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Culture Conditions Determine the Balance between Two Different Exopolysaccharides Produced by *Lactobacillus pentosus* LPS26[▽]

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Lactobacillus pentosus **LPS26, isolated from a natural fermentation of green olives, produces a capsular polymer constituted of two exopolysaccharides (EPS): EPS A, a high-molecular-weight (high-M_w) polysaccharide (1.9×10^6 Da) composed of glucose and rhamnose (3:1), and EPS B, a low-M_w polysaccharide (3.3×10^4 Da) composed of glucose and mannose (3:1). Fermentation experiments in a chemically semidefined medium with different carbon sources (glucose, fructose, mannitol, and lactose) showed that all of them except fructose supported EPS A production rather than EPS B production. The influence of temperature and pH was further analyzed. As the temperature dropped, increased synthesis of both EPS was detected. The control of pH especially enhanced EPS B production. With regard to this, the maximum total EPS production (514 mg liter^{-1}) was achieved at a suboptimal growth temperature (20°C) and pH 6.0. Continuous cultures showed that EPS A, synthesized mainly at low dilution rates, is clearly dependent on the growth rate, whereas EPS B synthesis was hardly affected. EPS production was also detected in supplemented skimmed milk, but no increase on the viscosity of the fermented milk was recorded. This could be linked to the high proportion of the low-M_w polysaccharide produced in these conditions in contrast to that observed in culture media. Overall, the present study shows that culture conditions have a clear impact on the type and concentration of EPS produced by strain LPS26, and consequently, these conditions should be carefully selected for optimization and application studies. Finally, it should be noted that this is, to our knowledge, the first report on EPS production by *L. pentosus*.**

Microbial exopolysaccharides (EPS) are a wide group of secreted polymers that can be tightly attached to the cell surface (capsular polysaccharides) or released as extracellular slime in the surroundings of the cell (20). In the natural environments, polysaccharides may be associated with virulence and cell protection against desiccation, osmotic stress, antibiotics, toxic compounds, and bacteriophage or protozoa attack (11, 40). Several food applications as emulsifiers, thickeners, viscosifiers, and stabilizers are known for these molecules (40). Among microbial EPS, those produced by lactic acid bacteria (LAB) are receiving increasing attention because these microorganisms have a 'food-grade' status. The rheological properties of EPS produced by LAB have found a major application in the manufacture of fermented dairy products such as yogurt, cheese, or fermented milks, although they also play a role in the elaboration of fermented meats and vegetables (2, 33, 42, 45). In addition, they have also been proven to have beneficial effects on human health such as cholesterol-lowering, antitumoral, and inmunomodulating activities and prebiotic effects (8, 29, 37). Some studies have also related the production of EPS in LAB with a partial resistance to phage infections, one of the main problems in the dairy industry (16, 24).

EPS from LAB can be composed of a single type of sugar monomer (homopolysaccharides) or several types of monomers (heteropolysaccharides [HePS]). Moreover, EPS produced by different strains vary in sugar composition, chain length, degree of branching, or sugar linkages. All of these factors determine the rheological and health-promoting properties of EPS (36, 37). EPS can be produced either in a bioreactor, followed by the appropriate downstream processing for their further use as food additives, or in situ during the fermentation process. Thus, they have great potential to become alternatives for the currently used stabilizers and thickeners. Thus far, the use of these EPS as bioingredients in the food industry depends on the economic yield of the process which, in many cases, is limited by the low quantities of polymers produced by LAB (40 to 600 mg liter^{-1}) compared to other polymers traditionally used such as xanthan or gellan (10 to 25 g liter^{-1}) produced by non-food-grade microorganisms (11). It should be also noticed that homopolysaccharides are produced to a much greater extent than HePS. In this regard, the conditions for efficient production of EPS in LAB should be optimized. It is known that the biosynthesis of EPS is strain dependent and influenced by fermentation conditions and medium composition (23). In fact, mesophilic strains seem to produce maximum levels of EPS in suboptimal conditions for the bacterial growth, whereas EPS production appears to be growth associated in thermophilic strains (10).

Lactobacilli are often used in mixed starters in the manufacture of dairy products. In addition, secondary microbiota of most ripened cheeses mainly consist of mesophilic lactobacilli that play an important role in flavor and texture development

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(47). Lactobacilli are also the predominant microbiota in "natural fermented" meat and vegetables and used as starters in controlled fermentations of these products (4). Within the lactobacillus group, *Lactobacillus pentosus* is a facultative heterofermentative microorganism that has been isolated from the adventitious nonstarter LAB in several cheese varieties (27, 39), and it is also involved in the fermentation of meat (13), fish (30), and vegetables (38).

EPS production by lactobacilli has been recently reported (1, 31, 41, 43). However, no EPS produced by *L. pentosus* has been characterized thus far. With regard to this, we have studied the production of two different EPSs by *L. pentosus* LPS26, a strain isolated from a natural fermentation of olives. The influence of culture conditions, the carbon source, and the growth rate on the yield and the composition of EPS are reported. According to our results, the ratio between these two EPSs can be modulated, and the production levels can be optimized by the culture conditions.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *L. pentosus* LPS26 was isolated from a Spanish-style green olive fermentation by J. L. Ruiz-Barba and R. Jiménez-Díaz at the Instituto de la Grasa-CSIC (Seville, Spain). This strain, formerly classified as *L. plantarum* according to its carbon fermentation profile (25), has been recently identified as *L. pentosus* (J. L. Ruiz-Barba, unpublished data) by molecular techniques (44). *Lactococcus lactis* subsp. *lactis* IPLA947 was isolated from an artisan cheese (9). Strains were routinely cultured at 30°C in MRS or M17 broth (Scharlab, Barcelona, Spain), respectively, and stored at −80°C with glycerol at 20% (vol/vol).

For EPS production, a modification of semidefined medium (SDM) was used (19). It contains Tween 80 (1 g liter^{-1}; Panreac, Barcelona, Spain), ammonium citrate (2 g liter^{-1}; Panreac), sodium acetate (5 g liter^{-1}; Sigma-Aldrich Chemie GmbH, Steinheim, Germany), $MgSO_4 \cdot 7H_2O$ (0.1 g liter^{-1}; Probus, Badalona, Spain), $MnSO_4$ (0.05 g liter^{-1}; Panreac), K_2HPO_4 (2 g liter^{-1}; Merck, Darmstadt, Germany), Difco yeast nitrogen base (5 g liter^{-1}; Becton Dickinson, Sparks, MD), and tryptone (10 g liter^{-1}; Scharlab, Beauvais, France). The medium (pH 6.0) was supplemented with the carbon source at the concentration indicated in each case.

Chemical treatment of cells surface. A method previously described was followed with slight modifications (16). Samples (each 10 ml) of overnight cultures at 30°C were washed and suspended in 5 ml of one of the following solutions, followed by incubation as indicated: 1% (wt/vol) sodium dodecyl sulfate, 30 min at 45°C; 0.05 M NaOH, 30 min at 20°C; and proteinase K (1.5 mg ml^{-1}), 30 min at 50°C.

Isolation and purification of EPS. A modification of the method previously described (41) was used. EPS from 10-ml samples of culture was dissociated from cells by sonication at 30 W for 5 min with pulses of 1 s using a Vibra Cell CV17 (Sonics & Materials, Inc., Danbury, CO) equipped with a 3-mm-diameter probe. Afterward, samples were treated with trichloroacetic acid (10% [wt/vol]) with agitation for 30 min and centrifuged at 10,000 × *g* for 10 min at 4°C for cell and protein removal. EPS was precipitated overnight with 2 volumes of ethanol at 4°C, redissolved in 1 ml of distilled water, and dialyzed (M_w cutoff of 12,000 to 16,000) for 48 h at 4°C with water replacement twice a day. Purified EPS was frozen or lyophilized for further quantification or composition analysis, respectively.

Quantification of EPS. To quantify each polymer produced by the strain *L. pentosus* LPS26, samples containing EPS were subjected to size-exclusion chromatography (SEC) in a high-pressure liquid chromatography (HPLC) system. Separation was carried out on a TSK-Gel G5000 PW_{XL} column protected by a TSK-Gel precolumn (Tosoh Corp., Tokyo, Japan). As the mobile phase, 0.1 M $NaNO_3$ was used with an operating temperature of 40°C, a flow rate of 0.6 ml min^{-1}, and an injection volume of 50 μl. The polymers were detected by refractive index using a 410 differential refractometer (Waters Corp., Milford, MA). A UV 996 detector (Waters) was used to detect the potential presence of proteins. Standard dextrans (Fluka-Chemie, Buchs, Switzerland) were used to calculate the molecular weights of the eluting peaks. The EPS concentration was determined by the integration of the refractive index signal with Millennium 2010 software (Waters Corp.) using calibration curves obtained with dextrans of 5×10^4 to 4.9×10^6 Da.

Monosaccharide analysis. Both EPS fractions (EPS A and EPS B) were separated by SEC from the EPS isolated from 400 ml of culture. The collected fractions were dialyzed for 48 h against distilled water to remove the $NaNO_3$ from the mobile phase and lyophilized. The sugar composition was determined by gas chromatography according to the alditol acetate method (48). Samples were dissolved in acetone (1 μl per μg of EPS) and analyzed by using a Series II Hewlett-Packard 5890 instrument coupled to a Hewlett-Packard 5972 selective mass detector and a fused silica capillary column (0.25 mm by 30 m; SPTM 2330) (Sigma-Aldrich) operated in splitless mode. The oven temperature was held at 220°C.

Fermentation conditions. All of the fermentation assays were performed in SDM in a working volume of 750 ml. Inoculation was at 2% (vol/vol) with overnight cultures grown at 30°C in SDM containing glucose (20 g liter^{-1}). Glass flasks were used for uncontrolled-pH fermentations. For batch and continuous fermentation at controlled pH, a 2-liter bioreactor vessel (Biostat B; Braun-Biotech International GmbH, Melsungen, Germany) was used. pH values were measured with an InPro 3000/225 (Mettler-Toledo, France) pH probe and controlled by the automatic addition of 5.7 N NH_4OH. The agitation speed was 150 rpm. Continuous cultures were started in batch mode. When the culture reached the exponential phase, continuous culture was established by pumping in fresh medium, starting at the lowest dilution rate (D). Dilution rates varied from (0.02 to 0.11 h^{-1}). A period of three residence times was found to be sufficient to obtain steady-state conditions.

Several carbon sources (glucose, lactose, fructose, and mannitol), sugar concentrations (5, 20, 30, and 40 g liter^{-1}), temperatures (20, 25, and 30°C), and pH levels (6.0, 5.0, and not controlled) were used depending on the assay.

Samples from batch and continuous cultures were taken, and the bacterial growth (expressed as CFU ml^{-1}), pH (in uncontrolled-pH cultures), and EPS production were determined. Decimal dilutions of culture samples were made in quarter-strength Ringer solution (Oxoid, Basingstoke, Hampshire, United Kingdom). Appropriate dilutions were plated in duplicate on MRS agar (Scharlab) and incubated for 48 h at 30°C. Sugars and organic acids were simultaneously determined by HPLC (5).

Kinetic parameters. In batch culture assays, the maximum specific growth rate (μ_{max}) was experimentally determined in the exponential growth phase as $\mu_{max} = [\ln(X_1/X_0)]/(t_1 - t_0)$, where X_0 and X_1 are the numbers of CFU ml^{-1} and t_0 and t_1 are the times along the exponential phase, respectively. The EPS yield (Y_{EPS}) was expressed as milligrams of EPS produced per gram of sugar consumed, and the volumetric productivity (P_v) was expressed as milligrams of EPS produced per liter per hour. For the calculation of these parameters, maximum bacterial growth (determined at the end of the exponential phase for each experiment) and maximum EPS production (determined at the end of the incubation period) were used. In continuous cultures, kinetic parameters were determined when steady-state conditions were assessed. In this case, P_v was calculated as milligrams of EPS produced per liter at each dilution rate (D).

Milk cultures. Pure and mixed cultures containing either *L. pentosus* LPS26 alone or both *L. pentosus* LPS26 and *L. lactis* subsp. *lactis* IPLA947 were prepared in UHT commercial skimmed milk supplemented with 2% of powder skimmed milk (Scharlab). Overnight cultures in MRS or M17, respectively, were used as inocula to give an initial population of about 2×10^7 CFU ml^{-1} for *L. pentosus* LPS26 and 7.5×10^6 CFU ml^{-1} for *L. lactis* subsp. *lactis* IPLA947, and cultures were incubated at 25°C for 72 h. When indicated, milk was supplemented with glucose (0.5% [wt/vol]) and yeast extract (0.5% [wt/vol]). LPS26 counts were performed by plating appropriate dilutions on MRS agar (Scharlab) supplemented with vancomycin (30 mg ml^{-1}). IPLA947 counts were performed on M17 agar (Scharlab). Plates were prepared in duplicate and incubated for 48 h at 30°C. Isolation and quantification of EPS was performed as described above. To discard any influence of yeast extract present in supplemented milk on EPS quantification, uninoculated samples were tested to check for the absence of interfering polymers. The viscosity of the fermented milks was determined according to the Posthumus method (46) using a pure culture of *L. lactis* subsp. *lactis* IPLA947 as a reference sample.

Statistical analysis. Data were analyzed with a computer software package (SPSS, Chicago, IL) using analysis of variance and the least-significance-difference test ($P < 0.01$).

RESULTS

L. pentosus LPS26 displayed a soft, loose pellet after centrifugation. In MRS-agar, colonies had a ropy phenotype, in-

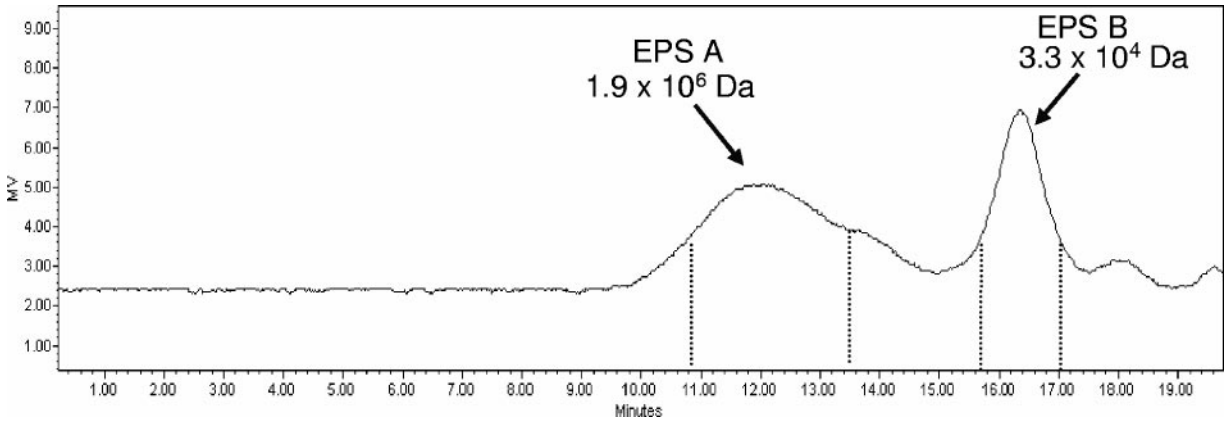

FIG. 1. Size exclusion chromatogram showing the two EPS produced by *L. pentosus* LPS26. Dotted lines flank the fractions collected for sugar composition analysis of the two EPS.

dicating the production of a polymer. In order to confirm its polysaccharidic nature, a preliminary assay consisting of a chemical treatment of the cells surface was carried out. Treatment with proteinase K or SDS did not affect the mucoid aspect of the pellet, whereas treatment with NaOH resulted in a nonviscous pellet, suggesting the presence of polysaccharides (16).

EPS isolation and sugar composition. *L. pentosus* LPS26 was grown in SDM supplemented with glucose (30 g liter^{-1}) at 30°C without pH control for 72 h. At first, no EPS could be isolated from culture supernatants, indicating that it should be entirely attached to the cell surface. As a matter of fact, the mild sonication of the cultures allowed the release of EPS from the cell surface and the formation of a hard, nonviscous pellet after centrifugation. These observations suggested that the EPS produced by *L. pentosus* LPS26 was a capsular polysaccharide. EPS was subsequently extracted from the supernatants and applied on an SEC column in an HPLC system. The analysis revealed the presence of two peaks corresponding to a high-molecular-weight (high-M_w) polymer (EPS A) and a low-M_w polymer (EPS B) with molecular sizes of 1.9×10^6 and 3.3×10^4 Da, respectively (Fig. 1).

Analysis by gas chromatography-mass spectrometry revealed differences in the monosaccharide composition of both EPS (Table 1). EPS A was composed of glucose and rhamnose in a molar ratio of approximately 3:1, whereas EPS B consisted of glucose and mannose in a similar proportion.

EPS production in batch cultures. Several parameters, such as carbon source and concentration, pH, and temperature, were tested to determine the optimal conditions for the production of each EPS.

TABLE 1. Composition of the two EPS produced by *L. pentosus* LPS26[a]

EPS	Sugar composition (%)		
	Glucose	Rhamnose	Mannose
A	73	27	0
B	76	0	24

[a] Incubation was performed in batch cultures at 30°C without pH control in SDM supplemented with glucose (30 g liter^{-1}) as the carbon source.

The influence of carbon source on growth and EPS production was studied in SDM with glucose, fructose, and mannitol (sugars present in olive flesh) and lactose. The concentration of carbon source was 30 g liter^{-1}, and the temperature of incubation was 30°C. Table 2 summarizes the results obtained. Mannitol and fructose provided the highest (2.4×10^9 CFU ml^{-1}) and the lowest (1.5×10^8 CFU ml^{-1}) viable counts, respectively, and no significant differences were detected between mannitol and glucose ($P > 0.01$). Fructose and mannitol supported the lower EPS synthesis ($P < 0.01$), whereas glucose and lactose were more favorable sugar sources for EPS production. The synthesis of the high-M_w EPS (EPS A) seemed to be more dependent on the carbon source than the low-M_w EPS (EPS B) since larger variations were detected in the former (Table 2). A higher ratio of EPS A to EPS B was obtained with mannitol, glucose, and lactose. However, an inverse ratio was detected with fructose (Table 2). Slight variations in the constituent monosaccharides of both EPS were observed within the different carbon sources. Glucose content ranged from 71 to 80%, and the rhamnose content ranged from 20 to 29% in EPS A, whereas the glucose content ranged from 60 to 76%, and the mannose content ranged from 24 to 30% in EPS B (data not shown). For subsequent assays, glucose was selected as the carbon source.

To determine any effect of glucose concentration, batch cultures were performed at 30°C for 72 h, and polymer synthesis

TABLE 2. Effect of carbon source on growth and EPS production by *L. pentosus* LPS26[a]

Carbon source	Amt of EPS (mg liter^{-1})[b]			Maximum growth[c] (CFU ml^{-1})	EPS A/EPS B ratio
	EPS A	EPS B	Total		
Fructose	26.3^{a}	55.6^{c}	81.9^{a}	1.5×10^{8a}	3:7
Mannitol	91.2^{b}	34.6^{a}	125.8^{b}	2.4×10^{9c}	7:3
Glucose	134.0^{d}	32.0^{a}	166.0^{c}	1.3×10^{9c}	8:2
Lactose	116.6^{c}	48.3^{b}	164.9^{c}	5.8×10^{8b}	7:3

[a] Incubation was performed in batch cultures at 30°C without pH control for 72 h in SDM supplemented with 30 g of each carbon source liter^{-1}.

[b] Data are mean values ($n = 3$). Within the same column, means with the same superscript letter are not significantly different ($P > 0.01$).

[c] Data are mean values ($n = 3$). Within the column, means with the same superscript letter are not significantly different ($P > 0.01$).

TABLE 3. Effect of glucose concentration on growth and EPS production by *L. pentosus* LPS26[a]

Glucose concn (g liter^{-1})	Amt of EPS (mg liter^{-1})[b]			Maximum growth[c] (CFU ml^{-1})	Y_{EPS}[c] (mg of EPS g of glucose^{-1})
	EPS A	EPS B	Total		
5	84.9^{a}	49.9^{b}	134.80^{a}	3.6×10^{8a}	41.3^{d}
20	101.8^{b}	47.3^{b}	149.2^{b}	8.5×10^{8b}	10.1^{b}
30	134.6^{d}	32.1^{a}	166.7^{c}	1.3×10^{9b}	12.4^{c}
40	125.5^{c}	48.4^{b}	173.8^{d}	7.2×10^{9c}	8.2^{a}

[a] Incubation was performed in batch cultures at 30°C without pH control for 72 h in SDM supplemented with glucose as the carbon source.
[b] Data are mean values ($n = 3$). Within the same column, means with the same superscript letter are not significantly different ($P > 0.01$).
[c] Data are mean values ($n = 3$). Within the column, means with the same superscript letter are not significantly different ($P > 0.01$).

was tested at different concentrations of glucose (Table 3). Higher viable counts and an extended exponential phase (12 to 24 h) were observed as the sugar concentration increased. No significant differences were observed between glucose at 20 and 30 g liter^{-1} ($P > 0.01$). Regarding the total EPS production, it was first detected after 8 h of incubation, and a clear increase in synthesis was observed as the initial glucose concentration increased ($P < 0.01$). In contrast, the highest EPS yield (41.3 mg of EPS g of glucose consumed^{-1}) was attained with glucose at 5 g liter^{-1}, whereas it was kept in a narrow range (8.2 to 12.4 mg of EPS g of glucose^{-1}) at between 20 and 40 g of glucose liter^{-1} ($P < 0.01$). The ratio of EPS A to EPS B remained practically unchanged whatever the glucose concentration. On the other hand, metabolic end products were also analyzed in these batch fermentations. Increasing concentrations of glucose in the culture medium led to a gradual increase in lactate production (70 to 140 mM). This metabolite accounted for 76 to 82% of the sugar consumed, although acetate (41 to 57 mM) and, to a lesser extent, formate (0.4 to 0.8 mM) were also accumulated at the end of the batch runs. A residual carbon source (between 26 and 55% of the initial concentration) was detected in all of the assays (data not shown).

Batch cultures were also performed with glucose (30 g liter^{-1}) as the carbon source at three different incubation temperatures (20, 25, and 30°C) for 72 h without pH control (Fig. 2). Temperature affected the production of both EPS, and an inverse relationship between temperature and EPS production was mainly observed with EPS A ($P < 0.01$). Consequently, total polymer synthesis determined after 72 h of incubation was about 1.2- and 1.6-fold higher at 20°C than at 25 and 30°C, respectively. The EPS yield was also higher at 20°C (23.1 mg g of glucose^{-1}) than at 25°C (17.6 mg g of glucose^{-1}) and 30°C (12.4 mg g of glucose^{-1}) ($P < 0.01$). In contrast, the lower temperature resulted in lower viable counts, with the differences being significant between 20 and 25°C.

The influence of pH (pH 6.0, 5.0, and no pH control) on bacterial growth and EPS synthesis was determined in batch cultures performed with glucose (30 g liter^{-1}) as the carbon source at 20°C for 72 h. A clear influence of pH EPS production was observed (Fig. 3). The maximum total EPS production reached under uncontrolled pH conditions (pH dropped to 3.88) was 265 mg liter^{-1} compared to 352 mg liter^{-1} at pH 5.0 and 511 mg liter^{-1} at pH 6.0. The control of pH favored the rise of both EPS ($P < 0.01$), but the increase of EPS B was more pronounced. Higher cell viability was also detected at pH 5.0 ($P < 0.01$). Regarding the EPS yield, it was notably higher at pH 5.0 (38.7 mg g of glucose^{-1}) than at pH 6.0 (22.4 mg g of glucose^{-1}) or at a uncontrolled pH (23.1 mg g of glucose^{-1}) ($P < 0.01$). It should be also mentioned that fermentation under controlled pH conditions showed a shift to a mixed-acid metabolism, resulting in a clear increase in formate production (108 mM) compared to the low level detected under an uncontrolled pH (0.55 mM) (data not shown).

We have, therefore, defined the growth conditions that could be picked from batch cultures with the purpose of EPS production by *L. pentosus* LPS26. These conditions consisted

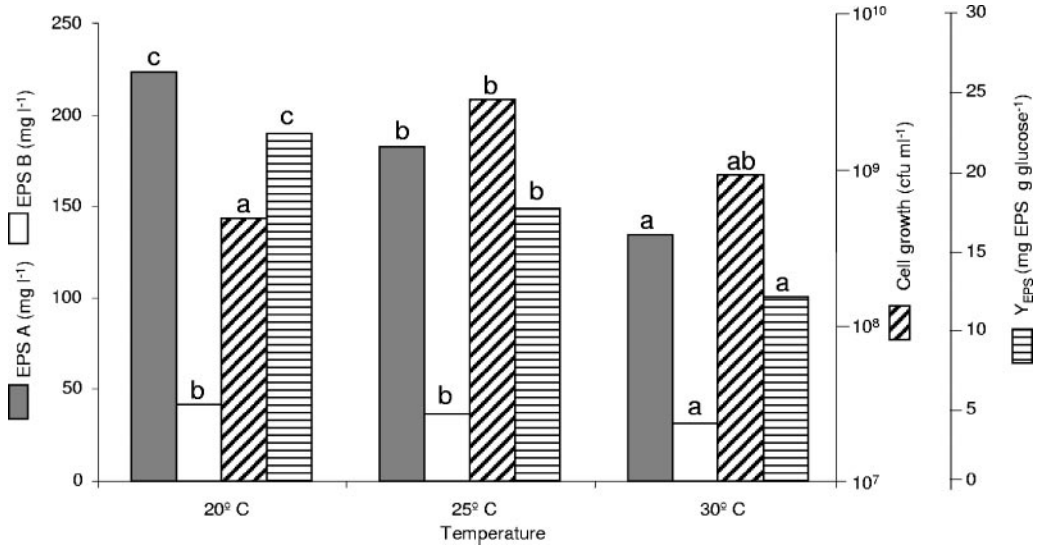

FIG. 2. Effect of temperature on growth and EPS production by *L. pentosus* LPS26. Incubation was performed in batch cultures without pH control in SDM supplemented with glucose (30 g liter^{-1}). The data are mean values ($n = 3$). Within the same variables (i.e., EPS production, cell growth, and EPS yield), means with the same lowercase letter are not significantly different ($P > 0.01$).

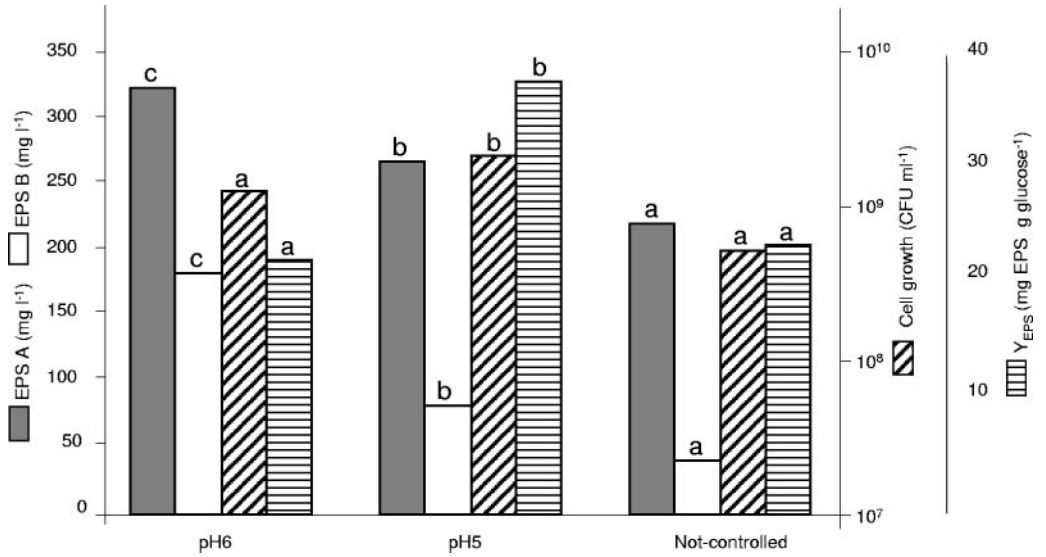

FIG. 3. Effect of pH on growth and EPS production by *L. pentosus* LPS26. Incubation was performed in batch cultures at 20°C in SDM supplemented with glucose (30 g liter^{-1}). The data are mean values ($n = 3$). Within the variables (i.e., EPS production, cell growth, and EPS yield), means with the same lowercase letter are not significantly different ($P > 0.01$).

of SDM with glucose at 30 g liter^{-1} (pH 6.0) and an incubation temperature of 20°C. In this way, we can ensure a total EPS production of 511 mg liter^{-1}.

EPS production in continuous culture. Continuous cultures were used to accurately study the influence of the growth rate on the synthesis of both EPS. Taking into account the growth conditions that provided the highest levels of EPS in batch cultures, a range of dilution rates (D) (0.02 to 0.11 h^{-1}) was assayed. As expected, steady-state growth, as assessed by the CFU ml^{-1}, was maintained at the different dilution rates since the critical growth rate (0.12 h^{-1}) corresponding to the μ_{max} calculated from batch cultures was not exceeded. The highest EPS A production (336.3 mg liter^{-1}) was noted at D = 0.02 h^{-1} (14% of μ_{max}), and higher dilution rates resulted in a considerable decrease (Fig. 4). On the other hand, the production of EPS B did not seem to be affected by the growth rate since a low amount (<50 mg liter^{-1}) was produced at dilution rates ranging from 0.02 to 0.07 h^{-1}. At the highest D value (92% of μ_{max}), EPS B was hardly detected (2.5 mg liter^{-1}). Consequently, the total EPS, mainly composed of EPS A,

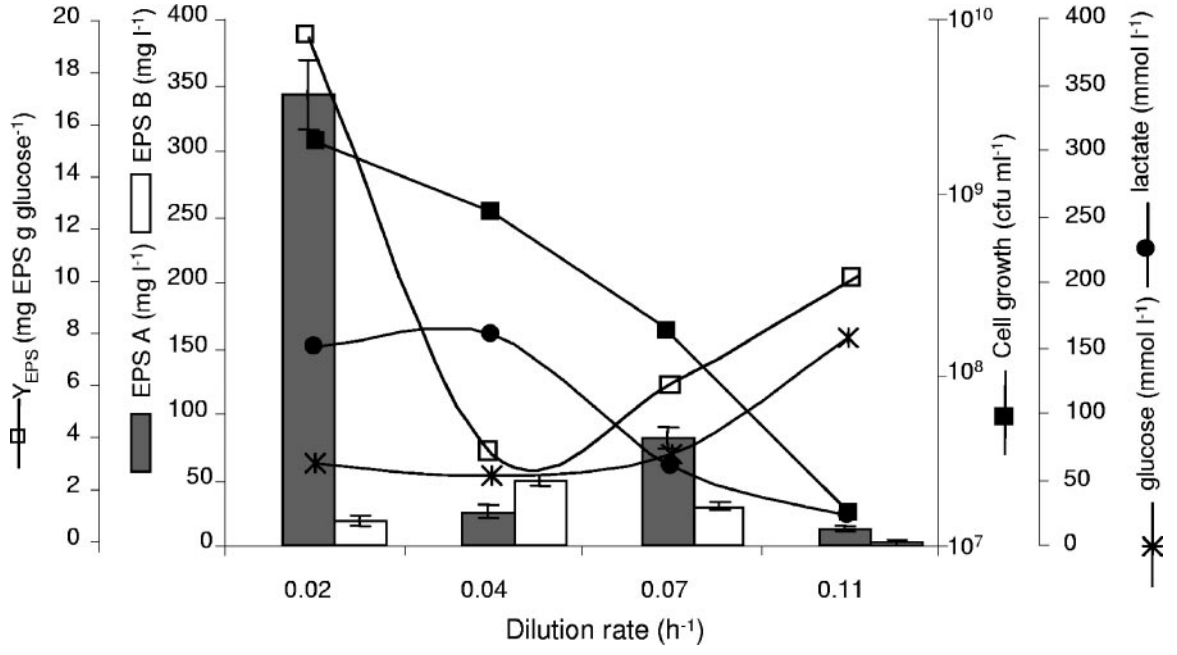

FIG. 4. Effect of dilution rate on growth and EPS production by *L. pentosus* LPS26 in continuous culture. Incubation was performed at 20°C and pH 6.0 in SDM supplemented with glucose (30 g liter^{-1}). The data are means of two independent experiments.

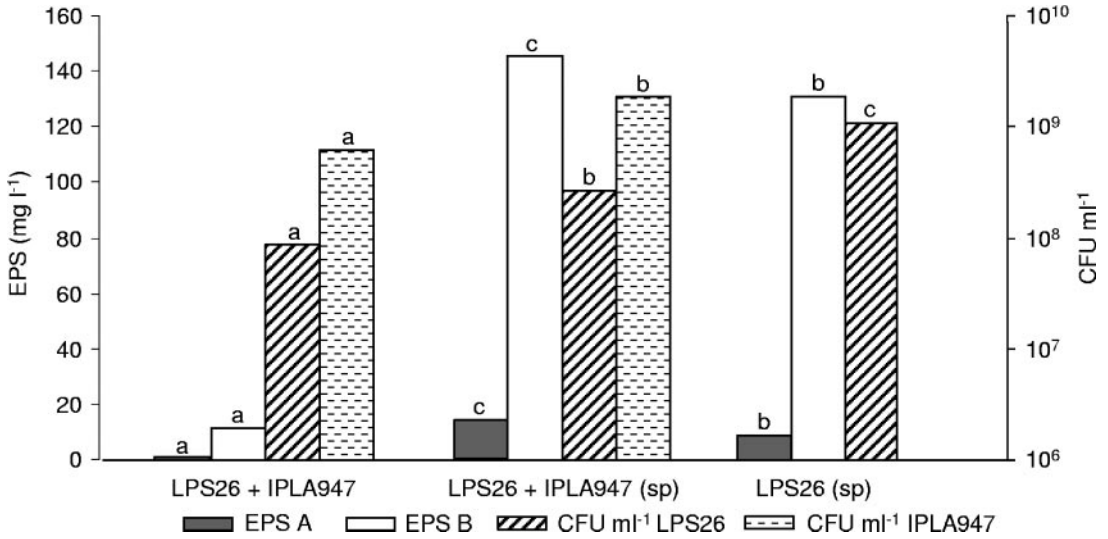

FIG. 5. Growth and EPS production of EPS by *L. pentosus* LPS26 in pure and mixed dairy cultures. Cultures were incubated at 25°C for 72 h. "(sp)" indicates milk supplemented with glucose (0.5% [wt/vol]) and yeast extract (0.5% [wt/vol]). The data are mean values ($n = 3$). Within the same variables (i.e., EPS production, cell growth, and EPS yield), means with the same lowercase letter are not significantly different ($P > 0.01$).

showed a maximum (354.3 mg liter^{-1}) at the lowest D value (0.02 h^{-1}).

Residual glucose was detected throughout the range of dilution rates. Similar sugar consumption (ca. 67% of initial concentration) and lactate production (150 mM) were observed at low D (0.02 to 0.04 h^{-1}). At higher D values (0.07 to 0.11 h^{-1}), increasing residual glucose and decreasing lactate concentrations were detected. Organic acids other than lactate, such as acetate (16.5 mM) and formate (4.1 mM), were also detected over the range of dilution rates, with no substantial variation on their concentrations (data not shown). It should be noticed that bacterial growth and, in particular, EPS production were notably higher at D = 0.02 h^{-1}. This D value provided also the maximum EPS yield (18.9 mg of EPS g of glucose^{-1}) and a volumetric productivity of 7.08 mg liter^{-1} h^{-1}.

Production of EPS in milk. To evaluate the potential of the EPS-producing strain *L. pentosus* LPS26 as an adjunct culture for the elaboration of fermented dairy products and to measure the amount and EPS ratio produced in milk, fermentations were carried out in milk. *L. pentosus* LPS26 alone was not able to grow in milk, most likely due to a low proteolytic activity on milk casein (data not shown). Therefore, supplementation of milk with glucose and yeast extract was required. In single cultures performed in supplemented milk, LPS26 reached 10^9 CFU ml^{-1}and produced 140.1 mg of total EPS liter^{-1}. Cocultures of LPS26 and the acidifying strain *L. lactis* IPLA947 were also performed in both milk and supplemented milk. In milk, EPS production was rather low (12.8 mg liter^{-1}), with EPS B being predominantly produced. LPS26 reached levels of 6.3×10^7 CFU ml^{-1}, whereas viable counts of 6.2×10^8 CFU ml^{-1} were detected for IPLA947. Supplementation of milk resulted in both a clear rise of the microbial counts (2.5×10^8 CFU of LPS26 ml^{-1} and 1.8×10^9 CFU of IPLA947 ml^{-1}) and a notable increase in the total EPS production (162.9 mg liter^{-1}), mainly due to the great increase showed by EPS B (Fig. 5). As a whole, supplementation of milk significantly affected both EPS production and viable counts ($P < 0.01$). Unfortunately, no differences in the viscosity of fermented milks were observed in the presence of the EPS-producing strain LPS26.

DISCUSSION

This is, to our knowledge, the first report on EPS produced by a strain of *L. pentosus*. The strain *L. pentosus* LPS26, involved in green-olive fermentation, produces a capsular polymer consisting of two EPS that differ in size and sugar composition. The synthesis of more than one type of EPS by LAB has been previously reported (11, 18, 26). For instance, the production of two polymers of 8.5×10^5 and 4×10^4 Da and different sugar compositions was observed in *L. plantarum* EP56 (41).

Glucose and rhamnose present in EPS A are usual components of many EPS characterized to date (22, 34), whose mechanisms for the synthesis of their sugar-nucleotide precursors and incorporation to the polysaccharide chain are well known (12). In contrast, there are no data in the literature regarding metabolic routes that could explain the incorporation of mannose units in HePS (as is the case for EPS B) produced by LAB, although the presence of this sugar has been already described (21). Some authors suggest that the detection of sugars such as mannose, arabinose, or xylose is due to contamination from material coming from cell wall components present in the medium composition, such as glucomannans from yeast extract or peptone, that interfere with the isolation, purification, and structural characterization of EPS (6). However, the use of a medium such as the SDM minimizes the presence of these interfering components (19). Furthermore, analysis performed in uninoculated medium processed in the same way as the cultures did not show the presence of any polymer (data not shown), confirming that mannose was a genuine component of EPS B.

Our results revealed that culture conditions have a clear

impact on growth and EPS production by *L. pentosus* LPS26. We were able to improve EPS production by up to 511 mg liter^{-1} in batch cultures, a relatively high value compared to those generally described for HePS produced by LAB, for which EPS production ranges between 150 and 600 mg liter^{-1} (7). Nevertheless, it is far from the EPS amount produced by *L. rhamnosus* RW-9595M (1,275 mg liter^{-1}), described as the highest EPS-producing strain among LAB (14). Remarkably, the tested conditions differently affected the production of the two EPS in our strain, and thus the ratio between both EPS can be modulated.

The carbon source has a marked influence on growth and EPS production by *L. pentosus* LPS26 and also on the ratio of both EPS. Glucose provided higher EPS production and clearly supported EPS A synthesis over EPS B. In contrast, fructose favored the synthesis of EPS B. Similar observations were reported for *L. delbrueckii* subsp. *bulgaricus* NCFB 2772, which produced a high-M_w EPS and a low-M_w EPS with a molar ratio of 1:1 in glucose, and the low M_w EPS was nearly the only one in fructose (18). In our case, certain uncoupling between growth and EPS production occurred in *L. pentosus* LPS26, since the maximum viable counts (detected after 12 to 24 h of incubation) did not correspond with the maximum EPS production (detected after 72 h of incubation). This has been also described in other *Lactobacillus* (15) and *Lactococcus* (23) species. We have only observed slight differences in EPS production using different glucose concentrations. Interestingly, the EPS yield, i.e., the amount of EPS produced per glucose consumed, was highest at the lowest sugar concentration, suggesting that an excess of the sugar source is not needed to stimulate EPS synthesis, but it may even decrease the efficiency of its synthesis.

The increase of EPS production at low temperatures, as happened with EPS A and EPS B, seems to be a common feature of mesophilic EPS-producing LAB, in which suboptimal growth conditions result in improved EPS production (10). To explain this, it has been suggested that slowly growing cells exhibit much slower cell wall polymer biosynthesis and that more lipid carrier precursor molecules are available for EPS biosynthesis (40). Control of the pH also enhanced the EPS production by *L. pentosus* LPS26. Control of the pH fostered mainly the synthesis of EPS B, although EPS A production was also favored. Again, certain uncoupling between growth and EPS production was observed, since the maximum viable counts were reached at pH 5.0, and pH 6.0 resulted in the maximum EPS production, in contrast to previous reports (17, 28).

The results obtained with continuous cultures indicate that EPS A and EPS B have different production kinetics. EPS A is mainly synthesized at a very low D value (0.02 h^{-1}); hence, its production would be considered non-growth associated. This finding is in agreement with the results obtained in batch cultures where the production of EPS A was mainly detected at the end of the exponential phase and maintained for at least 72 h (data not shown). In contrast, the production of EPS B did not seem to be affected by the growth rate. Similar sugar consumption and lactate production was observed at a lower D (0.02 to 0.04 h^{-1}), although great differences in EPS production were observed. This finding suggests that glucose consumed at the lowest dilution rate is more efficiently rerouted toward EPS synthesis. Thus, after sugar uptake an important proportion of carbon source is metabolized toward the formation of the sugar nucleotides precursors involved in EPS biosynthesis (3).

Continuous culture could outperform batch culture for the production of a potential bioingredient such as EPS produced by LAB by eliminating the inherent idle time for cleaning and sterilization and the long lags before the organisms enter a period of high productivity. Moreover, continuous culture allows a higher automation of processes and lowers labor costs. In this regard, the greatest volumetric productivity (7.08 mg liter^{-1} h^{-1}) obtained with a D of 0.02 h^{-1} was similar to that calculated for batch culture in the same conditions (7.13 mg liter^{-1} h^{-1}). However, the time required for cleaning and sterilization in batch cultures was not taken into consideration for this calculation, and thus the volumetric productivity obtained with continuous culture would be actually higher.

In the present study, we have also evaluated the potential application of *L. pentosus* LPS26 for the manufacture of dairy fermented products as an in situ EPS producer. The results showed that EPS B was the main polymer produced in milk cultures, in contrast to what we observed in SDM, where EPS A was the most abundant EPS type. This is another example of the effect that medium composition has on the ratio of different EPS produced by a particular strain. On the other hand, no effect of the EPS on the viscosity of the fermented milks was observed. This could be explained by an insufficient EPS production. However, similar or even lower EPS concentrations have been shown to be enough to have a significant effect on viscosity (36). Therefore, this lack of thickening effect may be related to the structural characteristics of EPS B. In this regard, molecular size, branching degree, or type of linkages are determining factors for the rheological properties of EPS (36). Mainly, molecular size seems to be a critical parameter for determining the viscosity-intensifying properties of EPS since high-M_w polymers possess a higher intrinsic viscosity (32, 35). In this assay, therefore, the absence of higher viscosity could be explained by the synthesis, almost exclusively, of EPS B. Culture conditions that enhance production of the high-M_w polysaccharide (EPS A) in milk should therefore be determined.

Further research is being performed to determine the structures of both EPS to get a deeper insight into the structure-function relationships regarding their applications as texturing agents for both dairy and nondairy products and their potential prebiotic effect.

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