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Comprehensive structural characterization of water-soluble and water-insoluble homoexopolysaccharides from seven lactic acid bacteria

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ABSTRACT

Several lactic acid bacteria are able to produce water-soluble and water-insoluble homoexopolysaccharides (HoEPS) from sucrose. In this study, structures of all HoEPS which were fermentatively produced by *Leuconostoc mesenteroides* subsp. *dextranicum* NRRL B-1121 and B-1144, *Leuconostoc mesenteroides* subsp. *mesenteroides* NRRL B-1149, B-1438 and B-1118, *Leuconostoc suionicum* DSM 20241, and *Liquorilactobacillus satsumensis* DSM 16230 were systematically analyzed. Monosaccharide analysis, methylation analysis, NMR spectroscopy, size-exclusion chromatography, and different enzymatic fingerprinting methods were used to obtain detailed structural information. All strains produced water-soluble dextrans and/or levans as well as water-insoluble glucans. Levans showed different degrees of branching and high molecular weights, whereas dextrans had comparable structures and broader size distributions. Fine structures of water-soluble HoEPS were analyzed after *endo*-dextranase and *endo*-levanase hydrolysis. Water-insoluble glucans were composed of different portions of 1,3-linkages (5 to 40 %). Hydrolysis with *endo*-dextranase and *endo*-mutanase yielded further information on block sizes and varying fine structures. Overall, clear differences between HoEPS yields and structures were observed.

1. Introduction

Several lactic acid bacteria produce exopolysaccharides (EPS) with different structural characteristics and physicochemical properties. These exopolysaccharides are of particular interest, because lactic acid bacteria are widely spread, considered safe, and used by humans to produce fermented foods. Apart from potential health promoting properties, exopolysaccharides have different potential applications as hydrocolloids and dietary fiber in foods (Monsan et al., 2001; Zannini, Waters, Coffey, & Arendt, 2016). Because their applicability depends on the chemical structure, elucidation of monosaccharide compositions, molecular weights and linkage types as well as fine structures of exopolysaccharides is important.

Although some lactic acid bacteria synthesize heteroexopolysaccharides (HeEPS), the formation of homoexopolysaccharides (HoEPS) from sucrose by extracellular glucan- und fructansucrases is most common. These enzymes belong to glycoside hydrolase (GH) families 70 and 68 and synthesize α-glucans and β-fructans, respectively (van Hijum, Kralj, Ozimek, Dijkhuizen, & van Geel-Schutten, 2006).

β-fructans can be classified into inulins, which are composed of β-1,2-linked fructofuranoses (Fru*f*) with ramifications at position *O*6, and levans, which are composed of β-2,6-linked Fru*f* with ramifications at position *O*2 (Monsan et al., 2001; van Hijum et al., 2006). Structure elucidation of selected bacterial fructans was performed in several studies (Bouallegue et al., 2020; Haddar et al., 2021; Han & Clarke, 1990; Hancock, Marshall, & Weigel, 1976; Pei, Ma, Chen, & Liu, 2020; Shi, Hou, Xu, Mørkeberg Krogh, & Tenkanen, 2019; Simms, Boyko, & Edwards, 1990).

Generally, bacterial α-glucans have a high structural diversity: They are composed of glucopyranoses (Glc*p*) which form a backbone with 1,3-, 1,4-, or 1,6-linkages. This backbone can be branched at different positions and to varying extents, whereas monomeric as well as di- and oligomeric side chains may occur (Ebisu, Misaki, Kato, & Kotani, 1974; Hare, Svensson, & Walker, 1978; Inoue, Yakushiji, Katsuki, Kudo, &

Abbreviations: HoEPS, homoexopolysaccharides; EPS, exopolysaccharides; TFA, trifluoroacetic acid; PMAA, partially methylated alditol acetate; HPAEC-PAD, high performance anion exchange chromatography with pulsed amperometric detection; RI, refractive index; GC, gas chromatography; FID, flame ionization detector; MS, mass spectrometry; ESI, electrospray ionization; HPSEC, high performance size exclusion chromatography; Fru*f*, fructofuranose; Glc*p*, glucopyranose; DMSO, dimethyl sulfoxide.

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Koga, 1988; Münkel et al., 2019; Münkel & Wefers, 2019; Wiater, Choma, & Szczodrak, 1999). Glucans with a 1,6-linked backbone are referred to as dextrans, whereas glucans which contain mainly 1,3-linked Glc*p* backbone units are often called mutans. The backbone structure significantly influences water solubility: while dextrans are water-soluble, a certain amount and distribution of α-1,3-linkages leads to water-insoluble glucans. Although the exact criteria are not yet fully understood, sections with consecutive 1,3-linkages seem to be an important aspect for water-insolubility (Côté, Ahlgren, & Smith, 1999; Côté & Leathers, 2009; Hare et al., 1978; Tsumuraya & Misaki, 1979; Wiater et al., 1999; Wiater, Pleszczyńska, Próchniak, & Szczodrak, 2012). A first comprehensive attempt to analyze and compare structures of water-soluble and water-insoluble glucans from multiple bacterial strains was performed by Jeanes et al. (1954). Later on, several comparative studies regarding the structure of different glucans were published (Jeanes, Haynes, & Wilham, 1955; Pearce, Walker, Slodki, & Schuerch, 1990; Slodki, England, Plattner, & Dick, 1986). However, these investigations were based on only one or two analytical methods, such as methylation analysis, appearance, viscosity, and the amount of main enzymatically released products. Nevertheless, details on the molecular structure were not obtained by using these methods. In particular, detailed structural characterization of water-insoluble glucans was only performed for a small number of selected strains and enzymes (Côté et al., 1999; Côté & Leathers, 2009; Côté & Skory, 2012; Ebisu et al., 1974; Funane et al., 2001; Hare et al., 1978; Inoue et al., 1988; Tsumuraya & Misaki, 1979; Wangpaiboon et al., 2020; Wiater et al., 1999).

Strains of the genera *Streptococcus*, *Lactobacillus*, and *Leuconostoc* produce water-insoluble α-glucans as well as water-soluble dextrans and/or fructans (Díaz-Montes, 2021; Hare et al., 1978; Korakli & Vogel, 2006; Kralj et al., 2004; Monsan et al., 2001; Morales-Arrieta, Rodríguez, Segovia, López-Munguía, & Olvera-Carranza, 2006; Münkel & Wefers, 2019; Zahnley & Smith, 1995). Thus, to understand the overall production of HoEPS by a single bacterial strain, investigations on glucan and fructan yields and structures are crucial. Birkhed, Rosell, and Granath (1979) for example investigated the water-soluble HoEPS from several *Streptococcus* strains. They analyzed monosaccharide compositions and glycosidic linkages by methylation analysis as well as molecular weight distributions by gel chromatography. They found both inulin-type and levan-type fructans as well as dextrans with different molecular weights. For HoEPS of *Lactobacillus* and *Leuconostoc* strains, comprehensive studies have not been conducted, yet. Therefore, the aim of this study was to extend our knowledge of the compositions and fine structures of water-soluble and water-insoluble HoEPS synthesized by different strains of *Lactobacillus* and *Leuconostoc*. Our hypothesis was that *Leuconostoc mesenteroides* subsp. *mesenteroides* and subsp. *dextranicum* as well as *Leuconostoc suionicum* and *Liquorilactobacillus satsumensis* produce different amounts of water-soluble and water-insoluble HoEPS which differ in their linkage compositions as well as their fine structures. We selected seven strains for which only limited information on HoEPS composition and structure has been published (reviewed in Table 1). For structural characterization, several analytical approaches, including enzymatic hydrolysis, mono- and oligosaccharide analysis, high performance size exclusion chromatography and methylation analysis were used. From the application and combination of these methods we obtained detailed and comprehensive information on the polysaccharide structures.

2. Experimental

2.1. Materials

The bacteria used in this study were obtained from two culture collections: Agricultural Research Service Culture Collection (NRRL), Peoria, IL, USA and German Collection of Microorganisms and Cell Cultures (DSMZ) GmbH, Braunschweig, Germany. If not stated otherwise, all chemicals used were of "p.a." grade or better and were purchased from Carl Roth (Karlsruhe, Germany), Merck (Darmstadt, Germany), Thermo Fisher Scientific (Waltham, MA USA), VWR (Darmstadt, Germany) and Grüssing GmbH (Filsum, Germany). *Endo*-dextranase (EC 3.2.1.11) from *Chaetomium* sp., 8000 U/mL was purchased from Megazyme (Bray, Ireland). *Endo*-levanase BT1760 (EC 3.2.1.65, accession number AAO76867.1) from *Bacteroides thetaiotaomicron* DSM 2079 (Mardo et al., 2017) and *endo*-mutanase SSAL4105 (EC 3.2.1.59, accession number AIY20934.1) from *Streptococcus salivarius* DSM 20560 were obtained by amplification of the corresponding

Table 1
Strains used in this study and literature data on their HoEPS. NRRL: Agricultural research service collection, DSM: German Collection of Microorganisms and Cell Cultures GmbH, Glc*p*: Glucopyranose, GH: Glycoside hydrolase.

Leuconostoc mesenteroides subsp. *dextranicum*		
NRRL B-1121	Glucans: 20–30 % 1,3-linked, water-insoluble, 50 % is solubilized by mutanase	(Jeanes et al., 1954; Pearce et al., 1990)
NRRL B-1144	Glucans: 5 % 1,3-linkages, water-soluble	(Jeanes et al., 1954)
Leuconostoc mesenteroides subsp. *mesenteroides*		
NRRL B-1149	Active glucan- and fructansucrase (identity of fructans unknown); water-insoluble glucans: 40 % 1,3-linkages, incompletely solubilized by mutanase (80 %), no 1,3,6-linked branched units, long sequences of 1,3-Glc*p*	(Jeanes et al., 1954; Pearce et al., 1990; Seymour & Knapp, 1980; Shukla et al., 2011; Shukla & Goyal, 2013; Slodki et al., 1986)
NRRL B-1438	Glucans: 10–20 % 1,3-linkages, water-insoluble & water-soluble, completely solubilized by mutanase	(Jeanes et al., 1954; Jeanes et al., 1955; Pearce et al., 1990)
NRRL B-1118	More water-insoluble than water-soluble glucans; water-insoluble glucans: 10–20 % 1,3-linkages, completely solubilized by mutanase; recombinant glucansucrase: water-insoluble glucans with equal amounts of 1,3 and 1,6-linkages, probably both in long sequences; recombinant levansucrase: levans (molecular weight: 10^6 Da)	(Côté & Skory, 2012, 2016; Côté, Skory, Unser, & Rich, 2013; Jeanes et al., 1954; Olvera, Centeno-Leija, & López-Munguía, 2007; Pearce et al., 1990; Slodki et al., 1986)
Leuconostoc suionicum		
DSM 20241	Active dextransucrase (products unknown)	(Chun, Lee, Jeon, Kim, & Jeon, 2017; Nolte, Kempa, Schlockermann, Hochgürtel, & Schörken, 2019)
Liquorilactobacillus satsumensis		
DSM 16230	Produces water-insoluble polysaccharide from sucrose; Encodes for GH70, GH13 enzymes, but no GH68 enzyme	(Côté et al., 2013; Sun et al., 2015)

genes by PCR (genomic DNA was purchased from DSMZ), molecular cloning of the genes, and heterologous expression of the recombinant proteins in *E. coli*. A detailed description of the protocol is given by Münkel et al. (2019). Only minor modifications were applied in this study. The annealed gene-vector product of the *endo*-levanase encoding gene was transformed to 5α-competent *E. coli* cells (High efficiency, NEB, USA), cell lysis was performed using a sonifier (SFX250, Branson Ultrasonics Corporation USA), and proteins were purified with HisPur Ni-NTA resin (Thermo Fisher Scientific).

2.2. Exopolysaccharide synthesis and isolation

Lactic acid bacteria were cultured in MRS medium (10 g/L soy peptone, 10 g/L meat extract, 5 g/L yeast extract, 1 g/L Tween80, 2 g/L dipotassium phosphate, 5 g/L sodium acetate, 2 g/L diammonium citrate, 0.2 g/L magnesium sulfate, 0.05 g/L manganese sulfate, pH 6.2) as described by Münkel et al. (2019). An overnight preculture was grown statically at 30 °C in MRS4 medium (MRS supplemented with 5 g/L glucose, 5 g/L fructose and 10 g/L maltose). HoEPS production was achieved by inoculating MRSSuc medium (MRS supplemented with 80 g/L sucrose) with 10 mL of the preculture in a 1 L Schott flask, filling up to the lip with MRSSuc, sealing, and incubating for 30 h at 30 °C and shaking at 70 rpm.

For HoEPS isolation, cultures were first centrifuged for 10 min at 3328 rcf. The resulting residues (containing water-insoluble HoEPS and cells) were washed with ultrapure water until the supernatants were colorless and clear. To precipitate water-soluble HoEPS from the combined supernatants two volumes of ethanol (96 % v/v) were added, the mixture was cooled to 4 °C and incubated overnight. Precipitated water-soluble HoEPS were collected by centrifugation as described above, redissolved in 1 M NaOH, and centrifuged to remove the water-insoluble residue. The supernatant was neutralized with HCl, dialyzed against water for 48 h (molecular weight cut off: 14 kDa) and lyophilized to obtain the water-soluble HoEPS fractions.

HoEPS in the water-insoluble residue containing cells obtained from the first centrifugation step were dissolved in 1 M NaOH and cell debris were removed by centrifugation (30 min, 3328 rcf). The supernatants were neutralized with HCl and precipitated as described above. Precipitates were recovered by centrifugation, dialyzed against water for 48 h (molecular weight cut off: 14 kDa), and lyophilized to obtain the water-insoluble HoEPS fractions. Yields of water-soluble and water-insoluble fractions were quantified by weighing the dried HoEPS.

2.3. Monosaccharide composition analysis

The monosaccharide composition of the isolated HoEPS was determined after two different trifluoroacetic acid (TFA) hydrolyses. In the case of water-soluble HoEPS, an aliquot (containing 20 µg polysaccharide) of an aqueous solution was dried prior to TFA hydrolysis. For water-insoluble HoEPS about 1 mg of the dried sample was directly hydrolyzed. The samples were hydrolyzed with 500 µL of 2 M TFA for 60 min at 121 °C for glucose determination and with 500 µL of 1 M TFA for 30 min at 70 °C for fructose determination. In both cases, TFA was removed by evaporation and subsequent co-evaporation with ethanol. The dried samples were redissolved in ultrapure water and analyzed by high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) on an ICS-3000 system (Thermo Fisher Scientific, Sunnyvale, CA, USA). Separation was achieved by using a CarboPac PA20 column (150 mm × 3 mm i.d., 6.5 µm particle size, Thermo Fisher Scientific) and quantification was performed by using an external calibration. The column temperature was 30 °C and the detector temperature was 25 °C. The following gradient with a flow rate of 0.4 mL/min was used: column equilibration with 10 mM NaOH for 15 min prior to every run; 0–20 min: Isocratic 10 mM NaOH; 20–30 min: Linear gradient from 10 mM NaOH to 200 mM NaOH; 30–40 min: Isocratic 200 mM NaOH + 200 mM sodium acetate; 40–50 min: Isocratic 200 mM NaOH.

2.4. Enzymatic hydrolyses and oligosaccharide analysis

2.4.1. Endo-dextranase hydrolysis

Small amounts (1 mg) of water-soluble HoEPS were hydrolyzed, whereas water-insoluble HoEPS were hydrolyzed on a larger scale (200 mg). This was necessary because the residue obtained from the hydrolysis of water-insoluble glucans was further investigated (see below). For the hydrolysis a 1 mg/mL sample solution/dispersion was incubated with *endo*-dextranase (5 U/mg sample) for 24 h at 40 °C and shaken at 400 rpm (small scale) or 150 rpm (large scale). The enzyme was inactivated by heating to 95 °C for 15 min. Samples were centrifuged and the supernatant was analyzed by HPAEC-PAD as described in Section 2.4.4. The water-insoluble residue after hydrolysis of the water-insoluble HoEPS was washed with ultrapure water, an aliquot was hydrolyzed with *endo*-mutanase (Section 2.4.2), and the remaining water-insoluble residue was lyophilized and weighed for semiquantification.

2.4.2. Endo-mutanase hydrolysis of water-insoluble products after endo-dextranase hydrolysis of water-insoluble HoEPS

About 1 mg of wet, water-insoluble residue from *endo*-dextranase treatment was suspended in 100 µL of ultrapure water and incubated with 60 µg of *endo*-mutanase (obtained from heterologous expression) for 24 h at 37 °C and 200 rpm. The enzyme was inactivated by the addition of two volumes of ethanol. Ethanol was evaporated for 2 h at 45 °C under vacuum and the sample was resuspended in 100 µL of ultrapure water. After centrifugation the supernatant was analyzed by HPAEC-PAD/mass spectrometry (MS) as described in Section 2.4.4.

2.4.3. Endo-levanase hydrolysis of water-soluble HoEPS

300 µL of a 3 mg/mL aqueous solution of water-soluble HoEPS was incubated with 40 µg of *endo*-levanase (obtained from heterologous expression) for 24 h at 40 °C and 400 rpm. Inactivation was achieved by heating to 95 °C for 15 min and protein was removed by centrifugation. The supernatant was analyzed by HPAEC-PAD/MS (Section 2.4.4).

2.4.4. Analysis of enzymatically liberated oligosaccharides

Enzymatically hydrolyzed samples were analyzed by HPAEC-PAD on an ICS-6000 system (Thermo Fisher Scientific) equipped with a CarboPac PA200 column (250 × 3 mm i.d., 5.5 µm particle size, Thermo Fisher Scientific). Temperatures and flow rate are described in Section 2.3. The following gradient composed of four eluents (A: ultrapure water, B: 10 mM NaOH, C: 200 mM NaOH, D: 200 mM NaOH + 500 mM sodium acetate) was used for separation: column equilibration with 100 % B for 20 min prior to every run; 0–10 min: Isocratic 100 % B; 10–20 min: Linear gradient to 50 % C + 50 % A; 20–45 min: Linear gradient to 50 % A, 30 % C + 20 % D; 45–55 min: Linear gradient to 50 % A + 50 % D; 55–65 min: Linear gradient to 100 % D; 65–80 min: Isocratic 100 % D; 80–95 min: Isocratic 100 % C.

Oligosaccharides from dextranase hydrolysis were identified via comparison of their retention times to the oligosaccharides described by Münkel et al. (2019). To identify unknown oligosaccharides released by mutanase and levanase, HPAEC-PAD was coupled to an LTQ-XL linear ion trap mass spectrometer (Thermo Fisher Scientific). A post column split allowed simultaneous PAD and MS analysis. Prior to MS analysis, desalting of the eluent was achieved by an AERS 500e suppressor (4 mm, Thermo Fisher Scientific). To facilitate ionization by electrospray ionization (ESI), 500 µM LiCl was added at a flow rate of 0.05 mL/min (AXP-MS pump, Thermo Fisher Scientific). The source temperature was 300 °C and lithium adducts of oligosaccharides were detected in positive mode.

2.5. Methylation analysis

Methylation analysis of water-soluble and water-insoluble fractions as well as water-insoluble residues after *endo*-dextranase hydrolysis of

water-insoluble glucans was carried out as described in detail by Ernst, Werner, and Wefers (2023). In brief, all samples were methylated by methyl iodide in dimethyl sulfoxide (DMSO)/NaOH(s). An ultrasonic treatment was used to dissolve/swell the sample in DMSO and a second ultrasonication was carried out after addition of NaOH. Methylation was repeated once and different hydrolysis procedures were used for the HoEPS fractions. Water-insoluble samples were hydrolyzed with 2 M TFA for 60 min at 121 °C and water-soluble HoEPS were methylated and hydrolyzed in two batches: One batch was hydrolyzed with 1 M TFA for 30 min at 70 °C to analyze partially methylated alditol acetates (PMAAs) derived from fructans. The second batch was hydrolyzed with 3 M TFA for 90 min at 121 °C to obtain information on glucans. Partially methylated monosaccharides were reduced by using $NaBD_4$ and acetylated by using acetic anhydride and 1-methylimidazole. PMAAs were separated by gas chromatography (GC), identified by their mass spectra and quantified by flame ionization detection (FID). The molar response factors described by Sweet, Shapiro, and Albersheim (1975) were used.

2.6. High performance size exclusion chromatography with refractive index detection (HPSEC-RI)

HPSEC-RI analysis of water-insoluble HoEPS and the water-insoluble residues, which resulted from *endo*-dextranase hydrolysis, was conducted on an HPLC-RI system (P6.1L, AS6.1L, RID 2.1L, Knauer, Germany) equipped with a GRAM column (300 × 8 mm, particle size 10 µm) (PSS, Germany). DMSO with 100 mM LiCl was used as eluent at a flow rate of 0.2 mL/min. Column temperature was 80 °C and RI temperature was 35 °C. Samples were solubilized as follows: 1 mg of lyophilized water-insoluble glucans and water-insoluble residues were swollen in 500 µL of DMSO by shaking overnight at 60 °C and 1500 rpm. Subsequently, 2 mg of LiCl(s) were added (resulting concentration: 100 mM LiCl) and the samples were shaken overnight (60 °C, 1500 rpm). A second LiCl(s) addition (2 mg) was conducted, the samples were again shaken overnight at 60 °C and 1500 rpm and finally 500 µL of DMSO was added to obtain a concentration of 100 mM LiCl. This procedure allowed for the dissolution of all water-insoluble glucans. The molecular weight of the glucan samples was estimated by using standard dextrans with known molecular weight (dextran blue, 670 kDa, 270 kDa and 12 kDa, Sigma Aldrich, Germany; 64.3 kDa, PSS, Germany).

For HPSEC-RI analysis of water-soluble HoEPS, an Agilent 1100 Series HPLC equipped with a 1260 Infinity II refractive index detector and a TSKgel G5000 PW_{XL} column (300 × 7.8 mm, particle size 10 µm; TOSOH, Japan) was used. Water-soluble HoEPS were dissolved in ultrapure water (final concentration: 1 mg/mL) and filtered (syringe filter 0,45 µm, Nylon). Ultrapure water supplemented with 0.5 g/L NaN_3 as preservative was used as eluent at a flow rate of 0.4 mL/min at 40 °C. RI detector temperature was 35 °C.

2.7. ^1H NMR spectroscopy

Water-soluble HoEPS were analyzed by ^1H NMR spectroscopy on a 400 MHz VNMRS or a 500 MHz DD2 spectrometer (Agilent, Santa Clara, CA, USA). Freeze-dried samples were solubilized in D_2O (10–20 mg/mL) and acetone was added as reference (referenced to 2.22 ppm according to Gottlieb, Kotlyar, and Nudelman (1997)).

3. Results and discussion

Seven lactic acid bacteria strains were selected for the production of water-soluble and water-insoluble HoEPS (Table 1). HoEPS synthesis was performed under identical conditions and absolute yields of water-soluble and water-insoluble HoEPS fractions varied from 0.2 to 15.5 g/L culture (Fig. 1). Total HoEPS yields varied from 5 to 22 g/L, which was equivalent to 13–55 % of the maximum yield of 40 g/L (calculated from the amount of available sucrose in the cultures). Jeanes et al. (1954 and 1955) described yields of up to 10 % of the available sucrose for strains

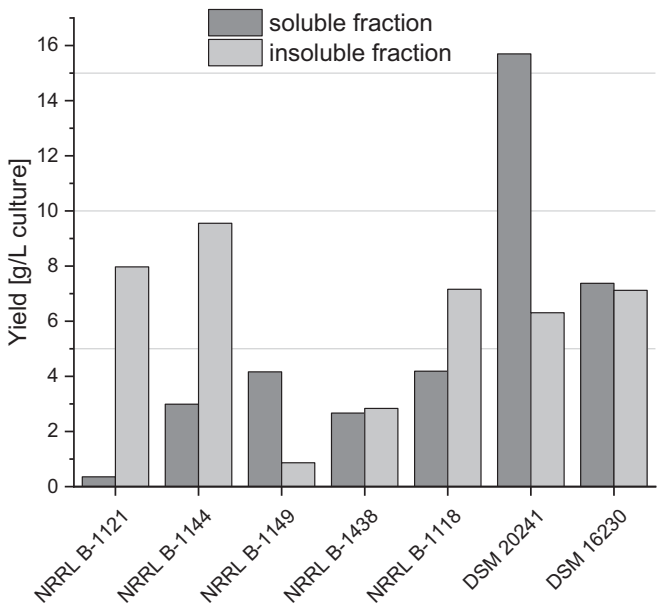

Fig. 1. Absolute yields of isolated water-soluble and water-insoluble HoEPS fractions obtained from 1 L of culture of the seven lactic acid bacteria strains used in this study (Table 1). Yields were determined gravimetrically.

NRRL B-1121, B-1144, B-1149, B-1438, and B-1118, whereas other strains used up to 30 % of the available sucrose for dextran synthesis. However, it must also be considered that HoEPS yields may vary considerably when the incubation conditions are modified. Nevertheless, high yields are a basic requirement for applications as hydrocolloids or dietary fiber. In this regard, strains NRRL B-1144, B-1118, DSM 20241, and DSM 16230 which in total used at least 25 % of the available sucrose for HoEPS production are promising candidates for HoEPS yield optimization.

Notably, varying relative yields of water-soluble and water-insoluble HoEPS were obtained for individual strains. NRRL B-1149 and DSM 20241 synthesized mainly water-soluble HoEPS. NRRL B-1438 and DSM 16230 produced equal amounts of water-soluble and water-insoluble HoEPS, whereas higher portions of water-insoluble HoEPS were obtained from NRRL B-1121, B-1144, and B-1118. For NRRL B-1118, Côté et al. (2013) also described the production of more water-insoluble than water-soluble glucans and for NRRL B-1438, Jeanes et al. (1955) found more water-insoluble than water-soluble dextrans. As described above, differences may occur due to different culture conditions. For the other strains used in this study, relative yields have not been described previously. However, water-soluble HoEPS production was known for NRRL B-1144, and water-insoluble HoEPS production has been described for NRRL B-1121, B-1149, B-1438, B-1118 and DSM 16230 (see Table 1). In this study, all strains produced water-soluble and water-insoluble HoEPS, and thus several HoEPS fractions have not been considered yet. Therefore, the water-soluble as well as the water-insoluble HoEPS were analyzed separately for their monosaccharide composition, linkage types, enzymatically released oligosaccharides, and molecular sizes.

3.1. Water-soluble HoEPS

3.1.1. Monosaccharide composition of water-soluble HoEPS

To obtain basic information on the identity of the water-soluble HoEPS, the monosaccharide compositions were analyzed by HPAEC-PAD after TFA hydrolysis. The results are shown in Fig. 2. All HoEPS were composed of glucose and/or fructose which demonstrated that water-soluble glucans and/or fructans were present. All detected monosaccharides were released from polysaccharides because no free

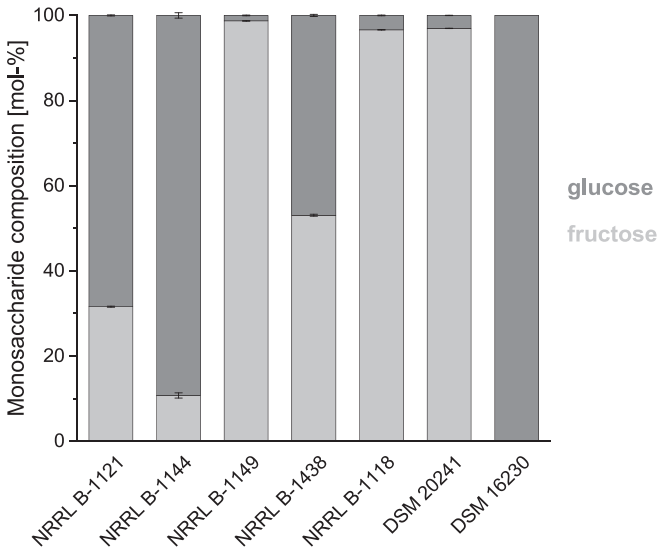

Fig. 2. Monosaccharide composition of water-soluble HoEPS obtained from the seven lactic acid bacteria strains used in this study (Table 1). Monosaccharides were analyzed by HPAEC-PAD after two TFA hydrolyses (glucose: 2 M TFA, 121 °C, 60 min; fructose: 1 M TFA, 70 °C, 30 min). All analyses were performed in duplicate.

monosaccharides or oligosaccharides were detected when the untreated HoEPS were analyzed by HPAEC-PAD. Galactose and mannose were only detected in trace amounts which demonstrates that media components were efficiently removed.

From the portions of the corresponding monosaccharides, it was concluded that glucans, fructans, and mixtures of both polysaccharides were present: Water-soluble HoEPS from strains NRRL B-1149, B-1118, and DSM 20241 consisted mainly of fructans, whereas HoEPS of DSM 16230 were glucans. Strains NRRL B-1121, B-1144 and B-1438 synthesized both, water-soluble glucans and fructans.

The expression of levansucrases is known for NRRL B-1149, and B-1118 (Table 1), but synthesis of fructans by strains NRRL B-1121, B-1144, B-1438, and DSM 20241 was not expected. According to the literature in Table 1, all seven strains were expected to synthesize water-soluble or water-insoluble glucans. While all strains indeed formed water-insoluble glucans (Section 3.2), water-soluble glucans were solely described for NRRL B-1144, B-1438, and B-1118. In particular, DSM 16230 produced high amounts of water-soluble glucans, whereas DSM 20241 produced high amounts of fructans (Figs. 1 and 2).

3.1.2. *Methylation analysis of water-soluble HoEPS*

Methylation analysis was carried out to obtain information on the glycosidic linkages of water-soluble HoEPS. Different conditions were used to analyze the individual HoEPS (see Section 2.5), because glucans, fructans, and mixtures of both polysaccharides were obtained. In a previous study, we showed that the applied hydrolysis conditions are suitable to determine the linkage composition of each HoEPS individually and in a mixture of both (Ernst et al., 2023). PMAAs derived from fructans were detected in all samples except for the water-soluble fraction of DSM 16230, which was in good agreement with the results from monosaccharide analysis (see Section 3.1.1). All fructans were composed of 2,6-linked, 1,2,6-linked, and terminal Fru*f* units which demonstrated that levan-type fructans were present. However, the portions of the individual PMAAs varied considerably. To describe the overall structure of the levans, the degree of branching was calculated from the ratio of branched backbone units (1,2,6-Fru*f*) and total backbone units (2,6-Fru*f* + 1,2,6-Fru*f*). The degree of branching varied between 7 % and 24 % which demonstrated considerable structural differences between levans (Fig. 3A).

The results for water-soluble glucans produced by NRRL B-1121, B-1144, B-1438, and DSM 16230 showed that all glucans were composed of comparable portions of 1,6-linked Glc*p* units and 1,3,6-linked Glc*p* units (Fig. 3B). Furthermore, small portions (about 5 %) of 1,3-linked Glc*p* units were detected. Thus, all water-soluble glucans were dextrans with side chains at position *O*3 and small portions of 1,3-linked units. ^{1}H NMR spectra (Fig. S1) also confirmed the presence of dextrans and levans in water-soluble HoEPS fractions. The linkage type composition of dextrans is comparable to that of many other dextrans from *Leuconostoc* and *Lactobacillus* (Côté et al., 2013; Jeanes et al., 1954; Monsan et al., 2001; Pearce et al., 1990; Slodki et al., 1986), wheares levans possessing high portions of branch points up to 24 % are rather rare (Bouallegue et al., 2020; Haddar et al., 2021; Han & Clarke, 1990;

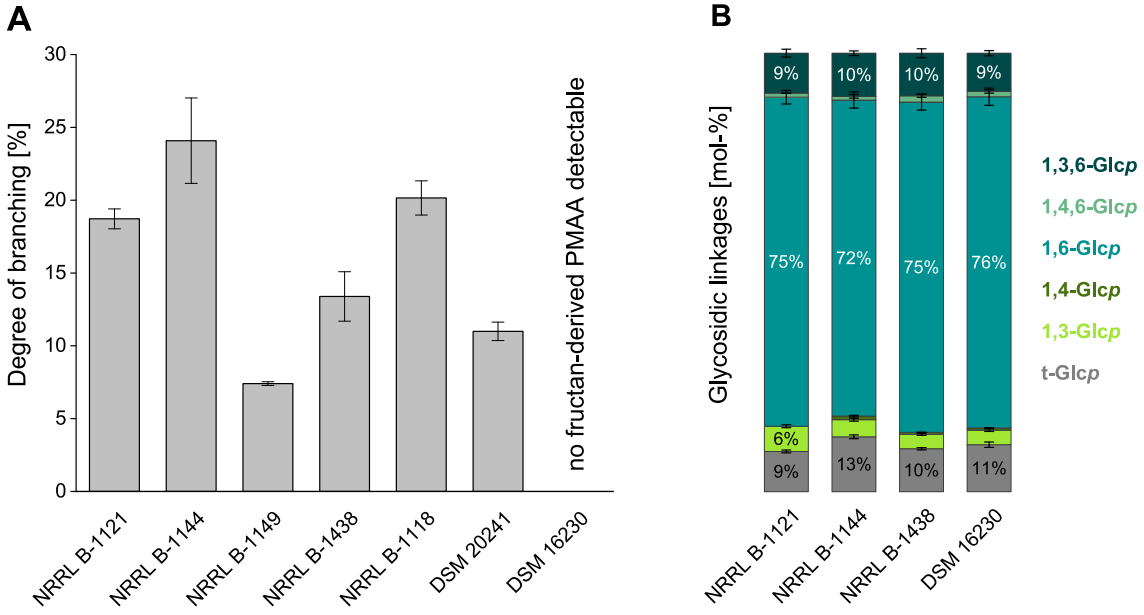

Fig. 3. Glycosidic linkages (determined by methylation analysis) of water-soluble HoEPS from seven lactic acid bacteria strains (see Table 1). A: Degree of branching of levans (calculated by dividing branched units (1,2,6-Fru*f*) by all backbone units (1,2,6-Fru*f* and 2,6-Fru*f*)). B: Portions of glucose glycosidic linkages. t: terminal, Glc*p*: glucopyranose, numbers indicate the position of substitution in the monosaccharide unit. All analyses were performed in duplicate.

Hancock et al., 1976; Simms et al., 1990). Thus, investigation of their physiochemical properties could improve the understanding of the effect of side chains in levans.

3.1.3. Endo-dextranase and endo-levanase hydrolysis of water-soluble HoEPS

To obtain information on the dextran and levan fine structures, HoEPS were hydrolyzed with *endo*-dextranase and *endo*-levanase, respectively. Released oligosaccharides were analyzed by HPAEC-PAD and identified by reference standard oligosaccharides in the case of gluco-oligosaccharides (Münkel et al., 2019; Münkel & Wefers, 2019) or by mass spectrometry in case of fructo-oligosaccharides. *Endo*-dextranase cleaves α-1,6-linked Glc*p* units which leads to the liberation of isomaltose and branched isomalto-oligosaccharides from dextrans (Münkel & Wefers, 2019; Pittrof, Kaufhold, Fischer, & Wefers, 2021). Isomaltose and *O*3-branched isomalto-oligosaccharides were liberated from all glucan containing water-soluble HoEPS (Fig. S2). This was in good agreement with the results from methylation analysis. However, the liberated oligosaccharides also demonstrated that monomeric, dimeric as well as oligomeric side chains were present in all glucans. The oligosaccharide pattern obtained was comparable to the ones obtained from other dextrans from *Leuconostoc* or *Lactobacillus* (Münkel et al., 2019; Münkel & Wefers, 2019).

The HPAEC-PAD chromatograms of *endo*-levanase hydrolysates of water-soluble HoEPS are shown in Fig. 4. The chromatograms of the HoEPS hydrolysates from NRRL B-1149, B-1438, B-1118, and DSM 20241 contained several peaks, whereas the chromatograms of NRRL B-1121 and B-1144 had low peak intensities and no peaks were detected for DSM 16230. Thus, significant amounts of oligosaccharides were only liberated from HoEPS fractions which contained high portions of levans. Among the liberated fructo-oligosaccharides, two disaccharides as well as two tri-, tetra-, penta- and hexasaccharides were identified by using MS. In addition, several oligosaccharides with higher molecular weights were detected between 50 and 65 min. Different elution times indicated that oligosaccharides with the same degree of polymerization had varying structures. Therefore, linear 2,6-linked oligosaccharides as well as fructo-oligosaccharides resulting from ramifications were present.

3.1.4. HPSEC-RI analysis of water-soluble HoEPS

For comparison of molecular weights, water-soluble HoEPS were analyzed by HPSEC-RI (Fig. 5). HoEPS from DSM 16230, NRRL B-1144, and B-1121 showed a rather broad RI elution pattern and thus a broad size distribution. However, the early eluting peak (~6 mL) in the elugram of dextrans from DSM 16230 indicated that these dextrans contained a fraction with a very high molecular weight. HoEPS from NRRL

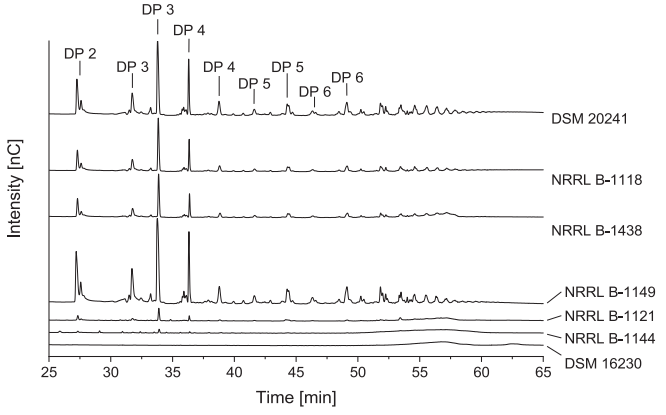

Fig. 4. HPAEC-PAD chromatograms of *endo*-levanase hydrolysates of water-soluble HoEPS from seven lactic acid bacteria strains (Table 1). Degree of polymerization (DP) of the individual oligosaccharides was determined by mass spectrometry and is given above the peaks.

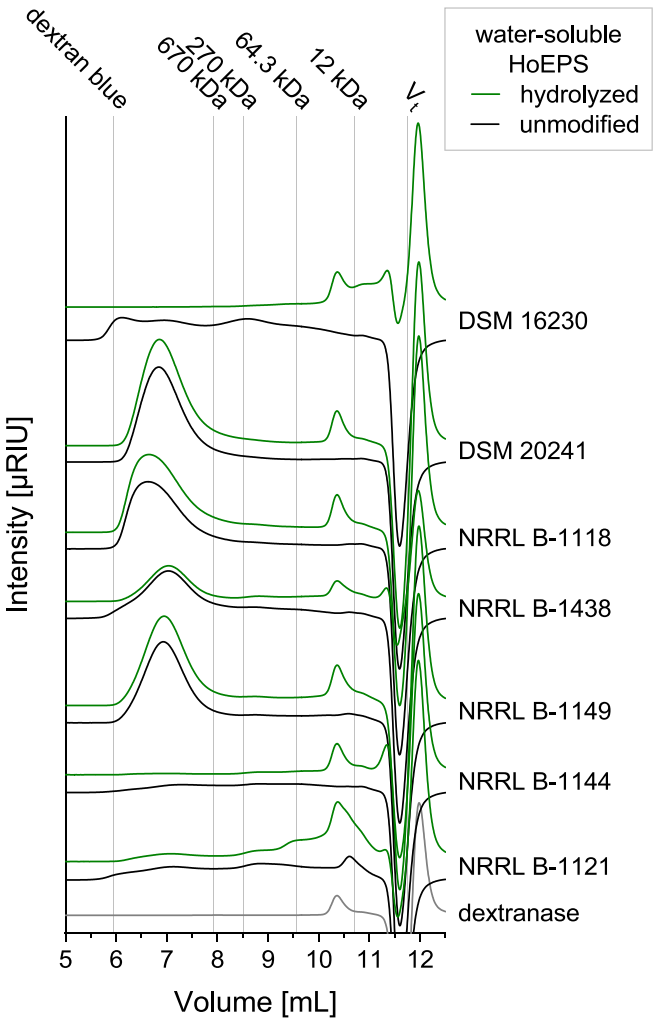

Fig. 5. HPSEC-RI elugrams of water-soluble HoEPS (black) obtained from seven lactic acid bacteria strains (Table 1) and their *endo*-dextranase hydrolysates (green). Peak maxima of size standards with known molecular masses are indicated by reference lines (V_t was determined by using glucose) and the elugram of *endo*-dextranase is given for comparison.

B-1149, B-1438, B-1118, and DSM 20241 showed an intense peak at elution volumes between 6 and 8 mL. Thus, these HoEPS were mainly composed of a high molecular weight fraction which is larger than 670 kDa. Notably, all of these HoEPS contained at least 50 % levans (see Section 3.1.1), whereas the HoEPS with a broader molecular weight distribution contained at least 50 % dextrans.

Endo-dextranase was used to identify which polysaccharide fractions can be assigned to dextrans. HoEPS from NRRL B-1121, B-1144 and DSM 16230 were hydrolyzed to a significant extent which led to the disappearance of early-eluting, high molecular weight material and to the formation of low molecular weight compounds. The elution profiles of HoEPS from NRRL B-1149, B-1118 and DSM 20241 after *endo*-dextranase hydrolysis were unchanged compared to untreated HoEPS, whereas a slight reduction in size was observed for NRRL B-1438. Minor variations in the peak intensities between the unmodified and hydrolyzed samples are most likely caused by slight variations in polysaccharide solubility before and after enzymatic hydrolysis. Altogether, the dextrans in the samples had a broad molecular weight distribution, and their size was reduced by *endo*-dextranase hydrolysis. Furthermore, the molecular sizes of levans from NRRL B-1149, B-1118 and DSM 20241 were in the range from 670 kDa to 2 million Da (dextran blue).

The estimated molecular weights of the levans were in accordance with the molecular weights of other microbial levans (Srikanth, Reddy,

Siddartha, Ramaiah, & Uppuluri, 2015), whereas most fermentatively synthesized dextrans of *Leuconostoc* and *Lactobacillus* strains showed higher molecular weights than the dextrans in this study (Díaz-Montes, 2021).

It is worth noting that in the characterized mixtures of levans and dextrans, levans showed higher molecular weights. Comparisons of fermentatively synthesized dextrans and levans molecular sizes have sparsely been found in literature. For *Lactobacillus reuteri* HoEPS, van Geel-Schutten et al. (1999) found α-glucans which showed a higher molecular weight than the levans produced by this strain. In contrast, levans from water kefir showed a higher molecular weight than dextrans (Fels, Jakob, Vogel, & Wefers, 2018).

3.1.5. Overall structure of water-soluble HoEPS

Water-soluble HoEPS synthesized by the seven lactic acid bacteria strains were obtained in yields ranging from 0.2 to 15.5 g/L culture. They consisted of dextrans and levans in different portions. Dextran structures were similar and all dextrans were hydrolyzed with *endo*-dextranase to isomaltose and isomalto-oligosaccharides with monomeric, dimeric as well as oligomeric side chains at position *O*3. Therefore, all dextrans had a comparable structure which was also comparable to other *O*3-branched dextrans. However, dextrans showed a broad size distribution ranging from 12 kDa to about 2 million Da (dextran blue) or more, whereas most dextrans in the literature show higher molecular weights. Apart from the molecular weight, the degree of branching and side chain length of dextrans are known to influence their physicochemical characteristics (Nachtigall, Hassler, Wefers, Rohm, & Jaros, 2023; Tirtaatmadja, Dunstan, & Boger, 2001). Despite the comparable degree of branching of the obtained dextrans, their broad size distribution may result in varying viscosities particularly when compared to dextrans with more narrow size distributions.

Levans showed different degrees of branching (7 % to 24 %) and *endo*-levanase hydrolysis released different, presumably linear and branched oligosaccharides from levans. This indicated a complex structural architecture. The impact of degrees and distributions of branching on the physicochemical properties of levans is unknown and should be subject of further investigations. Furthermore, levans had rather high molecular weights and narrow size distributions in the range of 670 kDa to 2 million Da (dextran blue).

3.2. Water-insoluble HoEPS

Only glucose was detected by monosaccharide analysis after TFA hydrolysis of the seven water-insoluble HoEPS, thus all water-insoluble HoEPS were glucans.

3.2.1. Methylation analysis of water-insoluble glucans

Water-insoluble glucans were analyzed by methylation analysis to obtain information on their glycosidic linkages. Results are shown in Fig. 6 and revealed that water-insoluble glucans were composed of 1,6-, 1,3- and 1,3,6-linked Glc*p* units (besides terminal units). Clear variations were observed for portions of 1,6-linkages (36 to 68 %) and 1,3-linkages (5 to 40 %). Strains NRRL B-1149 and DSM 20241 synthesized water-insoluble glucans with approximately equal portions of 1,6-linked and 1,3-linked Glc*p* units. For glucans of NRRL B-1121, B-1438, and B-1118 about 60 % 1,6-linkages and 20 % 1,3-linkages were determined. However, while the mentioned glucans all showed comparably high portions of 1,3-linkages, glucans from NRRL B-1144 and DSM 16230 were mainly 1,6-linked with 1,3-linkages accounting for less than 10 %. The portions of 1,3,6-linked Glc*p* units varied between 3 % and 12 % which demonstrated that all of the analyzed glucans are branched, but to a rather low extent. Notably, the portions of terminal glucans were higher than it would be expected from the portions of 1,3,6-linked Glc*p* units. However, this is most likely caused by residual sucrose or low molecular weight compounds which are embedded within the water-insoluble glucans and solubilized during methylation analysis (Münkel et al., 2019). Glycosidic linkages of water-insoluble glucans of NRRL B-1121, B-1149, B-1438 were in accordance with the literature (Jeanes et al., 1954; Jeanes et al., 1955; Pearce et al., 1990; Slodki et al., 1986). For strain NRRL B-1118 only the glucans synthesized by DsrI glucansucrase were described by Côté and Skory (2012) and for strains NRRL B-1144, DSM 20241, and 16230 water-insoluble glucans have not been described yet. Altogether, all water-insoluble glucans of this study were complex copolymers and contained significant portions of 1,6-linkages.

Therefore, *endo*-dextranase hydrolysis was applied to obtain more information about the different structural elements. Released isomalto-oligosaccharides in the supernatants were analyzed by HPAEC-PAD as described in Section 3.1.3 and results are shown in Fig. S2. Isomaltose and several branched isomalto-oligosaccharides were released by *endo*-dextranase. This clearly demonstrated that the 1,6-linked dextran blocks of the water-insoluble glucans were branched. Notably, comparable portions of the same compounds were detected in the water-soluble glucans. Therefore, the dextran blocks within the water-insoluble glucans contained a comparable structural architecture. However, water-insoluble glucans were incompletely hydrolyzed with *endo*-dextranase. The proportions of water-insoluble residues ranged from about 45–85 % and water-insoluble residues were additionally analyzed by methylation analysis (Fig. 6). Water-insoluble residues after *endo*-dextranase hydrolysis were mainly composed of 1,3-Glc*p* units with less than 10 % of 1,6-linkages. Thus, *endo*-dextranase efficiently hydrolyzed 1,6-linkages

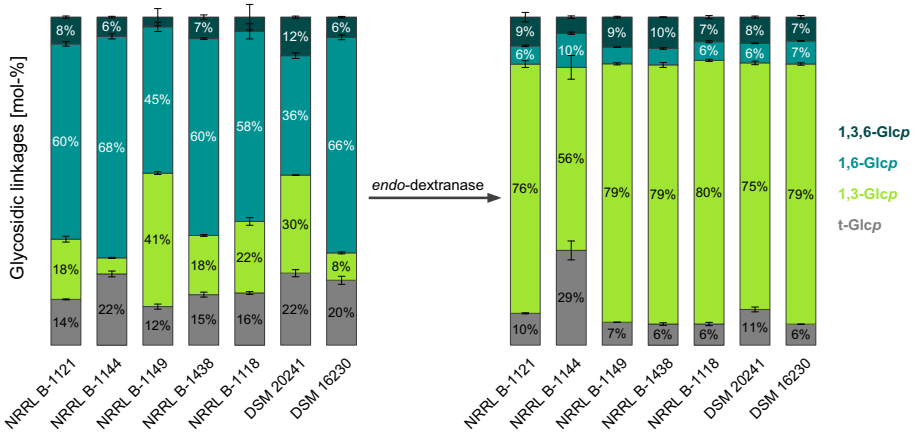

Fig. 6. Glycosidic linkages of water-insoluble glucans (left) obtained from seven lactic acid bacteria strains (Table 1) and their water-insoluble residues after *endo*-dextranase hydrolysis (right). Glycosidic linkages were determined by methylation analysis and all analyses were performed in duplicate. t: terminal, Glc*p*: glucopyranose, numbers indicate the position of substitution in the monosaccharide unit.

and the residue was mostly composed of the 1,3-linked blocks which render insolubility in water. Notably, the glucan residue of NRRL B-1144 showed an unusually high portion of terminal units. Although the exact portion was most likely overestimated due to methodological limitations (a tetrasaccharide would not be insoluble), these results indicated that the obtained 1,3-linked structural elements may have a lower molecular weight. All samples contained significant portions of 1,3,6-linked Glc*p* units which suggested that ramifications were also present in the 1,3-linked areas. Seymour and Knapp (1980) supposed a structure with segments of 1,3-linked Glc*p* units for water-insoluble glucans of NRRL B-1149, which was confirmed by the findings in this study.

3.2.2. *Endo*-mutanase hydrolysis

To obtain further information on the fine structures of the water-insoluble, mainly 1,3-linked residues from *endo*-dextranase hydrolysis, they were hydrolyzed with *endo*-mutanase. The enzymatically liberated oligosaccharides were analyzed by HPAEC-PAD/MS and the chromatograms are shown in Fig. 7. *Endo*-mutanase liberated oligosaccharides from all seven water-insoluble glucan residues, although they were not hydrolyzed completely. This confirmed that water-insoluble glucans contain α-1,3-linked Glc*p* units. In addition to linear nigero-oligosaccharides ($[\alpha\text{-}1,3\text{-}D\text{-}Glcp]_n$), different unknown oligosaccharides were released which were presumably branched: By using mass spectrometric detection we were able to identify two pentasaccharides. Different portions of these compounds were present in all hydrolysates, but the highest portions were released from glucan of NRRL B-1149. Therefore, the 1,3-linked areas could also show a varying structural architecture. The release of linear nigero-oligosaccharides by *endo*-mutanase was described for glucans from *Streptococcus* bacteria (Pleszczyńska, Boguszewska, Tchórzewski, Wiater, & Szczodrak, 2012; Tsumori, Shimamura, Sakurai, & Yamakami, 2011).

3.2.3. HPSEC-RI analysis of water-insoluble glucans

HPSEC-RI analysis was performed to analyze the molecular weight distribution of water-insoluble glucans. To enable HPSEC analysis, water-insoluble glucans were dissolved in DMSO with 100 mM LiCl and this solvent was also used as eluent. To gain information about the size of the 1,3-linked areas, the water-insoluble residues after *endo*-dextranase hydrolysis were also analyzed. Fig. 8 shows the elugrams of the water-insoluble glucans as well as the elugrams of the water-insoluble residues after *endo*-dextranase hydrolysis. All water-insoluble glucans eluted at comparable elution volumes than dextran blue (2 million Da) which indicated comparably high molecular weights. However, elugrams of water-insoluble glucans showed different size distributions. Notably, the early eluting peaks disappeared after *endo*-dextranase hydrolysis and the water-insoluble residues eluted between the standard compounds with 270 kDa and 12 kDa (peak maximum at about 50–60 kDa). Some differences between the individual glucans could be observed: The residue of glucans from DSM 16230 showed a peak maximum at about 64.3 kDa, and the residue of glucans from NRRL B-1144 showed a broader and flat size distribution with slightly smaller sizes than the other residues. This was in good agreement with the detection of higher portions of terminal Glc*p* units in the water-insoluble residue of NRRL B-1144 glucans (Section 3.2.1). Furthermore, these results confirmed that terminal units were overestimated for this sample. Notably, glucans from DSM 16230 and NRRL B-1144 contained high portions of 1,6-linked Glc*p* units and a different linkage distribution could be the reason for the different elution profiles. However, the overall results of this analysis suggested that all 1,3-linked areas were rather small and continuously interrupted by dextran structural elements. A block-wise structure was previously suggested for several water-insoluble glucans (Côté et al., 1999; Tsumuraya & Misaki, 1979; Wangpaiboon et al., 2020).

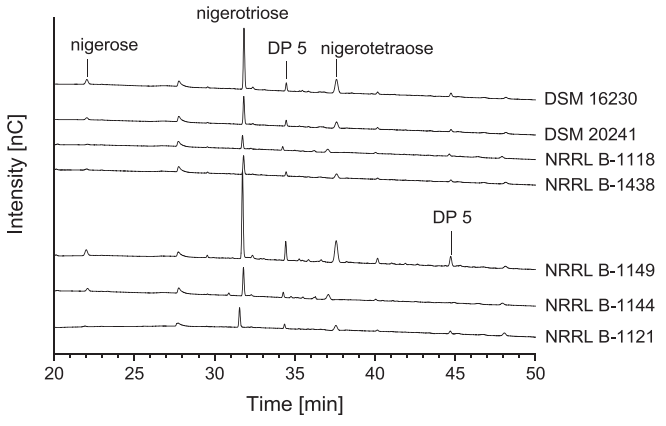

Fig. 7. HPAEC-PAD chromatograms of oligosaccharides liberated by *endo*-mutanase from water-insoluble residues after *endo*-dextranase hydrolysis of water-insoluble glucans from seven lactic acid bacteria strains (Table 1). Nigero-oligosaccharides were identified by external standard oligosaccharides and the degree of polymerization (DP) of some unknown oligosaccharides was determined by mass spectrometry. Slight retention time shifts in the area of 30–40 min were caused by different impurities as confirmed by standard compounds.

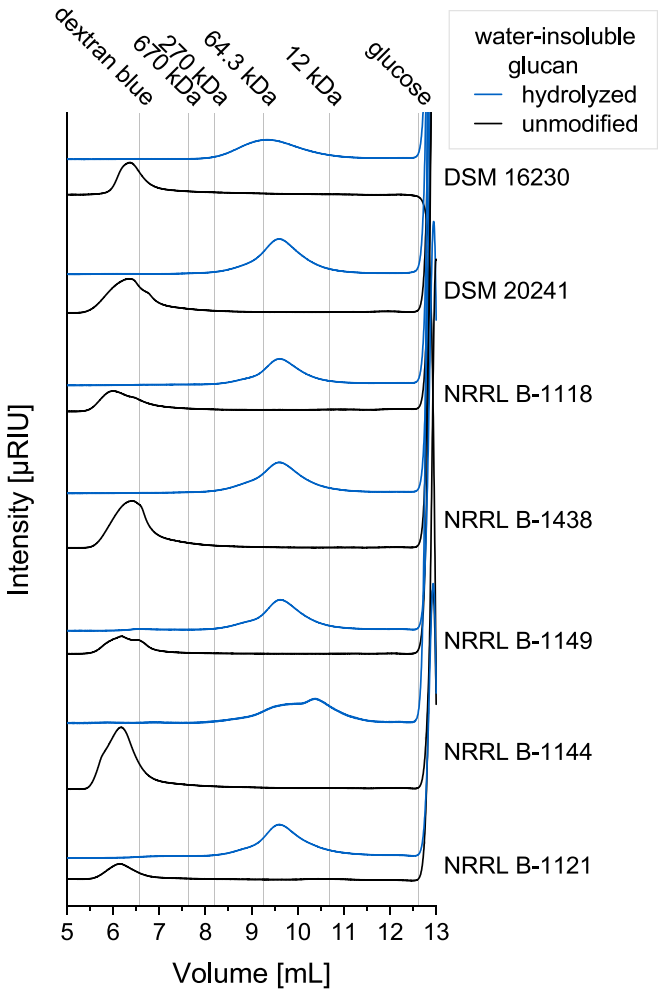

Fig. 8. HPSEC-RI elugrams of water-insoluble glucans (black) obtained from seven lactic acid bacteria strains (Table 1) and their water-insoluble residues after *endo*-dextranase hydrolysis (blue). Peak maxima of size standards with known molecular masses are indicated by reference lines. The elugram of the hydrolyzed glucans from NRRL B-1144 is enhanced by factor 3.5.

3.2.4. Overall structure of water-insoluble HoEPS

Water-insoluble HoEPS were glucans with different portions of 1,3-, 1,6-, and 1,3,6-linkages. Portions of 1,3-linked Glc*p* units ranged from 5 to 40 %. The products of *endo*-dextranase hydrolysis demonstrated that all glucans contained dextran blocks with mono-, di-, and oligomeric side chains. Methylation analysis, high performance size exclusion chromatography, and *endo*-mutanase hydrolysis of the water-insoluble products after *endo*-dextranase hydrolysis showed that water-insoluble glucans contained α-1,3-linked blocks of varying sizes and structural composition. Most likely, 1,3,6-linked units were also present in the 1,3-linked areas. Water-insoluble glucans had a high molecular weight in the range of dextran blue (about 2 million Da). Altogether, water-insoluble glucans contained 1,3-linked blocks which were interrupted by 1,6-linked blocks. For applications of water-insoluble α-glucans, the effect of these structural elements on their physicochemical properties remains to be elucidated.

4. Conclusion

The systematic and comparative characterization of the water-soluble and water-insoluble HoEPS of seven lactic acid bacteria yielded detailed information on the HoEPS production of the respective strains and on the fine structure of the individual polysaccharides. All bacteria used produced significant amounts of water-soluble HoEPS. Pure dextrans or levans as well as mixtures of both polysaccharides were identified and differences in the size distribution were observed: Levans showed a rather high molecular weight, whereas dextrans had a broad size distribution. Furthermore, differences in the structural variation between water-soluble HoEPS were observed: Dextrans showed similar linkage type portions and degrees of branching, whereas levans differed in their degree of branching which ranged from 7 to 24 %. By using *endo*-dextranase and *endo*-levanase hydrolysis, various oligosaccharides were released from all dextrans and levans. The elution patterns suggested that the different HoEPS had comparable fine structures. Furthermore, all seven strains produced water-insoluble glucans showing different portions of 1,3-linkages (5–40 %) and of 1,6-linkages (36–68 %). In addition to water-soluble, linear and branched isomalto-oligosaccharides, e*ndo*-dextranase hydrolysis released water-insoluble 1,3-linked polysaccharide blocks, which showed varying molecular weights. Furthermore, it is likely that the 1,3-linked blocks contain ramifications. Altogether, our results broaden the knowledge on α-glucan and levan structures and allow selection of appropriate HoEPS for further research and/or application.

CRediT authorship contribution statement

Luise Ernst: Conceptualization, Investigation, Methodology, Formal analysis, Validation, Visualization, Writing – original draft. **Hanna Offermann:** Investigation, Methodology, Formal analysis. **Annemarie Werner:** Investigation. **Daniel Wefers:** Conceptualization, Resources, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.carbpol.2023.121417.

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