Naturally occurring 2-substituted (1,3)-β-D-glucan producing *Lactobacillus suebicus* and *Pediococcus parvulus* strains with potential utility in the production of functional foods

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Running title**:** 2 substituted (1,3)-β-D-glucan role in lactic acid bacteria isolated from cider

ABSTRACT

We have isolated three lactic acid bacteria (*Lactobacillus suebicus* CUPV221, *Pediococcus parvulus* CUPV1 and *P. parvulus* CUPV22) that produced high levels of 2-substituted (1,3)-β-D-glucans which increased the viscosity of the growth media. The (1,3)-β-D-glucan consisted of two main molecular species, with masses of approximately 10^7 Da, and 10^4 Da, whose proportions varied among the strains. The three strains survived exposure to saliva and simulated gastric conditions at pH 5, with *P. parvulus* CUPV22 surviving at pH 3.1, and *L. suebicus* CUPV221 surviving at pH 1.8. All strains were resistant to pancreatin and bile salts. *P. parvulus* CUPV22 exhibited the highest adhesion (10.5%) to Caco-2 cells, which decreased to 1.2% after washing the cells. Finally, *P. parvulus* CUPV22 and *L. suebicus* CUPV221 induced the production of inflammation-related cytokines by polarized macrophages, and interestingly, *L. suebicus* stimulated production of cytokine IL 10. These results indicate that the three strains have potential utility for the production of functional foods.

Keywords: exopolysaccharide, lactic acid bacteria, (1,3)-β-D-glucan, probiotic, prebiotic.

1. Introduction

Lactic acid bacteria (LAB), bifidobacteria and certain streptococci are used industrially in food production, particularly in fermented milk products. Some of these bacteria are inhabitants of the human gastrointestinal tract and they are the most commonly used probiotic microorganisms in functional foods (Waldherr and Vogel, 2009). The benefits claimed for probiotic bacteria include minimizing symptoms of lactose intolerance, modulating the immune system, reducing cholesterol levels and hypertension, and preventing infectious and inflammatory diseases (De Vrese and Schrezenmeir, 2008).

Most commercialized probiotics produce exopolysaccharides (EPS) (de Vrese and Schrezenmeir**,** 2008). The EPS play an important role in the rheology, texture and 'mouthfeel' of fermented milks (yoghurt, viili, långfil, etc) and other fermented products. Most of the strains used in the production of functional dairy food synthesize heteropolysaccharides (Welman et al., 2003). Homopolysaccharide producers have been evaluated to a lesser extent and are used mainly for fermentation of non-dairy products. Several glucan or fructan producing *Lactobacillus* strains have been tested for sourdough fermentation (Tieking et al., 2003) and the 2-substituted (1,3)-β-D-glucan producer *P. parvulus* 2.6 (isolated from cider) has been tested for the production of a fermented oat product (Mårtensson et al., 2002). The (1,3)-β-D-glucans are attracting an increasing attention of the pharmaceutical and functional food industries (Zekovic et al., 2005). Human trials of the oat-based food elaborated with *P. parvulus* 2.6 resulted in a decrease of the serum cholesterol levels and increased counts of faecal *Bifidobacterium* spp. (Mårtensson et al., 2005). It has also been reported that an oat fibre concentrate fermented with *P. parvulus* 2.6 provoked changes in short-chain fatty acid formation in the caecum, distal colon and faeces of rats, which may be beneficial to colonic health (Lambo-Fodje et al., 2006). 2-substituted (1,3)-β-D-glucan is synthesized in *P. parvulus* 2.6 by the GTF glycosyltransferase which is encoded by the *gft* gene (Werning et al., 2008).

The role of surface polysaccharides in probiotic-host interactions has not yet been studied in great detail. The structural diversity of EPS among lactobacilli may determine strain-specific properties important for probiotic action, such as adhesion, stress resistance and interactions with specific receptors and effectors of the host defence system (Welman and Maddox, 2003). It has been shown that cloning the *P. parvulus* 2.6 *gtf* gene into *Lactobacillus paracasei* markedly increased the organism's survival under simulated gastro-intestinal stress conditions in comparison to the unmodified host strain (Stack et al, 2010). However, knowledge of the involvement of these biopolymers in the adhesion of bacteria to the intestinal epithelium is scarce. It was shown that the long galactose-rich EPS of *Lactobacillus rhamnosus* GG, (Lebeer et al, 2009) acted negatively on its adhesion and biofilm formation capacity. In contrast, wefound that the 2-substituted (1,3)-β-D-glucan produced by *P. parvulus* 2.6 contributed to bacterial adhesion to human intestinal epithelial cells (Fernández de Palencia et al., 2009). Moreover, the results of Dols-Lafargue et al. (2008) revealed the contribution of this β-glucan to biofilm formation by *P. parvulus* IOEB8801 (isolated from wine). The (1,3)-β-D-glucans from several bacteria, fungi and plants are considered to be biological response modifiers and numerous publications describe their biological activities and therapeutic uses (Sletmoen and Stokke, 2008). In this context, (1,3)-β-D-glucan producing lactic acid bacteria isolated from non dairy habitats could represent an opportunity to diversify and extent the number and applications of EPS producing probiotic strains.

To this end in this work we have characterized three 2-substituted (1,3)-β-D-glucan-producing LAB strains (*Pediococcus parvulus* CUPV1 and CUPV22 and *Lactobacillus suebicus* CUPV221, isolated from a non-dairy source (Basque Country natural cider), and their exopolysaccharides, with the aim of assessing their potential probiotic and viscosifying properties and hence their suitability for the production of functional foods.

2. Methods

2.1. Bacterial strains and media

The EPS producing LAB strains were previously isolated from Basque natural ciders (Dueñas et al. (1997) and identified as *Pediococcus parvulus* (strains CUPV1 and CUPV22) and *Lactobacillus suebicus* (strain CUPV221) by 16S rRNA sequencing (Accession numbers in GenBank: GQ923890, GQ923889 and GQ923888). These strains and the probiotic bacteria *Lactobacillus acidophilus* LA-5 and *Bifidobacterium animalis* subps. *lactis* BB-12 (Chr. Hansen A/S., Hørsholm, Denmark) were maintained at -80ºC in Man Rogosa Sharpe (MRS) broth (Pronadisa, Madrid, Spain) supplemented with 20% (vol/vol) glycerol.

For production and further characterization of the EPS, strains were grown in two semi-defined media buffered at pH 6.0: MST (Velasco et al., 2006) for *P. parvulus* strains and SMD (Velasco et al., 2009) for *L. suebicus* CUPV221. Both media contained glucose (20 g L^{-1}) as carbohydrate source. Batch fermentations without pH control were carried out in completely filled 250 mL screw-cap flasks for 181 h at 28ºC in an atmosphere containing 5% CO_2. At the end of the fermentations the pHs of the media were 3.47, 3.52 and 3.93 for *P. parvulus* CUPV1, *P. parvulus* CUPV22 and *L. suebicus* CUPV221, respectively.

For analysis of probiotic properties, the EPS-producing and probiotic bacteria were grown respectively at 30ºC or 37ºC in MRS broth supplemented with 0.05% (wt/vol) L-cysteine hydrochloride (Merck, Darmstadt, Germany) and 0.1% (wt/vol) Tween 80 (Pronadisa, Barcelona, Spain) and incubated in anaerobic jars with AnaeroGen™ (Oxoid Unipath Ltd. Basingstoke, Hampshire, UK).

2.2. Detection of the gtf *gene in genomic DNA of LAB*

The three EPS producing LAB strains were characterized as described below.

Total DNA for PCR detection of the gene (*gtf*) encoding the glycosyltransferase (GTF) responsible for the synthesis of the 2-substituted (1,3)-β-D-glucan, was extracted from cells harvested from 1 mL of MRS cultures using the DNeasy Blood and Tissue Kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's instructions. DNA samples were stored at -20ºC until used. For detection of the *gtf* gene of the EPS producing strains, specific primers GTFF and GTFR were used for PCR amplification as previously described by Werning et al. (2006). Total genomic DNA and total plasmidic DNA prepared as previously described (Werning et al., 2006), was used for characterization of the *gtf* gene, The entire genes were amplified with the primers 5´-ATGTTAAA TGATAATGATTCAGAACTAAAAAAATTTC-3´ (nucleotides 1-37 of *gtf*) and 5´-TTAATCATTCCAATCAACTGTT TCCGTGTT-3´ (nucleotides 1674-1704 of *gtf*) (Werning et al., 2006) and these as well as internal primers were used to determine the 1704 nt DNA sequence of both strands of the *gtf* genes of the three EPS-producing strains as previously described (Werning et al., 2006).

In addition, the DNA sequence of the first 37 nt and the last 30 nt of the genes of the *P. parvulus* strains was confirmed by DNA sequencing using total plasmidic DNA preparations (carrying several plasmids) as template and either 5´-AGCCCTGCGTGTTATCATA-3´ (nucleotides 419-400 of *gft*) or 5´-GCATGTATCGCAATTTCTGG-3´ (nucleotides 1516-1535 of gft). Prior to sequencing, single stranded DNA was generated by random priming of the templates using the DNA polymerase from bacteriophage ϕ29 and the MagniPhi™ kit (Xpolbiotech, Tres Cantos, Madrid, Spain) following the specifications of the suppliers.

The DNA sequences have been deposited in GenBank (*P. parvulus* CUPV1, GU174475; *P. parvulus* CUPV22, GU174476 and *L. suebicus* CUPV221, GU174474).

2.3. Purification and characterization of EPS

For purification and quantification of EPS**,** bacterial cells were removed from fermented media by centrifugation (16,000 × *g*, 4ºC, 30 min). The EPS present in the supernatant was precipitated by addition of two volumes of cold acetone and incubation overnight at 4ºC (Velasco et al., 2006). After centrifugation at 14,000 × *g* for 10 min at 4ºC, the precipitate was washed 3 times with 70% acetone and sedimented by centrifugation. Finally, the EPS was suspended in distilled water and the total sugar content was determined by the phenol-sulphuric acid method using glucose as standard (Dubois et al., 1956). Each reported value was the average of three independent determinations.

For EPS characterization, the exopolysaccharides were isolated from the fermented growth media after 96 h of culture. After removal of bacteria by centrifugation (as described above) the EPS present in the supernatant was precipitated by addition of three volumes of cold ethanol and incubation overnight at 4ºC. After centrifugation at 14,000 × *g* for 10 min at 4ºC, the precipitate was washed twice with 80% ethanol and sedimented by centrifugation. Then, the precipitated EPS was dissolved and dialyzed against deionized water, using a membrane (Medicell International, Ltd., London, U.K.) having a cut-off of 3.5 KDa, for 2–3 days (water changed twice daily). After dialysis, the precipitate was lyophilized.

For NMR analysis, 0.6 mg of each purified EPS sample was resuspended in 0.6 mL of deuterated water (99 atom% D, Aldrich) and the solutions were stirred to complete homogenization. The NMR analyses of the EPS were performed using an Avance 500 spectrometer (Bruker Instruments Inc.). ^{1}H monodimensional and 2D-COSY analyses were carried out at 30ºC according to the conditions described by Dueñas-Chasco et al. (1997).

The molecular masses of the exopolysaccharides were determined by high-performance size exclusion chromatography (HP-SEC, GPCV 2000, Waters) as described by Velasco et al.

(2009), using as molecular weight standards dextrans of 10^4, 4 x 10^4, 7 x 10^4, 1,5 x 10^5, 2,7 x 10^5, 4,1 x 10^5, 6,7 x 10^5, 1,4 x 10^6 y 2 x 10^6 Da (Fluka).

The viscosities of the cell-free fermented growth media were determined with a capillary Cannon-Fenske viscometer (0.3 mm Ø) totally immersed in a constant temperature bath at 26ºC. Viscosity, v=Kt, where K is the characteristic of the capillary (0.002017) was the time measured, was expressed in centistokes (cSt = mm^2 seg^{-1}) and all measurements were performed in triplicate.

2.4. Gastrointestinal transit tolerance assay

Bacterial suspensions in acidified skimmed-milk (pH 4.6) were incubated in conditions that simulated the major factors influencing the survival of the ingested microorganisms during their passage through the gastrointestinal tract. Several relevant factors were considered: the effect of lysozyme as well as pepsin in the range of pH values that can occur in the stomach (pHs 5.0, 4.0, 3.1, 2.0 and 1.8) under digestion conditions (gastric stress), and the further action of bile salts and pancreatin (gastrointestinal stress). Tolerance assays were performed with late exponential-phase cells sedimented and resuspended without washing in 15 mL of 10% skim-milk acidified with 1 M HCl to pH 4.6 (giving approximately 10^9 CFU mL^{-1}). Human gastrointestinal tract conditions were simulated as previously described (Fernández de Palencia et al., 2008) with the following modifications. For gastric stress (G) analysis, bacteria after exposure to lysozyme were treated with pepsin at pHs 5.0, 4.0, 3.1, 2.1 or 1.8 for 20 min. Gastrointestinal stress (GI) was mimicked by exposure of the G pH 5 samples to bile salts and pancreatin at pH 6.5 for 120 min. Cell suspensions were prepared as described above and samples of 2.5 mL were withdrawn to determine cell viability by the LIVE/DEAD® BacLight™ fluorescent stain (Fernández de Palencia et al., 2008). Prior to the analysis, it was established that a correlation exists for all strains between green (live bacteria)/red (dead bacteria) fluorescent

(G/R) ratio and viable cell numbers as determined by plate count. All experiments were performed in triplicate with three independent cultures of each strain.

2.5. Caco-2 cell culture and adhesion assay

The Caco-2 cell line from human colon adenocarcinoma was obtained from the cell bank of the Centro de Investigaciones Biológicas (Madrid, Spain). Cells were grown in Men-Alpha Medium (Invitrogen, Barcelona, Spain), supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum. The incubation was carried out at 37ºC in an atmosphere containing 5% CO_2. Caco-2 cells were seeded in 96-well tissue culture plates (Falcon Microtest^{TM}, Becton Dickinson, Franklin Lakes, NJ, USA) at a concentration of 1.25×10^4 cells per well ($1.25x10^5$ cells mL^{-1}) and grown for 15 days to obtain a monolayer of differentiated and polarized cells. The culture medium was changed every 2 days. Cell concentrations were determined before and after adhesion experiments in a counting chamber (Type Neubauer 0.100 mm Tiefe. 2. Depth Profondeur 0.0025 mm^2) using a Nikon Eclipse TS 100 microscope.

For adhesion experiments, late exponential phase cells of the three strains were sedimented by centrifugation (10 min, 12,000 x g, 4 ºC) resuspended in the appropriate volume of Dulbecco´s modified Eagle medium (DMEM, Invitrogen) to give 1.25×10^6 CFU mL^{-1}, and were added to Caco-2 cells in a final volume of 0.1 mL per well. In addition, for analysis of the influence of the EPS on the adhesion capability of the *P. parvulus* CUPV22, two sub-populations were used: (i) prepared as indicated above (CUPV22) and (ii) composed of bacterial cells washed with PBS prior to resuspension as above (CUPV22*), with the aim of removing the EPS attached to bacterial cells before analyzing their adhesion.

Adhesion experiments consisted of exposing Caco-2 cells to bacteria (ratio 1:10) for 1 h, at 37ºC. Then, unattached bacteria were removed by three-fold washing with 0.2 mL PBS pH 7.1, and then the Caco-2 cells were detached from the plastic surface by incubating for 10 min at

37ºC with 0.2 mL of 0,05% trypsin–EDTA per well. The detachment reaction was stopped by adding 0.1 mL of cold phosphate-buffered saline PBS pH 7.1 (10 mM Na_2HPO_4, 1 mM KH_2PO_4, 140 mM NaCl, 3 mM KCl, all purchased from Merck). To determine the number of cell-associated bacteria, appropriate dilutions were plated onto MRS agar plates. All adhesion assays were conducted in triplicate.

2.6. Detection of EPS by electron microscopy

The presence or absence of the EPS attached to the bacteria was determined using a JEOL 1230 transmission electron microscope operated at 100 KV. Samples were prepared as follows. Glow-discharged carbon-coated formvar grids were placed face-down over a droplet of each culture concentrated five-fold in 0.1 M $AcNH_4$ pH 7.0. After 1 min, each grid was removed, blotted briefly with filter paper and, without drying, negatively stained with 2% uranyl acetate for 40 s, then blotted quickly and air-dried.

2.7. Macrophage differentiation, culture and immune stimulatory assay

Human peripheral blood mononuclear cells (PBMC) were isolated from buffy coats from healthy blood donors by means of gradient centrifugation. Briefly, buffy coats were diluted 1:1 with PBS and 35 ml were layered over 15 ml of Lymphoprep (Nycomed Pharma, Oslo, Norway). After centrifugation at 400g for 30 minutes in a swinging bucket rotor, the mononuclear cell layer, containing lymphocytes and monocytes, was recovered and extensively washed with PBS before subsequent procedures. Monocytes were purified from PBMC by magnetic cell sorting using CD14 microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany). CD14+ cells (>95% monocytes) were cultured at 37ºC in an atmosphere containing 5% CO_2 for 7 days in RPMI (GIBCO, Invitrogen, NY, USA) supplemented with 10% fetal calf serum (FCS) (complete medium), containing 1,000U mL^{-1} granulocyte-macrophage colony-stimulating factor (GM-CSF)

or 10 ng mL^{-1} macrophage colony-stimulating factor (M-CSF) (ImmunoTools GmbH, Friesoythe, Germany) to generate M1 and M2 monocyte-derived macrophages, respectively, with cytokine addition every second day. M1 and M2 macrophages ($5x10^5$ cells mL^{-1} as determined by Neubauer-counting) were stimulated with LPS from *Escherichia coli* 055:B5 (Sigma, Barcelona, Spain) at 10 ng mL^{-1}, or exposed to $1.25x10^7$ CFU mL^{-1} (ratio 25:1, bacteria:Caco-2 cells) of *P. parvulus* CUPV22 or *L. suebicus* CUPV221 in an atmosphere containing 5% CO_2 for 18 h.

Supernatants from untreated (basal) and treated M1 and M2 macrophages were tested for the presence of cytokines using commercially available ELISA for IL-6, IL-8, IL-10, TNF-α (all purchased from ImmunoTools, Friesoythe, Germany), following the protocols supplied by the manufacturers. Each determination was performed in triplicate, and the mean and standard deviations are shown.

2.8. Statistical Analysis

Statistical analysis was performed using the SPSS-PC 17.0 Software (SPSS Inc., Chicago, Illinois, USA). Data were subjected to a one-way ANOVA, and where appropriate, the least significant difference test (LSD) was used for comparison of the means.

2.9. Prediction of protein topological location

Prediction of the trans-membrane helices of GTFs glycosyltransferase was performed with the TMpred program (http://www.ch.embnet.org/software/TMPRED_form.html).

3. Results and Discussion

3.1. LAB strains characterization and EPS production

Three LAB strains isolated from cider were investigated with the aim of identifying potential probiotic bacteria which synthesize 2-substituted (1,3)-β-D-glucans. They exhibited a ropy phenotype when cultured in MRS broth, suggesting that they were exopolysaccharide producers. 16S rRNA sequencing showed that they belonged to the species *P. parvulus* (strains CUPV1, CUPV22) and *L. suebicus* (CUPV221). RAPD finger printing of CUPV1 and CUPV22 genomes, revealed that they share a 72% similarity and therefore the two strains were not identical (Garai-Ibabe et al., 2010).

Previously it was observed that 2-substituted (1,3)-β-D-glucan producing LAB strains isolated from cider and wine carry the *gtf* gene, encoding the GTF glycosyltransferase, located on various plasmids or in the chromosome (Werning et al., 2006, Dols-Lafargue et al., 2008).Based on PCR analysis, the strains used in this study also carry the *gtf* gene. Sequencing of the 1704 nt of the amplified *gtf* genes and proximal upstream and downstream regions revealed that the genes and flanking sequences from CUPV1 and CUPV22 were identical. The *gtf* genes had 99% identity with that of the cider *P. parvulus* 2.6 strain, which is carried in the 35 kDa pPP2 plasmid (Werning et al., 2006). However, the flanking regions were different to those in pPP2 and identical to those in the 5.5 kb pF8801 plasmid (GenBank AF196967) detected in wine *Pediococcus* strains (data not shown).

In the case of the *Lb. suebicus gft* gene, we were unable to determine the DNA sequence of the flanking regions and therefore to establish the gene location by DNA sequencing, but we determined the DNA sequence of 1637 nt out of 1704 nt of the gene (see details in Methods). They differed in only three nucleotides from the gene of the *P. parvulus* strains. Comparison of the deduced amino acid sequences using the Blast program (NCBI, USA)

showed that the enzyme of CUPV221 only deviated by two amino acids (A91V and L126I) from that of *P. parvulus* 2.6 (Werning et al., 2006) within the overlap region of 545 amino acids. Moreover, the enzyme of *P. parvulus* 2.6 deviated in one amino acid (A489T) from those of the *P. parvulus* CUPV1 and CUPV22 strains. Furthermore, the enzymes from the two *P. parvulus* strains analyzed have 97% and 94% identity with their homlogues in *O. oeni* strains isolated from cider (Werning et al. 2006) and wine (Dols-Lafargue et al., 2008). In addition, they also share 31% homology with the putative GTF glycosyltransferase of dairy *Propionibacterium freudenreichii* subsp. *shermanii* strains (Deutsch et al. 2008) and 35% with the Tts glycosyltransferase from *S. pneumoniae* serotype 37, which synthesizes a capsular 2-substituted (1,3)-β-D-glucan (Llull et al., 2001) almost identical to the EPS of *P. parvulus* strains. This close homology, and the ropy phenotype, suggested that the *L. suebicus* strain like the *P. parvulus* isolates, was producing 2-substituted (1,3)-β-D-glucan.

GTF glycosyltransferase encoded by the *gtf* gene is a membrane-bound protein (Werning et al., 2008), which belongs to the glycosyltransferase super-family A (GT-A, cl11394 in the CDD data base of NCBI) and specifically to the glycosyltransferase family 2 (GT-2). This family include membrane-bound enzymes, which use as substrates sugar nucleotides to synthesize a polymer by inversion of the anomeric configuration (Karnezis et al., 2000).

Analysis of the GTFs with the TMpred program predicts that the glycosyltranferase active site is surrounded by a two and a four transmembrane region located respectively at the amino and carboxyl ends of the protein. Moreover, the analysis revealed that mutation A489T is positioned in the loop located between the fifth and the six transmembrane helices of the GTF glycosyltransferase whereas neither of the other two mutations is included in transmembrane regions.

Alignment of the GTF glycosyltransferase amino acid sequence of the EPS-producing strains with the members of the superfamily GT-A revealed that the homology extended from

amino acids 109 to 354. This positioned the L126I mutation within the homology region and indicated that it is located in the active site of the enzyme. The super-family includes diverse families of glycosyltransferases with a common GT-A type structural fold, which has two tightly associated beta/alpha/beta domains and a DXD motif, which is the putative metal ion binding site that coordinates the phosphates of the NDP-sugar in the active centre (Karnezis et al., 2000). In addition, some members of GT-2 family have a conserved motif $D_1D_2D_3$ QXXRW, in which D_2 correspond to the first aspartate of the DXD motif, D_1 is also implicated in coordination of the metal and QXXRW has been associated with repetitiveness (Karnezis et al., 2000). The four aspartates are present in the GTF of LAB (D143, D198, D200 and D306). However, interestingly the QXXRW motif is absent in these enzymes and a poorly conserved motif RHSKW is present in the GTF of P. *freudenreichii* (Deutsch et al. 2008) and in the pneumococcal Tts (Llull et al., 2001).

Since we previously detected that *P. parvulus* 2.6 is able to synthesize β-D-glucan using glucose as carbon source (Velasco et al., 2009), EPS production by the three LAB strains was analyzed during growth in semi-defined media supplemented with this sugar (Fig. 1). The three strains were able to produce and release EPS into the growth media, at final concentrations between 137 to 180 mg L^{-1}. All the strains synthesized EPS during the exponential phase of growth, though the pattern of production and secretion differed between the *Pedioccus* and *Lactobacillus* strains in the stationary phase. In *Pediococcus* CUPV1, the EPS concentration increased up to 123 mg L^{-1} in the early stationary phase (60 hours) with only a moderate increase in concentration to 167 mg L^{-1} after 181 hours of growth (Fig. 1A). Similarly, *Pediococcus* CUPV22 showed a rapid accumulation of EPS (159 mg L^{-1} at 60 hours) then only a slow increase to a concentration of 179 mg L^{-1} after 181 hours of growth (Fig. 1 B). By contrast, in the *Lactobacillus* strain, there was a markedly slower rate of EPS accumulation during the exponential growth phase, reaching a concentration of 48 mg L^{-1} in early stationary phase which

remained constant until a late, sharp increase to a concentration of 137 mg L^{-1} at 181 hours (Fig. 1C). In addition, production of EPS and viscosity increases of the growth media were statistically correlated ($P< 0.001$) for each strain (Fig. 1), with R^2 values of 0.792, 0.933 and 0.952 for CUPV1, CUPV22 and CUPV221, respectively. Interestingly, after 181 hours of incubation CUPV22 and CUPV221 had produced concentrations of EPS of 179 and 137 mg L^{-1} respectively, which resulted, in both cases, in a final viscosity of approximately 4 mm seg^{-1}, whereas in the case *P. parvulus* CUPV1, which produced a similar concentration of EPS (167 mg L^{-1} at 181h), the final viscosity of 1.5 mm^2 seg^{-1} was significantly lower ($P <0.001$). This difference in viscosity could have been due to differences in the molecular structure or the mass of the polymers synthesized by the different strains, and therefore this hypothesis was further investigated.

3.2. EPS characterization

The ^{1}H monodimensional NMR analysis of the three exopolysaccharides showed that the chemical shifts for the ^{1}H resonances of the two purified exopolysaccharides from *P. parvulus* CUPV1 and CUPV22 strains were identical to those of the *P. parvulus* 2.6 EPS (Fig. S1). However, the identity of the EPS from *L. suebicus* CUPV221 could not be established, due to the low resolution of the anomeric proton region. Therefore, the polysaccharides were subjected to 2D-COSY NMR analysis (Fig. S1) which revealed that indeed all three analyzed EPS were identical to the β-glucan obtained from *P. parvulus* 2.6 (Dueñas-Chasco et al., 1997 and Fig. S1), *Pediococcus* sp. (Llaubères et al., 1990), *L. diolivorans* (Dueñas-Chasco et al., 1998) and *O. oeni* (Ibarburu et al., 2007; Dols-Lafargue et al., 2008) which were isolated from ropy cider and wine. This fact clearly indicates that the three strains synthesize the same β-D-glucan, with a primary structure constituted by a trisaccharide repeating unit, with two (1,3)-β linked residues in the main chain, one of which is substituted in position 2 by a terminal glucose residue.

Molecular weight determination of the 2-substituted (1,3)-β-D-glucans was carried out by size exclusion chromatography. Apart from a minor fraction with a molecular mass below the smallest 10 kDa dextran standard (data not shown), two main fractions were detected for the polymers of the three strains (Fig. S2). The higher molecular mass fraction of the β-D-glucan was the more abundant for *P. parvulus* CUPV1 (10.24 x 10^6 Da) and *P. parvulus* CUPV22 (10.47 x 10^6 Da), constituting respectively 96% and 92% of the total polymer population. A lower molecular mass polymer fraction (ranging from 25 to 40 x 10^3 Da) was a minority for these strains, but constituted 88% of the EPS produced by *L. suebicus* CUPV221. Interestingly, these and the above results (Fig. 1C) indicate that the lower molecular mass polymer can confer a substantial viscosity to the growth medium. Moreover, they also indicated that the different viscosities of the two *Pediococcus* cultures were not attributable to differences in the primary structure or molecular mass of the EPS. These results are in agreement with those reported by Petry et al. (2003), who found no direct correlation between the amounts of EPS produced, the molecular mass ratio of EPS fractions and the rheological properties of fermented milk. These observations suggest that other factors such as EPS conformation or interactions between EPS and growth media microstructure, could also affect the rheological features of the fermented media (Vaningelgem et al., 2004).

3.3. Characterization of probiotic properties

The tolerance of the three β-glucan producing strains to digestive tract stresses was investigated in the simulated gastrointestinal tract model. Assays were performed in skimmed-milk for two reasons: i) fermented milks and yoghurt are widely used vehicles for delivering probiotic bacteria, and ii) it has been previously described that skimmed-milk enhanced the gastric stress tolerance of LAB strains (Charteris et al., 1998). The survival of the three strains was mainly affected during exposure to saliva and gastric conditions (Fig. 2). After exposure to

pH 5.0 (G pH 5.0 in Fig. 2), the three strains showed a reduction of viability, though with still a 23% to 30% of cell survival. Treatments at lower pHs caused a progressive reduction of viability of *P. parvulus* CUPV1 which dropped below the detection limits ($1x10^5$ cfu mL^{-1}) at pH 2.1 (Fig. 2A, G pH 2.1), although the strain still exhibited a 1.6% cell survival after exposure to pH 3.1 (Fig. 2A, G pH 3.1). Tolerance to acidic stress was better for the other two strains. Survival of *P. parvulus* CUPV22 remained at around 30% of cell viability after exposure to pHs from 5.0 to 3.1 (Fig. 2B), but dropped below the detection limit at more acidic pH values. *L. suebicus* CUPV221 was significantly more resistant to the gastric acidic stress (P <0.05) with 11% cell survival being detected at pH 1.8 (Fig. 2C), similar to that previously reported for the *Bifidobacterium animalis* subps. *lactis* BB-12 probiotic strain (Fernández de Palencia et al., 2008). To evaluate the tolerance of the strains to intestinal conditions, G pH 5 samples were incubated in the presence of bile salts and pancreatin at pH 6.5 (GI pH 5.0 in Fig. 2). Under these conditions, cell viability of the three strains was not further affected.

The ability of the strains to attach to Caco-2 cells was used as an *in vitro* model for adhesion to the colonic epithelium. The three test bacterial strains had no deleterious effect on the Caco-2 cells, as assessed by Neubauer-counting at the end of the adhesion assay. The number of epithelial cells present in the untreated control sample ($1.72\ x10^5 \pm 2.12x10^4$ cells mL^{-1}) was similar to that detected in presence of *P. parvulus* CUPV1 ($1.76\ x10^5 \pm 1.59x10^4$), *P. parvulus* CUPV22 ($1.99\ x10^5 \pm 4.7x10^4$) and *L. suebicus* CUPV221 ($1.43\ x10^5 \pm 1.06x10^4$). As shown in Figure 3 the adherence of the LAB strains to Caco-2 cells varied, with *P. parvulus* CUPV22 presenting the highest level of adhesion (10.5%). This level was similar to that reported for the probiotic *L. rhamnosus* GG (9.7%) (Toumola and Salminen, 1998), but higher than that detected for *P. parvulus* 2.6 (6.1%) (Fernández de Palencia et al., 2009). The adhesion level of *P. parvulus* CUPV22 was significant higher (P <0.05) than the *Lactobacillus acidophilus* LA-5 (5.5%) and *Bifidobacterium animalis* subsp. *lactis* BB-12 (1.2%) probiotic strains (Fig. 3), while

L. suebicus showed a higher level than that found for *Bifidobacterium animalis* subsp. *lactis* BB-12 (P <0.05) and similar to that of *L. acidophilus*.

In order to test the contribution of the β-glucan to the adhesion capability, an additional assay was performed with *P. parvulus* CUPV22, where the bacterial cells had been previously washed with PBS with the aim of removing the β-glucan attached to the cell surface (*P. parvulus* CUPV22* sub-population). Analysis of the washed bacteria by electron microscopy (insets in Fig. 3) revealed that indeed this treatment had removed the EPS. Moreover, it provoked a sharp decrease in the adhesion capability of the strain to Caco-2 cells (from 10.5 % to 1.2%) for the CUPV22* sub-population. This is in agreement with the previously reported finding that removal of the EPS of *P. parvulus* 2.6 EPS reduced its adherence to Caco-2 cells to levels close to that of its isogenic strain, which does not produce the (1,3)-β-D-glucan (Fernández de Palencia et al., 2009), This and the results of Dols-Lafargue et al. (2008) which showed the contribution of 2-substituted (1,3)-β-D-glucan on biofilm formation by LAB, support that this biopolymer could play an important role in cellular recognition and intestinal colonization by LAB strains.

The immunomodulatory capacity of *P. parvulus* CUPV22 and *L. suebicus* CUPV221 was evaluated by determining their ability to modulate cytokine production by GM-CSF-driven pro-inflammatory M1 macrophages and M-CSF-driven anti-inflammatory macrophages (Verreck et al., 2004). Both bacterial strains triggered production of IL-6, IL-8 and TNF-α pro-inflammatory cytokines from M1 macrophages (Fig. 4). By contrast, IL-10 production was only induced by *L. suebicus* CUPV221 in both pro-inflammatory M1 and anti-inflammatory M2 macrophages (Verreck et al., 2004). It is well recognized that the anti-inflammatory cytokine IL-10 has a critical role in the regulation of immunity against infections and the resolution of inflammatory responses, by virtue of its capacity to down-modulate the activity of the immune cells that cause tissue damage during pathogen clearance and inflammation (Couper et al., 2008). IL-10 action is

primarily on monocytes and macrophages, where it limits the production of pro-inflammatory cytokines (including IL-1, IL-6, IL-12 and TNF-α) (Moore et al., 2001). Therefore, the ability of *L. suebicus* CUPV221 to induce the expression of IL-10 by macrophages may indicate an ability to limit tissue damage. In this regard, given its higher IL-10-inducing capacity, strain *L. suebicus* CUPV221 would be better suited for favouring resolution of inflammation and maintenance of tissue homeostasis, two effects that would be compatible with a probiotic action. To our knowledge this is the first instance of a *L. suebicus* strain with potentially beneficial immunomodulatory properties.

3.4. Suitability for industrial use

Traditionally, LAB strains have been used to impart characteristic flavours to fermented food products and also to increase viscosity, especially in dairy products, though also in cereal derived products as described above. Basic requirements for candidate industrial strains are, therefore the ability to ferment the candidate food substrate, and the ability to produce a biothickening product (typically an EPS).

In order to detect the potential raw materials to be fermented, the carbohydrate consumption profile of the two *P. parvulus* and the *L. suebicus* strains has been analyzed. The three strains were able to grow on, and ferment, glucose, fructose, and trehalose (data not shown), making them suitable for use to elaborate fermented products. The ability of the two *P. parvulus* strains to also ferment maltose (data not shown), a disaccharide present in oat and other cereals, indicates a potential use as starter or adjunt cultures in non-dairy product fermentations.

The use of the 2-substituted (1,3)-β-D-glucan of *P. parvulus* 2.6 as a biothickener has been proven, and has been tested in human trials (Mårtensson et al., 2005). For this reason, we determined the increase in viscosity of the culture supernatants produced by the three strains studied in this work. All three strains produced significant levels (137-180 mg L^{-1}) of the EPS

during growth in semi-defined medium (Fig. 1). β-glucan production by the *Pediococcus* strains started during exponential growth phase, as has been demonstrated for other 2-substituted (1,3)-β-D-glucan producers, such as *P. parvulus* 2.6 (Velasco et al., 2006) and *P. damnosus* IOEB8801 (Walling et al., 2005). By contrast, *L. suebicus* CUPV221 produced the EPS during the stationary phase, and could be advantageously used as an adjunct during fermentations.

Given their fermentative and biothickening properties alone, these three LAB strains could have potential as industrially useful cultures. However, the strains also possess potential probiotic properties which enhance their attractiveness. All showed some degree of stability to simulated GI tract conditions, with the *L. suebicus* strain being the best equipped to pass through the stomach and survive in the lower intestine. All showed ability to bind to Caco-2 cells, suggesting that they could colonise the lower GI tract, and modulated the production of cytokines by pro-inflammatory M1 and anti-inflammatory M2 macrophages. *L. suebicus* CUPV221 was of particular interest as it induced the anti-inflammatory cytokine IL10.

4. **Conclusions**

From the results reported here we conclude that *Lactobacillus suebicus* CUPV221, *Pedioccocus parvulus* CUPV1 and *P. parvulus* CUPV22 are potential candidates for the production of functional foods. All strains produced high levels of 2-substituted (1,3)-β-D-glucan, and *P. parvulus* CUPV22 and particularly *L. suebicus* CUPV221 would probably be able to survive and colonize the lower parts of the GI tract. The three strains varied in their ability to induce the production of inflammation-related cytokines by polarized macrophages. Interestingly, *L. suebicus* CUPV221 induced the anti-inflammatory cytokine IL10 with could be advantageous in a probiotic strain.

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LEGENDS TO THE FIGURES

Figure 1. Analysis of EPS production by LAB. Growth of the indicated EPS-producing LAB in semi-defined medium was followed by OD measurements (left panels). Their EPS production (solid line) and the viscosity increases of culture medium (dashed line) are depicted in the right panels. The determinations were performed in duplicate and experiments were conducted in triplicate and the mean and standard deviations are shown. *The ratio viscosity/EPS at 181 hours for strain CUPV1 showed a significant difference (P<0.001) from the ratios detected with strains CUPV22 and CUPV 221.

Figure 2. Analysis of cell survival after gastric (G) and gastrointestinal (GI) stresses. The indicated bacterial strains were untreated (C) or subjected to various G or GI stresses as described in the text. Cell viability was analyzed by measurement of green and red fluorescence after appropriate staining. The determinations were performed in duplicate and the values are the mean of three independent experiments performed with three different cultures of each bacterium and are expressed as a percentage of control. 100% control values of the green/red fluorescence ratio for untreated *P. parvulus* CUPV1, *P. parvulus* CUPV22 and *L. suebicus* CUPV221 were respectively 7.3, 10.8 and 9.5 which corresponded to $2.2x10^8$, $2.3x10^8$ and $8x10^7$ cfu mL^{-1}.
* P <0.01 compared with *P. parvulus* CUPV 22 and *L. suebicus* CUPV221. ** P <0.05 compared with the *P. parvulus* CUPV1 and CUPV22 strains.

Figure 3. Adhesion of bacterial strains to Caco-2 cells after infection with ten bacteria per epithelial cell. Adhesion levels are expressed as the percentage of the total number of bacteria (adhered plus unadhered) detected after exposure for 1 hour to Caco-2 cells. Each adhesion assay was conducted in triplicate. The values are the mean of three independent experiments

performed with three different cultures of each bacterium and each experiment with a different Caco-2 culture, and for each of them, three independent determinations were performed. ** P <0.05 compared with *L. acidophilus* La-5 and *B. animalis* BB-12; * P <0.05 compared with *B. animalis* BB-12. The two insets show electron micrographs of *P. parvulus* CUPV22 cultures unwashed (CUPV22) or washed with PBS (CUPV22*) prior to the adhesion experiments.

Figure 4. Cytokine response of macrophages to *P. parvulus* CUPV22 and *L. suebicus* CUPV221 strains M1 and M2 macrophages were either untreated (Basal 18h) or stimulated with LPS from *Escherichia coli* 055:B5 at 10 ng mL^{-1} or the indicated LAB strains. The levels of IL-6 (A), IL-8 (B), TNF-α (C) and IL-10 (D) released were determined. Each determination was performed in triplicate and the mean and standard deviations are shown. * P <0.05 comparison with *P. parvulus* CUPV22.

Figure
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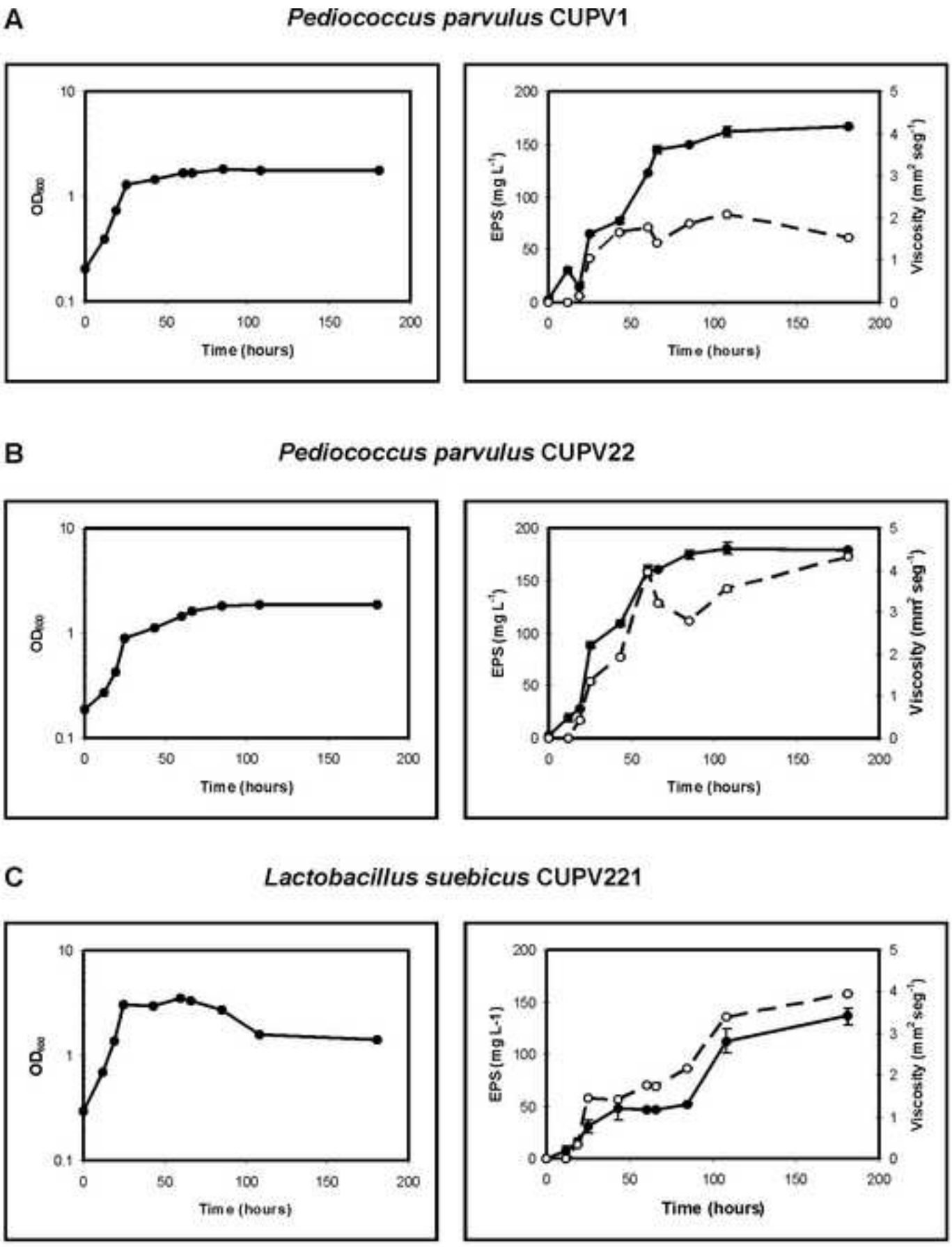

Figure 1

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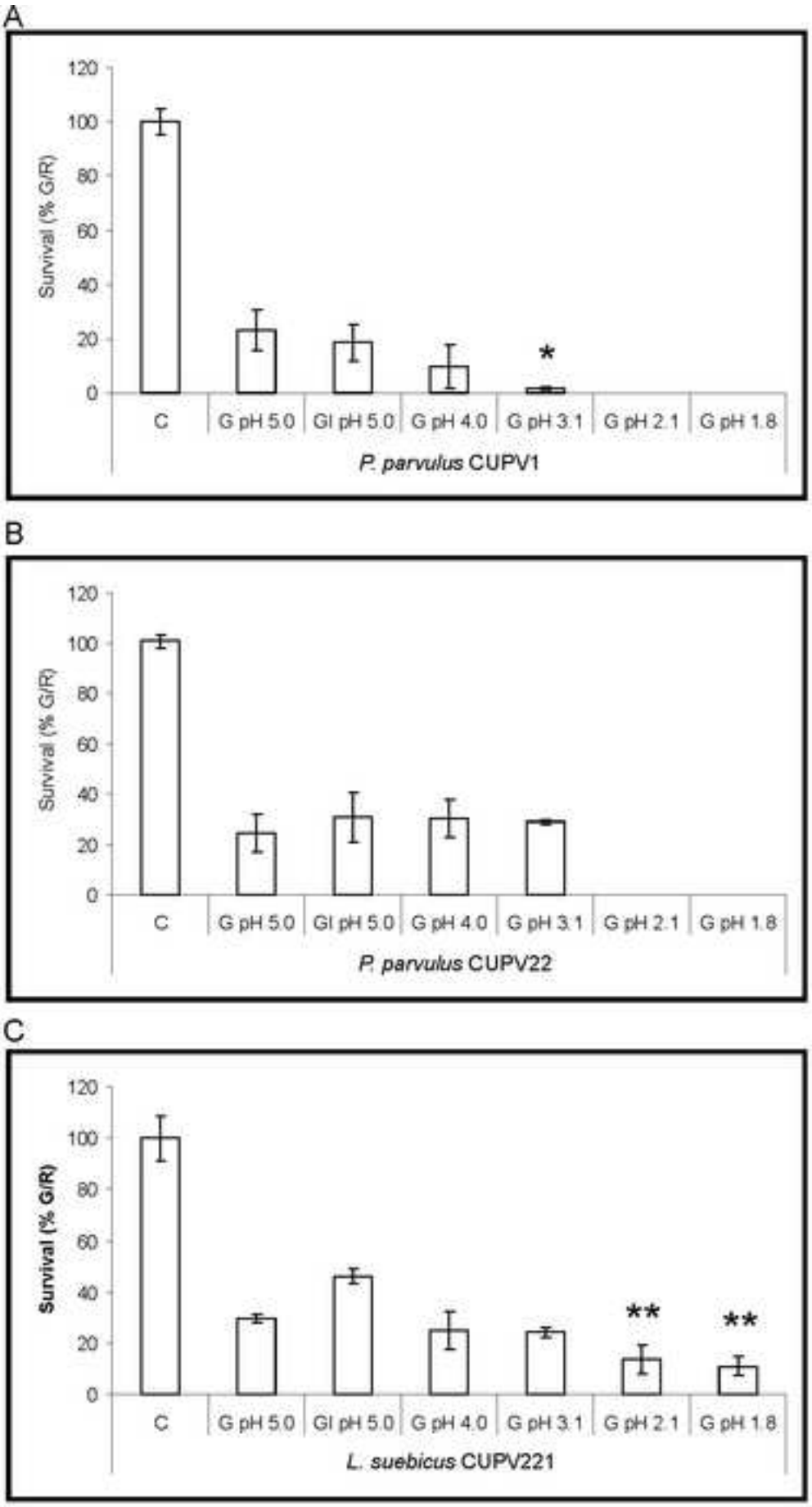

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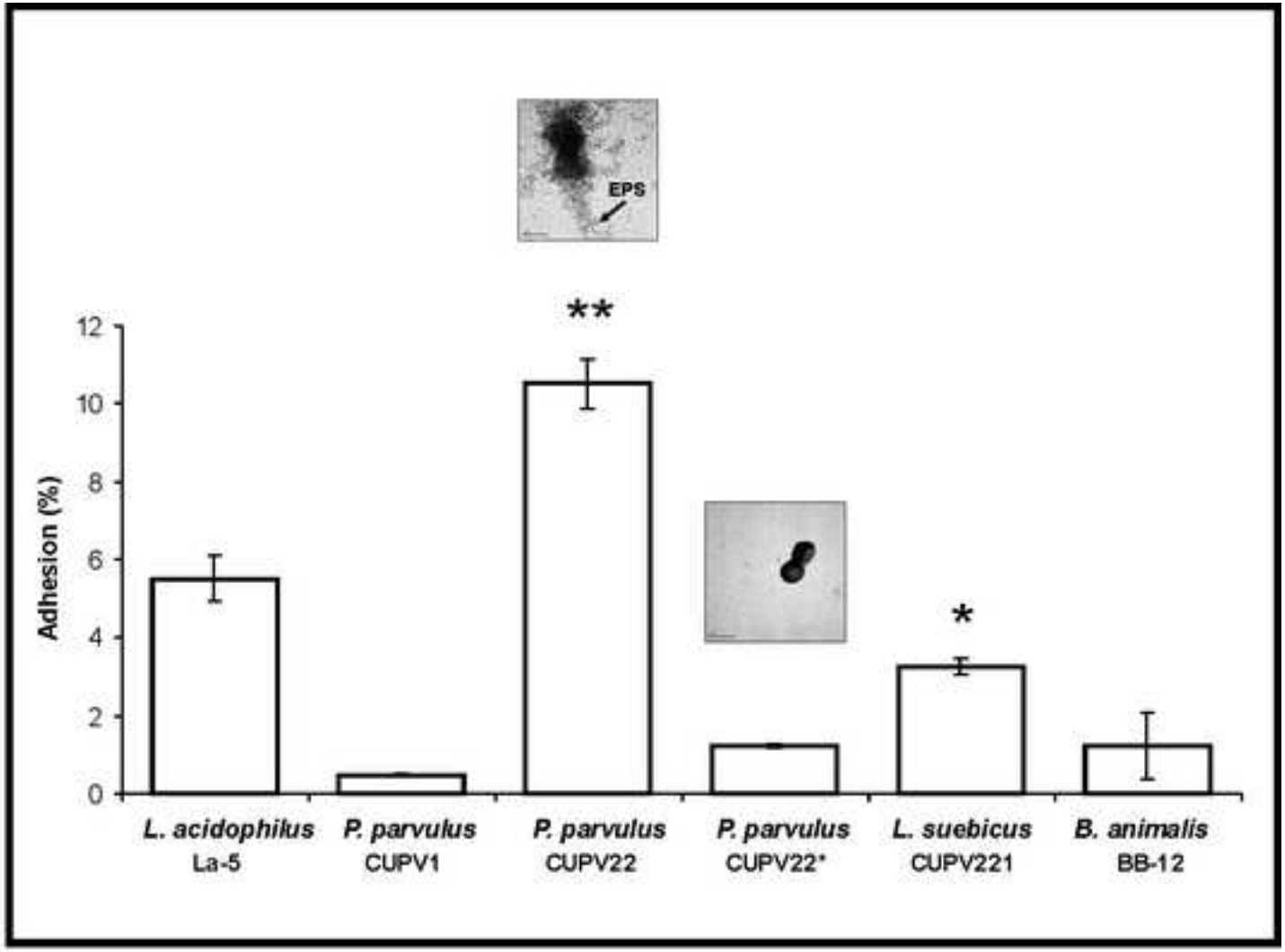

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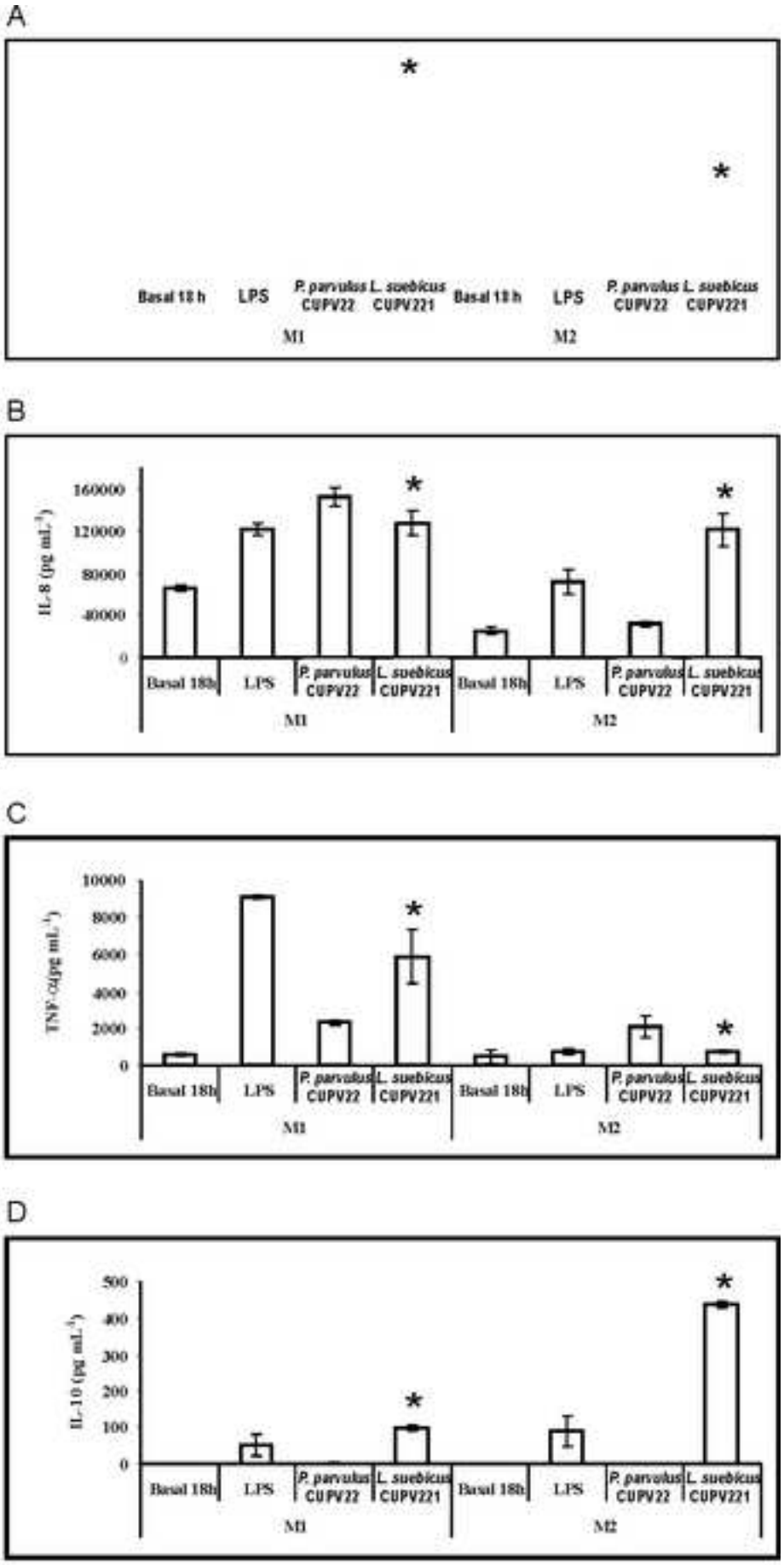

Figure 4

Electronic Annex

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SUPPLEMENTARY DATA

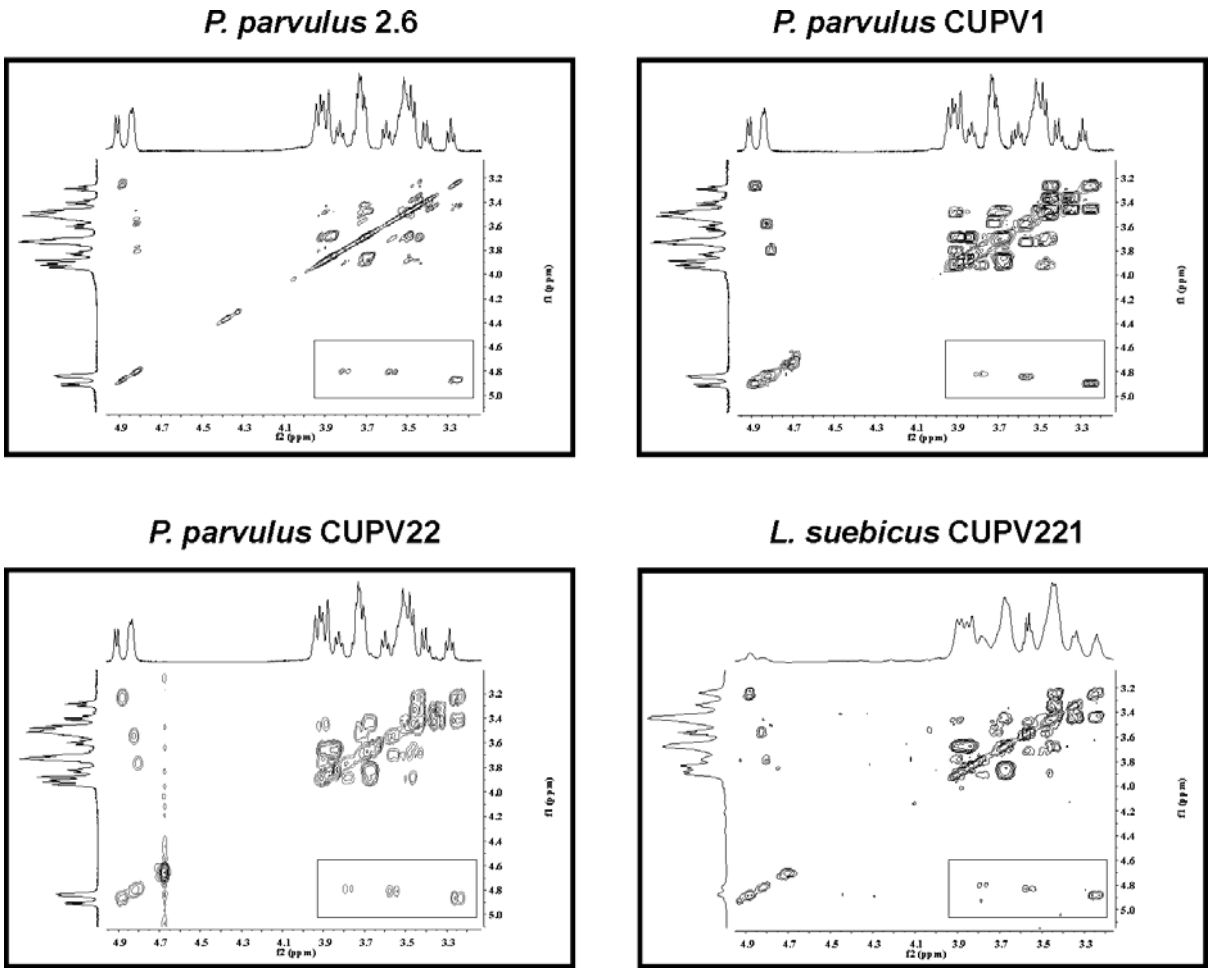

Figure S1. Analysis of the structure of EPS. 500 MHz ^{1}H NMR spectra of the polymers synthesized by the indicated strains were recorded in D_2O at 30ºC and the 500 MHz 2D homonuclear proton double-band filtered correlation spectrum (DBF-COSY) was obtained by double band selective excitation of the 3-5 ppm region. Couplings between anomeric protons and C2 protons ($H1_C/H2_C$, $H1_B/H2_B$ y $H1_A/H2_A$) of the 2-substituted (1,3)-β-D-glucan from *P. parvulus* 2.6 are boxed.

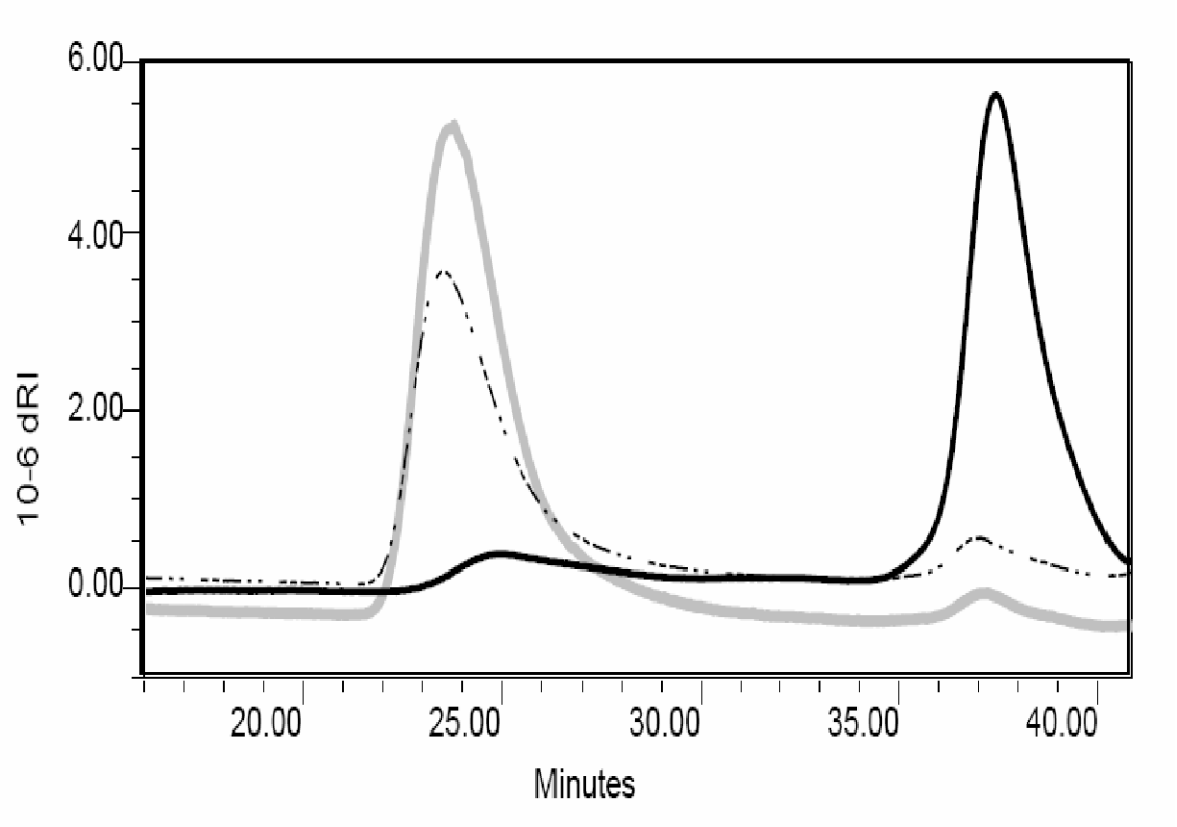

Figure S2. Determination of the molecular mass of EPS. Size exclusion HPLC chromatograms are shown for the β-glucans synthesized by *P. parvulus* CUPV1 (grey line), *P. parvulus* CUPV22 (dashed line) or *L. suebicus* CUPV221 (solid line).