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Physicochemical characterization of exopolysaccharides produced by *Lactobacillus rhamnosus* on various carbon sources

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ABSTRACT

The impact of five carbohydrate sources (glucose, maltose, galactose, sucrose, and lactose) on the chemical composition, structure, morphology, and physicochemical properties, as well as, viscosity of exopolysaccharides (EPSs) produced by *Lactobacillus rhamnosus* E/N was investigated. GLC-MS analysis and 2DNMR spectroscopy showed that the EPSs had the same primary structure independently of the carbon source used in the growth medium. The following EPS composition was elucidated: four rhamnose, two glucose, and one galactose residue with a pyruvate substituent. Molecular masses (M_w) were determined by gel permeation chromatography, which revealed differences in M_w distribution. EPS-Gal, EPS-Suc, and EPS-Lac showed heterogenic fractions of a high and low molecular weight, while EPS-Mal and EPS-Glc contained only a high-molecular-weight fraction. AFM microscopy revealed morphological differences in chain length, thickness, and branching. Differences in the M_w ratio and thickness of the polymer chain were correlated with high viscosity of EPS solutions. Our results indicate that a single bacterial strain, depending on the carbon source in the medium, can produce EPSs of different rheological properties.

1. Introduction

Exopolysaccharides (EPSs) from lactic acid bacteria (LAB) are widely used in the food industry as viscosifying, stabilizing, gelling, or emulsifying agents, due to their characteristic physical and rheological properties (De Vuyst & Degeest, 1999). EPSs can exert functional effects on foods and improve the rheology of fermented milk products. EPSs from LAB also have beneficial health effects. In particular, these polymers have immunostimulatory activities (Chabot et al., 2001; Duboc & Mollet, 2001; Jolly, Vincent, Duboc, & Neeser, 2002), enhance colonization of the gastrointestinal tract by probiotic bacteria and act as antioxidants (Badel, Bernardi, & Michaud, 2011; Hugenholtz & Smid, 2002; Polak-Berecka, Waśko, Szwajgier, & Choma, 2013). As far as their physiological role is concerned, EPSs from LAB have been claimed to protect cells from detrimental environmental conditions, such as dehydration, macrophages, antibiotics, and bacteriophages, to sequester essential cations, and to be involved in adhesion and biofilm formation (Looijesteijn, Trapet, de Vries, Abee, & Hugenholtz, 2001).

EPSs derived from *Lactobacillus* spp. show great variability in their monomer composition, charge, spatial arrangement, and rigidity (Duboc & Mollet, 2001). A majority of exopolysaccharide backbones have repeating units composed of glucose, galactose, and rhamnose, which occur in different ratios and different anomeric configurations 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 (Badel, Bernardi, & Michaud, 2011; De Vuyst & Degeest, 1999). Some authors have reported that the regulation of the EPS biosynthetic pathway in *Lactobacillus* and the EPS sugar composition is dependent on the carbohydrate source in the growth medium (Cerning et al., 1994; Grobben, Sikkema, Smith, & De Bont, 1995; Welman & Maddox, 2003). There is some evidence for a correlation between the structure of EPS and its ability to interact with proteins (Ruas-Madiedo, Hugenholtz, & Zoon, 2002). It has been found that sugar composition, sugar linkages, chain length, presence of repeated side chains, and substitutions

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determine the technological and biological properties of EPS (Jolly, Vincent, Duboc, & Neeser, 2002). Therefore, physicochemical characterization of novel EPSs is relevant for predicting their potential application.

The aim of this study was to analyze structures and determine some of the physicochemical properties of EPSs extracted from *Lactobacillus rhamnosus* E/N which had been cultured on media with five different carbon sources.

2. Materials and methods

2.1. Bacterial culture conditions

Lb. rhamnosus E/N, obtained from Biomed Serum and Vaccine Production Plant Ltd., (Lublin, Poland), was stored at −80 °C in MRS (de Man, Rogosa, & Sharpe, 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. 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 an appropriate sugar (20 g/L; glucose, galactose, sucrose, maltose, or lactose) as the only carbon source. In order to eliminate any contamination of the EPSs derived from the MRS medium (sugars and aminosugars), Yeast Nitrogen Base (Sigma-Aldrich, Germany) was used as a nitrogen source in the growth medium. Batch culture inoculated with an overnight culture of *Lb. rhamnosus* E/N (2.5%, v/v) was carried out for 68 h at 37 °C at a constant pH of 6.3. After isolation, the following EPS preparations were obtained: EPS-Glc, EPS-Gal, EPS-Mal, EPS-Suc, and EPS-Lac.

2.2. Extraction and purification of EPSs

After incubation, the cultures were heated at 100 °C for 15 min, and bacterial cells were removed by centrifugation (4000 × g, 30 min, room temperature). The supernatant volumes were reduced 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 (4000 Da molecular weight cut-off membranes, Roth, Karlsruhe, Germany) against distilled water for 48 h at 4 °C. The water was changed twice. The crude EPSs were purified by dissolving in 15% trichloroacetic acid (TCA), and precipitated materials were removed by centrifugation (20,000 × g). The sample was then diluted with deionized water (EPS: water 4:1 v:v) and ultrafiltered at 4 °C, for four days, using Vivaflow 50 cartridges (10 kDa cut-off, Sartorius Stedim, France) and a Masterflex L/STM Economy Drive pump (Cole-Palmer, USA). As a final step, the sample after ultrafiltration was 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, Gilles, Hamilton, Rebers, and Smith (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.

2.3. Molecular mass determination

The average molecular mass of the EPSs was determined by gel permeation chromatography (Dionex Ultimate 3000) on an OHpak SB-806 M HQ column (8 × 300 mm, Shodex), calibrated with dextran standards (Mw 12, 25, 50, 80, 150, 270 kDa) with 0.1 M ammonium acetate buffer as eluent. The column eluate was monitored with a refractive index detector (Shodex RI 102).

2.4. Compositional and methylation analyses

The estimations of sugar contents in purified EPSs were based on alditol acetate analysis. For this purpose, the EPSs were hydrolyzed with 2 M TFA (100 °C, 4 h), and, after drying, the samples were subjected to N-acetylation (Que, Lin, Cotter, & Raetz, 2000) followed by reduction with $NaBH_4$ and peracetylation (Sawardeker, Sloneker, & Jeans, 1965). For methylation analysis, the polysaccharide preparations were permethylated, solvolyzed, and converted to permethylated alditol acetates according to the modified Hakomori procedure, as described by York, Darvill, McNeil, Stevenson, and Albersheim (1985). Alditol acetates as well as permethylated alditol acetates were analyzed under identical conditions 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 min.

2.5. NMR spectroscopy

NMR spectra were obtained on a Varian Unity 500 plus spectrometer. Lyophilized probes (7–10 mg) were dissolved at ^2H_2O (99.98% atom % ^2H). 2DNMR spectra were recorded at 65 °C and processed using standard spectrometer software (Varian). ^1H and ^{13}C chemical shifts are reported in ppm using 2,2-dimethylsilapentane-5-sulfonate (DSS) as internal reference. The TOCSY experiments were carried out with a mixing time of 120 ms, ROESY – 300 ms, and NOESY – 100 ms and 300 ms. The HMBC spectrum was recorded with a 60 ms delay for evolution of long range spin couplings.

2.6. Atomic force microscopy (AFM)

Purified and dried EPS samples were used to prepare suspensions in ultra-pure water at a concentration of 1 μg/mL. Freshly cleaved mica was attached to microscope slides with adhesive pads. 10 μL of EPS suspension was pipetted/dispensed onto this base and allowed to dry in the air. After drying, the samples were stored in desiccators with silica gel in order to avoid dehydration.

AFM images were taken with the Bioscope Catalyst microscope (Bruker, Germany) in the ScanAsyst mode in air and in Scanasyst-AIR probes with a nominal resonance frequency 70 kHz and a nominal spring constant 0.40 N/m. The images were registered with Nanoscope 8.0 software and analyzed using SPIP 6.0.9 (Image Metrology A/S, Denmark).

2.7. Rheological studies

The rheological properties of the aqueous solutions of the EPSs (10 mg/mL) were performed at 20 °C using a dynamic rheometer RS 300 (ThermoHaake, Karlsruhe, Germany) with a C60/2°Ti cone – plate system. Flow curves were measured by recording shear stress values when shearing the samples at an increasing shear rate from 0.1 to 200 s^{-1} for a period of 60 s and in a reverse sequence for the same time in the case of thixotropic measurements. The thixotropic area (A_T) was determined using Rheowin Pro software (v. 3.61, Haake) as the area limited between the up-curve and the down-curve. Experimental data of descending flow curves were

Table 1
Average molecular mass of EPSs synthesized on different carbon sources.

Exopolysaccharide	Peak no. 1 (kDa) (% of total peak area)	Peak no. 2 (kDa) (% of total peak area)
EPS-Mal	195 (100)	nd*
EPS-Glc	548.7 (100)	nd*
EPS-Gal	3 901 (49)	46.5 (51)
EPS-Lac	7 027 (68)	41.9 (32)
EPS-Suc	11 130 (48)	42.6 (52)

*nd–not detected

matched to the Ostwald de Waele model equation using Rheowin Pro software (v. 3.61, Haake): $\sigma = K\gamma^n$, where σ = shear stress, K = consistency index, γ = shear rate, and n = flow behavior index. The apparent viscosity of the EPS solutions was determined at a shear rate 50 s^{-1}, at 20 °C.

The values from all the tests performed are the means of three separate experiments ± standard deviation. Tukey's HSD test (STATISTICA 8.0, StatSoft, Inc., USA) was used for the determination of statistical differences with the significance denoted at $P < 0.05$.

3. Results

3.1. Molecular mass determination

The average molecular masses extrapolated from the dextran calibration curves for the EPSs synthesized by *Lb. rhamnosus* E/N cultivated on five carbon sources were in the range from 195 to 11 130 kDa (Table 1). The EPSs produced by the bacteria in MRS-Gal, MRS-Lac, and MRS-Suc media contained additional lower-molecular-mass fractions, whose molecular masses were about 45 kDa. EPSs marked as EPS-Glc and EPS-Mal were eluted on a size exclusion chromatography column as single peaks with molecular masses close to 200 and 550 kDa, respectively.

3.2. NMR spectroscopy

The 1DNMR spectra of the investigated EPSs were all very similar. Each spectrum contained signals in the methyl region (1.1–1.6 ppm), the backbone sugar proton region 3.2–4.4 ppm), and the anomeric region (4.5–5.5 ppm). Fig. 1 provides a comparison of the anomeric signals (^{1}HNMR) of the five EPS preparations. All these signals, except one, represent unresolved doublets. The signal at the lowest chemical shift, at δ 4.655, has a large coupling constant ($J_{1,2}$ = 7.6 Hz) and, therefore, represents a β anomer of glucose or galactose. Further analysis of NMR data revealed that this signal belonged to the β-D-glucopiranose spin system. This residue was denoted as A (Table 2 and figures). We started a detailed structural analysis from the EPS-Lac preparation, because its spectra contained the smallest number of anomeric signals. Beside residue A, the anomeric region of EPS-Lac contained six additional signals. Other sugar spin systems were marked with capital letters from B to G according to the increasing δ_H value (Fig. 2). The assignment of ^{1}H and ^{13}C resonances was performed on the basis of homo- (^{1}H—^{1}H) and hetero- (^{1}H—^{13}C) two-dimensional correlation NMR experiments. Three spin systems denoted as **B**, **D,** and **G**, with chemical shifts of the anomeric proton/carbon at δ_H/δ_C 5.051/104.28, 5.078/104.20, and 5.187/102.74, respectively, represented →3)-α-L-Rha(1→. This conclusion was based on assigned chemical shifts and a ^{1}H—^{1}H correlation of H5 protons with protons from methyl groups **B**, **D**, **G** residues, respectively. Positions C-3 of these sugar residues were glycosidically substituted since significant downfield shifts for C-3 were observed as compared with the chemical shift of the corresponding carbon of non-substituted α-L-Rha*p*. These results were confirmed by methylation analysis, in which 1,3,5-tri-O-acetyl-2,4-di-O-methyl-rhamnitol was found

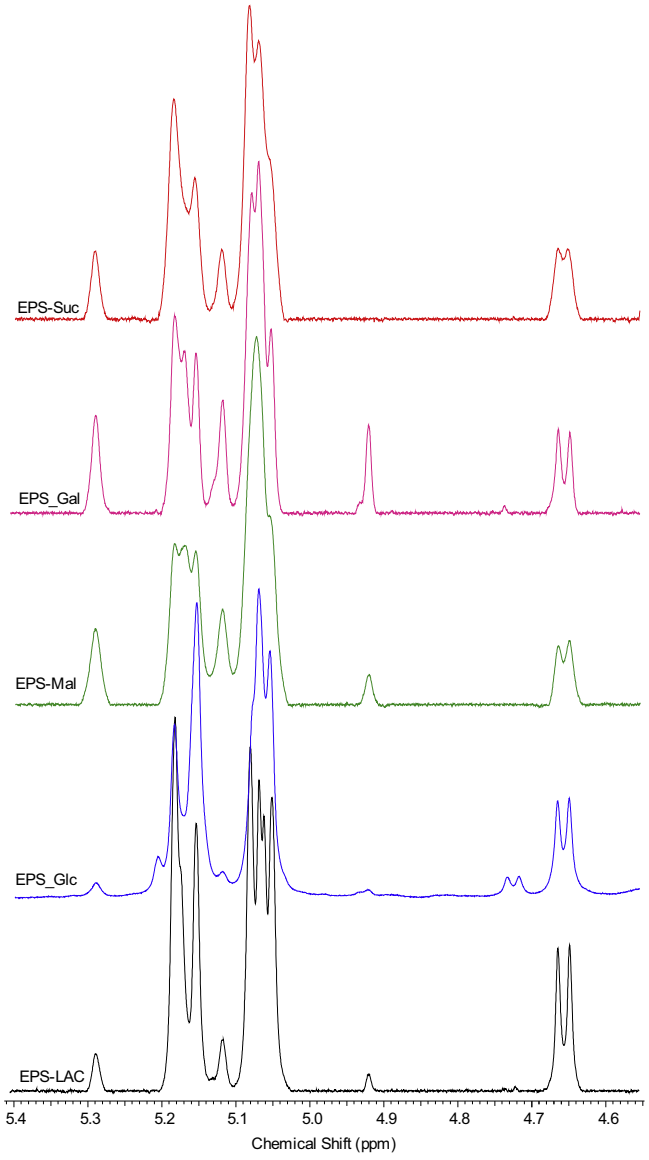


Fig. 1. Comparison of anomeric regions of ^{1}HNMR spectra of the five EPSs studied.

to be the main peak in the chromatogram of permethylated alditol acetates. Residue **E** had the anomeric proton resonance at δ_H 5.155, small $J_{1,2}$ (<3.0 Hz), and no correlation between EH1 and EH2 as well as some proton and carbon signals in high-field regions (δ_H ~1.300, δ_C ~20.00). Thus, this spin system also correspond to α-rhamnose. Downfield glycosylation shifts were observed for EC2 and EC3 (δ_C 78.28 and 81.17, respectively), indicating that this residue was a branching point within the polysaccharide polymer. The anomeric resonance for the proton of residue **F** was observed at δ_H 5.179 and showed $J_{1,2}$ = 3.8 Hz. Other backbone protons from this residue had large (~10 Hz) coupling constants typical of the *gluco*-configuration (data not shown). The data mentioned above, together with the FC1 resonance at δ_C 97.06, indicated the presence of α-D-Glc. The chemical shift of the carbon at position 2 in the piranoid ring was observed at δ_C 80.53, pointing to glycosidic substitution. Finally, the last spin system with an anomeric proton at δ_H 5.066 represented α-D-Gal, as proved by a detailed analysis of 2DNMR spectra. This residue had substituted hydroxyl groups at positions C-4 and C-6 (see Table 2– characteristic downfield chemical shifts for EC4 and EC6 carbons). A detailed analysis of ROESY and HMBC spectra showed that pyruvate was the substituent. The chemical shift of the pyruvate methyl group (27.65 ppm)

Table 2
Chemical shifts (proton/carbon) for sugar components in EPS-Lac.

Sugar residue	$^{1}H/^{13}C$						
	1 $J_{1,2}$ [Hz]	2	3	4	5	6	6′
→3)-β-D-Glc*p*-(1→ A	4.655/106.38 [7.6]	3.263/76.49	3.605/84.73	3.423/70.95	3.440/78.17	3.721/63.53	3.949/63.53
→3)-α-L-Rha*p*-(1→ B	5.051/104.28 [3.1]	4.179/72.23	3.883/80.18	3.627/72.80	3.290/71.65	1.326/18.93	–
→4,6)-α-D-Gal*p*-(1→ C	5.066/100.34 [3.5]	3.928/70.52	3.976/70.35	4.263/73.73	4.182/72.19	4.061/67.14	3.889/67.14
→3)-α-L-Rha*p*-(1→ D	5.078/104.20[~3.0]	4.185/72.23	3.949/80.73	3.573/73.67	3.885/71.59	1.344/19.24	–
→2,3)-α-L-Rha*p*-(1→ E	5.155/101.88 [<3.0]	4.332/78.28	4.085/81.17	3.654/73.66	3.917/71.66	1.328/18.92	–
→2)-α-D-Glc*p*-(1→ F	5.179/97.06[3.8]	3.685/80.53	3.948/74.72	3.526/72.10	3.980/74.10	3.791/62.99	3.865/62.99
→3)-α-L-Rha*p*-(1→ G	5.187/102.74 [<3.0]	4.238/69.62	3.910/77.93	3.625/73.59	4.058/71.25	1.297/19.83	–
Pyruvate	–/178.34	–/103.24	1.475/27.65				

indicated that chiral center of the pyruvate ligand (carbon C-2) had the *R*-configuration. This residue with *S*-configuration has axial methyl resonated at about 17 ppm. (Garegg et al., 1980, Jansson, Lindberg, & Widmalm, 1993). Absolute configurations of the monosaccharides identified in the EPSs were established using known regularities in the effects of glycosylation on ^{13}CNMR chemical shifts (Shashkov, Lipkind, Knirel, & Kochetkov, 1988). The spectroscopic data were confirmed by methylation analysis. Beside C-3-substituted Rha, the following permethylated sugars were identified: 1,2,3,5-tetra-O-acetyl-4-mono-O-methyl-rhamnitol, 1,2,5-tri-O-acetyl-3,4,6-tri-O-methyl-glucitol, 1,3,5-tri-O-acetyl-2,4,6-tri-O-methyl-glucitol, 1,4,5,6-tetra-O-acetyl-2,3-di-O-methyl-galactitol.

Taking all of the above data together, we can conclude that EPS-Lac is composed of four α-L-Rha*p*, two glucose residues (α- and β-D-Glc*p*), and one α-D-Gal*p* bearing pyruvate.

The sequence of the seven monosaccharide residues within the repeating unit of EPS-Lac was determined using 2-D ROESY and HMBC spectra. Inter-residue NOE correlations were found between: AH1and EH3, BH1 and FH2, CH1 and EH2, DH1 and BH3, EH1 and DH3, FH1 and GH3, and finally, between GH1 and AH3. The sequence deduced from the ROESY spectrum was confirmed by HMBC data showing cross-peaks characteristic of interactions between an anomeric proton and a carbon separated by glycosidic linkages (see Table 3). Therefore, the structure of the

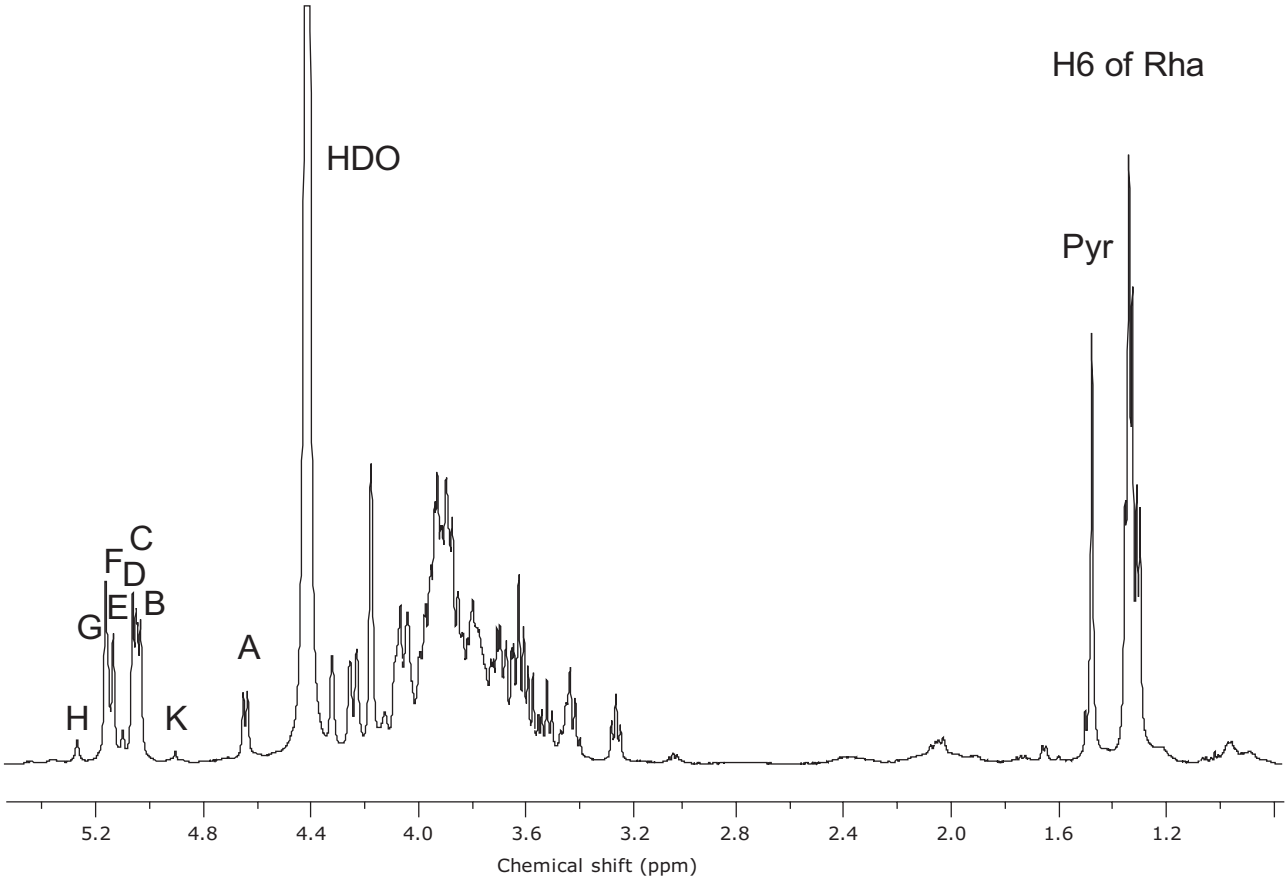
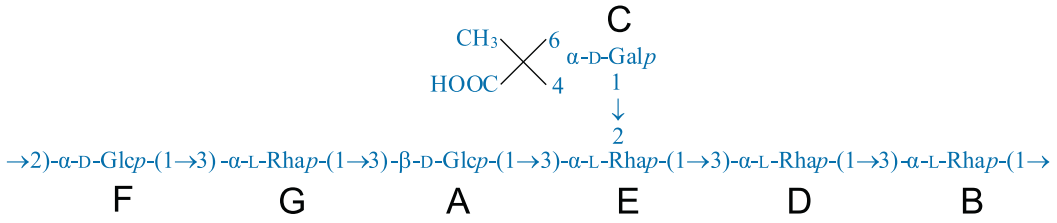


Fig. 2. ^{1}HNMR spectrum of the EPS-Lac preparation. Resonances from anomeric and methyl protons are marked with capital letters.

Table 3
ROESY and HMBC correlations to the anomeric protons in EPS-Lac.

Anomeric proton	ROESY		HMBC	
	Intraresidue	Interresidue	Intraresidue	Interresidue
AH1	**A**H3, **A**H5	**E**H3		**E**C3
BH1	**B**H2	**F**H2	**B**C3, **B**C5	**F**C2
CH1	**C**H2	**E**H2	**C**C3, **C**C5	**E**C2
CH6				**Pyr** C2
DH1	**D**H2	**B**H3	**D**C3, **D**C5	**B**C3
EH1	**E**H2	**D**H3	**E**C3, **E**C5	**D**C3
FH1	**F**H2	**G**H3	**F**C3, **F**C5	**G**C3
GH1	**G**H2	**A**H3	**G**C3, **G**C5	**A**C3

heptasaccharidic repeating unit of *Lb. rhamnosus* E/N was elucidated as follows:



Integration of methyl peaks on the 1DNMR spectrum confirmed this structure, indicating the presence of four methyls of rhamnose and one methyl with a chemical shift characteristic of pyruvate protons. Summarized integrals for anomeric protons gave a value of about 7, which allows us to assume that the analyzed material contained almost exclusively the polymer under study.

The structure of EPS-Lac synthesized by *Lb. rhamnosus* strain E/N growing on lactose (the only carbon source), as elucidated in this study, is identical with that of the polysaccharides produced by *Lb. rhamnosus* strains RW-9595M and R (van Caslteren, Pau-Roblot, Begin, & Roy, 2002). To emphasize this identity, we used the same letters as did van Calsteren et al., 2002to mark the same residues.

Extracellular material synthesized by *Lb. rhamnosus* E/N cultivated on glucose as the sole carbon source contained predominantly the EPS with the structure described above, with the exception that the total integrals of rhamnose methyl protons showed a value of 5.3 per one repeating units. In parallel, the ^{1}HNMR spectrum of EPS-Glc pointed to the presence of one pyruvate methyl per one repeating unit. It was concluded that the preparation contained some other polysaccharidic material composed of rhamnose. This preparation contained also β-D-Glc*p*, which was found in the spectrum at δ_H 4.718, glycosidically substituted at C-3 (data not shown). The residue represents the second spin system with the anomeric proton giving a doublet in the ^{1}HNMR spectrum (Fig. 1, spectrum EPS-Glc). Those two components, mentioned above, presumably are from second saccharide polymer. Thus one can assume that EPS-Glc is a mixture of polysaccharides and based on integrals, it was estimated that this preparation contained about 86% of EPS built of heptasaccharide repeating units and 14% of another EPS.

The most heterogenic material was synthesized by *Lb. rhamnosus* E/N cultivated on MRS broth supplemented with galactose. A comparison of the surface area of the β-D-Glc*p* anomeric signal at δ_H 4.655 with those of pyruvate and α-L-Rha*p* methyls showed that rhamnose as well as the pyruvate substituent (the ratio of H1s of β-D-Glc*p*/pyruvate methyl/α-L-Rha*p* methyls was 1/2.85/12.02) derived entirely from EPS built of heptasaccharide repeating units. From the other hand, a comparison of the surface area of the β-D-Glc*p* anomeric signal (δ_H 4.655) with those of other anomeric signals led to the conclusion that no more than a half of the preparation represented the EPS described above in this article. The remainder had a polysaccharidic character and was composed of at least five residues (see Fig. 3, red cross-peaks marked from **I** to **L**, and compare other preparations – Fig. 1). It was impossible to show the ratio among those signals using natural numbers, which means that they could represent at least one additional polysaccharide. A further, thorough analysis is needed to separate fractions from this material and determine their structures.

It seems that the polysaccharide composition of the polysaccharide material isolated from spent media and the size of the different polymers were not correlated. For instance, both EPS-Glc and EPS-Lac were composed exclusively of one type of polymer, but the former contained only a high-molecular-weight fraction while the latter was composed of both high- and low-molecular-weight fractions. On the other hand, the exopolysaccharides EPS-Mal and EPS-Gal, both containing at least two types of polysaccharides, had different molecular size distributions (Table 1 and Fig. 3).

3.3. Atomic force microscopy

Atomic force microscopy analysis revealed that EPSs produced by *Lb. rhamnosus* E/N on five different carbon sources varied in chain length. Loops were observed in EPS-Gal and EPS-Glc. EPS-Mal was the only polysaccharide with a branched chain. The cross-sectional heights (representing the thicknesses) of EPS-Suc, EPS-Lac, and EPS-Gal strands were in the range of 6.3–7.5 nm whereas the heights of EPS-Glc and EPS-Mal were 2.0–3.4 nm. The topographical AFM images of EPS-Mal and EPS-Suc are shown in Fig. 4.

3.4. Rheological properties

The viscosity function of the aqueous solutions of the EPSs produced by *Lb. rhamnosus* E/N in different carbon sources was determined. The results obtained at $T = 20\,^{\circ}C$ for the different EPS aqueous solutions are presented in Table 4. An increase in viscosity values and thixotropic area was a function of an increase in the molecular mass of the EPSs. The values of the pseudoplasticity index (n) were close to $n = 1$, which indicates non-Newtonian or pseudoplastic behavior. The flow curves of the solutions showed a non-Newtonian shear thinning flow. In Fig. 5, two groups of EPS samples can be seen: EPS-Lac, EPS-Suc, and EPS-Gal with a high shear stress value and EPS-Glc and EPS-Mal with a low shear stress value. In our study, we observed a positive correlation among the

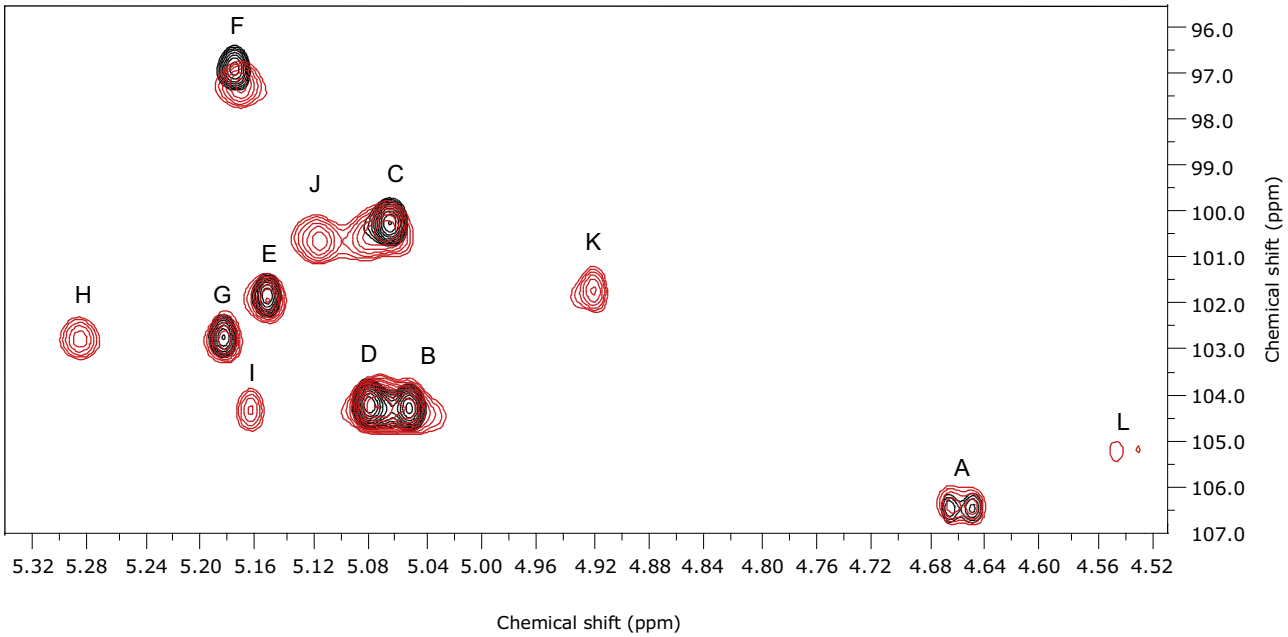


Fig. 3. Anomeric regions of HSQC spectra of the EPS-Lac (black cross-peaks) and EPS-Gal (red cross-peaks) preparations. Signals are marked with capital letters (see Table 2). Overlapping signals labeled A to G represent the repeating unit of the polysaccharide, common for all the preparations.

consistency index, viscosity and shear stress values of EPS suspensions.

4. Discussion

The literature provides descriptions of the primary structure of EPSs produced by several *Lb. rhamnosus* strains (Górska-Frączek et al., 2011; Landersjö, Yang, Huttunen, & Widmalm, 2002; Lipiński et al., 2003; van Calsteren et al., 2002; Vanhaverbeke et al., 1998). It has been reported that EPSs of *Lb. rhamnosus* most often contain glucose, galactose and rhamnose, but *N*-acetyl-glucosamine and *N*-acetyl-galactosamine have also been found. Sometimes, more than one polysaccharide can be secreted by a single strain (De Vuyst & Degeest, 1999; Lipiński et al., 2003). In some instances, the structure of EPS has been found to depend on the carbon source. Structural analyses of the EPS produced by *L. delbrueckii* subsp. *bulgaricus* NCFB 2772 grown in continuous culture have shown that it consisted of repeating units of glucose and galactose (in a 1:2.4 ratio) when grown on fructose; and of glucose, galactose, and rhamnose in a ratio of 1:7.0:0.8 when grown on a mixture of fructose and glucose (Grobben, Smith, Sikkema, & de Bont, 1996). This approach might not be applicable to all Lactobacilli strains. For example, the EPS composition of *Lactobacillus sake* strain 0–1 and *Lactobacillus fermentum* TDS030603 has been found to be independent of the type of carbon source used (Fukuda et al., 2010; Van Den Berg et al., 1995). In the present work, the structure of EPS synthesized by *Lb. rhamnosus* E/N irrespective of carbon sources in the growth medium was determined. This structure is fully described, including the number and type of residues, their absolute and anomeric configuration, linkage type, sequence, and substituent information. Presented herein chemical formula revealed that the EPSs are heteropolysaccharides composed of four rhamnose, two glucose, and one galactose residue with a pyruvate substituent. However, our study also demonstrates that the EPS materials synthesized by *Lb. rhamnosus* E/N cultivated on a medium with galactose, lactose, or sucrose contain heterogenic EPS fractions of high and low molecular weight, while those synthesized on a medium with maltose and glucose contain only a high-molecular-weight fraction. It was interesting to discover that the EPSs produced by *Lb. rhamnosus* E/N can be divided into four groups: (I) high-molecular-weight-fraction EPS composed of one type of polymer (EPS-Glc), (II) high-and-low-molecular-weight-fraction EPS composed of one type of polymer (EPS-Lac and EPS-Suc), (III) high-and-low-molecular-weight-fraction EPS composed of two types of polysaccharides (EPS-Gal), and (IV) high-molecular-weight-fraction EPS composed of two types of polysaccharides (EPS-Mal). Our results are in agreement with Fukuda et al. (2010), who also observed different molecular mass distributions but did not observe any differences in monosaccharide composition in EPS fractions produced by *Lb. fermentum* on different carbohydrate sources. It is still an open question what factors regulate EPS production and control polysaccharide chain length. One explanation could be the different pathways involved in sugar metabolism. The synthesis of heteropolysaccharides can be divided into three steps: assimilation of simple sugars and their conversion into nucleotide derivatives; assembly of pentasaccharide subunits attached to a lipid transporter, probably undecaprenyl phosphate or isoprenoid phosphate; and

Table 4
Values of the consistency index (K), flow index (n), thixotropic area (A_T), and viscosity of exopolysaccharides synthesized on different carbon sources.

Exopolysaccharide	K (mPa s)	n	A_T	Viscosity (mPa s)
EPS-Mal	6.982 ± 0.0001^{a}	0.92 ± 0.01^{a}	4.86 ± 0.35^{a}	5.1 ± 0.0002^{a}
EPS-Glc	8.952 ± 0.0002^{b}	0.93 ± 0.01^{ab}	4.99 ± 1.79^{a}	6.938 ± 0.0004^{b}
EPS-Gal	17.04 ± 0.0007^{c}	0.92 ± 0.01^{a}	11.87 ± 0.7^{b}	14.185 ± 0.0011^{c}
EPS-Lac	32.45 ± 0.0005^{e}	0.93 ± 0.01^{ab}	22.73 ± 0.78^{c}	24.935 ± 0.0001^{e}
EPS-Suc	17.44 ± 0.0003^{d}	0.98 ± 0.01^{b}	16.51 ± 0.73^{b}	16.37 ± 0.0002^{d}

In each column, different superscript letters denote a significant difference at $P < 0.05$.

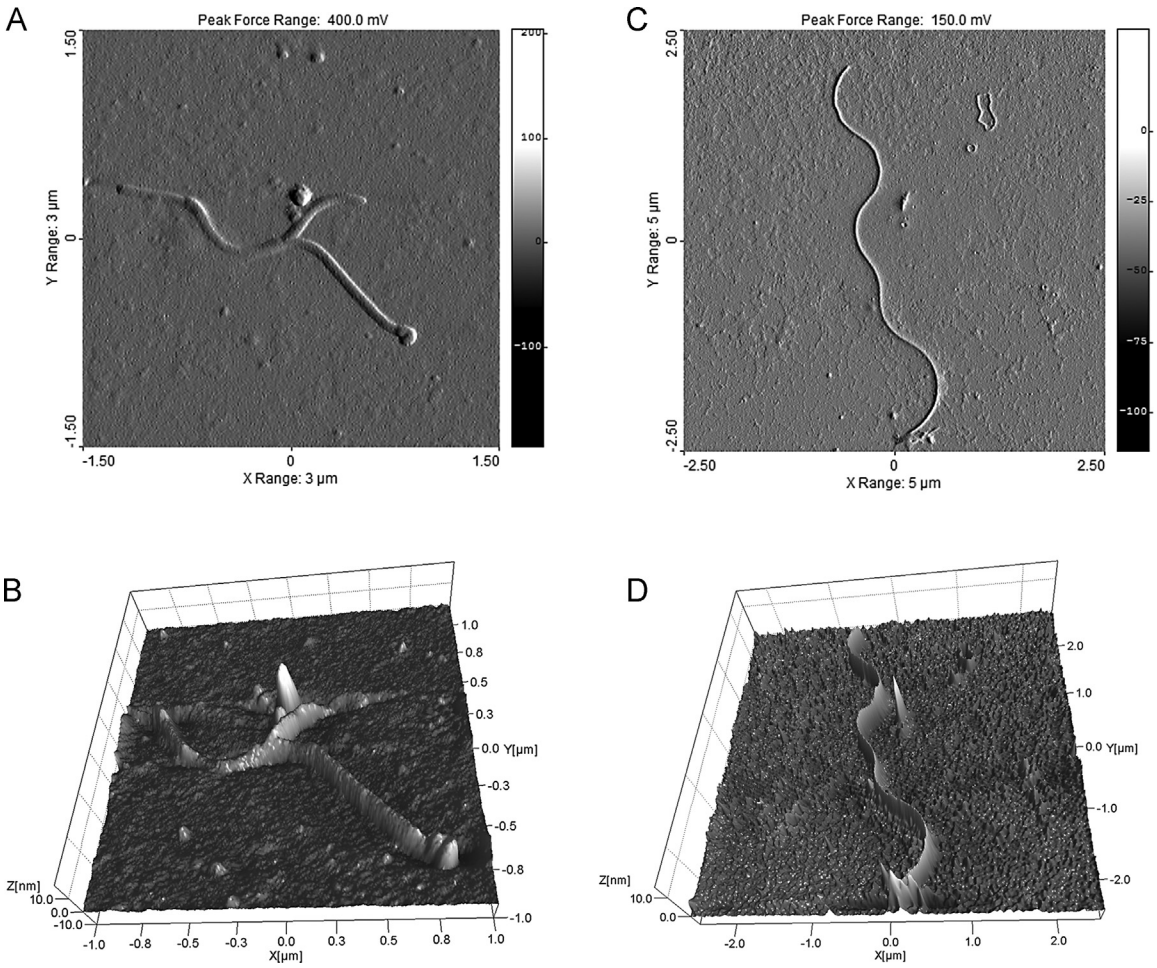


Fig. 4. Atomic Force Microscopy planar and cubic images of EPS-Mal (A and B) and EPS-Suc (C and D).

polymerization of the repeating units of the pentasaccharide and secretion of the polymer into the extracellular environment (Donot, Fontana, Baccou, & Schorr-Galindo, 2012). Sugar transport and its possible influence on EPS structure has been reviewed by De Vos (1996). It has been established that in Lactobacilli many monosaccharides and disaccharides are transported via the phosphoenolopyruvate-sugar phosphotransferase system (PEP-PTS). This mode of transport is energetically efficient since the sugars are translocated and phosphorylated in a single step at the expense of one ATP, which would otherwise be generated by PEP conversion into pyruvate catalyzed by pyruvate kinase (Barrangou et al., 2006; Boels, van Kranenburg, Hugenholtz, Kleerebezem, & de Vos, 2001). The lactose permease system has been reported to have the ability to import lactose in Lactobacilli (Barrangou et al., 2006). Depending on the mode of transport (PEP-PTS or the lactose permease system), lactose metabolism involves hydrolysis of lactose-6P by phospho-ß-galactosidase, yelding galactose-6P and glucose, or cleavage of the unmodified sugar by ß-galactosidase, generating glucose and galactose (Boels et al., 2001). Similarly, sucrose may enter the cell either in a phosphorylated state or after hydrolysis as fructose and glucose. Glucose is fermented via the glycolytic pathway or the phosphoketolase pathway. While galactose-6P is fermented by the tagatose-6P pathway, galactose is metabolized via the Leloir pathway. Grobben et al. (1996) suggested that the regulation of the biosynthetic pathway of EPS in *Lb. bulgaricus* may be dependent on the carbohydrate source. Heteropolysaccharide biosynthesis in Lactobacilli relies on the activity of specific enzymes and is regulated by specific genes, the organization of which is well-documented (Welman & Maddox, 2003; Péant et al., 2005). Genes encoding glycosyltransferases responsible for the structural organization of the heptasaccharide repeating unit are highly conserved and are co-transcribed with genes involved in the polymerization and export of the EPS. Two genes, *wzd* and *wze*, involved in chain-length determination are located in the 5′ region of the EPS cluster, and a third one, *wzb*, is located at the end of the cluster.

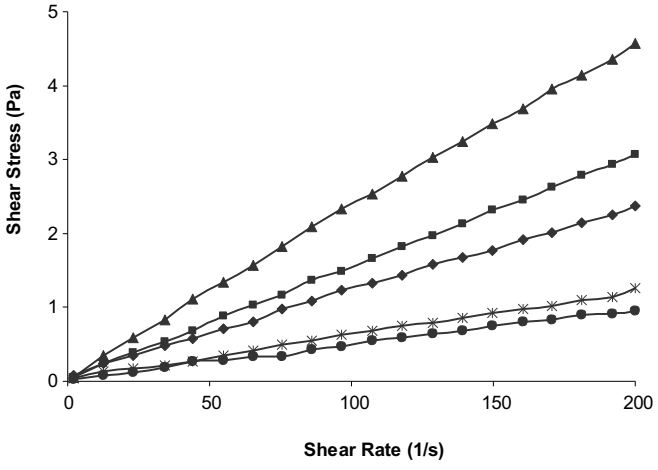


Fig. 5. Flow curves of exopolysaccharide aqueous solutions: EPS-Glc (×), EPS-Gal (♦), EPS-Mal (●), EPS-Suc (■), and EPS-Lac (▲).

Transcription of these genes may be driven by different promoters, indicating that a complex system is involved in the regulating of the expression of these genes in *Lb. rhamnosus*. Péant et al. (2005) have proposed a model of regulating EPS biosynthesis at the post-transcriptional level. The specific ATP-binding motifs, a tyrosine cluster and the residues involved in this model have been identified in *wze*. These motifs are usually found in protein kinases involved in EPS biosynthesis (Bender, Cartee, & Yother, 2003).

Rheological analysis revealed that the carbohydrates present in the growth medium had a significant effect on the viscosity of the EPS produced by *Lb. rhamnosus* E/N. EPS-Lac, EPS-Suc, and EPS-Gal, which contained high- and low-molecular-mass fractions showed a higher viscosity than EPS-Glc and EPS-Mal, in which only a high-molecular-mass fraction was observed. This indicates that heterogeneity of molecular mass distribution may cause variation in EPS viscosity. This observation is consistent with the findings of other authors, who have claimed that the viscosity of an EPS solution might be affected by the ratio of low-molecular-mass EPS (Pham, Dupont, Roy, Lapointe, & Cerning, 2000; Fukuda et al., 2010). The rheological properties of EPS are related to its molecular structure and its average molecular weight (M_w) (Laws, Leivers, Chacon-Romero, & Chadha, 2009). Due to its acidic nature, the viscosity of the EPS from *Lb. rhamnosus* E/N may also be affected by electrostatic interactions between charged residues. The AFM results presented in this study show that there is a correlation between the viscosity of an EPS solution and the thickness of the EPS chain rather than its length or branching. This is in line with Fukuda et al. (2010), who have observed that differences in EPS length did not cause variations in viscosity. Data on the viscosity of aqueous solutions of EPSs produced by *Lb. rhamnosus* E/N on different carbon sources can be used to select appropriate culture conditions for the production of EPS of the desired physicochemical properties. Viscosity data may help to explain differences in the textural properties of fermented milk products with different EPS-producing strains. Our results indicate that a single bacterial strain, depending on the carbon source in the medium, can produce EPSs which will variously influence the rheological properties of fermented products. Ruas-Madiedo, Hugenholtz, and Zoon (2002) have also stated that the production of a desired EPS could be achieved by controlling culture conditions.

5. Conclusion

EPSs isolated from *Lb. rhamnosus* E/N cultured on five carbon sources were investigated with regard to their structure and physicochemical and rheological properties. Our results indicate that the carbon source in the growth medium does not influence the primary chemical structure of the synthesized EPSs. Despite the same monomer composition, the examined EPSs showed different physicochemical and rheological properties. They had different molecular mass distributions and varied in chain length, branching, and thickness. A correlation was observed between the higher viscosity of the EPS solutions produced on lactose, sucrose, and galactose and their molecular mass ratio and polymer chain thickness. These results indicate that the choice of an appropriate carbon source allows one to obtain EPSs of particular physicochemical and rheological properties for particular food applications. Genetic and metabolic engineering tools for altering molecular weight and chain length can be employed to direct the synthesis of EPS so that desired properties are obtained.

References

Badel, S., Bernardi, T., & Michaud, P. (2011). New perspectives for *Lactobacilli* exopolysaccharides. *Biotechnology Advances*, *29*, 54–66.

Barrangou, R., Azcarate-Peril, M. A., Duong, T., Conners, S. B., Kelly, R. M., & Klaenhammer, T. R. (2006). Global analysis of carbohydrate utilization by *Lactobacillus acidophilus* using cDNA microarrays. *Proceedings of the National Academy of Sciences of the United States of America*, *103*(10), 3816–3821.

Bender, M. H., Cartee, R. T., & Yother, J. (2003). Positive correlation between tyrosine phosphorylation of CpsD and capsular polysaccharide production in *Streptococcus pneumoniae*. *Journal of Bacteriology*, *185*, 6057–6066.

Boels, I. C., van Kranenburg, R., Hugenholtz, J., Kleerebezem, M., & de Vos, W. M. (2001). Sugar catabolism and its impact on the biosynthesis and engineering of exopolysaccharide production in lactic acid bacteria. *International Dairy Journal*, *11*, 723–732.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, *72*, 248–254.

Cerning, J., Renard, C. M. G. C., Thibault, J. F., Bouillanne, C., Landon, M., Desmazeaud, M., et al. (1994). Carbon source requirements for exopolysaccharide production by *Lactobacillus casei* CG11 and partial structure analysis of the polymer. *Applied and Environmental Microbiology*, *60*, 3914–3919.

Chabot, S., Yu, H., De Léséleuc, L., Cloutier, D., Van Calsteren, M. R., Lessard, M., 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.

de Man, J. C., Rogosa, M., & Sharpe, E. (1960). A medium for the cultivation of the lactobacilli. *Journal of Applied Bacteriology*, *23*, 130–135.

De Vos, W. M. (1996). Metabolic engineering of sugar catabolism in lactic acid bacteria. *Antonie van Leeuvenhoek*, *70*, 223–242.

De Vuyst, L., & Degeest, B. (1999). Heteropolysaccharides from lactic acid bacteria. *Federation of European Microbiological Sciences Microbiology Reviews*, *23*, 153–177.

Donot, F., Fontana, A., Baccou, J. C., & Schorr-Galindo, S. (2012). Microbial exopolysaccharides: Main examples of synthesis, excretion, genetics and extraction. *Carbohydrate Polymers*, *87*, 951–962.

Duboc, P., & Mollet, B. (2001). Applications of exopolysaccharides in the dairy industry. *International Dairy Journal*, *11*, 759–768.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956). Colorimetric method for the determination of sugars and related substances. *Analytical Chemistry*, *28*, 350–356.

Fukuda, K., Shi, T., Nagami, K., Leo, F., Nakamura, T., Yasuda, K., et al. (2010). Effects of carbohydrate source on physicochemical properties of the exopolysaccharide produced by *Lactobacillus fermentum* TDS030603 in a chemically defined medium. *Carbohydrate Polymers*, *79*, 1040–1045.

Garegg, P. J., Jansson, P. E., Lindberg, B., Lindh, F., Lonngren, J., Kvarnstrom, I., et al. (1980). Configuration of the acetal carbon atom of pyruvic acid acetals in some bacterial polysaccharides. *Carbohydrate Research*, *78*, 127–132.

Górska-Frączek, S., Sandstrom, C., Kenne, L., Rybka, J., Strus, M., Heczko, P., et al. (2011). Structural studies of the exopolysaccharide consisting of a nonasaccharide repeating unit isolated from *Lactobacillus rhamnosus* KL37B. *Carbohydrate Research*, *346*(18), 2926–2932.

Grobben, G. J., Sikkema, J., Smith, M. R., & De Bont, J. A. M. (1995). Production of extracellular polysaccharides by *Lactobacillus delbrueckii* ssp. *bulgaricus* NCFB 2772 grown in a chemically defined medium. *Journal of Applied Bacteriology*, *79*, 103–107.

Grobben, G. J., Smith, M. R., Sikkema, J., & de Bont, J. A. M. (1996). Influence of fructose and glucose on the production of exopolysaccharides and the activities of enzymes involved in the sugar metabolism and the synthesis of sugar nucleotides in *Lactobacillus* delbrueckii subsp. bulgaricus NCFB 2772. *Applied Microbiology and Biotechnology*, *46*, 279–284.

Hugenholtz, J., & Smid, E. (2002). Nutraceutical production with food-grade microorganisms. *Current Opinion in Biotechnology*, *13*, 497–507.

Jansson, P.-E., Lindberg, J., & Widmalm, G. (1993). Syntheses and NMR studies of pyruvic acid4,6-acetals of some methyl hexopyranoses. *Acta Chemica Scandinavica*, *47*, 711–715.

Jolly, L., Vincent, S. J. F., Duboc, P., & Neeser, J. R. (2002). Exploiting exopolysaccharides from lactic acid bacteria. *Antonie Van Leeuwenholk*, *82*, 367–374.

Landersjö, C., Yang, Z., Huttunen, E., & Widmalm, G. (2002). Structural studies of the exopolysaccharide produced by *Lactobacillus rhamnosus* strain GG (ATCC 53103). *Biomacromolecules*, *3*, 880–884.

Laws, A. P., Leivers, S., Chacon-Romero, M., & Chadha, M. J. (2009). Variation in the molecular mass of exopolysaccharides during the time course of extended fermentations of skimmed milk by lactic acid bacteria. *International Dairy Journal*, *19*, 768–771.

Lipiński, T., Jones, C., Lemercinier, X., Korzeniowska-Kowal, A., Strus, M., Rybka, J., et al. (2003). Structural analysis of the *Lactobacillus rhamnosus* strain KL37C exopolysaccharides. *Carbohydrate Research*, *338*, 605–609.

Looijesteijn, P. J., Trapet, L., de Vries, E., Abee, T., & Hugenholtz, J. (2001). Physiological function of exopolysaccharides produced by *Lactococcus lactis*. *International Journal of Food Microbiology*, *64*, 71–80.

Vanhaverbeke, C., Bosso, C., Colin-Morel, P., Gey, C., Gamar-Nourani, L., Blondeau, K., et al. (1998). Structure of an extracellular polysaccharide produced by *Lactobacillus rhamnosus* strain C83. *Carbohydrate Research*, *314*, 211–220, acid.

Péant, B., LaPointe, G., Gilbert, C., Atlan, D., Ward, P., & Roy, D. (2005). Comparative analysis of the exopolysaccharides biosynthesis gene clusters from four strains of *Lactobacillus rhamnosus*. *Microbiology*, *151*, 1838–1851.

Pham, P. L., Dupont, I., Roy, D., LaPointe, G., & Cerning, J. (2000). Production of exopolysaccharide by *Lactobacillus rhamnosus* R analysis of its enzymatic degradation during prolonged fermentation. *Applied and Environmental Microbiology*, *66*, 2302–2310.

Polak-Berecka, M., Waśko, A., Szwajgier, D., & Choma, A. (2013). Bifidogenic and antioxidant activity of exopolysaccharides produced by *Lactobacillus rhamnosus* E/N on different carbon sources. *Polish Journal of Microbiology*, *62*(2), 181–189.

Que, N. L. S., Lin, S., Cotter, R. J., & Raetz, C. R. H. (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. *The Journal of Biological Chemistry*, *275*, 28006–28016.

Ruas-Madiedo, P., Hugenholtz, J., & Zoon, P. (2002). An overview on the functionality of exopolysaccharides produced by lactic acid bacteria. *International Dairy Science*, *12*, 163–171.

Sawardeker, J. S., Sloneker, J. H., & Jeans, A. R. (1965). Quantitative determinations of monosaccharides as their alditol acetates by gas liquid chromatography. *Analytical Chemistry*, *37*, 1602–1604.

Shashkov, A. S., Lipkind, G. M., Knirel, Y. A., & Kochetkov, N. K. (1988). Stereochemical factors determining the effects of glycosylation on the 13C chemical shifts in carbohydrates. *Magnetic Resonance in Chemistry*, *26*, 735–747.

van Calsteren, M. R., Pau-Roblot, C., Begin, A., & Roy, D. (2002). Structure determination of the exopolysaccharide produced by *Lactobacillus rhamnosus* strain RW-9595M and R. *Biochemical Journal*, *363*, 7–17.

Van Den Berg, D. J. C., Robijn, G. W., Janssen, A. C., Giuseppin, M. L. F., Vreeker, R., Kamerling, J. P., et al. (1995). Production of a novel extracellular polysaccharide by *Lactobacillus sake* 0-1 and characterization of the polysaccharide. *Applied and Environmental Microbiology*, *61*, 2840–2844.

Welman, A. D., & Maddox, I. S. (2003). Exopolysaccharides from lactic acid bacteria: Perspectives and challenges. *Trends in Biotechnology*, *21*(6), 269–274.

York, W. S., Darvill, A. G., McNeil, M., Stevenson, T. T., & Albersheim, P. (1985). Isolation and characterisation of plant cell walls and cell wall components. In A. Weissbach, & H. Weissbach (Eds.), *Methods in enzymology* (118) (pp. 3–40). Orlando: Academic Press, Inc.