

Structural characterization of an exopolysaccharide produced by *Lactobacillus plantarum* Ts isolated from Bulgarian wheat and rye flour

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ABSTRACT

An exopolysaccharide (EPS) was isolated from *Lactobacillus plantarum* Ts and purified by size exclusion chromatography train. *L. plantarum* Ts showed the highest production (7.4 ± 0.8 g/l) of EPS. The purified, homogeneous EPS by HPLS analysis had an average molecular weight of $9.03 \cdot 10^4$ Da. The FTIR spectrum of the EPS from *L. plantarum* Ts showing the functional groups in the $4,000\text{--}400\text{ cm}^{-1}$ region. TLC analysis indicated that the EPS was a heteropolymer composed of fructose, and sucrose as monomeric constituent units. The strain *L. plantarum* Ts and pathogenic *E. coli* 3398, *St. aureus* 745, *B. subtilis* 6633, *S. Typhi* 3591, *L. monocytogenes* 863 and *E. aerogenes* 3691 were tested for their growth utilizing the EPS from *L. plantarum* Ts as the sole carbon source for its possible use as a prebiotic. *L. plantarum* Ts exhibited growth in the EPS supplied medium compared with glucose as carbon source, whereas the pathogenic strains did not grow in the EPS-supplied medium. In vitro evaluations showed that, like other reported polysaccharides, this EPS displayed significant prebiotics properties.

Keywords: Exopolysaccharides, *Lactobacillus plantarum* Ts, prebiotic, lactic acid bacteria, pathogenic

Mathematics Subject Classification: cc:92C50

INTRODUCTION

A vast number of microbial EPSs were reported over the last decades, and their composition, structure, biosynthesis and functional properties have been extensively studied. In recent years the increased demand for natural polymers for pharmaceutical, food, and other industrial applications has led to a

remarkable interest in polysaccharides produced by microorganisms. The physiological role of EPS depends on the ecological niches and the natural environment in which microorganisms have been isolated. According to Moradali et.al. (2019), bacterial EPSs are believed to play an important role in the environment by promoting survival strategies such as bacterial attachment to surfaces and nutrient trapping, which facilitate processes of biofilm formation and development. *Lactobacillus* strains of food origin are regarded as safe and their EPSs can be used as prebiotics. Prebiotics are regarded as nondigestible food ingredients which affect the host beneficially by selectively stimulating of growth and/or activity of one or a number of bacterial species which already resident in the colon, and influence the host health (Gibson et.al., 2004). The EPSs can be classified into homopolysaccharides (HoPSs) and heteropolysaccharides (HePSs), depending on the monosaccharide composition. HoPSs has one type of monosaccharide (e.g. glucose or fructose), as HePSs has more than one types of monosaccharides (e.g. glucose, galactose, mannose, so on). In some EPSs, there are also small quantities of fucose, glucosamine or galactosamine. The properties of EPSs depend on the composition of the monosaccharides and the connections of the monomers to build the exopolymers (Kodali et.al., 2011).

For the commercial and therapeutic importance of the products, is important to provide information on the EPS isolated and purified from a *Lactobacillus plantarum* Ts isolated from Bulgarian Wheat and Rye Flour and has shown prebiotics potential.

MATERIALS AND METHODS

Materials, Bacterial Strains, and Culture Conditions

Strain *Lactobacillus plantarum* Ts was obtained from the collection of the Department of Biology, Shumen University. Molecular analysis in LAB (lactic acid bacteria) was performed by molecular identification (16S rRNA gene sequencing) in GeXP Genetic Analysis System (Beckman Coulter, USA) (Ignatova-Ivanova et.al., 2014). The *L. plantarum* Ts were cultured in de Man, Rogosa, and Sharpe (MRS) agar medium at 37°C and pH of media was adjusted to 6.5 with 1 M NaOH. The basic media was sterilized in an autoclave at 121 °C for 20 min. Afterwards the carbohydrates supplemented were sterilized by 0.22 µm filters (Manisart®). *Escherichia coli* 3398, *Staphylococcus aureus* 745, *Bacillus subtilis* 6633, *Salmonella typhimurium* 3591, *Listeria monocytogenes* 863 and *Enterobacter aerogenes* 3691 are from the Collection of the Department of General and Applied Microbiology at Sofia University. All of the isolated substances were checked for purity and maintained in slants of Luria Bertani (LB) agar. The pathogenic strains were culture in Luria Bertani (LB) agar at 37°C. Modified MRS (mMRS) medium (10 g/l casamino acids, 2 g/l Na₂HPO₄, 5 g/l sodium acetate, 2 g/l triammonium citrate, 0.2 g/l MgSO₄·7H₂O, 0.2 g/l MnSO₄·4H₂O, and 1 g/l Tween 80) was used for the study of EPS utilization by LAB strains. Additionally, carbohydrates supplemented 10% glucose were sterilized using 0.22 µm filters (Manisart®).

Extraction and Quantification of Exopolysaccharides from *L. plantarum* Ts

L. plantarum Ts strains were cultured in mMRS broth with 10% glucose at 37°C for 48 h. The culture was centrifuged at 5,000 ×g for 10 min to remove the cells and the supernatant was boiled for 10 min to inactivate enzymes. The supernatant was decolorized with activated carbon in a water bath at 40°C for 30 min (Chen et.al., 2013). Afterwards was deproteinized as described previously (Wang et.al., 2007). The deproteinized supernatant was subsequently centrifuged at 5,000 ×g for 10 min, mixed with 4 volumes of 95% ethanol, and kept overnight at 4°C. The mixture was centrifuged at 8,000 ×g for 10 min. The pellets were collected and dissolved in sterile distilled water. The solution was centrifuged at 3,000 ×g for 10 min, and so the supernatant was dialyzed against sterile distilled water for 24 h. The EPS solution was dried freeze-dried and stored at 4°C until used and the EPS content was measured by the phenol-sulfuric acid method (Masuko et.al., 2005).

Fourier Transform Infrared Spectra of the EPS

Functional groups present in the purified EPS were determined by Fourier transform infrared (FTIR) spectroscopy. The samples selected for infrared analysis were prepared by mixing with spectroscopy-grade KBr and then prepared in the form of pellets at a pressure of 1 MPa. All of the pellets were of about 10 mm diameter and 1 mm thickness. The FTIR spectral data were recorded via a Varian 2000 FTIR spectrometer (FT-IR Afinity-1 Shimadzu) as FTIR spectra were recorded covering the region of 4,000–400 cm⁻¹.

EPS Composition Analysis

The lyophilized EPS (10 mg) was hydrolyzed with 6 M trifluoroacetic acid (TFA) for 6 h at 100°C and TFA was removed using a rotary vacuum evaporator. The hydrolyzed solution was neutralized with 15 M ammonia solution (0.32 ml) (6). The resultant hydrolysate was analyzed by thin-layer chromatography (TLC) to determine the monosaccharide composition. The monosaccharide standard was also treated with 6 M TFA and as described above, neutralized with ammonia. The EPS hydrolysates were separated using Silica Gel 60 TLC. For this we used pre-coated plates (Merck, Germany) developed with a mobile phase of ethylacetate: n-propanol: acetic acid: water (4:2:2:1 (v/v/v/v)). The separated sugars were visualized with an orcinol-sulfuric acid spray reagent.

Carbohydrates Used in This Study.

We studied five types of commercially available carbohydrates presenting different degree of polymerization (DP): FOS (Raftilose P95 from Orafit, Tienen, Belgium) contained 5% glucose, fructose, and sucrose; and 12% DP2, 48% DP4 and 35% DP7; GalOS (TOS-P from Yakult, Tokyo, Japan) contained 2% DP2, 48% DP3, 38% DP4 and 12% DP5; GOS (BioEcolians from Solabia, Pantin, France) contained 6% glucose and leucrose, and 24% DP4, 56% DP5, 7% DP6 and 7% DP7; FOS

from chicory (Sigma) and commercial β -1,3 glucan from *Euglena gracilis* (Sigma). Glucose (purity 99%, Merck); Fructose (purity 99%, Merck), Sucrose (purity 99%, Merck), Lactose (purity 99%, Merck), Maltose (purity 99%, Merck) and raffinose (purity 99%, Fluka, Sigma Aldrich, Switzerland) were used as standards at EPS Composition Analysis.

3.9.3. HPLC analysis

The average numerous molecular weight (Mn) and average molecular weight (Mw) of the samples were determined by molecular sieve chromatography (HPLC-SEC) at the University of Food Technology, Plovdiv. The separation was performed on an HPLC ELITE LaChrome chromatograph (VWR Hitachi, Japan) equipped with a Shodex OHPAK 806 MHQ column (8 mm x 300 mm), (Shodex Co., Tokyo, Japan) and an RI detector (VWR Hitachi, Chromaster, 5450, Japan). (Murdzheva et.al., 2016).

Effect of EPSs on Growth of Lactic Acid Bacteria and Enteric Pathogens

The growth and EPS utilization by LAB such as *L. plantarum* Ts, and *Escherichia coli* 3398, *Staphylococcus aureus* 745, *Bacillus subtilis* 6633, *Salmonella Typhimurium* 3591, *Listeria monocytogenes* 863 and *Enterobacter aerogenes* 3691 were evaluated in mMRS medium with 1% EPS at 37°C for 24 h. LAB growth was also analyzed in mMRS with 1% glucose (positive control), as the growth of the pathogenic bacteria *Escherichia coli* 3398, *Staphylococcus aureus* 745, *Bacillus subtilis* 6633, *Salmonella Typhimurium* 3591, *Listeria monocytogenes* 863 and *Enterobacter aerogenes* 3691 was monitored by using LB medium supplemented with 1% EPS and 1% glucose (positive control) as the carbon source separately. Bacterial growth was measured by a turbidimetric method at 650 nm and calibrated against cell dry-weight using a spectrophotometer (UV/Vis Shimadzu, Japan) and for each experiment, data was analyzed using Excel statistical package.

Statistical Analyses

Experiments were repeated at least three times. The data are expressed as the mean $\pm$ standard deviation.

RESULTS AND DISCUSSION

Screening of EPS-Producing Microbes

The presence of EPS associated with bacterial cells can be recognized by the formation of colonies in mucous solid medium (Stadler et.al., 2010). Colonies showing a slimy appearance were picked and subcultured in mMRS with 10% glucose and incubated for 2 days at 37°C. Totally nine isolates were collected from the ganjang samples. Therefore, the presence of a translucent or creamy material involving a mucoid colony is indicative of EPS production potential. When cultivated in a media with high content of saccharides such as 10% glucose solutions, strain *L. plantarum* Ts synthesizes exopolysaccharides (Fig. 1).

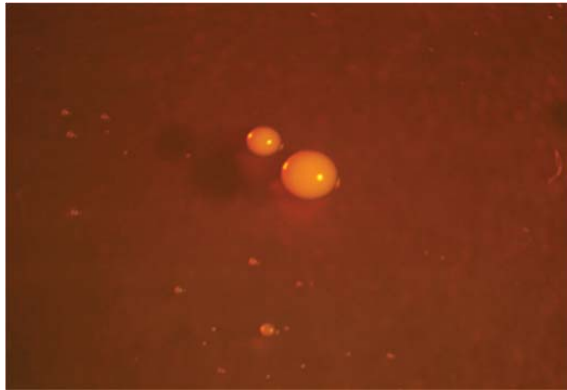


Figure 1. EPSs (exopolysaccharides) produced by *L. plantarum* Ts cultivated in a media containing 10% glucose, which are secreted in the culture medium.

Quantification of the EPS

The EPS produced by *L. plantarum* Ts strain was quantified by the phenol-sulfuric acid method (Dubois et.al., 1956). The results of the analysis showed that starin *L. plantarum* Ts produced EPS amount (7.4 ± 0.8 g/l).

FTIR Spectral Analysis of the EPS from *L. plantarum* Ts

The FTIR spectrum of the EPS from *L. plantarum* Ts showing the functional groups in the 4,000-400 cm^{-1} region is represented on Fig. 2.

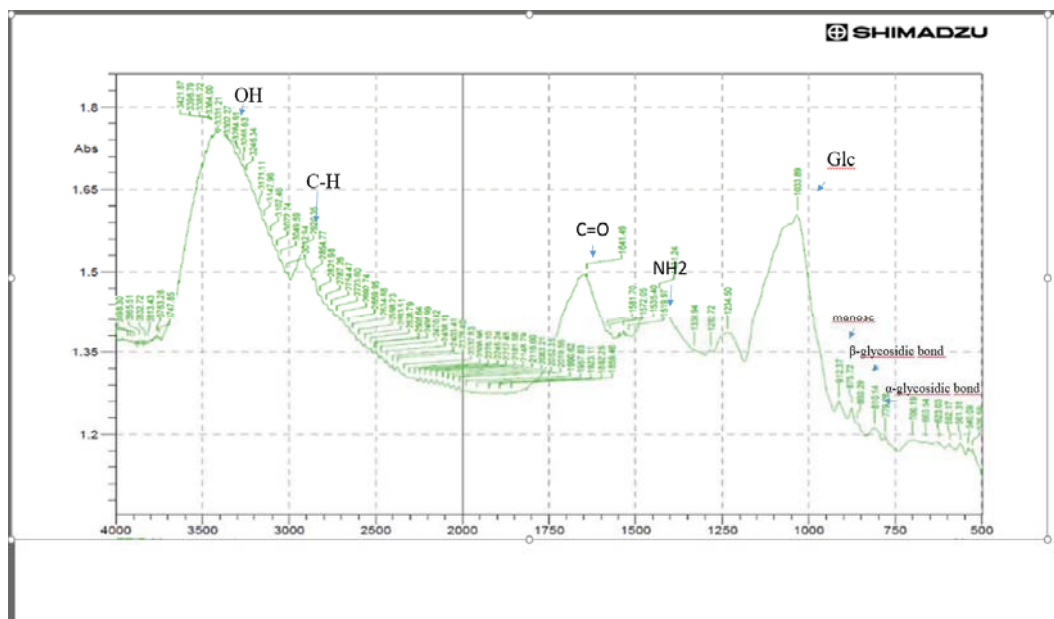


Figure 2. FTIR analysis of the exopolysaccharide preparation from *L. plantarum* Ts.

We performed a comparative analysis of the spectrum of the obtained EPS and the spectra of two known OSs - raffinose and glucooligosaccharide. The results are shown in Figure 3.

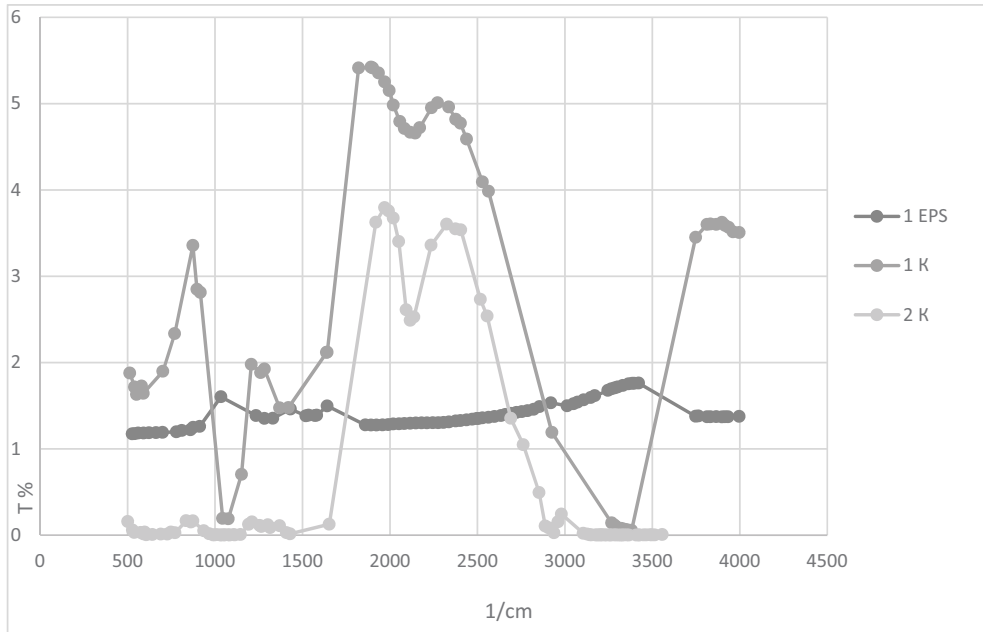


Figure 3. FTIR analysis of an EPS of *L. plantarum* Ts obtained by culturing the strain on 10% glucose compared with gluco-oligosaccharide-1K and raffinose-2K.

There is no complete match between the synthesized *L. plantarum* Ts species with the two standards tested. This suggests that the synthesized FTE has a new unique structure.

Monosaccharide Composition of the EPS from *L. plantarum* Ts.

Hydrolysis of the EPS with 6 M TFA and subsequent analysis of the EPS hydrolysate by TLC showed the presence of mannose and glucose as principal components of the EPS from *L. plantarum* (Fig. 4).

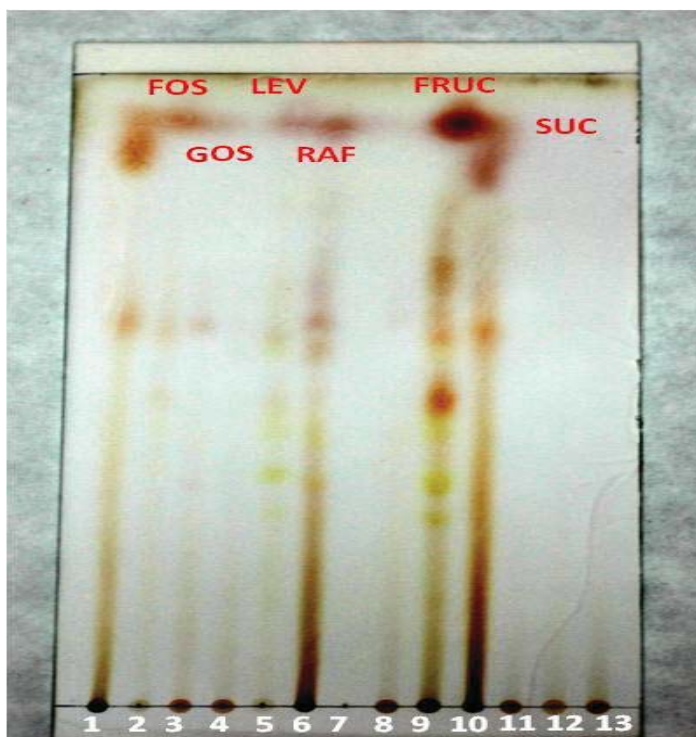


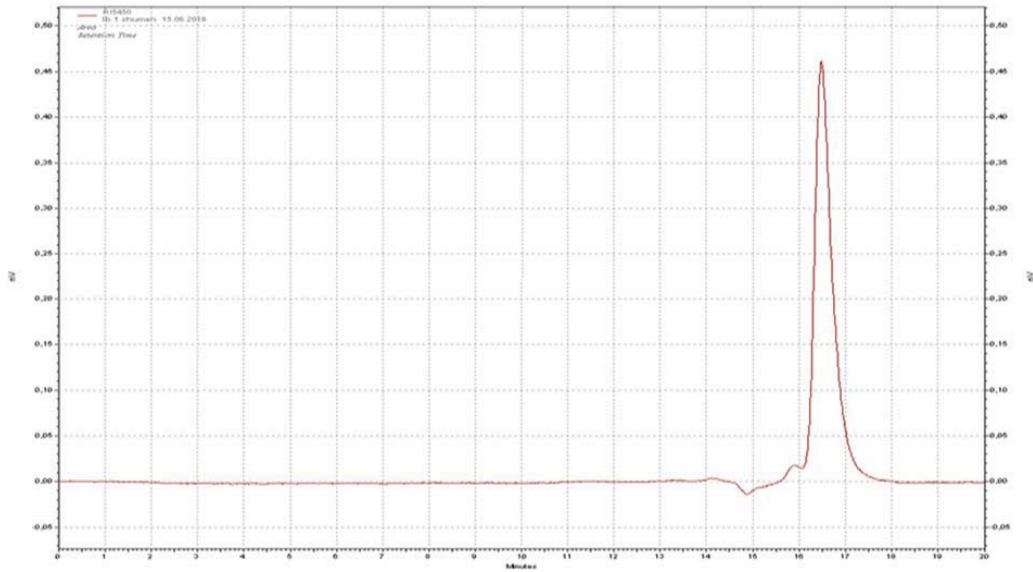
Figure 4. TLC analysis of monosaccharides from *L. plantarum* Ts. exopolysaccharide hydrolysate.

Where 1. EPS from *L. plantarum* Ts; 2. FOS; 3. GOS; 4 GalOS; 5 FOS from chicory (Sigma); 6 Raffinose; 7 comersial β -1,3 glucan from *Euglena gracilis* (Sigma); 8 Glucose; 9. Fructose; 10 Sucrose; 11. Galactose, 12. Lactose; 13 Maltose

Hydrolysis of the EPS with 6 M TFA and subsequent analysis of the EPS hydrolysate by TLC showed the presence of fructose and sucrose as principal components of the EPS from *L. plantarum* Ts (Fig. 3). Comparison with selected standards shows similarity as FOS (Raftilose P95) and raffinose, FOS and GOS (Figure 4).

HPLC analysis of EPS

The average numerous molecular weight (M_n) and average molecular weight (M_w) of the purified EPS were determined by molecular sieve chromatography (HPLC-SEC) at UCT Plovdiv. The molecular weights were calculated according to the relative molecular weight of the known molecular weight pullulans (P-5, P-10, P-20, P-50, P 100, P-200, P-400, P-800). The polydispersity index, which is a characteristic of polymers, is calculated by the formula: $I = M_w / M_n$ (11). The results obtained are shown in Figure 5.



Fraction	Mw, Da	Mn, Da	Polydispersity index
Fraction 1	$9,03 \cdot 10^4$	80091	1,13

Figure 5. HPLC-SEC.

The chromatogram of the EPS appeared as a single symmetrical narrow peak, confirming the homogeneity of the purified EPS sample. The molecular mass of the EPS was determined to be $9,03 \cdot 10^4$ Da.

Effect of the EPS on the Growth of Lactic Acid Bacteria and Enteric Pathogens

Growth of *L. plantarum* Ts strain in modified MRS broth supplemented with carbohydrates EPS utilization by LAB shown in Table 1. The growth of the pathogenic bacteria was monitored using LB medium supplemented with 1% EPS utilization by LAB and 1% glucose (positive control) as the carbon source separately. Growth was evaluated in terms of maximum optical density at 600 nm and specific growth rate achieved during 24 h fermentation. Growth kinetics on glucose were used as control.

Table 1. Oligosaccharide Utilization

Strains	growth on MRS	OD glucose ^a	growth on MRS-EPS	OD EPS ^a
<i>L. plantarum</i> Ts	+	5,9±0,12	+	3,2±0.01
<i>E. coli</i> 3398	+	0,2±0,02	-	0,2±0,05
<i>St. aureus</i> 745	+	0,2±0,01	-	0,1±0,02
<i>B. subtilis</i> 6633	+	0,3±0,02	-	0,3±0,03
<i>S. typhi</i> . 3591	+	0,1±0,01	-	0,1±0,02
<i>L. monocytogenes</i> 863	+	0,1±0,03	-	0,1±0,01
<i>E. aerogenes</i> 3691	+	0,1±0,03	-	0,2±0,02

^a Optical density (OD) at 600 nm after 24 h in MRS, MRS-EPS broths. For each experiment, data were analyzed using the Excel statistical package. The OD readings and standard deviations were calculated from duplicate samples from three separate experiments.

L. plantarum Ts strain fermented EPS utilization by LAB. Pathogenic strains *E. coli* 3398, *St. aureus* 745, *B. subtilis* 6633, *S. Typhi*. 3591, *L. monocytogenes* 863 and *E. aerogenes* 3691 did not show any growth in EPS-supplied medium compared with glucose-supplied medium (table 1), indicating that these strain could not utilize EPS as a carbon source.

L. plntarum is used in the food industry, because the EPS synthesized by it have unique properties. The functions of the synthesized EPS depend, on the one hand, on their molecular weight and, on the other, on the characteristics of the species that produces them. A similar relation has been reported by other authors (Wang et al., 2015; Ruas-Madiedo et al., 2010; Pryanika et al., 2014).

FT-IR is an effective method by which both functional groups and covalent bonds between them can be determined using the property of bonds and groups to vibrate at a certain frequency. The EPS synthesized by us (Fig. 2) shows a small peak at 1530 cm⁻¹ corresponding to an amino group. The absorption peak at 879.54 cm⁻¹ indicates the existence of a b-glycosidic bond. The range 1,030-944 cm⁻¹, with a minimum of 998 cm⁻¹, can be assigned to Glc. The absorption bands at 812 and 879 cm⁻¹ indicate that EPS contains both α and β type glycosidic bonds in its structure. Similar results have been published by other authors (Xu et.al., 2010). The peak at 3331 cm⁻¹ indicates the presence of an OH group and the peak of the carbonyl group (C = O) is at 1670 cm⁻¹. The peak between 2800 and 3000 cm⁻¹ indicates C-H bonds. The characteristic absorption at 812 cm⁻¹ corresponds to the presence of mannose. Such results have been also reported by Kodali et.al. (2011).

The presence of high absorption in the region of 1,200–950 cm⁻¹ is in the so-called “finger print region”, which is specific and enables the identification of EPS. The presence of glucose and sucrose indicate that the synthesized *L. plantarum* Ts EPS of our species is a heteropolysaccharide.

Mw is a major property of *L. plantarum* WLPL04 FTE that may affect probiotic properties. FW mw from *L. plantarum* WLPL04 is determined to be 6.61×10^4 Da, which is lower than FEV (1.1×10^5 Da) than *L. plantarum* YW11 (17) and FEV ($1, 15 \times 10^6$ Da) of *L. plantarum* C88 (24), but higher than *L. plantarum* BC-25 FEV (1.83×10^4 and 1.33×10^4 Da) (25). In a parallel study, the Mw of *L. plantarum* ZDY2013 was 5.17×10^4 Da by high performance chromatography except size (Zhang et.al., 2016).

The EPS from *L. plantarum* Ts showed potential prebiotic property by selectively supporting the growth of LAB. The pathogenic strains were unable to grow in the medium containing EPS as a carbon source, indicating that in the presence of EPS, LAB will be selectively enriched, whereas pathogenic Enterobacteriaceae members will be suppressed. A similar result was observed in the cases of EPS from *Weissella cibaria* A2, *W. confusa* A9, *L. plantarum* A3, and *Pediococcus pentosaceus* 5S4, which selectively enhanced the growth of *Bifidobacterium* and *Lactobacillus/Enterococcus* groups whereas *Clostridia* were suppressed (Hongpattarakere et.al., 2012).

It is well-known that while most bifidobacteria strains can use oligosaccharides easily, only a few strains from other genera, including lactobacilli, possess this ability. It has been demonstrated in this study that, in the presence of EPS strain *L. plantarum* Ts is capable of fermenting it. Despite significant commercial interest in using oligosaccharides as prebiotic substrates, little is known about how these oligosaccharides are metabolized by LAB and related bacteria. LAB accumulate sugars by secondary active transport (mainly by proton motive force, PMF), the PTS, or an ATP-mediated system. However, more studies should be performed in order to elucidate the pathways of utilization of oligosaccharides in these *Lactobacillus* strains.

CONCLUSION

In conclusion, *L. plantarum* Ts produced a water soluble heteropolysaccharide, which is composed of sucrose and glucose. *L. plantarum* Ts showed the highest production (7.4 ± 0.8 g/l) of EPS when cultivated on glucose. The purified, homogeneous EPS by HPLS analysis had an average molecular weight of 9,03,104 Da. Fourier transform infrared spectroscopy analysis showed the presence of hydroxyl and carboxyl groups and glycosidic linkages. TLC analysis indicated that the EPS was a heteropolymer composed of fructose, and sucrose as monomeric constituent units. Additionally, growth study using EPS as the sole carbon source showed that it could specifically support the growth of LAB while suppressing the growth of *E. coli* 3398, *St. aureus* 745, *B. subtilis* 6633, *S. Typhi*. 3591, *L. monocytogenes* 863 and *E. aerogenes* 3691. These results indicate that the EPS from *L. plantarum* Ts has potential application as a prebiotic in the food industry.

Informed consent: "Informed consent was obtained from all individual participants included in the study."

Disclosure statement

No potential conflict of interest was reported by the authors.

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