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Functional Characterization of an Exopolysaccharide Produced by *Lactobacillus plantarum* Ts Isolated from Bulgarian Wheat and Rye Flour.

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ABSTRACT

The present study focused on the production, characterization, and in vitro prebiotic evaluation of an exopolysaccharides (EPS) from *Lactobacillus plantarum* Ts isolated from Bulgarian wheat and rye flour. Strain *L. plantarum* Ts showed the highest production (6.4 ± 0.8 g/l) of EPS. Furthermore, *L. plantarum* Ts was cultured in 5 L of medium and the EPS was extracted by ethanol precipitation. Fourier transform infrared spectroscopy analysis showed the presence of hydroxyl and carboxyl groups and glycosidic linkages. The isolated EPS contained sucrose and glucose, as observed by thin-layer chromatography analysis of the EPS hydrolysate. 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 EPSsupplied medium compared with glucose as carbon source, whereas the pathogenic strains did not grow in the EPS-supplied medium. These findings indicate that the EPS from *L. plantarum* Ts has potential application in as a prebiotic in the food industry.

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

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INTRODUCTION

EPS (exopolysaccharide) is a term first used by Sutherland (Arrage A, et.al., 1995) to describe high-molecular-weight carbohydrate polymers produced by marine bacteria 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. 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 (Costerton WJ, et.al., 1987). Lactobacillus strains of food origin are regarded as safe and their EPSs can be used as prebiotics. Prebiotics are nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacterial species already resident in the colon, and thus attempt to improve host health (Glenn R, et.al., 1995). EPSs produced by the food-grade Lactic acid bacteria (LAB) with GRAS (Generally Recognized as Safe) status are an important source of natural alternatives to commercial additives of plant or animal origin. Most of those additives used are chemically modified to improve the rheological properties of the product and hence are not allowed in most European Union countries (German B, et.al., 1999). EPS from LAB have found their most valuable application in the improvement of the rheology, texture and 'mouth feel' of fermented milk products, such as yoghurt. An additional hypothesized physiological benefit is that EPS will remain for longer in the gastrointestinal tract, thus enhancing colonization by probiotic bacteria (German B, et.al., 1999). EPS produced by LAB are in a great variety, depending on the type of LAB strains, culture conditions, and medium composition (Looijesteijn PJ and Hugenholtz J., 1999). *L. plantarum* is a versatile, Gram-positive, bacterium that can be found in a wide range of habitats such as dairy, meat, and many plant fermentations. Furthermore, *L. plantarum* has the advantage that it can grow to high cell densities which is desirable for industrial applications (Bindhumol I and Nampoothiri KM., 2010). This study focused on characterizing the properties of an EPS produced by *L. plantarum* sonorensis strains Ts, such as emulsifying activity, carbohydrate composition, and the presence of functional groups by IR spectroscopy. Furthermore, the EPS was evaluated for its prebiotic potential by studying its effect as a carbon source for the growth of probiotic LAB and enteric pathogens.

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 Ts, et.al., 2014). The *L. plantarum* Ts were cultured in de Man, Rogosa, and Sharpe (MRS) agar medium at 37°C. The pH of media was adjusted to 6.5 with 1 M NaOH. The basic media was sterilized by autoclaving at 121 °C for 20 min. and carbohydrates supplemented were sterilized using 0.22 µM filters (Manisart®). *Escherichia coli* 3398, *Staphylococus aureus* 745, *Bacillus subtilis* 6633, *Salmonella Typhimurium* 3591, *Listeria monocytogenes* 863 and *Enterobacter aerogenes* 3691 were obtained from the Collection of the Department of General and Applied Microbiology, Sofia University. All the isolates werechecked for purity and maintained in slants of Luria Bertani (LB) agar. The pathogenic strains were routinely culture in Luria Bertani (LB) agar at 37°C. Modified MRS (mMRS) medium (10 g/l casamino acids, 2 g/l Na_2HPO_4, 5 g/l sodium acetate, 2 g/l triammonium citrate, 0.2 g/l $MgSO_4 \bullet 7H_2O$, 0.2 g/l$MnSO_4 \bullet 4H_2O$, and 1 g/l Tween 80) was used for the study of EPS utilization by LAB strains. Carbohydrates supplemented 10% sucrose 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% sucrose at 37°C for 48 h. The culture was centrifuged at 5,000 ×g for 10 min to remove cells. 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 30min (Chen Y-T, et.al., 2013) and then deproteinized as described previously (Wang X, 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 then centrifuged at 8,000 ×g for 10 min, and the pellet was collected

and dissolved in sterile distilled water. The solution was centrifuged at 3,000 ×g for 10 min, and the supernatant was dialyzed against sterile distilled water for 24 h. The EPS solution was freeze dried and stored at 4°C until used. The EPS content was measured by the phenol-sulfuric acid method (Masuko T, et.al., 2005) using glucose as a standard.

Fourier Transform Infrared Spectra of the EPS

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

EPS Composition Analysis

The lyophilized EPS (10 mg) was hydrolyzed with 6 M trifluoroacetic acid (TFA) for 6 h at 100°C. The TFA was removed using a rotary vacuum evaporator, the hydrolyzed solution was neutralized with 15 M ammonia solution (0.32 ml) (Kodali VP, et.al., 2011), and 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 neutralized with ammonia as described above. The EPS hydrolysates were separated using Silica Gel 60 TLC using 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.

Fife types of commercially available carbohydrates presenting different degree of polymerization (DP) were studied. FOS (Raftilose P95 from Orafti, Tienen, Belgium) contained 5% glucose, fructose, and sucrose; and 12% DP2, 48% DP4 and35%DP7; GalOS (TOS-PfromYakult,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 comersial 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 standarts at EPS Composition Analysis.

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, *Staphylococus 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). The growth of the pathogenic bacteria *Escherichia coli* 3398, *Staphylococus aureus* 745, *Bacillus subtilis* 6633, *Salmonella Typhimurium* 3591, *Listeria monocytogenes* 863 and *Enterobacter aerogenes* 3691 was monitored 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). 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 ± standard deviation.

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RESULTS

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 R, et.al., 2010). Colonies showing a slimy appearance were picked and subcultured in mMRS wiht 10% sucrose 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% sucrose solutions, solutions, strain *L. plantarum* Ts synthesizes exopolysaccharides (Fig. 1).

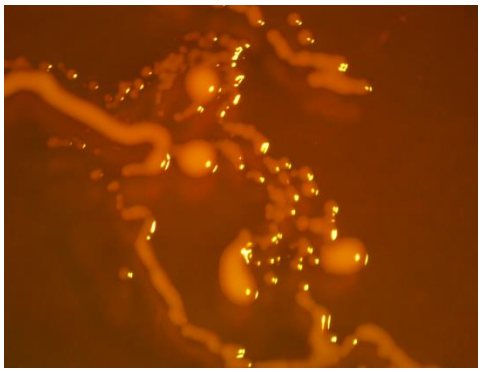

Fig 1: EPSs (exopolysaccharides) produced by *L. plantarum* Ts cultivated in a media containing 10% sucrose, which are secreted in the culture medium.

Quantification of the EPS

The EPS produced by *L. plntarum* Ts strain was quantified by the phenol-sulfuric acid method (Dubois M, et.al., 1956). The results of the analysis showed that starin *L. plantarum* Ts produced EPS amount (6.4 ± 0.8 g/l).

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

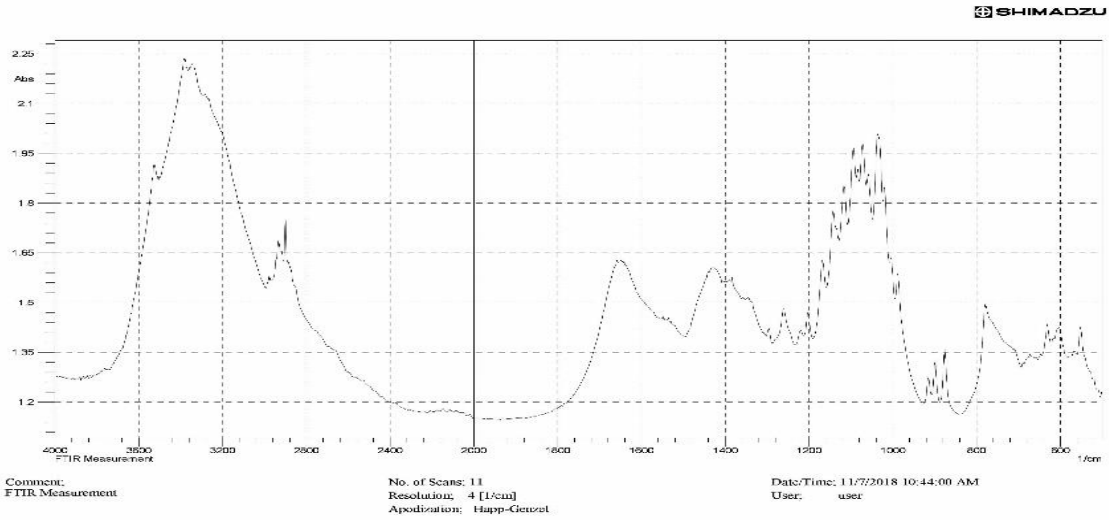

Fig. 2. FTIR analysis of the exopolysaccharide preparation from *L. plntarum* Ts.

The FTIR spectrum of the EPS from *L. plantarum* Ts showing the functional groups in the 4,000-400 cm^{-1} region is given in Fig. 2. In the IR spectrum, the peak at 3,420 cm-1corresponds to the OH stretch and the peak at 2,920 corresponds to the CH stretch. The broad stretch of C-O-C, C-O at between 1,000 and 1,200 cm^{-1}

corresponds ob carbohydrates (Wang Y, et.al., 2008). A peak at 1,366 cm-1 could be assigned to the C=O stretch of the COO− and C–O bond from COO− (Nikonenko NA, et.al., 2000). The band at 1,162 cm^{-1} corresponds to a glycosidic linkage, as 1,4-glycosidic linkage in polysaccharides gives absorption bands in the range of 1,175-1,140 cm^{-1} [16] (Fig. 2).

Monosaccharide Composition of the EPS from *L. plntarum 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. plntarum* (Fig. 3).

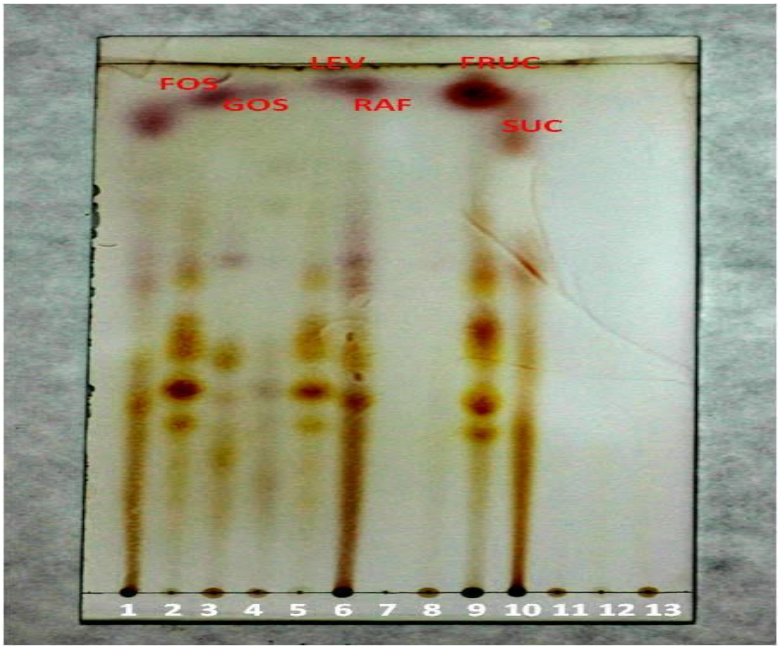

Fig. 3. TLC analysis of monosaccharides from *L. plntarum* Ts. exopolysaccharide hydrolysate. Where 1. EPS from *L. plntarum* Ts; 2. FOS; 3. GOS; 4 GalOS; 5 FOS from chicory (Sigma);6 Rafinose; 7 comersial β-1,3 glucan from Euglena gracilis (Sigma); 8 Glucose; 9. Fructose; 10 Sucrose; 11.Galactose, 12.Lactose; 13 Maltose

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

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

Table 1. Oligosaccharide Utilization

Strains	growth on MRS	OD glucose[a]	growth on MRS-EPS	OD EPS[a]
L. plantarum Ts	+	5,9±0,12	+	1,2±0.05
E. coli 3398	+	0,4±0,02	-	0,2±0,01
St. aureus 745	+	0,2±0,01	-	0,1±0,02
B. subtilis 6633	+	0,3±0,02	-	0,3±0,03
S. Typhi. 3591	+	0,1±0,01	-	0,1±0,01
L. monocytogenes 863	+	0,1±0,03	-	0,1±0,02
E. aerogenes 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.

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.

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.

DISCUSSION

FT-IR is an effective analytical instrument for detecting functional groups and characterizing covalent bonding information. The technique works on the fact that bonds and groups of bonds vibrate at characteristic frequencies. A small peak around 1,530 cm^{-1} corresponding to an amino group was also observed in the spectrum of EPS. The obvious absorption peak 879.54 cm^{-1} revealed the existence of b-glycosidic bond. It was explained by Braissant et al. (2009) that the peak at 898 cm^{-1} could be attributed to the b-glycoside linkage between sugar monomers. The band in the range 1,030–944 cm-1, with minimum at 998 cm^{-1} can be attributed to Glc (Nikonenko NA, et.al., 2000; C ˇerna ´ M., ET.AL., 2003) The absorption bands at 812, and 879 cm^{-1} indicated that the EPS contained both a and b-type glycosidic linkages in its structure (Barker SA, et.al., 1954). The carbohydrates show high absorbencies in the region 1,200–950 cm^{-1}, that is within the so-called fingerprint region, where the position and intensity of the bands are specific for every polysaccharide, allowing its possible identification (Filippov MP.,1992). Polysaccharides present the highest capacity for carrying biological information since they have the greatest potential for structural variability. Presence of glucose and sucrose indicated that the EPS produced by the isolate is a heteropolysaccharide. The biological activities of glucomannans implicate the importance of this polysaccharide extracted from the indigenous LAB strain which showed probiotic properties.

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 T, 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.

In conclusion, *L. plantarum* Ts produced a water soluble heteropolysaccharide, which is composed of sucrose and glucose. 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.

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