# Comparative analysis of production and purification of homo- and hetero-polysaccharides produced by lactic acid bacteria

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**Abstract**

Lactic acid bacteria (LAB) produce homopolysaccharides (HoPS) and heteropolysaccharides (HePS) with potential functional properties. In this work, we have performed a comparative analysis of production and purification trials of these biopolymers from bacterial culture supernatants. LAB strains belonging to four different genera, both natural as well as recombinant, were used as model systems for the production of HoPS and HePS. Two well characterized strains carrying the *gft* gene were used for β-glucan production, *Pediococcus parvulus* 2.6 (*P. parvulus* 2.6) isolated from cider, and the recombinant strain *Lactococcus lactis* NZ9000[pGTF] (*L. lactis* NZ9000[pGTF]). In addition, another cider isolate, *Lactobacillus suebicus* CUPV225 (*L. suebicus* CUPV225), and *Leuconostoc mesenteroides* RTF10 (*L. mesenteroides* RTF10), isolated from meat products were included in the study. Chemical analysis of the EPS revealed that *L. mesenteroides* produces a dextran, *L. suebicus* a complex heteropolysaccharide, and the β-glucan producing-strains the expected 2-substituted (1,3)-β-glucan.

**Keywords:** lactic acid bacteria, homopolysaccharides, heteropolysaccharides, production, purification, methods.

## 1. Introduction

Many LAB synthesize extracellular polysaccharides (exopolysaccharides, EPS), which remain attached to the outer cell wall forming a capsule or are released into the environment in the form of slimy, or ropy, EPS. These bacteria produce a wide variety of EPS with different composition, structure, molecular mass, and conformation. Most LAB synthesize HePS usually composed of D-galactose, D-glucose, and L-rhamnose. These polysaccharides are produced from sugar nucleotides by the activity of intracellular glycosyltransferases (Welman & Maddox, 2003). Other LAB produce HoPS containing only D-glucose (*e.g.*: dextran, reuteran, and mutan) or D-fructose (*e.g.*: levan and inulin) (Monsan, Bozonnet, Albenne, Joucla, Willemot & Remaud-Simeon, 2001). HoPS are usually synthesized by extracellular glycansucrases (glucan- or fructan-sucrases) using sucrose as the glycosyl donor. EPS formation by glycansucrases has been reported for strains of *Lactobacillus* (Kralj, van Geel-Schutten, Dondorff, Kirsanovs, van der Maarel & Dijkhuizen, 2004; Tieking, Korakli, Ehrmann, Ganzle & Vogel, 2003), *Leuconostoc* (Bounaix et al., 2010; Fraga Vidal et al., 2011; Seymour & Knapp, 1980), and *Weissella* (Galle, Schwab, Arendt & Gaenzle, 2010) species. LAB strains belonging to the *Pediococcus*, *Lactobacillus*, and *Oenococcus* genera, isolated from cider and wine, produce a 2-substituted (1, 3)-β-D-glucan (Dols-Lafargue, Lee, Le Marrec, Heyraud, Chambat & Lonvaud-Funel, 2008; Dueñas-Chasco et al., 1997; Dueñas-Chasco, Rodríguez-Carvajal, Tejero-Mateo, Espartero, Irastorza-Iribas & Gil-Serrano, 1998; Ibarburu et al., 2007; Llaubères, Richard, Lonvaud, Dubourdieu & Fournet, 1990). This β-glucan is synthesized in *Pedicoccus parvulus*, *Lactobacillus suebicus*, and *Oenococcus oeni* by a single GTF glycosyltransferase, which is a membrane-bound protein that polymerizes glucosyl residues from UDP-glucose (Garai-Ibabe et al., 2010; Velasco, Yebra, Monedero, Ibarburu, Dueñas & Irastorza, 2007; Werning, Corrales, Prieto, Fernández de Palencia, Navas & López, 2008; Werning et al., 2006).

The HePS from LAB play an important role in the rheology, texture and 'mouthfeel' of fermented milks (yoghurt, viili, långfil, etc.) (Welman & Maddox, 2003) and other fermented products, such as sorghum sourdoughs (Galle, Schwab, Arendt & Gaenzle, 2011). HoPS producers have been evaluated to a lesser extent and are used mainly for fermentation of non-dairy products. Dextrans from *L. mesenteroides* and *Weissella* spp. as well as levan from *Lactobacillus sanfranciscensis* positively affect dough rheology and bread texture (Di Cagno et al., 2006; Waldherr & Vogel, 2009). The analysis of the rheological properties of 2-substituted β-D-glucan showed that it has potential utility as a biothickener (Lambo-Fodje, Leeman, Wahlund, Nyman, Oste & Larsson, 2007; Velasco, Areizaga, Irastorza, Dueñas, Santamaría & Muñoz, 2009)**.** Testing of this β-glucan producing LAB for the production of a fermented oat product (Martensson, Oste & Holst, 2002), yogurt, and various beverages (Elizaquível et al., 2011; Kearney et al., 2011) indicated advantageous techno-functional properties of these strains.

Additionally, prebiotic effects of several EPS have also been demonstrated (Hongpattarakere, Cherntong, Wichienchot, Kolida & Rastall, 2011; Korakli, Ganzle & Vogel, 2002).

Furthermore, health benefits have been claimed for EPS from LAB because of their putative antitumoral, immunostimulatory, and blood cholesterol lowering activities (Liu, Tseng, Chiang, Lee, Hsua & Pan, 2011; Welman & Maddox, 2003). In addition, β-glucan-producing *Pediococcus* (Fernández de Palencia et al., 2009) and *Lactobacillus* (Garai-Ibabe et al., 2010) are able to immunomodulate macrophages, and human consumption of oat-based food prepared with it resulted in a decrease of serum cholesterol levels, boosting the effect previously demonstrated for (1,3)-β-D-glucans in oats (Martensson et al., 2005).

Therefore, EPS from LAB are of great interest for Agro-Food industries since they have a vast structural diversity, which opens the way to innovations. Moreover, several LAB species have a "Generally Recognized As Safe" status by the American Food and Drug Association or a

93 "Qualified Presumption of Safety" status by the European Food Safety Authority (Gueimonde,
94 Frias & Ouwehand, 2006). This fact facilitates the application of their polysaccharides either as
95 additives or as *in situ*-produced thickeners. On the other hand, the low production of
96 polysaccharides by the majority of LAB species has hampered both their molecular
97 characterization and their commercial exploitation.
98 Thus, optimized methodologies of EPS production and recovery are required in order to
99 facilitate their characterization, and thereby explore the structural diversity of LAB EPS and
100 potential applications. The aim of the present study is, therefore, to identify optimized
101 procedures for the synthesis and analysis of structurally different EPS produced by a variety of
102 LAB species isolated from meat fermented products and beverages as well as by recombinant
103 strains.
104

105 **2. Material and methods**

106 *2.1. Bacterial strains and growth conditions*

107 Strains used in this study were from the authors' culture collections and had been previously
108 characterized and identified into species. *L. mesenteroides* RTF10 was isolated from meat
109 products (Chenoll, Macián, Elizaquível & Aznar, 2007) at the Department of Food
110 Biotechnology**,** Institute of Agrochemistry and Food Technology (C.S.I.C., Valencia, Spain).
111 *P. parvulus* 2.6 (Dueñas-Chasco et al., 1997) and *L. suebicus* CUPV225 were isolated from
112 ropy cider at the Department of Applied Chemistry, Faculty of Chemistry (University of the
113 Basque Country UPV/EHU, San Sebastián, Spain). *P. parvulus* 2.6 carries the gene *gtf* that
114 encodes the GTF glycosyltransferase, which catalyzes the synthesis of the 2-substituted-β-D-
115 glucan. The recombinant strain *L. lactis* NZ9000[pNGTF] carries the plasmid pNGTF
116 containing the *gtf* gene under the control of the $P_{NisA}$ promoter, inducible by the addition of
117 nisin to the growth medium (Werning, Corrales, Prieto, Fernández de Palencia, Navas &

López, 2008). Strains were kept in Man Rogosa Sharpe (MRS) broth (Pronadisa, Madrid, Spain), except *L. lactis* NZ9000 [pNGTF], which was maintained in ESTY medium (Pronadisa, Madrid, Spain), supplemented with 20% (v/v) glycerol for long-term storage at -80 ºC.

*2.2. Production of EPS by natural LAB strains*

To obtain inocula for EPS production, *L. mesenteroides* RTF10 was grown in MRS supplemented with 2% sucrose to an $OD_{600}$=1.0 and *P. parvulus* 2.6, as well as *L. suebicus* strains, in MRS supplemented with 2% glucose, 0.05% (w/v) L-cysteine hydrochloride (Merck, Darmstad, Germany), and 0.1% (w/v) Tween 80 (Pronadisa, Barcelona, Spain) to an $OD_{600}$=2.0. For EPS production, the inocula were diluted 1:100 in fresh media. Cultures were grown in the following media: defined CDM (Sánchez et al., 2008) for *L. mesenteroides*, semi-defined MST (Velasco et al., 2006) for *P. parvulus* and SMD containing glucose (20 g $L^{-1}$) (Dueñas-Chasco et al., 1997) for *L. suebicus*. Batch fermentations without pH control were carried out for the two lactobacillus strains. For *P. parvulus* fermentation were performed in media containing glucose (50 g $L^{-1}$) and ethanol 4.9% (w/v), in a 3-L fermenter (Bioflo 110, New Brunswick Scientific), at 30 °C for 96 h. The pH was controlled at 5.2 with 5 M NaOH, the agitation was set at 50–70 rpm to keep the fermentation broth homogeneous, and nitrogen gas (0.2 L $h^{-1}$) was sparged through the headspace continuously to maintain anaerobic conditions.

EPS production by *L. mesenteroides* RTF10 was carried out in medium supplemented with sucrose (0.8%) instead of glucose as carbohydrate source, and cultures were incubated for 12 h at 30 ºC until $OD_{600}$=1.0. *L. suebicus* CUPV225 was grown in the SMD medium (pH 5.5) for 72 h at 28 ºC in an atmosphere containing 5% $CO_2$ until $OD_{600}$= 3.4.

143 *2.3. Production of EPS by* L. lactis *NZ9000[pNGTF]*

144 A frozen culture of the recombinant strain was used after thawing to inoculate 1 L of CDM
145 medium supplemented with glucose (0.5%) and chloramphenicol (5 µg $mL^{-1}$). The culture was
146 grown at 30 ºC to $OD_{600}$=0.6. Then, it was centrifuged at 6,816 ×*g* for 20 min at 4 ºC and re-
147 suspended in fresh CDM medium without antibiotic. For EPS production, expression of *gft*
148 gene was induced by the addition of nisin (0.25 ng $mL^{-1}$) and further incubation during 24 h at
149 30 ºC. Batch fermentation without pH control was performed.

150

151 *2.4. EPS isolation and purification from* P. parvulus *2.6 culture supernatant*

152 Bacterial cells were removed from fermented media by centrifugation at 16,000 ×*g* for 30 min
153 at 4 ºC. The clear supernatant was collected and the EPS precipitated by adding three volumes
154 of cold absolute ethanol, and maintained overnight at 4 ºC. The precipitate was recovered by
155 centrifugation at 14,000 ×*g* for 10 min at 4 ºC. The resulting EPS pellet was dissolved in
156 ultrapure water, and the EPS was recovered by precipitation with ethanol (three times). The
157 final precipitate was dissolved in and dialysed for 2 days against ultrapure water (changed
158 twice each day), using a dialysis membrane (Medicell International, Ltd., London, U.K.)
159 having a cut-off of 12-14 kDa. After dialysis, the solution was frozen at -80 ºC and lyophilised
160 (Telstar Cryodos equipment, Spain) at -50 ºC for up to 3 days to completely remove the
161 solvent. The lyophilised solid was then stored in a desiccator at room temperature. To
162 eliminate impurities the EPS preparation was fractionated by size-exclusion chromatography
163 (SEC). Dry EPS was dissolved in 0.3 M NaOH (to eliminate extra contaminants and to
164 improve the EPS dissolution) and centrifuged to eliminate insoluble material. The supernatant
165 was loaded into a column (60 cm x 2.6 cm) of Sepharose CL-6B equilibrated with 0.3 M
166 NaOH, which was also used as eluent (0.3 mL $min^{-1}$). Fractions were collected, and monitored
167 for carbohydrate content by the phenol-sulphuric acid method (Dubois, Gilles, Hamilton,

Rebers & Smith, 1956). A calibration curve was obtained by using standards (Blue Dextran; Dextrans: T500, T70, and T10 (Pharmacia), and vitamin B12). From this curve, the apparent molecular mass (Mr) of the EPS was estimated. The fractions corresponding to the same chromatographic peak were pooled together, concentrated to a small volume, dialysed against ultrapure water using a dialysis membrane (12-14 kDa cut-off) until neutrality, and lyophilised for up to 3 days.

*2.5. EPS isolation and purification from* L. suebicus *CUPV225 culture supernatant*

Bacterial cells were removed from fermented medium by centrifugation at 16,000 ×*g* for 30 min at 4 ºC. The clear supernatant was collected and the EPS precipitated by adding three volumes of cold absolute ethanol. A floating fraction was first removed by winding around a glass rod and resuspended in ultrapure water (fraction H). The remaining cloudy ethanol mixture was maintained overnight at 4 ºC, then centrifugation at 11,000 ×*g* for 30 min at 4 °C to collect the suspended polysaccharide material (fraction L). This fraction was washed 3 times with 70% (v/v) ethanol and centrifuged. The floating and precipitated polysaccharides were re-suspended in and dialysed against ultrapure water, using a membrane (12-14 kDa cutoff), for 2 days (changed twice). After dialysis, both fractions were frozen at -80 ºC and lyophilised for up to 3 days. The floating fraction (H) was very insoluble and was not further analyzed. Fraction L was subjected to SEC and the Mr of the EPS was estimated as described in section 2.4.

*2.6. EPS isolation and purification from* L. mesenteroides *RTF10 culture supernatant*

Cells were removed from culture supernatant by centrifugation at 16,000 ×*g* for 30 min at 4 ºC. Then, cold absolute ethanol (v/v) was added to the supernatant, allowing the EPS to precipitate at 4 ˚C for 24 h. After centrifugation at 10,651 ×*g* for 60 min at 4 ºC, the supernatant was

193 removed, and the EPS was re-suspended in and dialysed for 2 days against ultrapure water
194 (changed twice), using a membrane (12-14 kDa cutoff). The EPS was frozen at -80 ºC and
195 lyophilised for up to 3 days. The dry biopolymer was dissolved in ultrapure water and
196 centrifuged to eliminate insoluble material. The supernatant was loaded into a column (60 cm x
197 2.6 cm) of Sepharose CL-6B equilibrated with ultrapure water, which was also used as eluent
198 (0.3 mL $min^{-1}$). The Mr of the EPS was estimated as described in section 2.4.
199
200 *2.7. EPS isolation and purification from* L. lactis *NZ9000[pGTF] supernatants*
201 To improve the release of EPS from bacterial surface, the cultures were heated at 60 ºC for 20
202 minutes in a water bath. Then, for removal of the bacteria, the culture was centrifuged at
203 10,651 ×*g* for 60 min at 4 ºC. The EPS present in the supernatant was recovered by overnight
204 precipitation at -20 ºC with three volumes of absolute ethanol and re-suspension in ultrapure
205 water (three times). After each precipitation, the EPS was recovered by centrifugation at
206 10,651 ×*g* for 60 min at 4 ºC. Then, the precipitate was re-suspended in and dialysed for 2 days
207 against ultrapure water (changed twice) in a membrane (12-14 kDa cutoff). The EPS was
208 frozen at -80 ºC and lyophilised for up to 3 days. The dry biopolymer, after being dissolved in
209 0.3 M NaOH, was subjected to SEC and its Mr was estimated as described in section 2.4.
210
211 *2.8. Determination of EPS concentration and presence of contaminants*
212 The concentration of EPS in the different steps of purification was estimated as neutral
213 carbohydrate content determined by the phenol-sulphuric acid method (Dubois, Gilles,
214 Hamilton, Rebers & Smith, 1956). The amount of EPS was determined from culture
215 supernatants: i) after concentration, precipitation with two volumes of absolute ethanol (to
216 remove the carbon source added to the medium), and washing of the EPS with 70% (v/v)
217 ethanol, vacuum drying and suspension in water; ii) after precipitation with ethanol, dialysis,

freeze-drying and EPS suspension in water (solutions 1 or 2 mg $mL^{-1}$, depending on their solubility); and iii) after chromatographic purification, dialysis, freeze-drying and resuspension in 1 or 2 mg $mL^{-1}$ water solutions of the EPS. Contamination by DNA, RNA, and proteins was measured with the Qubit® 2.0 fluorometer in the same solutions for the purified samples or directly from the culture supernatant. This technique allows the detection of more than 0.5 µg $mL^{-1}$ of DNA, 20 ng $mL^{-1}$ of RNA, and 1 µg $mL^{-1}$ of proteins.

*2.9. Detection of EPS by electron microscopy*

The presence of the EPS attached to the bacteria either capsular, tightly associated with the cell surface, and/or as slime or rope secreted into the extracellular environment, was determined by transmission electron microscopy (TEM) using either the ruthenium red staining method (Akin & Rigsby, 1990) or by negative staining with uranyl acetate (Maeyama, Mizunoe, Anderson, Tanaka & Matsuda, 2004). i) The ruthenium red staining method was used for *L. mesenteroides* RTF10 basically as described by Akin & Rigsby (1990). The bacterium was grown to early stationary phase in MRS medium (Pronadisa, Madrid, Spain) at 30 ºC. Cells were sedimented by centrifugation, washed in phosphate buffer saline (PBS, pH 7.4), and concentrated fivefold in 0.1 M sodium cacodylate buffer (pH 7.4). Cells were fixed in freshly prepared 2.5% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4), embedded in 2% agarose, and sections were cut with a scalpel. Cells were post fixed in 1.5% (w/v) $OsO_4$ in 0.1 M cacodylate buffer (pH 7.4) containing 0.075% (w/v) ruthenium red. Then, they were washed three times in 0.1 M sodium cacodylate buffer (pH 7.4), dehydrated using a graded ethanol series (30, 50, 70, 95, and 100% ethanol, 5 min each), and embedded in epoxy resin. Thin sections (60 nm) were made with a Diatome glass knife, using an Ultracut Leica UC6 ultramicrotome and examined with a JEOL JEM1010 transmission electron microscope after uranyl acetate staining. Images were captured and digitized using a MegaView III camera with

Software "AnalySIS". ii) The negative staining with uranyl acetate was used for *P. parvulus* 2.6, *L. suebicus* CUPV225, *L. lactis* NZ9000[pNGTF], and for purified EPSs. *P. parvulus* 2.6 and *L. suebicus* CUPV225 were grown to early stationary phase in MRS medium at 30 ºC and *L. lactis* NZ9000[pNGTF] cultures were induced with nisin for 24 h as indicated in section 2.3. The EPS previously purified from *L. lactis* NZ9000[pNGTF] was re-suspended in PBS pH 7 at 1 mg $mL^{-1}$ prior to negative staining. Samples were prepared as follows. Glow-discharged carbon-coated Formvar grids were placed facedown over a droplet of each culture concentrated fivefold in 0.1 M $NH_4Ac$, pH 7 or PBS pH 7. After 1 min, each grid was removed, blotted briefly with filter paper, and without being dried, negatively stained with 2% uranyl acetate for 40 s and then blotted quickly and air-dried. Samples were examined using a JEOL 1230 transmission electron microscope operated at 100 kV. Images were digitalized using an Epson Perfection 4870 Photo scanner at 1200 dpi final resolution.

*2.10. Infrared (IR) spectroscopy*

IR spectra were obtained by the KBr technique. In brief, approximately 2 mg of dry sample were thoroughly mixed in a mortar with 300 mg of KBr and maintained in a desiccator. The pellet was prepared by using a hydraulic press, applying a pressure of 2 tons for 2 minutes and then 10 tons for 6 min. The spectra were recorded in a FTIR 4200 type A instrument (Jasco Corporation, Tokyo, Japan). Light source of transmittance was in the middle range infrared 400–4000 $cm^{-1}$. The detector used was triglycine-sulfate (TGS) with resolution 4 $cm^{-1}$.

*2.11. Determination of monosaccharide composition and phosphate content*

For analysis of neutral sugars, the polysaccharides (approximately 1 mg) were first hydrolyzed with 3M TFA (121 ºC, 1 h). The monosaccharides were converted into their corresponding alditol acetates by reduction with $NaBH_4$ and subsequent acetylation (Laine, Esselman &

Sweeley, 1972). Identification and quantification were performed by gas-liquid chromatography (GLC) on a 6890A instrument (Agilent) equipped with a flame-ionization detector, using a HP5 fused silica column (30 m x 0.25 mm I.D. x 0.2 μm film thickness) with He as the carrier gas. Injector and detector were set at 250 ºC. Samples (1 μL) were injected with a split ratio of 1:50, with a temperature program: 160 °C for 5 min, then 3.5 °C $min^{-1}$ to 205 °C and finally 210 ºC for 0.5 min. Identification was performed on the basis of the coincidence of the retention time of sample components with those previously measured for standards analyzed in identical conditions, using inositol as internal standard. Phosphate content was deduced from inorganic phosphate determination on a 5500 Inductively Coupled Plasma instrument (Perkin Elmer).

*2.12. Methylation analysis*

The polysaccharides (1-5 mg) were methylated according to the method of Ciucanu and Kerek (1984). The permethylated polysaccharides were hydrolyzed with 3M trifluoroacetic acid (TFA) at 121 ºC for 1 h. The resulting partially methylated monosaccharides were converted into their corresponding alditol acetates by reduction with $NaBD_4$ and subsequent acetylation with 250 μL of pyridine:acetic anhydride (1:1) for 1 h at 100 ºC, as described by Laine, Esselman & Sweeley (1972). The partially methylated alditol acetates obtained were analyzed by gas chromatography-mass spectrometry (GC-MS) on a 6890A/5975C instrument from Agilent, with He as the carrier gas. The injector was programmed at 250 ºC. Samples (1 μL) were injected with a split ratio of 1:50 and their components separated in a HP5MS (Agilent) fused silica column (30 m x 0.25 mm I.D. x 0.2 μm film thickness), with a temperature program starting at 160 ºC (1 min) and then rising 2 ºC $min^{-1}$ up to 200 ºC. An m/z range between 40 and 450 amu was scanned. Identification was done on the basis of the retention time and mass spectra of the compounds. Quantification was performed according to peak area.

## 3. Results and discussion

### *3.1. Production of EPS by LAB*

The amount and composition of the EPS produced by LAB is strongly influenced by culture and fermentation conditions such as pH, temperature, and medium composition (Dueñas, M., Munduate, Perea, & Irastorza, 2003). In general, higher yields of HoPS and HePS are obtained when complex media are used for bacterial growth. However, the use of these media results in EPS preparations with higher levels of contaminants (Ruas-Madiedo & de los Reyes-Gavilán, 2005). Thus, in this work, specific conditions for the production of each EPS produced by different LAB have been set up by using defined or semi-defined media, to diminish, during purification, the co-precipitation of interfering compounds (*e.g.* polysaccharides from the culture medium) together with the bacterial EPS.

The four strains analyzed secreted variable amounts of EPS into the culture media, as quantified by the phenol sulfuric acid method: *L. suebicus* CUPV225 144±1 mg $L^{-1}$, *P. parvulus* 2.6 378±3 mg $L^{-1}$, *L. lactis* NZ9000[pGTF] 561±18 mg $L^{-1}$, and *L. mesenteroides* RTF10 1,870±180 mg $L^{-1}$. The proportion of the EPS in the supernatants relative to protein, RNA, and DNA contaminants ranged from 9 to 97% from *L. suebicus* CUPV225 to *L. mesenteroides* RTF10 cultures, the highest contamination being due to proteins (Table 1).

The production of the exocellular polymers was also detected by TEM analysis of bacterial cultures (Fig. 1). *P. parvulus* 2.6 (Fig. 1A), *L. lactis* NZ9000[pGTF] (Fig. 1B), and *L. suebicus* CUPV225 (Fig. 1D) preparations revealed their EPS as amorphous masses, either attached to the bacterial cells or free in the preparation. Similarly, the EPS was visualized in aqueous solutions of the purified EPS. As an example, the EPS produced by the recombinant *L. lactis* strain is depicted in Fig. 1C. In the case of *L. mesenteroides* RTF10, the EPS was predominantly concentrated around the exterior of the cells in a putative capsule, with fibrous

structures interconnecting adjacent cells. There were also cell-free isolated clumps of EPS in the medium (Fig. 1E). The detection of a low proportion of molecules that were not attached to the cells and the high yield of EPS production by the bacterium indicated that most of the biomolecules produced by *L. mesenteroides* were lost by sedimentation of the bacterial culture prior to TEM analysis.

*3.2. Recovery and initial purification of EPS*

Bacterial EPS are generally recovered from culture broth by precipitation, by adding a water-miscible organic solvent (Kumar, Mody & Jha, 2007). Here, ethanol precipitation was used for the recovery and initial purification of EPS from bacterial culture supernatants. The concentration of neutral carbohydrates, protein, DNA, and RNA was analysed in aqueous solutions of the EPS preparations to check their purity after ethanol precipitation, dialysis, and lyophylisation (Table 1).

The amounts of absolute ethanol needed to achieve the recovery of the EPS from the culture supernatants depended on the type of polymer released. For example, the EPS from *P. parvulus* and the recombinant *L. lactis*, which are β-glucans, needed three volumes of ethanol, while the EPS from *L. mesenteroides* precipitated easily with only one volume of this solvent. In the case of *L. suebicus* two EPS fractions were separated from the culture supernatant, one that precipitated quickly after ethanol addition (fraction H) and other that flocculated after a longer time under cold conditions (fraction L). Fraction H turned out to be very insoluble after drying, and was not further analysed. In addition, the EPS from *P. parvulus* and *L. lactis*, either directly from supernatants or after initial purification or chromatographic fractionation (see below), generated gel-like solutions at concentrations higher than 3 mg $mL^{-1}$ and had to be diluted to 1 mg $mL^{-1}$ to get a homogeneous suspension. The EPS from *L. mesenteroides*, which was produced in the highest yield and precipitated with the lowest ethanol proportion (1:1 v/v),

343 contained a negligible amount of impurities. A similar yield was observed for the EPS
344 produced by the recombinant *L. lactis* strain, for which optimized polymer production was
345 better controlled. By contrast, the EPS preparations from *P. parvulus* and *L. suebicus* strains
346 had variable amounts of contaminating proteins and nucleic acids.
347 The results show that purification resulted in all cases in a decrease of protein contamination
348 and it had variable effects to remove the residual DNA and RNA contamination presumably
349 due to cell lysis.
350
351 *SEC of the EPS preparations and analysis of sugar composition of the purified EPS*
352 To further purify the polysaccharides and to estimate their average molecular mass, the
353 lyophilised samples were fractionated by SEC after being dissolved in the appropriate solvent,
354 according to their solubility. Thus, for *P. parvulus*, *L. lactis,* and *L. suebicus*, the use of 0.3 M
355 NaOH was required, in order to get a better dissolution of the samples. On the other hand, the
356 more soluble EPS from *L. mesenteroides* was analyzed using a water solution. Analysis of
357 neutral sugar content of EPS preparations, after SEC fractionation, revealed for all isolates a
358 single peak. EPS from *P. parvulus* 2.6, *L. lactis* NZ9000[pGTF], and *L. mesenteroides* RTF10
359 had a Mr higher than $10^6$ Da and were composed exclusively of glucose (results not shown).
360 However, the EPS collected in fraction L from *L. suebicus* CUPV225 had a Mr around 52,000
361 Da and contained galactose, glucose, and glucosamine in the proportions 1.1:1.8:1,
362 respectively, and 4.5% phosphate. Analysis of EPS, after SEC, did not reveal contamination by
363 RNA and DNA (Table 1). Moreover, protein contamination at the level of 3% was only
364 detected in the *L. suebicus* EPS preparation. As previously mentioned, this HePS was
365 composed of neutral sugars and glucosamine. Since the phenol-sulfuric acid method does not
366 give positive reaction with aminosugars, carbohydrate content in this sample (37.3%) was
367 underestimated by using this technique.

368 SEC is a chromatographic procedure currently used to purify in one step HePS from fungal cell
369 walls (Leal, Prieto, Bernabé & Hawksworth, 2010). Moreover, this method has been
370 successfully used to puri -
371 - , 2012). Our results validate the use of this
372 method to purify HoPS produced by LAB. However, they show that this method is not
373 sufficient to remove all impurities from the HePS from *L. suebicus*, probably due to
374 interactions of its aminosugars with the Sepharose bed and/or with other components of the
375 preparation. In any case, this fact did not prevent the characterization of the preparation by IR
376 spectroscopy and chemical analysis (see below). Nevertheless, if a high purity HePS is
377 required for further uses, other chemical or enzymatical deproteinization treatments and/or
378 other chromatographic fractionation methods, such as ion exchange chromatography, could be
379 used (Ruas-Madiedo & de los Reyes-Gavilán, 2005; Kumar, Mody & Jha, 2007; Freitas,
380 Alves, & Reis, 2011).
381
382 *3.3. IR spectra of exopolysaccharides*
383 The overall appearance of the spectra (Fig. 2) is typical of those from carbohydrates.
384 Observation of the signals showed similarities and differences among the EPSs analyzed in the
385 present work and allowed certain chemical characteristics of these compounds to be deduced.
386 The spectra from the EPS produced by *P. parvulus* 2.6 and *L. lactis* NZ9000[pGTF] were very
387 similar, displaying an absorption band around 890 $cm^{-1}$ attributable, as expected, to β-anomers.
388 On the other hand, the spectrum from the EPS from *L. mesenteroides* RTF10 did not show this
389 absorption band, but instead had a shoulder at 849 $cm^{-1}$ and a more intense absorption band at
390 916 $cm^{-1}$, both characteristic of α-anomers.
391 The spectrum of the EPS from *L. suebicus* CUPV225 showed two absorption bands, at 1,551
392 and 1,655 $cm^{-1}$, characteristic of the amide linkage, confirming that the glucosamine residues

393 detected by GC were N-acetylated. However, nothing could be clearly deduced regarding the
394 anomeric configuration of sugars, probably due to the presence of more than one anomeric type
395 in the EPS preparation. Only a slight band at 846 $cm^{-1}$ was observed in the IR spectrum.
396
397 *3.4. Methylation analysis of exopolysaccharides produced by LAB*
398 Methylation analysis of the polysaccharides (Table 2) proved the similarity of the biopolymers
399 produced by *L. lactis* NZ9000[pGTF] and *P. parvulus* 2.6, giving the expected products for a
400 (1→3)-glucan partially branched at positions *O*-2 in agreement with previous results (Dueñas-
401 Chasco et al., 1997; Werning et al., 2008).
402 The EPS from *L. mesenteroides* RTF10 gave the partially methylated and partially acetylated
403 derivatives of a (1→6)-glucan with approximately 6% of substitutions at positions *O*-3 by side
404 chains composed of a single residue of glucose. Since the band for α anomers was observed in
405 the IR spectrum of this polymer, it can be described as a dextran-type polysaccharide. The
406 production of dextrans from *Leuconostoc* strains grown in medium supplemented with sucrose
407 is well known and widely documented (Korakli & Vogel, 2006; Monsan, Bozonnet, Albenne,
408 Joucla, Willemot & Remaud-Simeon, 2001; Sarwat, Qader, Aman & Ahmed, 2008; Van
409 Hijum, Kralj, Ozimek, Dijkhuizen & Van Geel-Schutten, 2006), although this is the first report
410 on the production of dextrans from a *Leuconostoc* strain isolated from meat.
411 Finally, the EPS from *L. suebicus* CUPV225 gave a complicated pattern of derivatives from
412 methylation analysis, suggesting that the strain is producing a complex HePS. The possibility
413 of dealing with a mixture of polymers, which eluted together in SEC, cannot be ruled out.
414 Glucose residues were found to be mainly as terminal residues, or (1→6)-, (1→3)- and (1→4)
415 linked, although a small proportion of (1→3,6)-glucose was also detected. Galactose was
416 mostly found as galactofuranose (1→2,6)-linked. Aminosugars were detected as non-
417 methylated compounds, probably due to an incomplete dissolution of the sample in DMSO.

## 4. Conclusions

In this work we have used a combination of conditions to produce and purify high yields of homo- and hetero-polysaccharides from LAB. The strategy was based on identifying defined or semi-defined media as well as growth conditions to minimize contamination by other carbohydrates and bacterial molecules. Moreover, experimenting with different conditions of ethanol precipitation of high molecular mass EPS, which seems to depend on EPS composition and conformation, led to simple isolation methods suitable for determination of their primary structure. In addition, the method was combined with dialysis to remove low molecule weight contaminants such as residual media components and metabolites. The final purification of the EPS preparations was achieved by chromatographic SEC eluting with 0.3 M NaOH in all cases except for the α-glucan, which had good solubility in water. This method yielded pure HoPS and a partially purified HePS. In addition, chemical analyses have allowed us to describe a dextran-producing *L. mesenteroides* strain from a fermented meat product as well as a heteropolysaccharide-producing *L. suebicus* strain isolated from cider, whose HePS merits further investigation.

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597

598 **Legend to the figures**

599 **Figure 1. Transmission electron micrographs of EPS and bacterial cells.** Negative staining
600 with uranyl acetate of whole cells of *P. parvulus* 2.6 (A), *L. lactis* NZ9000[pNGTF] (B), and *L.*
601 *suebicus* CUPV225 (D) as well as of EPS purified from *L. lactis* NZ9000[pNGTF] (C).
602 Ultrathin section of *L. mesenteroides* RTF10 cell stained with ruthenium red (E). All bars
603 correspond to 500 nm except (C), 100 nm.

604 **Figure 2.** Infrared spectra of homo- and hetero-polysaccharides synthesized by LAB.

**Table 1. Detection of biomolecules in successive steps of the purification process.**

| Strain | Culture supernatant[2] | | | | After precipitation and dialysis[3] | | | | After SEC[3] | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | EPS (%) | Protein (%) | DNA (%) | RNA (%) | EPS (%) | Protein (%) | DNA (%) | RNA (%) | EPS (%) | Protein (%) | DNA (%) | RNA (%) |
| ***P. parvulus* 2.6** | 65.3 | 34.7 | 0 | 0 | 98.5 | <0.1 | 0 | 0 | 100 | 0 | 0 | 0 |
| ***L. lactis* NZ9000 [pGTF]** | 95.5 | 4.5 | <0.1 | <0.1 | 98.5 | 1.4 | <0.1 | 0 | 100 | 0 | 0 | 0 |
| ***L. suebicus* CUPV225[1]** | 9 | 83.3 | 0 | 7.7 | 29.6 | 4 | 0 | 7.9 | 37.3 | 3 | 0 | 0 |
| ***L. mesenteroides* RTF10** | 97 | 2.9 | <0.1 | <0.1 | 99.1 | 0.9 | <0.1 | <0.1 | 100 | 0 | 0 | 0 |

[1]In addition to neutral sugars, this sample contained phosphate and aminosugars. Consequently, low EPS levels were detected using the phenol-sulfuric acid method, which is appropriate for neutral sugars but not for aminosugars.

[2]Protein, DNA and RNA concentrations were measured directly from supernatants. EPS concentration was determined from neutral sugars estimation after ethanol precipitation from culture supernatants. 100 % corresponds to the total concentration of detected biomolecules (EPS, protein, RNA, and DNA) in each sample.

[3]Solutions were prepared in water at 2 mg $mL^{-1}$ for *L. mesenteroides* EPS and 1 mg $mL^{-1}$ for the other EPS.

**Table(s)**

Table 2. Linkage types and their percentages, deduced from methylation analysis of homo and hetero-polysacharides synthesized by LAB and purified by SEC.

| **Linkage type** | *P. parvulus* 2.6 | *L. lactis* NZ9000[pGTF] | *L. mesenteroides* RTF10 | *L. suebicus* CUPV 225 |
|---|---|---|---|---|
| **Gal*p*-(1→** | 0.0 | 0.0 | 0.0 | 6.4 |
| **Gal*f*-(1→** | 0.0 | 0.0 | 0.0 | 2.9 |
| **→2)-Gal*p*-(1→** | 0.0 | 0.0 | 0.0 | 3.4 |
| **→6)-Gal*f*-(1→** | 0.0 | 0.0 | 0.0 | 3.4 |
| **→2,6)-Gal*f*-(1→** | 0.0 | 0.0 | 0.0 | 14.4 |
| **Glc*p*-(1→** | 30.1 | 31.1 | 6.3 | 24.7 |
| **→3)-Glc*p*-(1→** | 37.9 | 36.7 | 0.0 | 17.5 |
| **→4)-Glc*p*-(1→** | 0.0 | 0.0 | 0.0 | 7.0 |
| **→6)-Glc*p*-(1→** | 0.0 | 0.0 | 86.5 | 15.4 |
| **→3,6)-Glc*p*-(1→** | 32.0 | 32.2 | 7.2 | 4.7 |

Figure(s)

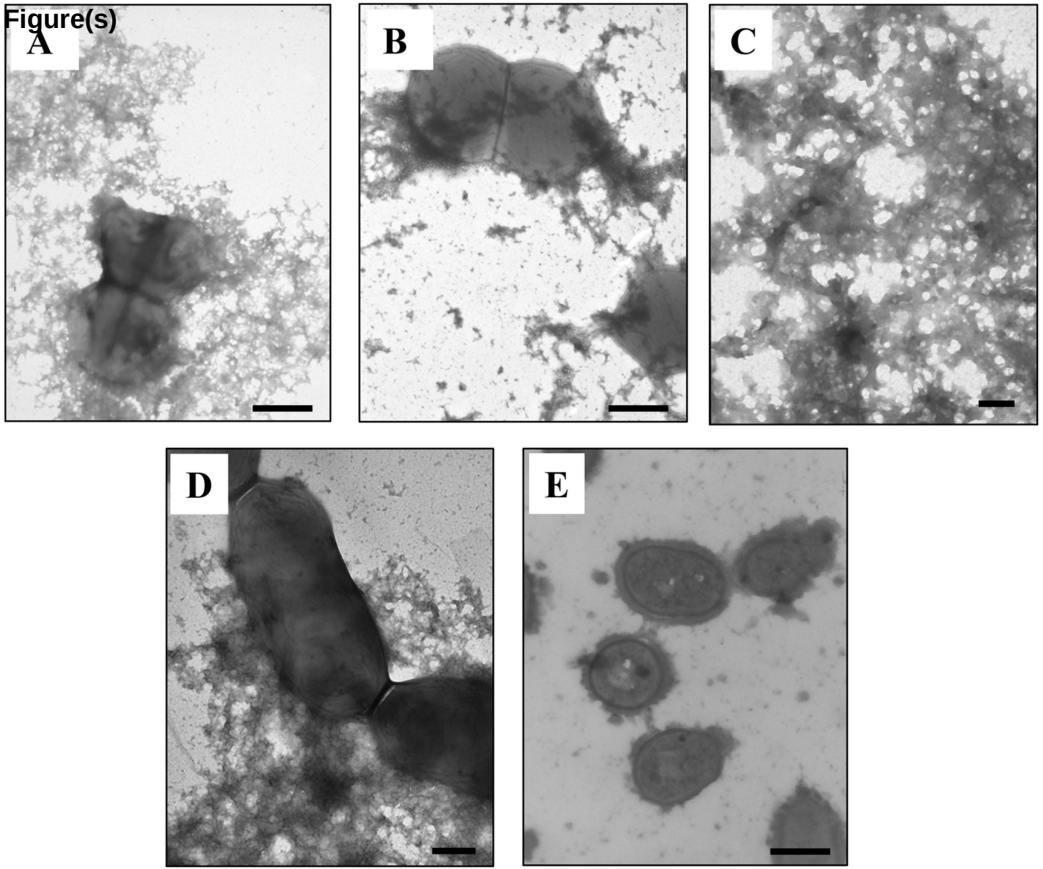


Figure 1

**Figure(s)**

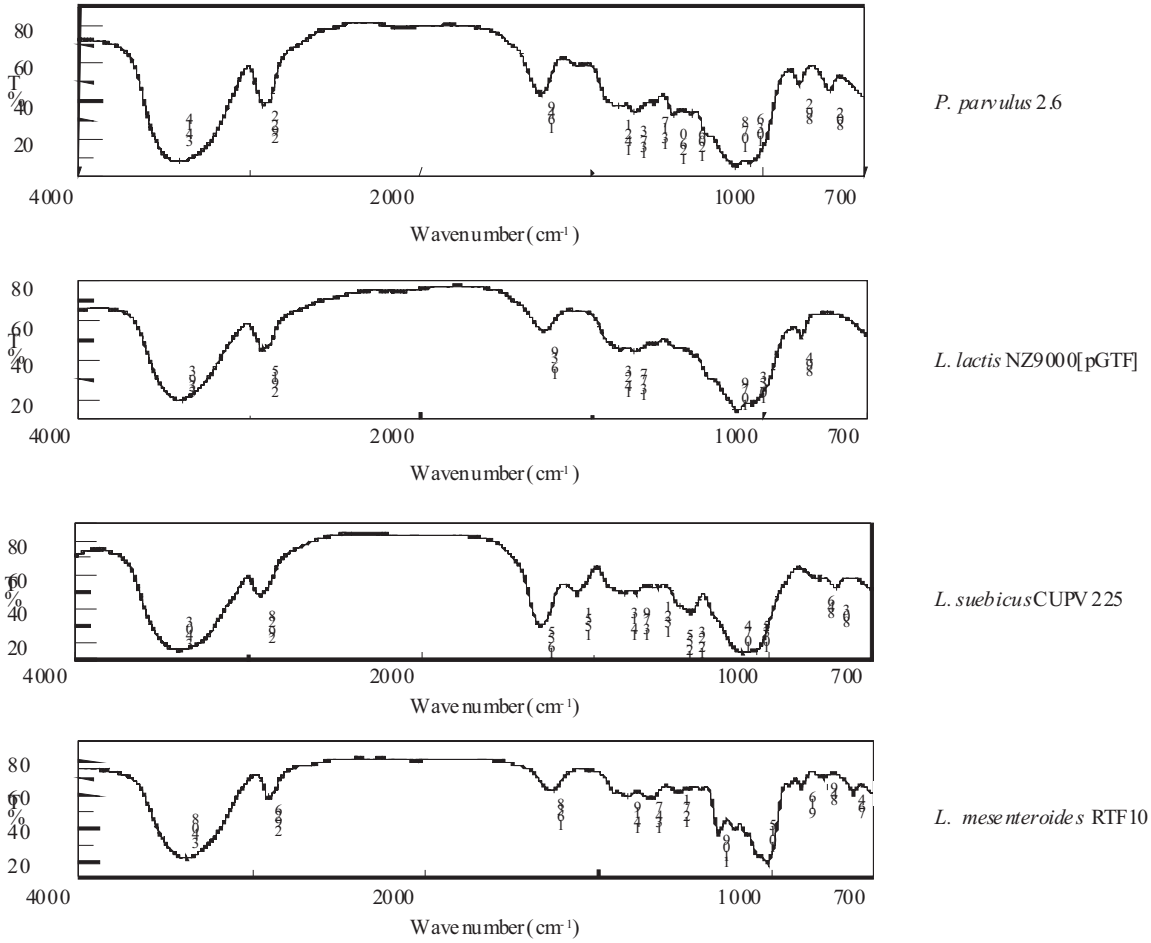


Figure 2