Metabolism of isomalto-oligosaccharides by *Lactobacillus reuteri* and bifidobacteria

Ying Hu[1,2], Ali Ketabi[1,] Amanda Buchko[1], and Michael G. Gänzle[1*].

[1)] Department of Agricultural, Food and Nutritional Science, University of Alberta, Edmonton, AB, Canada

[2)] present address, Key Laboratory of Fermentation Engineering (Ministry of Education), Hubei Provincial Cooperative Innovation Center of Industrial Fermentation, Hubei University of Technology, Wuhan 430068, China

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* corresponding author footnote

Michael Gänzle

University of Alberta, Department of Agricultural, Food and Nutritional Science

4-10 Ag/For Centre, Edmonton, AB, Canada, T6G 2P5

tel + 1 780 492 0774; fax, + 1 780 492 4265; e-mail, mgaenzle@ualberta.ca

Significance and impact of the study.

Isomalto-oligosaccharides (IMO) are applied as functional food ingredients but the composition and biological functionality of current commercial products is poorly documented.

This study is the first to analyse IMO metabolism by *Lactobacillus reuteri*. Bifidobacteria were used for comparison. Commercial IMO contained IMO with degre of polymerisation (DP) of up to four, and panose-series oligosaccharides with DP of up to 5. *L. reuteri* preferentially metabolized short chain oligosaccharides whereas bifidobacteria preferentially metabolized higher oligosaccharides. Results of this study allow the modification of the biological and technological functionality of commercial IMO by adjustment of the degree of polymerization, and will thus facilitate the application development for IMO.

Abstract.

Commercial isomalto-oligosaccharides (IMO) are functional food ingredients. They are composed of α(1→6) and α(1→4) linked oligosaccharides. IMO are partially indigestible and dietary IMO stimulate beneficial members of intestinal microbiota, including lactobacilli and bifidobacteria. However, data on IMO metabolism by lactobacilli are not available. It was the aim of this study to identify metabolic pathways of IMO metabolism in lactobacilli. This study focused on the host-adapted species *Lactobacillus reuteri*. Metabolism of bifidobacteria were analysed for comparison. Commercial IMO contained IMO with a degree of polymerization (DP) of up to four, and panose-series oligosaccharides (POS) with a DP of up to 5. Lactobacilli metabolised isomaltose preferentially over oligosaccharides with higher DP. Bifidobacteria preferentially metabolised oligosaccharides with higher DP and accumulated glucose. Metabolism of IMO and POS by *L. reuteri* was attributed to α(1→6)-specific glucanase DexB and maltose phosphorylase. Contribution of maltose phosphorylase was verified by quantification of IMO and POS phosphorolysis in crude cellular extracts of *L. reuteri* 100-23. In conclusion, metabolism of IMO by lactobacilli is limited to short chain oligosaccharides while bifidobacteria preferentially metabolise oligosaccharides with higher DP. The functionality of commercial IMO can thus be modified by degree of polymerization.

Key words: Lactobacillus, Bifidobacterium, isomalto-oligosaccharides (IMO), maltose phosphorylase

Introduction

Non-digestible oligosaccharides are used as functional food ingredients. Bulking properties and sweetness are technological properties relevant for food applications. Reduced cariogenicity, digestibility, caloric content, and selective colonic fermentation by specific bacterial groups, particularly bifidobacteria and lactobacilli, are relevant functional properties of oligosaccharides (Seibel and Buchholz, 2010, Goffin et al., 2011; Gänzle, 2012). Application development for oligosaccharides thus requires scientific data on their chemical properties as well as their digestibility and metabolism by intestinal microbiota. Extensive documentation on chemical composition and biological functionality is particularly available for fructo-oligosaccharides and galacto-oligosaccharides, and their metabolism by bifidobacteria and lactobacilli is well understood (reviewed by Macfarlane et al., 2007; Roberfroid, 2007, van den Broek et al., 2008; Gänzle and Follador, 2012).

Fructo-oligosaccharides and galacto-oligosaccharides are widely used in food products in European and North American markets (Seibel and Buchholz, 2010; Goffin et al., 2011). Isomalto-oligosaccharides (IMO) are applied as food ingredients in Asia but they increasingly find application in Western countries (Seibel and Buchholz, 2010, Goffin et al., 2011). Commercial IMO are partially indigestible and are fermented by human colonic microbiota (Kohmoto et al., 1992; Oku and Nakamura, 2003). Consumption of commercial IMO increased the proportion of bifidobacteria and lactobacilli in fecal microbiota of humans and rodents (Kohmoto et al., 1991; Yen et al., 2011; Ketabi et al., 2011). To understand mechanisms underlying the specific stimulation of beneficial intestinal bacteria, knowledge on bacterial oligosaccharide metabolism is necessary. The metabolism of α-glucans by bifidobacteria is mediated by an extracellular amyopullulanase, followed by transport and metabolism of glucose

and disaccharides (Ryan et al., 2006, Van den Broek et al., 2008). Data on the metabolism of commercial IMO by lactobacilli, however, are not available. It was the aim of this study to determine metabolism of IMO by lactobacilli. This study focused on *Lactobacillus reuteri*, a species that has adapted to specific intestinal habitats (Walter, 2008). Bifidobacteria were analysed for comparison. Commercial IMO preparations contain α(1→4)-, α(1→2)-, and α(1→3)-linked gluco-oligosaccharides and panose-series oligosaccharides (Goffin et al., 2011; Ketabi et al., 2011). In this communication, the terms "IMO" and "commercial IMO" are used to differentiate between α(1→6)-linked gluco-oligosaccharides (IMO) and commercial preparations containing panose-series oligosaccharides in addition to IMO, respectively.

Results and Discussion

Composition of a commercial IMO preparation. The IMO preparation was reported to contain isomaltose, isomaltotriose, panose, and 6'glucosyl-panose (Ketabi et al., 2011). This study additionally quantified isomaltotetra-, penta-, hexa- and heptaose with external standards. The IMO preparation contained two series of oligosaccharides (Figure 1). IMO result from transglucosylation with glucose as glucosyl-acceptor. Isomaltose, isomaltotriose, and isomaltotetraose were detected in decreasing concentration; isomaltopentaose and higher oligosaccharides were below the detection limit. Panose-series oligosaccharides result from transglucosylation with maltose as glucosyl-acceptor (Seibel and Buchholz, 2010). Panose, 6'glucosyl-panose, and 6'6'diglucosyl-panose were detected; higher panose-series oligosaccharides as well as glucose and maltose were below the detection limit. The concentration of isomaltose, isomaltotriose, and panose matched previous reports (Ketabi et al., 2011); isomaltotetraose accounted for 3.3 ± 2.6 % (w/w) of the IMO preparation. Panose series oligosaccharides with higher DP were not quantified due to the lack of standards. Minor peaks in

93 the chromatogram of commercial IMO may arise from transglucosylation of other acceptor
94 carbohydrates (e.g. maltotriose) or from formation of α(1→2) or α(1→3) linked oligosaccharides
95 (Goffin et al., 2011).

96 Commercial IMO are predominantly obtained from fungal glycosyltransferases using
97 maltodextrins as feedstock (Pan and Lee; 2005; Seibel and Buchholz, 2010). However,
98 commercial IMO differ substantially with regards to their composition, and thus in digestibility,
99 caloric content and utilisation by intestinal microbiota. Functional studies on IMO were
100 conducted with preparations containing up to 40% monosaccharides and digestible disaccharides.
101 In addition, oligosaccharides with higher DP were not characterized due to lack of authentic
102 standards (Kohmoto et al. 1992; Kaneko et al. 1994; Yen et al. 2010). The identification of all
103 major components of commercial IMO as achieved in this study is necessary to assess
104 digestibility and related functional properties of commercial products.

105 **Metabolism of IMO by lactobacilli and bifidobacteria.** To compare metabolism of IMO by
106 lactobacilli and bifidobacteria, cultures were grown in modified MRS containing commercial
107 IMO as sole carbon source. This initial comparison of chromatograms revealed major differences
108 in metabolism by lactobacilli and bifidobacteria. *L. reuteri* 100-23 metabolised only isomaltose in
109 the first 24 h of growth and metabolised tri- and tetrasaccharides in later stages of fermentation
110 (Figure 1 of the online supplementary material). *B. longum* ssp. *infantis* converted higher
111 oligosaccharides in the first 24 h of growth, and accumulated glucose (Figure 2 of the online
112 supplementary material).

113 The time course of oligosaccharide metabolism was determined for *L. reuteri* 100-23 and
114 LTH5795 (Figure 2A and 2B, respectively). Fermentations with *B. longum* ssp. *infantis*,
115 *B. longum*, and *B. breve* (Figure 3A, 3B, 3C) were performed for comparison. In Mal-MRS,

maltose was metabolised in the first 24 h of fermentation (data not shown). In IMO-MRS, both strains of *L. reuteri* metabolised 80% - 90 of isomaltose in the first 24 h of growth. Isomaltotriose and panose were degraded by 50% after 24 h of growth. Metabolism of isomaltotetraose was apparent only in later stages of fermentation (Figure 2). Metabolism of IMO produced equimolar amounts of lactate and ethanol (data not shown).

Analysis of the culture supernatants of bifidobacteria focused on major components, i.e. glucose, isomaltose, and isomaltotriose (Figure 3). Panose consumption was comparable to the time course of isomaltotriose consumption (Figure 3 and data not shown). Bifidobacteria metabolised oligosaccharides with higher DP and accumulated glucose in the first 24 h of growth (Figure 3). *Bifidobacterium longum* accumulated glucose and partially consumed isomaltose and isomaltotriose after 24 h. The concentrations of isomaltose and glucose remained essentially constant during subsequent fermentation (Figure 3A and 3B). *B. breve* accumulated less glucose and preferentially metabolized isomaltotriose. Isomaltose concentrations increased during fermentation, indicating its release from higher oligosaccharides rather than hydrolysis (Figure 3C). Isomaltose was detected in cultures of all three strains of *Bifidobacterium* spp. after 72 h of fermentation. *B. breve* but not *B. longum* or *B. longum* ssp. *infantis* metabolised glucose in later stages of growth (Figure 3). Consumption of IMO by bifidobacteria conforms to metabolism by extracellular amylopullulanase, followed by uptake of mono- and disaccharides (Ryan et al., 2006; van den Broek et al., 2008). Similarly, extracellular glycosyl hydrolases contribute to metabolism of galacto-oligosaccharides and inulin by bifidobacteria (Gopal et al., 2001; van den Broek et al., 2008; Møller et al., 2011). Bifidobacteria produced acetic and lactic acids in a molar ratio of 1.8 – 2.3 (data not shown), which was in agreement with prior studies using levan as

substrate (Korakli et al., 2002) and the metabolite profile produced from maltose (data not shown).

Metabolic pathways for IMO in *L. reuteri*. A substantial body of information is available on enzymes involved in metabolism of isomalto-oligosaccharides in *L. acidophilus* and *L. plantarum* (Nakai et al., 2009 and 2010; Gänzle and Follador, 2012). These organisms harbour an oligosaccharide transporter, several intracellular glycosyl hydrolases degrading α(1→4)- or α(1→6)-linked glucans, and maltose phorphorylase (Nakai et al., 2009 and 2010). Few lactobacilli additionally express extracellular amylopullulanases (Turpin et al., 2011; Gänzle and Follador, 2012). In contrast, DexB and MalP are the only α-glucan-active enzymes in *L. reuteri* 100-23 (Gänzle and Follador, 2012). The α-glucanase DexB is specific for α(1→6)-linked oligosaccharides but does not cleave α(1→4)-linkages (Møller et al., 2012). Maltose-phosphorylase MalP is highly specific for maltose (Ehrmann and Vogel, 1998; Nakai et al., 2010). The genes coding for maltose phosphorylase in *L. acidophilus* NCFM and *L. reuteri* 100-23 are 72% identical (Nakai et al., 2009) and the catalytic domain that determines substrate specificity of maltose phosphorylases is virtually identical in *L. reuteri* and *L. acidophilus* (Nakai et al., 2009 and 2010). When acting in concert, DexB and MalP completely convert IMO and panose-series oligosaccharides to glucose and glucose-1-phosphate (Figure 4). To confirm the involvement of maltose phosphorylase in IMO metabolism, MalP activity was assessed in crude cellular extract of *L. reuteri* grown in either IMO-MRS or Mal-MRS. With commercial IMO as substrate and a phosphate concentration of 0, 10, or 100 mmol L^{-1}, the specific maltose phosphorylase activity was 0.38 ± 0.18, 0.68 ± 0.12 and 1.19 ± 0.12, respectively. The maltose phosphorylase activity with maltose as substrate and 0, 10, or 100 mmol L^{-1} phosphate was 0.12 ±

0.06, 0.87 ±0.06, and 1.41 ± 0.12 respectively. Maltose phosphorylase activities were not significantly different when maltose or commercial IMO were used as substrate ($P > 0.05$).

Transport enzymes specific for α-glucans were not characterized in *L. reuteri* or annotated in *L. reuteri* genomes (Gänzle and Follador, 2012). In *L. sanfranciscensis*, a species harbouring identical enzymes for maltose and IMO metabolism, maltose is transported by proton symport (Neubauer et al., 1994). In this study, disaccharides were preferentially metabolised over tri- and tetrasaccharides, Metabolism of pentasaccharides was not observed. Since DexB preferentially hydrolyses oligosaccharides with a DP of 3 or higher, (Møller et al., 2012), this sequential metabolism likely reflects transport limitations.

Comparison of IMO metabolism in lactobacilli and bifidobacteria: implications for intestinal ecology and commercial applications of IMO. *L. reuteri* metabolised IMO and panose-series oligosaccharides exclusively by intracellular enzymes and metabolism was limited to di- tri- and tetrasaccharides. This transport-induced preference for short chain oligosaccharides matches the substrate preference of a majority of lactobacilli (Gänzle and Follador, 2012). In contrast, bifidobacteria hydrolyse α-glucans with an extracellular amylopullulanase and preferentially metabolise oligomeric- or polymeric carbohydrates (Ryan et al., 2006). These different substrate preferences reflect the adaptation of lactobacilli and bifidobacteria to the upper intestinal tract of animals and the colon of human and animals respectively (Bivati et al., 2000; van den Broek, 2008; Walter, 2008). Mono- and disaccharides are abundant in those intestinal ecosystems harboring stable populations of lactobacilli, e.g. the crop of poultry, the forestomach of rodents, and the pars oesophagus of swine (Walter, 2008, Tannock et al., 2012). However, the availability of carbohydrates in the colon is restricted to non-digestible oligo- and polysaccharides, and host secretions (van den Broek et al., 2008).

The different metabolic pathways of α-glucans in lactobacilli and bifidobacteria are reflected by divergent effects of commercial IMO and resistant starch on intestinal microbiota. Commercial IMO specifically increase the proportion of lactobacill in the intestine of rats (Ketabi et al., 2011) and increase the numbers of lactobacilli and bifidobacteria in human fecal microbiota (Kohmoto et al., 1991; Yen et al., 2011). In contrast, dietary resistant starch stimulated bifidobacteria but not lactobacilli in rodent and human fecal microbiota (Martinez et al., 2010; Rodriguez-Cabezas et al., 2010). It is thus likely that the biological functionality of IMO and related α-glucans can be modulated by adjusting the DP. Adjusting the DP of commercial IMO also alters other parameters that are relevant to current commercial applications, particularly their digestibility, caloric value and relative sweetness (Goffin et al., 2011).

Materials and methods

Strains and culture conditions. The rodent isolates *L. reuteri* 100-23 (Tannock et al., 2012) and *L. reuteri* LTH5795 were grown in modified de Man Rogosa Sharpe media (Galle et al., 2010). *L. reuteri* 100-23 was chosen because genome sequence data is available for this strain (Genebank Accession number AAPZ00000000.2). Media contained 10 g L^{-1} of maltose (Mal-MRS), or 10 g L^{-1} of a commercial IMO preparation (Vitasugar, BioNeutra Inc., Edmonton, Canada) (IMO-MRS) as sole carbon source. Agar plates inoculated with lactobacilli were incubated in anaerobic jars under an atmosphere of 1% O_2, 5% CO_2, 10% H_2, balance N_2. Bifidobacteria were incubated anaerobically.

Cultures were streaked on mMRS agar and subcultured twice in Mal-MRS broth with 1% inoculum. Lactobacilli and bifidobacteria were grown in IMO-MRS and compared to Mal-MRS as reference. For quantification of substrates and metabolites, cultures were sampled after 8, 24,

48, and 72 h of incubation. Cells were removed by centrifugation, and samples were analysed as indicated below. All fermentations were carried out in duplicate independent experiments.

Quantification of substrates and metabolites. Oligosaccharides in the supernatant were analysed by high performance liquid chromatography with pulsed amperometric detection (HPAEC-PAD) with a Carbopac PA20 column at 25°C combined with an ED40 chemical detector (Dionex, Oakville, Canada) as described (Ketabi et al., 2011). Isomaltose, isomaltotriose, panose, maltose and glucose were obtained from Sigma; authentic standards of isomaltoetraose, isomaltopentaose, isomaltohexaose, and isomaltoheptaose were obtained from Seikagaku Biobusiness Co. (Tokyo, Japan). 6'Glucosylpanose and 6'diglucosylpanose were qualitatively identified by comparison with oligosaccharides produced by dextransucrase of *Weissella* spp. with maltose as acceptor carbohydrate (Galle et al., 2010). Culture supernatants were diluted 100-fold and analysed in duplicate. To analyse organic acids, ethanol, and monosaccharides, 50 µL of perchloric acid (70%) was added to 1 mL of culture supernatants and incubated at 4°C overnight. Solids were removed by centrifugation. Samples were analysed in duplicate by HPLC (1200 series, Agilent Technologies, USA) equipped with an Aminex HPX 87H column (Biorad) (Dlusskaya et al., 2008). Glucose, maltose, lactate, acetate, fumarate, and ethanol (all from Sigma) were used as external standards.

Maltose phosphorylase activity of lactobacilli. Maltose phosphorylase activity of *L. reuteri* 100-23 and *l. reuteri* LTH5795 was quantified (Stolz et al. 1996). *L. reuteri* 100-23 was grown on Mal-MRS agar followed by two consecutive subcultures in Mal-MRS or IMO-MRS broth. Cells were harvested from overnight cultures in IMO-MRS broth. They were washed two times with 10 $mmoL^{-1}$ citrate buffer (pH 6.0), and resuspended in the same buffer in a tube containing silica beads. Cells were kept on ice and disrupted with mini bead beater (Biospec Products,

Bartlesville, USA) for three 1 min cycles. Cell debris was removed by centrifugation at 17000 x *g* for 8 min. The supernatant was collected as a crude cellular extract. The protein concentration of crude cellular extracts was quantified by the Bradford assay (Bio-Rad). Bovine serum albumin was used as external standard (Invitrogen, Burlington, ON, Canada).

Phosphorylase activities of crude cellular extracts were quantified in citrate buffer (100 mmol L^{-1}, pH 6.0) or potassium phosphate buffer (10 mmol L^{-1} and 100 mmol L^{-1}, pH 6.0) as indicated. Reactions contained 50 µL of 20 mmol L^{-1} magnesium sulphate, 25 µL of 4 g L^{-1} glucose 6-phosphate dehydrogenase from *Saccharomyces cerviseae*, 100 µL of 10 mmol L^{-1} NADP, 100 µL of 0.5 mmol L^{-1} maltose (all chemicals obtained from Sigma) or IMO-preparation, 20 µL crude cellular extract from *L. reuteri* 100-23 or LTH5795, and reaction buffer to a final volume of 1 mL. Control reactions did not contain crude cellular extracts. Reactions were carried out at 37ºC and the production of NADPH was measured at 340 nm. One unit of maltose phosphorylase activity was calculated as reduction of 1 µmol NADP (min x mg protein)$^{-1}$ (Stolz et al., 1996).

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Figure legends.

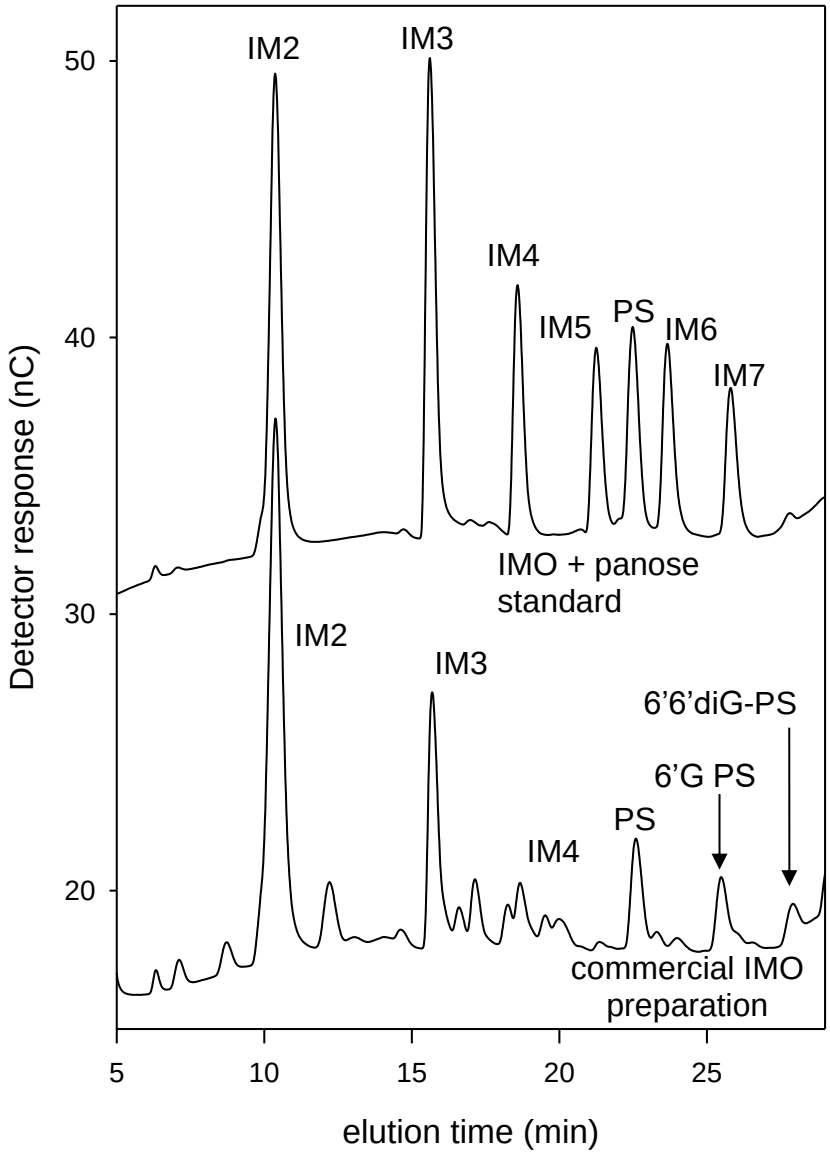
Figure 1. Separation of isomalto-oligosaccharides and panose (upper trace), and commercial IMO (lower trace) by high performance anion exchange chromatography coupled to pulsed amperometric detection. Compounds identified by external standards are labelled as follows: IM2, isomaltose; IM3, isomaltotriose; IM4, isomaltotetraose; IM5, isomaltopentaose; IM6, isomaltohexaose; IM7, isomaltoheptaose; PS, panose, 6'G-PS, 6'glucosyl-panose; 6'6'diG-PS, 6'6'-di-glucosyl-panose. Chromatograms are offset by 30 nC.

Figure 2. Concentration of carbohydrates during growth of *L. reuteri* 100-23 (Panel A) and *L. reuteri* LTH5795 (panel B) in IMO-MRS. Shown are the concentrations of isomaltose (❍), isomaltotriose (❑), panose (■), and isomaltotetraose (Δ). Concentrations of glucose, maltose, and isomaltopentaose were below detection limit in all samples. Data are shown as average of two independent fermentations analysed in duplicate.

Figure 3. Concentration of carbohydrates during growth of *B. longum* ssp. *infantis* ATCC15697 (Panel A), *B. longum* ATCC15707 (Panel B), and *B. breve* ATCC15700 (Panel C) in IMO-MRS. Shown are the concentrations of isomaltose (❍), isomaltotriose (❑), and glucose (▼). Data are shown as average of two independent fermentations analysed in duplicate.

Figure 4. Overview on IMO metabolism in *L. reuteri* 100-23. The strain harbours maltose phosphorylase MalP with high specificity for maltose, and the α-glucanase DexB with high specificity for a-(1→6) linked oligosaccharides as only α-glucan hydrolysing enzymes. Phosphoglucomutase PgmB converts glucose-1-phosphate to glucose-6-phosphate, hexokinase HK catalyzes the ATP-dependent phosphorylation of glucose. Oligosaccharide transport enzymes are not characterized in this strain or annotated in the genome.

362 **Figure 1.**



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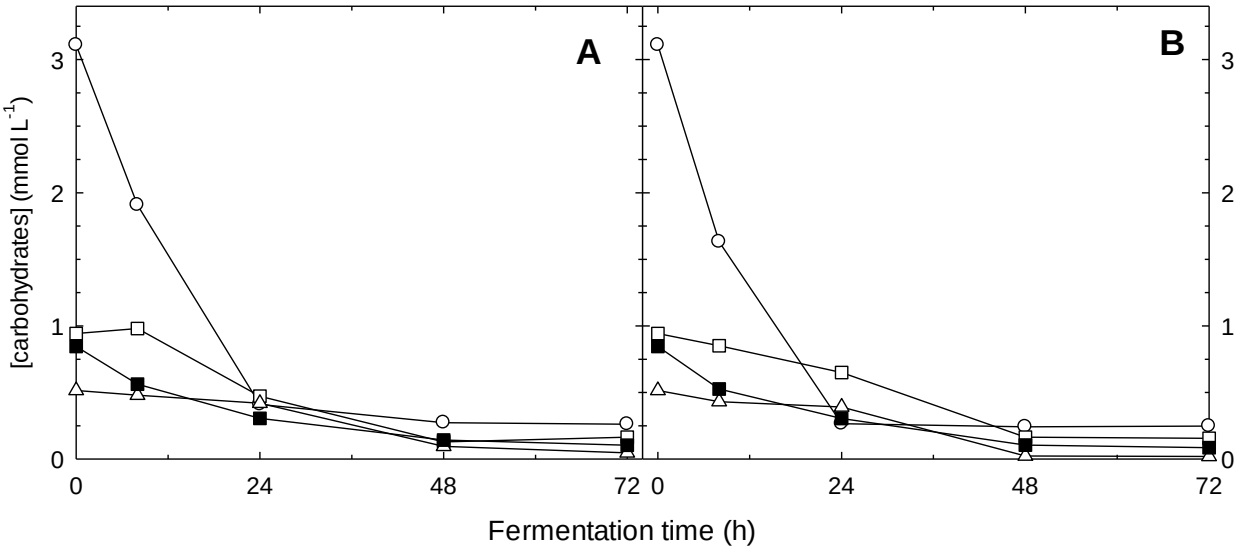
365 **Figure 2.**



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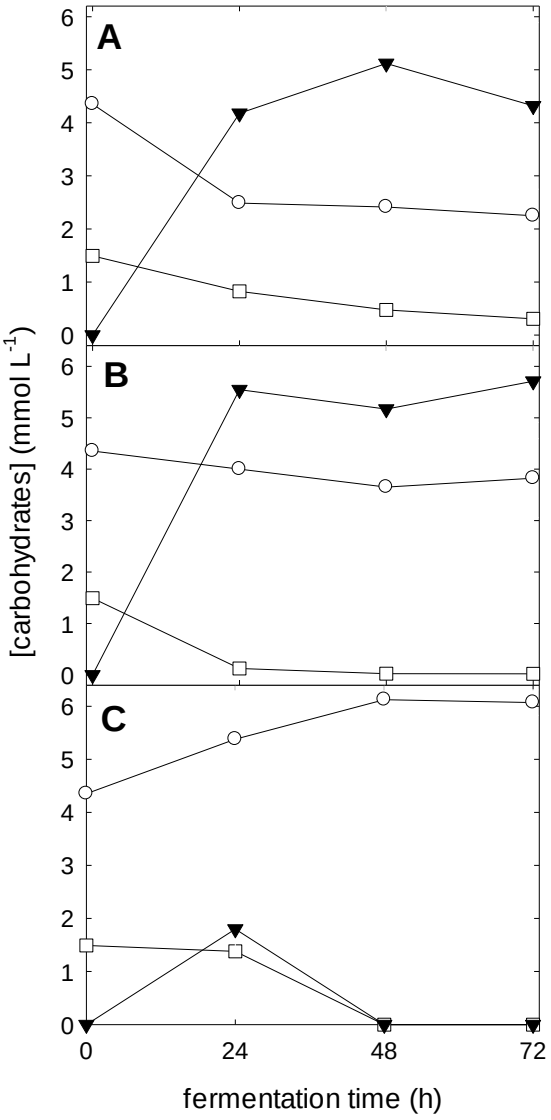
368 **Figure 3.**



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372 **Figure 4.**

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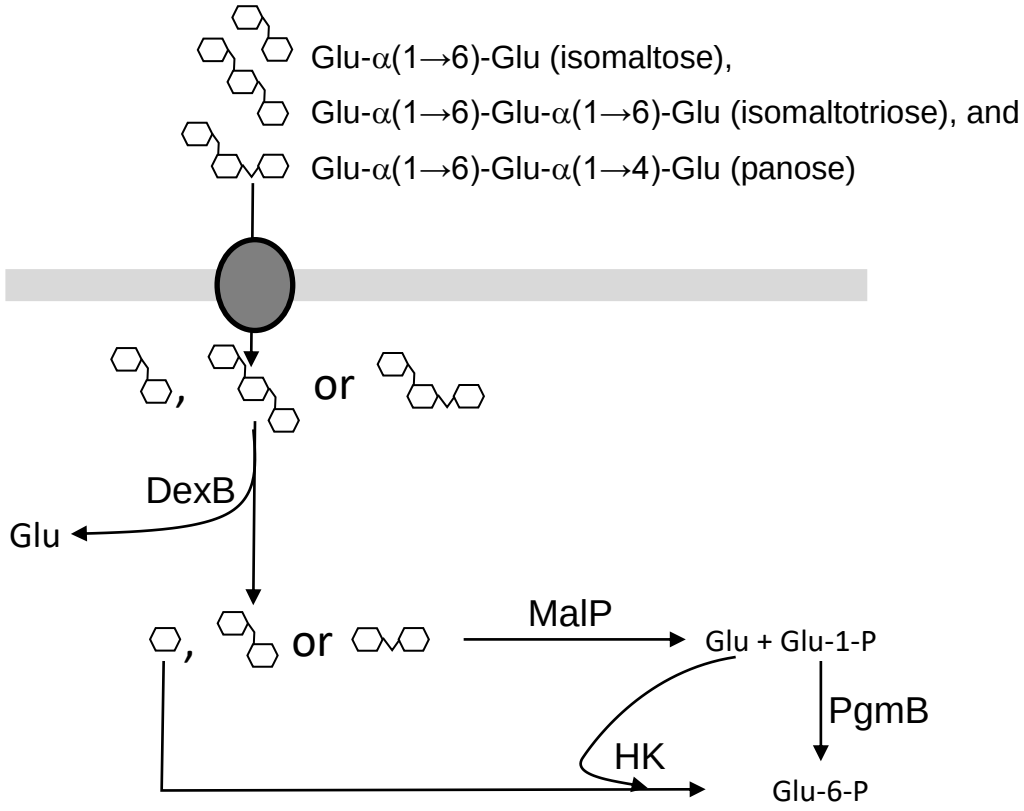

Figure 1 of the online supplementary material. HPLC chromatogram showing oligosaccharides profiles of IMO before (0 h), after 24 h, and after 72h of fermentation by *L. reuteri* 100-23 in mMRS. Chromatograms are offset by 8 nC and are representative for two independent fermentations.

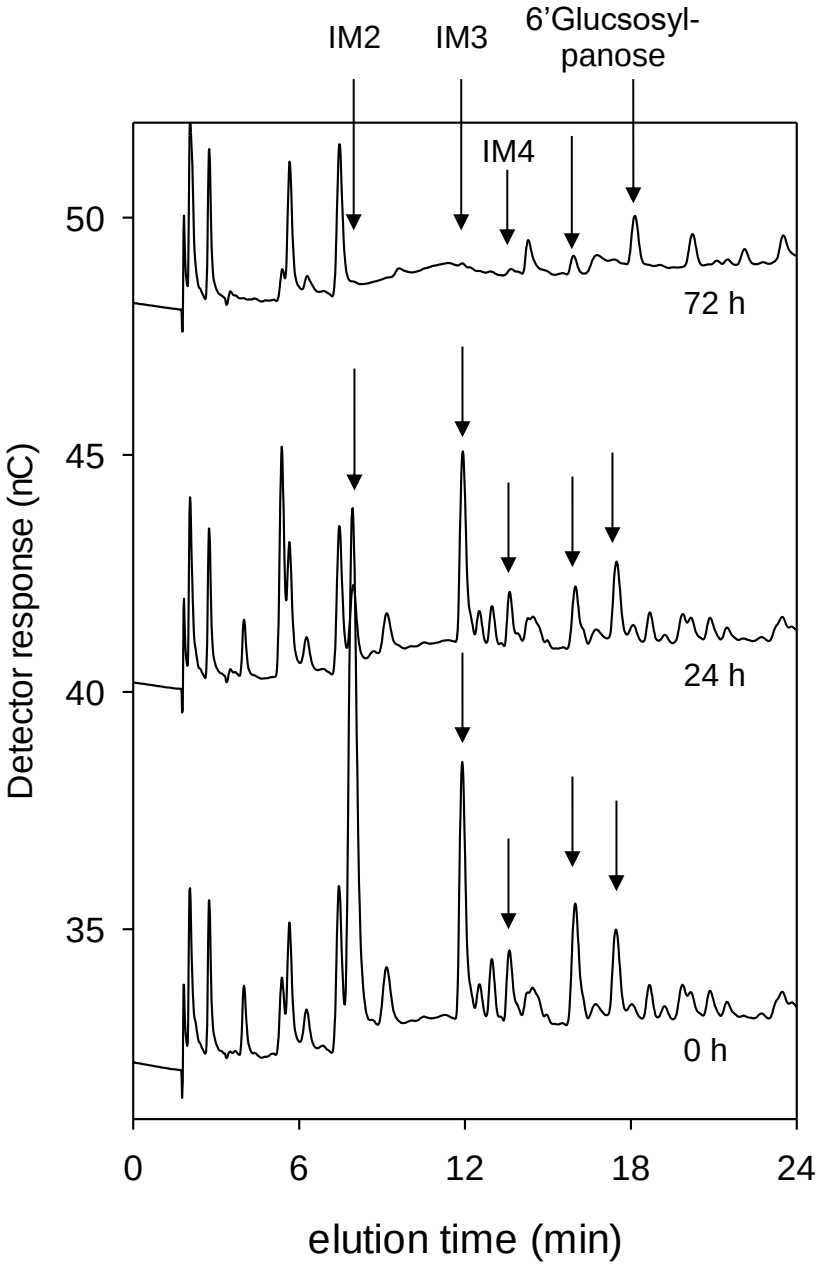

Figure 2 of the online supplementary material. HPLC chromatogram showing oligosaccharides profiles of IMO after 0h, 24h and 72 h of fermentation by *B. longum* spp. *infantis* ATCC 15697 in mMRS. Chromatograms are offset by 150 nC and are representative for two independent fermentations.