1 **Characterization of α-Galactooligosaccharides formed via Heterologous Expression of α-**
2 **Galactosidases from *Lactobacillus reuteri* in *Lactococcus lactis***
3
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18 **ABSTRACT**

19 *α*-Galacto-oligosaccharides (*α*-GOS) are produced by transgalactosylation reactions of α-
20 galactosidase (*α*-Gal), or by conversion of raffinose-family oligosaccharides by levansucrase.
21 Similarly to *β*-GOS, *α*-GOS have the potential to mimic glycan receptors on eukaryotic cells and act
22 as molecular decoys to prevent bacterial infection, however, data on transgalactosylation reactions of
23 *α*-Gal remain scarce. The α-Gal gene sequence from *Lactobacillus reuteri* was cloned into an α-Gal
24 negative strain of *Lactococcus lactis*. Transgalactosylation reactions were achieved using crude cell
25 extracts with melibiose or raffinose as galactosyl donor and fucose, N-acetylglucosamine or lactose
26 as galactosyl acceptor. The composition, sequence and most linkage types of *α*-GOS formed with
27 acceptors saccharides were determined by liquid chromatography-tandem mass spectrometry (LC-
28 MS/MS). *α*-Gal of *L. reuteri* formed (1→3)-, (1→4)-, or (1→6)-linked *α*-GOS but exhibited a
29 preference for formation of (1→6)-linkages. Fucose, N-acetylglucosamine as well as lactose were
30 suitable galactosyl acceptors for *α*-Gal of *L. reuteri,* resulting in formation of (1→3)-, (1→4)-, or
31 (1→6)-linked hetero-oligosaccharides. By determining structural specificity of *α*-Gal and increasing
32 the variation of oligosaccharides produced by introducing alternative acceptor sugars, this work
33 supports further studies to assess *α*-GOS pathogen adhesion prevention in mammalian hosts.

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35 KEY WORDS: (4-6 words) α-galactosidase, *Lactobacillus reuteri*, galacto-oligosaccharides, hetero-
36 oligosaccharides, LC/MS

37

38 **INTRODUCTION**

39 Bacterial infection is initiated by adherence to skin or mucosal surfaces in mammalian hosts
40 (Finlay and Falkov 1997). Adherence can be prevented by soluble oligosaccharides that resemble
41 surface glycans of eukaryotic cells and act as receptor analogues (Kulkarni et al. 2010). An improved
42 understanding of the interactions between surface glycans of eukaryotic cells and adhesion
43 mechanisms of pathogenic bacteria and their toxins has resulted in increased interest in applications
44 of oligosaccharides to treat infections in farm animals and humans (Arslanoglu et al. 2007; Bruzzese
45 et al. 2009; Liu et al. 2008; Rozeboom et al. 2005). Preventative treatments utilizing oligosaccharide
46 analogues are also motivated by the need to replace antibiotics as growth promoters in animal
47 production (Fox 1988; Verstegen and Williams 2002).

48 Human milk oligosaccharides (HMOS) inhibit bacterial adhesion to epithelial surfaces in
49 infants (Kunz et al. 2000). HMOS are constituted of glucose (Glc), galactose (Gal), N-
50 acetylglucosamine (GlcNAc), fucose (Fuc) and sialic acid and are joined by a variety of linkage
51 types and branching which collectively gives rise to highly diverse and complex structures (Bode
52 2006). *β*-Galacto-oligosaccharides (*β*-GOS) consist of *β*-linked galactose moieties with galactose or
53 glucose located at the reducing end. Although they differ structurally from HMOS, *β*-GOS were also
54 shown to prevent pathogen adhesion. For example, *β*-GOS were used to prevent the adhesion of
55 enteropathogenic *Escherichia coli* to tissue culture cells, and prevented *Salmonella enterica* adhesion
56 both in vitro and in vivo (Searle et al. 2010; Shoaf et al. 2006).

57 *β*-GOS are produced by transgalactosylation of glucose, galactose, or lactose, and the
58 mechanisms of enzymatic transgalactosylation with microbial *β*-galactosidases (*β*-Gal) are well
59 understood (Torres et al. 2010; Gänzle 2012). Transgalactosylation by microbial *β*-Gal produces
60 hetero-oligosaccharides (HeOS) with potential applications in food and therapeutics if suitable
61 acceptor carbohydrates or sugar alcohols are present (Gänzle 2012). *β*-Gal from lactic acid bacteria

62 and bifidobacteria were employed to produce *β*-HeOS with structural similarity to HMOS. These *β*-
63 HeOS have the potential to mimic receptor glycan structures of enteropathogenic *Enterotoxigenic*
64 *coli* (Black et al. 2012; Schwab et al. 2011). Similarly to *β*-Gal, *α*-galactosidases (*α*-Gal) [EC
65 3.2.1.22] cleave Gal*α*-(1→6)-Glc bonds and subsequently transfer the liberated galactose moiety to
66 suitable acceptors forming *α*-galacto-oligosaccharides (*α*-GOS). Comparable to *β*-GOS, *α*-GOS have
67 the potential to mimic glycan receptors on eukaryotic cells. For example, Gal*α*-(1→4)-Gal and Gal*α*-
68 (1→4)-Gal*β*-(1→4)-Glc act as receptors for P-fimbriae of *E. coli* and Shiga toxin I/II, respectively
69 (Lingwood 1996; Strömberg et al. 1991). *α*-Gal activity is relatively common among lactobacilli
70 (Gänzle and Follador 2012); however, applications for *α*-Gal from lactic acid bacteria almost
71 exclusively aimed to eliminate raffinose-and stachyose in food and feed fermentations (Alazzeh et al.
72 2009; Teixeira et al. 2012). Only few reports describe *α*-GOS formation by *α*-Gal (Mital et al. 1973;
73 Tzortzis et al. 2003). The resulting *α*-GOS, however, were not characterized on a structural level, and
74 the formation of HeOS with *α*-Gal was not explored.

75 It was therefore the aim of this study to employ *α*-Gal from lactobacilli for production of *α*-
76 GOS with different acceptor carbohydrates, and to structurally characterize the *α*-GOS formed from
77 various acceptor sugars. *Lactobacillus reuteri* was chosen as source of *α*-Gal because *α*-GOS
78 formation by enzymes of this organism was previously described (Tzortzis et al. 2003). Genes coding
79 for *α*-Gal in *L. reuteri* 100-16 and 100-23 were cloned into an *α*-Gal negative strain of *Lactococcus*
80 *lactis* and transgalactosylation was achieved using the crude cell extracts (CCE) of the transgenic *α*-
81 Gal active *L. lactis*. *α*-GOS were characterized by liquid chromatography/electrospray ionization
82 tandem – mass spectrometry (LC/ESI-MS/MS) (Black et al. 2012).

83 **MATERIALS AND METHODS**

84 **Chemicals and standards.** Oligosaccharide standards including melibiose, raffinose, and stachyose
85 were purchased from Sigma Aldrich (Oakville, Canada); globotriose was purchased from Carbosynth
86 (Berkshire, United Kingdom). Fisher Scientific (Ottawa, Canada) supplied HPLC grade acetonitrile,
87 methanol, and ammonium acetate. Other solvents were of analytical grade.
88 **Bacterial strains and growth conditions.** *Escherichia coli* TG1 was purchased from Stratagene
89 (Amsterdam, the Netherlands). *L. reuteri* 100-23 (FUA3030, DSM17509) and 100-16 (FUA 3032),
90 and *L. lactis* MG1363 (FUA3016) were obtained from the Food Microbiology strain collection of the
91 University of Alberta (FUA) and grown in modified deMan-Rogosa-Sharpe (mMRS) media
92 containing 10 g L^{-1} maltose, 5 g L^{-1} fructose and 5 g L^{-1} glucose with a pH of 6.5. *L. lactis* was grown
93 in M17 media with the addition of 0.5% glucose (mM17). *E. coli* was grown in Luria-Bertani media.
94 Agar plates contained 15 g L^{-1} agar for each medium and strains were incubated at 37 °C.
95 **Cloning of *α*-galactosidase and transformation of *E. coli* and *L. lactis*.** The *E. coli* – *L. lactis*
96 expression shuttle vector pAMJ586 was used for cloning the *α*-Gal gene (*aga*) (Israelsen et al.,
97 1995). *α*-Gal genes from the genome-sequenced strain *L. reuteri* 100-23 and *L. reuteri* 100-16
98 (Accession numbers NZ_AAPZ02000002, Supplementary Table S1) were amplified by PCR using
99 primers listed in the Supplementary Table S1. Restriction digest of the PCR products was then
100 conducted to generate the insert with FastDigest® restriction enzymes *Sma*I and *Sal*I (Fermentas,
101 Burlington, Canada). The same restriction digest was done for the pAMJ586 shuttle vector and all
102 *Sma*I and *Sal*I restriction fragments were gel purified using the PureLink™ Quick Gel Extraction Kit
103 (Life Technologies Inc., Burlington, Canada) after electrophoresing in a 1% agarose gel at 90 V for
104 45 min. Ligation using the T4 DNA ligase (Fermentas) was done to produce the final constructs,
105 which were named as pAMJ586-*aga*23 and pAMJ586-*aga*16.

106 Recombinant plasmids were electro-transformed into *E. coli* TG1 and subsequently electro-
107 transformed into the *α*-Gal negative host strain *L. lactis* MG1363. The electroporation conditions
108 used were 25 μF, 1.7 kV, and 200 Ω in 0.1 cm Gene Pulser® cuvettes (Biorad, Mississauga,
109 Canada). Electroporated *E. coli* were recovered in SOC medium (Life Technologies); electroporated
110 *L. lactis* were recovered in mM17. Both strains were incubated for at least 2 h at 37 °C. After
111 recovery, transformed strains were grown on their respective media with erythromycin as the
112 selective agent at 5 mg L^{-1} in LB for *E. coli* and at 100 mg L^{-1} in mM17 for *L. lactis*. Competent cells
113 of *L. lactis* were prepared as described (Schwab et al. 2010), except overnight cultures were
114 inoculated 2% in 500 mL mM17 supplemented with 1% glycine. Cloning of the genes was confirmed
115 by PCR and sequencing of the amplicon (Macrogen, Rockville, MD, U.S.A.). The sequence of *aga23*
116 matched the corresponding sequence in the genome of *L. reuteri* 100-23; the sequence of *aga16* was
117 deposited to Genebank with the accession number KF410950. Gene expression was confirmed with
118 the observation of blue coloured transformants growing on mM17 agar supplemented with
119 erythromycin at 5 mg L^{-1} and 20 μL of 20 g L^{-1} 5-bromo-4-chloro-3-indoxyl-*α*-D-galactopyranoside
120 (X-*α*-D-Galactoside) (Gold Biotechnology Inc., St. Louis, MO, U.S.A.) that forms blue colour upon
121 hydrolysis by *α*-Gal. The transformants carrying *aga*23 and *aga*16 from *L. reuteri* 100-23 and *L.*
122 *reuteri* 100-16, respectively, were designated as *L. lactis* FUA 3376 and FUA 3377.

123 **Preparation of crude cell extracts.** Single colonies of *L. lactis* MG1363, FUA 3376 or FUA 3377
124 were used to inoculate 10 mL cultures in mM17 with 0.0274% manganese sulphate added (Ibrahim
125 et al. 2010). The cultures were incubated overnight and were used to inoculate 500 mL of the same
126 medium at 2%. The cultures were then incubated until the pH was reduced to values between 5.0 and
127 5.2, and cells were harvested by centrifugation at 5525 x g for 20 min. Cells were washed once in
128 McIlvaine buffer (0.1 M citric acid and 0.2 M disodium phosphate, pH 5.66), and resuspended in

129 approximately 10 mL of the same buffer additionally supplemented with 10% glycerol and 0.0274%
130 manganese sulphate. Cell were disrupted using a bead beater at 4 °C and crude cell extracts were
131 collected removing cellular debris by centrifuging at 12, 000 x g for 10 min at 4 °C.

132 **Determination of *α*-galactosidase activity of AGA23 and AGA16 in crude cell extracts.**
133 Enzymatic assays were conducted as described (Church et al. 1980) by adding 5 µL of CCE to 95 µL
134 of 4 g L^{-1} 4-nitrophenyl-α-D-galactopyranoside (PNPG). The reaction was stopped after 1, 2, 3, 4 or
135 5 min by the addition of 130 µL of 1 M sodium carbonate. Absorption at 400 nm was determined
136 using a Varioskan Flash Multimode Reader (Fisher Scientific Limited, Ottawa, Canada). Relative *α*-
137 Gal activity of each CCE was defined as the liberation of 1 mmol PNPG per min per mg of CCE at
138 35 °C and pH 4.7. The protein contents of CCEs were determined with the Bio-Rad Protein Assay
139 reagent (Bio-Rad, Missisauga, Canada) and bovine serum albumin (New England Biolabs,
140 Missisauga, Canada) as standard.

141 The optimal pH was determined using McIlvaine buffer with PNPG dissolved at 4 g L^{-1}. The pH of
142 the buffer was adjusted to 3.34, 3.64, 4.03, 4.47, 4.64, 4.75, 5.48, 6.10, 6.67, and 7.03. The optimal
143 temperature was determined by conducting the reaction at temperatures between 20 °C to 55 °C. In
144 all enzymatic reactions, CCE from the α-Gal negative *L. lactis* MG1363 was used as negative
145 control.

146 **Synthesis of oligosaccharides in acceptor reactions.** Reactions were conducted at 30 °C for 24 h
147 with 600 g L^{-1} melibiose or raffinose, or with 300 g L^{-1} melibiose or raffinose and 300 g L^{-1} lactose,
148 fucose or GlcNAc. Reactions without CCE, or reactions with CCE from *L. lactis* MG1363 were
149 included as negative controls. CCE of *L. lactis* FUA 3376 or FUA 3377 were added to achieve a final
150 *α*-Gal activity of 151 ± 39 µmol min^{-1} mg^{-1}. All carbohydrates were dissolved in McIlvaine buffer at
151 pH 4.7.

152 **High performance anion exchange chromatography with pulsed amperometric detection.** Sugar
153 standards of glucose, galactose, melibiose, raffinose, fucose, GlcNAc and lactose were prepared by
154 dissolving 0.6 g L^{-1} of each sugar in distilled water. Standards and samples were analyzed with high
155 performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD)
156 using a CarboPac PA-20 column (3 x 150 mm; Dionex, Oakville, Canada) as described (Black et al.
157 2012). Because GlcNAc co-eluted with glucose and galactose, acceptor reactions with GlcNAc were
158 analyzed with an Aminex HPX-87H column (7.8 x 300 mm, 9 µm; BioRad, Mississauga, Canada)
159 coupled to an RI detector eluted at 70 ºC with 5 mM sulfuric acid at 0.4 mL min^{-1} (Schwab et al.
160 2007).

161 **Combined liquid chromatography/electrospray ionization tandem mass spectrometry.**
162 Oligosaccharides were analyzed by LC/ESI-MS/MS. Separations were conducted on an Agilent
163 1200 series LC system (Agilent Technologies, Palo Alto, CA, U.S.A.) at 25 °C using a Supelcosil
164 LC-NH_2 column (250 mm x 4.6 mm, 5 µm; Sigma Aldrich). Samples were injected onto the column
165 at 3 µL each time, using an isocratic flow of acetonitrile/water 80:20 (v/v) at a rate of 1 mL min^{-1}.
166 The effluent from the column was split at a ratio of 1:3 (v/v). A post-column addition of ammonium
167 acetate (40 mM in methanol) at 0.03 mL min^{-1} was delivered by an Agilent 1200 series isocratic
168 pump that was added to the ESI source.

169 Negative ion ESI-MS and collision induced dissociation tandem mass spectrometry (CID-
170 MS/MS) were performed on a QStar® Elite hybrid orthogonal Q-TOF mass spectrometer coupled to
171 a TurboIon Spray® source with Analyst® QS 2.0 software (Applied Biosystems/MDS Sciex,
172 Concord, Canada). The conditions were as follows: nebulizer gas, 50 (arbitrary units); auxillary gas,
173 60 (arbitrary units); ionspray voltage, -4500 V; curtain gas, 25 (arbitrary units); declustering potential
174 (DP), -50 V; focusing potential, -150 V; DP2, -10 V; and a source temperature of 400 °C, scanning

175 over a mass range of *m/z* 100-1000. Fragmentation was achieved using nitrogen as a collision gas at a
176 collision energy that varied between -10 to -35 eV and optimized for each saccharide.

177 **RESULTS**

178 **Enzyme Sequence Alignment.** *α*-Gals from *L. reuteri* 100-16 and 100-23 were initially compared to
179 other *α*-Gals from lactic acid bacteria that are characterized on the protein level. Alignment of the
180 protein sequences with CLUSTALW multiple sequence Alignment software (version 2.1) revealed
181 that AGA23 and AGA16 are 97% identical (data not shown). AGA23 had 62% sequence identity
182 with *melA* of *L. plantarum* (Silvestroni et al. 2002); 42% with the *α*-Gal of *L. brevis* (Q03PP7), 37%
183 with *melA* of *L. fermentum* (Q6IYF5), and 51% match with *aga*A of *Carnobacterium*
184 *maltaromaticum* (AAL27305.1) (Supplementary Fig. S1 and data not shown). Overall, AGA23 and
185 AGA16 were highly similar in sequence, had comparable enzyme activities, and acceptor reaction
186 results. While CCE containing both types of enzymes were used in most cases during our study,
187 results obtained with either one of the enzymes are presented.

188 **Optimum pH and temperature for activity of *α*-galactosidase from *L. reuteri*.** The pH and
189 temperature for optimum activity of *α*-Gal was determined with PNPG as substrate. *α*-Gal activities
190 of AGA23 and AGA16 were optimal at a pH 4.7 and at temperatures between 30 °C – 35 °C (Fig. 1
191 and data not shown). In order to determine whether the presence of sugars alters the optimal
192 temperature of *α*-Gal, sucrose or melibiose were incorporated into enzyme assays at a concentration
193 of 300 g L^{-1}. Sucrose and melibiose reduced enzyme activities below 35 °C when compared to the
194 control, but enzymes were stabilized in the presence of the sugars at higher temperatures. Addition of
195 melibiose shifted the optimal temperature of *α*-Gal from 35 °C to 40 °C.

196 **Acceptor reactions.** Transgalactosylation of acceptor carbohydrates was initially assessed by
197 analysis of acceptor reactions with HPAEC-PAD. The formation of putative products was observed
198 in the reactions with lactose, fucose, or GlcNAc as galactosyl-acceptors (Fig. 2, Supplementary Fig.

199 S2 and data not shown). Transferase and hydrolase activities were calculated from the release of
200 glucose (representing total enzyme activity) and the release of galactose (representing hydrolase
201 activity), and the difference between glucose and galactose release (representing transferase activity)
202 (Table 1). No significant differences were observed when different acceptor carbohydrates were
203 present. However, when melibiose was present at 600 g L^{-1}, the total activity and contribution of
204 transferase activity was higher, likely in consequence of the higher substrate concentration.

205 **Characterization of oligosaccharides produced from melibiose with liquid**
206 **chromatography/electrospray ionization tandem mass spectrometry**. LC/ESI-MS was employed
207 to confirm the presence of oligosaccharides formed by the transferase reaction of *α*-Gal. All exact
208 masses of observed compounds are shown in Table 2. In order to determine the structural identity of
209 oligosaccharides, tandem mass spectrometry (MS/MS) was performed. This allowed the comparison
210 of fragmentation patterns from substrates or products in the samples to those of known standards, in
211 cases where these were available. Additionally, matching retention times between analytes and
212 standards confirmed structural identity (Supplementary Fig. S3). Based on the exact mass
213 measurements and the MS/MS spectra, a total of five oligosaccharides were identified that were
214 formed by *α*-Gal with melibiose as the substrate. The linkage identifications were based partly on the
215 standards used within this study and by adherence to the fragmentation rules described earlier in
216 Black et al. (2012). Specifically, the absence of both $^{0,2}A(-H_20)$ and $^{0,3}A$ cross-ring fragment ions
217 indicates a *β*/*α*-1→3 linkage; the presence of $^{0,2}A(-H_20)$ and the absence of $^{0,3}A$ cross-ring fragment
218 ions indicates a *β*/*α*-1→4 linkage; and the presence of $^{0,3}A$ and the absence of $^{0,2}A(-H_20)$ cross-ring
219 fragment ions indicate *β*/*α*-1→6 linkage. Thus, the disaccharide Gal*α*-(1→3)-Gal/Glc at retention
220 time 16.3 min was identified by the absence of both *m/z* 263 $^{0,2}A_2(-H_20)$ and *m/z* 251 $^{0,3}A_2$ ions;
221 Gal*α*-(1→4)-Gal/Glc at retention time 18.2 min was identified by the presence of *m/z* 263 $^{0,2}A_2(-H_20)$

222 and the absence of *m/z* 251 $^{0,3}A_2$ ions; Galα-(1→6)-Gal at retention time 19.1 min was identified by
223 the presence of *m/z* 251 $^{0,3}A_2$ and the absence of *m/z* 263 $^{0,2}A_2$(-H_20) ions (Fig. 3A-C). The linkages
224 for the trisaccharide products were determined similarly; Galα-(1→3)-Galα-(1→6)-Gal/Glc eluted at
225 39.9 min; Galα-(1→6)-Galα-(1→6)-Gal/Glc eluted at retention time 53.3 min (Fig. 3D-E).

226 **Characterization of oligosaccharides produced from melibiose and fucose, lactose, or GlcNAc.**
227 When lactose, fucose or GlcNAc were present as acceptor sugars, α-Gal formed hetero-
228 oligosaccharides in addition to the products produced from melibiose alone. In the presence of
229 fucose, a total of eleven oligosaccharides were obtained. The disaccharides resulting from
230 transgalactosylation of fucose were identified as Galα-(1→3)-Fuc, which appeared at a retention time
231 of 11.5 min, and Galα-(1→4)-Fuc at a retention time of 14.0 min (Fig. 4 and Table 2). The low
232 abundance of ions in the LC/MS/MS analysis of trisaccharides formed by galactosylation of fucose
233 was sufficient for the monosaccharide sequence to be established but did not provide enough
234 information to positively determine the linkages (Supplementary Fig. S4).

235 In the presence of lactose, one additional trisaccharide eluting at 37.5 min was formed; this
236 product was identified as Galα-(1→6)-Galβ-(1→4)-Glc (Fig. 5A). Disaccharides formed as the result
237 of reactions with melibiose and lactose could not be identified, due to co-elution with lactose. In
238 addition, the intense peak assigned as Galα-(1→6)-Galβ-(1→4)-Glc partly co-eluted with the peak
239 due to Galα-(1→3)-Galα-(1→6)-Gal/Glc (retention time 39.9 min) formed from melibiose and could
240 not be distinguished in an extracted ion chromatogram of *m/z* 503 (Table 2).

241 Finally, in reactions with GlcNAc, one new disaccharide eluting at 15.6 min was identified as
242 Galα-(1→6)-GlcNAc (Fig. 6). As with Galβ-(1→6)-GlcNAc, Galα-(1→6)-GlcNAc did not display
243 the characteristic *m/z* 251 fragmentation ion (Black et al. 2012).

244 **Compositional analysis of oligosaccharides produced from raffinose.** α-Gal formed a total of four
245 products were formed when raffinose was present as substrate. Trisaccharides were identified as
246 Galα-(1→3)-Glcα-(1→2)-Fru (retention time 24.2 min) and Galα-(1→4)-Glcα-(1→2)-Fru (retention
247 time 33.0 min) (Fig. 7A-B). Likely, α-(1→6)-linkages also formed, producing raffinose (retention
248 time 31.2 min); in this case, reactant and product were indistinguishable. Tetrasaccharides were
249 identified as Galα-(1→3)-Galα-(1→6)-Glcα-(1→2)-Fru product (retention time 48.5 min) (Fig. 7C)
250 and stachyose (Galα-(1→6)-Galα-(1→6)-Glcα-(1→2)-Fru, retention time 75.7 min) (Fig. 8).
251 Stachyose production by α-Gal from raffinose was confirmed by comparison of the retention time
252 and the fragmentation pattern of acceptor carbohydrates with an authentic standard (Fig. 8).

253 **DISCUSSION**

254 Lactic acid bacteria are excellent sources for food grade α-Gal (Alazzeh et al. 2009; Donkor et al.
255 2007). α-Gal gene sequences are present in genomes of most lactobacilli (Gänzle and Follador 2012)
256 but α-Gal has been characterized only in few species, including *L. reuteri*, *L. plantarum*, *L.*
257 *fermentum*, *L. brevis*, and *L. buchneri* (Mital et al. 1973; Tamura and Matsushita 1992; Garro et al.
258 1993). Past studies reported α-Gal activity and oligosaccharide formation but no connection between
259 observed activity and protein sequences were made, and the potential of transgalactosylation of
260 acceptor carbohydrates with α-Gal was not explored. Silverstroni et al. (2002) previously aligned the
261 putative active site for α-Gal, which was biochemically and physiologically characterized in *L.*
262 *plantarum*. The comparison of the amino acid sequences of the uncharacterized hydrolases of *L.*
263 *reuteri* 100-16 and 100-23, in combination with biochemical characterization, confirmed that
264 AGA16 and AGA23 are fully functional α-Gal.

265 The optimum pH of α-Gal from *L. reuteri* 100-23 and 100-16 match prior reports on α-Gal
266 activity of lactobacilli (Mital et al., 1973; Garro et al., 1993; Carrera-Silva et al., 2006) as well as the
267 growth optimum of the organism. However, the observed temperature optima of α-Gal from *L.*

268 *reuteri* were below the temperature optima of *α*-Gal from *L. fermentum* and other lactobacilli (Mital
269 et al., 1973; Garro et al., 1993; Carrera-Silva et al., 2006), and below the growth optimum of *L.*
270 *reuteri*, 37 °C – 42 °C (Gänzle et al. 1995; van Hijum et al. 2002). Moreover, optimal temperature of
271 *α*-Gal shifted when high concentrations of melibiose were incorporated into the enzyme reactions.
272 Therefore, melibiose and sucrose may stabilize heat-labile enzymes above the optimal temperature
273 and behave as compatible solutes for microorganisms in a physiological context (Brown and
274 Simpson 1972).

275 Past studies on oligosaccharide formation of *α*-Gal have employed melibiose or raffinose as
276 galactosyl-donor and galactosyl-acceptor (Mittal et al. 1973; Tzortzis et al. 2003). Manninotriose
277 (Gal*α*-(1→6)-Gal*α*-(1→6)-Glc) was tentatively identified as acceptor product, indicating that
278 oligosaccharide formation by *α*-Gal introduces *α*-(1→6) linkages (Mittal et al., 1973). The present
279 study confirmed the formation of *α*-(1→6) in oligosaccharides by identification of manninotriose and
280 stachyose produced from melibiose and raffinose, respectively. Additionally, *α*-(1→3) and *α*-(1→4)
281 linkages were formed by *α*-Gal. Comparable to *β*-Gal of lactic acid bacteria (Black et al. 2012;
282 Gänzle 2012), *α*-Gal of lactobacilli thus produce different linkages in the transgalactosylation
283 reaction. While the production of *α*-GOS or raffinose family oligosaccharides with *α*-Gal of lactic
284 acid bacteria is possible, it is unlikely to have practical applications. Raffinose family
285 oligosaccharides widely occur in nature, and the conversion of raffinose-family oligosaccharides to
286 *α*-GOS by levansucrase activity is straightforward and attained with a high yield (Teixeira et al.
287 2012).

288 This study is the first to clearly demonstrate the formation of oligosaccharides using acceptor
289 sugars such as fucose, lactose and GlcNAc by *α*-Gal in lactic acid bacteria. HPLC analyses not only
290 allowed quantification of hydrolase and transferase activities but also demonstrated the ability of *α*-

291 Gal from *L. reuteri* to produce oligosaccharides. From LC/ESI-MS/MS analyses, the composition,
292 sequence and in many cases the linkage types of formed *α*-GOS were determined. Linkage-type
293 information of reaction products was achieved since the characteristic fragment ions differentiating
294 (1→2)-, (1→3)-, (1→4)- and (1→6)-linkages are similar for both *β*-GOS and *α*-GOS (Dallinga and
295 Heerma 1991; Mulroney et al. 1995). The linkage-types of HeOS formed by *β*-Gal were previously
296 characterized using a the same LC/ESI-MS/MS method along with authentic *β*-HeOS standards
297 (Black et al. 2012). Hence, these fragmentation patterns could be applied in the present work in order
298 to characterize the linkage types of HeOS formed with *α*-Gal. In case of stachyose, the MS/MS
299 spectra of acceptor products could also be compared to authentic standards, further confirming that
300 the characteristic ions to determine linkage-type are transferable between *β*-GOS and *α*-GOS. Similar
301 to HeOS produced by *β*-Gal (Black et al. 2012), *α*-Gal of *L. reuteri* exhibited a preference for the
302 formation of (1→6)-linkages, although (1→3)- and (1→4)-linkages were also observed in acceptor
303 reactions with fucose or lactose. The formation of (1→3)-, (1→4)-, and (1→6)-linkages in
304 transgalactosylation of melibiose and acceptor carbohydrates may allow the targeted synthesis of
305 oligosaccharides for use as receptor analogues to prevent pathogen adhesion. For example, the
306 formation of *α*-(1→4) linkages by *α*-Gal may allow formation of Gal*α*-(1→4)-Gal*β*(1→4)-Glc
307 (globotriose) by transgalactosylation of lactose. Globotriose and globo-series oligosaccharides are
308 abundantly present in on the surface of animal and human mucosal cells, and are widely used by
309 several bacterial pathogens or their toxins for establishing adherence (Jacewicz et al. 1986;
310 Stromberg et al. 1990; Samuel et al. 1990; Leach et al. 2005) However, in the present study, the
311 formation of globotriose from melibiose and lactose could not be confirmed.

312 In conclusion, heterologously expressed *α*-Gal from *L. reuteri* was used to produce novel
313 oligosaccharides; additionally, LC/ESI-MS/MS was used to characterize composition,

314 monosaccharide sequence, and the linkage type of (most) acceptor products. Our work leads to
315 improving industrial processes to produce oligosaccharides with physiological functionality,
316 particularly with applications to prevent pathogen adhesion to mammalian hosts. Our findings may
317 contribute to support the future incorporation of *α*-GOS as therapeutic functional food ingredients to
318 reduce the incident of gastrointestinal infections and to improve the health of farm animals and
319 humans.

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426 **Figure legends**

427 **Fig. 1. Panel A.** Relative activity of AGA16 in *L. lactis* at 35°C and different pH values. Data are
428 means of three independent replicates and the experimental error is smaller than symbol size. **Panel**
429 **B.** Relative activity of AGA16 in *L. lactis* at pH 4.7 and temperatures ranging from 20 - 50°C.
430 Reactions were carried out with PNPG as substrate in McIlvaine buffer (▲), McIlvaine buffer
431 containing 300 g L^{-1} melibiose (■), or McIlvaine buffer containing 300 g L^{-1} sucrose (●). Data
432 represent means ± standard deviation of three independent experiments.

433 **Fig. 2.** HPAEC-PAD chromatograms of acceptor reactions with AGA23. Reactions were carried out
434 at pH 4.7 and 30 °C for 24 h a total carbohydrate concentration of 600 g L^{-1} with sugars indicated to
435 the right (Raf, raffinose; Mel, melibiose; Fuc, fucose; Lact, lactose; GlcNAc, N-acetylglucosamine).
436 Sugars that were identified by external standards are indicated by arrows (Gal, galactose; Glc,
437 glucose); unknown peaks representing putative acceptor carbohydrates are indicated by asterisks.
438 Data are representative of three independent experiments. Comparable results were obtained with
439 AGA16 (data not shown).

440 **Fig. 3.** ESI-MS/MS spectra of $[M\text{-}H]^-$ ions of oligosaccharide products from *α*-Gal with melibiose as
441 galactosyl-donor and -acceptor. (A) Gal*α*-(1→3)-Gal/Glc *m/z* 341 at retention time 16.3 min; (B)
442 Gal*α*-(1→4)-Gal/Glc *m/z* 341 at retention time 18.2 min; (C) Gal*α*-(1→6)-Gal *m/z* 341 at retention
443 time 19.1 min; (D) Gal*α*-(1→3)-Gal*α*-(1→6)-Gal/Glc product *m/z* 503 at retention time 39.9 min; (E)
444 Gal*α*-(1→6)-Gal*α*-(1→6)-Gal/Glc product *m/z* 503 at retention time 53.3 min. Melibiose eluted at
445 21.9 min, its spectrum is shown in Supplementary Figure S2.

446 **Fig. 4.** ESI-MS/MS spectra of $[M\text{-}H]^-$ ions of oligosaccharides from *α*-Gal with melibiose as
447 galactosyl-donor and fucose as galactosyl-acceptor. (A) Gal*α*-(1→3)-Fuc *m/z* 325 at retention time
448 11.5 min; (B) Gal*α*-(1→4)-Fuc *m/z* 325 at retention time 14.0 min.

449 **Fig. 5.** ESI-MS/MS spectra of $[M-H]^-$ ions of oligosaccharides from *α*-Gal with melibiose as
450 galactosyl-donor and lactose as galactosyl-acceptor. (A) Gal*α*-(1→6)-Gal*β*-(1→4)-Glc *m/z* 503 at
451 retention time 37.5 min; (B) Gal*α*-(1→6)-Gal*α*-(1→6)-Gal/Glc *m/z* 503 at retention time 53.2 min.
452 **Fig. 6.** ESI-MS/MS spectra of $[M-H]^-$ ions of Gal*α*-(1→6)-GlcNAc *m/z* 382 at retention time 15.6
453 min from *α*-Gal with melibiose as galactosyl-donor and GlcNAc as galactosyl-acceptor.
454 **Fig. 7.** ESI-MS/MS spectra of $[M-H]^-$ ions of oligosaccharides from *α*-Gal with raffinose galactosyl-
455 donor and -acceptor. (A) Gal*α*-(1→3)-Glc*α*-(1→2)-Fru *m/z* 503 at retention time 24.2 min; (B) Gal*α*-
456 (1→4)-Glc*α*-(1→2)-Fru *m/z* 503 at retention time 33.0 min; (C) Gal*α*-(1→3)-Gal*α*-(1→6)-Glc*α*-
457 (1→2)-Fru *m/z* 665 at retention time 48.5 min. Raffinose reactant eluted at 31.2 min, its spectrum is
458 shown in Supplementary Figure S2.
459 **Fig. 8.** ESI-MS/MS spectra of $[M-H]^-$ ions representative of stachyose. (A) stachyose standard; (B)
460 Gal*α*-(1→6)-Gal*α*-(1→6)-Glc*α*-(1→2)-Fru (*m/z* 665 at retention time 75.7 min) produced by *α*-Gal
461 with raffinose galactosyl-donor and -acceptor.
462

463 **Table 1.** Comparisons between enzyme activities among different acceptor reactions.

Acceptor reactions {n = 3}	**Glucose liberated (mol L^{-1})**	**Percent hydrolase activity**	**Percent transferase activity**
M + AGA16[a)]	0.52 ± 0.17	55.1 % ± 0.5	44.9 % ± 0.5
M + Fuc + AGA16	0.26 ± 0.13	80.2 ± 6.3	19.8 % ± 6.3
M + Lactose + AGA16	0.36 ± 0.12	65.0 % ± 1.1	35.0 % ± 1.1
M + GlcNAc + AGA16	0.22 ± 0.03	77.9 % ± 13.7	22.1 % ± 13.7

464 [a)] Note: M, melibiose, Fuc, fucose, GlcNAc, N-acetylglucosamine. Reactions were conducted at a
465 total sugar concentration of 600 g L^{-1} (300 g L^{-1} melibiose and 300 g L^{-1}acceptor carbohydrate). For
466 reactions with melibiose only, 600 g L^{-1} melibiose were used.

467

468

469 **Table 2.** Mass accuracy of deprotonated molecular ions and retention times of oligosaccharides

470 formed between samples with either GlcNAc, fucose or lactose added as acceptor carbohydrates.

Acceptor	Compound	Retention Time (min)	Measured Mass (Da)	Error (mDa)
		Melibiose		
None	$C_{12}H_{21}O_{11}$	16.3; 18.2; 19.1; 21.9	341.1099	1.0
	$C_{18}H_{32}O_{16}$	39.9; 53.3	503.1637	1.9
+ Fuc	$C_{12}H_{21}O_{10}$	11.5; 14.0	325.1156	1.6
	$C_{12}H_{21}O_{11}$	16.3; 18.2; 19.1; 21.9	341.1076	-1.3
	$C_{18}H_{31}O_{15}$	26.1; 33.4; 36.4	487.1689	2.0
	$C_{18}H_{32}O_{16}$	39.9; 53.0	503.1609	-0.9
+ Lactose	$C_{12}H_{21}O_{11}$	18.7; 21.8	341.1081	-0.8
	$C_{18}H_{32}O_{16}$	37.5; 53.2	503.1632	1.4
+ GlcNAc	$C_{14}H_{24}O_{11}N$	15.6	382.1375	2.0
	$C_{12}H_{21}O_{11}$	16.3; 18.3; 19.1; 21.8	341.1099	1.0
	$C_{18}H_{32}O_{16}$	39.5; 53.2	503.1638	2.0
		Raffinose		
None	$C_{18}H_{32}O_{16}$	24.2; 31.2; 33.0	503.1629	1.1
	$C_{24}H_{41}O_{21}$	48.5; 75.7	665.2164	1.8
		Standards		
N/A	Lactose	18.8	341.1082	-0.7
	Melibiose	21.9	341.1095	0.6
	Raffinose	31.3	503.1628	1.0
	Globotriose	33.9	503.1626	0.8
	Stachyose	75.8	665.2155	0.9

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472

473 **Figure 1.**
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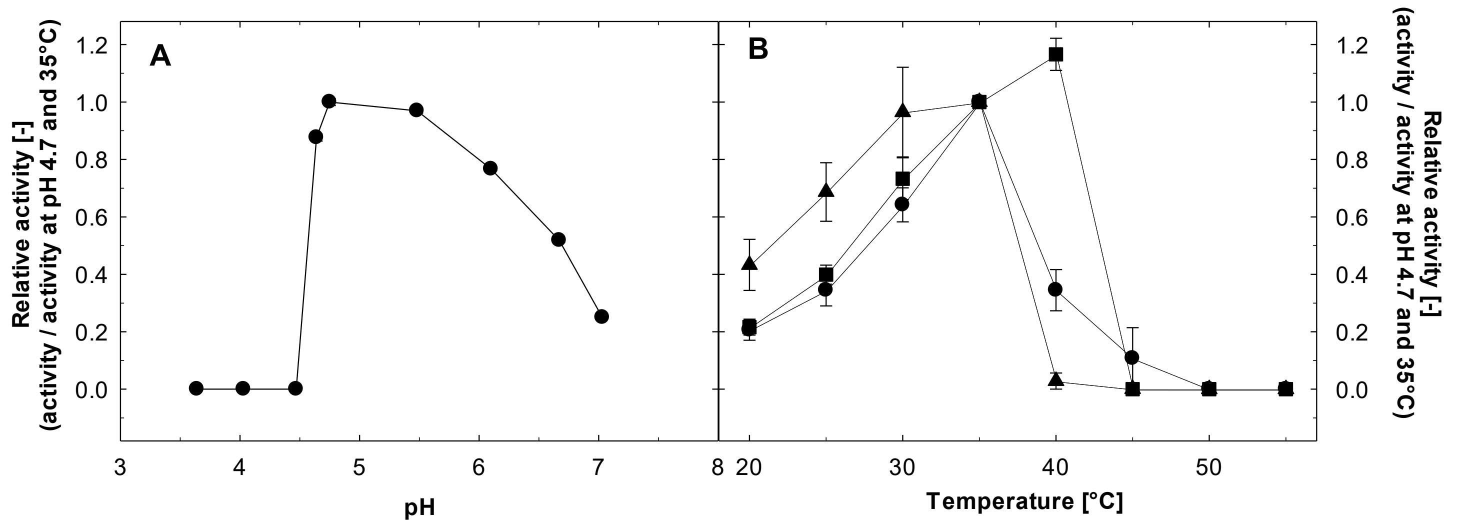
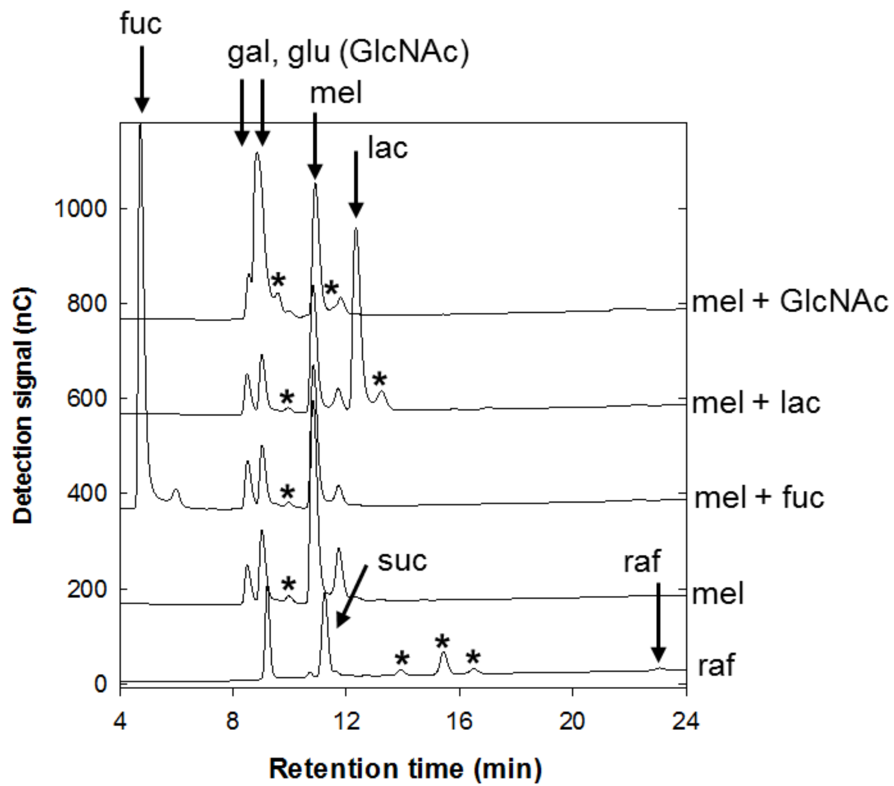
476



477 **Figure 2.**



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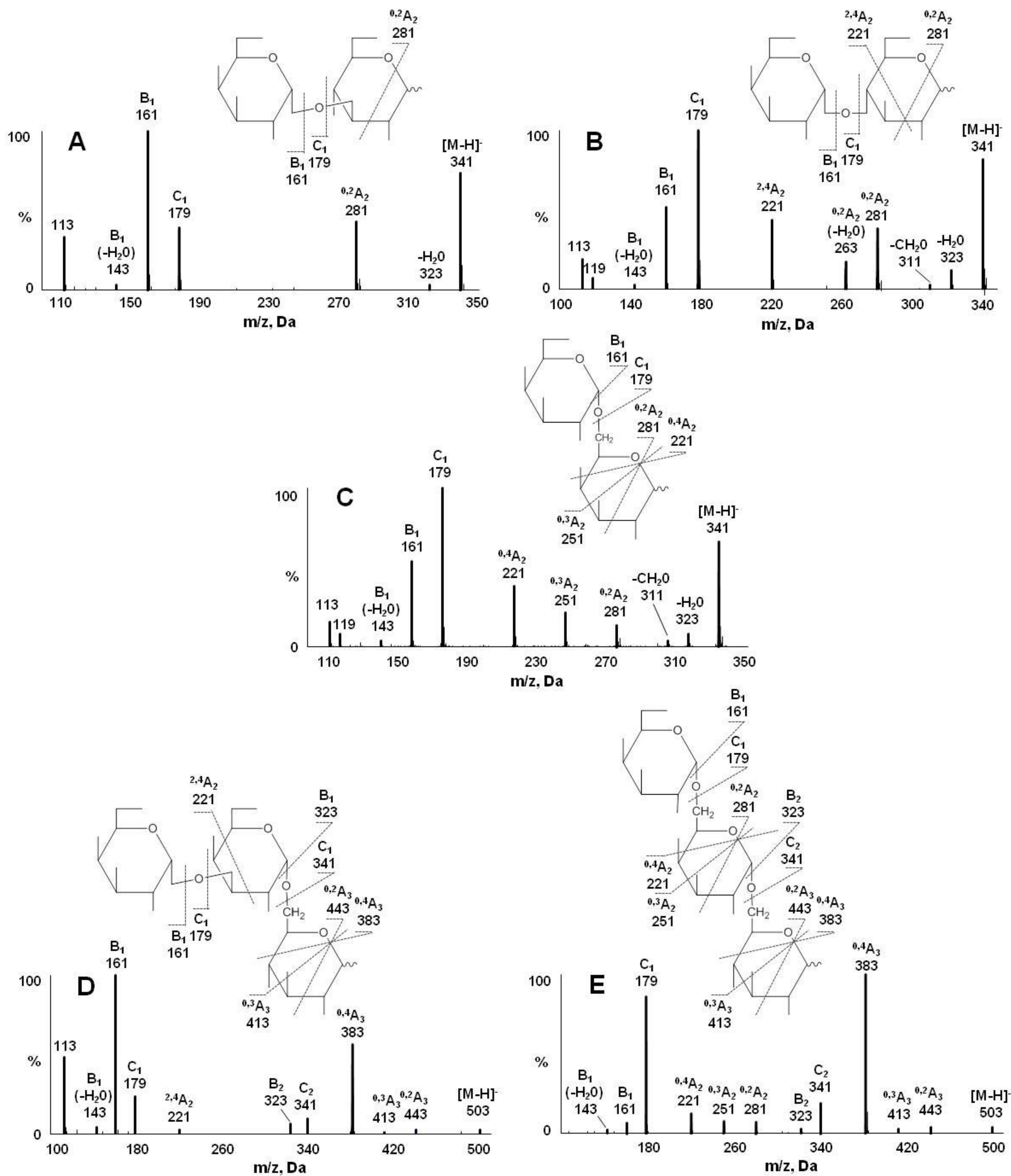

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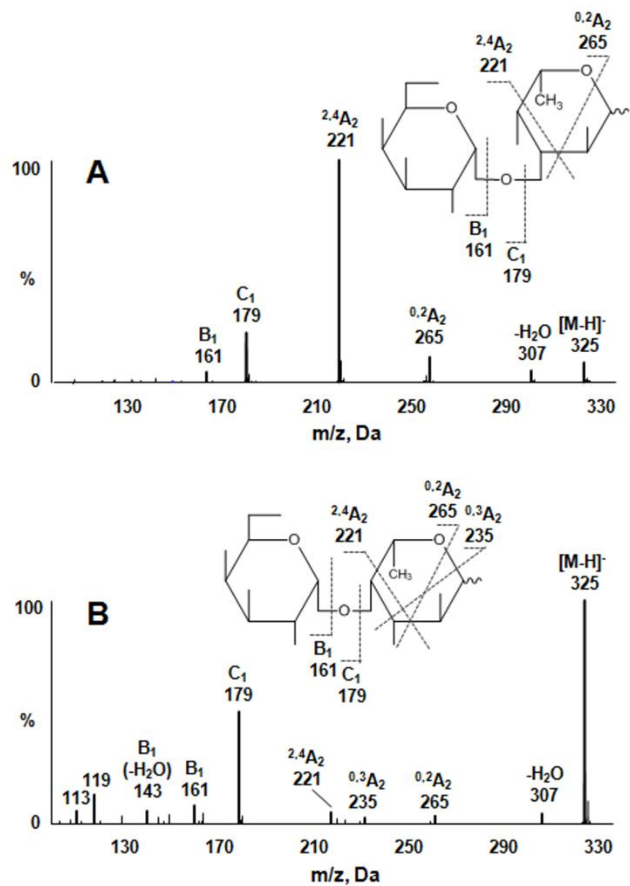

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484 **Figure 4.**

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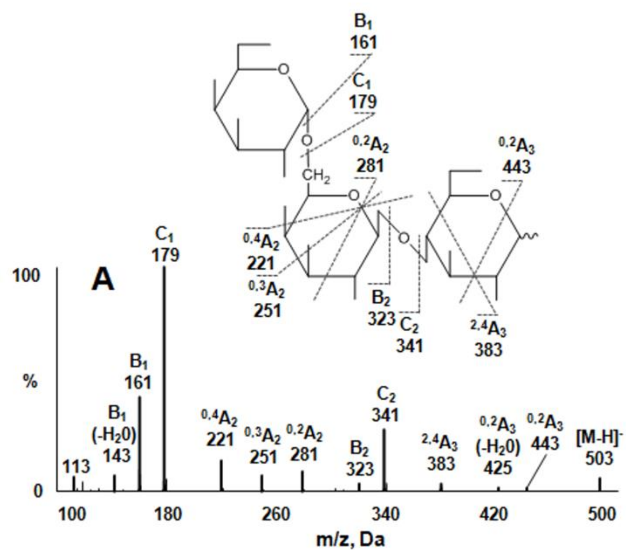

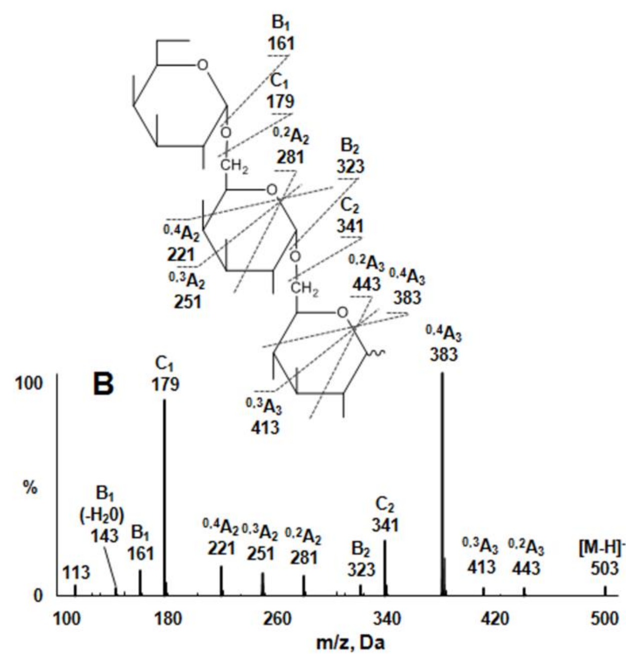

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488 **Figure 5.**

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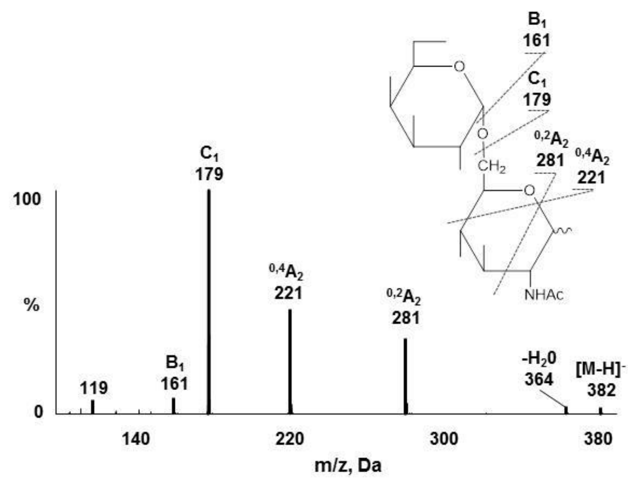

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492 **Figure 6.**

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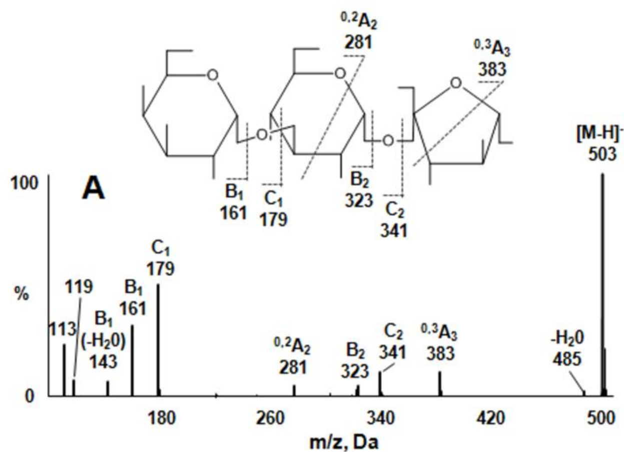
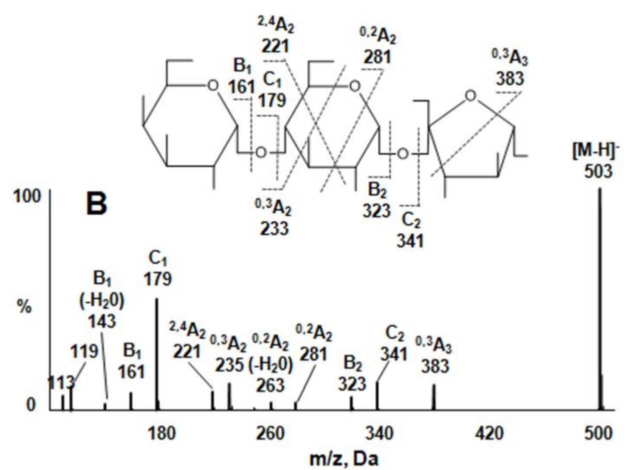
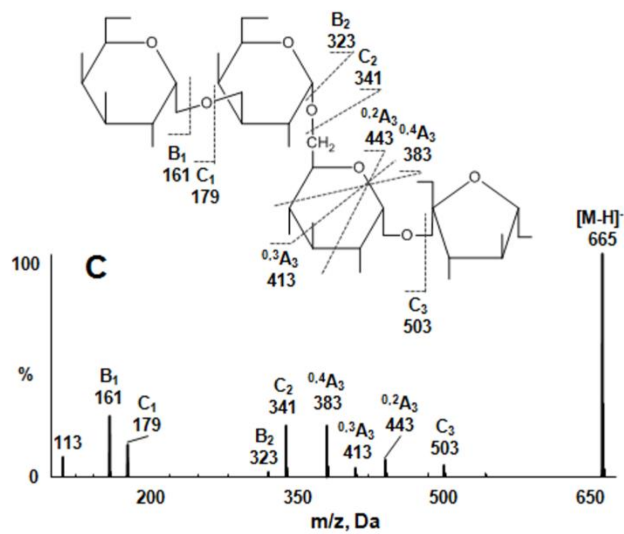
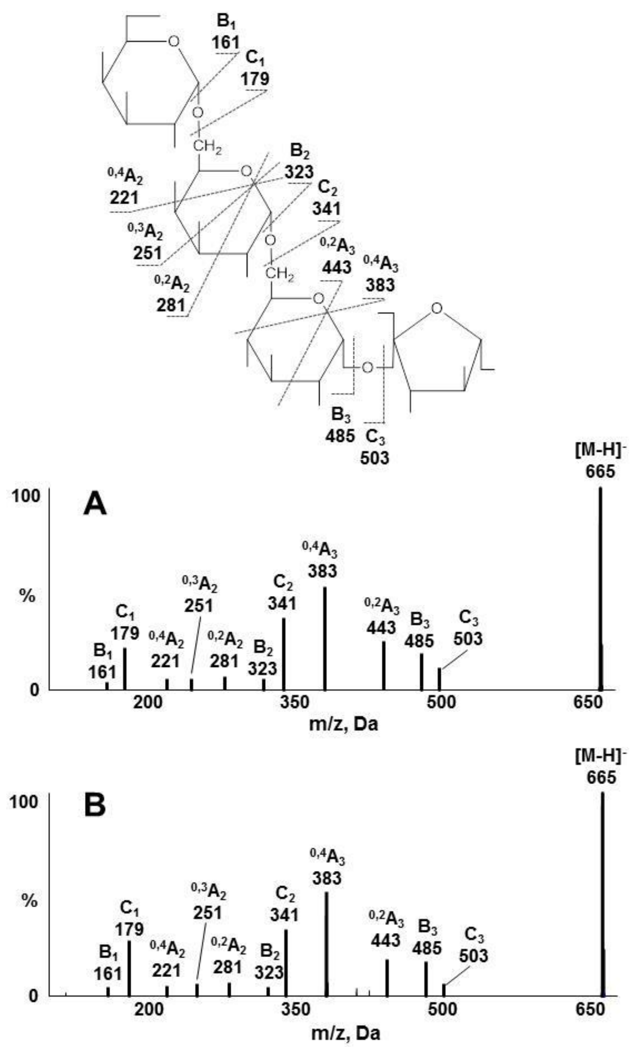






495 **Figure 7.**



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497 **Figure 8.**