Polish Journal of Microbiology
2013, Vol. 62, No 2, 181–189

ORIGINAL PAPER

Bifidogenic and Antioxidant Activity of Exopolysaccharides Produced by *Lactobacillus rhamnosus* E/N Cultivated on Different Carbon Sources

MAGDALENA POLAK-BERECKA[1]*, ADAM WAŚKO[1], DOMINIK SZWAJGIER[1] and ADAM CHOMA[2]

[1] Department of Biotechnology, Human Nutrition and Science of Food Commodities, University of Life Sciences in Lublin, Skromna 8, 20-704 Lublin, Poland
[2] Department of Genetics and Microbiology, Institute of Microbiology and Biotechnology, Maria Curie-Skłodowska University, Akademicka 19, 20-033 Lublin, Poland

Received 11 September 2012, revised 7 February 2013, accepted 12 March 2013

Abstract

Lactobacillus rhamnosus E/N is a probiotic bacterium, which synthesizes exopolysaccharides (EPS) with significant bifidogenic and antioxidant activities. The sugar composition of the EPSs produced depended on carbohydrates used as a carbon source in the growth media. Five *Bifidobacterium* strains were tested *in vitro* for their ability to utilize all the EPSs studied. The highest bifidogenic activity was revealed by EPSs obtained from *Lactobacillus* cultures supplemented with Gal, Lac, and Mal as the only carbon source, while significant antioxidant effects were observed in EPSs isolated from growth media enriched with galactose, lactose, and sucrose.

Key words: *Lactobacillus rhamnosus,* exopolysaccharides (EPSs), antioxidant activity, bifidogenic effect

Lactobacillus rhamnosus E/N belongs to lactic acid bacteria (LAB) and is a probiotic microorganism, which secretes significant amounts of extracellular polysaccharides. Exopolysacharides (EPSs) from LAB can exert functional effects on foods, improve the rheology of fermented milk products, and have beneficial health effects. In particular, LAB EPSs have immunostimulatory activities (Chabot *et al.,* 2001; Duboc and Mollet, 2001; Jolly *et al.*, 2002). Moreover, these polymers enhance colonization of the gastrointestinal tract by probiotic bacteria and act as antioxidants (Hugenholtz and Smid, 2002; Badel *et al.*, 2011). Dal Bello and coworkers (2001) as well as Korakli and coworkers (2002) pointed to the influence of EPS from *Lactobacillus sanfranciscensis* on bifidobacterial metabolism. A large group of food-grade bacteria are producers of nondigestible carbohydrates, which have bifidogenic activity (Hugenholtz and Smid, 2002). Moreover, EPS obtained from a culture of *Lactococcus lactis* subsp. *lactis*, mainly composed of fructose and rhamnose, showed strong antioxidant capacity, *i.e.* the ability to inhibit hydroxyl and superoxide anion radical activities (Pan and Mei, 2010). A majority of the bifidogenic compounds described so far were carbohydrates of plant origin (Su *et al.*, 2007). However, until now, no data have been published on selective stimulation of the growth of *Bifidobacterium* or on the antioxidant action of *Lb. rhamnosus* EPSs.

Heteropolysaccharides derived from *Lactobacillus* spp. show great variability in their monomer composition, charge, spatial arrangement, and rigidity (Duboc and Mollet, 2001). A majority of exopolysaccharide backbones have repeating units composed of glucose, galactose, and rhamnose, which occur in different ratios, different anomeric configuration and are connected by different linkages. Occasionally, aminosugars such as *N*-acetyl-D-glucosamine and *N*-acetyl-D-galactosamine as well as non-carbohydrate substituents (*sn*-glycerol-3-phosphate, phosphate, and acetyl groups) may also be present in EPSs (De Vuyst and Degeest, 1999; Badel *et al.*, 2011). There is some evidence for a correlation between the structure of EPS and its ability to interact with proteins (Ruas-Madiedo *et al.*, 2002). It can also be assumed that the structure-function relationships in these biopolymers are crucial for their specific biological actions such as the bifidogenic and antioxidant effects. The knowledge of this correlation is particularly important as it provides a foundation for producing functionally valuable exopolysaccharides. Growth conditions and the medium components may

* Corresponding author: M. Polak-Berecka, Department of Biotechnology, Human Nutrition and Science of Food Commodities, University of Life Sciences in Lublin, Skromna 8, 20-704 Lublin, Poland: phone: +48 81 46 23 356; fax: +48 81 46 23 400, **e-mail: 3mj@wp.pl**

affect the yield and sugar composition of EPSs (Looijesteijn *et al.*, 2000). The carbon source seems to be one of the most important factors influencing the monomeric composition and variations in the glycosidic bonds of EPS (De Vuyst and Degeest, 1999). Grobben *et al.* (1995; 1996) have reported that the composition of EPS produced by *Lactobacillus delbrueckii* subsp. *bulgaricus* depended on the culture components and conditions of cultivation of bacteria.

In the present paper, the exopolysaccharides synthesized by *L. rhamnosus* E/N growing on different sugar-supplemented media were tested in respect of their composition. The bifidogenic potential and antioxidant effects of these EPSs were estimated as well.

Experimental

Materials and Methods

Bacterial strains and growth conditions. *L. rhamnosus* E/N, obtained from Biomed Serum and Vaccine Production Plant Ltd., (Lublin, Poland), was stored at −80°C in the MRS (de Man *et al.*, 1960) medium supplemented with 20% (v/v) glycerol. This strain is deposited at the Institute of Biochemistry and Biophysics of the Polish Academy of Sciences under number 2594 and is one of the components of Lakcid®, a pharmaceutical product containing viable probiotic bacteria. The strain was revitalized in MRS broth at 37°C for 24 h before use. *Bifidobacterium infantis* ATCC1567 was obtained from the American Type Culture Collection (ATCC, Manassas, Va., U.S.A., http://www.atcc.org/). *Bifidobacterium bifidum* B 41410, *Bifidobacterium animalis* subsp. *lactis* B 41406, *B. animalis* BI 30, and *Bifidobacterium longum* KN 29 were kindly provided by Prof. M. Bielecka (Department of Food Microbiology, Institute of Animal Reproduction and Food Research, Polish Academy of Sciences in Olsztyn, Poland). The strains were cultured on Garche's medium (Rasic 1990) (10 ml) at 37°C in test tubes. Anaerobic conditions were obtained by injecting 0.5 ml of 15% (m/v) $NaHCO_3$ and 0.5 ml of 20% (m/v) pyrogallol solutions in water into cotton stoppers followed by aseptic sealing of the tubes with parafilm.

For EPS extraction, *Lb. rhamnosus* E/N was cultivated in a bioreactor (Sartorius, Goettingen, Germany) in 1.5 l of the MRS medium supplemented with appropriate sugar (20 g/l; glucose, galactose, sucrose, maltose, and lactose) as the only carbon source. Batch culture inoculated with the *Lb. rhamnosus* E/N overnight culture (2.5%, v/v) was carried out for 68 h at 37°C at a constant pH of 6.3. After isolation (by precipitation with 96% ethanol, see below) and lyophilisation, the following EPS preparations were obtained: EPS-Glc, EPS-Gal, EPS-Mal, EPS-Suc, and EPS-Lac.

Preparation of exopolysaccharides. After incubation, the cultures were heated at 100°C for 15 min, and bacterial cells were removed by centrifugation (4,000 × *g*, 30 min, room temperature). The supernatant volumes were reduced (3 times) by evaporation using a vacuum rotary evaporator (Büchi R210: 0.093 MPa, 40°C). The EPSs were precipitated by adding three volumes of chilled 96% ethanol. The mixtures were left for 24 h at 4°C. The exopolysaccharides were collected by centrifugation at 20,000 × *g* for 30 min at 4°C, dissolved in distilled water, and dialyzed (4,000 Da molecular weight cutoff membranes, Roth, Karlsruhe, Germany) against distilled water for 48 h at 4°C. The water was changed twice. The crude freeze-dried EPSs were purified by dissolving in 15% trichloroacetic acid (TCA), and precipitated materials were removed by centrifugation (20,000 × *g*). The purified EPSs were exhaustively dialyzed and finally lyophilized.

Production of exopolysaccharides by *Lb. rhamnosus* E/N was determined by measuring protein and carbohydrate concentrations. The total sugar content was determined by the method of Dubois *et al.* (1956), using glucose as a standard. The protein content was determined with the method of Bradford (1976), using bovine serum albumin (Sigma Company, USA) as a standard.

In order to eliminate any contamination of the EPSs derived from the MRS medium (sugars and aminosugars), the same procedures were used for precipitation of high molecular weight substances from the non-inoculated medium.

Compositional analysis. For qualitative analysis, the exopolysaccharides were subjected to methanolysis in 1 M HCl in MeOH (0.5 ml, 80°C, 20 h). The samples were dried and acetylated with a mixture of Ac_2O and pyridine (1:1, v:v, 50 µl, 100°C, 30 min). The quantitative estimations of neutral and amino sugars isolated from the EPSs were based on analysis of alditol acetates. To this point, the exopolysaccharides were hydrolyzed with 2 M TFA (100°C, 4 h) and, after drying, the samples were subjected to N-acetylation (Que *et al.*, 2000) followed by reduction with $NaBH_4$ and peracetylation (Sawardeker *et al.*, 1965). Alditol acetates as well as acetylated methyl glycosides were analyzed using combined gas chromatography-mass spectrometry. GLC-MS analyses were carried out on a Hewlett-Packard gas chromatograph (model HP5890A) equipped with a capillary column (HP-5MS, 30 m × 0.25 mm) and connected to a mass selective detector (MSD model HP 5971). Helium was the carrier gas, and the temperature program was initially 150°C for 5 min, then raised to 310°C at a ramp rate of 3°C/min, final time 20 minutes.

Bifidogenic effect of exopolysaccharides (*in vitro* assay). Prior to testing the effect of EPS on the growth of bifidobacterial strains, an optimal basal medium was chosen. Several media were tested: 152.CECT

Bifidobacterium sp. medium without a carbon source (Espinosa-Martos and Rupérez, 2009) and modified Garche's media (González *et al.*, 2008) containing peptone 20 g/l, yeast extract 2 g/l, HCl cysteine 0.4 g/l, sodium acetate 6 g/l, $MgSO_4$*$7H_2O$ 0.12 g/l, Na_2HPO_4* $12H_2O$ 2.5 g/l, K_2HPO_4 2 g/l, and lactose in concentrations from 0.1 to 3 g/l.

Lyophilized exopolysaccharides were added to the basal medium to a final concentration of 0.5% (w/v). The growth rate of each strain of *Bifidobacterium* on these media was monitored by measuring optical density (OD_{600}) using a Bioscreen C instrument (LabSystem, Finland). Three hundred and fifty µl of the test media were inoculated with an overnight culture of actively growing *Bifidobacterium* cells (50 µl). The surfaces of the cultures were covered with 20 µl of sterile paraffin. All the experiments were carried out in honeycomb plates at 37°C for 96 h.

Antioxidant activities of EPS (measured using ABTS). The atioxidant properties of EPSs were estimated using a slightly modified method of Miller *et al.* (1993). The reagents (in 5 mM phosphate buffer, pH 7.0) were present in the reaction mixture at final concentrations of 0.15 mM ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); 0.0025 mM myoglobin, and 0.375 mM H_2O_2. The EPS solution in double distilled (DDI) water (0.03 ml, 5 mg/ml) was mixed with 0.03 ml of the buffer and 0.28 ml of the ABTS/myoglobin reagent followed by addition of 0.023 ml of H_2O_2 solution in water (5.92 mM/dm^3) to start the reaction. Absorbance was measured every minute (700 nm, 20°C) using a 96-well microplate reader (Tecan Sunrise, Austria). Reaction time of 15 minutes was selected for the calculations of the TEAC value. The synthetic antioxidant Trolox, a water-soluble vitamin E analogue, was used as an antioxidative standard. Fifteen Trolox standard solutions in the buffer (0.041 mM–0.615 mM) replaced 0.03 ml of the tested sample. Total antioxidant activity was expressed in mM of Trolox (TEAC value), which inhibited the increase in absorbance to the same degree as the EPS solution. Blanks containing EPS and without EPS were run simultaneously for subtraction of background absorbances of the tested samples.

The method of Miller *et al.* (1993) in modifications proposed by Re *et al.* (1999) was also used. A solution of ABTS (7 mM) containing potassium persulphate (2.45 mM) was prepared and left for 24 h at ambient temperature. Directly prior to analysis, the absorbance of this solution (at 700 nm) was adjusted to 0.70 ± 0.02. For measurements, 0.06 ml of the ABTS/persulphate solution, 0.1 ml of DDI water, and 0.15 ml of the tested sample (5 mg/ml) were mixed. Absorbance was read at 700 nm after 10 min of the reaction (20°C, microplate reader, Tecan Sunrise, Austria). Twenty Trolox standard solutions in DDI water (0.4155 mM–8.31 mM) were used for the calculation of the TEAC values as described above. Blanks were run as described above.

Statistical analysis. For each experiment, the optical density was given as mean ± SD of triplicate samples from two independent experiments. The data were analyzed using the Excel statistical package. Statistical significances were determined by the Student's *t*-test and set at $P < 0.01$.

Results

Compositional analyses of exopolysaccharides. The yields of EPS produced by *Lb. rhamnosus* E/N varied and were dependent on the carbon source in the growth medium (Table I). Lyophilized EPS contained from 92.05% to 97.45% carbohydrates and from 2.51% to 7.95% proteins, depending on the carbon source.

Chromatographic separation (GLC-MS) of acetylated methyl glycosides and alditol acetates obtained after acidic degradation of the carbohydrate polymers was the basic method of qualitative and quantitative analyses of the isolated EPSs. Glucose, galactose, mannose and 6-deoxyhexose (rhamnose) were found to be constituents of the investigated preparations. The results were corrected by subtraction of sugar amounts characteristic for carbohydrate polymers precipitated from the non-inoculated MRS medium (data not shown). The contents of sugars in each EPS preparation are expressed in mol% and listed in Table II. The

Table I
Yields of exopolysaccharides synthesized by *Lactobacillus rhamnosus* E/N on media with different carbon sources

Carbon source	EPS, mg/1 l	% of carbohydrates*	% of proteins**
Galactose	81.08	96.12	3.88
Lactose	219.25	97.18	2.82
Glucose	130.08	97.41	2.59
Saccharose	31.55	92.64	7.36
Maltose	37.37	93.18	6.82

* – estimated by the Dubois method
** – estimated by the Bradford method

Table II
Carbohydrate composition of exopolysaccharides produced by *Lactobacillus rhamnosus* E/N cultivated on different carbon sources

EPS (carbon sources)	Sugars (mol%)			
	Rha	Man	Glc	Gal
EPS-Gal	47.9	5.9	33.9	12.3
EPS-Glc	63.1	0.0	32.6	4.2
EPS-Suc	100	0.0	0.0	0.0
EPS-Lac	32.6	32.7	25.1	9.5
EPS-Mal	17.6	65.4	16.9	0.0

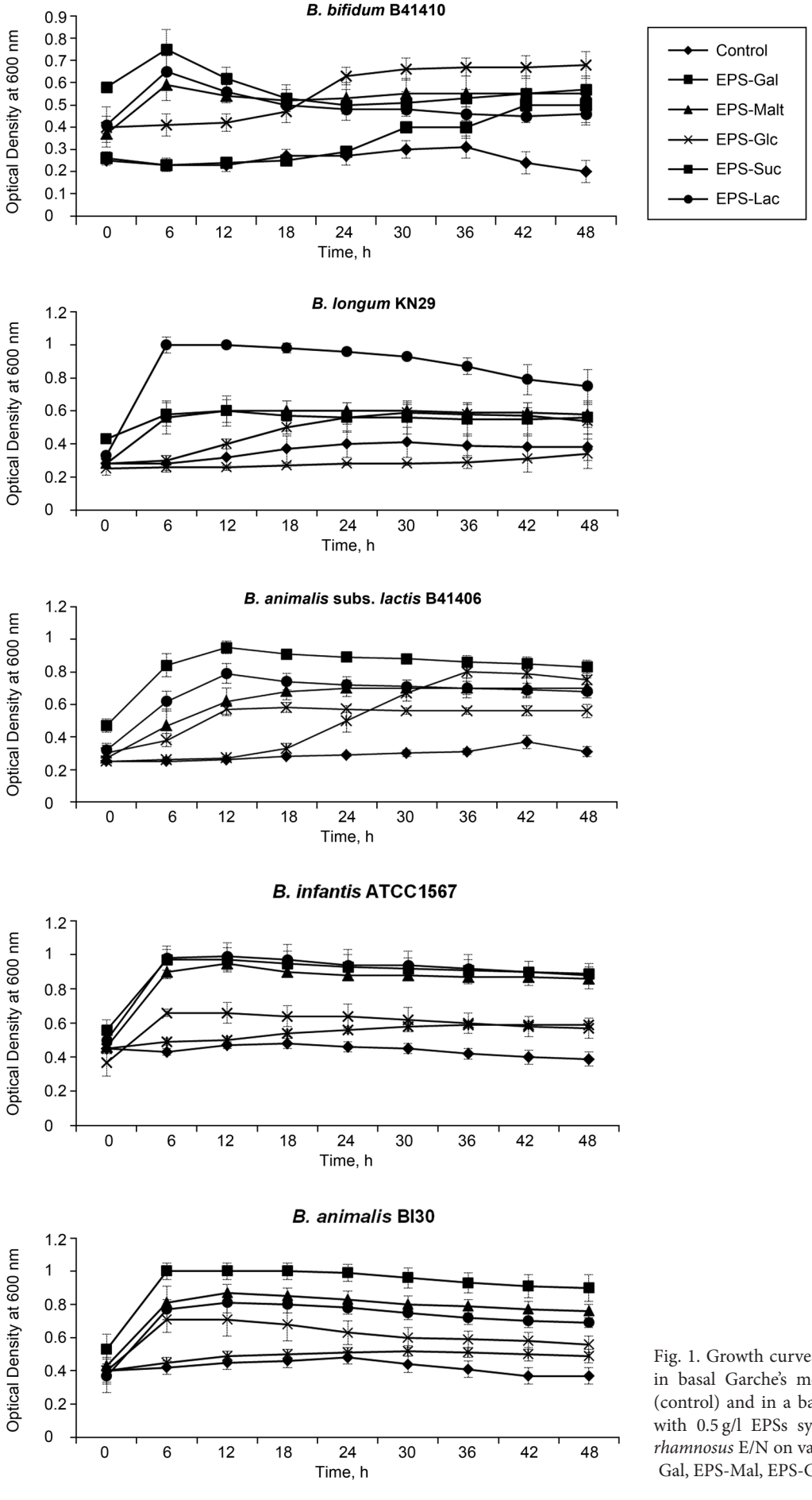

Fig. 1. Growth curves of *Bifidobacterium* grown in basal Garche's medium with 0.1 g/l lactose (control) and in a basal medium supplemented with 0.5 g/l EPSs synthesized by *Lactobacillus rhamnosus* E/N on various carbon sources (EPS-Gal, EPS-Mal, EPS-Glc, EPS-Suc, and EPS-Lac)

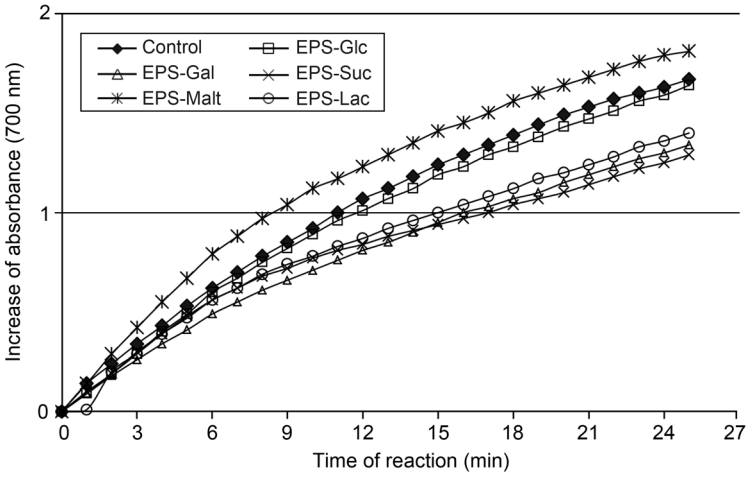

Fig. 2. Increase in absorbance at 700 nm due to the generation of ABTS radicals by myoglobin and H_2O_2 in the presence of EPS (mean values)

results obtained unequivocally indicate that the carbon source in the growth medium significantly influences the production and composition of exopolysaccharides synthesized by *Lb. rhamnosus* E/N. The EPS preparations have distinctly different sugar compositions and, presumably, different structures. EPS-Suc was identified as a homopolysaccharide composed of rhamnose. Both rhamnose and glucose were the major components of the other EPS preparations. Exopolysaccharides denoted as EPS-Mal contained a substantial amount of mannose; hence, they can be classified as mannans. Rhamnose was present in an amount comparable to the glucose and mannose content only in the EPS-Lac preparation. Galactose was always a minor component of the EPS preparations. Especially EPS-Glc contained a low amount of galactose; therefore, this sugar should be treated as a nonstoichiometric branching residue.

Bifidogenic effect of exopolysaccharides (*in vitro* assay). The basal media tested showed differences in supporting the growth of bifidobacteria (results not shown). The best results were obtained for Garche's modified medium containing 0.1 g/l of lactose. Therefore, it was chosen as a basal medium for further experiments. *Bifidobacterium* microcultures grown in this medium reached an OD_{600} of 0.3 to 0.4, whereas those grown in original Garche's medium had an OD_{600} of about 3.0.

The results of the analysis of bifidobacterial growth with Garche's basal medium supplemented with different EPS preparations showed that the growth of *B. bifidum* B 414010, *B. animalis* subsp. *lactis* B 41406, and *B. animalis* Bi30 was stimulated by all the EPSs tested. However, the growth of *B. longum* KN29 was not stimulated by EPS-Glc (Fig. 1). Generally, the EPS-Gal, EPS-Lac, and EPS-Mal preparations showed high bifidogenic activity. For *B. bifidum* B 414010 grown in a medium with EPS-Glc, a long lag phase was observed followed (after 18 h) by active cell multiplications causing the highest optical density of the culture, indicating the best bifidogenic effect of EPS-Glc on this bacterial species. A similar growth curve was observed for *B. animalis* B41406 cultivated in a medium supplemented with EPS-Suc.

Antioxidant activities of EPS. The antioxidant activities of the EPS polymers were studied by two methods. The method utilizing the ABTS/myoglobin reagent is rapid and suitable for measurement of both antioxidant and prooxidant activities. The method with ABTS/potassium persulphate is suitable for detection of the free radical "scavenging" ability but not for measurement of the prooxidant action.

The antioxidant activities of the EPS-Glc, EPS-Gal, EPS-Suc, and EPS-Lac preparation s were proved using the ABTS/myoglobin method, whereas EPS-Mal exhibited prooxidant activity over the whole time range (Fig. 2).

The TEAC values were calculated on the basis of the absorbance readings within 15 min after the beginning of the reaction (Fig. 3). EPS-Suc and EPS-Gal exhibited the highest TEAC values (0.50 mM and 0.49 mM, respectively) with an insignificant ($p > 0.05$) difference between the two polymers. EPS-Lac exhibited

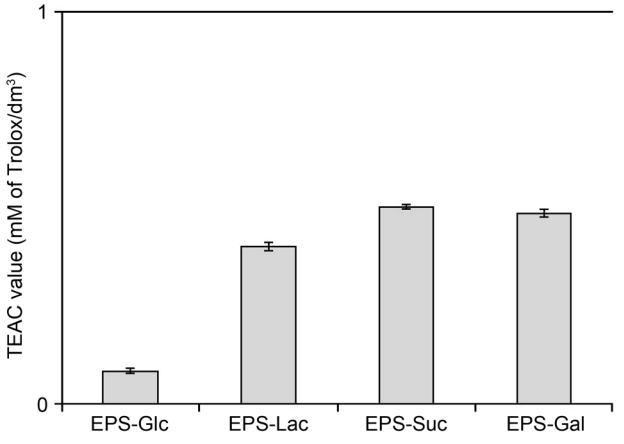

Fig. 3. Antioxidant activities of EPS measured using ABTS/myoglobin and H_2O_2 (mean ± SD)

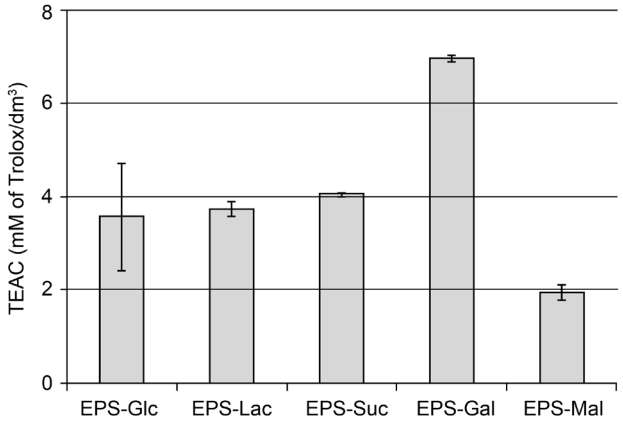

Fig. 4. Antioxidant activities of EPSs measured using the method with ABTS/potassium persulphate (mean ± SD)

significantly ($p < 0.05$) lower antioxidant activity (0.40 mM) than EPS-Suc and EPS-Gal, but significantly ($p < 0.05$) higher than EPS-Glc (0.08 mM).

Each exopolysaccharide polymer effectively "scavenged" ABTS radicals (Fig. 4). However, free radicals were "scavenged" to a different extent by the EPSs tested. It is interesting to note that among the polysaccharides studied, EPS-Gal exhibited the highest ability to remove ABTS radicals (TEAC 6.96 mM).

Discussion

Our study clearly showed that the amount of exopolysaccharides produced by *Lb. rhamnosus* E/N depended on the type of the carbohydrate source in the growth medium. A similar relationship was reported by other authors, who suggested that the regulation of the EPS biosynthetic pathway in *Lactobacillus* and the EPS sugar composition was dependent on the carbohydrate source (Cerning *et al.*, 1994; Grobben *et al.*, 1995; Welman and Maddox, 2003). It has been found that the monosaccharides occurring most frequently in EPS from various *Lactobacillus* strains were glucose, galactose, and rhamnose (Grobben *et al.*, 1995). Our results confirmed the fact that exopolysaccharides produced by *Lb. rhamnosus* E/N are mainly heteropolymers consisting of rhamnose, glucose, and mannose when the bacteria were grown on galactose, lactose, or maltose. Rhamnose was present in amounts comparable to the contents of glucose and mannose only when the bacteria were grown in media supplemented with lactose. *Lb. rhamnosus* E/N growing on saccharose as the only carbon source in the medium was able to synthesize homopolysaccharides composed of rhamnose. This is the first report about the capability of the *Lactobacillus* strain to biosynthesize both homo- and heteropolysaccharides.

Hongpattarakere *et al.* (2012) have proved that EPSs from marine lactic acid bacteria can be used as a prebiotic ingredient in food industry to modulate intestinal microbiota for health benefits. These exopolysaccharides were human non-digestible carbohydrates, which might be used as an additional energy source for intestine microbiota. Bielecka *et al.* (2002) have shown that a range of bifidobacteria were able to utilize saccharides with a high degree of polymerization. Other authors reported that the broad range of carbohydrates (soybean oligosaccharides, galactooligosaccharides, *etc.*) were similar to or better than glucose in supporting the growth of bifidobacteria (Su *et al.*, 2007; González *et al.*, 2008). It has been confirmed that carbohydrate metabolism varies considerably among bifidobacterial strains (Pokusaeva *et al.*, 2011). In the present study, the biodegradability of EPSs from *Lb. rhamnosus* E/N was tested *in vitro* using different species of bifidobacteria. The growth curves on media supplemented with EPSs were compared with bacterial growth on a modified basal Garche's medium containing a minimal carbon source. Our observations indicate that EPSs, which differ in monosaccharide composition, stimulate the growth of bifidobacteria at different rates. Ruas-Madiedo *et al.* (2002) related the susceptibility to biological degradation directly to the primary structure of the EPSs. EPSs substituents make their molecules more or less accessible to degradative enzymes. The recently characterized bifidobacterial strains possess extracellular enzymes belonging to glycoside hydrolase families 2, 13, 36, and 42, such as α-galactosidases, ß-galactosidases, and an enzyme active towards glucooligosaccharides (Pokusaeva *et al.*, 2011). Presence of these hydrolytic enzymes supported the metabolic and nutritional dependencies among colon microorganisms. González *et al.* (2008) described a novel galactose/lacto-*N*-biose operon with associated sugar transporters in bifidobacteria. They reported that the bifidogenic effect of human milk was based on complex oligosaccharides. The exopolysaccharides produced by *Lb. rhamnosus* E/N exhibited similar monosaccharide composition as that of the oligosaccharides described previously. In our study, we identified the particular EPSs that significantly enhanced the growth of *B. bifidum*, *B. animalis*, *B. longum*, and *B. infantis.* The present findings suggest that among the EPSs tested, EPS-Gal and EPS-Lac have the potential to promote the activity of bifidobacterial strains.

Several studies have shown that polysaccharides from bacterial and fungal sources act as antioxidants (Kodali and Sen, 2008; Guo *et al.*, 2010; Pan and Mei, 2010). Very limited information is available concerning the mechanisms of the antioxidant action of polysaccharides at the molecular level. Our results suggest that antioxidant activity is strongly influenced by the substrate used in the oxidative reaction, but also show that various EPSs from *Lb. rhamnosus* E/N trigger diverse

antioxidant effects. These properties may be dependent on the arrangement of monosaccharides, as well as the type of glycosidic linkages of the polysaccharide and its conformation (Kumar *et al.*, 2007). However, with the use two methods, ABTS/myoglobin and ABTS/potassium persulphate, a significant antioxidant effect was observed for EPS-Gal, EPS-Lac, and EPS-Suc.

In a conclusion, the EPSs produced by *Lb. rhamnosus* E/N grown on five different carbon sources showed bifidogenic and antioxidant activities. It was also found that the level of biological activity was depended on the type of exopolysaccharide. Modification of the growth medium by changing the carbohydrate source is not only a way to optimize the yield of EPS, but also to modify EPS composition. One of the valuable applications of lactic acid bacteria is the possibility to use them to compose complex starter cultures containing probiotic LAB and *Bifidobacterium* strains, which exhibit health-promoting characteristics. The relationship between the physicochemical characteristics of bacterial exopolysaccharides and their biological function still remains to be elucidated. Further investigations are required to determine the physiological mechanisms of their prebiotic, bifidogenic, and antioxidant effects.

Literature

Badel S., T. Bernardi and P. Michaud. 2011. New perspectives for Lactobacilli exopolysaccharides. *Biotechnol. Advances* 29: 54–66.

Bielecka M., E. Biedrzycka and A. Majkowska. 2002. Selection of probiotics and prebiotics for synbiotics and confirmation of their *in vivo* effectiveness. *Food Res. Int.* 35: 125–131.

Bradford M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72: 248–254.

Cerning J., C.M.G.C. Renard, J.F. Thibault, C. Bouillanne, M. Landon, M. Desmazeaud and L. Topisirovic. 1994. Carbon source requirements for exopolysasccharide production by *Lactobacillus casei* CG11 and partial structure analysis of the polymer. *Appl. Environ. Microbiol.* 60: 3914–3919.

Chabot S., *et al.* 2001. Exopolysaccharides from *Lactobacillus rhamnosus* RW-9595M stimulate TNF, IL-6 and IL-12 in human and mouse cultured immunocompetent cells, and IFN-γ in mouse splenocytes. *Lait* 81: 683–697.

Dal Bello F., J. Walter, C. Hertel and W.P. Hammes. 2001. *In vitro* study of prebiotic properties of levan-type exopolysaccharides from Lactobacilli and non-digestible carbohydrates using denaturing gradient gel electrophoresis system. *Appl. Microbiol.* 24: 232–237.

de Man J.C., M. Rogosa and E. Sharpe. 1960. A medium for the cultivation of the lactobacilli. *J. Appl. Bacteriol.* 23: 130–135.

De Vuyst L. and B. Degeest. 1999. Heteropolysaccharides from lactic acid bacteria. *FEMS Microbiol. Rev.* 23: 153–177.

Duboc P. and B. Mollet. 2001. Applications of exopolysaccharides in the dairy industry. *Int. Dairy J.* 11: 759–768.

Dubois M., K.A. Gilles, J.K. Hamilton, P.A. Rebers and F. Smith. 1956. Colorimetric method for the determination of sugars and related substances. *Anal. Chem.* 28: 350–356.

Espinosa-Martos I. and P. Rupérez. 2009. Indigestible fraction of okara from soybean: composition, physicochemical properties and in vitro fermentability by pure cultures of *Lactobacillus acidophilus* and *Bifidobacterium bifidum*. *Eur. Food Res. Technol.* 228: 685–693.

González R., E.S. Klaassens, E. Malinen, W.M. de Vos and E.E. Vaughan. 2008. Differential transcriptional response of *Bifidobacterium longum* to human milk, formula milk, and galactooligosaccharide. *Appl. Environ. Microbiol.* 74(15): 4686–4694.

Grobben G.J., J. Sikkema, M.R. Smith and J.A.M. De Bont. 1995. Production of extracellular polysaccharides by *Lactobacillus delbrueckii* ssp. *bulgaricus* NCFB 2772 grown in a chemically defined medium. *J. Appl. Bacteriol.* 79: 103–107.

Grobben G.J., M.R. Smith, J. Sikkema and J.A.M. De Bont. 1996. Influence of fructose and glucose on the production of exopolysaccharides and the activity of enzymes involved in the sugar metabolism and the synthesis of sugar nucleotides in *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2772. *Appl. Microbiol. Biotechnol.* 46: 279–284.

Guo S., W. Mao, Y. Han, X. Zhang, *et al.* 2010. Structural characteristics and antioxidant activities of the exracellular polysaccharides produced by marine bacterium *Edwardisiella tarda*. *Bioresour. Technol.* 101: 4729–4732.

Hugenholtz J. and E. Smid. 2002. Nutraceutical production with food-grade microorganism. *Curr. Opinion Biotechnol.* 13: 497–507.

Hongpattarakere T., N. Cherntong, S. Wichienchot, S. Kolida and R.A. Rastall. 2012. In vitro prebiotic evaluation of exopolysaccharides produced by marine isolated lactic acid bacteria. *Carbohydrate Polymers* 87: 846– 852.

Kodali V.P. and R. Sen. 2008. Antioxidant and free radical scavenging activities of an exopolysaccharides from a probiotic bacterium. *Biotechnol J.* 3: 245–251.

Korakli M., M.G. Gänzle and R.F. Vogel. 2002. Metabolism by bifidobacteria and lactic acid bacteria of polysaccharides from wheat and rye, and exopolysaccharides produced by *Lactobacillus sanfranciscensis*. *J. Appl. Microbiol.* 92: 958–965.

Kumar A.S., K. Mody and B. Jha. 2007. Bacterial exopolysaccharides – a perception. *J. Basic Microbiol.* 47: 103–117.

Jolly L., S.J.F. Vincent, P. Duboc and J.R. Neeser. 2002. Exploiting exopolysaccharides from lactic acid bacteria. *Antonie Van Leeuwenhoek* 82: 367–374.

Liu C.-F., K.-C. Tseng, S.-S. Chiang, B.-H. Lee, W.-H. Hsu and T.-M. Pan. 2011. Immunomodulatory and antioxidant potential of *Lactobacillus* exopolysaccharides. *J. Sci. Food Agric.* 91: 2284–2291.

Looijesteijn P.J., W.H.M. van Casteren, R. Tuinier, C.H.L. Doeswijk-Voragen and J. Hugenholtz. 2000. Influence of different substrate limitations on the yield, composition and molecular mass of exopolysaccharides produced by *Lactococcus lactis* subsp. *cremoris* in continuous cultures. *J. Appl. Microbiol.* 89: 116–122.

Miller N.J., C. Rice-Evans, M.J. Davies, V. Gopinathan and A. Milner. 1993. A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. *Cli. Sci.* 84: 407–412.

Pan D. and X. Mei. 2010. Antioxidant activity of an exopolysaccharide purified from *Lactococcus lactis* subsp. *lactis* 12. *Carbohydrate Polymers* 80: 908–914.

Pieretti G., G. Puopolo, S. Carillo, A. Zoina, R. Lanzetta, M. Parrilli, A. Evidente and M.M. Corsaro. 2011. Structural characterization of the O-chain polysaccharide from an environmentally beneficial bacterium *Pseudomonas chlororaphis* subsp. *aureofaciens* strain M71. *Carbohydr. Res.* 346: 2705–2709.

Pokusaeva K., G.F. Fitzgerald and D. van Sinderen. 2011. Carbohydrate metabolism in Bifidobacteria. *Genes Nutr.* 6: 285–306.

Que N.L.S., S. Lin, R.J. Cotter and C.R.H. Raetz. 2000. Purification and mass spectrometry of six lipid A species from the bacterial endosymbiont *Rhizobium etli*. Demonstration of a conserved distal unit and a variable proximal portion. *J. Biol. Chem.* 275: 28006–28016.

Rasic J.L. 1990. Culture media for detection and enumeration of the bifidobacteria in fermented milk products. *Bulletin of IDF* 252: 24–31.

Re R., N. Pellegrini, A. Proteggente, A. Pannala, M. Yang and C. Rice-Evans. 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radic. Biol. Med.* 26: 1231–1237.

Ruas-Madiedo P., J. Hugenholtz and P. Zoon. 2002. An overview on the functionality of exopolysaccharides produced by lactic acid bacteria. *Int. Dairy Sci.* 12: 163–171.

Sawardeker J.S., J.H. Sloneker and A.R. Jeans. 1965. Quantitative determinations of monosaccharides as their alditol acetates by gas liquid chromatography. *Anal. Chem.* 37: 1602–1604.

Su P., A. Henriksson and H. Mitchell. 2007. Selected prebiotics support the growth of probiotic mono-cultures *in vitro*. *Anaerobe* 13: 134–139.

Welman A.D. and I.S. Maddox. 2003. Exopolysaccharides from lactic acid bacteria: perspectives and challenges. *Trends Biotechnol.* 21(6): 269–274.