In vitro digestibility of commercial and experimental isomalto-oligosaccharides

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Abstract

Isomalto-oligosaccharides (IMO) significantly contribute to the global oligosaccharide market. IMO are linear α-(1→6) linked oligosaccharides with isomaltotriose as the representative trisaccharide. Commercial IMO preparations ypically also contain panose-series oligosaccharides as a major component. In humans, IMO are partially digestible but the digestibility of specific components of commerical IMO preparations remains unknown. This study aimed to compare the *in vitro* digestibility of reference compounds, experimental α-gluco-oligosaccharides and commercial IMO. Experimental α-gluco-oligosaccharides were synthesized with the recombinant dextransucrase DsrM. Two *in vitro* digestion methods were used, a reference method matching the AOAC method for dietary fibre, and a protocol that uses brush border glycosyl hydrolases from the rat intestine. The α-gluco-oligosaccharides patterns after hydrolysis remain were analyzed by high performance anion exchange chromatography coupled to pulsed amperometric detection. Panose-series oligosaccharides were hydrolysed more rapidly by amylase and amyloglucosidase when compared to hydrolysis by rat intestinal enzymes. The rate of hydrolysis by rat intestinal enzymes decreased in the order panose > isomaltose, kojibiose or nigerose. Hydrolysis of panose-series oligosaccharides but not the hydrolysis of isomalto-oligosaccharides was dependent on the degree of polymerization. Qualitative analysis of oligosaccharides remaining after hydrolysis indicated that rat small intestinal enzymes hydrolyse their substrates from the non-reducing end. Taken together, results inform on the modification or optimization of current production processes for IMO to obtain tailored oligosaccharide preparations with reduced digestibility and an increased content of dietary fibre.

Keywords. Isomaltose, panose, isomalto-oligosaccharides; digestibility, brush border enzymes, dextransucrase, prebiotic.

1. Introduction

Non-digestible oligosaccharides resist digestion in the small intestine and are fermented to short chain fatty acids by intestinal microbiota. The conversion of carbohydrates to short chain fatty acids benefit human health and support the use of non-digestible oligosaccharides as functional food ingredients (Bindels, Delzenne, Cani, & Walter, 2015; Yan, Hu, & Gänzle, 2018). Commercial oligosaccharides including fructo-oligosaccharides, galaco-oligosaccharides, xylo-oligosaccharides and isomalto-oligosaccharides are extracted from natural sources or synthesized enzymatically (Courtois, 2009; Nakakuki, 2002; Seibel & Buchholz, 2010). The composition, the linkage type and the degree of polymerization are key characteristics that determine the *in vivo* digestibility of oligosaccharides by brush border enzymes in the small intestine (Hooton, Lentle, Monro, Wickham, & Simpson, 2015; Sanz, Gibson, & Rastall, 2005). Isomalto-oligosaccharides (IMO) are significant contributors to the global oligosaccharide market (Nakakuki, 2002) but their *in vivo* digestibility is poorly characterized. The method of production determines the degree of polymerization (DP) as well as the ratio of α-(1→4) to α-(1→6) linkages in IMO and hence the digestibility. Isomaltose-series oligosaccharides are linear α-(1→6) linked oligosaccharides with isomaltotriose as the representative trisaccharide. Commercial IMO preparation additionally contain substantial proportions of panose-series oligosaccharides, which consist of two α-(1→4) linked glucose units at the reducing end which are extended by α-(1→6) linked glucose units. In addition, commercial isomalto-oligosaccharides contain oligosaccharides with α-(1→2) and α-(1→3) linkages as well as branched oligosaccharides (Madsen, Stanley, Swann, & Oswald, 2017).

Production of IMO uses enzymes from diverse glycosyl hydrolase (GH) families including GH13, GH31, GH57, GH66 and GH70 to produce IMO from starch or sucrose (Casa-Villegas, Marín-Navarro, & Polaina, 2018; Gangoiti, Lamothe, Van Leeuwen, Vafiadi, & Dijkhuizen, 2017;

Gutiérrez-Alonso et al., 2016). Commercial IMO often contain several series of oligosaccharide (Madsen et al., 2017), which impedes *in vitro* or *in vivo* experimentation to determine the digestibility of individual components. Dextransucrases of *Weissella* spp. synthesize homologous series of linear oligosaccharides with different acceptor carbohydrates (Hu, Winter, Chen, & Gänzle, 2017; Shukla et al., 2014). The degree of polymerization is controlled by the choice of the biocatalyst and by the ratio of glycosyl-acceptor and glycosyl-donors in the transglycosylation reactions (Hu et al., 2017; Robyt & Eklund, 1983). Equimolar addition of acceptor molecules and sucrose in reactions catalyzed by dextransucrase DsrM from *Weissella cibaria* 10M resulted in homologous series of linear oligosaccharides as a virtually exclusive product (Hu et al., 2017). Experimental oligosaccharides that are produced in the acceptor reaction with dextransucrase are thus a suitable tool to probe the digestibility of specific components of commercial IMO.

IMO are partially hydrolyzed *in vivo* by brush border isomaltase and maltase / glucoamylase after transport across the brush border membrane (Hooton et al., 2015; Lee et al., 2016). Major components of commercial IMO preparations, particularly panose, are readily hydrolyzed by brush border sucrase / isomaltase and maltase / glucoamylase, however, the digestion of IMO with DP3 and higher is poorly documented *in vitro* or *in vivo* (Hu, Heyer, Wang, Zijlstra, & Gänzle, 2020; Kohmoto et al., 1992; Oku, Tanabe, Ogawa, Sadamori, & Nakamura, 2011). The analysis of IMO digestion in ileal cannulated swine indicated that indigestible compounds in a commercial IMO preparation accounted for about 50% of the preparation (Hu et al., 2020); however, the digestibility of IMO is modulated by other dietary components (Koleva, Ketabi, Valcheva, Gänzle, & Dieleman, 2014; Lim, Kim, Shin, Hamaker, & Lee, 2019). Despite their partial digestibility, prebiotic properties of commercial isomalto-oligosaccharides preparations were consistently

89 demonstrated in animal and human studies (Goffin et al., 2011; Ketabi, Dieleman, & Gänzle, 2011;
90 Likotrafiti, Tuohy, Gibson, & Rastall, 2014; Lin et al., 2014).

91 Current *in vitro* digestibility assays for dietary fiber including resistant starch and non-digestible
92 oligosaccharides use pancreatic amylase and fungal amyloglucosidase (Table 1), which hydrolyze
93 linear oligosaccharides with α-(1→4) and α-(1→6) linkages (McCleary, 2019; Pazur & Ando,
94 1960). The difference in the substrate specificity of intestinal brush border enzymes and those
95 enzymes that are used in determination of dietary fibre (Table 1), and the lack of knowledge on
96 oligosaccharide degradation by intestinal brush border enzymes impedes the targeted modification
97 of commercial IMO preparations to reduce their digestibility. It was therefore the aim of this study
98 to compare the *in vitro* digestion of commercial and experimental α-gluco-oligosaccharides.
99 Oligosaccharides were digested with amylase and glucoamylase, or with rat brush border enzymes,
100 and oligosaccharides that were obtained after partial hydrolysis were analyzed by high
101 performance anion exchange chromatography coupled to pulsed amperometric detection
102 (HPAEC-PAD).

103 **2. Materials and methods**

104 2.1. Source and synthesis of oligosaccharides.

105 The composition and suppliers of the commercial oligosaccharide substrates (digestible
106 maltodextrin, resistant maltodextrin, and different commercial isomaltooligosaccharides (ISO,
107 IMO, IMO-DP3) are listed in Table 2. Maltose was obtained from Sigma; resistant starch was
108 provided by MSPrebiotic Inc (Carberry, Canada).

109 Enzymatic synthesis of different isomalto-oligosaccharides was performed as described (Chen &
110 Gänzle, 2016; Hu et al., 2017) with 50 nM recombinant dextransucrase DsrM from *Weissella*

111 *cibaria* 10M using isomaltose, maltose, nigerose, maltotriose, or IMO as the acceptor
112 carbohydrates and sucrose as donor incubated at 30°C in 25 mM sodium acetate buffer (pH 5.2)
113 containing 1 mM $CaCl_2$ for 24 h. The enzyme was inactivated by heating at 90°C for 10 min. All
114 enzymatic syntheses and analyses were carried out in duplicate or triplicate biological repeats.
115 Sucrose, maltose, glucose, and fructose were removed by addition of 10 % (v/v) of alginate-
116 immobilized commercial baker's yeast (*Saccharomyces cerevisiae*), representing 1% dry yeast
117 biomass, followed by incubation for 24 h at 30°C. Alginate encapsulated yeasts were employed to
118 facilitate removal of yeast by centrifugation at 7000 x g; the supernatant containing
119 oligosaccharides was collected and freeze-dried. The linkage type of glycosidic bonds formed by
120 DsrM was previously confirmed by NMR (Chen & Gänzle, 2016); the removal of mono- and
121 disaccharides was confirmed by HPAEC-PAD as described (Hu et al., 2017).

122 2.2. *In vitro* digestibility of commercial and experimental isomalto-oligosaccharides

123 The *in vitro* digestibility was determined with two methods that are based on different enzymes
124 (Table 1). The first method was from modified from the AOAC 2009.01 dietary fiber method for
125 starch digestibility which uses pancreatic amylase, invertase and fungal amyloglucosidase (van
126 Kempen, Regmi, Matte, & Zijlstra, 2010). The second method uses commercially available brush
127 border enzymes from the rat intestinal mucosa (Tsunehiro, Okamoto, Furuyama, Yatake, &
128 Kaneko, 1999). The enzyme solution was freshly prepared for each digestion. The AOAC method
129 but not the method using rat small intestinal enzymes includes a hydrolysis step with pepsin.
130 Because pepsin has no activity on carbohydrates, the omission of a pepsin hydrolysis step to mimic
131 gastric digestion is unlikely to alter the hydrolysis of pure carbohydrate preparations.

132 2.3. Oligosaccharide digestion with a modified AOAC method

Freeze dried oligosaccharides (1.000 g) were transferred to a 50 mL tube containing 10 mL pepsin solution, containing 50 mg pepsin (250U/mg), and 50 mg guar gum in 0.05 M hydrogen chloride (HCl) (Englyst, Englyst, Hudson, Cole, & Cummings, 1999; van Kempen et al., 2010); 5-10 glass beads (5 mm diameter) were also added to each tube. The first digestion step, mimicking the gastric digestion, lasted 30 min at 37°C with agitation at 200 rpm. Then 10 mL of 0.25 M sodium acetate solution and 5 mL of enzyme mixture containing 0.7 g pancreatin from porcine pancreas (Sigma-Aldrich) (45 U/mg lipase, 42 U/mg amylase and 3.0 U/mg protease), 3 mg invertase from *Saccharomyces cerevisiae* (Sigma-Aldrich) and 50 µL amyloglucosidase from *Aspergillus niger* (aqueous solution, ~300U/mL) (Sigma-Aldrich) were added. For the relative assessment of the *in vitro* digestibility of commercial and experimental IMO, the solution was further incubated for 4 h (Englyst et al., 1999; van Kempen et al., 2010). Aliquots (500 µL) were taken at intervals and mixed with 0.5mL absolute ethanol. The glucose content was measured by a glucose oxidase kit (Megazyme, Bray, Ireland). The time course of oligosaccharide digestion is shown in Figure S1 of the online supplementary material.

2.4. Oligosaccharide digestion with rat small intestinal enzymes

This digestion method uses acetone extract of rat intestinal mucosa (Oku et al., 2011; Tsunehiro et al., 1999). The reaction mixture containing 1 mL sample dissolved in water to 10 g/L, 1 mL of 50 mM sodium maleate buffer (pH 6.0) with 10 g/L intestinal acetone powder from rat (Sigma-Aldrich), and 3-7 glass beads (5 mm diameter) was incubated at 37 °C for 4 h with agitation at 200 rpm. The reaction was stopped by heating to 90 °C for 5 min. The samples were cooled on ice and subsequently centrifuged for 3 min at 5000 x g. The glucose concentration was measured with a glucose oxidase kit.

155 2.5. Analysis of oligosaccharides and digestion samples by high-performance anion-exchange
156 chromatography with pulsed amperometric detection (HPAEC-PAD)

157 Samples were diluted with water and separated with a Dionex ICS-3000 Ion Chromatography
158 System (Dionex, Oakville, Canada) equipped with a Carbopac PA20 column. Water (A), 0.2 M
159 NaOH (B) and 1 M NaOAc (C) were used as eluents at 0.2 mL/min with the following gradient: 0
160 min, 68.3% A, 30.4%B and 1.3%C; 25 min, 54.6% A, 30.4% B and 15.0% C; 28min, 50% A and
161 50% C; 31min, 10% A, 73% B and 17%C; followed by re-equilibration. Glucose, fructose,
162 sucrose, isomaltose, nigerose, maltose, isomaltotriose, panose, and maltotriose were used as
163 external standards. Consistent with IUPAC nomenclature, all oligosaccharides that could be
164 assigned a precise DP by HPAEC-PAD, i.e. oligosaccharides with a DP of up to 30, were termed
165 oligosaccharides to differentiate these from polymeric dextran.

166 2.6. Statistical analysis

167 The *in vitro* digestibility was determined relative to maltose in triplicate experiments; results are
168 presented as means ± SEM. Data analysis was performed with one way or two way Analysis of
169 Variance (ANOVA) and Tukey post hoc analysis (PASW Statistics 18.0, Quarry Bay, HK, China)
170 and assessed at a 5% probability of error ($P<0.05$).

171 **3. Results**

172 3.1. Comparison of the *in vitro* hydrolysis by two digestion methods

173 This study used a modified AOAC method and a digestion method using rat small intestinal
174 enzymes (Table 1). The digestibility of different commercial and experimental isomalto-
175 oligosaccharides was calculated relative to the digestibility of maltose (Fig. 1). Enzymatically
176 prepared α-(1→6)- extended maltotriose, nigerose, and isomaltose were used only with the method

using rat small intestinal enzymes because of their synthesis did not provide sufficient quantities for use in the modified AOAC protocol. Maltodextrins were fully digestible in both methods; resistant starch and resistant maltodextrins were essentially indigestible with both methods. IMO-DP3 were highly digestible, followed by IMO and ISO-Thrive (Fig. 1). The digestibility of IMO was reduced after extension with α-(1→6) linked glucose. The digestibility of experimental IMO produced by extension of maltose, isomaltose, or maltotriose with α-(1→6) linked glucose with the method using rat small intestinal enzymes was comparable to each other and to commercial IMO. With the modified AOAC method, the digestibility of panose-series IMO was higher when compared to isomaltose-series IMO (Fig. 1). The relative digestibility of nigerose-series IMO in the method using rat small intestinal enzymes was lower than the digestibility of panose-series IMO.

3.2. *In vitro* digestion of reference compounds

The digestibility of pure oligosaccharides was evaluated with rat intestinal enzymes using maltose and maltodextrins as digestible controls (Fig. 2). The selection of oligosaccharides included compounds that are present in commercial IMO preparations, analogous α-gluco-oligosaccharides, and trehalose, which is substrate for brush border trehalase (Table 1 and Table 2). The digestibility was highest for panose and lowest for cellobiose. The digestibility of kojibiose and isomaltose was comparable and the digestibility of isomaltose was lower than the digestibility of panose. Remarkably, the degree of polymerization of isomaltose-series IMO did not have a major effect on their digestibility (Fig. 2).

3.3. Qualitative assessment of oligosaccharide hydrolysis by brush border enzymes and amylase / amyloglucosidase

Oligosaccharide profiles before and after hydrolysis were analysed by HPAEC-PAD to assess qualitative differences between the two *in vitro* digestion methods. The HPAEC-PAD profiles of maltose, maltodextrin and resistant maltodextrin are shown in Fig. 3. All profiles obtained with the modified AOAC method generated an unknown peak around 10 min, indicating that this peak is not related to the substrates but originates from the enzyme preparation. Maltose and maltodextrins were completely hydrolyzed after both *in vitro* digestion methods (Fig. 3A and 3B). Resistant maltodextrins with higher DP (around 10 to 15) and complex branches were resistant to digestion by rat intestinal enzymes and only a small amount of glucose was released (Fig. 3C). However, a higher amount of glucose eluting at 7.5 min was released from resistant maltodextrin after digestion with modified AOAC method.

3.4. *In vitro* digestion for commercial isomalto-oligosaccharides

Three commercially available isomalto-oligosaccharides IMO, IMO-DP3 and ISO were also compared with two *in vitro* digestion methods. IMO and IMO-DP3 are comprised of two series oligosaccharides (isomaltose-series DP2 to DP5) and panose-series (DP3' to DP5'). IMO showed higher contents of isomaltose (DP2) and isomaltotriose (DP3) than IMO-DP3; the latter showed a higher content of panose (DP3') (Fig. 4A and 4B). Digestion of IMO and IMO-DP3 with rat small intestinal enzymes decreased the levels of isomaltotetraose and panose-series oligosaccharides but increased the levels of isomaltose and isomaltotriose. Most of the higher oligosaccharides eluting between 24 to 32 min were completely or partially hydrolyzed (Fig. 4A and 4B). Digestion with the modified AOAC method decreased the levels of all oligosaccharides, including isomaltose and isomaltotriose. ISO contained maltose and of linear panose-series oligosaccharides (DP3-7') (Fig. 4C); peak shoulders in the DP4' to DP7' peaks indicate the presence of other oligosaccharides with a different linkage. Digestion of ISO with the method using rat small intestinal enzymes

222 hydrolysed all panose-series oligosaccharides and accumulation of isomaltose was not observed
223 (Fig. 4C); a series of peaks remained which likely represents a homologous series of
224 oligosaccharides with linkages other than α-(1→4) or α-(1→6) linkages. Digestion with the
225 modified AOAC method reduced the levels of all oligosaccharides including the unknown peak
226 series, and accumulated isomaltose.

227 3.4. *In vitro* digestion for experimental isomalto-oligosaccharides

228 Commercial IMO preparations contain more than one series of oligosaccharides. To further study
229 oligosaccharide hydrolysis by intestinal enzymes, experimental oligosaccharides were synthezised
230 with DsrM and different acceptor carbohydrates. DsrM extends the acceptor carbohydrates
231 maltose, isomaltose, nigerose, and maltotriose almost exclusively by addition of one or more
232 α-(1→6) linked glucose moieties (Hu et al., 2017). The part of the maltose that remained after
233 yeast hydrolysis and panose-series oligosaccharides were almost completely hydrolysed by rat
234 small intestinal enzymes (Fig. 5A); only few peaks that represent higher oligosaccharides or
235 α-(1→3) linkages remained. Hydrolysis of isomaltose-series (IM) oligosaccharides by rat small
236 intestinal enzymes was less extensive; isomaltose (IM2) levels were unchanged; the level of
237 isomaltotriose (IM3) decreased slightly while IM6 and IM7 were almost completely hydrolysed
238 (Fig. 5B). Nigerose is not as efficient as maltose or isomaltose as acceptor in the DsrM mediated
239 oligosaccharide synthesis; therefore, glucose transfer to nigerose is slower than glucose transfer to
240 α-(1→6) extended nigerose and the average degree of polymerization of oligosaccharides is higher
241 when compared to maltose and isomaltose (Fig. 5C). After digestion with rat small intestinal
242 enzymes, the level of nigerose decreased; oligosaccharides with DP 3 and 4 increased while
243 oligosaccharides with DP 5-6 decreased (Fig. 5C). Isomalto-oligosaccharides generated with
244 maltotriose as acceptor were rapidly hydrolysed by rat small intestinal enzymes; hydrolysis of low

DP oligosaccharides was more extensive than hydrolysis of high DP oligosaccharides (Fig. 5D). Two peaks were generated after hydrolysis with rat small intestinal enzymes; isomaltose and an unknown peak with a retention time of 22 min (Fig. 5D). In summary, hydrolysis of IMO depended on the linkage type at the reducing end and increased in the order of α-(1→4) > α-(1→6) >> α-(1→3). Accumulation of isomaltose was observed after hydrolysis of oligosaccharides with α-(1→4) linkages at the reducing end. These results conform to the relative digestibility that was calculated from the glucose release (Fig. 1).

3.6. *In vitro* digestion of dextransucrase extended commercial IMO

We also evaluated the hydrolysis of commercial IMO after their DP was increased through extension with α-(1→6) linked glucose moieties in the acceptor reaction with DsrM. The DsrM acceptor reaction particularly increased the DP of isomaltose series oligosaccharides from DP2 – 4 to DP3 – DP7 (Fig. 6 and Fig. 3). Hydrolysis by rat small intestinal enzymes decreased the levels of higher oligosaccharides; the level of isomaltotriose remained unchanged and the level of isomaltose increased (Fig. 6). After hydrolysis with the modified AOAC method, the level of all oligosaccharides decreased; isomaltotriose and isomaltotetraose as well as the panose-series oligosaccharides DP5' and DP6' were most resistant to hydrolysis. Accumulation of isomaltose was not observed.

4. Discussion

Commercial isomalto-oligosaccharides contribute substantially to the global market of functional food ingredients. The digestibility if IMO is a critical determinant of their prebiotic properties, their classification as dietary fibre, and their physiological benefits in humans, which are predominantly mediated by conversion of IMO to short chain fatty acids (Bindels et al., 2015; Yan et al., 2018). Discrepant results on digestibility and functional benefits of commercial IMO relate

to their partial digestibility and to the different composition of commercial IMO preparations (Madsen et al., 2017). The lack of knowledge on the *in vivo* digestion of α-gluco-oligosaccharides (Hu et al., 2020) hampers efforts to modify existing production methods to adjust sweetness, fibre content, or functional benefits. This study provides a comparative analysis of experimental oligosaccharide, commercial IMO, and reference disaccharides to determine how linkage type and degree of polymerization impact the digestibility of IMO. A modified AOAC method was used for comparison (McCleary, 2019).

Compared to *in vivo* digestion models, *in vitro* digestion techniques are less expensive, less time consuming, and allow testing of food or feed ingredient samples with an adequate number of replicates. *In vitro* methods that use pancreatic α-amylase in combination with fungal amyloglucosidase and yeast invertase were validated to predict the starch digestibility in humans, swine, and chicken (Englyst et al., 1999; van Kempen et al., 2010; Weurding, Veldman, Veen, van der Aar, & Verstegen, 2001). These enzymes are also used in the AOAC method 2009.01 and updated versions of this method that are used as official methods for determination of the dietary fiber content of food (McCleary, 2019; McCleary, Sloane, Draga, & Lazewska, 2013). The AOAC method 2009.01 has limitations, however, with respect to the quantification of non-digestible oligosaccharides (Tanabe, Nakamura, & Oku, 2014). Accordingly, the *in vitro* digestibility of non-digestible oligosaccharides has been increasingly evaluated with brush border enzymes that include maltase/ glucoamylase, sucrase/ isomaltase, lactase-phlorizin hydrolase and trehalase activities (Ferreira-Lazarte, Olano, Villamiel, & Moreno, 2017; Lee et al., 2016; Tanabe et al., 2014). In particular, the brush border enzymes partially hydrolyse fructo-oligosaccharides and β-galacto-oligosaccharides that are not hydrolysed in the AOAC 2009.01 protocol (Ferreira-Lazarte et al., 2017). A majority of studies on the activity of brush border enzymes has used a

mixture of enzymes that were extracted from the small intestine of animals. Cloning and expression of human brush border maltase / glucoamylase and sucrase / isomaltase demonstrated that isomaltose, which is readily hydrolysed by fungal amyloglucosidase, is hydrolysed only slowly by brush border sucrase / isomaltase (Lee et al., 2016). We observed that panose-series oligosaccharides are hydrolysed more rapidly by amylase / amyloglucosidase than isomaltose; moreover, a different pattern of products was obtained after hydrolysis of commercial and experimental IMO with amylase / amyloglucosidase and brush border enzymes. These results further document that oligosaccharide hydrolysis in these two protocols and hence the accuracy of the prediction of the *in vivo* digestibility of oligosaccharides is different. The use of rat small intestinal enzymes determined the *in vitro* digestibility of a commercial IMO preparation as about 50% (this study), matching *in vivo* observations in an ileal-cannulated swine model (Hu et al., 2020).

In vitro digestion with rat small intestinal enzymes was assayed the digestibility of commercial oligosaccharide preparations, experimental oligosaccharides that were prepared in the acceptor reaction with DsrM of *Weissella cibaria* (Hu et al., 2017) and commercially available disaccharides. The *in vitro* digestibility of oligosaccharides decreased in the order maltodextrins > panose and panose series oligosaccharides > isomaltose and isomaltose series oligosaccharides. The *in vitro* digestibility of nigerose and kojibiose was comparable to panose and isomaltose, respectively. These results confirm and extend previous reports on the digestion of glycemic disaccharides with purified brush border enzymes (Lee et al., 2016). Digestion of isomaltose-oligosaccharides and panose series oligosaccharides accumulated isomaltose. Isomaltose accumulation reflect the slow hydrolysis of isomaltose when compared to panose. In addition, brush border maltase / glucoamylase hydrolyses oligosaccharides from the non-reducing end

(Hooton et al., 2015; Sim et al., 2010) and thus does not accumulate isomaltose from panose-series oligosaccharides. Brush border sucrase / isomaltase hydrolyses α-(1→4) as well as α-(1→6) glycosidic bonds; the crystal structure of brush border sucrase / isomaltase suggests that, in contrast to maltase / glucoamylase, binding occurs at the reducing end of the substrate (Sim et al., 2010). Panose hydrolysis from the reducing end by sucrase / isomaltase may account for the accumulation of isomaltose. Remarkably, the hydrolysis of isomalto-oligosaccharides was not substantially impacted by the degree of polymerization while quantitative and qualitative analysis suggested that panose is hydrolysed much faster than panose-series oligosaccharides with a higher DP (Fig. 1, Fig. 2 and Fig. 5). These data indicate that the linkage type of IMO is a more significant determinant of digestibility than the degree of polymerization.

IMO are produced commercially from diverse glycosyl hydrolases and sucrose or starch hydrolysates as alternative substrates (Casa-Villegas et al., 2018; Dobruchowska et al., 2012; Madsen et al., 2017). Commercial IMO preparations that are produced by different enzymatic processes and differ substantially with respect to their composition. Current commercial IMO preparations are produced mainly by using glycosyl transfer of starch hydrolysates with maltose as glycosyl donor and glycosyl acceptor (Casa-Villegas et al., 2018; Sorndech, Sagnelli, & Blennow, 2017). The degree of polymerization, the linkage type and the content of digestible α-(1→4) linked oligosaccharides depends on the conditions for starch hydrolysis, the transferase activity, and downstream purification to remove maltose, glucose and other digestible carbohydrates (Pan & Lee, 2005; Sorndech et al., 2017). Dextransucrases of lactic acid bacteria or food-grade lactic acid bacteria expressing dextransucrase are also used commercially for production of IMO with sucrose as glucosyl donor and maltose as glucosyl acceptor (Madsen et al., 2017). Enzymes of food-grade lactobacilli offer additional avenues for production of IMO e.g.

by using the reuteransucrase GtfA for conversion of sucrose or the glucanotransferase GtfB that synthesizes linear α-(1→4) and α-(1→6)-linked oligosaccharides from maltodextrins (Dobruchowska et al., 2012, 2013; Kralj et al., 2011).

In conclusion, current knowledge provides an extensive enzymatic toolset for production and modification of IMO for tailored nutritional and technological properties. The present study provides data on the influence of the degree of polymerization and the linkage type on the hydrolysis of IMO by brush border enzymes. Together with studies on the sweetness of oligosaccharide preparations (Ruiz-Aceituno, Hernandez-Hernandez, Kolida, Moreno, & Methven, 2018) as well as other functional aspects that relate to food applications, these data can inform the modification or optimization of current production processes to obtain tailored oligosaccharide preparations with reduced digestibility, increased content of dietary fibre, or improved sensory properties for specific food applications.

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Figure legends

Figure 1. Relative digestibility of different carbohydrates after *in vitro* digestion with rat small intestinal enzymes (black bars) or a modified AOAC protocol (gray bars). The digestibility is expressed relative to maltose; resistant maltodextrins were used as non-digestible control and maltodextrin was used as a negative digestible control. Data are shown as means ± standard deviation of triplicate independent experiments. Significant differences were analysed by two-way ANOVA. Data that were obtained with the same *in vitro* method are significantly different ($P<0.05$) if bars do not share a common superscript. An asterisk indicates significant differences ($P<0.05$) between results obtained for the same substrate with the two hydrolysis protocols.

Figure 2. Relative digestibility of reference compounds by rat small intestinal enzymes. The digestibility is expressed relative to maltose; cellobiose was used as non-digestible control and maltodextrin was used as a digestible control. Significant differences were analysed by one way ANOVA. Data are shown as means ± standard deviation of triplicate or quadruplicate independent experiments. Data are significantly different ($P<0.05$) if bars do not share a common superscript.

Figure 3. HPAEC-PAD profiles of maltose (A), maltodextrins (B) and resistant maltodextrins (C) before (black line) and after 4 h digestion with 10 g/L rat small intestinal enzymes in 50 mM sodium maleate buffer (pH 6.0) (dark gray line) and after 4h digestion with pancreatic enzymes and amyloglucosidase (light gray line). The HPAEC-PAD profiles shown in the insets depict peaks with low intensity from 16 to 32 min.

Figure 4. HPAEC-PAD profiles of commercial isomalto-oligosaccharides IMO (A), IMO-DP3 (B) and ISO (C) before (black line) and after 4 h of digestion with 10 g/L rat small intestinal enzymes in 50 mM sodium maleate buffer (pH 6.0) (dark gray line) and after 4h digestion of digestion with pancreatic enzymes and amyloglucosidase (light gray line). The HPAEC-PAD

524 profiles shown in the small chromatographs shows peaks with low intensity from 20 to 32 min.
525 Isomaltose series oligosaccharides are designated with DP2, DP3 etc; panose series
526 oligosaccharides are designated with DP3', DP4' etc.

527 **Figure 5.** HPAEC-PAD profiles of α-(1→6) extended maltose (A), α-(1→6) extended isomaltose
528 (B), α-(1→6) extended nigerose (C) and α-(1→6) extended maltotriose (D) before (black line) and
529 after 4 h of digestion (gray line) with 10 g/L rat small intestinal enzymes in 50 mM sodium maleate
530 buffer (pH 6.0). The HPAEC-PAD profiles shown in the insets depict peaks with low intensity
531 from 22 to 32 min.

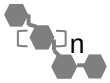
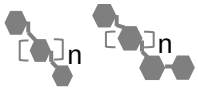
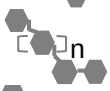
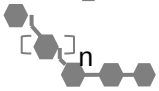
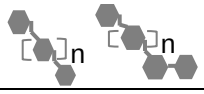
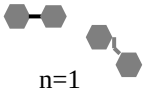
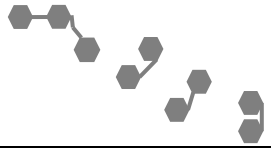
532 **Figure 6.** HPAEC-PAD profiles of α-(1→6) extended IMO before (gray line) and after 4 h of
533 digestion with 10 g/L rat small intestinal enzymes in 50 mM sodium maleate buffer (pH 6.0) (dark
534 gray line) and after 4 h of digestion with pancreatic enzymes and amyloglucosidase (light gray
535 line). The HPAEC-PAD profiles shown in the insets depict peaks with low intensity from 20 to 32
536 min.

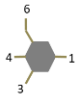
537

Table 1. Composition of enzymes and buffers of the two *in vitro* digestion methods. Based on information from (Oku et al., 2011; Tsunehiro et al., 1999; van Kempen et al., 2010)

Digestion with rat small intestinal enzymes	**Modified AOAC method for starch digestibility**
maltase/ glucoamylase (MGAM, EC 3.2.1.20/ 3.2.1.3)	pepsin (EC 3.4.23.1) in 0.05 M HCl
sucrase/ isomaltase (SIM, EC 3.2.1.48/ 3.2.1.10)	porcine pancreatic enzymes (amylase, lipase, protease, etc.)
lactase-phlorizin hydrolase (LPH, EC 3.2.1.108/ 3.2.1.62)	amyloglucosidase from *Aspergillus niger* (EC3.2.1.3)
trehalase (EC 3.2.1.28)	invertase from *Saccharomyces cerevisiae* (EC 3.2.1.26)
25mM sodium maleate buffer(pH 6.0)	0.1M sodium acetate buffer (pH 6.0)

Table 2. Composition of the α-glucan and isomalto-oligosaccharides

Substrates	Main structure(s)	DP	source
Commercial products			
Digestible maltodextrin		DE=3-20%	Protein Co. (USA)
Resistant maltodextrin	-	DE=8-12.5%	Matsutani America Inc. (USA)
Isomalto-oligosaccharides (ISO)		DP 3-8 branched at greater DP	ISOThrive™ Inc. (USA); extension by DSR from *Leuconostoc citreum* NRRL B-742 with maltose
Isomalto-oligosaccharides (IMO)		DP 2-5	BioNeutra North America Inc. (Canada); hydrolysis and transglucosylation of starch
Isomalto-oligosaccharides (IMO-DP3)		DP 2-5	
Experimental oligosaccharides synthesized in this study			
Isomaltose-series		DP 3-6	extension of isomaltose with DsrM
Panose-series		DP 3-8	extension of maltose with DsrM
Nigerose-series		DP 2-25	extension of nigerose with DsrM
Maltotriose-series		DP 3-7	extension of maltotriose with DsrM
Extended IMO		DP 3-7 DP' 4-6	extension of IMO with DsrM
Reference compounds used in this study			
Maltose		DP2	Sigma (Canada)
Panose		DP3	Megazyme (Ireland)
Maltotriose		DP3	Sigma (Canada)
Cellobiose		DP2	Sigma (Canada)
Isomaltose		DP2	Carbosynth (UK)
Isomaltotriose	n=1	DP3	Carbosynth (UK)
Isomaltotetraose	n=2	DP4	Carbosynth (UK)
Isomaltopentaose	 n=3	DP5	Carbosynth (UK)
Isomaltoheptaose	n=5	DP7	Carbosynth (UK)
Isopanose		DP3	Megazyme (Ireland)
Kojibiose		DP2	Carbosynthn (UK)
Nigerose		DP2	Carbosynth (UK)
Trehalose		DP2	Sigma (Canada)



Symbol for glucose and linkage types. α-Linkages are printed in gray, β-linkages in black

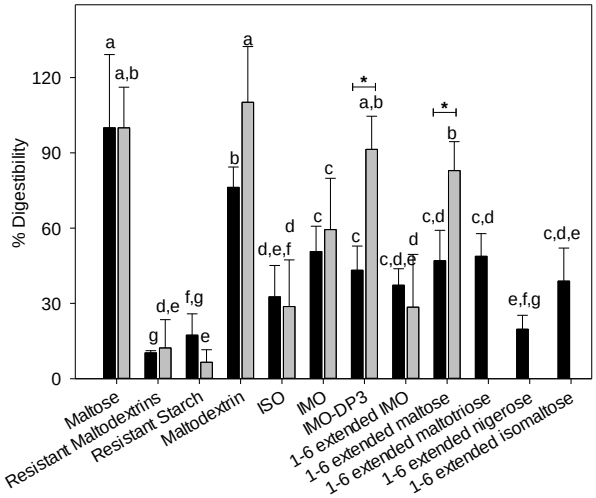
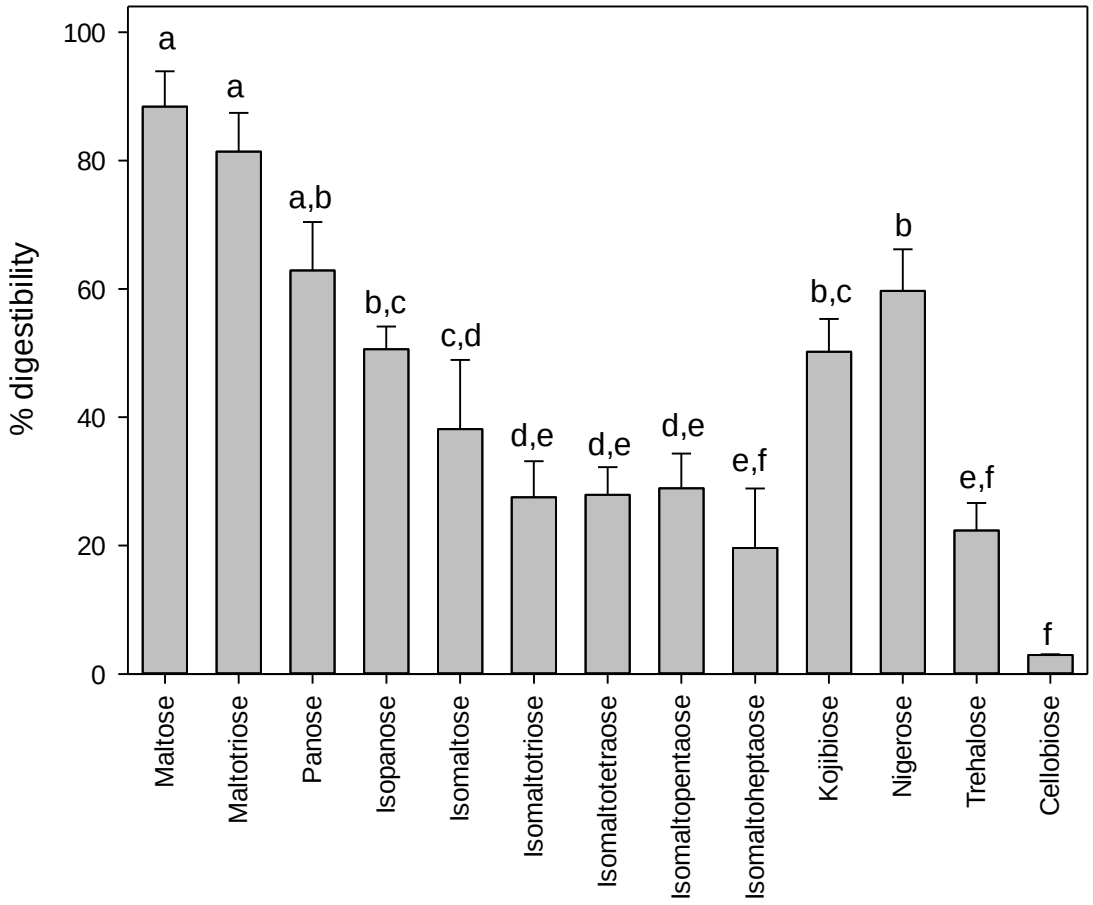

% Digestibility
0
30
60
90
120
a
a,b
g
d,e
f,g
e
b
a
d,e,f
d
c
c
c
a,b
*
c,d,e
d
c,d
b
*
c,d
e,f,g
c,d,e
Maltose
Resistant Maltodextrins
Resistant Starch
Maltodextrin
ISO
IMO
IMO-DP3
1-6 extended IMO
1-6 extended maltose
1-6 extended maltotriose
1-6 extended nigerose
1-6 extended isomaltose

Figure 1.

Fig. 2


% digestibility
100
80
60
40
20
0
a
a
a,b
b,c
c,d
d,e
d,e
d,e
e,f
b,c
b
e,f
f
Maltose
Maltotriose
Panose
Isopanose
Isomaltose
Isomaltotriose
Isomaltotetraose
Isomaltopentaose
Isomaltoheptaose
Kojibiose
Nigerose
Trehalose
Cellobiose

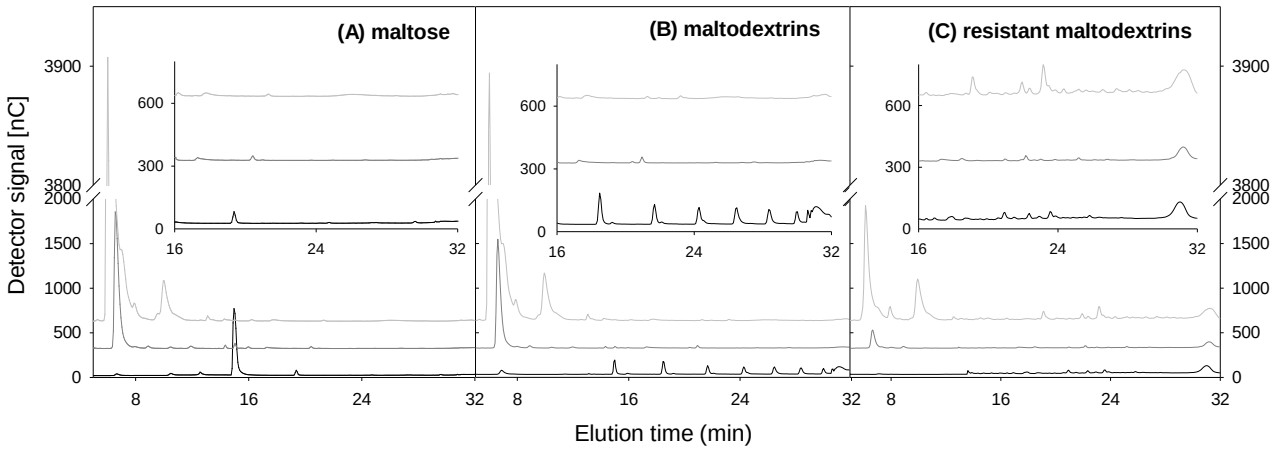

(A) maltose
(B) maltodextrins
(C) resistant maltodextrins
Detector signal [nC]
Elution time (min)
3900
3800
2000
1500
1000
500
0
600
300
8
16
24
32

Fig. 3.

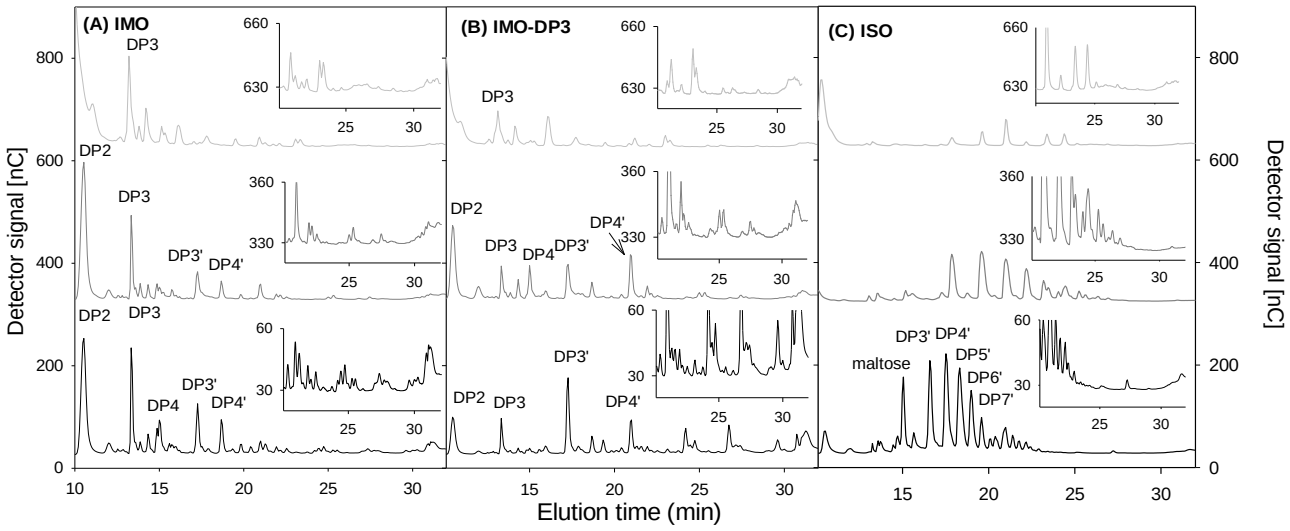

(A) IMO
(B) IMO-DP3
(C) ISO
DP2
DP3
DP3'
DP4
DP4'
DP5'
DP6'
DP7'
maltose
Detector signal [nC]
Elution time (min)

Fig. 4.

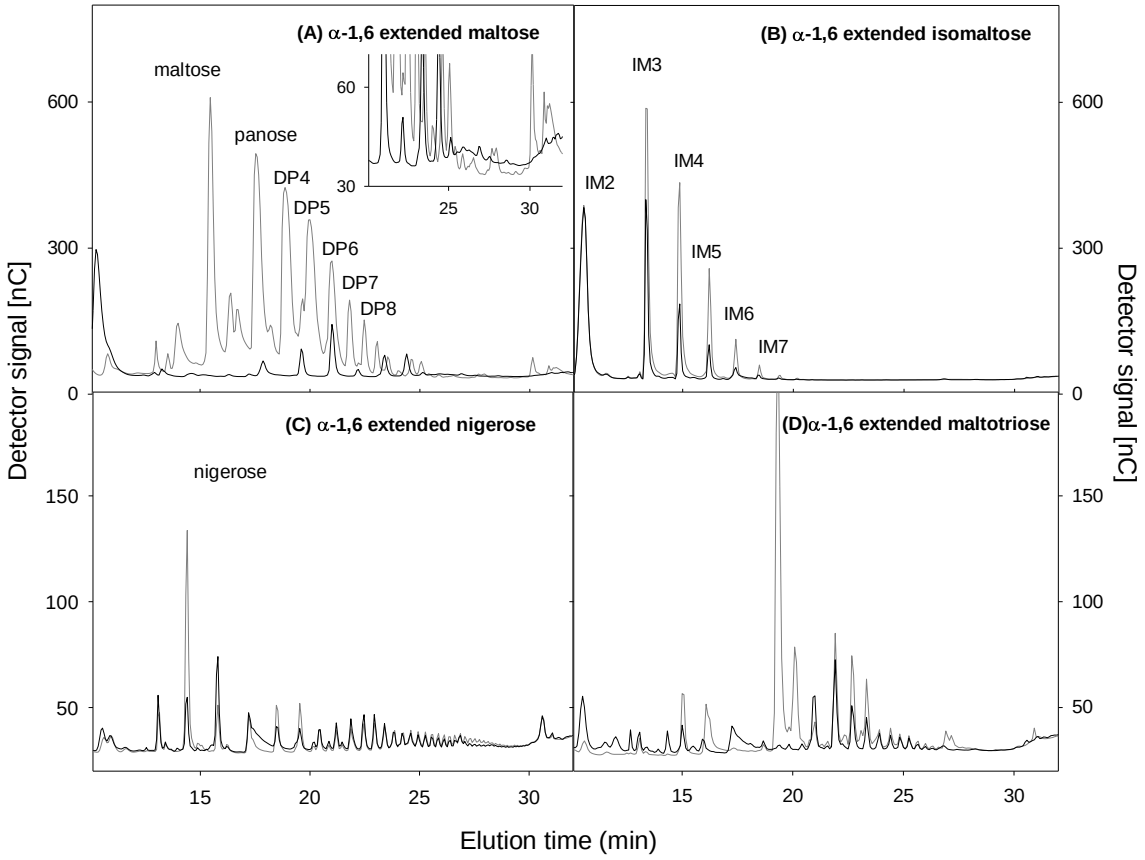

(A) α-1,6 extended maltose
maltose
panose
DP4
DP5
DP6
DP7
DP8
(B) α-1,6 extended isomaltose
IM2
IM3
IM4
IM5
IM6
IM7
(C) α-1,6 extended nigerose
nigerose
(D)α-1,6 extended maltotriose
Detector signal [nC]
Elution time (min)

Fig. 5.

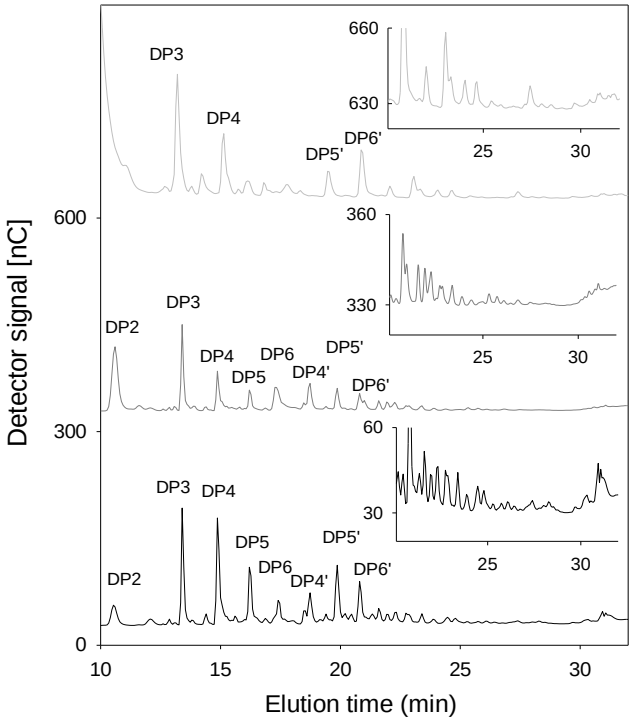

Detector signal [nC]
Elution time (min)
DP2
DP3
DP4
DP5
DP6
DP4'
DP5'
DP6'
660
630
360
330
60
30
600
300
0
10
15
20
25
30

Fig. 6.