Structure and function relationships of the binding of β- and α-galactosylated oligosaccharides to K88 fimbriae of enterotoxigenic *Escherichia coli*

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Abstract

Galactosylated oligosaccharides are synthesized by retaining glycosyl hydrolases with lactose as galactosyl donor. Previous studies reported that chitosan-oligosaccharides (COS) are suitable acceptors for β-galactosidase, and that galactosylated-COS prevented adhesion of enterotoxigenic *Escherichia coli* (ETEC) K88 to porcine erythrocytes. The present study aimed to determine the structure and functional relationship of oligosaccharides binding to ETEC K88 fimbriae. β- Or α-galacto-oligosaccharides (βGOS and αGOS), β-galactosylated melibiose, α-galactosylated lactose and β-/α-galactosylated COS were produced with β-/ α-galactosidase using lactose or melibiose as galactosyl donor, respectively. Fructosylated COS were produced by levansucrase with sucrose and COS. Oligosaccharides were fractionated with cation exchange chromatography and size exclusion chromatograph. The ability of oligosaccharides to prevent pathogen adhesion was measured with a hemagglutination assay, ELISA with ETEC cells, or with purified K88 fimbriae. High molecular weight β-GalCOS, β-galactosylated melibiose, and βGOS had strong anti-adhesion activity. Other oligosaccharides had weak or no anti-adhesive effects against ETEC K88. The ability of oligosaccharides to prevent binding of ETEC K88 or purified K88 fimbriae decreased with decreasing molecular weight. In conclusion, β-linked galactosyl moieties were necessary for preventing ETEC K88 attachment to porcine erythrocytes, the addition of *N*-glucosamine moieties improved the anti-adhesive ability against ETEC K88. Results of this study provide an avenue for valorization of lactose from whey to produce oligosaccharides with specific biological activity.

1. Introduction

Lactose is a disaccharide composed of galactose and glucose and occurs almost exclusively in the milk of mammals (Gänzle, Haase, Jelen, 2008). Whey containing lactose as main component is a by-product of cheese production, and lactose is thus an abundant carbohydrate resource (Fox, Guinee, Cogan, & McSweeney, 2017). Conversion of lactose to oligosaccharides is increasingly used for valorization of lactose from cheese whey (Fox, et al., 2017; Gänzle et al., 2008). β-Galactosidase hydrolyzes lactose and transfers galactose moieties to produce galacto-oligosaccharides (GOS) (Gänzle et al., 2008). Galactosylated oligosaccharides are synthesized when lactose is used as galactosyl donor for transgalactosylation of acceptor sugars including mannose, fructose, maltose, and chitosan-oligosaccharides (Gänzle, 2012; Yan, Hu, Simpson, & Gänzle, 2017). Other glycosyl hydrolases including sialidase, glucansucrase, levansucrase and fucosidase use lactose as acceptor carbohydrate to produce diverse oligosaccharides containing lactose at the reducing end (Chen & Gänzle, 2017). Sialyllactose and 3'-fucosyllactose were synthesized with lactose as acceptor sugar by sialidase and α-(1→3)-fucosyltransferase, respectively (Choi, Kim, Park, & Kim, 2016; Michalak, et al. 2014).

Dietary oligosaccharides have multiple biological activities. Oligosaccharides may directly modulate the immune system (Vogt et al., 2013), and prevent adherence of pathogens to intestinal epithelia (Chen & Gänzle, 2017; Kulkarni, Weiss, & Iyer, 2010). Colonic fermentation of oligosaccharides generates health beneficial short chain fatty acids but too vigorous fermentation induces osmotic diarrhea. Moreover, reduced consumption of fermentable oligosaccharides, disaccharides, monosaccharides and polyols (FODMAPS) relieved the symptoms of irritable bowel syndrome (Halmos, Power, Shepherd, Gibson, & Muir, 2014).

Pathogen adhesion through specific interaction between host glycan receptors and bacterial glycan binding proteins is a critical step for infection or intoxication (Shoaf-Sweeney & Hutkins, 2008). Specific glycan binding domains in bacterial proteins recognize specific oligosaccharide structures to mediate attachment. For instance, *Escherichia coli* with P-fimbriae specifically recognise galactobiose or molecules with galactobiose structures (Nizet, Varki, & Aebi, 2017). Receptor analogs saturate bacterial lectins and thus prevent pathogen adherence and infection (Shoaf-Sweeney & Hutkins, 2008). The concept is well supported by *in vivo* data for human milk oligosaccharides (HMOs), which prevent enteropathogenic *Escherichia coli* infection *in vitro* and in *vivo* by decreasing the attachment of EPEC (Manthey, et al., 2014). The commercial availability of biotechnologically produced HMOs (Öhrlein, 1999; Nishimoto & Kitaoka, 2007) allowed verification of their anti-adherence ability *in vivo*. HMOs prevented *Vibrio cholera*, *Campylobacter jejuni, and E.coli* adherence *in vivo* (Chen & Gänzle, 2017, Ruiz-Palacios, et al., 2003). GOS may have comparable activity for specific pathogens; for example, GOS reduced adherence of *Cronobacter sakazakii* to HEp-2 human cells (Quintero et al., 2011).

The specificity of pathogens adherence to specific host species allows the design of specific and effective intervention strategies; however, it also impedes concept validation through use of cell lines, (surgical) animal models, and *in vivo* pathogen challenge trials. Enterotoxigenic *E.coli* (ETEC) causes diarrhea in humans and piglets; human and animal ETECs produce identical toxins but employ host-specific fimbriae for adhesion (Jin & Zhao, 2000; Van den Broeck, Cox, Oudega & Goddeeris, 2000; Wolf, 1997). The use of piglets nevertheless allows validating *in vitro* studies with suitable *in vivo* model systems

because *in vitro* experimentation and *in vivo* validation with invasive surgical models can be done with the same host species (Chen, Woodward, Zijlstra, & Gänzle, 2014). β-Galactosylated chitosan oligosaccharides (β-GalCOS) were shown to prevent ETEC K88 adherence to porcine erythrocytes (Yan et al., 2017), however the specificity of oligosaccharide binding to K88 fimbriae remains unknown. This study therefore aimed to determine the influence of oligosaccharide composition, linkage type, and degree of polymerization (DP) on binding affinity to ETEC K88. Transglycosylation with β-galactosidase (Yan et al., 2017), α-galactosidase (Wang, Black, Curtis & Gänzle, 2013) and levansucrase (Tieking, Kühnl, & Gänzle, 2005) was employed to synthesize β-galactosylated COS, α-galactosylated COS, fructosylated COS, and β-/α-linked GOS. Oligosaccharides were separated by cation exchange and/or size exclusion chromatography and their binding affinity to was determined with cells of ETEC as well as purified K88 fimbriae.

2. Materials and Methods

2.1 Preparation of β-galactosidase and α-galactosidase crude cell extracts

Lactococcus lactis MG 1363 expressing the LacLM type β-galactosidase of *Lactobacillus plantarum* (Schwab, Sørensen, & Gänzle, 2010) were streaked onto M17 agar plates supplemented with 5% glucose and 5mg L^{-1} erythromycin (mM17) and incubated anaerobically at 30ºC for 48h. *L. lactis* MG 1363 harbouring the α-galactosidase of *Lb. reuteri* 100-23, Aga23 (Wang et al., 2013) was streaked onto mM17 with 0.0274% $MnSO_4$ and incubated anaerobically at 37ºC for 24h. Crude cell extract with β-galactosidase activity and α-galactosidase activity was prepared as described (Black, et al., 2014; Wang, et al., (2013). Protein content for both crude cellular extracts was

determined with Bradford protein assay. β-Galactosidase and α-galactosidase activities were measured with o-nitrophenyl-β-galactoside and 4-nitrophenyl-α-D-galactopyranoside, respectively, and were expressed as conversion of μmol substrate per min and mg protein in the crude cellular extract. (Schwab et al., 2011, Wang et al., 2013)

2.2 Overexpression and purification of levansucrase

Escherichia coli JM109 DE3 (pLEV1) harbouring *levS* coding for levansucrase of *Lb.sanfranciscensis* TMW 1392 (Tieking, et al., 2005) was streaked onto Luria-Bertani (LB) plate containing 100mg L^{-1} ampicillin at 37ºC. Overexpression of LevS by induction with isopropyl-β-D-thiogalactopyranoside was performed as described (Tieking et al., 2005). Levansucrase was purified from crude cellular extract by affinity chromatography (Tieking et al., 2005). The purification protocol was verified by SDS-PAGE; the protein concentration was quantified with the Bradford assay. Levansucrase activity was assay as previously described (Tieking, et al., 2005).

2.3 Synthesis of oligosaccharides with β-galactosidase

β-Galactosylated oligosaccharides were synthesized with lactose, melibiose or chitosan-oligosaccharides (COS; Glycobio Company, Dalian, P.R. China) as acceptors, and reactions contained 1M lactose, 0.5M lactose and 0.5M melibiose, or 180g L^{-1} lactose and 180g L^{-1} COS, respectively. Reaction were started by addition of 20% (v/v) crude cellular extract to achieve β-galactosidase activity of 28– 32μmol min^{-1} mg^{-1}, incubated 45ºC for 16h, and terminated by precipitation of proteins by addition of perchloric acid to a final concentration of 3.5% (v/v). All carbohydrates were dissolved in phosphate buffer at pH 6.8.

2.4 Synthesis of oligosaccharides with α-galactosidase

130 α-Galactosylated oligosaccharides were produced with melibiose, lactose, or COS as
131 acceptor carbohydrates. Reactions contained 1M melibiose, 0.5M melibiose and 0.5M
132 lactose, or 180g L^{-1} melibiose and 180g L^{-1} COS, respectively. Reactions were started by
133 addition of crude cellular extract to achieve α-galactose activity of 150 ± 10μmol min^{-1}
134 mg^{-1}, incubated at 37ºC for 24h, and terminated as described above. All carbohydrates
135 were dissolved in McIlvaine buffer at pH 4.7.

136 *2.5 Synthesis of oligosaccharides with levansucrase*

137 COS were β-fructosylated by incubation of 130g L^{-1} sucrose as fructosyl-donor and
138 130g L^{-1} COS with LevS as biocatalyst at 37ºC for 20h. Reactions were performed with
139 an enzyme concentration of 1μg mL^{-1} in 10mM sodium acetate buffer with 1mmol L^{-1}
140 $CaCl_2$, pH 5.4, and terminated as described above. Levansucrase enzyme activity was
141 adjusted to 90-100μmol min^{-1} mg^{-1}. Fructo-oligosaccharides as controls were also
142 synthesized in the acceptor reaction with sucrose at 260g L^{-1}.

143 *2.6 Oligosaccharides purification and fractionation*

144 β-Galactosylated COS (β-GalCOS), α-galactosylated COS (α-GalCOS) and fructosylated
145 COS (Fruc-COS) were purified by cation exchange chromatography (Yan et al., 2017). In
146 brief, reaction mixtures were adjusted to a total carbohydrate concentration of 10g L^{-1}
147 with 0.1% trifluoroacetic acid (TFA) and loaded on pre-conditioned SCX columns (55μm,
148 70A, Phenomenex, USA). Columns were washed with 0.1% TFA, charged
149 oligosaccharides were eluted with 0.2% trimethylamine and lyophilized.

150 The lyophilized fractions as well as the uncharged βGOS and αGOS were further
151 fractionated by size exclusion chromatography (SEC) (Yan et al., 2017). Five fractions
152 were collected for each sample and concentrated by lyophilization. Samples were

153 discarded when visible browning occurred during drying. Oligosaccharides were re-
154 dissolved in phosphate–buffer saline (PBS) (pH 7.2) to a concentration of 10g L^{-1}.

155 *2.7 High Performance Anion Exchange Chromatography with Pulsed Amperometric*
156 *Detection (HPAEC-PAD).*

157 Oligosaccharides were diluted to 1g L^{-1} prior to analysis on a HPAEC-PAD ICS-3000
158 system (Dionex, Oakville, Canada). Samples (10µL) were separated on a CarboPac PA-
159 20 column using water (A), 200mM NaOH (B), and 1M sodium acetate (C) as eluent.

160 *2.8 Hemagglutination assay to determine the effect of oligosaccharides on ETEC K88*
161 *adhesion to porcine erythrocytes.*

162 The hemagglutination assay was performed with the porcine ETEC ECL 13795
163 expressing $K88_{ac}$ fimbriae (Yan et al., 2017). *E. coli* were cultivated on Minca agar
164 overnight and washed with 1mL of PBS. *E.coli* cell suspensions with an optical density
165 (600 nm) of 70 were diluted 2-fold microtiter plates (Corning, Fisher Scientific, CA). The
166 same volume of PBS or oligosaccharide fractions was added. Oligosaccharides were used
167 at 10g L^{-1} and in two-fold serial dilutions to the exhaustion of biological activity or to
168 0.1g L^{-1}. Erythrocytes were prepared by 2-fold dilution of a 10% suspension of porcine
169 red blood cells (Innovative Research, Novi, MI, USA). The plate was incubated at 4ºC
170 overnight before visually inspection of hemagglutination. The lowest sugar concentration
171 corresponding to four fold difference to the control was noted.

172 *2.9 Purification of $K88_{ac}$ fimbriae from enterotoxigenic* E. coli *(ETEC) K88*

173 ETEC K88 fimbriae were extracted as described (Peng, Ling, Ning, & Deng, 2014) with
174 some modifications. ETEC were cultivated in tryptic soy broth medium for 16h at 37ºC.
175 Cells were collected by centrifugation, washed 3 times with phosphate buffer saline (PBS,

176 pH 7.4), and incubated at 60ºC for 30min. Cell debris was removed by centrifugation and
177 K88 fimbriae were precipitated with 60% (w/v) ammonium sulphate. Precipitates were
178 collected by centrifugation, re-solubilized in PBS and dialyzed against PBS overnight.
179 SDS-PAGE and Western blot verified purification. The antibody used for western blot
180 was *E.coli* K88A antibody (Biorad, USA) and a goat anti-mouse IgG (H+L) secondary
181 antibody conjugated to horseradish peroxidase (Invitrogen, Fisher Scientific, CA). The
182 protein concentration of the solution containing K88 fimbriae was determined with
183 Bradford assay and adjusted to 0.37 ± 0.02mg L^{-1} prior to ELISA assay as described
184 below.
185 *2.10 Enzyme-linked immunosorbent assay (ELISA) to determine the effect of*
186 *oligosaccharides on adhesion of ETEC K88 cells or K88 fimbriae to porcine erythrocytes.*
187 The ability of oligosaccharides to inhibit adhesion of *E.coli* K88 cells or K88 fimbriae to
188 porcine erythrocytes was also assayed by ELISA. A 96-well high bind microtiter plate
189 (Corning, Fisher Scientific, CA) was coated overnight with 100µL of 5% porcine red
190 blood cells (Innovative Research, Novi, MI, USA) and blocked with 200µL of 3% bovine
191 serum albumin for 1h at 4ºC. Oligosaccharides were re-dissolved in PBS to a
192 concentration of 10g L^{-1}. The same volume of ETEC with OD_{600nm} of 1.0, or of K88
193 fimbriae with a protein concentration of 0.1mg L^{-1} were mixed with PBS or
194 oligosaccharides; 100µL of the mixture was added into plates and incubated for 1h 4ºC.
195 Red blood cells without addition of ETEC or K88 fimbriae, red blood cells with addition
196 of ETEC or K88 fimbriae only, and wells without red blood cells but addition of ETEC
197 or K88 fimbriae served as controls. The wells were then treated with mouse anti *E.coli*
198 K88A antibody (Biorad, USA) at a dilution of 1:2000. After 1h incubation, wells were

199 treated with a goat anti-mouse IgG (H+L) secondary antibody conjugated to horseradish
200 peroxidase (Invitrogen, Fisher Scientific, CA) for 1h. After addition of TMB-ELISA
201 substrates and incubation for 30min, the reaction was stopped by 2M sulphuric acid, and
202 the absorbance at 450 nm was measured on a microplate reader (Thermo Scientific, CA).

203 *2.11 Statistical analysis*

204 Purification and fractionation of each oligosaccharide was conducted in three
205 independent enzymatic reactions. Bioassays were performed in triplicated technical
206 repeats. Results of bioassay are presented as means ± standard error of the mean.

207 **3. Results**

208 *3.1 Enzymatic synthesis of oligosaccharide libraries with β-galactosidase,*
209 *α-galactosidase, and levansucrase*

210 The formation of oligosaccharides through acceptor reactions with β-galactosidase (Fig
211 1A and Fig 1C), α-galactosidase (Fig 1B and Fig 1D) and levansucrase (Fig 2) were
212 analyzed by HPAEC-PAD. Chromatograms indicated that COS (data not shown) and
213 melibiose (Figure 1C) were suitable acceptors for β-galactosidase; COS (data not shown)
214 and lactose (Fig 1D) were suitable acceptors for α-galactosidase (Figure 1, Black, et al.,
215 2014; Lu, Xiao, Li, Li, & Wang, 2009; Wang et al., 2014**)**. In analogy, the pattern of
216 oligosaccharides produced by levansucrase after addition of COS as acceptor suggested
217 formation of fructosylated COS (Fig.2A). Enzymatic reactions with COS as acceptor
218 were purified by ion exchange chromatography to remove uncharged reaction products,
219 and to remove contaminants (Yan et al., 2017).

220 *3.2 Effect of oligosaccharides on adhesion of* E. coli *K88 to porcine erythrocytes.*

221 Oligosaccharide fractions obtained by cation exchange chromatography were tested for
222 anti-adhesion activity against ETEC K88 with hemagglutination assay and their activity
223 was compared to the corresponding COS fractions (Table 1). COS separated by cation
224 exchange chromatography inhibit ETEC K88 adhesion at 6.7g L^{-1} (Yan et al., 2017 and
225 data not shown). Glycosylation of COS to obtain β-GalCOS, α-GalCOS or β-FrucCOS
226 enhanced their ability to prevent ETEC K88 binding to erythrocytes about five-fold
227 (Table 1).

228 *3.3 Fractionation of oligosaccharides by size exclusion chromatography*

229 β-GalCOS, α-GalCOS and β-FrucCOS were further fractionated by SEC. The molecular
230 weight cut off was F2, Mw 1500-2500 Da; F3, Mw 900-1500 Da; F4, Mw 500-1200 Da;
231 and F5, less than 500 Da. The formation of fructosylated COS was further confirmed
232 with HPAEC-PAD analysis of fractions obtained after cation exchange separation and
233 SEC fractionation. Analysis of oligosaccharide fractions derived from levansucrase
234 reactions with sucrose and COS as acceptor by HPAEC-PAD identified oligosaccharide
235 peaks that were absent in COS, or in reactions without COS (Fig 2B and 2C), indicating
236 formation of fructosylated COS.

237 β-GalCOS F2 had strongest anti-adherence activity followed by α-GalCOS F2 and β-
238 FrucCOS F2 (Table 1). The anti-adhesion activity decreased with decreasing molecular
239 weight (Table 1). All fractions containing glycosylated COS were more active when
240 compared to the corresponding fractions obtained with COS. β-Galactolylated COS
241 consistently exhibited the highest activity (Table 1).

242 GOS with mixed β- or α-linkages were also fractionated by SEC. Due to the low yield of
243 β- or α-galactosylated melibiose and lactose derived oligosaccharides with high

molecular weight, fractions 2 and 3 in each oligosaccharides group were combined. All fractions were analyzed with HPAEC-PAD (Figure 3) and evaluated with respect to their ability to prevent ETEC adhesion (Table 2). Different pattern of peaks were observed which indicated the formation of various oligosaccharides (Fig 3). Anti-adhesive activity was observed only with β-galactosylated oligosaccharides with a relative molecular weight of more than 900, corresponding to a DP of more than 6 (F2 and F3, Table 2). The activity of β-galactosylated melibiose oligosaccharides in (F2 + F3) was not significantly higher than the activity of β-galactosylated lactose (β-GOS) in (F2 and F3) (Table 2). Fractions of β-galactosylated melibiose or β-GOS with small molecular weight and fractions of α-galactosylated lactose or melibiose (α-GOS) did not prevent adherence of ETEC K88 to porcine erythrocytes (Table 2).

3.4 Quantification of ETEC adhesion to porcine blood cells by ELISA

The anti-adhesive activities of fractions were confirmed by quantification of ETEC K88 adhesion to porcine erythrocytes by ELISA and K88 specific antibodies (Figure 4). Results obtained by ELISA with β-GalCOS were similar to the hemagglutination assay; β-GalCOS F2 strongly reduced ETEC adhesion and the inhibitory effect decreased with decreasing molecular weight (Fig.4). Different from hemagglutination assay, the effect of α-GalCOS and β-FrucCOS was largely independent of the molecular weight (Fig 4).

The anti-adhesive activity of non-charged oligosaccharides against ETEC adhesion is shown in Figure 5. β-Galactosylated melibiose oligosaccharides with high molecular weight (F2 and F3) showed the strongest anti-adhesion activity. β-Galactosylated lactose (β-GOS) (F2 and F3), and α-galactosylated melibiose (F2 and F3) exhibited weaker

activity (Fig 5). Other oligosaccharide fractions and all fractions containing oligosaccharides with DP < 6 (F4 and F5) had only weak anti-adhesive activity (Fig. 5).

3.5. Quantification of the binding of K88 fimbriae to porcine blood cells by ELISA

To determine whether the activity of oligosaccharides is based on interference with binding of K88 fimbriae, the anti-adhesive activity of oligosaccharides were evaluated with purified K88 fimbriae. Western blot (Fig 6A) and SDS-PAGE (Fig 6B) confirmed that the protocol for purification of fimbriae provided a relatively pure preparation of fimbriae. The ability of oligosaccharides to prevent binding of these K88 fimbriae to porcine erythrocytes was quantified by ELISA (Figure 7). Similar to the other two assays, high molecular weight β-GalCOS (F2) had the highest activity in blocking K88 fimbriae; other glycosylated COS or β-GalCOS with a DP of less than 8 (F3, F4) were significantly less active (Figure 7). Uncharged oligosaccharides had lower anti-adherence activity; β-galactosylated melibiose (F2 + F3) blocked adhesion of K88 fimbriae getter than the corresponding fraction with α-galactosylated melibiose (Figure 7).

4. Discussion

This study assayed the glycan receptor specificity of ETEC K88 fimbriae with oligosaccharide libraries synthesized with three retaining glycosyl hydrolases and multiple acceptor carbohydrates for each glycosyl hydrolase.

Lactose and melibiose were used as glycosyl donor in reactions with β-galactosidase and α-galactosidase, respectively, using melibiose, lactose and COS as acceptor sugars. Oligosaccharide synthesis with levansucrase was conducted with sucrose as fructosyl donor and COS as acceptors. An overview on the reactions and the respective products is provided in Figure 8. Lactose and melibiose are known to be suitable acceptor

289 carbohydrates for β- as well as α-galactosidases (Lu et al., 2009; Wang, et al. 2014) but
290 the synthesis of fructosylated COS with levansucrase was not previously reported. The
291 synthesis of β-GalCOS by β-galactosidase was previously confirmed by MS/MS and LC-
292 MS/MS analysis of products (Black et al., 2014; Yan et al., 2017). The present study used
293 cation-exchange separation, SEC fractionation, HPACE-PAD analysis and the evaluation
294 of the biological activity as indication the formation of α-GalCOS and β-FrucCOS
295 formation. The structural analysis of α-GalCOS and β-FrucCOS, however, remains
296 subject to future investigations. Taken together, the present study demonstrates that the
297 use of retaining glycosyl hydrolases (Gänzle, 2012) with reciprocal acceptor / donor pairs
298 is a suitable tool to synthesize oligosaccharide libraries for studies aiming to elucidate
299 structure/function relationships.
300 Hemagglutination is a relatively simple assay to quantify ETEC binding to host cells
301 (Coddens et al., 2011; Monens et al., 2015; Sun, Gänzle & Wu, 2017), and results
302 obtained with the hemagglutination assay have been verified *in vivo* (Chen et al., 2014).
303 The assay is sensitive, however, to the presence of salts introduced by oligosaccharide
304 purification and concentration, necessitating the use of buffers composed of volatile
305 components for oligosaccharide fractionation to allow removal of buffer components by
306 freeze-drying (Sun et al., 2017; Yan et al., 2017). Moreover, COS are reducing
307 carbohydrates containing primary amines; the formation of Maillard products that
308 interfere with hemagglutination is observed even in the temperature range of 20 – 50°C
309 (Hrynets, Ndagijimana, Betti, 2015; Yan et al., 2017). The present study therefore
310 complemented the hemagglutination assay with the quantification of the binding of
311 ETEC and purified K88 fimbriae to porcine erythrocytes by ELISA. The three assays

312 provided consistent results with exception of the determination of the activity of
313 α-GalCOS and β-FrucCOS (Tab. 1, Fig. 4 and Fig. 7). The discrepancies may relate to
314 non-K88 binding molecules, or to Maillard reaction products that were present in low
315 molecular weight fractions prepared from of α-GalCOS and β-FrucCOS. Owing to longer
316 incubation times for the enzymatic reaction, discoloration caused by the Maillard reaction
317 was more pronounced in reactions with α-galactosidase and levansucrase. Fractionation
318 with cation exchange chromatography and SEC to obtain high molecular weight
319 oligosaccharide fractions, however, removed interferences. The use of multiple assays to
320 determine pathogen binding including the use of purified $K88_{ac}$ fimbriae, however,
321 demonstrated that the activity of oligosaccharide fractions directly relates to binding to
322 the lectin domain of K88 fimbriae (Moonens et al., 2015).
323 K88 fimbriae of *E. coli* occur in three variants, $K88_{ab}$, $K88_{ac}$, and $K88_{ad}$, which have
324 overlapping yet distinct receptor specificities (Coddens et al., 2011; Guinée & Jansen,
325 1979; Moonens et al., 2015). β-Galactosylated melibiose and lactose with DP > 6, β-
326 GalCOS with DP > 8 prevented binding of ETEC K88 as well as purified $K88_{ac}$ fimbriae
327 to porcine erythrocytes (Table 2, Fig. 5 and Fig. 7). Fructosylation of COS or
328 α-galactosylation of lactose and melibiose did not greatly enhance increase the anti-
329 adhesive activity of acceptor carbohydrates. β-Linked galactosyl moieties were thus
330 promoting anti-adhesive activity; the presence of multiple *N*-glucosamine moieties at the
331 reducing end also increased activity. Our results conform to prior studies demonstrating
332 that β-linked galactosidase and /or *N*-acetylglucosamine residues are important for
333 receptor binding of all three variants of K88 fimbriae, including the most common type
334 $K88_{ac}$ (Coddens et al., 2011; Grange et al., 2002; Moonens et al., 2015, Van den Broeck

et al., 2000), and extend the array of oligosaccharides binding to K88 fimbriae to oligosaccharides that are readily synthesised with β-galactosidase, lactose, and COS or melibiose.

We observed that high molecular weight oligosaccharides prevented K88 binding to porcine erythrocytes more effectively than analogous oligosaccharides with a lower DP, confirming and extending previous observations with β-GalCOS (Yan et al., 2017). This observation may relate to the low affinity of the glycan binding domain of bacterial fimbriae to the glycan receptors. High molecular weight oligosaccharides may allow multiple interactions with the bacterial lectins. Likewise, a high density of glycan moieties thus favours bacterial adherence with multiple fimbriae per cell (Nizet, Varki, & Aebi, 2017). In analogy, Shiga toxin subunit B, which mediates glycan recognition, has three glycan binding sites for a total of 15 glycan-protein receptor events for a Shiga toxin molecule composed of one StxA and five StxB units (Pina, and Johannes, 2005). Bacteriophage binding to host glycans also requires a multiplicity of the weak protein-glycan interactions (for review, see Mahony, Cambillau, & van Sinderen, 2017).

Because glycan binding proteins of different pathogens recognize different glycan receptors, the development of oligosaccharides libraries may allow interventions targeting multiple pathogens. *Salmonella* and type I fimbriated *E.coli* strains are specific to mannose (Nizet, Varki, & Aebi, 2017). Glycosylated mucin (MUC 1) from cow's milk inhibited binding of *E.coli*, *Salmonella enterica* serovar Typhimurium, *Staphylococcus aureus* and *Bacillus subtilis* to human cell lines (Parker, et al., 2010). Yeast-derived β-galactomannan blocked ETEC K88 binding to porcine intestinal epithelial cells (Badia, et al., 2012). β-Galactosides with or without *N*-glucosamine also prevent adhesion of other

pathogens including P-fimbriated *E.coli* and *Pseudomonas aeruginose* (Kulkarni, Weiss, & Iyer, 2010). *N*-acetylgalactosamine and *N*-acetylglucosamine reduced adhesion of *E.coli* (O-, H48) to Caco-2 cells (Parker, et al., 2010). COS prevented adhesion of enteropathogenic *E.coli* (Quintero-Villegas, et al., 2013), and chitosan reduced the adherence of human uropathogens (Campana, et al., 2017). The oligosaccharide libraries generated in the present study thus may prevent adhesion of pathogens other than ETEC K88, and may be useful in preventing human disease.

In conclusion, the present study generated oligosaccharide libraries by using three glycosyl hydrolases and lactose, melibiose or COS as acceptor carbohydrates, and evaluated the effect of oligosaccharide mixtures and fractions on adherence of *E. coli* K88 to porcine erythrocytes. β-Galactosylated oligosaccharides produced with lactose as galactosyl donor were superior to α-galactosylated or fructosylated oligosaccharides with respect to their inhibition of K88 adherence. The ability of β-galactosylated oligosaccharides to prevent pathogen adhesion may complement the specific synthesis of oligosaccharides produced by glycosyltransferases in genetically modified organisms (Nishimoto & Kitaoka, 2007; Öhrlein, 1999). Infants and weaned animals are particularly prone to infection by diarrheal pathogens; this study may thus provide novel avenues for valorization of lactose from whey by oligosaccharide synthesis for applications in infant formula and in feed formulation for calves and weaned piglets.

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References

381 Badia, R., Zanello, G., Chevaleyre, C., Lizardo, R., Meurens, F., Martínez, P.,martínez,
382 J.Brufau & Salmon, H. (2012). Effect of *Saccharomyces cerevisiae* var. Boulardii and β-
383 galactomannan oligosaccharide on porcine intestinal epithelial and dendritic cells
384 challenged in vitro with *Escherichia coli* F4 (K88). *Veterinary Research*, *43*, 4.
385 Black, B. A., Yan, Y., Galle, S., Hu, Y., Curtis, J. M., & Gänzle, M. G. (2014).
386 Characterization of novel galactosylated chitin-oligosaccharides and chitosan-
387 oligosaccharides. *International Dairy Journal*, *39*, 330-335
388 Campana, R., Casettari, L., Ciandrini, E., Illum, L., & Baffone, W. (2017). Chitosans
389 inhibit the growth and the adhesion of *Klebsiella pneumoniae* and *Escherichia coli*
390 clinical isolates on urinary catheters. *International Journal of Antimicrobial Agents*, *50*,
391 135-141.
392 Chen, X. Y., Woodward, A., Zijlstra, R .T., & Gänzle, M. G. (2014). Exopolysaccharides
393 synthesised by *Lactobacillus reuteri* protect against enterotoxigenic *Escherichia coli* in
394 piglets. *Applied Environmental Microbiology, 80*, 5752-5760.
395 Chen, X. Y., & Gänzle, M. G. (2017). Lactose and lactose-derived oligosaccharides:
396 More than prebiotics? *International Dairy Journal*, 67, 61-72.
397 Choi, Y. H., Kim, J. H., Park, B. S., & Kim, B. G. (2016). Solubilization and iterative
398 saturation mutagenesis of α1, 3-fucosyltransferase from *Helicobacter pylori* to enhance
399 its catalytic efficiency. *Biotechnology and Bioengineering*, *113*, 1666-1675.
400 Coddens, A., Valis, E., Benktander, J., Ångström, J., Breimer, M.E., Cox, E. & Teneberg,
401 S. (2011). Erythrocyte and porcine intestinal glycosphingolipids recognized by F4
402 fimbriae of enterotoxigenic *Escherichia coli. PLoS One, 6*, e23309.

403 Fox, P. F., Guinee, T. P., Cogan, T. M., & McSweeney, P. L. (2017). Whey and whey
404 products. In *Fundamentals of Cheese Science* (pp. 755-769). Springer US.
405 Gänzle, M. G. (2012). Enzymatic synthesis of galacto-oligosaccharides and other lactose
406 derivatives (hetero-oligosaccharides) from lactose. *International Dairy Journal*, *22*, 116-
407 122.
408 Gänzle, M. G., Haase, G., & Jelen, P. (2008). Lactose: crystallization, hydrolysis and
409 value-added derivatives. *International Dairy Journal*, 18, 685-694.
410 Grange, P. A., Mouricout, M. A., Levery, S. B., Francis, D. H., & Erickson, A. K. (2002).
411 Evaluation of receptor binding specificity of *Escherichia coli* K88 (F4) fimbrial adhesin
412 variants using porcine serum transferrin and glycosphingolipids as model receptors.
413 *Infection and Immunity*, *70*, 2336-2343.
414 Guinée, P. A., & Jansen, W. H. (1979). Behavior of *Escherichia coli* K antigens K88ab,
415 K88ac, and K88ad in immunoelectrophoresis, double diffusion, and hemagglutination.
416 *Infection and Immunity, 23,* 700–705.
417 Halmos, E. P., Power, V. A., Shepherd, S. J., Gibson, P. R., & Muir, J. G. (2014). A diet
418 low in FODMAPs reduces symptoms of irritable bowel syndrome. *Gastroenterology*, *146*,
419 67-75.
420 Hrynets, Y., Ndagijimana, M. & Betti, M. (2015). Studies on the formation of Maillard
421 and caramelization products from glucosamine incubated at 37 °C. *Journal of*
422 *Agricultural and Food Chemistry, 63,* 6249−6261.
423 Jin, L. Z., & Zhao, X. (2000). Intestinal receptors for adhesive fimbriae of
424 enterotoxigenic *Escherichia coli* (ETEC) K88 in swine--a review. *Applied Microbiology*
425 *and Biotechnology, 54,* 311-318.

426 Kulkarni, A. A., Weiss, A. A., & Iyer, S. S. (2010). Glycan-based high-affinity ligands
427 for toxins and pathogen receptors. *Medicinal Research Reviews*, *30*, 327-393.
428 Michalak, M., Larsen, D. M., Jers, C., Almeida, J. R., Willer, M., Li, H., Kirpekar, F.,
429 Kjærulff, L., Gotfredsen, C.H., Nordvang, R.T. & Meyer, A. S. (2014). Biocatalytic
430 production of 3'-sialyllactose by use of a modified sialidase with superior *trans*-sialidase
431 activity. *Process Biochemistry*, 49, 265-270.
432 Lu, L. L., Xiao, M., Li, Z. Y., Li, Y. M., & Wang, F. S. (2009). A novel
433 transglycosylating β-galactosidase from *Enterobacter cloacae* B5. *Process Biochemistry*,
434 *44*, 232-236.
435 Mahony, J., Cambillau, C., & van Sinderen, D. (2017). Host recognition by lactic acid
436 bacterial phages. *FEMS Microbiology Reviews*, *41*, S16-S26.
437 Manthey, C. F., Autran, C. A., Eckmann, L., & Bode, L. (2014). Human milk
438 oligosaccharides protect against enteropathogenic *Escherichia coli* attachment in vitro
439 and EPEC colonization in suckling mice. *Journal of Pediatric Gastroenterology and*
440 *Nutrition*, *58*, 165-168.
441 Moonens, K., Van den Broeck, I., De Kerpel, M., Deboeck, F., Raymaekers, H., Remaut,
442 H. & De Greve, H. (2015). Structural and functional insight into the carbohydrate
443 receptor binding of F4 fimbriae-producing enterotoxigenic *Escherichia coli. Journal of*
444 *Biological Chemistry, 290,* 8409-8419.
445 Nishimoto, M., & Kitaoka, M. (2007). Practical preparation of lacto-N-biose I, a
446 candidate for the bifidus factor in human milk. *Bioscience, Biotechnology, and*
447 *Biochemistry*, 71, 2101-2104.

448 Nizet, V., Varki, A., & Aebi, M. (2017). Microbial Lectins: Hemagglutinins, adhesins,
449 and toxins. In: Varki, A., Cummings, R.D., Esko, J.D., Stanley, P., Hart, G.W., Aebi, M.,
450 Darvill, A.G., Kinoshita, T., Packer, N.H., Prestegard, J.H., Schnaar, R.L., Seeberger,
451 P.H (eds). Essentials of glycobiology, 3rd Ed. Cold Spring Harbor (NY): Cold Spring
452 Harbor Laboratory Press; 2015-2017.
453 Öhrlein R. Glycosyltransferase-catalyzed synthesis of non-natural oligosaccharides.
454 *Topics in Current Chemistry* (*Biocatalysis: from discovery to application*), vol. 200.
455 Germany: Springer-Verlag; 1999. p. 227–254.
456 Parker, P., Sando, L., Pearson, R., Kongsuwan, K., Tellam, R. L., & Smith, S. (2010).
457 Bovine Muc1 inhibits binding of enteric bacteria to Caco-2 cells. *Glycoconjugate Journal*,
458 *27*, 89-97.
459 Peng, Z., Ling, M., Ning, Y., & Deng, L. (2014). Rapid fluorescent detection of
460 *Escherichia coli* K88 based on DNA aptamer library as direct and specific reporter
461 combined with immuno-magnetic separation. *Journal of Fluorescence*, *24*, 1159-1168.
462 Pina, D. G., & Johannes, L. (2005). Cholera and Shiga toxin B-subunits: thermodynamic
463 and structural considerations for function and biomedical applications. *Toxicon*, *45*, 389-
464 393.
465 Quintero, M., Maldonado, M., Perez-Munoz, M., Jimenez, R., Fangman, T., Rupnow, J.
466 Wittke, A., Russell, M.., & Hutkins, R. (2011). Adherence inhibition of *Cronobacter*
467 *sakazakii* to intestinal epithelial cells by prebiotic oligosaccharides. *Current*
468 *Microbiology*, *62*, 1448-1454.
469 Quintero-Villegas, M. I., Aam, B. B., Rupnow, J., Sorlie, M., Eijsink, V. G., Hutkins, R.
470 W. (2013). Adherence inhibition of enteropathogenic *Escherichia coli* by

chitooligosaccharides with specific degrees of acetylation and polymerization. *Journal of Agricultural and Food Chemistry, 61*, 2748-2754.

Ruiz-Palacios, G. M., Cervantes, L. E., Ramos, P., Chavez-Munguia, B., & Newburg, D. S. (2003). *Campylobacter jejuni* binds intestinal H (O) antigen (Fucα1, 2Galβ1, 4GlcNAc), and fucosyloligosaccharides of human milk inhibit its binding and infection. *Journal of Biological Chemistry*, 278, 14112-14120.

Schwab, C., Lee, V., Sørensen, K. I., & Gänzle, M. G. (2011). Production of galactooligosaccharides and heterooligosaccharides with disrupted cell extracts and whole cells of lactic acid bacteria and bifidobacteria. *International Dairy Journal*, *21*, 748-754.

Schwab, C., Sørensen, K. I., & Gänzle, M. G. (2010). Heterologous expression of glycoside hydrolase family 2 and 42 β-galactosidases of lactic acid bacteria in *Lactococcus lactis*. *Systematic and Applied Microbiology*, *33*, 300-307.

Shoaf-Sweeney, K. D., & Hutkins, R. W. (2008). Adherence, anti-adherence, and oligosaccharides: Preventing pathogens from sticking to the host. *Advances in Food and Nutrition Research*, *55*, 101-161.

Sun, X., Gänzle, M., & Wu, J. (2017). Identification and characterization of glycopeptides from ovomucin with anti-agglutinating activity against porcine K88 enterotoxigenic *Escherichia coli* strains. *Journal of Agricultural and Food Chemistry, 65,* 777–783.

Tieking, M., Kühnl, W., & Gänzle, M. G. (2005). Evidence for formation of heterooligosaccharides by *Lactobacillus sanfranciscensis* during growth in wheat sourdough. *Journal of Agricultural and Food Chemistry*, *53*, 2456-2461.

494 Van den Broeck, W., Cox, E., Oudega, B., & Goddeeris, B.M. (2000). The F4 fimbrial
495 antigen of *Escherichia coli* and its receptors. *Veterinary Microbiology*, *71*, 223-244.
496 Vogt, L., Ramasamy, U., Meyer, D., Pullens, G., Venema, K., Faas, M. M., Schols, H. A.
497 & de Vos, P. (2013). Immune modulation by different types of β-(2→1)-fructans is toll-
498 like receptor dependent. *PLoS One*, 8, e68367.
499 Wang, Y., Black, B. A., Curtis, J. M., & Gänzle, M. G. (2014). Characterization of
500 α-galacto-oligosaccharides formed via heterologous expression of α-galactosidases from
501 *Lactobacillus reuteri* in *Lactococcus lactis*. *Applied Microbiology and Biotechnology*, *98*,
502 2507-2517.
503 Wolf, M. K. (1997). Occurrence, distribution, and associations of O and H serogroups,
504 colonization factor antigens, and toxins of enterotoxigenic *Escherichia coli*. *Clinical*
505 *Microbiology Reviews, 10,* 569-584.
506 Yan, Y. L., Hu, Y., Simpson, D. J., & Gänzle, M. G. (2017). Enzymatic synthesis and
507 purification of galactosylated chitosan oligosaccharides reducing adhesion of
508 enterotoxigenic *Escherichia coli* K88. *Journal of Agricultural and Food Chemistry*.

Figure legends.

Figure 1. HPAEC-PAD diagrams of oligosaccharides synthesized through the acceptor reactions of α-galactosidase and β-galactosidase. Melibiose and lactose were used as glycosyl donors in all reactions with α-galactosidase and β-galactosidase, respectively. **Panel A.** Separation of reactions with β-galactosidase, lactose and COS; **Panel B.** Separation of with α-galactosidase, melibiose and COS; **Panel C,** Separation of reactions with β-galactosidase and lactose or lactose and melibiose; **Panel D,** Separation of reactions with α-galactosidase and melibiose or melibiose and lactose. Lactose, melibiose, and COS were used as external standards.

Figure 2. HPAEC-PAD diagrams of oligosaccharides synthesized through the acceptor reaction of levansucrase and oligosaccharides fractions collected with SEC chromatography. **Panel A**. levansucrase with sucrose alone or with sucrose and COS; **Panel B**. Fruc-COS fractions; **Panel C.** COS fractions. Sucrose and COS were used as external standards.

Figure 3. HPAEC-PAD diagrams of galactosylated oligosaccharides fractions collected with SEC chromatography. Panel A. β-GalCOS; Panel B: α-GalCOS; Panel C. β-galactosylated lactose; Panel D: α-galactosylated melibiose; Panel E. β-galactosylated melibiose; Panel F. α-galactosylated lactose.

Figure 4. Reduction of adherence of ETEC K88 ECL 13975 (O149, virotype STb:LT:EAST1:F4) to porcine erythrocytes by galactosylated or fructosylated chitosan oligosaccharides. The sugar concentration was 10 g L -1. ELISA was performed with ETEC cells and K88-specific antibodies. Results are shown as means ± standard

deviation of three independent assays. Bars that do not share a common superscript are significantly different ($P< 0.05$).

Figure 5. Reduction of adherence of ETEC K88 ECL 13975 (O149, virotype STb:LT:EAST1:F4) to porcine erythrocytes by α-, β-, or α/β-linked galacto-oligosaccharides as measured by ELISA. The carbohydrate concentration was adjusted to 10 g L -1. ELISA was performed with ETEC cells and K88-specific antibodies. Results are shown as means ± standard deviation of three independent assays. Bars that do not share a common superscript are significantly different ($P< 0.05$).

Figure 6. Panel A**:** Western blot of K88 fimbriae purified from ETEC ECL 13975. The molecular weight marker was applied on lane 1 and two independent extractions were applied on lane 2 and 3. Panel B: SDS-page of K88 fimbriae purified form ETEC ECL 13975. The molecular weight marker was applied on lane 1 and two independent extractions were applied on lane 2 and 3.

Figure 7. Reduction of binding of $K88_{ac}$ fimbriae purified from ETEC ECL 13975 (O149, virotype STb:LT:EAST1:F4) to porcine erythrocytes by galactosylated or fructosylated chitosan oligosaccharides, or by α-, β-, or α/β-linked galacto-oligosaccharides. The carbohydrate concentration was 10 g L^{-1}. ELISA was performed with K88-specific antibodies. Results are shown as means ± standard deviation of three independent assays. Bars that do not share a common superscript are significantly different ($P< 0.05$).

Figure 8. Schematic representation of acceptor reactions conducted in the study. β-Galactosylated or fructosylated oligosaccharides were synthesized with β-galactosidase and lactose as glycosyl donor (Panel A) or levansucrase and sucrose as glycosyl donor

(Panel B), respectively. α-Galactosylated oligosaccharides were synthesized with α-galactosidase and melibiose as glycosyl donor (Panel C).

Table 1. Inhibition of erythrocyte agglutination by ETEC K88 strain with glycosylated COS. Hemagglutination was determined with *E.coli* K88 ECL 13975 (O149, virotype STb:LT:EAST1:F4). Results are shown as means ± standard deviation of three independent assays. Values that do not share a common superscript are significantly different ($P< 0.05$).

Oligosaccharides	**Minimum inhibitory concentration for erythrocyte agglutination with ETEC (g L $^{-1}$)**
COS F2*	10.00 ± 0.00^{a}
COS F3	3.33 ± 1.2^{b}
COS F4	5.00 ± 0.0^{b}
β-GalCOS elution	1.25 ± 0.0^{bc}
β-GalCOS F2	0.17 ± 0.1^{d}
β-GalCOS F3	1.67 ± 0.6^{b}
β-GalCOS F4	3.33 ± 1.2^{b}
α-GalCOS elution	1.25 ± 0.0^{bc}
α-GalCOS F2	0.63 ± 0.0^{c}
α-GalCOS F3	4.17 ± 1.2^{b}
α-GalCOS F4	10.00 ± 0.0^{a}
β-FrucCOS elution	1.25 ± 0.0^{bc}
β-FrucCOS F2	0.63 ± 0.0^{c}
β-FrucCOS F3	1.25 ± 0.0^{bc}
β-FrucCOS F4	>10.00

*The molecular weight cut off was F2, Mw 1500-2500 Da; F3, Mw 900-1500 Da; F4, Mw 500-1200 Da; and F5, less than 500 Da.

Table 2. Inhibition of erythrocyte agglutination by ETEC K88 strain with α-galacto-oligosaccharides, β-galacto-oligosaccharides and α/β-galacto-oligosaccharides. Hemagglutination was determined with *E.coli* K88 ECL 13975 (O149, virotype STb:LT:EAST1:F4). Results are shown as means ± standard deviation of three independent assays.

Oligosaccharides	**Minimum inhibitory concentration for agglutination with ETEC (g L $^{-1}$)**
β-GOS F2+F3	8.33 ± 2.4
β-GOS F4	>10.00
β-GOS F5	>10.00
β-galactosylated melibiose F2+F3	5.00 ± 0.0
β-galactosylated melibiose F4	>10.00
β-galactosylated melibiose F5	>10.00
α-galactosylated lactose F2+F3	10.00 ± 0.0
α-galactosylated lactose F4	>10.00
α-galactosylated lactose F5	>10.00
α-GOS F2+F3	>10.00
α-GOS F4	>10.00
α-GOS F5	>10.00

Fig 1. Yan et al.

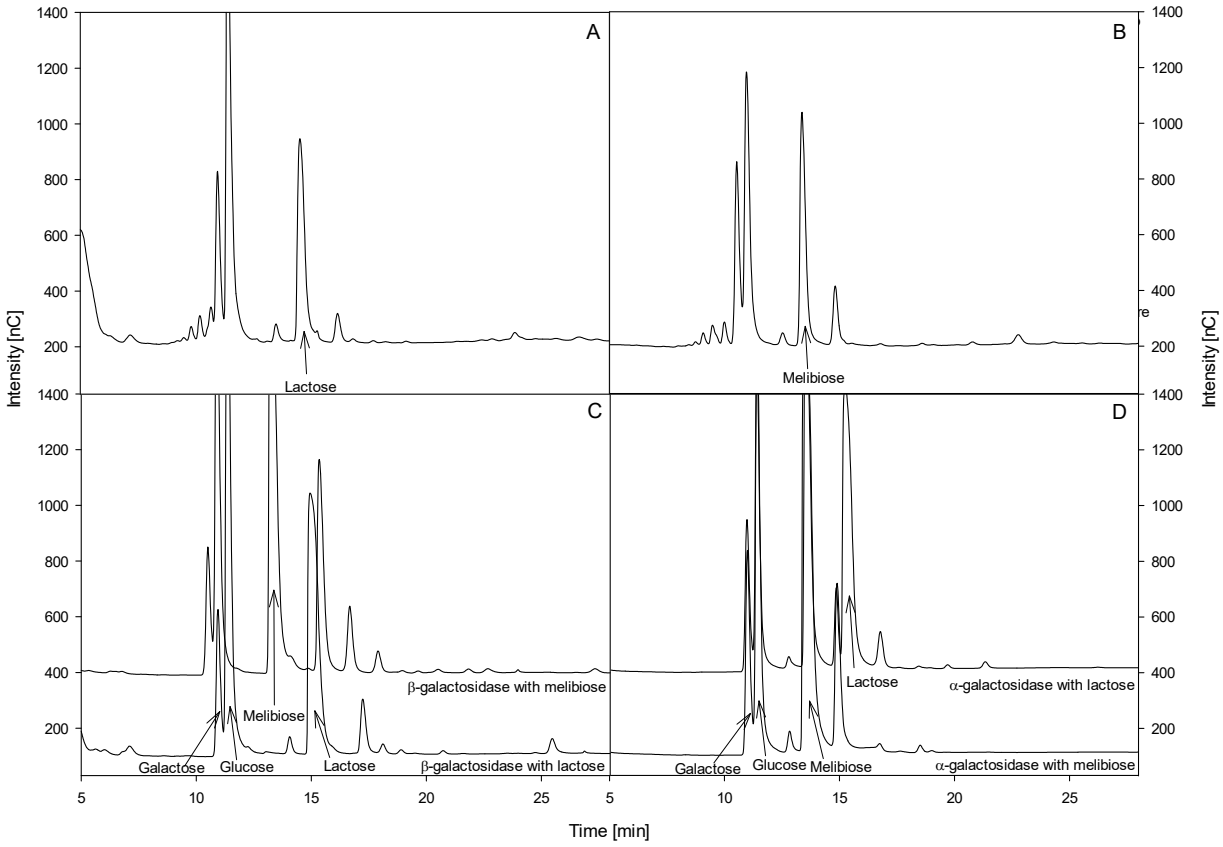
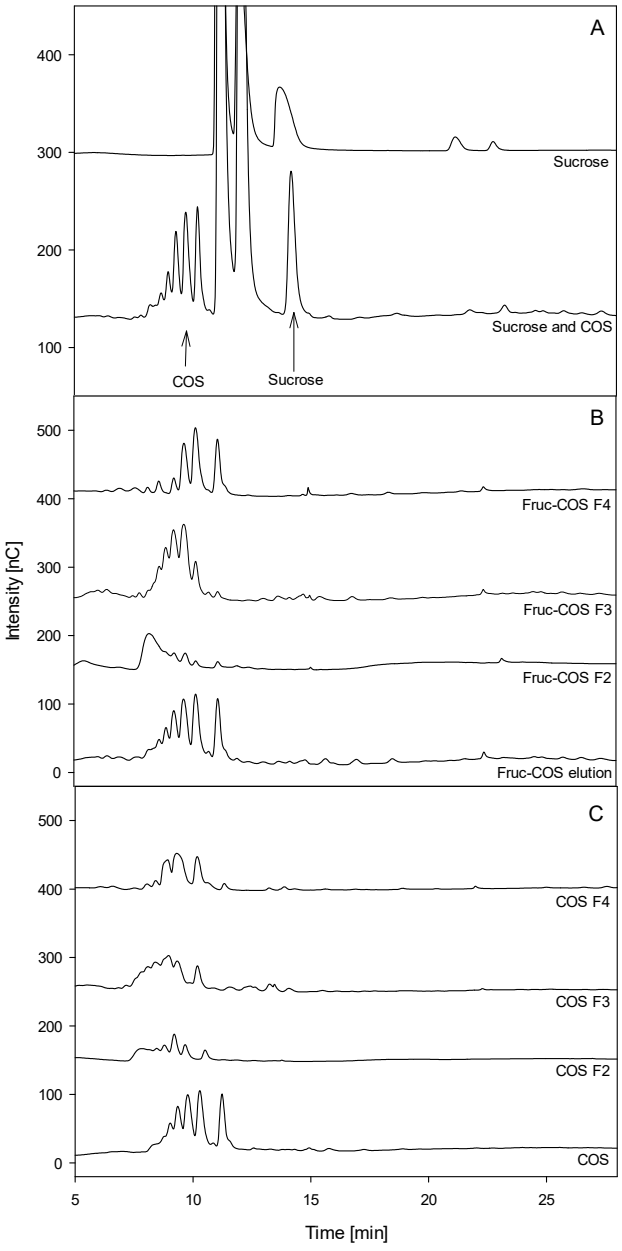


Fig 2. Yan et al.



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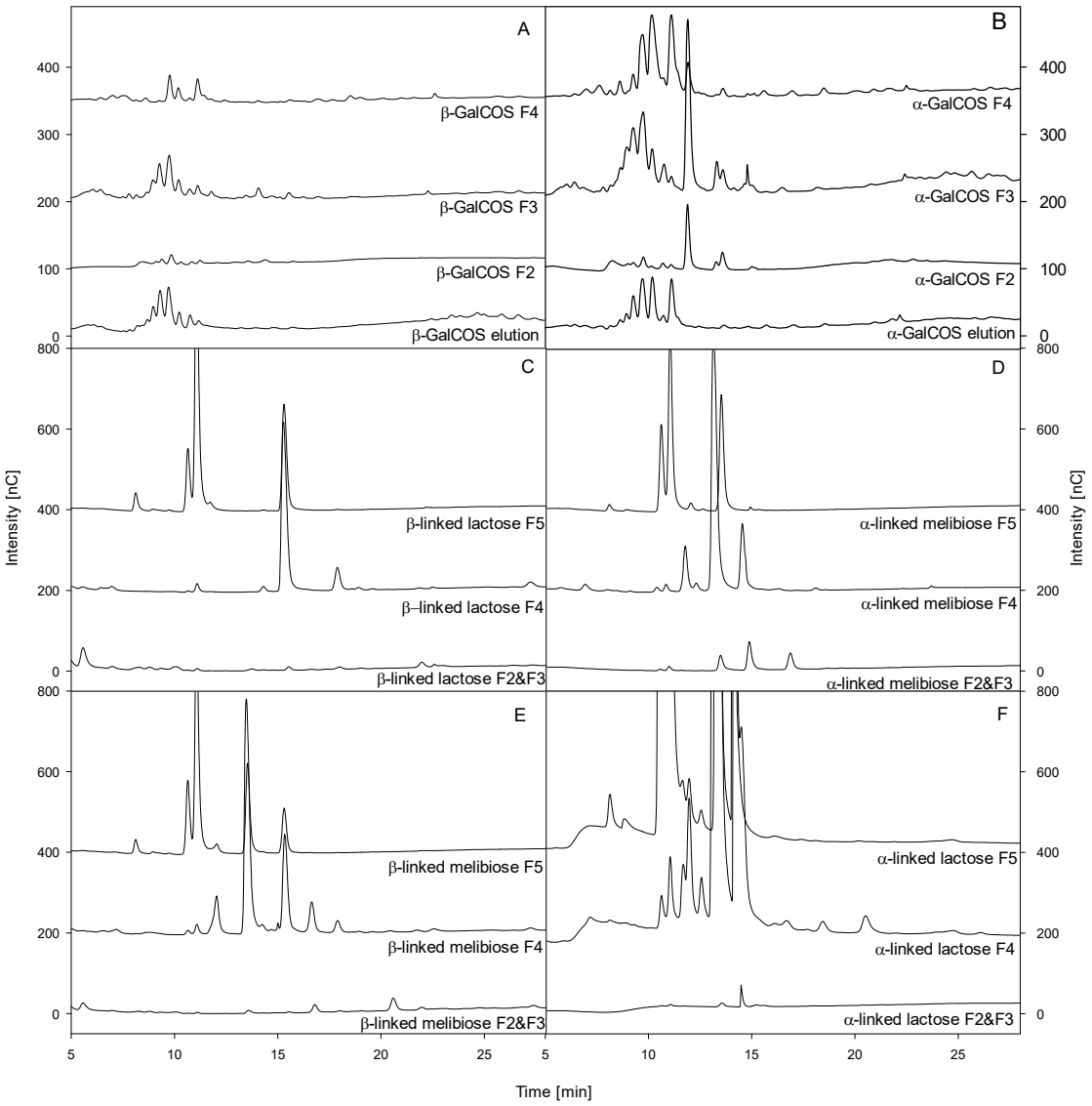

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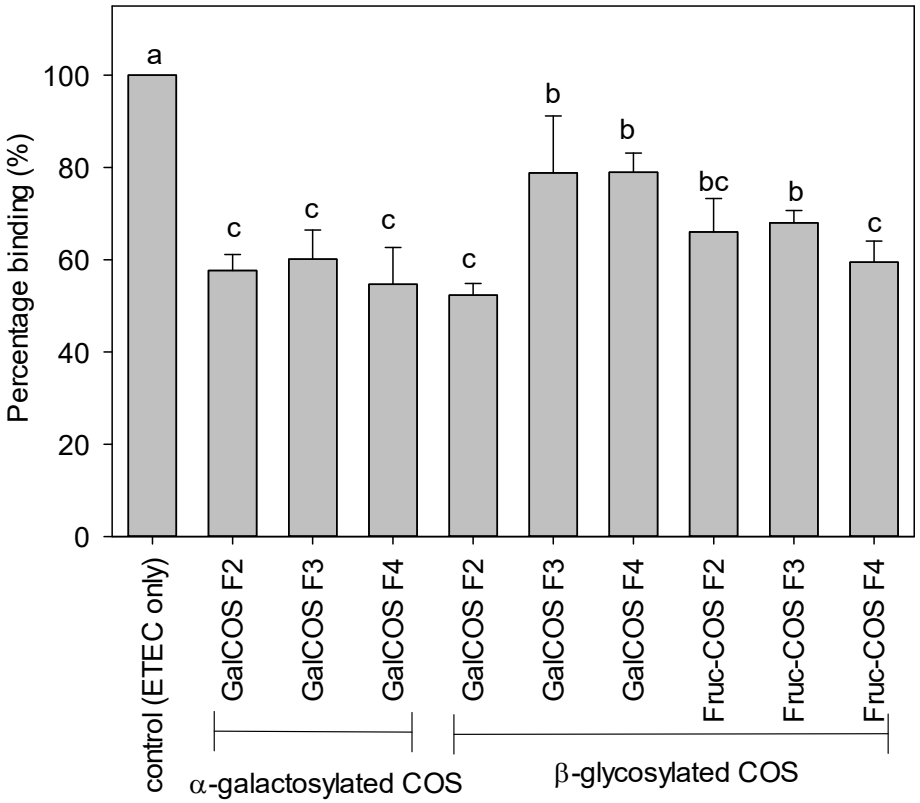

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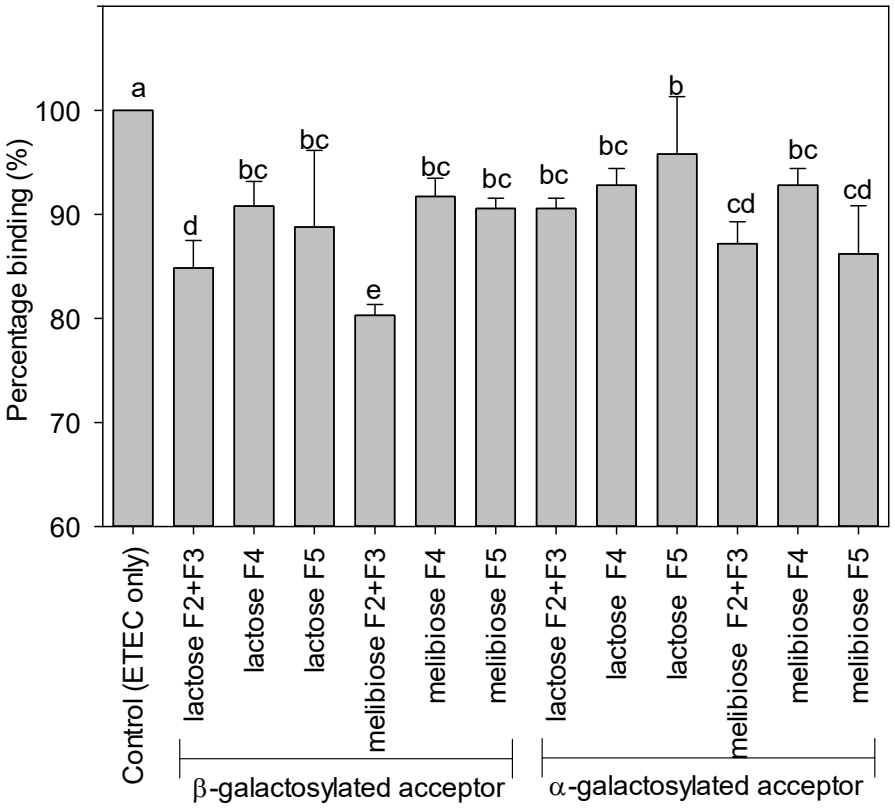

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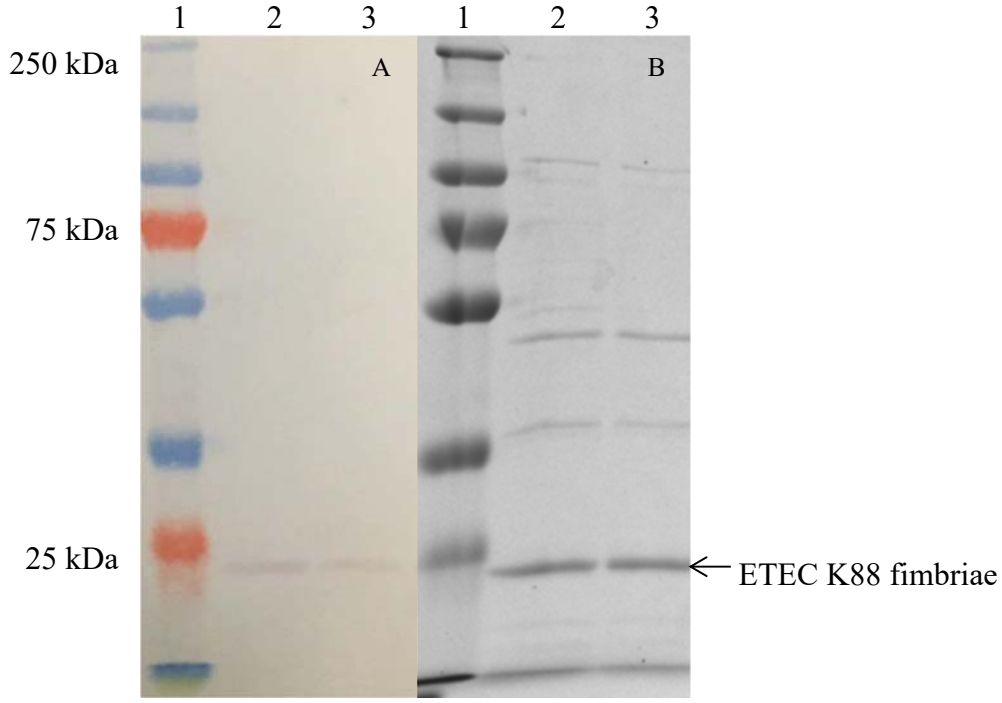

Figure 7. Yan et al.