30 °C or without pH control at 37 °C. This could be explained considering that the optimal growth temperature for L. *fermentum* Lf2 is 37 °C, hence, when pH was controlled and the acid accumulation did not inhibit its growth, higher rate in glucose consumption and the highest levels of lactic and acetic acid were observed, in comparison with cultivations at 30 °C. It is important to

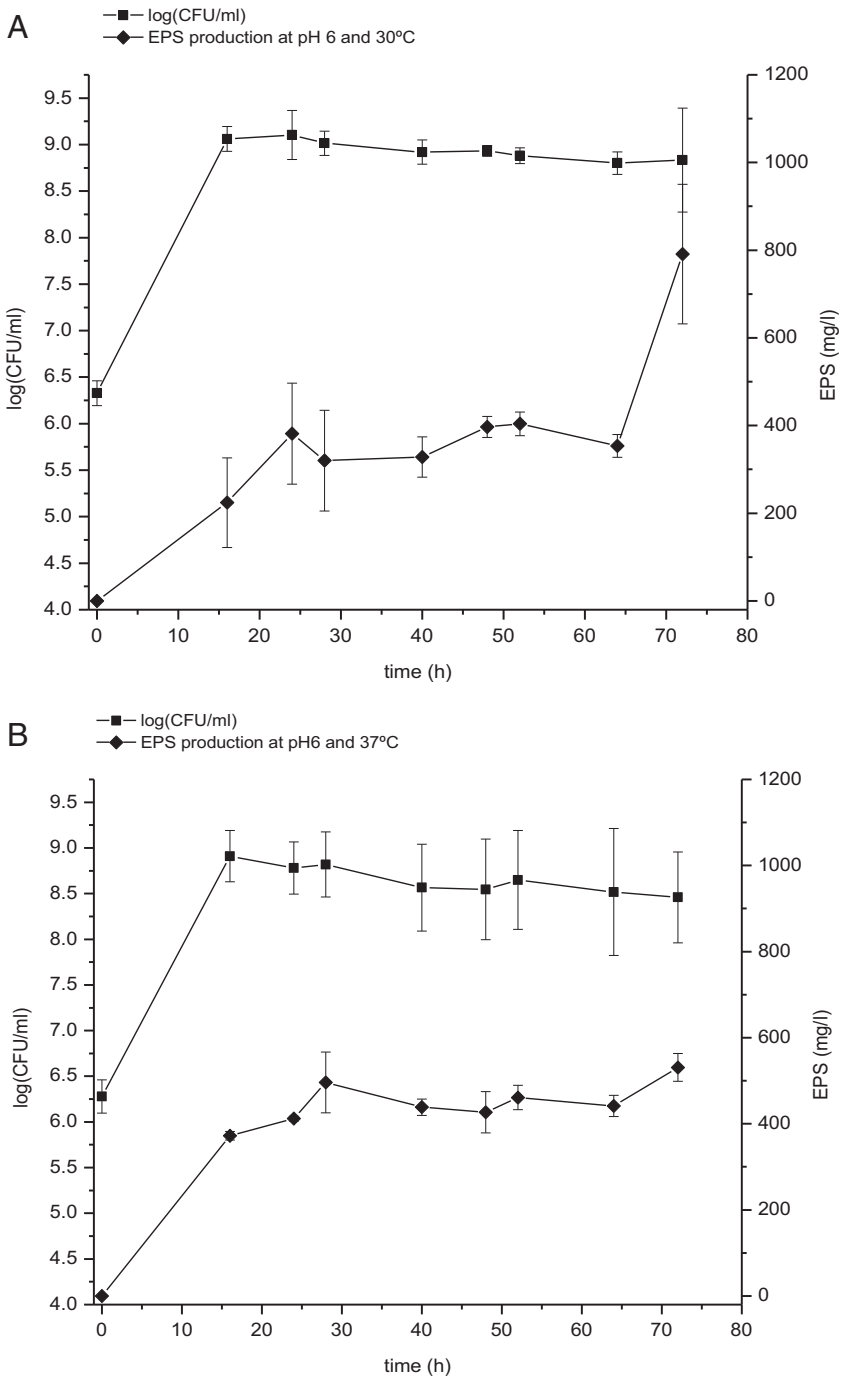

Fig. 1. Growth of L. *fermentum* Lf2 in SDM broth, at pH 6.0, at 30 °C (A) and 37 °C (B). Evolution of cell counts and EPS yield. Values represent mean values ± standard deviations.

highlight that the crude extract had 0.9% protein (Bradford method), a value lower than the limit recommended for EPS when added to food matrices (<3%, de Vuyst et al., 2003).

It should be noticed that the highest production of EPS is observed in the late stationary phase, and while no changes in the glucose consumption, lactic and acetic acids production and cell counts were observed after 40 h, the EPS extract amount increased at the end of fermentation at pH 6 and 30 °C. Vaningelgem, Zamfir, Adriany, and De Vuyst (2004) had similar results, since they observed for a strain of *Streptococcus thermophilus* that EPS production incremented with time of fermentation at controlled pH and temperature (milk medium, 37 °C and pH 5.8). The highest EPS production was at 35 h, with cell counts of approximately 2×10^9 CFU/ml. When the medium was supplemented with different nitrogen sources, the EPS yield increased considerably (3 times with casitone, 7 times with tryptone and 5 times with whey protein hydrolysate, approximately, when compared to the production in milk with no nitrogen source added). They observed that, although lactose was completely consumed at 10 h, the highest EPS production was at the end of fermentation (24 h of incubation). Besides, Zisu and Shah (2003) observed that the growth of *S. thermophilus* 1275 and EPS production were affected by the addition of WPC 392 (0.5%, wt/vol) at pH 5.5 and 37 °C. After 24 h of growth, the amount of EPS produced significantly increased ($P < 0.05$) from 458 mg/L (without WPC) to an amount of 1029 mg/L with 0.5% WPC supplement. Similarly, in a study about the optimization of EPS production from L. *fermentum* TDS030603, Shi et al. (2014) reported the best EPS yield when the strain was cultivated microaerobically at 30 °C in a chemically defined medium supplemented with glucose and 1% ammonium citrate, and after

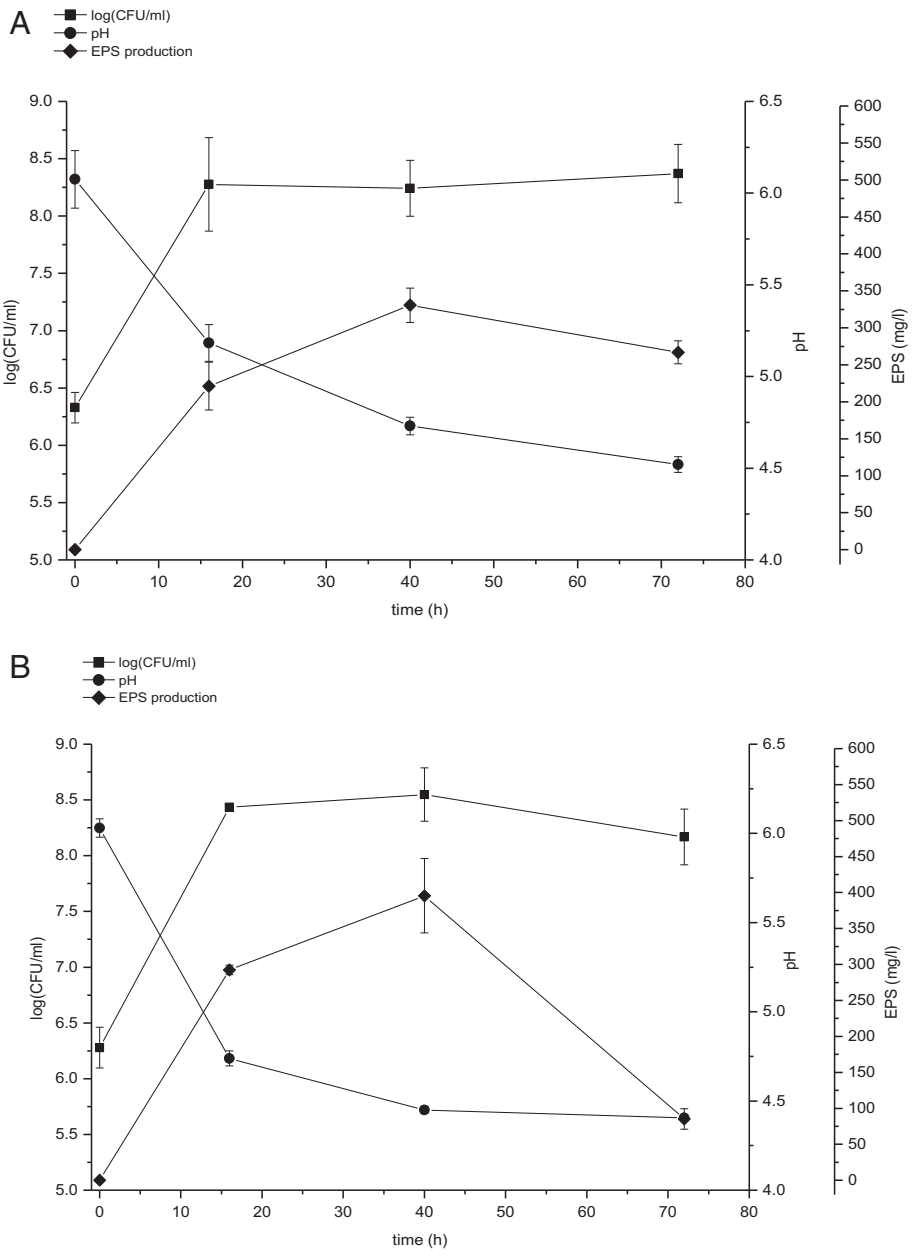

Fig. 2. Growth of L. *fermentum* Lf2 in SDM broth without pH control, at 30 °C (A) and 37 °C (B). Evolution of pH, cell counts and EPS yield. Values represent mean values ± standard deviations.

48 h incubation, when glucose was consumed. It seems that availability of a nitrogen source plays a very important role in EPS production.

3.2. Monosaccharide composition of the polysaccharide

The EPS released in SDM at pH 6.0 consisted of D-glucose and D-galactose, in a relation 2:1 approximately. No other monosaccharide was detected among the standards analyzed. This result was similar to the EPS from L. *fermentum* TDS030603, also composed by D-glucose and D-galactose, in molar ratios ranging from 2.6 to 2.8, depending on the growth media (Fukuda et al., 2010). Another previous report described the strain L. *fermentum* MR3 which produces EPS composed by 99.2% D-galactose and 0.8% glucose (Savadogo et al., 2004). The most important heteropolysaccharides from industrial LAB species usually contain D-galactose, D-glucose, and L-rhamnose in different ratios. In a few cases, fucose, ribose, acetylated amino sugars (*N*-acetylglucosamine and *N*-acetylgalactosamine), uronic acid, as well as other non-carbohydrate compounds such as phosphate, acetate, and glycerol, are also present (De Vuyst & Degeest, 1999; De Vuyst, De Vin, Vaningelgem, & Degeest, 2001).

3.3. Technological characterization of EPS-added yogurts

3.3.1. Rheological analysis

Table 1 shows the values obtained for the rheological parameters evaluated. Consistency index (K) was significantly higher ($p < 0.05$) in yogurts made with 300 mg/L of EPS when compared with the control group at both times evaluated, and a slight tendency to increase this value along time was observed. It is not the first time that an unclear relationship between the EPS concentration and the rheological characteristics of yogurt was observed. According to Doleyres, Schaub, and Lacroix (2005), the physicochemical characteristics of EPS and their interactions with milk proteins would be more important than the amount of EPS added for the rheological properties of the product. In

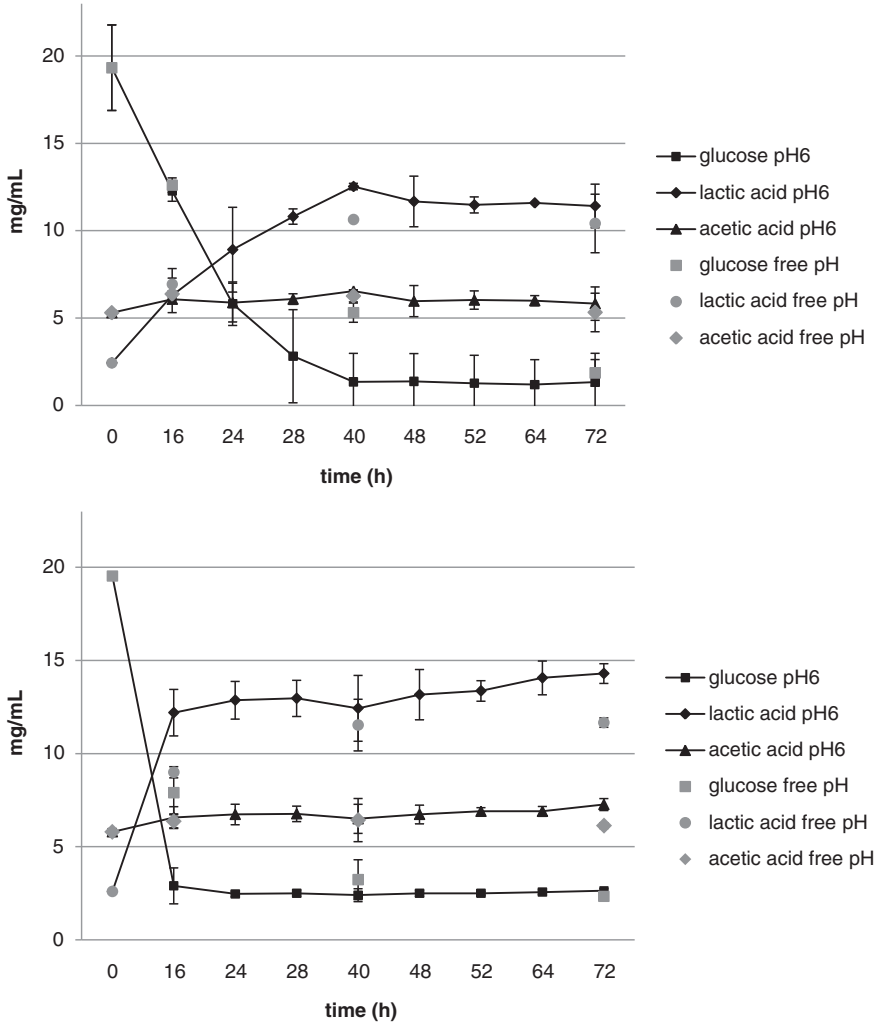

Fig. 3. Evolution of consumed glucose and production of lactic and acetic acids during growth of L. *fermentum* Lf2 in SDM broth at 30 °C (A) and 37 °C (B) at pH 6.0 and without pH control. Values represent mean values ± standard deviations.

that work, they described a similar behavior for a commercial EPS-producing culture added to yogurts, which showed higher consistency index in comparison with the control group, but EPS-added yogurts were similar to the control group. These authors reported lower yield stress and viscoelastic moduli in all cases (EPS or EPS-producing culture), but these differences could be attributed to the composition of milk used in yogurt formulation, mainly to the high levels of fat and proteins (26%), and the supplementation with milk protein concentrate reported in that case. In a similar study, but using buffalo milk, Yang et al. (2014) described that addition of EPS reduced the viscosity in yogurts, contrary to our observations. Authors hypothesized that the composition of buffalo milk, mainly the levels of dry matter, caused this behavior. In our study, and for 3 days of storage, the minimal value of flow behavior index (n) was found in samples made with 300 mg/L of crude EPS ($p < 0.05$). Values of n below 1 indicate non-Newtonian behavior, typical of this type of product. The behavior index of yogurts with 600 mg/L of EPS significantly decreased with time (from 0.22 to 0.157), resulting similar to the yogurts with 300 mg/L. Hence, a significant difference between yogurts with EPS (at both concentrations) and the control group, at 25 days of storage, was observed for this index. A high degree of hysteresis (TI) in EPS-containing yogurts was observed at 3 days, although the difference was not significant in comparison to the control samples. That would indicate that these products have a lower ability to recover their structures after the shear-induced structure breakdown at 3 days of storage. Along the storage time, the thixotropic index significantly increased for yogurts with 300 mg/L, leading

Table 1
Rheological parameters and syneresis evaluated for control and EPS-added yogurts (with 300 and 600 mg EPS/L) at 3 and 25 days of storage at 4 °C. Values represent average values ± standard deviations (SD). Different letters indicate differences among groups at initial and final times. Asterisks indicate differences among the same sample during storage time. Determinations were done in duplicate for the rheological parameters (consistency index, flow behavior and thixotropic index) and in triplicate for the syneresis analysis.

	EPS concentration (mg/L)	Consistency index (K) (Pa s^n)	Flow behavior index (n)	Thixotropic index (TI) (Pa s^{-1})	Syneresis (%)
3 days	0	$5^{b} \pm 1$	$0.2516^{a} \pm 0.0001$	$1177^{a} \pm 316$	$72.3^{a,b} \pm 0.8$
	300	$9.74^{a} \pm 0.09$	$0.17^{b} \pm 0.01$	$1661^{a} \pm 31$	$70.8^{b} \pm 0.4$
	600	$7.2^{a,b} \pm 0.2$	$0.22^{a*} \pm 0.01$	$1500^{a} \pm 102$	$72.8^{a*} \pm 0.9$
25 days	0	$6.1^{b} \pm 0.2$	$0.25^{a} \pm 0.01$	$1838^{a} \pm 32$	$72.9^{a} \pm 0.4$
	300	$10^{a} \pm 1$	$0.12^{b} \pm 0.03$	$1827^{a*} \pm 0.9$	$72.0^{a*} \pm 0.4$
	600	$7.8^{a,b} \pm 0.2$	$0.157^{b} \pm 0.009$	$1644^{b} \pm 20$	$69.4^{b} \pm 0.3$

to significant differences between yogurts with 600 mg/L of EPS, which presented the lowest TI values ($p < 0.05$) at the end of shelf-life, and the rest of the samples. The only rheological parameter that did not change with the storage time was the consistency index for all the samples evaluated.

3.3.2. Texture profile analysis

From TPA analysis, no significant differences ($p > 0.05$) were detected for cohesiveness, adhesiveness and gumminess among the samples, independently of the storage period (Fig. 4), and, although adhesiveness increased with the EPS extract concentration at 3 days of storage, the differences were not significant ($p > 0.05$). The control group showed the highest masticability values at 3 days of storage, while elasticity was, as expected, similar between samples, presenting values between 0.999 and 1.002 at both times of storage. In contrast, yogurts with different concentrations of EPS showed higher hardness values than the control group at both periods of time ($p < 0.05$), evidencing no differences between samples with 300 and 600 mg EPS/L ($p > 0.05$). The interaction between the protein matrix and EPS could improve the hardness of the products due to the presence of a dense network between the EPS and casein micelles and other milk components as whey proteins (Hassan, 2008). Our observations were similar to those reported by Yang et al. (2014) for buffalo milk-fermented yogurts with EPS added. In control samples, the storage did not modify TPA parameters. On the other hand, EPS-added yogurts showed higher values of hardness ($p < 0.05$) after 25 days at 4 °C, in comparison with the values they presented at the third day of storage. The gumminess and masticability were significantly incremented during conservation only for yogurts with 300 mg/L. It is important to highlight that the only texture parameter that showed significant differences between yogurts with and without EPS added after the storage period was hardness. This result is consistent with those obtained with the viscometer.

3.3.3. Sensory analysis

Fig. 5 shows evolution in texture descriptors of EPS-added yogurts after 3 and 25 days of storage. Yogurts made with 600 mg EPS/L showed high ($p < 0.05$) levels of consistency when compared to the control group at 3 days of storage, but this effect was not observed at the end of the shelf life period. All samples presented low (<2.5) values of graininess, and creaminess was more noticeable in those samples with the higher EPS concentration at day 3, but a tendency to achieve similar creamy textures at the end of shelf life was observed. High scores for visual lumps presence were obtained for all samples; lumps are protein aggregates that may occur due to excessive production of acid at high

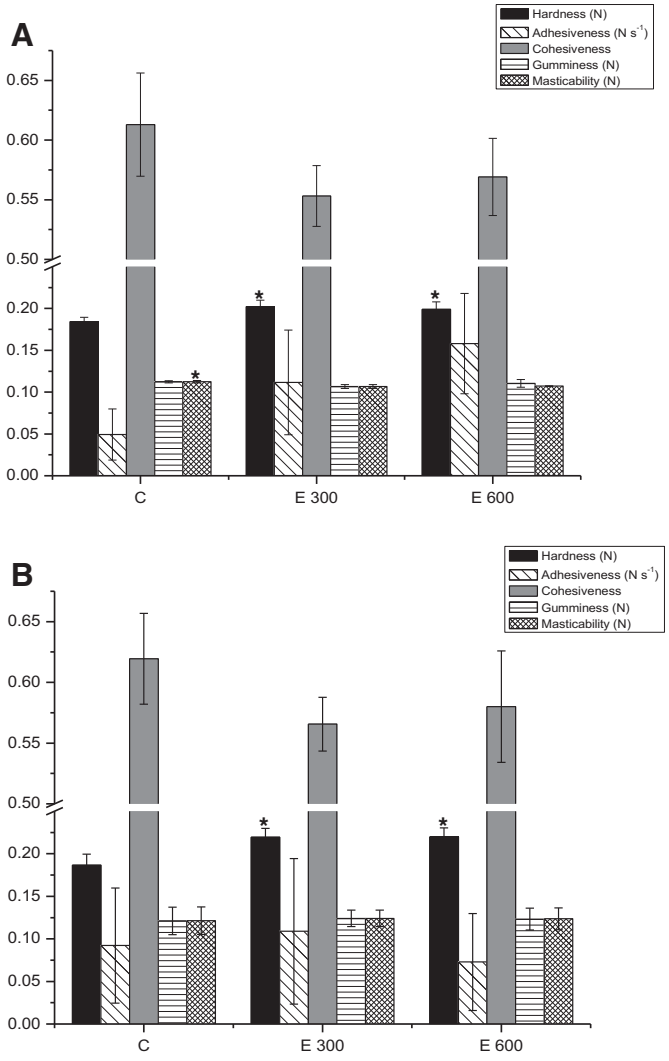

Fig. 4. Evolution in TPA parameters of control and EPS-added (300 (E300) and 600 (E600) mg EPS/L) yogurts after 3 (A) and 25 (B) days of storage. Bars represent mean values ± standard deviations. Asteriks indicate significant ($p < 0.05$) differences among samples.

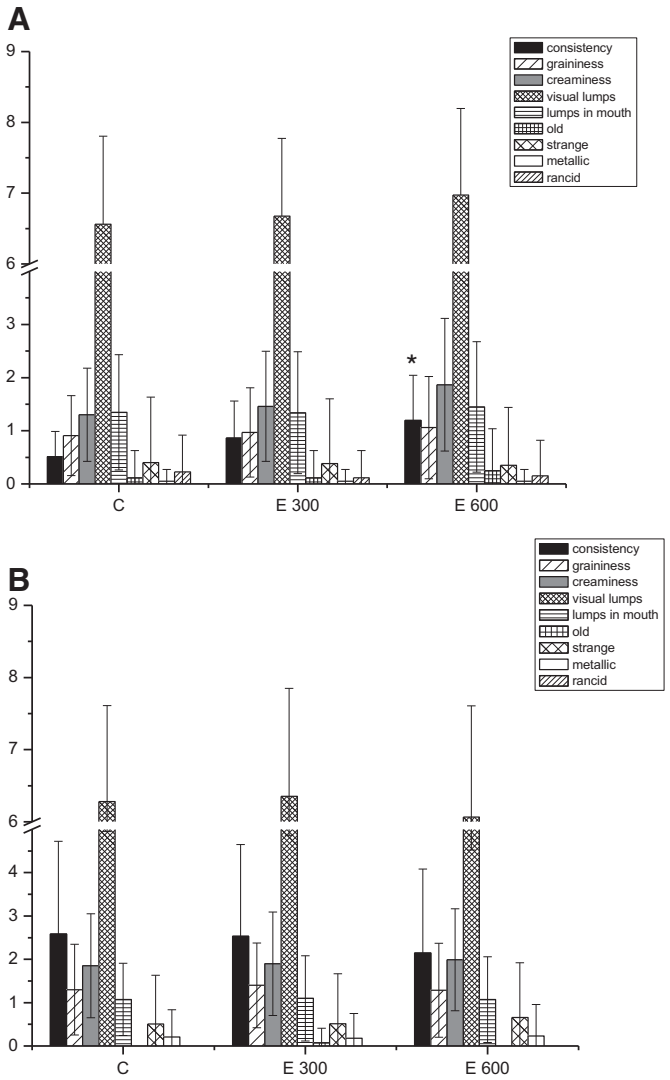

Fig. 5. Evolution in texture descriptors (consistency, smoothness, creaminess and lumps presence) and defects (old, strange, metallic and rancid tastes) of control and EPS-added (300 (E300) and 600 (E600) mg EPS/L) yogurts after 3 (A) and 25 (B) days of storage. Bars represent mean values ± standard deviations. Asteriks indicate significant ($p < 0.05$) differences among samples.

incubation temperatures, excessive heat treatment of milk and the addition of high levels of whey proteins (Humphreys & Plunkett, 1969). In our case, the excessive heat treatment could be the main factor that caused this defect, since milk was treated at 115 °C for 30 min. However, when the assessors evaluated the presence of lumps in the mouth during yogurt consumption, they were not perceptible. Regarding the effect of time for each group, consistency significantly increased for yogurts with no EPS added and those with 300 mg/L, resulting in similar values for all the samples at the end of storage. Besides, visual lumps decreased with time ($p < 0.05$) for yogurts with 600 mg EPS/L. Defects, such as old, strange, metallic and rancid tastes, presented low values (<2) for all the samples, and at both times evaluated.

Table 2 shows the weighted averages and perceived percentages for flavor attributes at 3 and 25 days of storage, for each experience. It should be noticed that, in general, "bitter", "cooked milk" and "milk powder" tastes had a variable behavior among samples, since weighted averages ranged between 0 and 0.6 for both periods of time, reaching low intensities for <30% of evaluators. "Acid" taste was variable between samples but the tendency was that yogurt with 300 mg EPS/L presented the highest intensity in this attribute at both times evaluated, that may be a consequence of post-acidification. This fact is in accordance to the pH values obtained at 25 days of storage, because, although all samples reached the same pH at the end of fermentation (around 4.8), yogurts with 300 mg/L of EPS extract presented lower pH values than yogurts with 600 mg/L of EPS extract and control samples (4.65 *vs.* 4.81 and 4.70, respectively). A typical acidity for this type of yogurts was detected, for which panelists considered acceptable acidity scores between 3 and 5 ("little" and "moderately" perceptible) at both times, and >60% of the assessors found this attribute in all samples. As expected, "sweet" taste was not found in high levels and only a few panelists (<30%) perceived it.

In general, the sensory evaluation revealed that the addition of EPS extract produces effects when it is added in the highest concentration and during the initial period of storage, influencing consistency. On the other hand, after 25 days, the effects observed were similar among samples. It is noticeable that the EPS extract did not negatively affect the yogurts, since no significant differences were detected for the defects evaluated (old, strange, metallic and rancid tastes). Besides, the flavor analysis showed scores lower than 5 points for all the samples and flavor attributes (moderately perceptible), with a perceived percentage lower than 30%, except for acidity, which reached 90%. This last result was expected because of the type of yogurt made, a plain yogurt without the addition of any additives. Considering the results of the sensory analysis altogether, it could be concluded that the application of this EPS extract as a food additive would be possible, with positive effects on the texture and without the consequent appearance of unwanted effects. It would be interesting to test a higher concentration of EPS extract, in order to see if these effects could be improved and persist over time of storage. Similar results were found in other studies; Folkenberg, Dejmek, Skriver, Guldager, and Ipsen (2006) reported that yogurts made with EPS-producing cultures presented higher mouth thickness, ropiness and creaminess than yogurts made with non-EPS- producing cultures, after 3 days of storage. In another work, Folkenberg, Dejmek, Skriver, and Ipsen (2005) described two different sensory profiles which depended on the protein-EPS interactions. These profiles were explained considering two types of microstructures: in one of them the EPS were associated with the proteins surrounding the pores, and in the other one incompatibility between the EPS and the protein existed, causing the EPS to be placed inside the pores. Yogurts with the first type of microstructure presented high ropiness, low serum separation and were more resistant to stirring, while yogurts with the second type of microstructure were less ropy, had high serum separation and showed increased mouth thickness as a result of stirring. Further analysis should be addressed to describe the type of interaction between the EPS produced by L. *fermentum* Lf2 and the protein network by confocal laser scanning microscopy, in order to explain the sensory characteristics observed but from a molecular and structural point of view.

3.3.4. Syneresis of yogurts

Determination of the syneresis evidenced (Table 1) that yogurts made with 300 mg EPS/L presented lower ($p < 0.05$) syneresis than yogurts with 600 mg EPS/L, but no differences with the control group, at 3 days of storage. While at the end of the shelf life, yogurts with 600 mg EPS/L presented lower syneresis ($p < 0.05$) than both, yogurts with 300 mg/L and the control group. The improvement of the water holding capacity of yogurts with EPS has been found in previous studies, for example Zhang, Folkenberg, Amigo, and Ipsen (2016) reported that an EPS-producing strain of *L. bulgaricus* improved the water holding capacity in low fat stirred yogurts. The same tendency was observed by Amatayakul et al. (2006) for set yogurts with 14% solids. Besides, Buldo et al. (2016) concluded that stirred yogurts made with a starter culture that produces high levels of EPS presented decreased syneresis in comparison to the control. The increase of the water holding capacity of yogurts with EPS could be explained by Hassan et al. (2003) for yogurts made with EPS^+ strains, who reported that an incompatibility between the proteins and EPS exists, leading to a phase separation where the proteins form a dense network and the EPS locate in large pores rich in whey. On the other hand, yogurts with EPS^- cultures, presented a homogenous phase, with small and evenly distributed pores. Qin et al. (2011) had similar results, since they found that yogurt fermented with EPS-producing *S. thermophilus* 05–34 exhibited lower susceptibility to whey separation than the control group. Considering the changes occurred during time, the control group did not present significant differences between 3 and 25 days of storage, while yogurts with 300 mg EPS/L slightly increased and samples with 600 mg EPS/L decreased ($p < 0.05$) syneresis with time. This result indicates that the highest concentration of EPS could have a positive impact on the water holding capacity of the samples during time of storage.

All the analysis done showed the potential of this extract to be used as technofunctional natural ingredient, and it should be considered that its impact on human health is being evaluated too, with promising preliminary results (Ale et al., 2016). Supplementary studies based on changes in the matrix (increasing the level of total solids in milk or fat content, for example) are mandatory to optimize its application in dairy products, and to understand its behavior in samples more similar

Table 2
Weighted averages and perceived percentages of flavor attributes for control and EPS-added yogurts (with 300 and 600 mg EPS/L) at 3 and 25 days of storage at 4 °C.

	3 days						25 days					
	Control		300 mg/L		600 mg/L		Control		300 mg/L		600 mg/L	
	Weighted average	Perceived %	Weighted average	Perceived %	Weighted average	Perceived %	Weighted average	Perceived %	Weighted average	Perceived %	Weighted average	Perceived %
Milk powder	0.10	10	0.10	10	0.05	5	0.15	15	0.10	10	0.25	15
Acid	2.90	80	3.50	80	2.60	60	3.00	90	3.35	85	2.30	80
Sweet	1.30	30	0.85	25	1.40	20	0.60	20	0.20	10	0.25	15
Bitter	0.20	10	0.10	10	0.05	5	0.20	10	0.35	15	0.60	20
Cooked milk	0.35	15	0.40	20	0.45	15	0.30	20	0.25	25	0.30	10

to the industrial yogurts available in the market. Furthermore, the primary structure of this polymer, together with its interaction with the food matrix, will be confirmed in future studies in order to explain its influence on texture parameters from a molecular point of view.

4. Conclusions

The EPS from L. *fermentum* Lf2, used as an additive and at a level of 300 mg/L, provided yogurt with increased hardness and consistency index, and a lower flow behavior index than control samples, allowing the production of a yogurt with no sensory defects. Considering our results, we recommend using the EPS extract at a level of 300 mg/L, concentration that demonstrated beneficial effects on health in previous studies, and with the advantage of presenting affordable production costs. We conclude that this EPS could replace artificial additives commonly used in the industry, in order to improve yogurt texture and functionality.

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