Characterization of 4,6-α-glucanotransferase enzymes and their functional role in Lactobacillus reuteri

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Chapter 4

Lactobacillus reuteri strains convert starch and maltodextrins into homo-exopolysaccharides using a cell-associated 4,6-α-glucanotransferase enzyme

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Abstract

Exopolysaccharides (EPS) of lactic acid bacteria (LAB) are of interest for food applications. LAB use sucrose as substrate for α-glucan synthesis by extracellular glucansucrase enzymes. Various *Lactobacillus reuteri* strains also possess 4,6-α-glucanotransferase (4,6-α-GTase) enzymes. Purified 4,6-α-GTases were shown to act on starches (hydrolysates), cleaving α1→4 linkages and synthesizing α1→6 linkages, yielding isomalto-/malto- polysaccharides (IMMP). Here we report that also *L. reuteri* cells with these extracellular, cell-associated 4,6-α-GTases synthesize EPS (α-glucan) from starches (hydrolysate). NMR, SEC, and enzymatic hydrolysis of EPS synthesized by *L. reuteri* 121 cells showed that these have similar linkage specificities but generally are much bigger in size than IMMP produced by 4,6-α-GTase GtfB enzyme. The various IMMP-like EPS are efficiently used as growth substrate by probiotic *Bifidobacterium* strains possessing amylopullulanase activity. These IMMP-like EPS thus have potential prebiotic activity and may contribute to the application of probiotic *L. reuteri* strains grown on maltodextrins or starches as synbiotics.

Introduction

Lactic acid bacteria (LAB) are a group of Gram-positive, non-sporulating bacteria, of which *Lactobacillus* and *Bifidobacterium* strains are regarded as probiotics. Some LAB are "Generally Recognized As Safe" (GRAS) strains and have been used in fermented foods such as cheese and yoghurt since ancient time. LAB produce lactic acid, diacetyl/acetoin, and carbon dioxide that contribute to the texture, flavor and shelf life of fermented foods (1-3). Some LAB also produce exopolysaccharides (EPS) which find application in the improvement of food texture. They also may have interesting health effects for humans, e.g. antitumor activity and immune stimulation (4,5).

Depending on their composition and mechanism of biosynthesis, EPS produced by LAB can be classified into two groups: hetero-EPS and homo-EPS (6). Hetero-EPS consist of more than one carbohydrate moieties, and generally are synthesized intracellularly from activated sugars (7). LAB homo-EPS consist of a single type of monosaccharide, generally D-glucose or D-fructose, and can be divided into α-glucans (e.g. dextran, mutan, alternan and reuteran) and β-fructans (e.g. inulin and levan) (8-10). Most LAB homo-EPS known are produced from sucrose by extracellular glucansucrase and fructansucrase enzymes (6, 11, 12). *Lactobacillus reuteri* 121 uses the GtfA glucansucrase to convert sucrose into an α-glucan (reuteran) with both α1→4 and α1→6 linkages, mostly alternating (9, 13-15). Glucansucrases are glycoside hydrolase enzymes of family GH70 (www.CAZy.org).

Compared to sucrose, starch is much more abundant and is a cheaper carbon source for LAB industrial cultivation and product formation (16). In the last 30 years, a number of amylolytic LAB strains have been isolated and their starch-acting enzymes investigated (17, 18). Most of these are starch-hydrolyzing enzymes and only few enzymes with starch transglycosylation activity have been characterized (19-24). Recently, various 4,6-α-glucanotransferase (4,6-α-GTase) enzymes have been characterized from *L. reuteri* strains (25, 26). These transglycosylating enzymes constitute a GH70 subfamily. These 4,6-α-GTases produce isomalto-/malto- polysaccharides (IMMP), which is a new type of soluble dietary fiber, using starch or starch hydrolysates as substrate rather than sucrose (27, 28). The *L. reuteri* 121 strain GtfB enzyme has been purified and its

IMMP products have been characterized in detail (27, 28). The *gtfA* and *gtfB* genes in *L. reuteri* 121 are located next to each other in the genome (26, 29).

In this study, 4 *Lactobacillus reuteri* strains possessing 4,6-α-GTase genes are shown to produce homo-EPS from maltodextrins *in vivo*. The model strain *L. reuteri* 121 with the *gtfB* gene was found to produce homo-EPS from starch or starch hydrolysates. The EPS products of *L. reuteri* 121 from different types of starch were further investigated with respect to structures and sizes, and compared to the IMMP produced by the GtfB enzyme *in vitro*. Finally, the *L. reuteri* 121 EPS derived from different maltodextrins as well as from sucrose (reuteran) were tested as carbon source for growth of various probiotic *Bifidobacterium* strains in order to evaluate their potential prebiotic activity.

Materials and Methods

Growth conditions

Lactobacillus reuteri strains, including *L. reuteri* 121 (LMG 18388), *L. reuteri* DSM20016, *L. reuteri* TMW1.106 (kindly provided by Prof. Rudi F. Vogel in Technische Universität München) (30), *L. reuteri* ML1 (LMG 20347), *L. reuteri* 180 (LMG 18389), and *L. reuteri* ATCC5573 were subcultured from stocks stored at -80 °C in 10 ml of sugar-free MRS medium (liquid culture or agar plate) supplemented with 1% glucose. The fresh cultures were inoculated anaerobically in modified sugar-free MRS medium with different carbon sources with or without 1% glucose (w/v). One liter medium contained 10 g bactopeptone, 4 g yeast extract, 5 g sodium acetate, 2 g tri-ammonium citrate, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.05 g $MnSO_4 \cdot 7H_2O$; and 1 ml Tween 80, supplemented with 5 g starch or 50 g maltodextrins. For agar medium, 15 g/l agar was added. The native starches, maltodextrins and amylose V were provided by AVEBE (Veendam, The Netherlands) (**Table 1**).

The *Bifidobacterium* strains *B. breve* DSM20091, *B. adolescentis* DSM 20083 and *B. dentium* DSM20436 were subcultured from stocks stored at -80 °C in 5 ml of *Bifidobacterium* medium (BM) supplemented with 1% glucose (31). One liter BM contained 10 g trypticase peptone, 2.5 g yeast extract, 3 g tryptose, 3 g K_2HPO_4, 3 g KH_2PO_4, 2 g triammonium citrate, 0.3 g pyruvic acid, 1 ml Tween

80, 0.574 g $MgSO_4 \cdot 7H_2O$, 0.12 g $MnSO_4 \cdot 7H_2O$ in 1 l distilled water), 5 g NaCl. After autoclaving BM was supplemented with 0.05% (w/v) filter-sterilized cysteine-HCl, and strains were grown at 37 °C under anaerobic conditions maintained by GasPak EZ anaerobe container system (BD, New Jersey, US). Aliquots (1%) from overnight cultures were inoculated into 5 ml of BM supplemented with different EPS (0.5%, w/v). BM without an added carbon source and BM with 0.5% (w/v) glucose were used as negative and positive controls, respectively.

Extraction of exopolysaccharides (EPS)

EPS were produced by *L. reuteri* strains in the sugar-free MRS medium supplemented with various maltodextrins (5.0%, w/v) or starch (0.5%, w/v). Reuteran was produced by *L. reuteri* 35-5, a mutant derived from *L. reuteri* 121, using the sugar-free MRS medium supplemented with sucrose (5.0%, w/v) (32). Strains were grown anaerobically at 37 °C for 72 h. EPS were extracted as described below.

For cultures grown with maltodextrins, EPS were extracted from supernatants obtained by centrifugation of liquid MRS cultures (33). Two volumes of cold ethanol (-20 °C) were added to supernatants and held at 4 °C overnight. Precipitates were harvested by centrifugation (12 000 ×g, 30 min, 4 °C) and re-dissolved in 1 volume of ddH_2O. EPS were re-precipitated with 2 volumes of ethanol at 4 °C overnight. After centrifugation, the harvested precipitates were dissolved in water, and then dialyzed (10 kDa MWCO, Thermo Scientific) against water for 48 h with changes of ddH_2O every 12 h (34).

For cultures grown with starch, the EPS were collected according to a modified method based on the protocol of Lee et al. (35). Total EPS including soluble and insoluble EPS, and cells were precipitated with 2 volumes of cold ethanol (-20 °C) at 4 °C overnight. Samples collected from tubes were centrifuged at 10000 ×g for 10 min. The polysaccharides were solubilized by addition of 10 ml 2 M NaOH. The cells were separated by centrifugation at 10000 ×g for 15 min. EPS present in the supernatant was neutralized by the addition of an appropriate amount of 2 M HCl (36). The ethanol precipitation procedure described above was used to further purify EPS.

Monosaccharide analysis

EPS samples (0.5 mg) were dissolved in 200 µl Milli-Q water in a glass reaction tube. An equal volume of 4 M trifluoroacetic acid (TFA) was added to the sample. The sample was hydrolyzed for 4 h at 100 °C. Samples were dried under a flow of dry nitrogen. The dry samples were dissolved in 100 µl isopropanol and dried again by evaporation under dry nitrogen. Samples were dissolved in 1 ml dimethyl sulfoxide (DMSO) and used for HPAEC analysis.

Expression of truncated GtfB-ΔN, full-length GtfA and full length GtfB

Truncated GtfB-ΔN and full-length GtfA were produced as described before (37, 38). The full-length GtfB protein was produced in *E. coli* BL21 Star (DE3) carrying the plasmids pRSF-GtfB and pBAD22-GroELS as described previously (27). All three enzymes were first purified by His-tag affinity chromatography using a 1-ml Hitrap IMAC HP column (GE Healthcare) followed by anion exchange purification using Hitrap Q FF (GE Healthcare). Protein Purity was checked on 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein concentration was measured by Bradford reagent (Bio-Rad, Hercules, CA) using bovine serum albumin as standard.

Activity staining of EPS synthesizing enzymes

After SDS-PAGE, the gels were washed 3 times with dd H_2O (30 min each), allowing protein renaturation, and incubated overnight at 37°C in sodium acetate buffer (25 mM, pH 5.0, 1 mM $CaCl_2$) with 5% (w/v) maltodextrins (AVEBE MD20). The activity of EPS synthesizing enzymes in the gel was detected by staining for the polysaccharides by a Periodic Acid-Schiff (PAS) procedure (29). The periodic acid oxidizes the vicinal diols in sugars, creating a pair of aldehydes, which react with the Schiff reagent to give a purple-magenta color. The full-length GtfB expressed in and purified from *E.coli* was loaded as a reference.

Amylose V conversion by washed *L. reuteri* 121 cells

The *L. reuteri* 121 cells were collected by centrifugation (5 000 ×g, 30 min, 4 °C). Cell pellets were washed twice using sodium acetate buffer (25 mM, pH 5.0, 1 mM $CaCl_2$, 0.02% (w/v) NaN_3) in order to remove any unbound proteins. The cells were resuspended in the same sodium acetate buffer and were incubated with amylose V (0.25%, w/v) on a shaker at 40 °C to measure enzyme activity

and to synthesize the enzyme products (37, 39). For amylose V conversion, cells (0.03 U of total GtfB activity per ml reaction volume) with an activity equivalent to that of 60 nM GtfB-ΔN enzyme, were incubated with amylose V (0.25%, w/v) on a shaker at 40 °C and pH 5.0 for 24 h.

Preparation of isomalto/malto-polysaccharides (IMMP) with GtfB-ΔN enzyme

IMMP were produced according to Leemhuis et al. (27). Different types of starches and maltodextrins (0.5%, w/v) were incubated with GtfB-ΔN enzyme (1.4 U per gram substrate) in 10 mM sodium acetate buffer pH5.0, supplemented with 1 mM $CaCl_2$ and NaN_3 (0.02% w/v) at 37 °C. Native starches were pregelatinized by autoclaving (15 min, 121 °C).

Debranching of polysaccharides

Starches, and the IMMP and EPS products derived, were suspended in citrate buffer (50 mM, pH 4.0, 0.02% NaN_3) to a final concentration of 10 mg/ml, and slowly heated to 99 °C with a thermomixer (Thermo Fisher Scientific Inc., Waltham, US). The suspension was left at 99 °C for 1 h while shaking. Afterwards, the polymers were debranched at 40 °C for 16 h with 20 U of isoamylase from *Pseudomonas* sp. (Megazyme, Wicklow, Ireland). The debranched samples were dialyzed (1000 Da MWCO tube, Sigma-Aldrich, US) against Milli-Q and freeze-dried.

NMR analysis

EPS samples produced by the GtfB enzyme *in vitro* and by *L. reuteri* 121 cells *in vivo* were analyzed by NMR according to Leemhuis et al. (27). NMR spectroscopy resolution-enhanced 1D 500-MHz ^1H NMR spectra were recorded in D_2O on a Varian Inova Spectrometer (NMR Center, University of Groningen) at probe temperatures of 300 K. The samples were exchanged twice with D_2O (99.9 atm% D, Cambridge Isotope Laboratories, Inc.) with intermediate lyophilization and then dissolved in 0.6 ml D_2O.

Enzymatic hydrolysis

Samples of 1 mg EPS were dissolved in 100 µl citrate buffer (50 mM, pH 3.5-5.0 based on the instructions for these commercial enzymes) and excess amount of

the following enzymes were added: pullulanase M1 from *Klebsiella planticola* (Megazyme, Ireland), dextranase from *Chaetomium erraticum* (Sigma-Aldrich, US), α-amylase from *Aspergillus oryzae* (Megazyme, Ireland), isoamylase from *Pseudomonas* sp. (Megazyme, Ireland), and isopullulanase from *Aspergillus niger* (Megazyme, Ireland), as well as α-amylase plus isoamylase. Samples were incubated for 72 h at 40 °C. The samples were boiled for 10 min to terminate the enzymatic hydrolysis and used for further analysis. The products after α-amylase and isoamylase treatment were fractionated by size-exclusion chromatography on a Bio-Gel P-2 column (100 x 0.9 cm) (Bio-Rad) using ammonium bicarbonate (10 mM) as eluent at a rate of ~10 ml/h and then subjected to NMR analysis.

Size exclusion chromatography (SEC)

DMSO-LiBr (0.05M) was prepared by stirring for 3 h at room temperature followed by degassing for 15 min using an ultrasonic cleaner (Branson 1510, Branson, Danbury, CT). Samples were dissolved at a concentration of 4 mg/ml in DMSO-LiBr by overnight rotation at room temperature, followed by 30 min heating in an oven at 80 °C, obtaining clear sample solutions. The samples were cooled to room temperature and filtered through a 0.45-µm Millex PTFE membrane (Millipore Corporation, Billerica, MA). The SEC system set-up (Agilent Technologies 1260 Infinity) from PSS (Mainz, Germany) consisted of an isocratic pump, auto sampler, an online degasser, an inline 0.2 µm filter, a refractive index detector (G1362A 1260 RID Agilent Technologies), viscometer (ETA-2010 PSS, Mainz) and MALLS (SLD 7000 PSS, Mainz). WinGPC Unity software (PSS, Mainz) was used for data processing. Samples (100 µl) were injected into a PFG guard column using an autosampler at a flow rate of 0.5 ml/min and DMSO-LiBr as eluent. The separation was done by three PFG-SEC columns with porosities of 100, 300 and 4000 Å. The columns were held at 80˚C, the refractive index detector at 45 °C and the viscometer was thermostatted at 60 °C. A standard pullulan kit (PSS, Mainz, Germany) with molecular masses from 342 to 805000 Da was used. The specific RI increment value dn/dc was measured by PSS and is 0.072 (obtained from PSS company).

High-pH anion-exchange chromatography (HPAEC)

The products of GtfB were injected onto a 4×250 nm CarboPac PA-1 column connected to a Dionex DX500 workstation (Dionex). Samples were run with a

gradient of 30-600 mM NaAc in 100 mM NaOH (1 ml/min), and detected by an ED40 pulsed amperometric detector. A mixture with known concentrations of glucose, isomaltose, isomaltotriose, maltose, panose, maltotriose, maltotetraose, maltopentaose, maltohexaose and maltoheptaose was used as reference.

Thin-layer chromatography (TLC)

The TLC silica gel 60F254 plates (Merck) were run with butanol/acetic acid/water (2:1:1, v/v/v) as solvent. After running for 6 h, TLC plates were developed with 10% (v/v) H_2SO_4 and 2 g/l orcinol in methanol and then heated by oven at 100 °C for 30 min. Glucose, maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose, and maltoheptaose were used as markers.

Total carbohydrate measurements

Total carbohydrates were measured using the total carbohydrate colorimetric assay kit (Biovision, Milpitas, USA). Of each liquid sample, 5-15 µl was added into a 96-well plate. The total volume was adjusted to 30 µl per well by adding ddH_2O. Then, 150 µl of concentrated H_2SO_4 (98%, v/v) was added per well. The plate was mixed for 1 min on a shaker at room temperature and incubated in an oven at 90 °C for 15 min. Afterwards, 30 µl of developer reagent was added and mixed with sample for 5 min on a shaker at room temperature. The optical density was measured at 490 nm. A calibration curve was made using 0, 4, 8, 12, 16, and 20 µg glucose per well.

Results and Discussion

Lactobacillus reuteri strains produce homo-exopolysaccharides (EPS) from maltodextrins

Protein blast searches in the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST/) using GtfB of *L. reuteri* 121 as a query sequence revealed that approx. 28 *Lactobacillus* strains carry a 4,6-α-GTase encoding gene. Eight out of these 28 are *L. reuteri* strains. Previously, we have expressed and purified some of these 4,6-α-GTases in *E. coli*, e.g. GtfB from *L. reuteri* 121, GtfW from *L. reuteri* DSM20016, and GtfML4 from *L. reuteri* ML1, and shown that they produce IMMP from maltodextrins (**Fig. 1A**)

(25-27). The question remained whether also *in vivo* these *L. reuteri* strains and their genes/enzymes support formation of IMMP-like EPS.

Using maltodextrins 13-17 (dextrose equivalent 13-17, Sigma-Aldrich) as carbon sources for growth, *L. reuteri* 121 (GtfB), *L. reuteri* DSM20016 (GtfW), *L. reuteri* TMW1.106 (Gtf106b), and *L. reuteri* ML1 (GtfML4), which all possess a 4,6-α-GTase gene, clearly produced ethanol-precipitable EPS, while *L. reuteri* strains lacking a 4,6-α-GTase gene, such as *L. reuteri* 180 and *L. reuteri* ATCC55730, did not. The yields of EPS produced by the *L. reuteri* DSM20016 (GtfW) and *L. reuteri* ML1 (GtfML4) strains were relatively low (data not shown), probably because the relatively high hydrolytic activity (39) of their enzymes impaired the formation of polysaccharides that can be precipitated by cold ethanol. These EPS are all composed of glucosyl units (monosaccharide analysis, data not shown) linked by α1→4 and α1→6 linkages (**Fig. 1B**). Thus, these *L. reuteri* strains are able to produce α-glucan EPS from maltodextrins, which may be highly relevant for their survival and activities in natural and industrial environments.

4,6-α-GTase enzymes are essential for EPS formation by *L. reuteri in vivo*

Using the previously established 4,6-α-GTase amylose-iodine assay with amylose V as substrate (39), no activity was detectable in cell-free culture supernatants. However, cell-associated activities were detected in the washed and resuspended cell pellets of all 4 *L. reuteri* strains with a (putative) 4,6-α-GTase enzyme (**Fig. 2A**). Amylose V is too large a molecule to pass the *L. reuteri* cell membrane, therefore intracellular enzymes are not involved here. The combined data indicate that the amylose V modifying enzymes are extracellular and cell-associated and not released into the culture fluid during growth. This is in accordance with the primary structures of 4,6-α-GTases, showing the presence of signal peptides for secretion (40). The 4,6-α-GTases, like glucansucrases, also have variable N-terminal regions. Although the functional role of this N-terminal region has not been established, glucansucrases with this region are always cell wall-bound in lactic acid bacteria (32). The same appears to be true for 4,6-α-GTases.

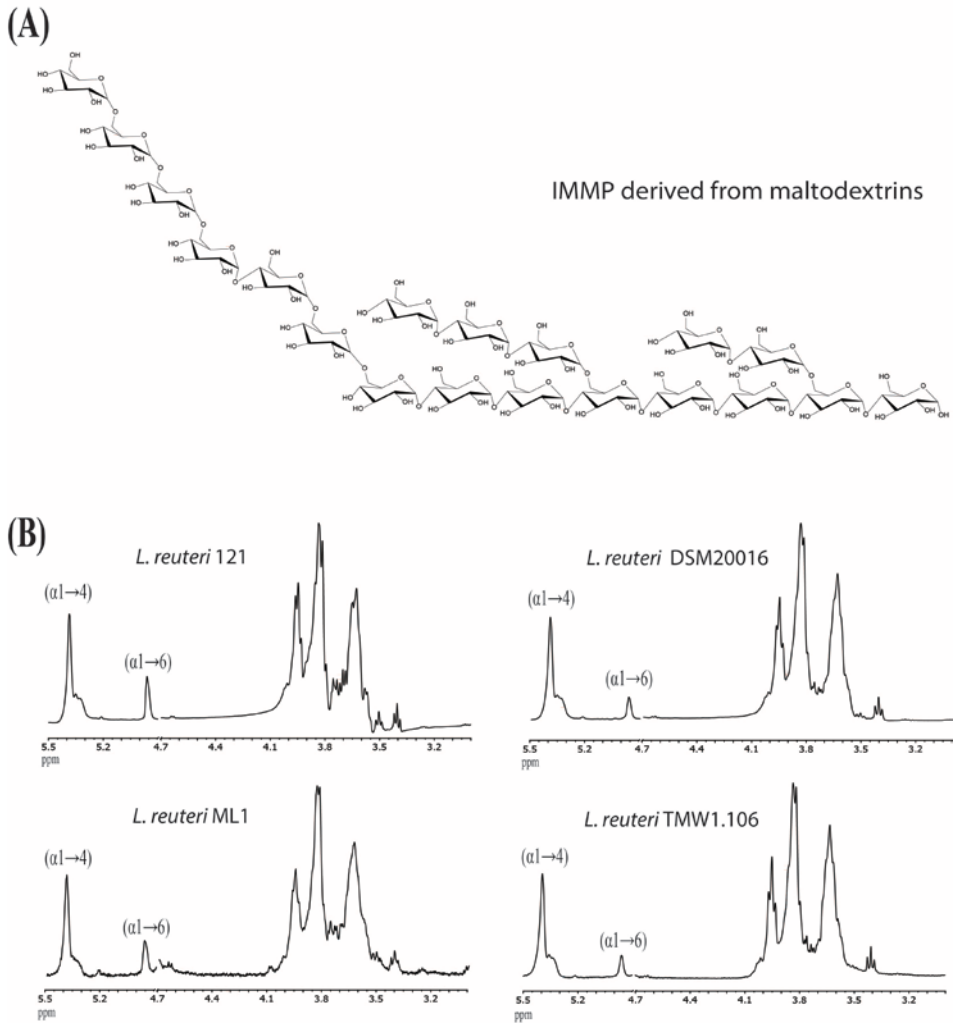

Fig. 1. (A) Representation of the IMMP products of 4,6-α-GTase (e.g. GtfB) with maltodextrins (28). (B) NMR spectra of the EPS isolated from *L. reuteri* 121, *L. reuteri* DSM20016, *L. reuteri* ML1, and *L. reuteri* TMW1.106 cultures grown on maltodextrins AVEBE MD20 (5%, w/v)-MRS medium.

To further analyze the functional *in vivo* role of 4,6-α-GTase, the *L. reuteri* 121 system was studied in detail as model strain. Periodic Acid-Schiff staining (PAS) is a well-established method that has been used to quickly detect polysaccharides produced by glucansucrase enzymes. Under mild conditions, denatured glucansucrases can be refolded and regain activity in SDS-PAGE gels (29). Previously, we already reported that also the denatured GtfB enzyme can be

easily renatured in double distilled water (37). PAS staining (**Fig. 2B, lane 2**) showed that *E. coli* expressed and purified GtfB protein (~179 kDa) in SDS-PAGE gels produced polysaccharides from maltodextrins. Following SDS-PAGE of *L. reuteri* 121 total cellular proteins and protein renaturation, incubation with maltodextrins and PAS staining revealed a band, representing an enzyme with a similar molecular mass, (**Fig. 2B, lane 1**). Previously we reported that *L. reuteri* 121 GS GtfA (~199 kDa) activity also can be detected by PAS staining of SDS-PAGE gels when using sucrose as substrate (resulting in reuteran formation) (29). GtfA is the only carbohydrate-acting protein in the genome of *L. reuteri* 121 that has a similar molecular mass as GtfB (Gangoiti et al., in preparation). Unlike the PAS results with sucrose as substrate, no SDS-PAGE band was observed following incubation of *E. coli* expressed and purified GtfA protein with maltodextrins (**Fig. 2B, lane 3**). In addition, the resuspended *L. reuteri* 121 cell pellet was incubated with amylose V (0.25%, w/v) until all substrate was consumed as no color formed when using the iodine-staining assay developed previously (39). NMR analysis revealed that an α-glucan product was formed with 54% of α1→4 linkages and 46% of α1→6 linkages (**Fig. 2C**). The *L. reuteri* 121 cells associated 4,6-α-GTase enzyme and the purified GtfB enzyme thus have the same product specificity, cleaving α1→4 linkages and forming α1→6 linkages. The combined data shows that the GtfB enzyme is responsible for EPS formation by *L. reuteri* 121 cells.

NMR analysis of EPS produced by *L. reuteri* 121 cells from maltodextins or starches

Subsequently, we investigated whether *L. reuteri* 121 cells in liquid cultures also are able to convert starch. Various starches were supplied at a concentration of 5 g/l in order to prevent retrogradation (27). Cultures were supplemented with glucose as carbon and energy source because *L. reuteri* 121 does not grow on starch alone, while in the case of maltodextrins, no extra glucose (**Table 1**) was added. The isolated EPS were subjected to NMR analysis. All starches tested were modified during the growth of *L. reuteri* 121 as is apparent from the increased percentages of α1→6 linkages obtained in all cases (**Table 1**). Growth of *L. reuteri* 121 in the presence of starch with a high amylose content and a lower degree of branching resulted in formation of EPS with higher percentages of α1→6 linkages. Highest conversion of α1→4 into α1→6 linkages was

obtained with wrinkled pea starch, which contains the highest amylose (52.8%, w/w) content. This is in accordance with the *in vitro* data for starch conversion by GtfB, showing that the amylose content is positively, but the degree of branching is negatively correlated with the percentage of α1→6 linkages in the produced IMMP (27). Compared to these IMMP (27), the *L. reuteri* 121 EPS derived from the same starch or maltodextrin substrates always have around 0-20% lower percentages of α1→6 linkages (**Table 1**). The *L. reuteri* 121 whole cell system provides more complicated reaction conditions, e.g. with pH variations, and the presence of other enzymes and components. Also, in whole cell systems the GtfB enzyme is cell wall-bound. These factors are likely to influence product formation, compared to incubations of the purified free GtfB enzyme with a pure substrate.

(A)

	L. reuteri 121	*L. reuteri* DSM20016	*L. reuteri* ML1	*L. reuteri* TMW1.106
Cell activity	+	+	+	+
Supernatant activity	-	-	-	-

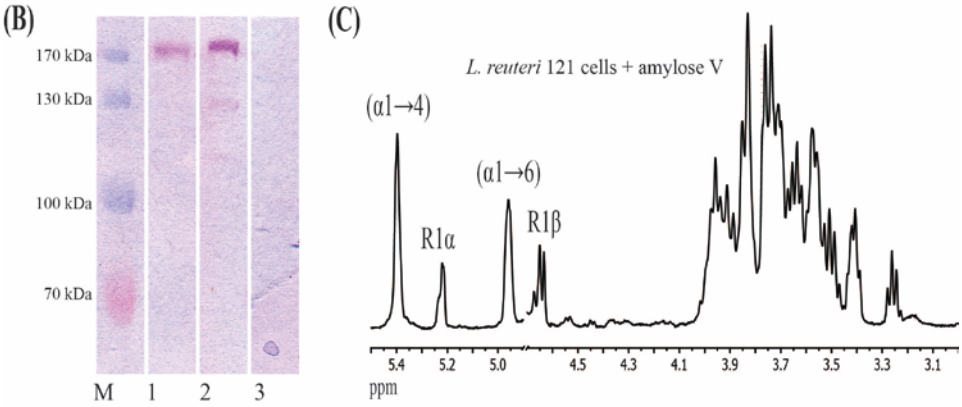

Fig. 2. (A) 4,6-α-GTase activity associated with cells of *L. reuteri* strains using amylose V (0.25%, w/v) as substrate (39). "+" and "-" indicate the presence and absence of activity. (B) Periodic Acid-Schiff (PAS) stained SDS-PAGE gels of whole-cell-extracted proteins of *L. reuteri* 121 (lane 1), *E. coli* expressed and purified GtfB (lane 2) and GtfA (lane 3) after incubating with maltodextrins (AVEBE MD20, 5.0%, w/v) overnight at pH 5.0 and 40 °C. (C) 1D ^{1}H NMR analysis of the products (with 54% of α1→4 linkages and 46% of α1→6 linkages) derived from amylose V (0.25%, w/v) incubated with *L. reuteri* 121 cell pellets. R1α/β represent the reducing –(1→4)-D-Glc*p* units and D-Glc*p* units.

Table 1. Percentages of α1→6 linkages in EPS formed during growth of *L. reuteri* 121 in MRS-medium with or without 1% of glucose in the presence of different types of starch and maltodextrins, determined by ^{1}H NMR spectroscopy. (n/a, not applicable). The final pH values of the *L. reuteri* 121 cultures with 1% glucose all were in the range of pH 4.5-4.6.

Substrate	Amylose content (%) (apparent)	Average DP[a]	Degree of branching (α1→4,6) (%)	% (α1→6) in IMMP produced by GtfB enzyme[d]	% (α1→6) in EPS from *L. reuteri* 121 grown with 1% glucose	% (α1→6) in EPS from *L. reuteri* 121 grown without 1% glucose
AVEBE Maltodextrins						
Paselli SA2	n/a	50		32		**35**
Paselli MD6	n/a	20		34		**19**
AVEBE MD20	n/a	6		25		**26**
AVEBE SPG30	n/a	4		24		**31**
AVEBE Starches						
Etenia 457	n/a	n/a	-[c]	33	**22**	
Arrow root	20.8	n/a	-	24	**14**	
Barley	25.5	n/a	-	21	**15**	
Corn	29.4	n/a	3.6	21	**22**	
Corn, waxy	0	n/a	4.8	7	**11**	
Mung bean	37.9	n/a	-	34	**21**	
Pea, yellow	30.9	n/a	-	35	**26**	
Pea, wrinkled	52.8	n/a	-	71	**52**	
Potato	36.0	n/a	3.1	28	**17**	
Rice, round grain	25.0	n/a	4.1	13	**12**	
Rice, waxy	0	n/a	4.9	7	**11**	
Sorghum	23.7	n/a	-	22	**18**	
Tapioca	23.5	n/a	3.9	20	**12**	
Wheat	28.8	n/a	3.7	22	**23**	

[a]Degree of polymerization; [b]n/a, not applicable; [c]not available in literature; [d]data adapted from Leemhuis et al. (27).

Size exclusion chromatography analysis of EPS and IMMP derived from maltodextrins

The sizes of the IMMP products of the GtfB enzyme have not been reported before. Therefore the IMMP-like EPS (**Table 1**) produced by *L. reuteri* 121 cells and the IMMP produced by the GtfB enzyme from different maltodextrins were analyzed by SEC in comparison with their original substrate. EPS derived from AVEBE SPG30 (< 1.0 kDa) and AVEBE MD20 (approx. 1.0 kDa) (**Table 1**) both contain two peaks of much bigger polymers (**Fig. 3A** and **B**) of relatively low (Region II, 132 kDa for EPS SPG30, 706 kDa for EPS MD20) and relatively high (Region I, 1.7 MDa for EPS SPG30, 3.3 MDa for EPS MD20) apparent average molar mass (MM) (**Fig. 3**). The high MM products in all cases represented only a small percentage (2.5%). In general, these EPS thus are much larger than the IMMP produced by the GtfB enzyme from the same maltodextrins (**Fig. 3**). A possible explanation is that the reaction conditions for the *L. reuteri* 121 cell-associated GtfB enzyme and the free purified GtfB enzyme differ. Any glucose and maltose produced by the *L. reuteri* 121 cell-associated GtfB enzyme can be consumed by the cells as carbon source and thus cannot be used as acceptor substrate in further reaction. On the contrary, the glucose and maltose produced in the *in vitro* GtfB reaction can be used as acceptor substrate (26), resulting in relatively high yield of oligosaccharides, which have no clear increase in chain length compared to maltodextrin substrate.

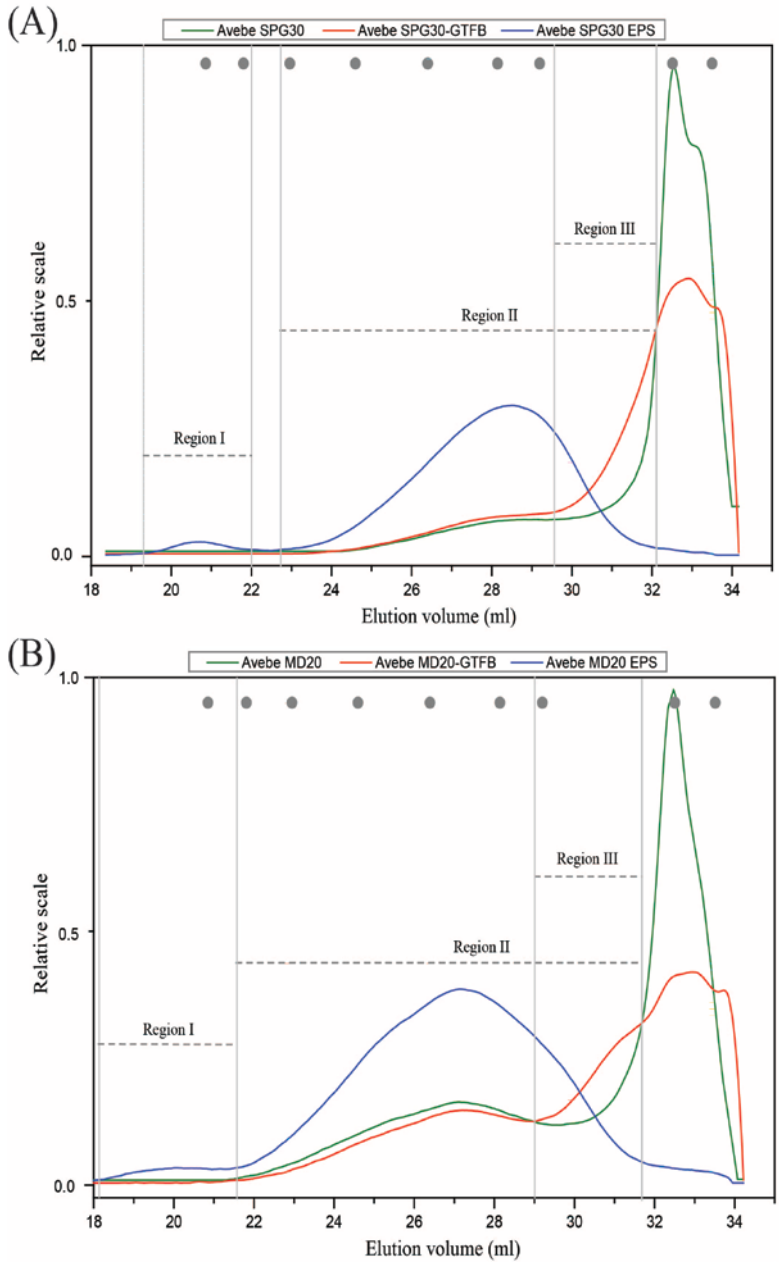

Fig. 3. SEC chromatograms of the different maltodextrin substrate (A, SPG30, B, MD20), the EPS produced by *L. reuteri* 121 cells, and IMMP produced by the free GtfB-ΔN enzyme. Elution times of the pullulan standards corresponding to 366, 200, 113, 48.8, 21.7, 10, 6.2, 1.32 and 0.342 kDa, are shown as gray dots.

SEC analysis of EPS and IMMP derived of Etenia 457 and wheat starch

NMR analysis showed that *L. reuteri* 121 cells convert various starches into EPS with increased percentages of α1→6 linkages in linear chains (**Table 1**). To compare starch conversion by the *L. reuteri* 121 cell-associated GtfB enzyme and by the free GtfB enzyme, Etenia 457 and wheat starch (28.8% amylose) were used as substrates (**Table 1**). The size of these substrates, and the EPS and IMMP produced were analyzed using SEC.

Etenia 457 is produced from potato starch by amylomaltase (4-α-glucanotransferase) treatment in which the amylose part gradually is transferred to the non-reducing ends of the side chains of amylopectin, resulting in elongated external chains (41). As shown in **Fig. 4A**, the *L. reuteri* 121 EPS derived from Etenia 457 (approx. 350-400 kDa) has almost the same SEC profile as Etenia 457 itself (thus no clear change in MM) although the NMR results (**Table 1**) showed that this EPS has more α1→6 linkages. By contrast, the SEC profile of the IMMP produced by GtfB from Etenia 457 clearly shows that Etenia 457 was converted into two main products with smaller MM (approx. 200 kDa in Region II and approx. 10 kDa in III). The material present in region II most likely represents Etenia 457 derived products with shortened non-reducing end side chains, used as donor substrate or hydrolyzed by GtfB. Region III represents Etenia 457 derived smaller MM products.

As any size difference between Etenia 457 and the IMMP and EPS product derived might be covered by their relatively broad size distribution in the SEC analysis, these three polymers were debranched by isoamylase and subsequently analyzed again by SEC (**Fig. 4B)** (42). After debranching, the side chains of Etenia 457/isoamylase all eluted in region II'. The debranched products from IMMP and EPS were both smaller than those from the Etenia 457 substrate, indicating that the original amylopectin side chains were modified. However, the EPS and IMMP differed as isoamylase treated EPS formed by *L. reuteri* 121 cells contained some larger MM material, eluting in Region I′.

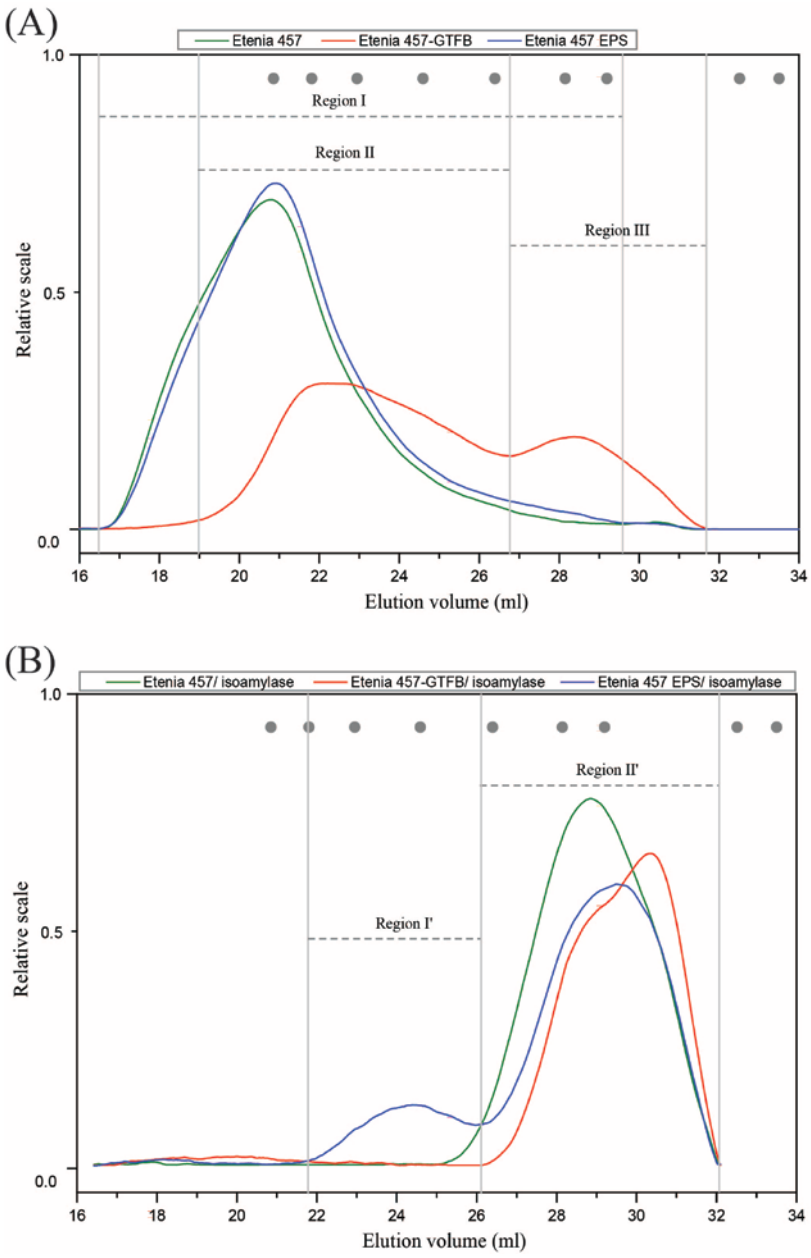

Fig. 4. SEC chromatograms of Etenia 457, the EPS produced from Etenia 457 by *L. reuteri* 121 and IMMP produced from Etenia 457 by the free GtfB-ΔN enzyme, before (A) and after (B) isoamylase treatment. Elution times of the pullulan standards corresponding to 366, 200, 113, 48.8, 21.7, 10, 6.2, 1.32 and 0.342 kDa, are shown as gray dots.

Starches with high amylose content have been described as preferred substrates for GtfB.[27] In *L. reuteri* 121 cultures, starches with a higher amylose content also yielded higher percentages of α1→6 linkages (**Table 1**). For instance, the EPS and IMMP derived from wheat starch both have approx. 22% of α1→6 linkages, which is much higher than the original 3.7% (**Table 1**). As shown in **Fig. 5A**, the graph representing wheat starch shows a peak in region I representing amylopectin content and a peak in region II representing amylose which are overlapping (42). After fermentation with *L. reuteri* 121, the amylopectin content decreased with an increase of products in region II. The SEC profile of the IMMP produced by GtfB from wheat starch is largely different from that of the wheat starch substrate. Wheat starch clearly was converted into two main products with smaller MM (approx. 300 kDa and approx. 15 kDa). The data indicates that GtfB also is capable to modify the amylopectin part in native wheat starch.

After debranching, the SEC curve of wheat starch was divided into two parts: one is the amylose part, which cannot be processed by isoamylase (Region I'); the other is the bimodal peak representing the amylopectin branches including side and inner chains (**Fig. 5b**) (42). For EPS derived from wheat starch, the SEC curve shows significant differences. The peak in region III' representing the long branches of amylopectin in wheat starch decreased a lot, suggesting that *L. reuteri* 121 also modifies the side chains of amylopectin in wheat starches. The peak representing amylose in region I' (> 400 kDa) shifted dramatically and formed a new peak in region II' (approx. 200 kDa), indicating that the amylose content of wheat starch was converted by *L. reuteri* 121 and new, smaller products were generated. By contrast, the amylose part in region I' was completely converted *in vitro* by the GtfB enzyme as the peak representing amylose disappeared. The amount of the short chains after debranching process increased.

To conclude, *L. reuteri* 121 cells and the GtfB enzyme are capable of modifying the amylose molecule as well as the side chains of amylopectin molecules in starches, introducing higher percentages of α1→6 linkages. The EPS produced have similar size distribution as the starch substrates although the percentages of α1→6 linkages increased significantly compared to the starch substrates. The produced IMMP are clearly different from the produced EPS and starch

substrates with regard to their size distribution. This may result from the minor endo-acting activity (Bai et al., to be submitted) of the GtfB enzyme. However, during the *L. reuteri* 121 fermentations, the interactions of the starch substrate with the cell-associated GtfB enzyme clearly are different from those with the purified GtfB enzyme. Conceivably, the endo-acting activity of the cell-associated GtfB enzyme is impaired compared to the free GtfB enzyme. In addition, any glucose released by the *L. reuteri* 121 cells may be consumed whereas the free GtfB enzyme is able to use glucose as acceptor substrate (26), resulting in smaller size products. This might influence product synthesis, resulting in the size differences between IMMP and EPS.

Structural analysis of EPS derived from maltodextrins by enzymatic digestion

IMMP produced by the GtfB enzyme from maltodextrin substrates were characterized as soluble fibers (27). They are structurally different from the known starch and maltodextrin molecules as GtfB builds linear consecutive (1→6)-α-glucan chains onto the non-reducing end of (1→4)-α-glucan chains. Thus IMMP derived from maltodextrins are mainly composed by sequences of -α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→4)-, -α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→6)-, and -α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-, as well as some branching -α-D-Glc*p*-(1→4,6)-α-D-Glc*p*-(1→4)- originating from the substrates (**Fig. 6B**) (27).

To reveal whether the IMMP-like EPS produced by *L. reuteri* 121 cells and cultures are structurally similar to IMMP produced by the GtfB enzyme, the AVEBE MD6 derived EPS were subjected to hydrolysis by several enzymes that are typical for structural analysis of polysaccharides. α-Amylase (**Fig. 6B**) is an endo-acting enzyme cleaving α1→4 glucosidic linkages inside starch molecules mainly producing maltose. Dextranase of *Chaetomium erraticum* is an (1→6)-α-glucan 6-glucanohydrolase that endo-hydrolyzes (α1→6)-α-glucosidic linkages (**Fig. 6B**) in dextran mainly producing glucose and isomaltose. As shown in **Fig. 6A**, glucose and maltose were released by α-amylase hydrolysis of EPS, suggesting that this EPS contains -α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)- sequences. Likewise, the presence of -α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→6)- sequences were also confirmed because dextranase hydrolysis generated glucose and isomaltose.

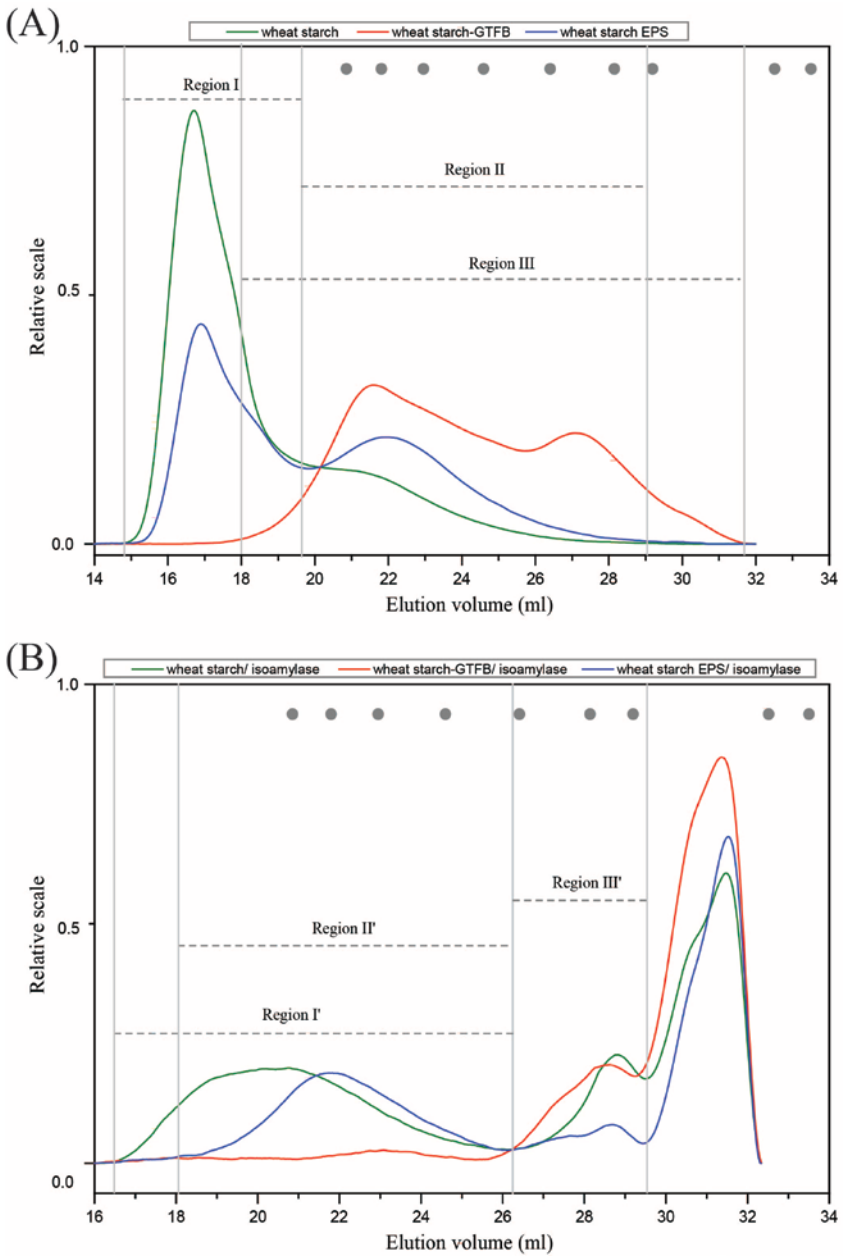

Fig. 5. SEC chromatograms of wheat starch and the EPS produced from wheat starch by *L. reuteri* 121, and IMMP produced from wheat starch by the free GtfB-ΔN enzyme, before (A) and after (B) isoamylase treatment. Elution times of the pullulan standards corresponding to 366, 200, 113, 48.8, 21.7, 10, 6.2, 1.32 and 0.342 kDa, are shown as gray dots.

Pullulanase type I specifically attacks α1→6 linkages in both -α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→4)- sequences (**Fig. 6B**) in pullulan and -α-D-Glc*p*-(1→4,6)-α-D-Glc*p*-(1→4)- branch points (**Fig. 6B**) in starch molecules (43). Isoamylase exclusively cleaves the branching α1→4,6 linkages (44). AVEBE MD6 derived EPS were hydrolyzed by both pullulanase and isoamylase, forming similar products (**Fig. 6A and Fig. S2**). These EPS thus possess -α-D-Glc*p*-(1→4,6)-α-D-Glc*p*-(1→4)- branch points which also exist in IMMP (27). Isopullulanase from *Aspergillus niger* exclusively cleaves the α1→4 linkages in -α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→4)- sequences (**Fig. 6B**) in pullulan but has no activity on starches (45). After treatment of EPS with isopullulanase, oligomers appeared (**Fig. 6A**), confirming the presence of linear -α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→4)- sequences. These enzymatic hydrolysis results thus showed that the EPS produced by *L. reuteri* 121 and IMMP produced by GtfB are structurally similar since both contain the same fragments.

To further investigate the presence of consecutive α1→6 linkages that are typical in IMMP, the EPS derived from AVEBE MD6 was treated with a combination of pullulanase and α-amylase to remove the consecutive α1→4 linkages, and the α1→6 linkages in connecting -α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→4)- sequences and -α-D-Glc*p*-(1→4,6)-α-D-Glc*p*-(1→4)- branches. The hydrolyzed products were fractionated on Bio-Gel P2, yielding nine fractions, denoted F1-F9. F8 and F9 were mainly composed of maltose and glucose (TLC, data not shown) that resulted from consecutive α1→4 linkages hydrolyzed by α-amylase. With the increase of DP, the percentage of α1→6 linkages increased (**Table S1**). In fractions 1 and 2, the polymers were mainly composed of α-glucan with 94% α1→6 linkages. This shows that linear consecutive α1→6 linked fragments inside EPS are present with a broad DP distribution.

Fermentation of *L. reuteri* EPS by probiotic *Bifidobacterium* strains

The EPS produced by *L. reuteri* 121 has a special structure, which is similar to IMMP but different from known α-glucans such as starch, dextran and pullulan. Previously, IMMP were shown to be soluble dietary fibers (27). EPS thus may partially escape digestion in the upper part of the gastrointestinal tract and small intestine, to serve as typical carbon sources for bacterial fermentation in the large intestine. Alternatively, uptake of probiotic *L. reuteri* 121 most likely results in

formation of EPS rich in α1→6 linkages in the upper gastrointestinal tract by 4,6-α-GTase enzymatic modification of the available (partially hydrolyzed) starch. The *in vivo* synthesized EPS may selectively stimulate the growth of (other) probiotic bacteria in the large intestine.

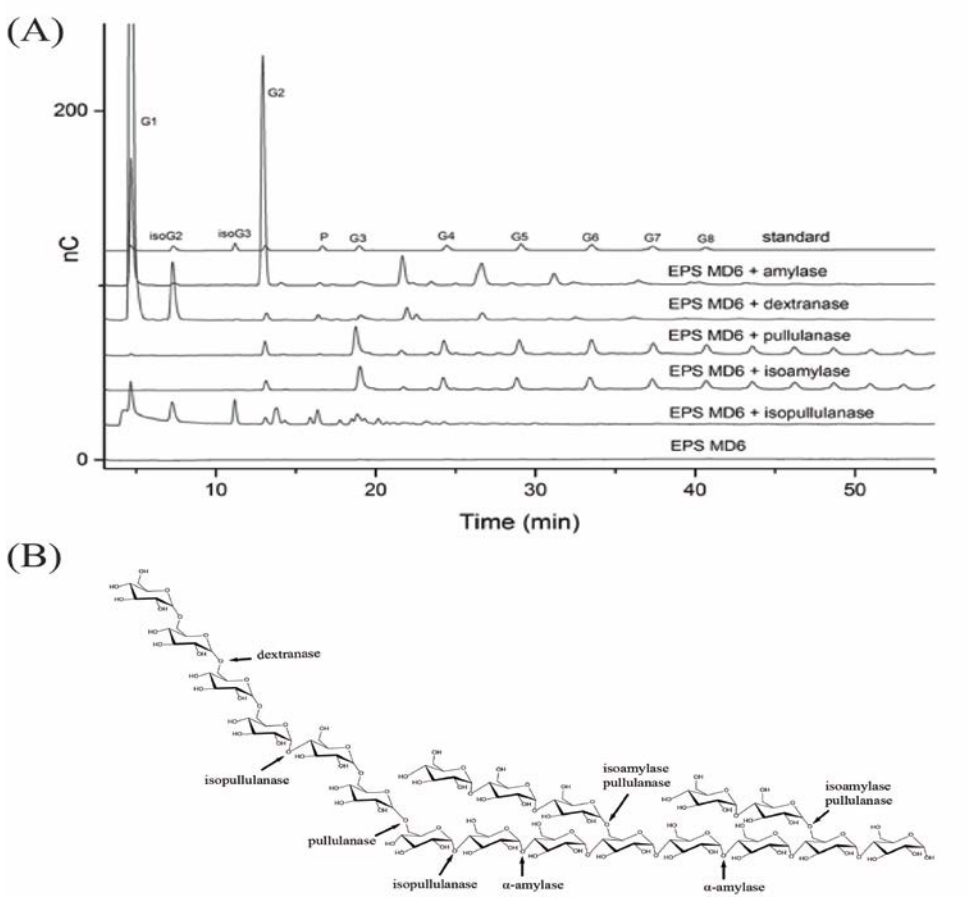

Fig. 6. (A) HPAEC analysis of the hydrolysis products of EPS (from AVEBE MD6) following incubation with an excess of different enzymes, including α-amylase, dextranase, pullulanase, isoamylase, and isopullulanase. A combination of glucose (G1), isomaltose (isoG2), isomaltotriose (isoG3), maltose (G2), panose (P), maltotriose (G3), maltotetraose (G4), maltopentaose (G5), maltohexaose (G6), maltoheptaose (G7), and maltooctaose (G8), were used as standard. EPS without enzymatic treatment was applied as negative control. (B) Representation of the IMMP products of 4,6-α-GTase (e.g. GtfB) with starch and maltodextrins. GtfB builds (1→6)-α-glucan chains with occasionally internal 1→4 linkages onto the non-reducing end of (1→4)-α-glucan chains (28). Schematic diagram of the α-amylase, dextranase, pullulanase, isoamylase, and isopullulanase enzymatic cleavage points in IMMP.

Bifidobacterium is one of the major genera of bacteria that make up the colon flora. Some of them are used as probiotics in food industry as they may exert a range of beneficial health effects (46, 47). To date, many type II pullulanases (amylopullulanases) with both α-amylase and pullulanase activity have been identified in probiotic *Bifidobacterium* species from the human gut (31, 48, 49). It was also reported that amylopullulanase is the only extracellular polysaccharide hydrolyzing enzyme in lactobacilli (18). Based on the above data (**Fig. 6** and **Fig. S2**), *L. reuteri* 121 EPS derived from maltodextrins can be partially hydrolyzed by both α-amylase and pullulanase. Previously, we reported that reuteran derived from sucrose can be hydrolyzed by pullulanase (9). Thus, the *Bifidobacterium* strains producing extracellular amylopullulanase have strong potential to degrade both *L. reuteri* 121 EPS derived from maltodextrins, and reuteran, and to use the degradation products as carbon- and energy source. Growth of *Bifidobacterium breve* DSM 20091 and *Bifidobacterium dentium* DSM 20436, both containing amylopullulanase activity (48), was studied using EPS of *L. reuteri* 121 as carbon source. Both *B. breve* DSM20091 (**Fig. 7b**) and *B. dentium* DSM20436 (**Fig. 7c**) grew well with different EPS. After 12 h of growth, OD_{600} values of *B. breve* DSM20091 all reached 80% with EPS derived from MD20, MD6, or SPG30 (**Table 1**), and 67.5% with EPS derived from MD4-7, compared to a 100% control grown on glucose. Growth curves of *B. dentium* with EPS followed closely the glucose-control reaching all a final OD_{600nm} value of 2.00 after 8.5 h. The exponential growth phases with the EPS growth substrate did not show any differences to the glucose-control indicating that these EPS are highly suitable as growth substrates. In addition, total carbohydrate measurements and determination of OD_{600} values and pH values (**Table 2**), as well as TLC detection (**Fig. S1**) also showed that EPS was partially or completely used by strains.

As reported, the EPS produced by *L. reuteri* 121 from maltodextrins is hardly degraded by human gut symbiont *Bacteroides thetaiotaomicron* (50). In this study, *Bifidobacterium adolescentis* DSM 20083 that cannot degrade starch (data not shown) was also investigated, showing that it cannot grow with any of the EPS derived from maltodextrins (**Fig. 7A**). Therefore, EPS of *L. reuteri* 121 derived from both maltodextrins and sucrose are potential prebiotics which typically stimulate growth of *Bifidobacterium* strains which have amylopullulanase activity.

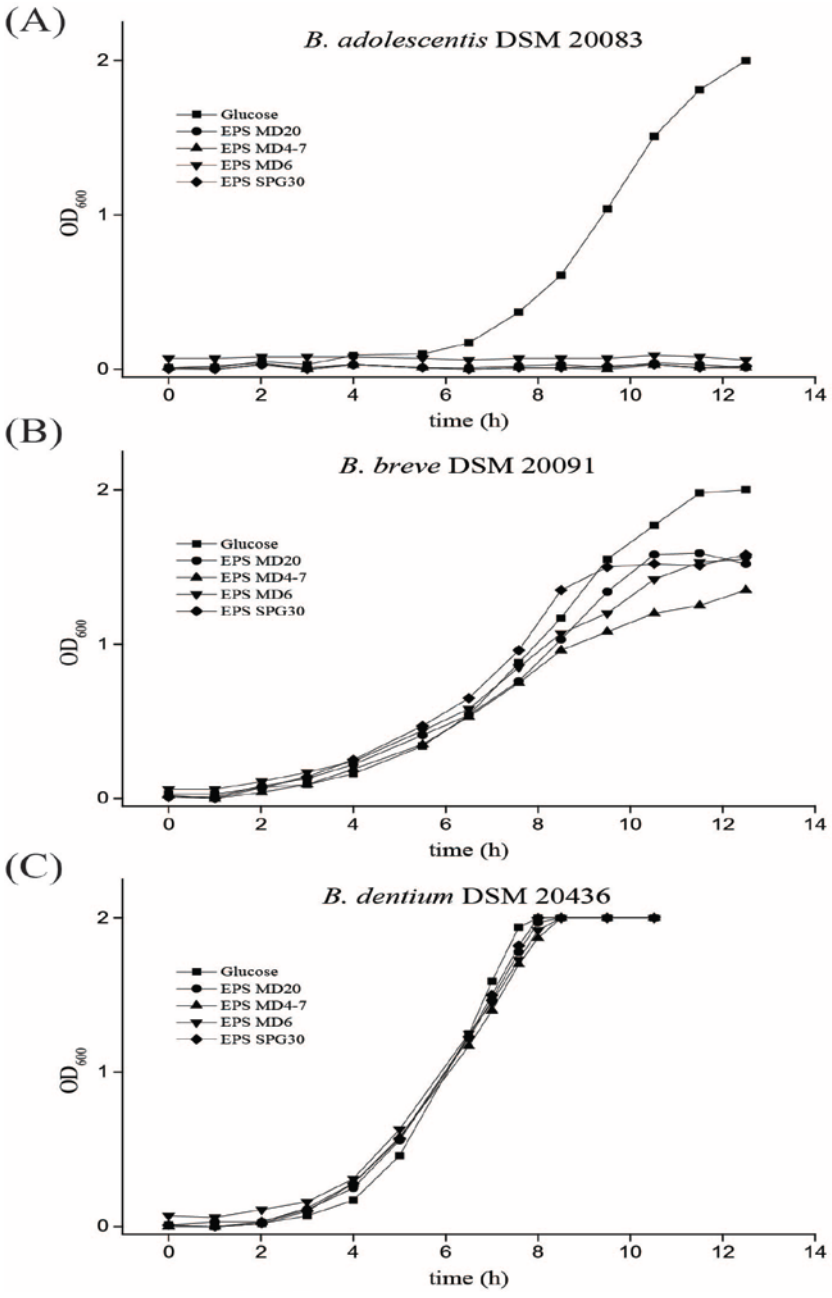

Fig. 7. Growth of *B. adolescentis* DSM 20083 (a), *B. breve* DSM 20091 (b) and *B. dentium* DSM 20436 (c) on different EPS (5 mg/ml) derived from different maltodextrins. Glucose (5 mg/ml) served as positive control.

Table 2. OD_{600}, pH values and total carbohydrate consumption values were determined after growth of *B. breve* DSM 20091 and *B. dentium* DSM 20436 for 24 h with *L. reuteri* 121 EPS derived from different maltodextrins, and reuteran derived from sucrose. Experiments were done in duplicate and the average data are shown.

	Bifidobacterium breve DSM 20091			*Bifidobacterium dentium* DSM 20436		
	OD_{600}	pH	Total carbohydrate consumption (%)	OD_{600}	pH	Total carbohydrate consumption (%)
Control	0.03	6.8	-	0.03	6.8	-
Glucose	1.68	4.9	100	1.85	5.0	100
EPS Avebe SPG30	1.54	5.2	74	1.68	5.2	84
EPS Avebe MD20	1.46	5.3	81	1.56	5.3	85
EPS Avebe MD6	1.63	5.2	91	1.68	5.1	88
EPS MD 4-7	1.62	5.2	82	1.70	5.1	86
Reuteran	1.56	5.0	84	1.80	5.0	95

To conclude, in this study, novel homo-EPS produced by probiotic bacterium *L. reuteri* strains from starch or maltodextrins as substrates were characterized as IMMP-like EPS. The data show that the cell-associated 4,6-α-glucanotransferase enzyme plays an important role in the formation of such IMMP-like EPS in *L. reuteri* strains. The *L. reuteri* 121 EPS are structurally similar to the IMMP produced by the free GtfB enzyme, with percentages of α1→6 linkages ranging between 11% to 52% (**Table 1**). However, the *L. reuteri* 121 EPS derived from either maltodextrins or starches are generally much larger than the IMMP produced by the *in vitro* GtfB enzyme. The SEC results also demonstrated that *L. reuteri* 121 is capable to modify starches by converting both the amylose part and the side chains of amylopectin molecules. Finally, the EPS derived from different maltodextrins were shown to be excellent growth substrates for some probiotic *Bifidobacterium* strains that have amylopullulanase activity. These EPS thus have potential prebiotic activity and may contribute to the application of probiotic *L. reuteri* strains grown on maltodextrins or starch as synbiotics.

Acknowledgements

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Fig. S1. TLC analysis of the saccharides in the cell-free supernatant before (1-5) and after (1'-5') growth of *Bifidobacterium breve* DSM 20091 and *Bifidobacterium dentium* DSM 20436 incubated with *L. reuteri* 121 EPS derived from different maltodextrins (1, SPG30, 2, MD20, 3, MD6, 4, MD4-7), and reuteran (5) derived from sucrose. BM medium without carbon source and with glucose was spotted as negative control (C) and positive control before (6) and after (6') fermentation.

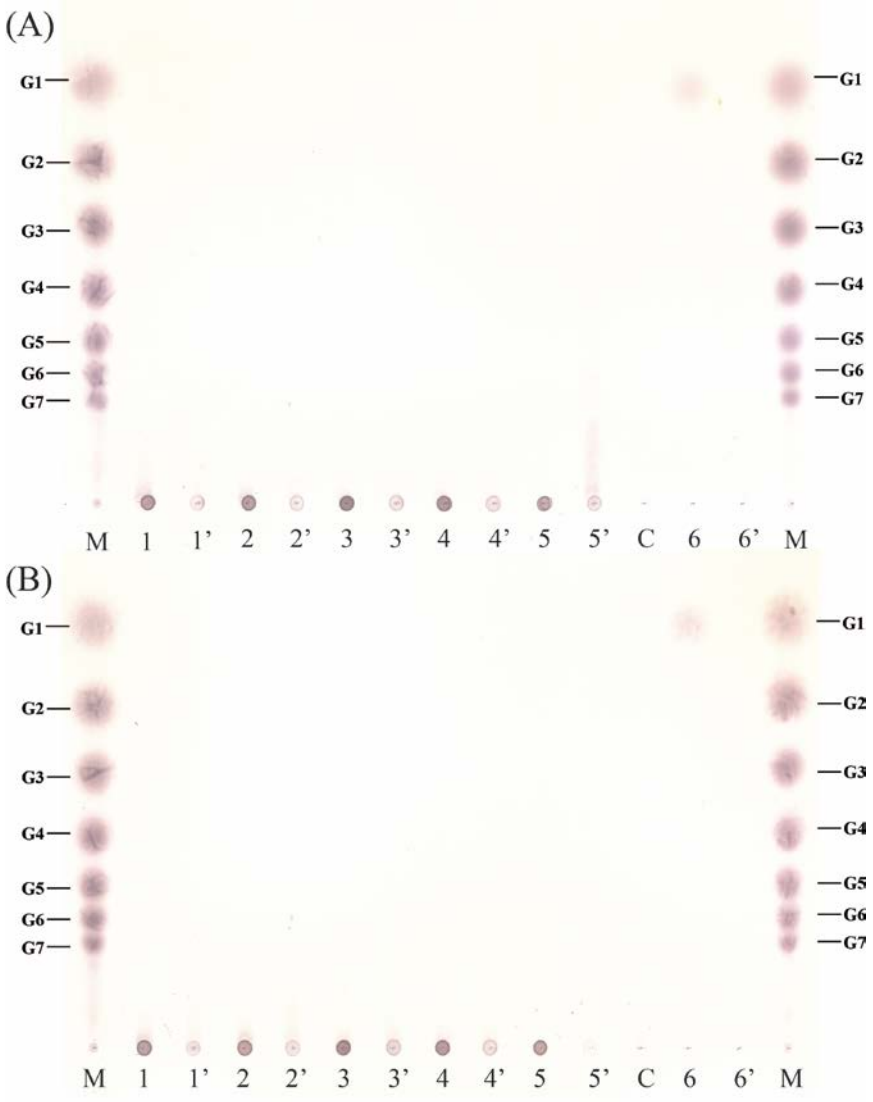

Fig. S2. TLC analysis of the enzymatic hydrolysis of EPS (from AVEBE MD6) by different enzymes, α-amylase, L1; dextranase, L2; pullulanase, L3; isoamylase, L4; and isopullulanase, L5; control (EPS), C.

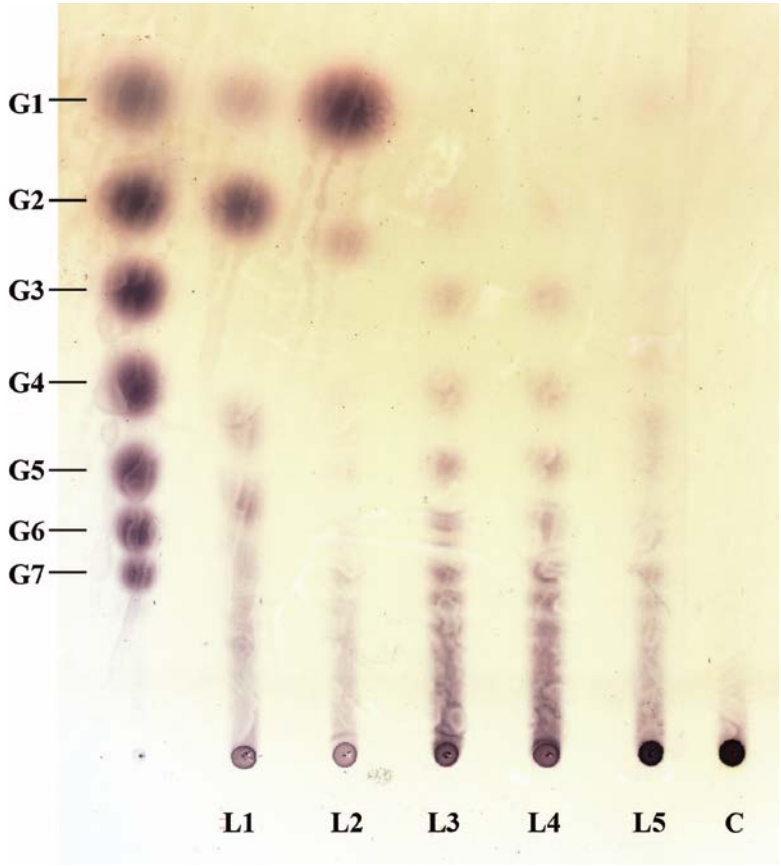

Table S1. The amylase-pullulanase treated EPS produced from Paselli MD6 was fractionated by Biogel P2 column into 9 fractions (fraction 1 represents the saccharides with highest DP and fraction 9 represents the saccharides with lowest DP). The linkage specificity of each fraction was recorded by ^{1}H NMR spectroscopy.

Fractions	α-1,4 (%)	α-1,6 (%)
1	6	94
2	6	94
3	11	88
4	20	80
5	51	49
6	69	31
7	94	6
8	98	2
9	100	0