A specific immunological method to detect and quantify bacterial 2-substituted (1,3)- β -D-glucan

María Laura Werning¹, Adrián Pérez-Ramos¹, Pilar Fernández de Palencia¹, Maria Luz Mohedano¹, María Teresa Dueñas², Alicia Prieto¹ and Paloma López^{1*}

¹Centro de Investigaciones Biológicas, C.S.I.C., Ramiro de Maeztu 9. 28040 Madrid, Spain. ²Departamento de Química Aplicada, Facultad de Ciencias Químicas, Box 1072, 20080 San Sebastian, Spain.

*Corresponding author. Mailing address: Centro de Investigaciones Biológicas, Ramiro de Maeztu 9. 28040 Madrid, Spain. Tel: 34-918373112. Fax: 34-915360432. e-mail: plg@cib.csic.es.

Abbreviations: DM, defined medium; ELISA, Enzyme Linked Immuno-Sorbent Assay; EPS, exopolysaccharide; GLC, gas-liquid chromatography; GLC-MS, gas-liquid chromatography-mass spectrometry; HP-SEC, high-performance size exclusion chromatography; LAB, lactic acid bacteria; PBS, Phosphate buffered saline; SDM, semi-defined medium.

Abstract

Exopolysaccharides synthesized by lactic acid bacteria have prebiotic properties and contribute to the rheology and texture of fermented foods. Here, we have standardized an immunological method for the specific detection of 2-substituted (1,3)- β -D-glucans. The method allows direct detection and quantification of this exopolysaccharide in culture supernatants containing other mono- and poly-saccharides. Moreover, it allows specific detection of the biomolecules synthesized *in vitro* in enzymatic reactions. Thus, this method allows the fast identification of producing bacteria, as well as biochemical characterization of the glycosyltransferases responsible for their synthesis.

Keywords: exopolysaccharide, lactic acid bacteria, β -D-glucan, probiotic, prebiotic.

1. Introduction

Most commercialized probiotic microorganisms are lactic acid bacteria (LAB) and some produce exopolysaccharides (EPS) (Ruas-Madiedo, Abraham, Mozzi & de los Reyes-Gavilán, 2008; Salminen et al., 1998; Ventura, Canchaya, Fitzgerald, Gupta, & van Sinderen, 2007). In addition, some LAB strains belonging to the *Pediococcus*, *Lactobacillus* and *Oenococcus* genera, isolated from cider and wine, are able to produce a 2-substituted (1,3)- β -D-glucan EPS (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). Analysis of the rheological properties of the 2-substituted (1,3)- β -D-glucan synthesized by *Pediococcus parvulus* 2.6R showed that it has potential utility as a biothickener (Lambo-Fodje, Leeman, Wahlund, Nyman, Öste, & Larsson, 2007; Velasco, Areizaga, Irastorza, Dueñas, Santamaría, & Muñoz, 2009). *Pediococcus* (Fernández de Palencia et al., 2009) and *Lactobacillus* (Garai-Ibabe et al., 2010) strains that produce this EPS have increased adherence to Caco-2 cells. *P. parvulus* 2.6R is able to immunomodulate macrophages (Fernández de Palencia et al., 2009) and production of the 2-substituted (1,3)- β -D-glucan confers to the intestinal *Lactobacillus. paracasei* NFBC 338 higher resistance to gastrointestinal and technological stresses (Stack, Kearney, Stanton, Gerald, Fitzgerald, & Ross, 2010). In addition, human consumption of oat-based food prepared with *P. parvulus* 2.6R resulted in a decrease of serum cholesterol levels, boosting the effect previously demonstrated for (1,3)(1,4)- β -D-glucans of oat-based products (Mårtensson et al., 2005). Moreover, the production of yogurt and various beverages with 2-substituted (1,3)- β -D-glucan producing LAB indicate the advantageous techno-functional properties of these strains (Elizaquível et al., 2011;

Kearney et al., 2011). Furthermore, the purified EPS from *P. parvulus* 2.6R increased the growth, viability and adherence capability of probiotic LAB (Russo et al., 2012). Therefore, LAB producing this EPS could have potential as probiotic strains and the 2-substituted (1,3)- β -D-glucan itself appears to have prebiotic properties.

The specific detection and quantification of EPS produced by LAB, especially in a background that contains other carbohydrates, is laborious and time-consuming. The colorimetric phenol-sulphuric method (Dubois, Gilles Hamilton, Rebers & Smith, 1956) to quantify the total polysaccharide content of samples, requires the removal of any disaccharides or monosaccharides present in the media by ultrafiltration, dialysis or ethanol precipitation (Ruas-Madiedo, Abraham, Mozzi & de los Reyes-Gavilán, 2008; Werning, Notararigo, Nácher, Fernández de Palencia, Aznar & López, 2012). A complete characterization of the polysaccharide then usually requires further purification, followed by relatively sophisticated, derivatisation, degradation and analytical techniques. Therefore a simple direct method for the specific and quantitative detection of 2-substituted (1,3)- β -D-glucan synthesized by bacteria would facilitate the identification of new potential probiotic strains, and the quantification of 2-substituted (1,3)- β -D-glucan production.

LAB 2-substituted (1,3)- β -D-glucans are synthesized by GTF glycosyltransferase (Werning, Ibarburu, Dueñas, Irastorza, Navas & López, 2006; Werning, Corrales, Prieto, Fernández de Palencia, Navas & López, 2008), which has significant identity (33%) only with the Tts glycosyltransferase of *Streptococcus pneumoniae* serotype 37 (Llull, Muñoz, López & García, 1999). This latter enzyme synthesizes this organism's capsule (Llull, García, & López, 2001), which is a β -D-glucan similar to that synthesized by *Pediococcus* and *Lactobacillus* strains. Anti-serotype 37 antibodies also agglutinate *Lactococcus lactis* and *Lb. paracasei* NFBC 338 strains that over-produce

1 GTF glycosyltransferase ((Dols-Lafargue, Lee, Le Marrec, Heyraud, Chambat, &
2 Lonvaud-Funel, 2008; Stack, Kearney, Stanton, Gerald, Fitzgerald, & Ross, 2010;
3 Werning, Corrales, Prieto, Fernández de Palencia, Navas & López, 2008) as well as
4 LAB strains naturally carrying this gene ((Dols-Lafargue, Lee, Le Marrec, Heyraud,
5 Chambat & Lonvaud-Funel, 2008; Werning, Ibarburu, Dueñas, Irastorza, Navas &
6 López, 2006). Therefore, in this work, we report on the design and validation of a
7 competition Enzyme Linked Immuno-Sorbent Assay (ELISA) method for the specific
8 detection of 2-substituted (1,3)- β -glucans, based on *S. pneumoniae* serotype 37
9 antibodies.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The following bacteria were used in this work. The 2-substituted (1,3)- β -D-glucan-producer strains isolated from Basque natural cider: *Lactobacillus diolivorans* G-77 (Dueñas-Chasco, Rodríguez-Carvajal, Tejero-Mateo, Espartero, Irastorza-Iribas, & Gil-Serrano, 1998), *Oenococcus oeni* I4 (Ibarburu et al., 2007), *P. parvulus* 2.6R, and its isogenic non-producing strain (2.6NR) (Dueñas et al. 1997). The 2-substituted (1,3)- β -D-glucan-producing *L. lactis* NZ9000[pNGTF] recombinant strain, which carries in the pNGTF plasmid, based on the pNZ8048 vector, the *P. parvulus* 2.6R *gft* gene under control of the PnisA promoter, and its isogenic β -D-glucan non-producing strain *L. lactis* NZ9000[pNZ8048] (Werning, Corrales, Prieto, Fernández de Palencia, Navas & López, 2008).

The *Pediococcus* strains were routinely grown at 30 °C in Man Rogosa Sharpe (MRS) broth (De Man, Rogosa & Sharpe, 1960) supplemented with 0.5% glucose and 0.1% tween 80. *O. oeni* I4 was routinely grown at 30 °C under anaerobic conditions in MRS supplemented with 0.5% fructose and 1% glucose. *Lb. diolivorans* G-77 was routinely grown at 30 °C in MRS supplemented with 0.5% glucose. The *Lactococcus* strains were routinely grown at 30 °C in M17 broth (Terzaghi & Sandine, 1975) supplemented with chloramphenicol (5 $\mu\text{g mL}^{-1}$).

For EPS production, batch fermentations without pH control were carried out in a semi-defined medium (SDM) supplemented with glucose (2%) (Dueñas et al. 1997) or MRS for *P. parvulus* 2.6R and *Lb. diolivorans* G-77 as well as in the same media supplemented with fructose (1%) for *O. oeni* I4 or in a defined medium (DM) (Sánchez et al., 2008) or M17, both supplemented with glucose (0.5%) and chloramphenicol (5 $\mu\text{g mL}^{-1}$) for *L. lactis* NZ9000[pNGTF]. *Pediococcus*, *Lactobacillus* and *Oenococcus*

strains were grown to stationary phase to increase production of the EPS. *L. lactis* strains were grown in the indicated media until OD₆₀₀= 0.6. Then, for *L. lactis* NZ9000[pNGTF] only, expression of the *gtf* gene encoding the GTF glycosyltransferase was induced with nisin (0.25 ng mL⁻¹) 24 h prior to testing. The strains were stored at -80°C in either MRS or M17 media supplemented with 20% (v/v) glycerol.

2.2. Isolation, purification and characterization of EPS synthesized by LAB

2-substituted (1,3)-β-glucan samples from *P. parvulus* 2.6R and *L. lactis* NZ9000[pNGTF] were isolated and purified from culture supernatants by ethanol precipitation and dialysis as previously described (Notararigo et al., 2013). Stock solutions of both purified EPS were prepared at 1 mg mL⁻¹ in phosphate buffered saline (PBS) pH 7.0 and stored at -20°C. For ELISA assays, EPS stock solutions were diluted in PBS pH 7.0 prior to analysis. The molecular masses of the EPS were determined by high-performance size exclusion chromatography (HP-SEC, GPCV 2000, Waters), as previously described (Velasco, Areizaga, Irastorza, Dueñas, Santamaría, & Muñoz, 2009).

2.3. ELISA assays

ELISA assays were carried out in 96-well Nunc Maxisorp microtitre plates (Thermo Fisher Scientific, USA). The samples (cultures supernatants or purified polysaccharides) and a negative control without biopolymers were tested in duplicate in each assay. In the standard assay the 2-substituted (1,3)-β-D-glucan synthesized by *L. lactis* NZ9000[pNGTF] or by *P. parvulus* 2.6R was used at 62.5 ng mL⁻¹ as immobilized EPS as well as at various concentrations as competitor for binding to the primary antibody (dilution 1:800 of anti-serotype 37, Statens Serum Institut, Denmark). In addition, to test specificity of the method, laminarin from *Laminaria digitata*; curdlan from

Alcaligenes faecalis and xanthan from *Xanthomonas campestris* (all from Sigma-Aldrich, Germany) were used as competitors at concentrations from 3.9 $\mu\text{g mL}^{-1}$ to 2.5 mg mL^{-1} .

To optimize the ELISA assay, various 2-substituted (1,3)- β -glucan solutions (31.25, 62.5, 125, 250 and 500 ng mL^{-1}) were used to coat the wells of microtitre plates (200 $\mu\text{l/well}$) by incubation for 16 hours at 4°C. The wells were washed twice with 200 μl of solution I (PBS pH 7.0 and 0.05% Tween 20), and then 300 μl of blocking solution (solution I plus 0.5% gelatin) were added, followed by 3 hours of incubation at 21°C. To perform the competition assay 150 μl of each dilution of the primary antibody (1:200, 1:400, 1:800 or 1:1600) was incubated for 30 min at 21°C with 150 μl of each EPS dilution (from 2.4 ng mL^{-1} to 5.0 $\mu\text{g mL}^{-1}$). Then, 200 μl of the mixture were added to each activated well of the microtitre plates and after 1.5 h of incubation at 21°C (to allow binding of free antibody to the immobilized 2-substituted (1,3)- β -glucan) the wells were washed three times with solution I. The amount of primary antibody bound to the wells was detected by addition of 200 μl of the secondary antibody (polyclonal Anti-IgG against rabbit alkaline phosphatase, Sigma-Aldrich, Germany) diluted 1:25000 in blocking solution, incubation for 2 h at 21°C, four washes of the wells with solution I followed by the addition of 200 μl of developing solution (*p*-nitrophenylphosphate at 1 mg mL^{-1}) in diethanolamine buffer (0.2 M diethanolamine pH 9.8 and 5 mM MgCl_2 , Sigma) and a final incubation for 30 min at 37°C. Then, the reactions were stopped by addition of 2 N NaOH (50 μl) and the absorbance at 415 nm was measured in a microtitre plate reader (BioRad model 680).

2.4. Phenol-sulphuric method

To determine the 2-substituted (1,3)- β -D-glucan concentration by this method, two replicates of 0.5 mL culture supernatants were precipitated overnight with 1 mL of

ethanol at -20°C and sedimented by centrifugation (13,600 x g, 20 min 4°C). The precipitates were twice resuspended in 0.5 mL of distilled water and ethanol reprecipitated and resedimented as described above. Finally, after resuspension in distilled water, the concentration of the biopolymer present in the precipitates was determined by the phenol-sulphuric method as reported by Dubois, Gilles Hamilton, Rebers & Smith (1956).

2.5. Analysis of GTF glycosyltransferase activity

Membrane vesicles displaying GTF glycosyltransferase activity, were prepared from *L. lactis* NZ9000[pNGTF] as previously described (Werning, Corrales, Prieto, Fernández de Palencia, Navas & López, 2008) and the enzymatic reactions were carried out as follows. Duplicate 100 µl reactions were prepared containing 12.5, 25 or 50 µg of membrane proteins as well as a range of UDP-glucose (Sigma) concentrations (from 0.04 to 25 mM) , 1 mM CaCl₂, 9 mM MgCl₂ and 50 mM NaCl. These were incubated for 30 min at 30°C then the reactions were stopped by addition of 900 µl of 100% ethanol. After 2 h incubation at 4°C, samples were centrifuged at 13,226 x g at 4°C for 20 min, the precipitates were air dried, and resuspended in 500 µL of PBS buffer pH 8.0. The reaction product was quantified by the ELISA method. The kinetic parameter, apparent K_m and apparent V_{max} were determined from Lineweaver Burk plots.

2.6. Chemical analysis of the enzymatic reaction product

The neutral sugar composition and linkage type was determined using polysaccharide synthesized in duplicate 1 mL reactions containing membrane vesicles (50 mg of total proteins) with or without 25 mM UDP-glucose. The ethanol precipitates, after air drying, were resuspended in 1 mL of distilled H₂O plus 5 µl of 10% SDS, incubated for 15 min at 37°C and dialyzed (molecular mass cut-off 3.5 kDa) against distilled H₂O for

1 24 h. Then, 100 μ l aliquots containing the membrane vesicles and the reaction product
2 were hydrolyzed, acetylated and analyzed for the presence of neutral sugars by gas-
3 liquid chromatography (GLC) or after methylation, hydrolysis, reduction and
4 acetylation for the determination of the structural unit of the polysaccharide by gas-
5 liquid chromatography-mass spectrometry (GLC-MS) as previously described
6 (Werning, Corrales, Prieto, Fernández de Palencia, Navas & López, 2008).

3. Results

3.1. Sensitivity of the ELISA assay

To determine the sensitivity of the method two samples of 2-substituted (1,3)- β -glucans synthesized by either *L. lactis* NZ9000[pNGTF] or *P. parvulus* 2.6R were purified and their molecular masses determined by HP-SEC. The average molecular masses of the two main fractions were 6.6×10^6 Da and 1.8×10^4 Da for *L. lactis* (Fig. 1A) and of 9.6×10^6 Da and 3.4×10^4 Da for *P. parvulus* (Fig. 1B). The higher molecular mass fraction was the most abundant for both strains constituting, respectively, 96% and 92% of the total polymer population. Both EPS preparations were used for testing as immobilized β -glucans and as competitors for binding to the primary antibody. Both EPS preparations were immobilized efficiently (Fig. 2A versus Fig. 2B) and both purified β -D-glucans could be quantified within a range of 20 to 2500 ng mL⁻¹, independently of the origin of the immobilized polymer (Fig. 2).

3.2. Specificity of the assay

Using the purified β -D-glucan from *L. lactis* (described above) as the immobilized substrate, we tested the specificity of the method using three polysaccharides as competitors: i) laminarin, a 6-substituted (1,3)- β -D-glucan (Fig. 3A); ii) curdlan, a linear (1-3)- β -D-glucan (Fig. 3B) and iii) xanthan, a branched heteropolysaccharide (Fig. 3C). Xanthan was unable to bind to the primary antibody even at very high concentration (500 μ g mL⁻¹) and curdlan also showed a very low affinity for the antibody at 250 μ g mL⁻¹. Laminarin was able to compete with the immobilized 2-substituted (1,3)- β -D-glucan in a linear mode, when tested in the range 10 to 250 μ g mL⁻¹.

3.3. Quantification of EPS in growth media by using the ELISA assay or the phenol-sulphuric method

The suitability of the immunological method for directly quantifying (1-3)- β -D-glucan in LAB growth media was tested as follows. The β -D-glucan purified from *L. lactis* NZ9000[pNGTF] was dissolved in either the DM (defined) or SDM (semi-defined) medium or in M17 and MRS complex media, and the EPS concentration was determined by the competition ELISA assay. In all cases the method was able to quantify EPS in the range of 30 to 500 ng mL⁻¹ (Supplemental material S1). However, examination of the different slopes of the curves (see panels A; B, C and D of Supplemental material S1) in comparison with Figure 2, indicated that for correct quantification of 2-substituted (1,3)- β -D-glucan in different buffers or growth media, the corresponding standard curve should be prepared using the corresponding solvent. Therefore, the method used to quantify β -D-glucan present in culture supernatants of LAB grown in the above mentioned media always used the appropriate standard curve. Thus, *L. lactis* NZ9000[pNGTF] and the isogenic β -D-glucan non-producing *L. lactis* NZ9000[pNZ8048] were grown in M17 medium as described previously (Werning, Corrales, Prieto, Fernández de Palencia, Navas & López, 2008). *P. parvulus* 2.6R and the isogenic β -D-glucan non-producing strain (2.6NR) as well as *O. oeni* I4 and *Lb. diolivorans* G-77, and two other 2-substituted (1,3)- β -D-glucan producers, were grown in MRS medium to stationary phase as described above. The cultures were then centrifuged and, after removal of the supernatants, the EPS still bound to the cells were released by resuspension into one volume of PBS and the bacterial cells were removed by centrifugation. Then, the supernatants, after appropriate dilution in PBS, were directly used in the ELISA assay. The results revealed that the 2-substituted (1,3)- β -D-

1 **glucan secreted** by the four producing-LAB could be detected in the media used,
2 whereas only background levels were observed in the supernatants of the non-producing
3 strains (Fig. 4).

4 To compare the inhibition enzyme immunoassay and the phenol-sulphuric method,
5 culture supernatants of *L. lactis* NZ9000[pNGTF] grown in DM medium and *P.*
6 *parvulus* 2.6R, *Lb. diolivorans* G-77 and *O. oeni* I4 grown in SDM medium were
7 analyzed using both methods. Only these media were tested for detection of the 2-
8 substituted (1,3)- β -D-glucan, because the presence of other polysaccharides in M17 and
9 MRS media impairs the usage of the phenol-sulphuric method. The concentration of 2-
10 substituted (1,3)- β -D-glucan was determined directly in the supernatants by the ELISA
11 test or in their ethanol precipitates by the ELISA and phenol-sulphuric methods (Table
12 1). Determination of the EPS concentration in the precipitates showed that for all the
13 strains tested the ELISA assay revealed a higher level of production (from 61% for *Lb.*
14 *diolivorans* G-77 to 36% for *L. lactis* NZ9000[pNGTF]). In addition, comparison of
15 EPS detection by **the** ELISA method prior **to, or after**, precipitation demonstrated that
16 indeed precipitation results in a moderate loss of 2-substituted (1,3)- β -D-glucan in a
17 range from 38% to 20% for *P. parvulus* 2.6R and *O. oeni* I4 biopolymers. These
18 differences were probably due to low molecular weight EPS that was not precipitated by
19 ethanol. Therefore, these results indicate that the ELISA method allows a more reliable
20 determination of the biopolymer after its synthesis by bacteria.

21 3.4. Detection of 2-substituted (1,3)- β -D-glucan produced in an enzymatic reaction

22 Membrane vesicles containing the GTF glycosyltransferase, were prepared from *L.*
23 *lactis* NZ9000[pNGTF] and the enzymatic activity was analyzed *in vitro* using UDP-
24 glucose as substrate. The reaction product was detected and quantified by the ELISA

1 method. Kinetic analysis of the results (Supplemental material S2) indicated that the
2 GTF glycosyltransferase has an affinity for UDP-glucose of $K_m = 123 \pm 3 \mu\text{M}$ and a $V_{\max}$
3 $= 18 \pm 0.8 \text{ ng of EPS/mg of total protein/min}$. This K_m is almost identical to that reported
4 for the cellulose synthase BscA ($125 \mu\text{M}$) (Ross et al. 1991) which, like GTF, belongs
5 to the GT-2 family of glycosyltransferases.

6 To confirm the nature of the enzymatic reaction product, reactions performed with
7 membrane vesicles and containing UDP-glucose (test) or lacking the substrate (control)
8 were analyzed. In the presence or absence of the substrate, the GLC analysis detected
9 rhamnose, glucose and galactose, in the ratios 1:2.9:0.4 and 1:1.4:0.5 for the test (Fig.
10 5A) and control (Fig. 5B) reactions respectively. Thus, the *L. lactis* membrane vesicle
11 preparations contained three monosaccharides, whose origin could be due to
12 polysaccharides bound to them and as expected only glucose was highest in the test
13 reaction. The GLC-MS analysis (Figs. 5C and 5D) detected, in the test reaction only
14 (Fig. 5C), the three peaks that are characteristic of 2-substituted-(1,3)- β -glucan,
15 corresponding to terminal glucopyranose, 3-*O*-substituted glucopyranose, and 2,-di-*O*-
16 substituted glucopyranose. Thus, the product synthesized *in vitro* by the GTF
17 glycosyltransferase was indeed 2-substituted (1,3)- β -D-glucan, confirming the ELISA
18 detection of the product.

4. Discussion

ELISA tests have been developed previously for the detection of both linear and 6-substituted (1,3)- β -D-glucans (Douwes, Doekes, Montijn, Heederik & Brunekreef, 1996; Sander, Fleischer, Borowitzki, Brüning & Raulf-Heimsoth, 2008) as well as for the specific detection of 6-substituted (1,3)- β -D-glucans (Milton, Udeni Alwis, Fisette & Muilemberg, 2001; Rao et al. 2007; Sander, Fleischer, Borowitzki, Brüning & Raulf-Heimsoth, 2008). However, as far as we know, this is the first instance of an ELISA test developed for the detection of 2-substituted (1,3)- β -D-glucan. In the inhibition enzyme immunoassay developed in this work the commercially available antibody (anti-*S. pneumoniae* serotype 37) has been used. Our results show that this primary antibody specifically detects 2-substituted (1,3)- β -D-glucan, has very low binding affinity for 6-substituted (1,3)- β -D-glucan and does not bind linear (1,3)- β -D-glucan even at a concentration as high as 500 $\mu\text{g mL}^{-1}$. The ELISA method allows detection of 30 ng mL^{-1} of the biopolymer in growth medium, a level of sensitivity similar to that observed for other β -D-glucan, such as (1, 6)- β -D-glucan (Vink et al., 2004) or (1,3)- β -D-glucan (Douwes, Doekes, Montijn, Heederik & Brunekreef, 1996), when polyclonal antibodies were used for their detection. This method is less sensitive than that obtained for the detection of other polysaccharides with monoclonal antibodies (in the range of pg mL^{-1}) or than the amoebocyte lysate assays for which commercial kits, such as GlucateLL® (Associates of Cape Cod, USA) or Fungitic-G (Seikagaku, Japan) are available. However, the first mentioned type of assay cannot be applied to the detection of 2-substituted (1,3)- β -D-glucan and the second type does not discriminate between different types of (1,3)- β -D-glucans. In addition, our results show that the sensitivity of our ELISA test permits the detection of novel 2-substituted (1,3)- β -D-glucan-producing

bacteria by direct analysis of culture supernatants even after growth in complex media. Moreover, the method can be used for the biochemical characterization of the glycosyltransferases involved in their synthesis. These findings will facilitate the identification of new probiotic strains and the optimization of the production of this EPS.

Acknowledgements. We thank Dr Stephen Elson for critical reading of the manuscript. This work was supported by the Spanish Ministry of Economy and Competiveness grants AGL2009-12998 and AGL2012-40084.

References

- De Man, J. C., Rogosa, M. & Sharpe, M. E. (1960). A medium for the cultivation of lactobacilli. *Journal of Applied Bacteriology*, 23(1), 130–135.
- Dols-Lafargue, M., Lee, H. Y., Le Marrec, C., Heyraud, A., Chambat, G. & Lonvaud-Funel, A. (2008). Characterization of *gtf*, a glucosyltransferase gene in the genomes of *Pediococcus parvulus* and *Oenococcus oeni*. *Applied Environmental Microbiology*, 74(13), 4079-4090.
- Douwes, J., Doekes, G., Montijn, R., Heederik, D. & Brunekreef, B. (1996). Measurement of β (1,3)-glucans in the occupational and home environment with an inhibition enzyme immunoassay. *Applied Environmental Microbiology*, 62(9), 3176–3182.
- Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. & Smith, F. (1956). Colorimetric methods for determination of sugars and related substances. *Analytical Chemistry*, 28(3), 350-356.
- Dueñas-Chasco, M. T., Rodríguez-Carvajal, M. A., Mateo, P. T., Franco-Rodríguez, G., Espartero, J., Irastorza-Iribas, A., et al. (1997). Structural analysis of the exopolysaccharide produced by *Pediococcus damnosus* 2.6. *Carbohydrate Research*, 303(4), 453-458.
- Dueñas-Chasco, M. T., Rodríguez-Carvajal, M. A., Tejero-Mateo, P., Espartero, J. L., Irastorza-Iribas, A. & Gil-Serrano, A. M. (1998). Structural analysis of the exopolysaccharides produced by *Lactobacillus* spp. G-77. *Carbohydrate Research*, 307(1-2), 125-133.
- Elizaquível, P., Sánchez, G., Salvador, A., Fiszman, S., Dueñas, M. T., López, P., et al. (2011). Evaluation of yogurt and various beverages as carriers of lactic acid bacteria producing 2-branched (1,3)- β -D-glucan. *Journal of Dairy Science*, 94(7), 3271-3278.

1 Fernández de Palencia, P., Werning, M. L., Sierra-Filardi, E., Dueñas, M. T., Irastorza, A.,
 2 Corbí, A. L., et al. (2009). Probiotic properties of the 2-substituted (1,3)- β -D-glucan
 3 producing *Pediococcus parvulus* 2.6 . *Applied Environmental Microbiology*, 75(14), 4887-
 4 4891.

5 Garai-Ibabe, G., Dueñas, M. T., Irastorza, A., Sierra-Filardi, E., Werning, M. L., López,
 6 P. et al. (2010). Naturally occurring 2-substituted (1,3)- β -D-glucan producing
 7 *Lactobacillus suebicus* and *Pediococcus parvulus* strains with potential utility in the
 8 production of functional foods. *Bioresource Technology*, 101(23), 9254–9263.

9 Ibarburu, I., Soria Díaz, M. E., Rodríguez-Carvajal, M. A., Velasco, S. E., Tejero-
 10 Mateo, P., Gil-Serrano, A. M., et al. (2007). Growth and exopolysaccharide (EPS)
 11 production by *Oenococcus oeni* I4 and structural characterization of their EPSs. *Journal*
 12 *of Applied Microbiology*, 103(2), 477-486.

13 Kearney, N., Stack, H. M., Tobin, J. T., Chaurin, V., Fenelon, M. A., Fitzgerald, G. F.,
 14 et al. (2011). *Lactobacillus paracasei* NFBC 338 producing recombinant β -glucan
 15 positively influences the functional properties of yogurt. *International Dairy Journal*,
 16 21(8), 561-567.

17 Lambo-Fodje, A. M., Leeman, M., Wahlund, K. G., Nyman, M., Öste, R. & Larsson, H.
 18 (2007). Molar mass and rheological characterisation of an exopolysaccharide from
 19 *Pediococcus damnosus* 2.6. *Carbohydrate Polymers*, 68(3), 577-586.

20 Llaubères, R. M., Richard, B., Lonvaud, A., Dubourdieu, D. & Fournet, B. (1990).
 21 Structure of an exocellular β -D-glucan from *Pediococcus* sp., a wine lactic bacteria.
 22 *Carbohydrate Research*, 203(1), 103-107.

23 Llull, D., García, E. & López, R. (2001). Tts, a processive β -glucosyltransferase of
 24 *Streptococcus pneumoniae*, directs the synthesis of the branched type 37 capsular

polysaccharide in pneumococcus and other gram-positive species. *Journal of Biological Chemistry*, 276(24), 21053-21061.

Llull, D., Muñoz, R., López, R., & García, E. (1999). A Single Gene (*tts*) located outside the *cap locus* directs the formation of *Streptococcus pneumoniae* type 37 capsular polysaccharide: Type 37 Pneumococci are natural, genetically binary strains. *Journal of Experimental Medicine*, 190(2), 241-252.

Mårtensson, O., Biörklund, M., Lambo, M. A., Dueñas-Chasco, M. T., Irastorza, A., Holst, O., et al. (2005). Fermented rye, oat-based products reduce cholesterol levels and stimulate the bifidobacteria flora in humans. *Nutrition Research*, 25(5), 429-442.

Milton, D. K., Udeni Alwis, K., Fiset, L., & Muilenberg, M. (2001). Enzyme-linked immunosorbent assay specific for (1,6) Branched, (1,3)- β -D-Glucan detection in environmental samples. *Applied Environmental Microbiology*, 67(12), 5420-5424.

Notararigo, S., Nácher-Vázquez, M., Ibarburu, I., Werning, M. L., Fernández de Palencia, P., Dueñas, M. T., et al. (2013). Comparative analysis of production and purification of homo- and hetero-polysaccharides produced by lactic acid bacteria. *Carbohydrate Polymers*, 93(1), 57-64.

Rao, C. Y., Riggs, M. A., Chew, G. L., Muilenberg, M. L., Thorne, P.S., van Sickle, D., et al., (2007). Characterization of airborne molds, endotoxins, in homes in New Orleans after Hurricanes Katrina and Rita. *Applied Environmental Microbiology*, 74(8), 1630-1634.

Ross, P., Mayer, R., & Benziman, M. (1991). Cellulose biosynthesis and function in bacteria. *Microbiological Molecular Biology Review*, 55(1), 35-58.

Ruas-Madiedo, P. & de los Reyes-Gavilán CG (2005). Invited Review: Methods for the screening, isolation, and characterization of exopolysaccharides produced by lactic acid bacteria. *Journal of Dairy Science*, 88(3), 843–856.

1 Ruas-Madiedo, P., Abraham, A., Mozzi, F. & de los Reyes-Gavilán, C. G. (2008).
 2 Functionality of exopolysaccharides produced by lactic acid bacteria, p. 137-166. In B.
 3 Mayo B, López P, Pérez-Martín G (ed), Molecular aspects of lactic acid bacteria for
 4 traditional and new applications. Research Signpost, Kerala, India.

5 Russo P, López P, Capozzi V, Fernández de Palencia P, Dueñas MT, Spano G, et al.
 6 (2012). Beta-glucans improve growth, viability and colonization of probiotic
 7 microorganisms. *International Journal of Molecular Science*, 13(5), 6026-6039.

8 Salminen, P., von Wright, A., Morelli, L., Marteau, P., Brassart, D., de Vos, W. M., et
 9 al. (1998). Demonstration of safety of probiotics- a review. *International Journal of*
 10 *Food Microbiology*, 44(1-2), 93-106.

11 Sander, I., Fleischer, C., Borowitzki, G., Brüning, T. & Raulf-Heimsoth, M. (2008).
 12 Development of a two-site enzyme immunoassay based on monoclonal antibodies to
 13 measure airborne exposure to (1→3)-β-D-glucan. *Journal of Immunological Methods*,
 14 337(1), 55–62.

15 Sánchez, C., Neves, R. A., Cavaleiro, J., Santos, M., García-Quintáns, N., López, P., et
 16 al. (2008). The contribution of citrate metabolism to the growth of *Lactococcus lactis*
 17 CRL264 at low pH. *Applied Environmental Microbiology*, 74(4), 1136-1144.

18 Stack, H. M., Kearney, N., Stanton, C., Gerald, F., Fitzgerald, G. F. & Ross, R. P.
 19 (2010) Association of β-glucan endogenous production with increased stress tolerance
 20 of intestinal lactobacilli. *Applied Environmental Microbiology*, 76(2), 500-507.

21 Terzaghi, B. E. & Sandine, W. E. (1975). Improved medium for lactic streptococci and
 22 their bacteriophages. *Applied Microbiology*, 29(6), 807-813.

23 Velasco, S. E., Areizaga, J., Irastorza, A., Dueñas, M. T., Santamaría, A. & Muñoz, M.
 24 E. (2009). Chemical and rheological properties of the β-glucan produced by
 25 *Pediococcus parvulus* 2.6. *Journal of Agricultural Food Chemistry*, 57(5), 1827-1934.

1 Ventura, M., Canchaya, C., Fitzgerald, G. F., Gupta, R. S. & van Sinderen, D. (2007).
2 Genomics as a mean to understand bacterial phylogeny and ecological adaptation: the
3 case of bifidobacteria. *Antonie van Leeuwenhoek*, 91(4), 351-372.

4 Vink, E., Rodríguez-Suárez, R. J., Gérard-Vincent, M., Ribas, J. C., de Nobe, H., van
5 den Ende, H., et al. (2004). An *in vitro* assay for (1 → 6)-beta-D-glucan synthesis in
6 *Saccharomyces cerevisiae*. *Yeast*, 21(13), 1121-1131.

7 Werning, M. L., Corrales, M. A., Prieto, A., Fernández de Palencia, P., Navas, J. &
8 López, P. (2008) Heterologous expression of a 2-substituted-(1,3)-β-D-glucan in
9 *Lactococcus lactis*. *Applied Environmental Microbiology*, 74(16), 5259-5262.

10 Werning, M. L., Ibarburu, I., Dueñas, M. T., Irastorza, A., Navas, J. & López, P. (2006).
11 *Pediococcus parvulus* *gtf* gene encoding the GTF glycosyltransferase and its application
12 for specific PCR Detection of β-D-glucan-producing bacteria in foods and beverages.
13 *Journal of Food Protection*, 69(1), 161-169.

14 Werning, M. L., Notararigo, S., Nácher, M., Fernández de Palencia, P., Aznar, R. &
15 López, P. (2012). Biosynthesis, purification and biotechnological use of
16 exopolysaccharides produced by lactic acid bacteria. In: El-Samragy Y (ed) Food
17 additives, InTech, Croatia, pp. 83-114.

Legend to the figures

Figure 1. Analysis of purified EPS produced by *L. lactis* NZ9000[pNGTF] (A) and *P. parvulus* 2.6R (B) by high-performance size exclusion chromatography using as molecular weight standards dextrans of 10^4 , 4×10^4 , 7×10^4 , $1,5 \times 10^5$, $2,7 \times 10^5$, $4,1 \times 10^5$, $6,7 \times 10^5$, $1,4 \times 10^6$ and 2×10^6 Da (Fluka).

Figure 2. Range of detection of purified 2-substituted (1,3)- β -D-glucan by the immunological method. EPS produced by *L. lactis* NZ9000[pNGTF] (A) and *P. parvulus* 2.6R (B) immobilized in microtitre plates and purified EPS from *L. lactis* NZ9000[pNGTF] (●) and *P. parvulus* 2.6R (○) were tested as competitors. The values are the mean of three independent experiments. Standard deviations are indicated as vertical bars over dots.

Figure 3. Specificity of the immunological method. Laminarin (A), curdlan (B) and xanthan (C) were tested as competitors. The values are the mean of three independent experiments. Standard deviations are indicated as vertical bars over dots.

Figure 4. Detection of 2-substituted (1,3)- β -D-glucan in culture supernatants. (A) The indicated *L. lactis* strains were grown and expression of the EPS induced in either M17 medium. *P. parvulus* 2.6R and 2.6NR, *Lb. diolivorans* G-77 and *O. oeni* I4 were grown in MRS medium. Triplicates of these cultures were then centrifuged and the supernatants were directly used for quantification of the β -D-glucan present by the ELISA assay. The values are the mean of three independent experiments. Standard deviations are indicated as vertical bars over bars.

Figure 5. Analysis by GLC of monosaccharides (A and B) and by GLC-MS (C and D) of linkage types between glucose units of the EPS synthesized *in vitro* by GTF glycosyltransferase. Analysis of reactions containing (A and C) or lacking (B and D) UDP-glucose.

Table(1)

Table 1. Comparative analysis of exopolysaccharide production by ELISA and phenol-sulphuric methods.

Method	ELISA			Phenol-sulphuric	Phenol-sulfuric/ELISA
<div>Bacteria</div> <div>EPS concentration</div>	Total ^a (μg mL ⁻¹)	Precipitated ^b (μg mL ⁻¹)	Precipitated ^b /Total ^a (%)	Precipitated ^b (μg mL ⁻¹)	Precipitated ^b /Precipitated ^b (%)
<i>P. parvulus</i> 2.6R	322 ± 15	200 ± 35	62	117 ± 21	58
<i>L. lactis</i> NZ9000/pNGTF	235 ± 35	183 ± 19	78	117 ± 8	64
<i>O. oeni</i> I4	118 ± 7	94 ± 9	80	59 ± 5	63
<i>L. diolivorans</i> G-77	211 ± 40	166 ± 24	79	64 ± 6	39

^aTotal: concentration of the EPS was determined directly in culture supernatants.

^bPrecipitated: concentration of the EPS present in culture supernatants was determined after ethanol precipitation.

A

B

Figure 1

A **B**

Figure 2

A **B** **C** **Figure 3**

Figure 4

A **B** **C** **D** **Figure 5**

A

B

C

D

Figure S1. Detection limits of purified 2-substituted (1,3)- β -D-glucan added to M17 (A), MD (B), MRS (C) or MSD (D) media. The values are the mean of three independent experiments.

Figure S2. Double reciprocal Lineweaver-Burk plot of the production of EPS at different concentrations of UDP-glucose. Activity of GTF glycosyltransferase was tested in membrane vesicle of *L. lactis* NZ9000[pNGTF] containing 12.5 ($\circ$), 25 ($\bullet$) or 50 ($\square$) μg of total proteins. The values are the mean of three independent experiments.