Structure and Function Relationships of Galactosylated Oligosaccharides and Their Anti-adhesive Effects Against Enterotoxigenic *Escherichia coli* K88

by

Yalu Yan

A thesis submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in

Food Science and Technology

Department of Agricultural, Food and Nutritional Sciences
University of Alberta

Abstract

β-Galactosidase is used in the production of galacto-oligosaccharides (GOS), lactosucrose, and other oligosaccharides. GOS prevented enteropathogenic *Escherichia coli* adherence to human epithelial cells, and the anti-adhesive effects of GOS depended on their chemical structures, such as linkage type and degree of polymerization. This research aimed to investigate the relationship between the structures of galactosylated oligosaccharides produced by β-galactosidase and their anti-adhesive effects against enterotoxigenic *Escherichia coli* (ETEC).

ETEC K88 causes diarrhea in weaned piglets, resulting an economic loss in swine industries. ETEC adheres to intestinal mucosa by fimbriae and releases toxins. This thesis investigated the effects of galactosylated chitosan-oligosaccharides (Gal-COS) against ETEC K88 adherence to porcine erythrocytes. Gal-COS were synthesized through transgalactosylation of β-galactosidase with lactose and COS. Fractions of Gal-COS were obtained through cation exchange and size exclusion chromatography. Fractions of acetylated Gal-COS were obtained through chemical acetylation followed by size exclusion fractionation. Hemagglutination assay and ELISA assay were used to measure the anti-adhesive ability of oligosaccharides against ETEC K88. Gal-COS F2 containing the largest oligosaccharides composed of 8 or more monosaccharides had the highest anti-adhesion activity with the minimum inhibotry concentration of 0.22g/L, followed by F3 and F4. Adhesion of ETEC K88 to procine erythrocytes was significantly decreased by Gal-COS F2 to more than 50% when compared to ETEC control. Acetylation of Gal-COS decreased their ability against ETEC K88. The compositions of active oligosaccharides were determined by LC-MS.

The structure and functional relationship of oligosaccharides binding to ETEC K88 fimbriae were also studied. 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. Anti-adhesive effect of oligosaccharides against ETEC K88 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. The ability of oligosaccharides to prevent binding of ETEC K88 or purified K88 fimbriae decreased with decreasing molecular weight. 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.

In conclusion, galactosylation of COS produces oligosaccharides which reduce ETEC K88 adhesion; moreover resulting oligosaccharides match the composition of human milk oligosaccharides, which prevent adhesion of multiple pathogens.

Preface

This thesis is an original work by Yalu Yan.

Chapter 1 is partially included in a manuscript which has been published as Ya Lu Yan, Ying Hu and Michael G. Gänzle. (2018). "Prebiotics, FODMAPs and dietary fibre –conflicting concepts in development of functional food products?" *Current Opinion in Food Science*, 20, 30-37. I was responsible for the sections related to lactose intolerance, fructose intolerance, FODMAPs, whole grain foods and polyphenols, and writing the manuscript with Dr. Hu and Dr. Gänzle. Table 1 in chapter 1 was made by Dr. Gänzle.

Chapter 2 has been published as Yan, Y. L., Hu, Y., Simpson, D. J., and Gänzle, M. G. (2017). "Enzymatic synthesis and purification of galactosylated chitosan oligosaccharides reducing adhesion of enterotoxigenic *Escherichia coli* K88". *Journal of Agricultural and Food Chemistry*, 65, 5142-5150. I was responsible for conducting the experiments and writing the manuscript. Dr. Hu and Dr. Simpson contributed to the development of methods in acetylation of chitosan-oligosaccharides and ELISA assay, respectively and to manuscript revision. Dr. Gänzle contributed to the hypothesis development, experimental design, manuscript composition and revision.

Chapter 3 has been published as Ya Lu Yan and Michael G Gänzle (2018) "Structure and function relationships of the binding of β- and α-galactosylated oligosaccharides to K88 fimbriae of enterotoxigenic *Escherichia coli*". *International Dairy Journal*. I was responsible for conducting the experiments and writing the manuscript. Dr. Gänzle contributed to the hypothesis development, manuscript composition and revision.

Chapter 4 contains experimental work performed by myself with the supervision of Dr. Gänzle. I was responsible for synthezing galactosylated COS with β-/α-galactosidase and exopolysaccharides for the animal study.

Acknowledgements

I would like to express my deepest appreciation to my supervisor, Dr. Michael Gänzle, for accepting me as a NUFS 401 student at the very beginning. The day you accepted me as a Ph.D. student was the best day of my life, your support, guidance, patience, and encouragement throughout my program motivated me to pursue my Ph.D. degree. I feel that I am fortunate to have you as my supervisor for my doctoral program. You have taught me more than just knowledge, and I will benefit from things I learned from you throughout my lifetime.

I would also like to thank my supervisory committee members Dr. Jonathan Curtis and Dr. Jianping Wu for the valuable feedback and suggestions during committee meetings. My appreciation goes to my examining committee members Dr. Catherine Field and Dr. Steve Cui for your time and support. My appreciation goes to my chair Dr. Feral Temelli for all the guidance, help, and encourage before and during tge defense.

My sincere thanks to Dr. Lynn McMullen. Thank you for all the help, support, and encouragement you provided during my entire program. I learned so many things from you, and your help and support motivated me to continue my research during my Ph.D. program.

Thanks to Dr. RandyWhittal, Jing Zheng and Béla Reiz in Mass Spectrometry Facility. Without your patience and help, I would not be able to understand how to process the data from LC-MS analysis.

Thanks to Susan Gibson for giving me the opportunity to work as a teaching assistant for NUFS 374. I enjoyed working with you, and I learned a lot from you and the students. I will never forget and will always be greatful for the experience. Thanks to Dr. Marleny D. Aranda Saldana for giving me the opportunity to be a marker for NUFS 283, it was my first experience with marking and I enjoyed it immensely. Thanks to Dr. Lynn McMullen and Dr. Michael Gänzle for

recruiting me as a marker for NUFS 361/363, I was very excited when I learned I was chosen as a marker.

My life as a Ph.D. student would not be a successes without the help from so many people helping me in different ways. My special thanks goes to Dr. Sandra Galle. Thank you for being a mentor and a friend, and taught me how to do experiments step by step. Thank you to my lab-mates in lab 2-50 for your caring support, and encouragement. My especial thanks goes to Danielle Balay, Dr. Urmila Basu, Dr. Xiaoyan Chen, Dr. Yuanyao Chen, Xu Dan, Gautam Gaur, Dr. Simmon Hofstetter, Dr. Ying Hu, Ziyi Hu, Tingting Ju, Dr. Petya Koleva, Dr. Xiaoxi (Brook) Lin, Dr. Alma Sanchez-Maldonado, Aleicia Mushins, Lisa N Nikolai, Dr. David J Simpson, Dr. Januana Teixeira, Kaixing Tang, Heather Vandertol-Vanier, Weilan Wang, and Bowen Yan. Moreover, I would like to express my appreciation and deepest gratitude for all the people who helped and supported me during my stay in Canada.

I am very thankful to Department of Agricultural, Food and Nutritional Sciences and Graduate Students' Association at the University of Alberta for providing me with travel awards and allowing me to present my work at international conferences. Thank you to Alberta Livestock and Meat Agency for funding my research projects.

Lastly, my deepest appreciation goes to my parents, Zaizai Yan and Yingtang Su. Thank you for providing me the opportunity to study abroad; thank you for supporting me through my studies and stay in Canada, and thank you for all your understanding and encouragement throughout my studies.

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List of Abbreviation

a_w	Water activity
CCE	Crude cell extract
COS	Chitosan-oligosaccharides
DP	Degree of polymerization
EHEC	Enterohemorrhagic *Escherichia coli*
ELISA	Enzyme-linked immunosorbent assay
EPEC	Enteropathogenic *Escherichia coli*
EPS	Exopolysaccharides
ETEC	Enterotoxigenic *Escherichia coli*
FODMAPs	Fermentable oligosaccharides, disaccharides, monosaccharides, and polyols
FOS	Fructooligosaccharide
FT	Flow through
Gal	Galactose
Gal-COS	Galactosylated chitosan-oligosaccharides
β-GalCOS	β-Linked galactosylated chitosan-oligosaccharides
α-GalCOS	α-Linked galactosylated chitosan-oligosaccharides
Gal	Galactose
GI tract	Gastrointestinal tract
Glc	Glucose
GlcN	*N*-glucosamine
GlcNAc	*N*-acetyl-glucosamine
GOS	Galacto-oligosaccharides

α-GOS	α-linked Galacto-oligosaccharides
β-GOS	B-linked Galacto-oligosaccharides
HMOs	Human milk oligosaccharides
HPAEC-PAD	High-performance anion exchange chromatography with pulsed amperometric detection
HPLC	High performace liquid chromatography
LC-ESI-MS	Liquid chromatography−electrospray ionization-mass spectrometry
LC-MS	Liquid chromatography –mass spectrometry
MW	Molecular weight
OD	Optical density
PB	Phosphate buffer
PBS	Phosphate buffer saline
RI	Refractive index
SCFAs	Short-chain fatty acids
SDS-page	Sodium dodecyl sulfate polyacrylamide gel electrophroesis
SEC	Size exclusion chromatography
RS	Resistant starch
RS 2	Type 2 resistant starch
RS 4	Type 4 resistant starch
TFA	Trifluoroacetic acid
UPEC	Uropathogenic *Escherichia coli*

Chapter 1. Introduction

Overview

Dietary carbohydrates play an important role in maintaining human health and regulating the composition of gut microbiota. Dietary fiber was first defined as non-digestible constituents of plant cell walls in 1953, and the updated definition of dietary fiber is expanded to include both polysaccharides and oligosaccharides (degree of polymerization ≥3) (Dreher, 2018a; Yan, Hu, & Gänzle, 2018). Inadequate fiber intake results in altered microbiota composition and low production of bacteria metabolites, which raise concerns in human health. Low fiber intake increases the risk of chronic constipation and diabetes in children and women, respectively (Morais, Vítolo, Aguirre, & Fagundes-Neto, 1999; Salmeron et al., 1997). Increased fiber intake reduces the risk of several diseases including type 2 diabetes, cardiovascular disease, and cancer (Dahl & Stewart, 2015).

Dietary fiber promotes laxation, decreases transit time, reduces total cholesterol level in serum, and improves colonic health (Dreher, 2018b). Dietary fiber, such as wheat bran, has high water binding capacity which results in an increased fecal bulk, soft stool, and decreased transit time (Dreher, 2018b). Consumption of 15g/day of guar gum decreased 10.6% LDL-cholesterol level (Anderson et al., 2009). Reduction of LDL-cholesterol level lowers the occurrence of heart attack, revascularization, and ischaemic stroke (Baigent et al., 2010). The hypocholesterolemic effect of dietary fiber was proposed primarily because of their bile acids binding capacity which increases bile acids excretion in the feces (Anderson et al., 2009). Bacterial metabolites produced through fermentation, such as propionate, also contribute to lowering the cholesterol level in serum (Anderson et al., 2009).

Dietary fiber is one of the critical determinants of composition and function of gut microbiota. Dietary fiber alters gut microbiota composition and diversity, which play an important role in host health (Sonnenburg & Sonnenburg, 2014). A higher diversity of intestinal microbiota diversity and a higher production of short-chain fatty acid (SCFAs) were observed in non-modern societies when compared to western groups (Sonnenburg & Sonnenburg, 2014). Studies illustrated that metabolic diseases were associated with the diversities of gut microbiota. For example, high LDL-cholesterol and fasting triglycerides level, high insulin resistant and high inflammation markers were found in individuals with low microbiota diversity (Cotillard et al., 2013). SCFAs produced through bacterial fermentation appear to be one of the critical mediators to the health effects of dietary fiber. Accumulating studies revealed the impact of dietary fiber and SCFAs to human health. A summary of SCFAs and host health will be discussed in the present chapter.

Recent studies demonstrated fermentation-independent activities of dietary fiber, such as modulating the immune system and preventing pathogen adhesion. Human milk oligosaccharides (HMOs) exhibited anti-inflammatory effects by attenuating of CD14 induction which is essential cascades for LPS-induced inflammation (He et al., 2014). Galacto-oligosaccharides (GOS) also had direct immune-modulation effects by activating the TLR4-NFκB pathway which leads to the production of various cytokines (Chen & Gänzle, 2017). Dietary fiber and SCFAs influence mucosal immune system by altering the enzyme activity in dendritic cells which resulted in an increased amount of intestinal regulatory T cells and increased IgA production (Goverse et al., 2017). The mechanism of immune modulation effects and anti-adhesive effects needs further investigation. The anti-adhesive effects of dietary carbohydrates will be discussed in the present chapter.

Dietary fiber modulates the composition and diversity of gut microbiota by regulating the SCFAs production through bacterial fermentation, by direct or indirect interactions with the immune system, and by preventing pathogen adherence, which in turn will impact human health.

Human gut microbiota and human health

Gut microbiota play an essential role in human health, such as the production of short-chain fatty acids (SCFAs) through fermentation and modulation of the immune system (Postler & Ghosh, 2017). In estimation, a human adult individual harbors an average of 10^{14} bacterial cells and the most populated area is the gastrointestinal (GI) tract (Sekirov, Russell, Antunes, & Finlay, 2010). Number and species of bacteria vary from the proximal to the distal part of GI tract. For example, few bacteria, such as *Veillonella* and *Helicobacter,* are able to colonize the stomach due to the acidic environment. In contrast, more than 70% of gut bacteria colonize the colon as it has relatively mild growth conditions (Sekirov et al., 2010).Three dominant phyla have been identified in the human gut: Bacteroidetes, Firmicutes, and Actinobacteria (Maukonen & Saarela, 2015; Sekirov et al., 2010). Other phyla, such as Proteobacteria and Verrucomicrobia, are presented in lower quantities (Sekirov et al., 2010).

Gut microbiota are associated with several diseases, such as type 2 diabetes, cardiovascular disease, and inflammatory bowel disease (Boulangé, Neves, Chilloux, Nicholson, & Dumas, 2016; Postler & Ghosh, 2017; Shen, 2017). Gut microbiota are also associated with GI disorders such as irritable bowel syndrome (IBS). In several clinical studies, IBS patients had a change in gut microbiota composition and diversity when compared with healthy controls. Decreased microbiota diversity, reduced the abundance

of *Bifidobacterium* and *Faecalibacterim prausnitzii,* and an increased amount of *Enterobacteriaceae* were reported (Carroll, Ringel-Kulka, Siddle, & Ringel, 2012; Kassinen et al., 2007; Zhuang, Xiong, Li, Li, & Chen, 2017). Therapeutic treatments targeting to modulate gut microbiota composition have been proposed for IBS, such as diet interventions and usage of probiotics (Dupont, 2014). Clinical studies demonstrated diets low in fermentable oligosaccharides, disaccharides, monosaccharides, and polyols (FODMAPs) reduced symptoms of IBS during the short-term intervention (Böhn et al., 2015; Halmos, Power, Shepherd, Gibson, & Muir, 2014; Staudacher et al., 2012). Three bifidobacteria species, *Bifidobacterium infantis, B. breve,* and *B.longum*, used in a probiotic trial resolved abdominal pain in children with IBS (Giannetti et al., 2017).

Various factors, such as host genetic background and age, influenced gut microbiota (Biagi et al., 2010; Stewart, Chadwick, & Murray, 2005). However, diet is the dominant factor in gut microbiota modulation. Carmondy (2015) demonstrated that changes in gut microbiota were closely related to the diet in a mice model. Diets high in fiber content resulted in a high abundance of Bacteroidetes in African children, but not for European children, who had a higher abundance of Firmicutes (De Filippo et al., 2010).

Whole grain diet and human health

Whole grains foods are commonly recognized as products containing the outer bran, endosperm, and germ of the grain (Ferruzzi et al., 2014; Seal, Nugent, Tee, & Thielecke, 2016). But no global agreement has been drawn on the definition of whole grain foods, thus resulting in various dietary recommendations (Ferruzzi et al., 2014; Seal, Nugent, Tee, & Thielecke, 2016). Three meta-analysis studies summarized 97 studies on the health benefits of whole grains foods on human health. Health effects of whole grain

diets in lowering risk of some diseases were dose-dependent. Specifically, a 90g/day intake of whole grains reduced the risk of heart disease and cardiovascular disease by 20% and 22%, respectively (Aune et al., 2016). An inverse linear correlation was found between whole grain food intake and risk of incidence of type 2 diabetes (Chanson-Rolle et al., 2015). Cereal fiber and whole grains consumption also lowered the risk of colorectal cancer by 10% with a 10g daily dose (Aune et al., 2011).

Dietary fiber and prebiotics

Dietary fibers present in whole grains contribute significantly to the health benefits of a whole grain diet. The definition of dietary fiber initially included non-digestible carbohydrates and polysaccharides from plant cell wall (Cummings & Stephen, 2007). Codex Alimentarius Commission had developed a more detailed definition. Dietary fibers are carbohydrate polymers with ten or more monomers which are resistant to endogenous enzymes in the human small intestine (Codex Alimentarius Commission, 2009). The Codex definition includes naturally occurring carbohydrates in food matrixs and synthetic carbohydrate polymers, if they demonstrate beneficial effects on human health (Codex Alimentarius Commission, 2009). The European Commission and other countries expanded the definition of dietary fiber by including non-digestible oligosaccharides with 3 to 9 monomeric units (Verspreet et al., 2016).

The definition of prebiotics remains under revision since its first published by Gibson and Roberfroid in 1995. Bindels et al. (2015) proposed a definition for prebiotics as non-digestible compounds with beneficial effects to host health as a result of microbial metabolism and modulation of the composition and/or activity of the gut microbiota. Based on the definition proposed by Bindels et al. (2015), prebiotics are not limited to

carbohydrates; however, most recognized prebiotics are oligosaccharides and polysaccharides (Bindels et al., 2015). Thus, most prebiotics are included in the definition of dietary fiber.

Table1. 1. Beneficial and adverse health effects of prebiotic carbohydrates*

General health benefits of carbohydrates with low small intestinal digestibility	
Low cariogenicity	
Reduced caloric content (60 – 75% of glucose)	[1]
Health benefits related to microbial production of short chain fatty acids	
Intestinal motility	[2]
Energy supply and proliferation of colonic mucosal cells	[3]
Satiety, glucose homeostasis and insulin sensitivity	[4]
Immune-modulation and improved epithelial barrier function	[5]
Reduced luminal pH and pathogen exclusion	[6]
Improved iron absorption	[7]
Adverse health effects	
Induction of osmotic diarrhea by oligosaccharides when oligosaccharide load exceeds ~ 0.3 g / kg bodyweight	[8]
Excessive gas formation and intestinal bloating after rapid intestinal fermentation of carbohydrates	[8]
Specific health benefits of dietary glycans that are unrelated to microbial metabolism	
Prevention of pathogen adhesion to intestinal cells	[9]
Direct interaction with immune system	[10]
Change of viscosity of intestinal content, increased secretion of bile salts and reduced cholesterol levels	[11]

*Prepared by Dr. Michael Gänzle for the publication Ya Lu Yan, Ying Hu and Michael G. Gänzle. (2018). "Prebiotics, FODMAPs and dietary fibre –conflicting concepts in development of functional food products?" *Current Opinion in Food Science*, 20, 30-37.
1, (Oku & Nakamura, 2002); 2,(Cherbut et al., 1998); 3,(Sakata, 1987); 4,(Byrne et al., 2015; Canfora et al., 2015; Tolhurst et al., 2012); 5,(Kelly et al., 2015; Smith et al., 2013); 6,(Fukuda et al., 2011; Holtug et al., 1992); 7,(Bougle et al., 2002); 8,(Murray et al., 2014; Oku & Nakamura, 2003); 9,(Chen & Gänzle, 2017); 10,(Jeurink et al., 2013); 11,(Whitehead et al., 2014; Wang et al., 2017)

The influence of dietary glycans on gut microbiota and human health was summarized in Table 1.1. The effects of dietary fiber were on the following aspects (Table 1.1): i) utilization of dietary fiber by gut microbiota; ii) production of short-chain fatty acids (SCFAs) and other metabolites via bacterial metabolism; iii) and anti-adhesive effects of oligosaccharides against various pathogens. However, the effects of dietary fiber on gut microbiota are partly depended on individual dietary history and habits (Brahma et al., 2017). A relatively stable microbiota diversity and higher butyrate yield were found in participants with habitual diets high in beneficial nutrients, such as dietary fiber (Brahma et al., 2017).

The utilization of dietary fiber by gut microbiota

Dietary fiber increased colonic fermentation resulting in increased SCFAs production and altered gut microbiota composition (Louis, Flint, & Michel, 2016). A 4-week whole grain dietary intervention study reported an increased bacterial diversity (Martínez et al., 2013). Increased *Lachnospira, Bacteroidetes, Ruminococcus,* and *Clostridiaceae* and decreased *Enterobacteriaceae, Firmicutes,* and *Fusobacteria* were observed in short-term whole grain diet substitution studies (De Angelis et al., 2015; Vanegas et al., 2016; Vanegas et al., 2017; Zhong, Nyman, & Fåk, 2015). Whole grain barley consumption results in changes in microbiota composition, specifically in populations of *Roseburia, Bifidobacterium,* and *Dialister* (Martínez et al., 2013). Taken together, consumption of various dietary fiber stimulates diverse microbiota and enhances microbiota diversity.

The digestibility of dietary fiber in the small intestine affects bacterial fermentation and in turn influences gut microbiota and human health.

The digestibility of dietary fiber is dependent on the food matrix. Starch is hydrolyzed by amylase, brush border maltase-glucoamylase, and sucrose-isomaltase into smaller oligomers and glucose for absorption (Dhital, Warren, Butterworth, Ellis, & Gidley, 2017). However, limited enzyme accessibility due to the structure of starch granules, formation of crystalline structures within starch, and formation of complexes with lipids (the amylose-lipid complex) can make starch resistant to intestinal digestive enzymes; thus, such starch is said to become resistant starch (RS) which is fermented in the colon and produces SCFAs, gases, and other metabolites (Fuentes-Zaragoza et al., 2011). RS increased the abundance of *Bacteroides, Ruminococcus, Lactobacillus,* and *Bifidobacterium* (Upadhyaya et al., 2016; Zeng et al., 2017). Gut modulation effects also depend on the type of fiber available in a food matrix. Type 2 RS (RS2) favored the growth of *Ruminococcus bromii* and *Eubacterium rectale,* while Type 4 RS (RS4) promoted the growth of *Bifidobacterium adolescentis* and *Parabacteroides distasonis* (Martinez, Kim, Duffy, Schlegel, & Walter, 2010).

Polyphenols influence the digestibility of carbohydrates

Polyphenols in the food matrix influence the digestibility of carbohydrates by inhibiting digestive enzymes in human or by crosslinking with polysaccharides (Figure 1.1).

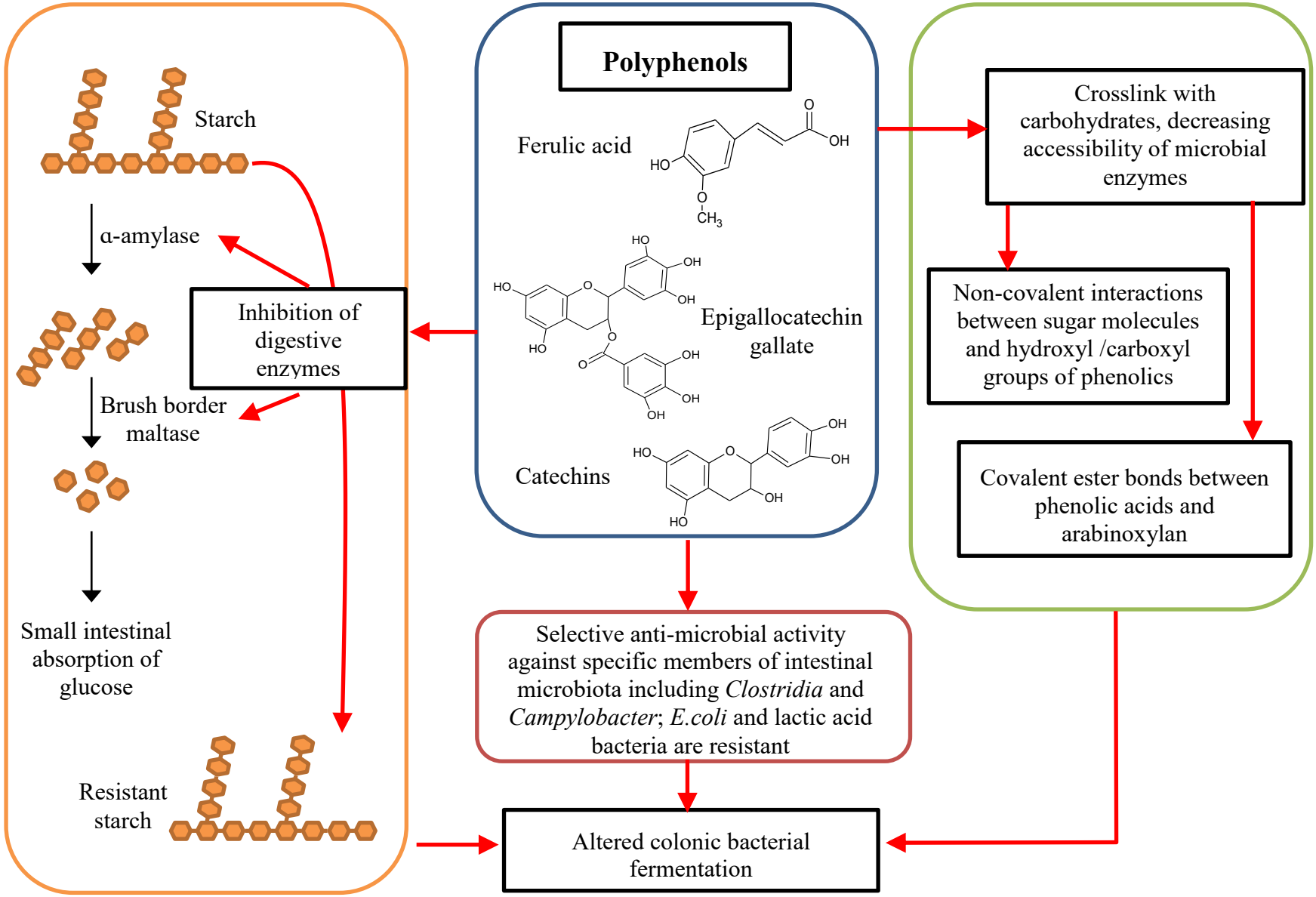
Starch
α-amylase
Inhibition of digestive enzymes
Brush border maltase
Small intestinal absorption of glucose
Resistant starch
Polyphenols
Ferulic acid
Epigallocatechin gallate
Catechins
Crosslink with carbohydrates, decreasing accessibility of microbial enzymes
Non-covalent interactions between sugar molecules and hydroxyl /carboxyl groups of phenolics
Covalent ester bonds between phenolic acids and arabinoxylan
Selective anti-microbial activity against specific members of intestinal microbiota including *Clostridia* and *Campylobacter*; *E.coli* and lactic acid bacteria are resistant
Altered colonic bacterial fermentation

Figure1.1. Influence of phenolic compounds on carbohydrate digestion. **Left panel.** Phenolic compounds, particularly hydrolysable and condensed tannins, inhibit small intestinal glycosyl hydrolases and shift starch digestion from small intestinal absorption to colonic fermentation. **Middle panel.** Some phenolic compounds including tannins and phenolic acids have highly selective antimicrobial activity against members of intestinal microbiota. **Right panel.** Covalent and non-covalent crosslinking of cell-wall polysaccharides with phenolic acids decreases the rate of colonic fermentation. Drawn with information from Barrett et al., 2018; Hamaker & Tuncil, 2014; Miao et al., 2015; Quiros-Sauceda et al., 2014; Selma, Espín, & Tomás-Barberán, 2009; Engels, Schieber, & Gänzle, 2011; Sánchez-Maldonado, Schieber, & Gänzle, 2011.

Polyphenols inhibit α-amylase and α-glucosidase activities through hydrophobic interactions or hydrogen bonding (Barrett, Farhadi, & Smith, 2018). Several compounds were suggested to be associated with amylase inhibitory effects, such as (-)-epigallocatechin gallate, tannic acid, and catechins with a galloyl at 3-position (Nyambe-Silavwe et al., 2015; Sun, Warren, Netzel, & Gidley, 2016). Extracts of seaweed, Omija fruit, and cranberries have all been shown to exhibit inhibitory effects on both α-amylase and α-glucosidase (Barrett et al., 2018). Approximately 50% reduction of α-amylase activity was reported when tested with oat phenolic extracts at 500 gallic acid equivalent μM (Li, Koecher, Hansen, & Ferruzzi, 2017). Green tea extracts containing epicatechin, epigallocatechin gallate, and epicatechin gallate inhibited more than 60% pancreatic α-amylase (Miao, Jiang, Jiang, Zhang, & Li, 2015). Miao (2015) conducted a docking study on green tea extracts containing epigallocatechin gallate and epicatechin gallate with human pancreatic α-amylase. The inhibitory effects of green tea extracts were demonstrated to be due to the occupation of catalytic centers and interaction with the active site (Miao et al., 2015). The modulation of carbohydrate digestion by polyphenols is not only limited to the inhibitory effects of digestive enzymes, but also contributes to polyphenol-carbohydrate interactions (Figure 1.1). Polysaccharides are cross-linked with gallic, ellagic acids, and caffeic acid through hydrogen bonds and hydrophobic interactions (Quiros-Sauceda et al., 2014). Covalent bonds between polyphenols and polysaccharides were also reported, such as ferulic acid and arabinoxylan conjugate (Jakobek, 2015). Proanthocyanidins strongly interacted with amylose and the linear portion of amylopectin in starch, which decreased starch digestibility *in vitro* and increased resistant starch content by approximately 50% (Barros, Awika, & Rooney,

2012). The influence of interactions between carbohydrates and polyphenols on microbiota was observed in rat feeding trials with fructooligosaccharide (FOS), monomeric ellagitannin, and dimeric ellagitannin-rich extracts (Jurgoński et al., 2017). Crosslink between FOS and ellagitannins decreased the total bacteria counts especially in *Bifidobacterium* and *Enterococcus* (Jurgoński et al., 2017). Polyphenols also decrease pathogen adherence to host cell surface but the mechanism is poorly described.

Dietary fiber digestibility also depends on individuals. Approximately 75% of the world's population are lactose intolerant due to a genetic decline in lactase activity in most children aged 5 - 10 years, although lactase persistence was found in the rest of the population because of a mutation in lactase gene (Gerbault et al., 2011; Mattar, de Campos Mazo, D F, & Carrilho, 2012). Around 50% of the healthy subjects have limited ability to absorb 25-40 g of fructose at one time (Latulippe & Skoog, 2011; Murray et al., 2014); a higher percentage of fructose malabsorption or fructose intolerance was observed in IBS patients (Wilder-Smith, Materna, Wermelinger, & Schuler, 2013). Lactose and fructose are not included in the definition of dietary fiber, but they exhibit a similar effect of GOS, FOS, and other dietary fiber to host. The undigested lactose and fructose are rapidly fermented in the large intestine and may result in GI tract discomfort (Gänzle, 2012; Chen & Gänzle, 2017). Moreover, lactose, fructose and other small molecules, such as FODMAPs, exert osmotic pressure in the GI tract which can result in watery diarrhea (Mattar et al., 2012). However, when those compounds are provided in appropriate amounts to avoid adverse effects, they can be beneficial to the host because they increase colonic fermentation by gut microbiota.

SCFAs produced by dietary fiber fermentation

The health benefits of whole grains are not linked to direct nutritional effects on the growth of bacteria but relate to the production of bacterial metabolites (Louis et al., 2016). Colonic fermentation of dietary fiber by gut bacteria produces SCFAs, gases, and other metabolites. Production of SCFAs, such as acetic acid, propionate, and butyrate, were increased with the consumption of whole grain products (De Angelis et al., 2015; Nielsen et al., 2014; Vetrani et al., 2016; Zhong, Marungruang, Fåk, & Nyman, 2015). SCFAs were suggested to have multiple beneficial effects (Table 1.1). SCFAs lower the intestinal pH and inhibit the growth of pathogens (Fukuda et al., 2011; Holtug, Clausen, Hove, Christiansen, & Mortensen, 1992). SCFAs serve as energy sources for colonic cell and epithelial cell growth (Sakata, 1987). For example, butyrate promoted the growth and proliferation of host colonocytes (Koh, De Vadder, Kovatcheva-Datchary, & Bäckhed, 2016). SCFAs are recognized by free fatty acid receptor 2 (GPR43), free fatty acid receptor 3 (GPR41), and GPR109A receptor on intestinal brush border (Koh et al., 2016). The interactions between these receptors and SCFAs influenced immunity, inflammation, satiety and glucose homeostasis (Koh et al., 2016). SCFAs could modulate gut microbiota through metabolic cross-feeding mechanisms. For example, utilization of lactate or acetate produced by *Bifidobacterium* fermentation increased the abundance of butyrate-production strains, such as *Faecalibacterium prausnitizii* (Bindels, Delzenne, Cani, & Walter, 2015; Koh et al., 2016).

Oligosaccharides and anti-adhesion activity

Besides exhibiting prebiotic effects, oligosaccharides play an important role in pathogen adhesion and infection in humans. Adhesion is the initial and critical step in pathogen

infection (Shoaf-Sweeney & Hutkins, 2008). Adherence between pathogenic bacteria and host has several advantages for the pathogen: i) Adherence to host cell surfaces prevents bacterial cells from being washed away by host mechanical processes; ii) Adherence provides advantage access to nutrients; iii) Adherence facilitates the release of toxins and/or other virulence factors to host cells (Shoaf-Sweeney & Hutkins, 2008; Moonens & Remaut, 2017; Ribet & Cossart, 2015). Pathogen adhesion is established through specific interactions between host carbohydrate receptors and bacterial glycan-binding proteins (Shoaf-Sweeney & Hutkins, 2008). Specific carbohydrate binding domains on bacterial binding proteins recognize complementary sugar structures on host cell surfaces to initiate attachment (Shoaf-Sweeney & Hutkins, 2008). Several known oligosaccharides structures have been identified to be pathogen binding receptors. For example, galabiose or sugar molecules with galabiose moieties were reported to be receptors for P-fimbriae on *E. coli* (Nizet, Varki, & Aebi, 2017). Uropathogenic *E.coli* (UPEC) initiated adherence to urinary tract through the recognization of α-linked mannose oligosaccharides by type 1 pili (Moonens & Remaut, 2017). UPEC also adhered to kidney cells through the interaction between P-fimbriae and S-fimbriae with receptor Gal-α-(1→4)-Gal and sialyl-α-(2→3)-Gal-β-(1→4)-Glc moieties, respectively (Moonens & Remaut, 2017). Sialylated oligosaccharides, such as sialyl-3'-lactose, were the receptors for *Helicobacter pylori* (Sharon, 2006).

Oligosaccharides were suggested to be promising tools to prevent pathogen adhesion by acting as carbohydrate receptor decoys (Sharon, 2006). The anti-adhesive effects of oligosaccharides against pathogen adhesion are not well understood. The concept is supported by studies of human milk oligosaccharides (HMOs) on pathogen adhesion.

HMOs decreased enteropathogenic *E.coli* (EPEC) attachment *in vitro* and *in vivo* (Manthey, Autran, Eckmann, & Bode, 2014). Another *in vitro* study showed the anti-adhesive abilities of HMOs against *Vibrio cholera* and *Salmonella fyris* (Coppa et al., 2006). Moreover, α-(1→2)-fucosylated HMOs, such as Fuc-α-(1→2)-Gal-β-(1→4)-GlcNAc, inhibited the adherence of *Campylobacter jejuni in vitro* (Ruiz-Palacios, Cervantes, Ramos, Chavez-Munguia, & Newburg, 2003). Oligosaccharides from other sources also had anti-adhesive effects against pathogens. Commercial galacto-oligosaccharides (GOS) reduced adherence of *Cronobacteria sakazakii* to HEp-2 human epithelial cells (Quintero et al., 2011). Xylo-oligosaccharides inhibited *Listeria monocytogenes* adherence to Caco-2 cells *in vitro* and prevented *Listeria* infection in guinea pigs *in vivo* (Ebersbach, Andersen, Bergstrom, Hutkins, & Licht, 2012; Ebersbach et al., 2010).

The specificity of pathogen adhesion depended on their respective adhesins, and bacterial fimbriae are examples of bacterial adhesins (Shoaf-Sweeney & Hutkins, 2008). Fimbriae mediate specific adherence to host species and host tissue (Shoaf-Sweeney & Hutkins, 2008). Enterotoxigenic *E.coli* (ETEC) expressing type 1 fimbriae were one of the causative bacteria for diarrhea in young children in developing countries, and binding was determined by the binding adhesin FimH encoded by *fimH* on the tip of type-1 fimbriae (Sheikh et al., 2017). Mutation of *fimH* impaired the ability for type 1 fimbriae production and decreased adherence ability to intestinal epithelia *in vitro* (Sheikh et al., 2017). ETEC expressing fimbriae F4, F5, and F6 were recognized as causing factors for animal illness (Xia et al., 2015). Different adhesin resulted in different infection areas. For example, *Salmonella* Typhi and *S.* Gallinarum were reported to cause systemic

infections in human and poultry, respectively (Yue et al., 2015). However, *E.coli* was commonly associated with infection in the intestine (Shoaf-Sweeney & Hutkins, 2008). Bacteria host adaptations also influence bacteria-host specificity. High degree of allelic variation was observed in *S. enterica* serovar Typhimurium, and a single mutation in amino acid composition (from valine to alanine) on type 1 fimbriae adhesin FimH changed *S enterica* host specificity from human to bovine (Yue et al., 2015). Host-species specificity makes *in vivo* studies for human pathogens significantly challenging. *In vitro* cell line studies and *in vivo* animal studies were used as proof-of-concept for applying carbohydrate decoys to prevent pathogen adhesion to host cell surfaces (Badia, Lizardo, Martínez, & Brufau, 2013; Chen, Woodward, Zijlstra, & Gänzle, 2014; Chen & Gänzle, 2017). Multiple binding adhesins were found for some pathogens. For instance, type 1 fimbriae and CFA/I fimbriae were identified as ETEC adhesins (Sheikh et al., 2017). Type 1 fimbriae, type IV pili, plasmid-encoded fimbriae, and long polar fimbriae were all identified in *Salmonella* as adhesins (Shoaf-Sweeney & Hutkins, 2008). Multiple binding adhesins suggested the necessity of using various oligosaccharides to prevent pathogen adherence.

Oligosaccharides can be synthesized with glycosyltransferases and retaining glycosyl hydrolases. Glycosyltransferases transfer monosaccharides moieties from sugar nucleotides to suitable acceptors (Lairson, Henrissat, Davies, & Withers, 2008). Sugars, lipids, and proteins are demonstrated to be suitable acceptors of glycosyltransferase for the formation of oligo-/poly-saccharides, glycolipids and glycoproteins (Lairson et al., 2008; Palcic, 2011). For example, β-1,4-galactosyltransferase produces *N*-acetyllactosamine by transferring galactose moieties from UDP-Gal to *N*-

acetylglucosamine (Palcic, 2011). β-1,3-*N*-acetylglucosaminyltransferase and β-1,3-galactosyltransferase produce lacto-*N*-tetraose which is a oligosaccharide found in human milk (Baumgärtner, Conrad, Sprenger, & Albermann, 2014). Protein engineering has been used to alter the specificities and activities of glycosyltranferases. Mutations on sialyltransferase from *Pasteurella multocida* increased enzymatic activities of the formation of 6'-sialyllactose and abolished the synthesis of 3'-sialyllactose (Guo et al., 2015).

Retaining glycosyl hydrolases, such as β-galactosidase, are also widely used in the production of oligosaccharides. Galactosylated oligosaccharides can be synthesized by transferring galactose moieties by galactosidase through transgalactosylation reactions (Gänzle, 2012). Transgalactosylation by β-galactosidase undergoes a double-displacement mechanism with the formation of galactosyl-enzyme intermediate, thus the stereochemistry of the products remains the same as the substrates (Gänzle, 2012). Lactose is a disaccharide composed of galactose and glucose and is widely used as a galactosyl donor in transgalactosylation reactions with β-galactose to synthesize galactosylated oligosaccharides, such as GOS, lactulose, lactosucrose and other hetero-oligosaccharides (Gänzle, 2012). The yield of galactosylated oligosaccharides is highly depended on the sources of β-galactosidase (Chen & Gänzle, 2017). Moreover, β-galactosidase from different sources produces galactosylated oligosaccharides with different linkage types and degree of polymerization. For example, β-galactosidase from *Kluyveromyces lactis* produces a trisaccharide Gal-β-(1→6)-Gal-β-(1→4)-Glc; while β-galactosidase form *Bacillus circulans* favors the production of a trisaccharide Gal-β-(1→4)-Gal-β-(1→4)-Glc and a tetrasaccharide Gal-β-(1→4)-Gal-β-(1→4)-Gal-β-(1→4)-

Glc (for review, see Gänzle, 2012 and Chen & Gänzle, 2017). Other glycoside hydrolases, such as trans-sialidase, glucansucrase, and fructansucrase, use lactose as acceptor to produce hetero-oligosaccharides (for review, see Chen & Gänzle, 2017). Galactosylated oligosaccharides were demonstrated to be acceptors for various toxins and pathogens, such as cholera toxin, heat labile enterotoxin, Shiga toxin 1, *Streptococcus suis,* and *E.coli* (Kulkarni, Weiss, & Iyer, 2010). For example, heat labile enterotoxin recognizes glycan receptor with the structure of Gal-β-(1→4)-Glc and *Streptococcus suis* interacts with glycan receptor with the structure of Gal-α-(1→4)-Gal-β-OMe (Kulkarni, Weiss, & Iyer, 2010). Galactosylated oligosaccharides are promising carbohydrate decoys to prevent pathogen adherence to host cell surface. GOS prevented enteropathogenic *E.coli* adhesion to HEp-2 and Caco-2 cells (Shoaf, Mulvey, Armstrong, & Hutkins, 2006). However, the anti-adhesive specificity of galactosylated oligosaccharides only has been demonstrated in GOS and lactose. Specificity of other galactosylated oligosaccharides against pathogens remains unknown. This thesis aimed to synthesize oligosaccharides with lactose and β-galactosidase and to investigate the anti-adhesive specificity of synthesized oligosaccharides.

Hypotheses and Objectives:

Hypotheses:

- Chitosan-oligosaccharides (COS) are suitable acceptor sugars for β-galactosidase in transgalactosylation reaction with lactose as galactosyl moieties donor.
- Galactosylated COS (Gal-COS) prevent the adhesion of enterotoxigenic *Escherichia coli* K88 to porcine erythrocytes.

- β-Galactosylated structures are necessary for ETEC K88 adhesion to porcine erythrocytes.

Objectives:

- To conduct acceptor reactions with β-galactosidase using lactose and chitosan-oligosaccharides as galactosyl donor and acceptor, respectively. (Chapter 2)
- To develop purification and fractionation methods to extract synthesized Gal-COS, and to determine their structural composition by LC-MS. (Chapter 2)
- To detect the anti-adhesive effects of Gal-COS against ETEC K88 by bio-assays (Chapter2).
- To investigate the necessity of β-galactosylated oligosaccharides against ETEC K88 (Chapter 3).
- To investigate the scale-up production of Gal-COS (Chapter 4)

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Chapter 2. Enzymatic synthesis and purification of galactosylated chitosan oligosaccharides reducing adhesion of Enterotoxigenic *Escherichia coli* K88

2.1 Introduction

Indigestible dietary oligosaccharides including galacto-oligosaccharides (GOS) have several biological activities that improve host health by modulating composition and activity of intestinal microbiota, by preventing the adhesion of pathogens to intestinal tissues, and by specific interactions with the immune system (Chen & Gänzle, 2017). The conceptual template for the food applications of oligosaccharides with specific health benefits is human milk oligosaccharides (HMOs). HMOs comprise over 150 different oligosaccharides, and their concentration in human milk ranges from 5 to 20g/L (Bode et al., 2016). HMOs consist of five monosaccharides, galactose, glucose, *N*-acetyl-glucosamine (GlcNAc), fucose, and *N*-acetyl-neuraminic acid (sialic acid) (Bode et al., 2016). HMOs shape the development of infant microbiome, block adhesion of pathogens including *Escherichia coli*, *Salmonella fyris*, and *Campylobacter jejuni* to host cells and thus prevent infections (Coppa et al., 2006; Morrow et al., 2004; Sela & Mills, 2010).

In food and feed applications, bifidogenic properties of HMOs are currently substituted by other nondigestible oligosaccharides including galacto-oligosaccharides (GOS), fructo-oligosaccharides (FOS), inulin, and lactulose, which stimulate growth and activity of intestinal microbiota (Ackerman, Craft, & Townsend, 2017; Davis, Martinez, Walter, Goin, & Hutkins, 2011). The ability of oligosaccharides to inhibit adhesion of pathogen, however, is highly specific for the target organism and the oligosaccharide structure and HMO are thus not readily replaced by other glycans (Chen & Gänzle, 2017; Shoaf-Sweeney & Hutkins, 2008). The structural diversity of HMOs, a prerequisite for their interference with adhesion of multiple pathogens, also impedes the production at the commercial scale. A commercial GOS preparation also inhibited the adherence of

enteropathogenic *Escherichia coli* (EPEC) to HEp-2 and Caco-2 cells (Shoaf, Mulvey, Armstrong, & Hutkins, 2006). However, the use of GOS to reduce adhesion of enterotoxigenic *Escherichia coli*, a major cause of childhood diarrhea, has not been reported.

GOS are synthesized by transgalactosylation of lactose with β-galactosidase (Gänzle, 2012). In response to the demand for improved HMOs substitutes, transgalactosylation with β-galactosidase was extended to acceptor carbohydrates other than lactose to synthesize a diversity of oligosaccharides (Chen & Gänzle, 2017). Fructose, mannose, sucrose, *N*-acetyl-glucosamine, and fucose have been used in transgalactosylation reactions as acceptor sugars to synthesize diverse galactosylated oligosaccharides (Gänzle, 2012; Schwab, Sørensen, & Gänzle, 2010; Schwab, Lee, Sørensen, & Gänzle, 2011). Of these acceptor carbohydrates, *N*-acetyl-glucosamine (GlcNAc) has received particular attention because it is a constituent of HMO (Bode et al., 2016), binds to fimbriae of *E. coli* that mediate adhesion to the intestinal mucosa (Rhen, Klemm, & Korhonen, 1986), and occurs in nature as the constituent monosaccharide of chitin, an abundant polysaccharide occurring in the shell of arthropods and the cell wall of fungi. Chitin and chitin-oligosaccharides are poorly soluble in water; commercial conversion of chitin from shrimp generally yields the deacetylated chitosan or chitosan-oligosaccharides (COS). COS exhibit high solubility in water; however, they are also highly reactive as they contain aldehyde and amino groups as reactants in the Maillard reaction, which occurs even during incubation at ambient temperature (Hrynets, Ndagijimana, & Betti, 2015).

Chitobiose, chitotriose, and COS are acceptor sugars for β- galactosidase to produce di-, tri-, tetra-oligosaccharides with galactose and GlcNAc/glucosamine (Black et al., 2014). GOS as well as COS reduce adhesion of pathogenic *E. coli* to intestinal mucosal cells; however, the activity of galactosylated COS remains unknown (Quintero-Villegas et al., 2013; Shoaf et al., 2006). Therefore, this study aimed to determine whether galactosylated COS inhibit adhesion of enterotoxigenic *E. coli* expressing K88 fimbriae to porcine cells (Chen, Woodward, Zijlstra, & Gänzle, 2014; Wang, Gänzle, & Schwab, 2010). ETEC K88 is an important cause for diarrhea in weaning piglets (Xia et al., 2015); different from human ETEC strains, is a model system that readily allows validation of *in vitro* results *in vivo* (Chen et al., 2014; Wang et al., 2010). To determine whether galactosylation influences the biological activity, oligosaccharides were fractionated by cation exchange and size exclusion chromatography. The influence of acetylation was determined by chemical acetylation.

2.2 Materials and methods

2.2.1 Preparation of β-galactosidase.

Lactococcus lactis MG1363 expressing the LacLM type β-galactosidase of *Lactobacillus plantarum* were streaked onto modified M17 (mM17) agar plates that were supplemented with 5% glucose and 5mg/L erythromycin and incubated at 30°C under anaerobic condition for 48h (Black et al., 2014; Schwab et al., 2010). Crude cell extract (CCE) was prepared as previously described (Black et al., 2014). Cells were harvested by centrifugation, washed, and resuspended in 50mM phosphate buffer (PB) (pH 6.5) with 10% glycerol and 1mM magnesium chloride. Cells were disrupted with a Mini Beadbeater (model 693, Biospec, Bartlesville, OK) for 2min and cell debris was removed

by centrifugation at 15,300g for 10min at 4°C. Protein content and β-galactosidase activity of CCE were measured by Bradford protein assay and *o*-nitrophenyl-*β*-galactoside, respectively. When necessary, CCE was diluted with PB containing 10% glycerol and $MgCl_2$ to standardize the activity to 25−30μmol/(min × mg protein) (Schwab et al., 2011).

2.2.2 Transgalactosylation reaction of β-galactosidase with lactose and COS.

COS with a degree of polymerization (DP) ranging from 2 to 6 and a degree of deacetylation of >95% were provided by Glycobio (Dalian, China). The size distribution of the COS was confirmed by size exclusion chromatography as described below (data not shown). Transgalactosylation reactions were conducted by the addition of 20% (v/v) of standardized CCE into 180g/L of COS and 180g/L of lactose, followed by incubation for 16h at 45°C. Reactions were terminated by addition of perchloric acid to a concentration of 3.5% (v/v). Control reactions contained 360g/L lactose with 20% CCE (control without COS), 360g/L COS with 20% CCE (v/v) (control without GOS), or 180g/L COS and 180g/L lactose without CCE (control without CCE). The transgalactosylation and control reactions were monitored by measuring the UV−vis absorbance in the range of 200−600nm for 16h with 1h intervals. Oligosaccharide samples were lyophilized prior to analyses described below.

2.2.3 Preparation of acetylated COS and acetylated galactosylated COS.

The acetylation of COS and Gal-COS was performed as described with some modifications (Hu, Du, Wang, & Feng, 2009). COS or Gal-COS were dissolved in 50:50 (v/v) methanol−water at the concentration of 40g/L. Acetic anhydride (0.5 equiv/glucosamine unit of COS) was added to COS or Gal-COS with continuous stirring

at room temperature for 4h. The acetylation was terminated by 40-fold dilution with water. Oligosaccharides were lyophilized. The degree of acetylation of acetylated oligosaccharides was measured by titration with 0.1M of NaOH by dissolving acetylated oligosaccharides into 0.1M HCl as described (D. Liu, Wei, Yao, & Jiang, 2006; Zhang, Xue, Xue, Gao, & Zhang, 2005). All reactions were conducted in triplicates.

Lyophilized acetylated Gal-COS were further separated by size exclusion chromatography as described below. Five fractions were collected. Acetylated COS, acetylated Gal-COS, and five collected fractions were lyophilized and redissolved to a concentration of 10g/L prior to analysis by HPAEC-PAD and LC−MS and tested for antiadhesive activities with the methods described below.

2.2.4 High-performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD).

Oligosaccharides were diluted to 1g/L prior to analysis on a HPAEC-PAD ICS-3000 system (Dionex, Oakville, Canada). Samples (10μL) were separated on a CarboPac PA-20 Dionex carbohydrates column using water (A), 200mM NaOH (B), and 1M sodium acetate (C) as eluent at flow rate of 0.25mL/min with the following gradient: 0 min 6% B, 20 min 100% B. GOS were further analyzed using gradient starting at 30.4% B, 1.3% C and increasing to 30.4% B, 11.34% C at 22 min.

2.2.5 Hemagglutination assay to determine the effect of oligosaccharides on ETEC K88 adhesion to porcine erythrocytes.

All samples used in the assay were lyophilized and re-dissolved in phosphate-buffer saline (PBS) (pH 7.2). The hemagglutination assay was performed with the porcine ETEC ECL 13795 with K88 fimbriae as described with some modifications (Wang et al.,

2010). *E. coli* ECL13795 were cultivated on Minca agar overnight and washed with 1mL of PBS. A volume of 25μL of *E. coli* cell suspensions with an OD600nm of 5−10 were diluted 2-fold in V-bottom 96-well polystyrene microtiter plates (Corning). The same volume of PBS or tested oligosaccharides with different concentrations was added, and the suspensions were incubated for 5min before the addition of 25μL of erythrocyte suspension into each well. Erythrocytes were prepared by 2-fold dilution of 10% porcine red blood cells (Innovative Research, Novi, MI). The plate was incubated at 4°C overnight prior to visual inspection. Activity was noted when the sample carbohydrate increased the cell density of ETEC presenting hemagglutination and lack of a defined pellet of erythrocytes at least 4-fold. All samples were diluted from 10g/L to exhaustion of the biological activity or to 0.1g/ L.

2.2.6 Enzyme-Linked Immunosorbent Assay (ELISA) to determine the effect of oligosaccharides on ETEC K88 adhesion to porcine erythrocytes.

The ability of oligosaccharides to inhibit *E. coli* K88 adhesion to porcine erythrocytes was also assayed by ELISA. Each step of the following assay was separated by washing the wells three times with PBS. A 96-well high bind microtiter plate (Corning) was coated overnight with 5% porcine red blood cells (Innovative Research, Novi, MI) and blocked with 3% bovine serum albumin for 1h at 4°C. Oligosaccharides were dissolved in PBS at the concentration of 10g/L. ETEC were mixed with PBS or oligosaccharides and then added into plates with 1h incubation at 4°C. Red blood cells without ETEC addition, red blood cells with only ETEC addition, and no red blood cells but with ETEC addition were used as controls. The wells were then treated with mouse anti *E. coli* K88A antibody (Biorad) at a dilution of 1: 2000. After 1h incubation, the wells were treated

with a goat antimouse IgG (H+L) secondary antibody conjugated to horseradish peroxidase (Invitrogen, Fisher Scientific, CA) for 1h. After addition of TMB substrates and incubated for 30min, the reaction was stopped with 2M sulfuric acid, and the absorption at 450nm was measured on a Varioscan Flash Microplate reader (Thermo Scientific, CA).

2.2.7 Separation of charged oligosaccharides by cation exchange chromatography and size exclusion chromatography

To separate charged oligosaccharides from lactose and GOS, reaction mixtures obtained with COS, GOS, and galactosylated COS (Gal-COS) were separated through solid phase extraction method with cation exchange column Strata SCX (55µm, 70A, Phenomenex). The SCX column was conditioned with 3mL of methanol prior to extraction. The column was equilibrated with 0.1% trifluoroacetic acid (TFA). Sugar samples were diluted with 0.1% TFA to a concentration of 10g/L and loaded onto the column. Charged oligosaccharides, containing COS and Gal-COS, were eluted with 0.2% triethylamine. Fractions collected during loading of the column were referred to as Gal-COS flow through (Gal-COS FT), and fractions collected after elution were referred to as Gal-COS. The collected fractions were lyophilized and re-dissolved to a concentration of 10g/L. All fractions were analyzed by HPAEC-PAD and hemagglutination assay.

Lyophilized COS, GOS, and Gal-COS fractions obtained by cation exchange chromatography were further separated by size exclusion chromatography on a Superdex peptide 10/300 GL column (10mm × 240mm, 13µm, GE Healthcare Life Sciences) eluted with 0.2M ammonium acetate at 0.3mL/min. Separations were carried out on a Agilent 1200 HPLC system coupled to refractive index (RI) and multiple wavelength detector.

Glucose (Fisher), lactose (Sigma), raffinose (Sigma), stachyose (Sigma), inulin from chicory (Sigma), and dextran (100 000−200 000 Da, Sigma) were used as external standards for molecular weight calibration. Five fractions were collected for each sample. All collected fractions were lyophilized and redissolved to a concentration of 10g/L prior to analysis by HPAEC-PAD, LC−MS, and antiadhesive activities.

2.2.8 Liquid Chromatography–Electrospray Ionization-Mass Spectrometry (LC–ESI–MS).

LC−ESI−MS analysis of oligosaccharides was conducted by Mass Spectrometry Facility of University of Alberta. LC−MS was performed using an Agilent 1200 SL HPLC system with GlycanPac AXH-1 column (2.1mm × 150mm, 1.9μm, Thermo Scientific, Sunnyvale, CA), thermostated at 40°C, with a buffer gradient system composed of 96:4 (v/v) acetonitrile (ACN)−water as mobile phase A and 80mM ammonium formate in water, pH 4.4 as mobile phase B. Oligosaccharides were separated using the following gradient: 10% B for 2 min, 10% to 30% B over a period of 10 min, 30% to 75% B over of 9 min, 75% to 80% B over a period of 1 min, 80% to 10% B over a period of 2 min, and held at 10% B for 3 min.

Mass spectra were acquired in positive mode ionization using an Agilent 6220 Accurate-Mass TOF HPLC−MS system (Santa Clara, CA) equipped with a dual sprayer electrospray ionization source with the second sprayer providing a reference mass solution. Mass spectrometric conditions were drying gas 10L/min at 300°C, nebulizer 30psi, and mass range 100−3200Da. Analysis of the HPLC−MS data was done using the Agilent Mass Hunter Qualitative Analysis software (version B.07.00). Previous study demonstrated the formation of Gal-COS in transgalactosylation reaction of β-

galactosidase (Black et al., 2014). Cation exchange purification removed glucose, galactose, lactose, and GOS from charged oligosaccharides (COS and Gal-COS). Based on the above information, a molecular weight library of expected oligosaccharides was generated using the different ratios, from DP1 to DP 20, of galactose, glucosamine and *N*-acetyl-glucosamine. This library was then used to screen for compounds of interest. Because they were not the results of enzymatic synthesis, COS were used as negative controls and their masses were eliminated in the analysis of Gal-COS and acetylated Gal-COS fractions. The isotope pattern of each screened mass was compared with the specific ratio of C, H, O, and N from the predicted isotope patterns of each oligosaccharide in the library. Moreover, ion abundance and signal to noise ratio were also taken into consideration during data analysis.

2.2.9 Stability of COS, acetylated-COS, Gal-COS, and acetylated Gal-COS during storage

Freeze-dried COS, acetylated-COS, Gal-COS, and acetylated Gal-COS (0.01−0.05g/vial) were placed into individual HPLC vials and stored in closed containers at 37 and 6°C for 7 days. The water activity (aW) in the containers was maintained by addition of saturated sodium chloride, saturated sodium bromide, and silica gel beads to achieve a constant aW of 0.75, 0.55, and 0.1, respectively. UV−vis absorbance spectra of tested oligosaccharides were measured before and after 7 days storage. The absorbance was recorded in the range of 200−600nm. Tested sugars were dissolved to a concentration of 10g/L prior to analysis. The compositions of tested sugar before and after storage were analyzed by HPAEC-PAD with the method described above.

2.2.10 Statistical analysis

Purification and fractionation was performed from three independent enzymatic reactions. Bioassays were performed in triplicate technical repeats. Results of the bioassayguided fractionation are presented as means ± standard error of the mean.

2.3. Results

2.3.1. Transgalactosylation and separation of COS

Transgalactosylation of COS and lactose with β-galactosidase were analyzed by HPAEC-PAD (Figure 2.1). The formation of Gal-COS was identified as additional peaks not present in the control reactions described in section 2.2.2 (Fig 2.1A and data not shown). Transgalactosylation activity of β-galactosidase was apparent but galactosylated oligosaccharides were partially obscured by the presence of glucose, galactose, COS, and galacto-oligosaccharides (Figure 2.1A, B). Previously, Gal-COS were synthesized via transgalactosylation of galactose from lactose to the non-reducing end of COS by β-galactosidase (Black et al., 2014; Schwab et al., 2010). Charged oligosaccharides including COS and Gal-COS were separated from the reaction mixture by cation exchange chromatography. In the separation of transgalactosylation reactions with COS as the acceptor, COS and Gal-COS were bound to the column and eluted with triethylamine (Figure 2.1B); glucose, galactose, lactose, and galacto-oligosaccharides did not bind to the columns (data not shown)

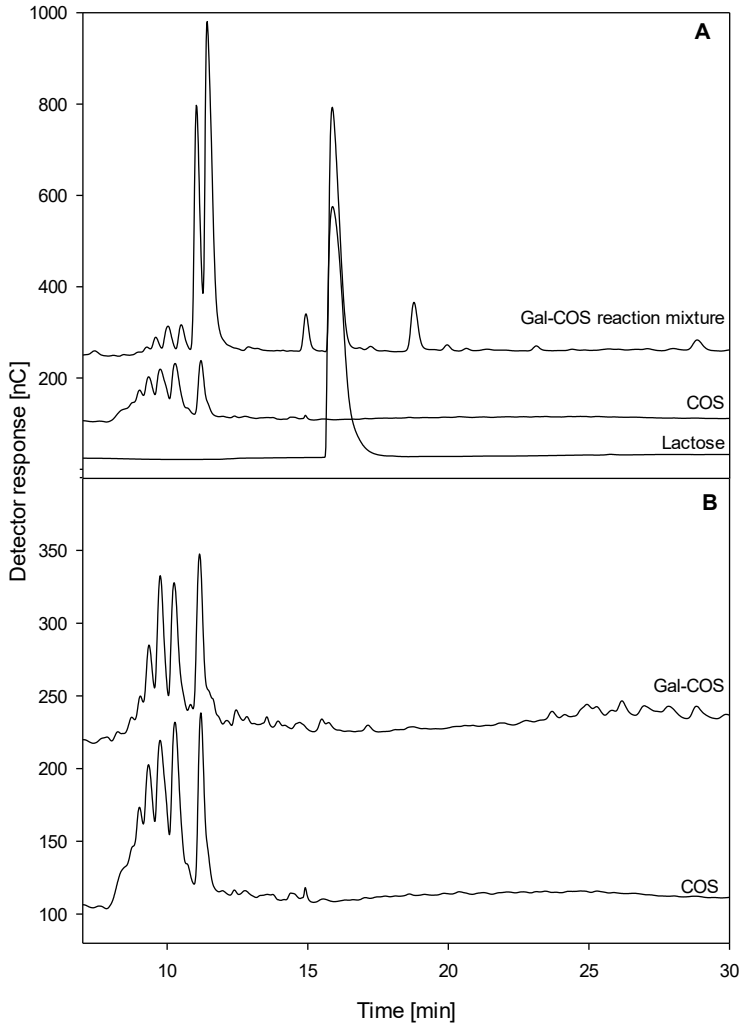

Figure 2.1. HPAEC-PAD results of transgalactosylation reaction with lactose and COS and purification of the active oligosaccharide fractions. (Panel A) Chromatographic trace of unpurified enzymatic reactions; chromatograms of lactose and COS are shown for comparison. (Panel B) Chromatographic traces of charged oligosaccharides purified by cation exchange chromatography. The corresponding fraction obtained with separation of COS is shown for comparison. Chromatograms are representative of three independent reactions. Galactose, glucose, and lactose elute at 11.2, 11.6, and 15.9 min, respectively.

The enzymatic reaction mixture and all fractions obtained by separation with cation exchange chromatography were tested for anti-adhesion activity against ETEC K88. All samples were adjusted to 10g/L and diluted to exhaustion of the biological activity. Enzymatic reactions without further purification had the strongest antiadhesive activities, followed by Gal-COS purified by cation exchange columns (Table 2.1). Glucose, galactose, lactose, COS, and GOS had no effect on agglutination of ETEC to porcine erythrocytes. Monitoring the transgalactosylation reactions with lactose or lactose and COS by measuring UV absorbance and comparison to reactions with COS and CCE suggested that the presence of COS leads to formation of UV-absorbing compounds. Oligosaccharide mixtures generated by enzymatic synthesis in the presence of COS thus require further separation before conclusions on their biological activity can be drawn. COS reduced K88 adhesion after separation on cation exchange columns; however, the effective concentration was twice as high as the effective concentration of Gal-COS (Table 2.1). The results indicate that Gal-COS have higher anti-adhesion activity than COS or GOS.

Table 2.1 Inhibition of erythrocyte agglutination by ETEC K88 strain with oligosaccharides at various concentrations[a].

Sample	Minimum concentration for agglutination with ETEC (g/L)
COS	> 10
COS elution [b]	6.67 ± 0.96[a]
COS FT [c]	9.44 ± 0.00[b]
Glucose	> 10
Galactose	> 10
Glucosamine	> 10
Lactose	> 10
Gal-COS mixture [d]	0.69 ± 0.00[c]
Gal-COS FT [c]	5.00 ± 0.00[a]
Gal-COS [b]	2.92 ± 0.48[d]
GOS	> 10

a: Hemagglutination was determined with *E. coli* K88 ECL13795 (O149, virotype STb:LT:EAST1:F4), and results were based on three independent assays. Different letters indicate statistical differences (P <0.05).
b: Fraction of charged oligosaccharides after purification by cation exchange chromatography.
c: Fraction of uncharged enzymatic reaction products that did not bind to cation exchange column.
d:Unpurified enzymatic reaction products or equivalent incubation of enzyme and COS.

2.3.2 Fractionation of oligosaccharides by SEC

In order to determine the effect of molecular weight of oligosaccharides on anti-adhesion activities against ETEC K88, COS, GOS, and Gal-COS were separated based on their molecular weight. Five different fractions were collected for each sample and designated F1 to F5 in order of decreasing molecular weight. Most Gal-COS eluted in F4 and F3, followed by F2 and F5 (Figure 2.2, upper trace). The carbohydrate concentration in Gal-COS F1 was below the detection limit of the RI detector. The molecular weight range of fractions was estimated as follows: F2, MW 1500−2500Da, corresponding to DP8 and higher; F3, MW 900−1500Da, corresponding to DP6-8; F4, MW 500−1200Da,

corresponding to DP 3-6; F5, less than 500Da and DP of less than 3. All fractions were tested for anti-adhesive activities against ETEC K88 (Table 2.2). Fractions containing Gal-COS were 2−8 times more active when compared to the corresponding factions containing COS only. The activity of Gal-COS decreased with decreasing molecular weight (Table 2.2).

In addition to carbohydrates, F5 contained compounds with UV absorbance at 254 and 280nm. This confirmed that compounds generated from non-enzymatic browning reactions influenced the results of the hemagglutination assay as Gal-COS elution fraction 5 (Table 2.2) and COS-containing control reactions (data not shown) inhibited *E. coli* adhesion to porcine red blood cells at 10g/L.

F1 and F5 were excluded from further analysis owing to the low yield, the low activity, and the presence of UV-absorbing compounds. F2, F3, and F4 of Gal-COS were analyzed by HPAEC-PAD. Different patterns of oligosaccharides were observed in each fractions; however, the lack of galactosylated COS standards prevented identification (data not shown).

The effect of Gal-COS on adhesion of *E. coli* was confirmed with an additional bioassay based on ELISA detection of K88 fimbriae (Figure 2.3). Consistent with results obtained with the hemagglutination assay, the highest antiadhesive activity was observed with Gal-COS after purification with cation exchange columns; Gal-COS F2 and Gal-COS F3 also exhibited strong anti-adhesion effects (Figure 2.3). Lactose and COS did not reduce ETEC K88 attachment onto porcine red blood cells. Different from the hemagglutination assay, however, the Gal-COS reaction mixture had no antiadhesive activity (Figure 2.3),

further indicating that products from non-enzymatic browning reactions interfere with the hemagglutination assay.

Taken together, ELISA confirmed that the Gal-COS and the Gal-COS fractions F2, F3, and F4 inhibit adherence of ETEC K88 to porcine red blood cells and that compounds from non-enzymatic browning reactions interfere with the hemagglutination assay.

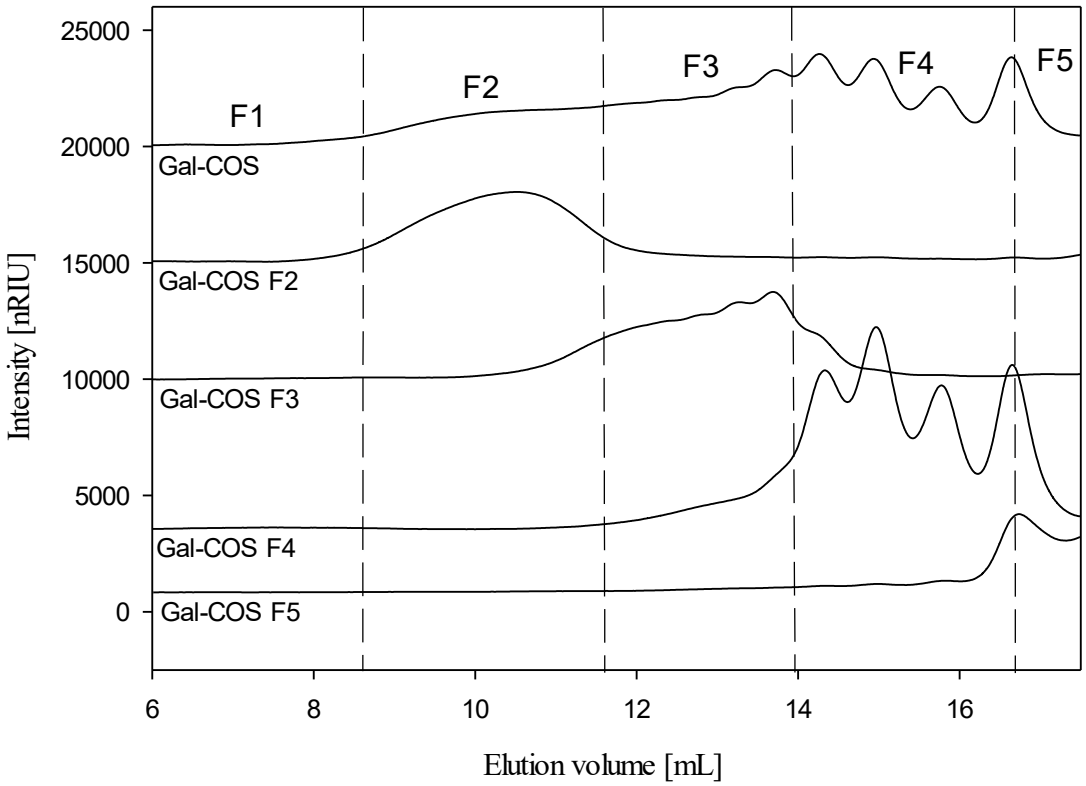

Figure 2.2. Separation and fractionation of Gal-COS by size exclusion chromatography. The upper trace represents charged oligosaccharides after cation exchange purification, other traces represent chromatography of isolated fractions. Fraction 2 is the largest followed by fraction 3 and followed by fraction 4 and fraction 5 was the smallest fraction collected. LMW dextran (450 000−650 000), inulin, stachyose (DP 4), raffinose (DP3), and lactose (DP 2) were used as molecular weight standards. Chromatography was based on three independent reactions.

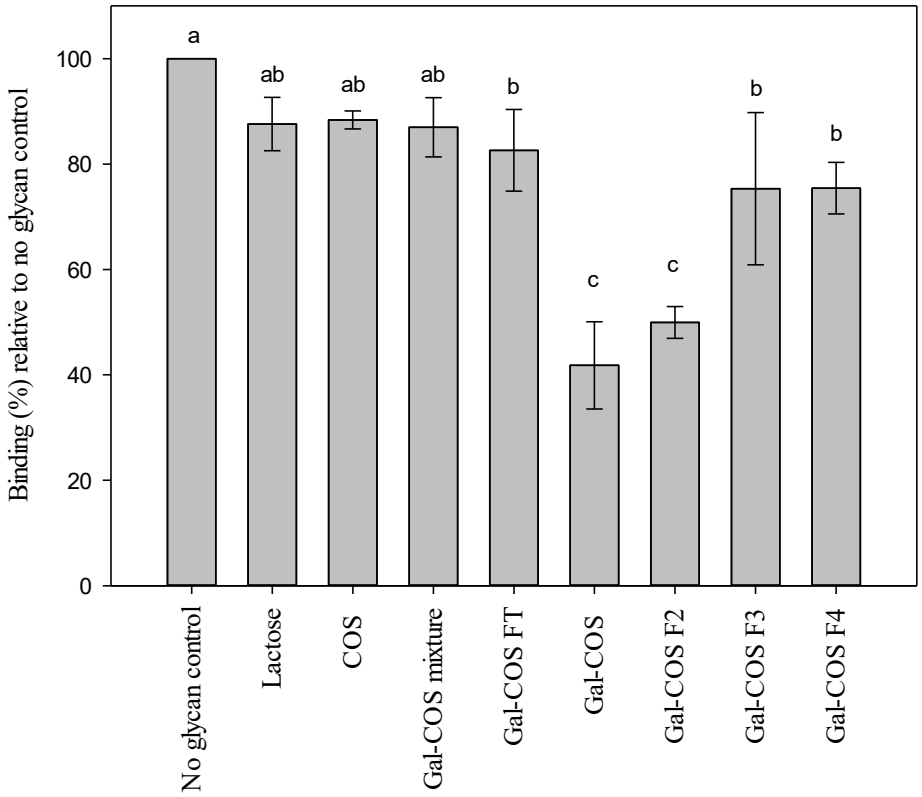

Figure 2.3. Quantification of *E. coli* K88 ECL13795 binding to porcine erythrocytes with ELISA targeting K88 antibodies. ETEC were incubated with erythrocytes without addition of glycans (no glycan control) or with addition of 10g/L of the carbohydrates as indicated on the x-axis. Results are reported as means ± standard deviation of three independent assays. The values of columns that do not share a common superscript differ significantly ($P < 0.05$).

Table 2.2. Inhibition of erythrocyte agglutination by ETEC K88 strain with Gal-COS elution fractions at various concentrations[a]

Oligosaccharides	Minimum concentration for agglutination with ETEC (g/L)
COS F1[b]	> 10
COS F2	8.33 ± 0.96[a]
COS F3	2.78 ± 0.48[b]
COS F4	6.11 ± 0.96[a]
COS F5	> 10
Gal-COS F2	0.22 ± 0.00[c]
Gal-COS F3	1.88 ± 0.24[d]
Gal-COS F4	3.06 ± 0.48[b]
Gal-COS F5	> 10
GOS fractions	> 10 in all tested fractions

a: The corresponding COS fractions were used for comparison. Hemagglutination was determined with *E. coli* K88 ECL13795 (O149, virotype STb:LT:EAST1:F4), and results were based on three independent assays. Values in the same column that do not share a common superscript are significantly different ($P < 0.05$).
b: Fractions numbers 1−5 correspond to fractions with decreasing molecular weight after separation with size exclusion chromatography.

2.3.3 Identification of oligosaccharides by LC−ESI-MS.

Active fractions were analyzed by LC−MS to achieve an accurate determination of Gal-COS. The high-resolution MS provided information on the chemical formula; the presence of Gal-COS was deduced from the enzymatic method of synthesis and the absence of the corresponding products in the control reactions. Galactosylated COS were present in all active fractions but absent in the corresponding fractions containing fractionated COS (Table 2.3 and data not shown). The predominant degree of polymerization in the respective fractions corresponded to the separation range of the SEC column used for fractionation (Table 2.3 and Figure 2.2). Galactosylated COS were the major compounds in each of the fractions tested (Table 2.3); Gal-GOS were composed of glucosamine and GlcNAc with one to four galactosyl-residues.

Table 2.3. List of compounds identified in Gal-COS elution fractions and their mass accuracy

Oligosaccharides fractions	Compounds[a,b]	Measured Mass (Da)	Error (ppm)
Gal-COS F2	Gal-Gal-$(GlcN)_4(GlcNAc)_6$	2204.8849	7.81
	Gal-$(GlcN)_6(GlcNAc)_5$	2161.8854	5.69
	Gal-$(GlcN)_7(GlcNAc)_4$	2119.8750	5.90
	Gal-$(GlcN)_8(GlcNAc)_3$	2077.8624	5.04
	Gal-$(GlcN)_5(GlcNAc)_5$	2000.8215	8.62
	Gal-Gal-$(GlcN)_6(GlcNAc)_3$	1917.7775	5.40
	Gal-$(GlcN)_8(GlcNAc)_2$	1874.7822	5.12
	Gal-Gal-$(GlcN)_6(GlcNAc)_2$	1714.6989	6.46
	Gal-Gal-$(GlcN)_7$(GlcNAc)	1672.6779	0.42
Gal-COS F3	Gal-Gal-Gal-Gal-$(GlcN)_5$	1471.5760	6.89
	Gal-Gal-Gal-$(GlcN)_6$	1470.5855	2.43
	Gal-Gal-$(GlcN)_7$	1469.5893	-5.86
	Gal-Gal-Gal-Gal-$(GlcN)_4$	1310.5067	7.33
	Gal-Gal-Gal-$(GlcN)_5$	1309.5378	0.38
	Gal-Gal-$(GlcN)_6$	1308.5296	0.42
	Gal-Gal-Gal-$(GlcN)_4$	1148.4479	3.14
	Gal-$(GlcN)_6$	1146.4698	-5.59
	$(GlcN)_7$	1145.4932	0.81
	Gal-Gal-$(GlcN)_4$	986.3933	1.84
	Gal-$(GlcN)_5$	985.4063	-1.15
	$(GlcN)_6$	984.4209	-2.59
Gal-COS F4	Gal-Gal-Gal-$(GlcN)_4$	1148.4457	1.27
	Gal-Gal-$(GlcN)_5$	1147.4640	3.24
	Gal-Gal-Gal-$(GlcN)_3$	987.3800	4.65
	Gal-Gal-$(GlcN)_4$	986.3921	0.66
	Gal-$(GlcN)_5$	985.4026	-4.86
	Gal-Gal-$(GlcN)_3$	825.3326	-0.06
	Gal-$(GlcN)_4$	824.3348	-4.63
	$(GlcN)_5$	823.3563	2.04
	Gal-Gal-$(GlcN)_2$	664.2489	-7.46
	$(GlcN)_4$	662.2852	-0.88
	$(GlcN)_3$	501.2179	1.8

a. The position of *N*-acetyl-glucosamine groups does not corresponding to their positions in different oligosaccharides; however, as a result of enzymatic catalysis, galactose units are at the non-reducing end.

b. Nomenclature: Gal, Galactose; GlcN, *N*-glucosamine; GlcNAc, *N*-acetyl-glucosamine

2.3.4 Preparation of acetylated COS and acetylated galactosylated COS.

Gal-COS contained both glucosamine and GlcNAc as constituting monosaccharides. To determine whether the degree of acetylation influences activity, Gal-COS were separated by cation exchange chromatography and acetylated chemically. Titration of Gal-COS before and after acetylation estimated the degree of acetylation as 50%. Analysis of acetylated Gal-COS by LC−MS confirmed the synthesis of *N*-acetyl-glucosamine containing oligosaccharides. LC−MS results (Table 2.4) showed galactosylated oligosaccharides with a different degree of acetylation. Acetylated Gal-COS fractions differed in their anti-adhesive activities (Table 2.5). The largest fraction (Figure 2.4) with molecular weight from 1700 to 2500Da exhibited the strongest activity, followed by the fractions with molecular range of 1000 to 2000Da and 700 to 1300Da. However, acetylation decreased the antiadhesive activity 3-10-fold when compared to the corresponding fractions containing Gal-COS (Tables 2.2 and 2.5).

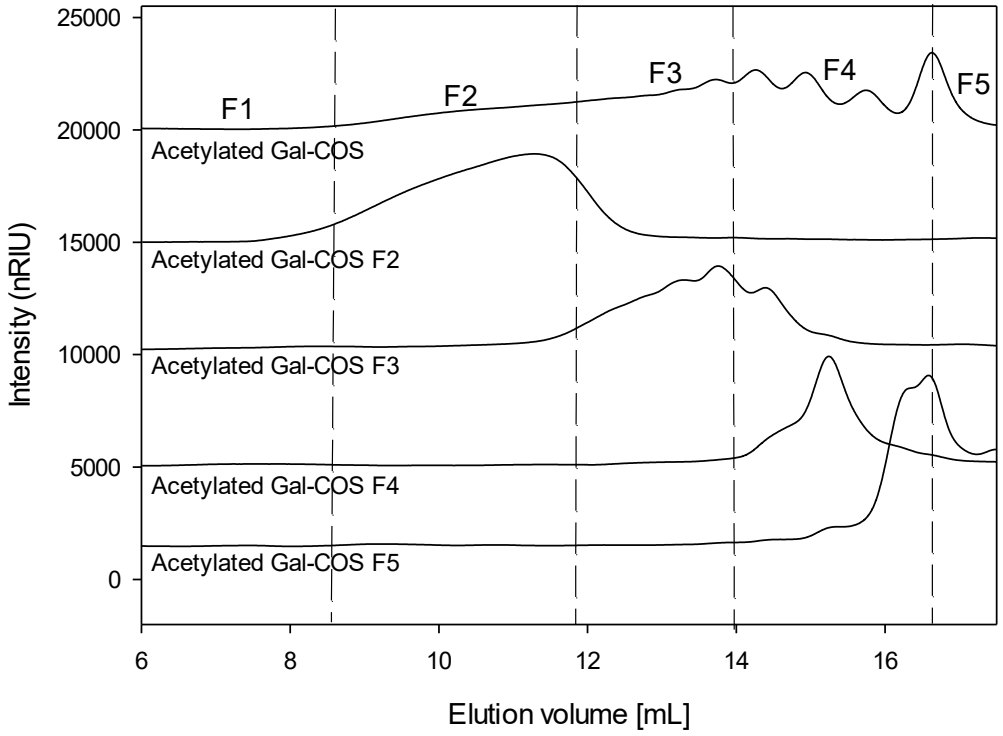

Figure 2.4. Separation and fractionation of acetylated Gal-COS by size exclusion chromatography. The upper trace represents Gal-COS after acetylation, other traces represent the chromatography of isolated fractions. Fraction 2 is the largest followed by fraction 3 and followed by fractions 4 and 5 was the smallest fraction collected. LMW dextran (450 000−650 000), inulin, stachyose (DP 4), raffinose (DP3), and lactose (DP 2) were used as molecular weight standards. Chromatography was based on three independent reactions.

Table 2.4. List of compounds identified in acetylated Gal-COS elution fractions and their mass accuracy

Oligosaccharides fractions	Compounds[a,b]	Measured mass (Da)	Error (ppm)	Compounds	Measured mass (Da)	Error(ppm)
Acetylated Gal-COS F2	Gal-(GlcN)$(GlcNAc)_{10}$	2371.9303	1.85	Gal-Gal-Gal-(GlcN)-$(GlcNAc)_7$	2086.8016	3.89
	Gal-Gal-Gal-(GlcN)$(GlcNAc)_8$	2289.8933	8.95	Gal-$(GlcNAc)_9$	2007.7821	2.17
	Gal-$(GlcNAc)_{10}$	2210.8655	3.80	Gal-(GlcN)$(GlcNAc)_8$	1965.7672	0.02
	Gal-Gal-$(GlcNAc)_9$	2169.8457	6.99	Gal-Gal-Gal-(GlcN)$(GlcNAc)_6$	1883.7253	5.98
	Gal-(GlcN)$(GlcNAc)_9$	2168.8497	1.44	Gal-(GlcN)$(GlcNAc)_7$	1762.6841	-2.11
	Gal-Gal-Gal-$(GlcNAc)_8$	2128.8214	8.15	Gal-Gal-(GlcN)$(GlcNAc)_6$	1721.6646	1.94
	Gal-Gal-(GlcN)$(GlcNAc)_8$	2127.8300	4.68			
Acetylated Gal-COS F3	Gal-Gal-(GlcN)$(GlcNAc)_8$	2127.8303	4.86	Gal-Gal-Gal-(GlcN)$(GlcNAc)_3$	1274.4845	6.69
	Gal-Gal-GlcN-$(GlcNAc)_7$	1924.7427	1.06	$(GlcNAc)_6$	1236.4851	-1.39
	Gal-Gal-Gal-(GlcN)$(GlcNAc)_4$	1477.5656	6.97	Gal-$(GlcNAc)_5$	1195.4652	4.17
	$(GlcNAc)_7$	1439.5653	-0.64	Gal-(GlcN)$(GlcNAc)_4$	1153.4464	-2.84
	Gal-$(GlcNAc)_6$	1398.5427	2.17	Gal-Gal-$(GlcN)_2(GlcNAc)_2$	1070.4117	-0.79
	Gal-Gal-$(GlcNAc)_5$	1357.5210	5.83	Gal-Gal-(GlcN)$(GlcNAc)_3$	1112.4238	0.63
	Gal-Gal-(GlcN)$(GlcNAc)_4$	1315.5033	0.63			
Acetylated Gal-COS F4	Gal-Gal-(GlcN)$(GlcNAc)_4$	1315.5069	3.37	Gal-Gal-(GlcN)$(GlcNAc)_2$	909.3452	1.6
	Gal-Gal-(GlcN)$(GlcNAc)_3$	1112.4237	0.50	Gal-Gal-Gal-(GlcN)(GlcNAc)	868.3246	8.49
	Gal-Gal-Gal-(GlcN)$(GlcNAc)_2$	1071.4047	7.53	$(GlcNAc)_4$	830.3283	0.25
	Gal-(GlcN)$(GlcNAc)_3$	950.3716	1.31	Gal-Gal-(GlcN)(GlcNAc)	706.2670	3.7

a: The position of *N*-acetyl-glucosamine groups does not correspond to its position in different oligosaccharides; however, as a result of enzymatic catalysis, galactose units are at the non-reducing end.
b: Nomenclature: Gal, galactose; GlcN, *N*-glucosamine; GlcNAc, *N*-acetyl-glucosamine.

Table 2.5. Inhibition of erythrocyte agglutination by ETEC K88 strain with acetylated Gal-COS elution fractions at various concentrations

Oligosaccharides	minimum concentration for erythrocyte agglutination with ETEC $(g/L)^{-1}$)
Acetylated Gal-COS F1	> 10
Acetylated Gal-COS F2	5.83 ± 0.96^{a}
Acetylated Gal-COS F3	6.11 ± 0.96^{a}
Acetylated Gal-COS F4	> 10
Acetylated Gal-COS F5	> 10

a: Hemagglutination was determined with E. coli K88 ECL13795 (O149, virotype STb:LT:EAST1:F4), and results were based on three independent assays. Different letters indicate statistical differences ($P < 0.05$)

2.3.5 Stability of Gal-COS and Acetylated Gal-COS during Storage

Past studies and observations during purification of Gal-COS in this study indicated that handling of COS at ambient temperature may result in formation of UV-absorbing products which affect their activity (Hrynets et al., 2015). To determine whether acetylation or galactosylation influences the reactivity during dry storage, the degradation of Gal-COS was monitored after lyophilization. Gal-COS were compared to COS, acetylated COS, and acetylated Gal-COS. Samples were stored at different temperatures and water activities. The change in UV-vis absorbance after 7 days of storage was used as a simple tool to monitor major changes. The differences in the UV−vis spectra were plotted to monitor the stability over time. An increased absorption was observed for COS storage under 37°C for all three water activities conditions (Figure 2.5 and appendix A). A minor increase in absorbance was observed in acetylated COS, Gal-COS, and acetylated Gal-COS under all conditions. No changes were observed in COS stored at 6 °C with all tested water activities. An increased UV absorption between 340 and 360 nm was observed for Gal-COS maybe because the non-enzymatic browning reaction

products formed during enzymatic reaction. COS were not stable when stored at 37 °C; however, galactosylation, acetylation, or both increased their storage stability. The lower UV absorption of acetylated Gal-COS after 7 days storage when compared to Gal-COS indicated a lower reactivity.

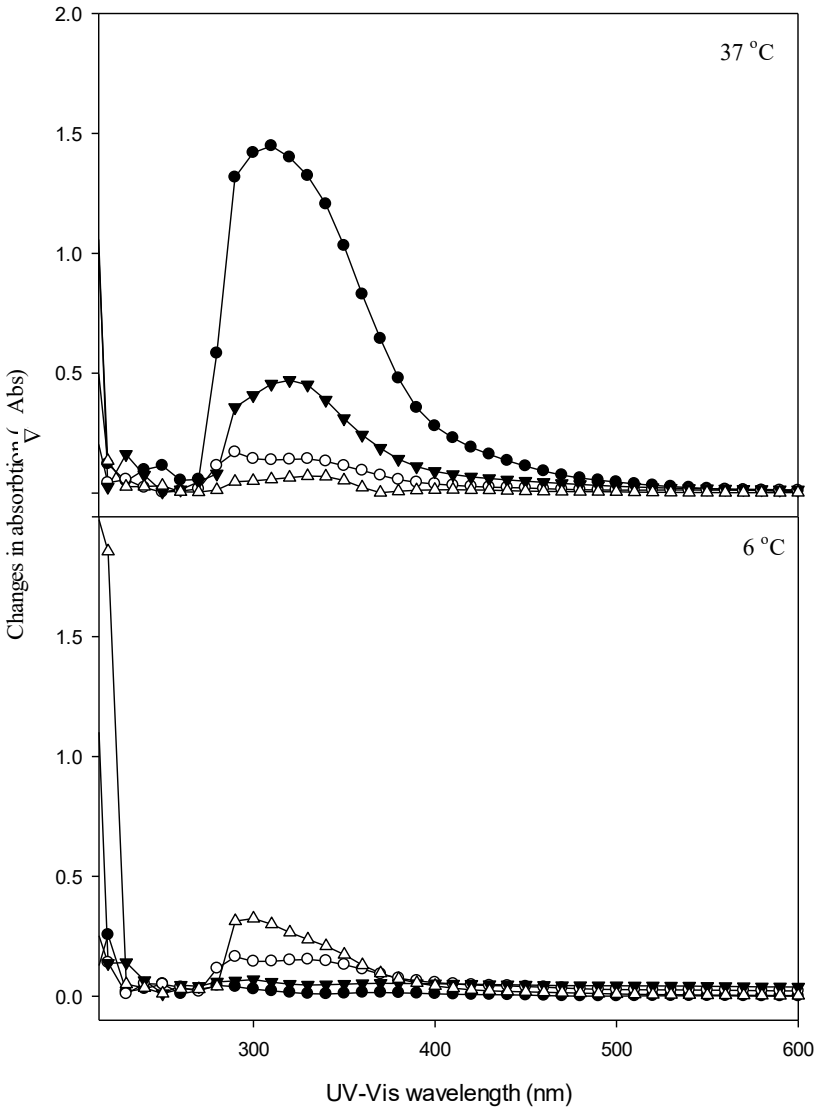

Figure 2.5. UV−vis absorbance difference for COS (●), acetylated COS (○), Gal-COS (▼), and acetylated Gal-COS (Δ) after 7 days storage with 2 storage temperatures 37°C (upper panel) and 6 °C (lower panel) at water activities 0.55.

2.4 Discussion

This study demonstrates that Gal-COS inhibit adhesion of ETEC K88 to porcine red blood cells and may thus be used to inhibit pathogen adhesion *in vivo*. The activity was confirmed by two bioassays, a hemagglutination assay and an ELISA assay targeting K88 fimbriae. Transgalactosylation strongly increased the anti-adhesive activity of COS; moreover, high molecular weight oligosaccharides had higher biological activity while acetylation decreased the effect on pathogen adhesion.

Transgalactosylation of COS with β-galactosidase as catalyst and lactose as galactosyl-donor provides an inexpensive approach to synthesize oligosaccharides that contain GlcNAc or glucosamine and exhibit biological activity (Chen & Gänzle, 2017). The production of glucosamine or COS currently employs acid hydrolysis of chitin from crab and shrimp shell or enzymatic hydrolysis of chitosan or chitin using chitosanase or chitinase, respectively (Hossain et al., 2016; Thadathil & Velappan, 2014; L. Liu et al., 2013). COS inhibited adhesion of EPEC (Quintero-Villegas et al., 2013) and ETEC (this study) to eukaryotic cells; however, COS are highly reactive and form UV-absorbing products during storage or in enzymatic reactions. These products interfere with their biological activity. Previous study demonstrated the formation of UV-absorbing compounds from COS were likely because of the occurrence of Maillard reactions (Hrynets, Ndagijimana, & Betti, 2015). The UV-absorbing compounds formed in enzymatic reactions might also because of Maillard reactions. Cation exchange and size exclusion purification excluded the effects of UV-absorbing compounds from non-enzymatic browning on hemagglutination assay. ELISA assay confirmed the inhibition of ETEC adhesion by Gal-COS. Moreover, Gal-COS with a DP ranging from 8−12

exhibited the highest activity; these compounds have a lower reactivity in the Maillard reaction when compared to short-chain COS.

Fimbriae mediated pathogen−host interactions are highly specific for host species, body site, and in several cases, also the age of the animals (Shoaf-Sweeney & Hutkins, 2008). For example, host adaptation of *Salmonella enterica* Typhimurium was related to allelic variations in the glycan binding domain of FimH (Yue et al., 2015). Likewise, host specificity of ETEC relates to the fimbriae that mediate adhesion. ETEC expressing class 1b and class 5 fimbria cause childhood diarrhea while ETEC with fimbria F4 (K88) cause disease in animals (Mortezaei et al., 2015; Xia et al., 2015). Porcine aminopeptidase N, a membrane bound glycoprotein, was recently identified as receptor for F4 fimbriae. Binding of F4 fimbriae to aminopeptidase N was dependent on decoration of the protein with sialic acid (Melkebeek et al., 2012). ETEC K88 fimbriae bind to GlcNAc, N-acetyl-galactosamine, N-acetylmannosamine, and D-galactosamine (Jin & Zhao, 2000) and β-linked oligosaccharides consisting of GlcNAc and galactose residues at the non-reducing end were suggested to be essential for adherence of K88 fimbriae to host cells (Grange, Mouricout, Levery, Francis, & Erickson, 2002; Jin & Zhao, 2000; Moonens et al., 2015; Sarabia-Sainz, Ramos-Clamont, del Carmen Candia-Plata, Ma Maria, & Vazquez-Moreno, 2009). In agreement with the GlcNAc and galactose-containing target glycans of K88 fimbriae, the effect of Gal-COS containing β-linked galactosyl and GlcNAc or glucosamine residues on ETEC K88 attachment to porcine red blood cells was stronger when compared to nongalactosylated COS (Table 2.2). However, acetylation to match the acetylation in porcine glycans recognized by K88 fimbriae reduced activity (Table 2.5).

ETEC induced diarrhea in suckling and post-weaning piglets cause significant economic losses in swine production. ETEC initiate host colonization through receptor−fimbriae interaction; diarrhea is caused by heat-labile or heat-stable enterotoxins secreted after colonization (Jin & Zhao, 2000). ETEC with fimbriae K88 (F4) and F18 infect suckling and post-weaning piglets, respectively (Moonens et al., 2015; Xia et al., 2015). Casein glycomacropetide, a glycoprotein obtained from cheese processing, reduced K88 attachment to swine epithelia *in vivo* and *in vitro* (Gustavo Hermes et al., 2013). Exopolysaccharides extracted form *Lactobacillus reuteri* also reduced ETEC K88 adherence *in vitro* and *in vivo* (Wang et al., 2010; Chen et al., 2014). Other food- or feed-derived glycans including wheat bran, galactomannan, or phenolic compounds from locust bean, or glycopeptides derived from ovomucin interfered with adhesion of ETEC K88 (Gonzalez-Ortiz et al., 2014), however, the active compounds have not been identified. Bioassay-guided separation and fractionation of Gal-COS as performed in this study allowed one to relate molecular weight and degree of acetylation of Gal-COS to their anti-adhesion activities. Moreover, the use of two complementary bioassays confirmed that the oligosaccharides target K88 fimbriae (Table 2.2 and Figure 2.3). The activity of Gal-COS decreased with decreasing molecular weight and acetylation decreased activity. This result matches observations on the antiadhesive activity of COS against EPEC, which also decreased with reduced DP and increased acetylation (Quintero-Villegas et al., 2013). Remarkably, the galactosylation of COS increased anti-adhesion activity when compared to COS. Gal-GOS are promising alternatives to antibiotic therapy for the swine industry. Bacterial pathogens other than ETEC K88 also

recognize β-linked galactosylated oligosaccharides, and Gal-COS may thus target additional human or animal pathogens (Shoaf-Sweeney & Hutkins, 2008).

The concept of using oligosaccharides for therapeutic applications is increasingly validated by *in vivo* studies (Chen et al., 2014). Oligosaccharide synthesis with glycosyl hydrolases requires high concentrations of both donor and acceptor sugars and generates oligosaccharide mixtures differing in the site of glycosylation, the linkage type, and the degree of polymerization (Black et al., 2014; Chen et al., 2014; this paper). In addition to β-galactosidases, oligosaccharides were produced with other retaining glycosyl hydrolases including α-galactosidase, fucosidase, trans-sialidase, and *N*-acetyl-glucosaminidase (Wang, Black, Curtis, & Gänzle, 2014; Holck et al., 2014; Chen & Gänzle, 2017). Metabolic engineering of microorganisms to synthesize oligosaccharides with glycosyl transferases provides an alternative approach for oligosaccharide production. Fucosyllactose has recently become commercially available through fermentation of fucose and lactose with *E. coli* harboring fucosyltransferase (Weichert et al., 2013). Oligosaccharide synthesis with intracellular glycosyl transferases generates lower yields when compared to glycosyl hydrolases but produces defined oligosaccharides (Crout & Vic, 1998). Site-directed mutagenesis can improve the transferase activity and the preference for the linkage type of retaining glycosyl hydrolases (Jorgensen, Hansen, & Stougaard, 2001; Meng et al., 2015; Zakariassen, Hansen, Joranli, Eijsink, & Sorlie, 2011). Relating the selectivity of oligosaccharide synthesis to the specificity of glycan recognition of pathogens, however, makes the use of oligosaccharide mixtures preferential as these may target multiple pathogens. Human

milk oligosaccharides are a mixture of more than 150 oligosaccharides targeting adhesion of multiple pathogens (Coppa et al., 2006).

In conclusion, this study demonstrated that partially purified galactosylated chitosan-oligosaccharides strongly interfered with adhesion of ETEC K88 to porcine erythrocytes. In addition, the influence of the molecular weight and degree of acetylation was elucidated, which is necessary to address the low solubility of chitin oligosaccharides and the high reactivity of chitosan oligosaccharides. Acetylation of COS or Gal-COS increases stability but decreases antiadhesive activity.

Acknowledgements

The Alberta Livestock and Meat Agency is acknowledged for funding (Grant No. 2015B009R).

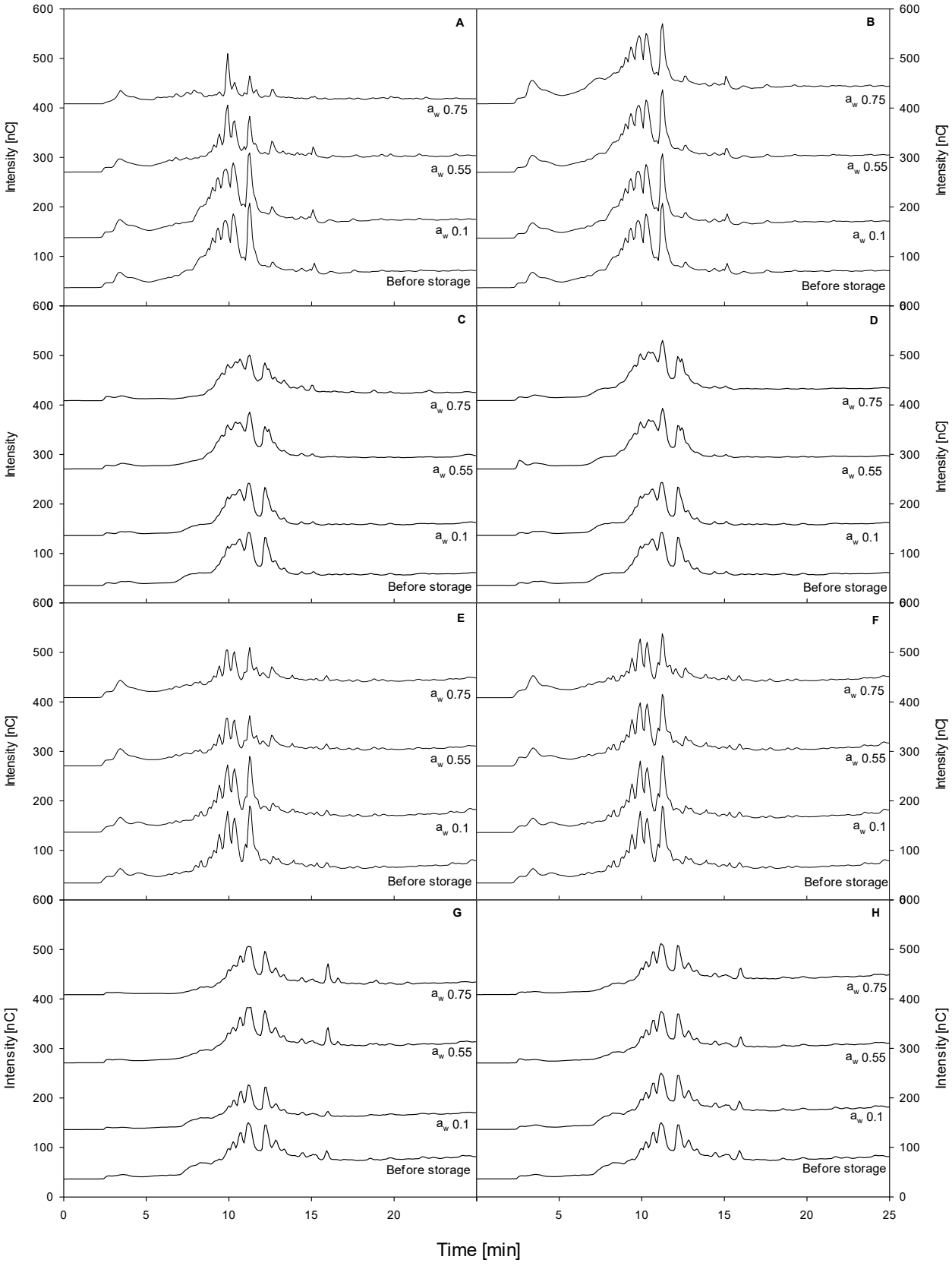
A
B
C
D
E
F
G
H
a_w 0.75
a_w 0.55
a_w 0.1
Before storage
Intensity [nC]
Intensity
Time [min]

Figure S2.1. HPAEC -PAD chromatography of COS before and after storage under 37 ºC (A) and 6 ºC (B) with 3 different water activities; Acetylated COS before and after storage under 37 ºC (C) and 6 ºC (D) with 3 different water activities; Gal-COS before and after storage under 37 ºC (E) and 6 ºC (F) with 3 different water activities ;Acetylated Gal-COS before and after storage under 37 ºC (G) and 6 ºC (H) with 3 different water activities. All chromatography are based on three independent reaction

COS, Gal-COS, acetylated COS and acetylated Gal-COS before and after storage test were analyzed by HPAEC-PAD (Fig. S2.1). Due to the lack of standards, only peaks at same retention time were compared as an indication of changes in oligosacchaieds composition during storage. No significant changes on chromatographs were observed for all the tested oligosaccharides at all conditions with the expectation of COS stored at 37 ºC (Fig S2.1A). A different separate pattern of oligosaccharides was observed in COS when stored at 37 ºC.

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Chapter 3. Structure and function relationships of the binding of β- and α-galactosylated oligosaccharides to K88 fimbriae of enterotoxigenic *Escherichia coli*

3.1. 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 vitro* and *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, Autran, Eckmann, & Bode, 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, Cervantes, Ramos, Chavez-Munguia, & Newburg, 2003). GOS may have comparable activity for specific pathogens; for example, GOS reduced adherence of *Cronobacter sakazakii* to HEp-2 human cells (Quintero-Villegas, Wittke, & Hutkins, 2014).

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, 2014) and levansucrase (Tieking, Kuhnl, & 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.

3.2 Materials and Methods

3.2.1. 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 erythromycin (mM17) and incubated anaerobically at 30ºC for 48h. *L. lactis* MG 1363 harboring the α-galactosidase of *Lb. reuteri* 100-23, Aga23 (Wang et al., 2014) 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., 2014). 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, Lee, Sørensen, & Gänzle, 2011; Wang et al., 2014).

3.2.2 Overexpression and purification of levansucrase

Escherichia coli JM109 DE3 (pLEV1) harboring *levS* coding for levansucrase of *Lb.sanfranciscensis* TMW 1392 (Tieking et al., 2005) was streaked onto Luria-Bertani (LB) plate containing 100mg/L 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).

3.2.3 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 lactose and 180g/L COS, respectively. Reaction were started by addition of 20% (v/v) crude cellular extract to achieve β-galactosidase activity of 28– 32µmol/(min x mg protein), 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.

3.2.4 Synthesis of oligosaccharides with α-galactosidase

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

3.2.5 Synthesis of oligosaccharides with levansucrase

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

3.2.6 Oligosaccharides purification and fractionation

β-Galactosylated COS (β-GalCOS), α-galactosylated COS (α-GalCOS) and fructosylated COS (Fruc-COS) were purified by cation exchange chromatography (Yan et al., 2017). In brief, reaction mixtures were adjusted to a total carbohydrate concentration of 10g/L with 0.1% trifluoroacetic acid (TFA) and loaded on pre-conditioned SCX columns (55μm,

70A, Phenomenex, Torrance, CA, USA). Columns were washed with 0.1% TFA, charged oligosaccharides were eluted with 0.2% trimethylamine and lyophilized.

The lyophilized fractions as well as the uncharged βGOS and αGOS were further fractionated by size exclusion chromatography (SEC) (Yan et al., 2017). Five fractions were collected for each sample and concentrated by lyophilization. Samples were discarded when visible browning occurred during drying. Oligosaccharides were re-dissolved in phosphate–buffer saline (PBS) (pH 7.2) to a concentration of 10g/L.

3.2.7 High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD).

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

3.2.8 Hemagglutination assay to determine the effect of oligosaccharides on ETEC K88 adhesion to porcine erythrocytes.

The hemagglutination assay was performed with the porcine ETEC ECL 13795 expressing $K88_{ac}$ fimbriae (Yan et al., 2017). *E. coli* were cultivated on Minca agar overnight and washed with 1mL phosphate buffer saline (PBS). *E.coli* cell suspensions with an optical density (600 nm) of 70 were diluted 2-fold microtiter plates (Corning, Fisher Scientific, CA). The same volume of PBS or oligosaccharide fractions was added. Oligosaccharides were used at 10g/L and in two-fold serial dilutions to the exhaustion of biological activity or to 0.1g/L. Erythrocytes were prepared by 2-fold dilution of a 10% suspension of porcine red blood cells (Innovative Research, Novi, MI, USA). The plate

was incubated at 4ºC overnight before visually inspection of hemagglutination. The lowest sugar concentration corresponding to four fold difference to the control was noted.

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

ETEC K88 fimbriae were extracted as described (Peng, Ling, Ning, & Deng, 2014) with some modifications. ETEC were cultivated in tryptic soy broth medium for 16h at 37ºC. Cells were collected by centrifugation, washed 3 times with PBS (pH 7.4), and incubated at 60ºC for 30min. Cell debris was removed by centrifugation and K88 fimbriae were precipitated with 60% (w/v) ammonium sulfate. Precipitates were collected by centrifugation, re-solubilized in PBS and dialyzed against PBS overnight. SDS-PAGE and Western blot verified purification. The antibody used for western blot was *E.coli* K88A antibody (Biorad, USA) and a goat anti-mouse IgG (H+L) secondary antibody conjugated to horseradish peroxidase (Invitrogen, Fisher Scientific, CA). The protein concentration of the solution containing K88 fimbriae was determined with Bradford assay and adjusted to 0.37 ± 0.02mg/L prior to ELISA assay as described below.

3.2.10 Enzyme-linked immunosorbent assay (ELISA) to determine the effect of oligosaccharides on adhesion of ETEC K88 cells or K88 fimbriae to porcine erythrocytes.

The ability of oligosaccharides to inhibit adhesion of *E.coli* K88 cells or K88 fimbriae to porcine erythrocytes was also assayed by ELISA. A 96-well high bind microtiter plate (Corning, Fisher Scientific, CA) was coated overnight with 100µL of 5% porcine red blood cells (Innovative Research, Novi, MI, USA) and blocked with 200µL of 3% bovine serum albumin for 1h at 4ºC. Oligosaccharides were re-dissolved in PBS to a concentration of 10g/L. The same volume of ETEC with OD_{600nm} of 1.0, or of K88

fimbriae with a protein concentration of 0.1mg/L were mixed with PBS or oligosaccharides; 100μL of the mixture was added into plates and incubated for 1h 4ºC. Red blood cells without addition of ETEC or K88 fimbriae, red blood cells with addition of ETEC or K88 fimbriae only, and wells without red blood cells but addition of ETEC or K88 fimbriae served as controls. The wells were then treated with mouse anti *E.coli* K88A antibody (Biorad, USA) at a dilution of 1:2000. After 1h incubation, wells were treated with a goat anti-mouse IgG (H+L) secondary antibody conjugated to horseradish peroxidase (Invitrogen, Fisher Scientific, CA) for 1h. After addition of TMB-ELISA substrates and incubation for 30min, the reaction was stopped by 2M sulfuric acid, and the absorbance at 450 nm was measured on a microplate reader (Thermo Scientific, CA).

3.2.11 Statistical analysis

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

3.3. Results

3.3.1 Enzymatic synthesis of oligosaccharide libraries with β-galactosidase, α-galactosidase, and levansucrase

The formation of oligosaccharides through acceptor reactions with β-galactosidase (Fig 3.1A and Fig 3.1C), α-galactosidase (Fig 3.1B and Fig 3.1D) and levansucrase (Fig 3.2) were analyzed by HPAEC-PAD. Chromatograms indicated that COS (data not shown) and melibiose (Figure 3.1C) were suitable acceptors for β-galactosidase; COS (data not shown) and lactose (Fig 3.1D) were suitable acceptors for α-galactosidase (Figure 3.1) (Black et al., 2014; Lu, Xiao, Li, Li, & Wang, 2009; Wang et al., 2014). In analogy, the

pattern of oligosaccharides produced by levansucrase after addition of COS as acceptor suggested formation of fructosylated COS (Fig.3.2A). Enzymatic reactions with COS as acceptor were purified by ion exchange chromatography to remove uncharged reaction products, and to remove contaminants (Yan et al., 2017).

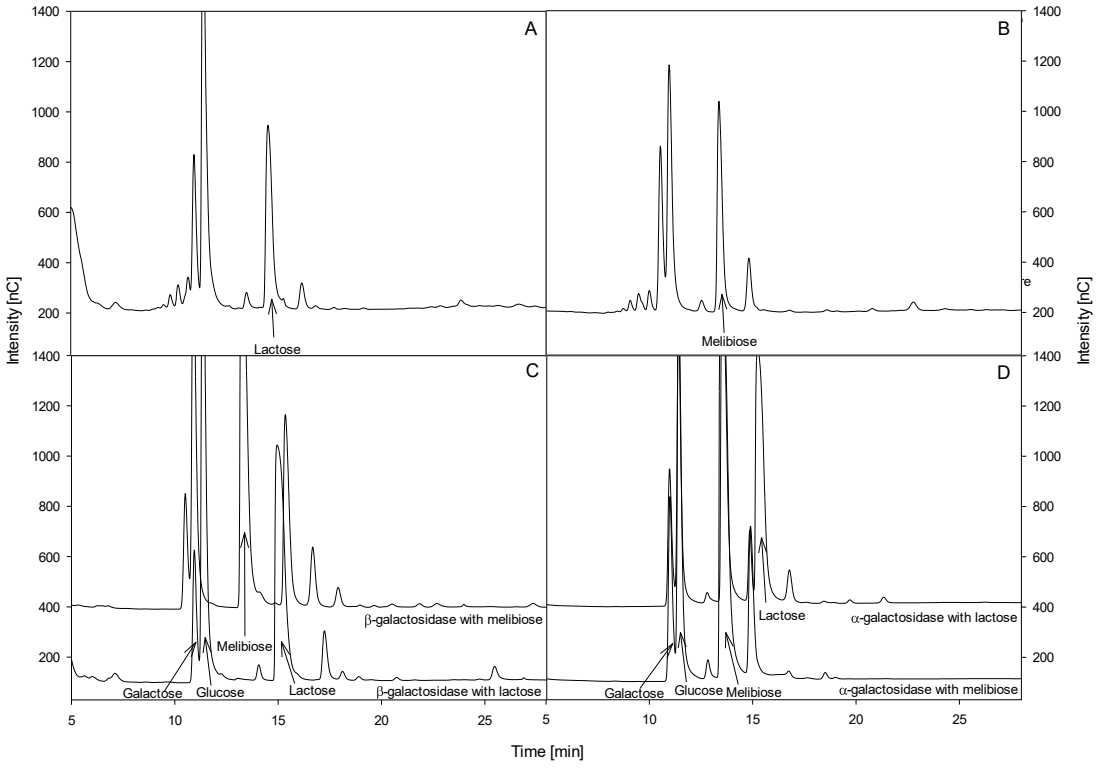

Figure 3.1. HPAEC-PAD diagrams of oligosaccharides synthesized through the acceptor reactions of α-galactosidase and β-galactosidase. Melibiose and lactose were used as glycosyl donors in all reactions with α-galactosidase and β-galactosidase, respectively. **Panel A.** Separation of the acceptor reactions with β-galactosidase, lactose, and COS; **Panel B.** Separation of the acceptor reactions with α-galactosidase, melibiose, and COS; **Panel C.** Separation of the acceptor reactions with β-galactosidase and lactose or with β-galactosidase , lactose, and melibiose; **Panel D.** Separation of the acceptor reactions with α-galactosidase and melibiose or with α-galactosidase, melibiose, and lactose. Lactose, melibiose, and COS were used as external standards

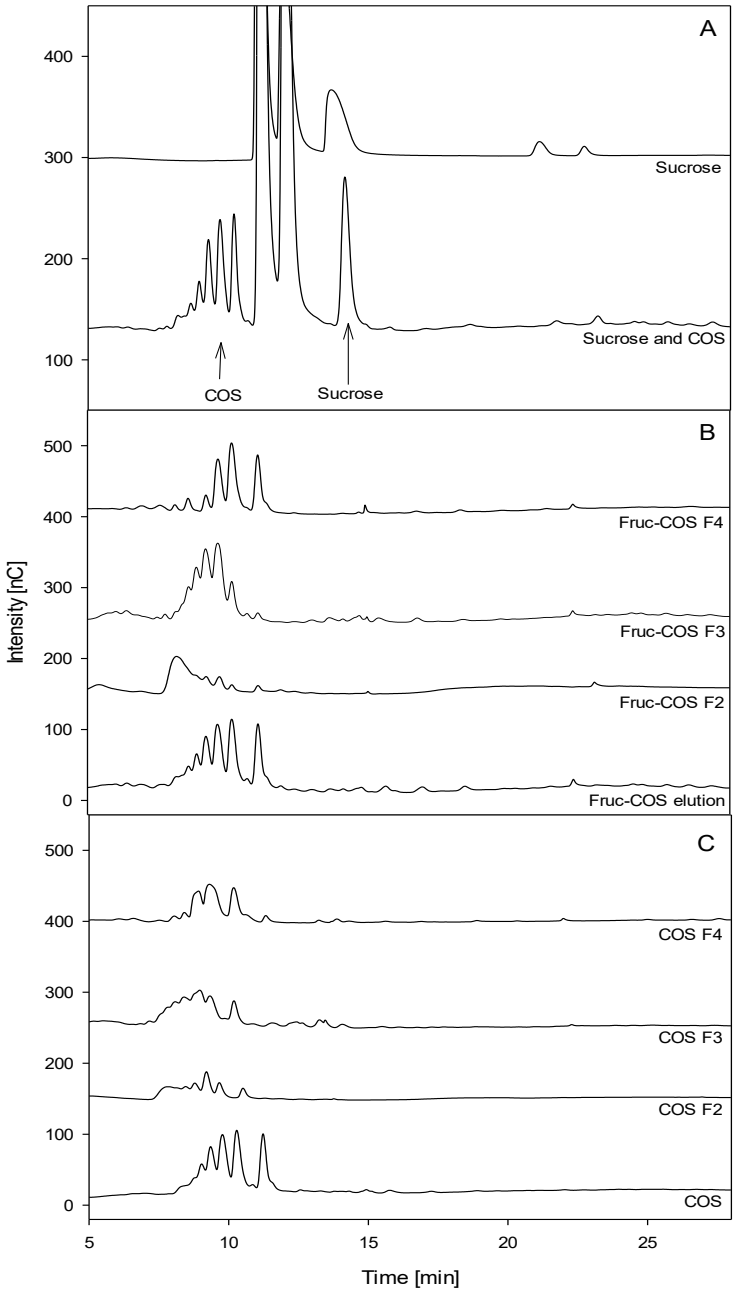

Figure 3.2. HPAEC-PAD diagrams of oligosaccharides synthesized through the acceptor reaction of levansucrase and oligosaccharides fractions collected with SEC chromatography. **Panel A**. levansucrase with sucrose alone or with sucrose and COS; **Panel B**. Fruc-COS fractions; **Panel C.** COS fractions. Sucrose and COS were used as external standards

3.3.2 Effect of oligosaccharides on adhesion of E. coli K88 to porcine erythrocytes.

Oligosaccharide fractions obtained by cation exchange chromatography were tested for anti-adhesion activity against ETEC K88 with hemagglutination assay and their activity was compared to the corresponding COS fractions (Table 3.1). COS separated by cation exchange chromatography inhibited ETEC K88 adhesion at 6.7g/L (Yan et al., 2017 and data not shown). Glycosylation of COS to obtain β-GalCOS, α-GalCOS or β-FrucCOS enhanced their ability to prevent ETEC K88 binding to erythrocytes about five-fold (Table 3.1).

Table 3.1. 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)
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, M_W 1500-2500Da; F3, M_W 900-1500Da; F4, M_W 500-1200Da; and F5, less than 500Da.

Table 3.2. 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)
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

3.3.3 Fractionation of oligosaccharides by size exclusion chromatography

β-GalCOS, α-GalCOS and β-FrucCOS were further fractionated by SEC. The molecular weight cut off was F2, M_W 1500-2500Da; F3, M_W 900-1500Da; F4, M_W 500-1200Da; and F5, less than 500Da. The formation of fructosylated COS was further confirmed with HPAEC-PAD analysis of fractions obtained after cation exchange separation and SEC fractionation (Fig 3.2B). Analysis of oligosaccharide fractions derived from levansucrase reactions with sucrose and COS as acceptor by HPAEC-PAD identified oligosaccharide peaks that were absent in COS, or in reactions without COS (Fig 3.2B and 3.2C), indicating formation of fructosylated COS.

β-GalCOS F2 had strongest anti-adherence activity followed by α-GalCOS F2 and β-FrucCOS F2 (Table 3.1). The anti-adhesion activity decreased with decreasing molecular weight (Table 3.1). All fractions containing glycosylated COS were more active when compared to the corresponding fractions obtained with COS. β-Galactosylated COS consistently exhibited the highest activity (Table 3.1).

GOS with mixed β- or α-linkages were also fractionated by SEC. Due to the low yield of 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 3.3) and evaluated with respect to their ability to prevent ETEC adhesion (Table 3.2). Different pattern of peaks were observed which indicated the formation of various oligosaccharides (Fig 3.3). 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 3.2). 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 3.2). 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 3.2).

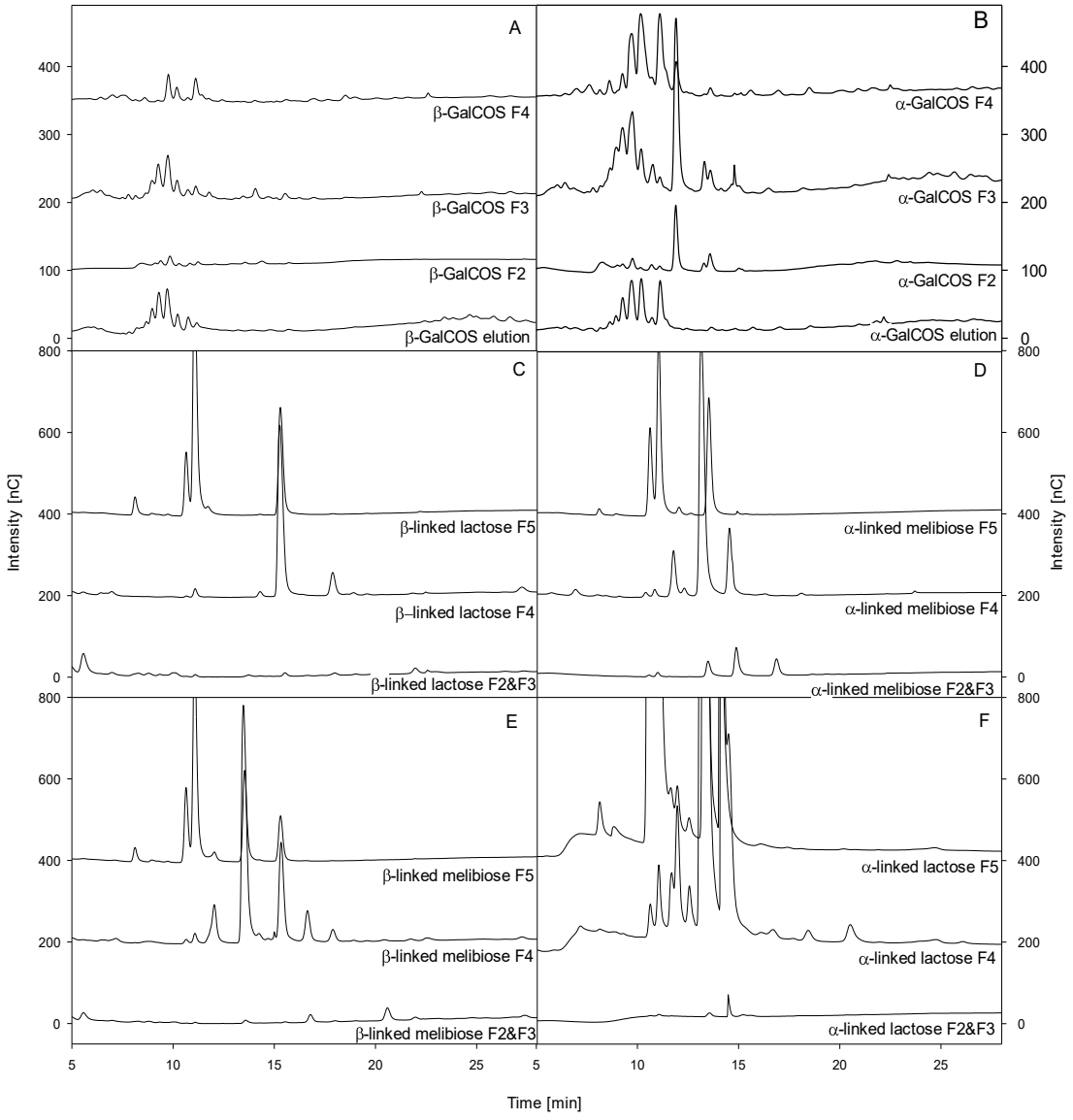

Figure 3.3. HPAEC-PAD diagrams of galactosylated oligosaccharides fractions collected with SEC chromatography. Panel A. β-GalCOS; Panel B: α-GalCOS; Panel C. β-galactosylated lactose; Panel D: α-galactosylated melibiose; Panel E. β-galactosylated melibiose; Panel F. α-galactosylated lactose.

3.3.4 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 3.4). 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.3.4). Different from hemagglutination assay, the effect of α-GalCOS and β-FrucCOS was largely independent of the molecular weight (Fig 3.4). The anti-adhesive activity of non-charged oligosaccharides against ETEC adhesion is shown in Figure 3.5. 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 3.5). Other oligosaccharide fractions and all fractions containing oligosaccharides with DP < 6 (F4 and F5) had only weak anti-adhesive activity (Fig. 3.5)

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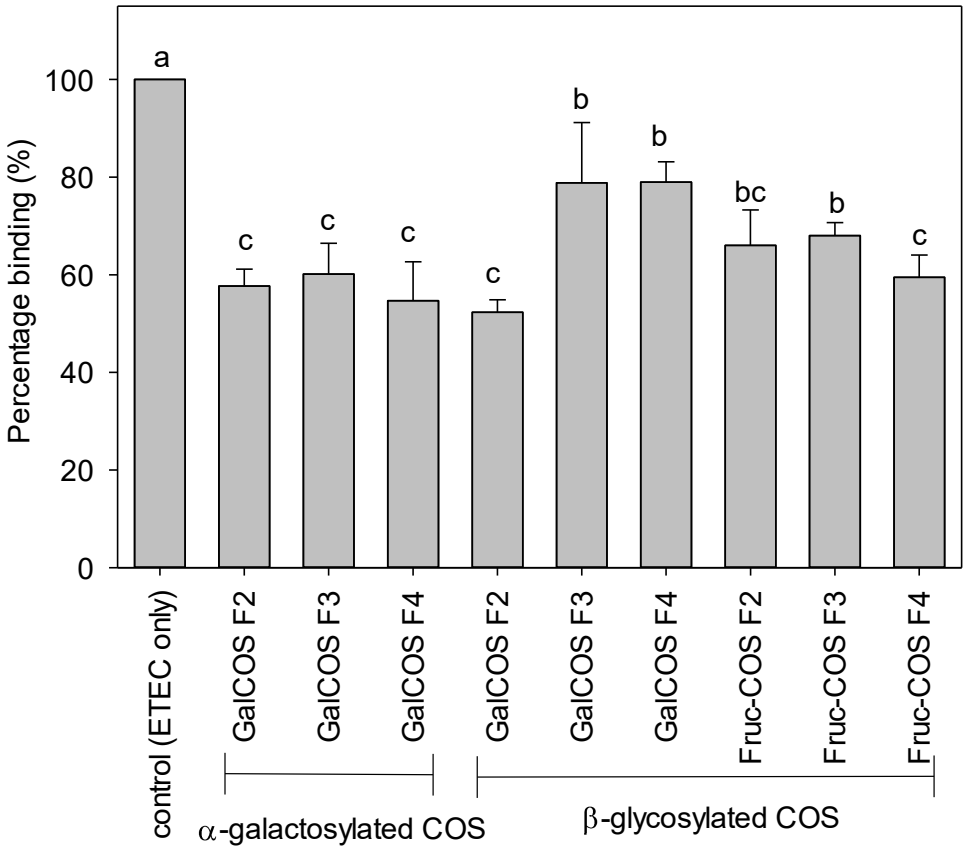

Figure 3.4. Reduction of adherence of ETEC K88 ECL 13975 (O149, virotype STb:LT:EAST1:F4) to porcine erythrocytes by galactosylated or fructosylated chitosan oligosaccharides. The sugar concentration was 10g/L. ELISA was performed with ETEC cells and K88-specific antibodies. Results are shown as means ± standard deviation of three independent assays. Bars that do not share a common superscript are significantly different ($P< 0.05$).

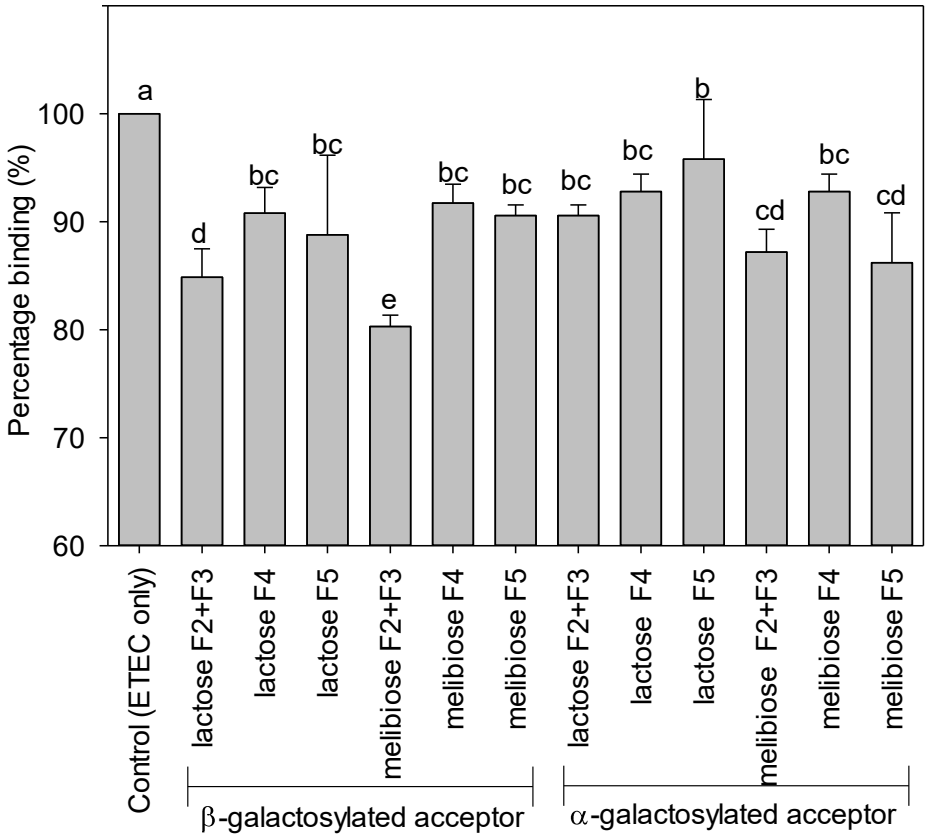

Figure 3.5. Reduction of adherence of ETEC K88 ECL 13975 (O149, virotype STb:LT:EAST1:F4) to porcine erythrocytes by α-, β-, or α/β-linked galacto-oligosaccharides as measured by ELISA. The carbohydrate concentration was adjusted to 10g/L. ELISA was performed with ETEC cells and K88-specific antibodies. Results are shown as means ± standard deviation of three independent assays. Bars that do not share a common superscript are significantly different ($P< 0.05$).

3.3.5. 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 3.6A) and SDS-PAGE (Fig 3.6B) 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 3.7). 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 3.7). Uncharged oligosaccharides had lower anti-adherence activity; β-galactosylated melibiose (F2 + F3) blocked adhesion of K88 fimbriae better than the corresponding fraction with α-galactosylated melibiose (Figure 3.7).

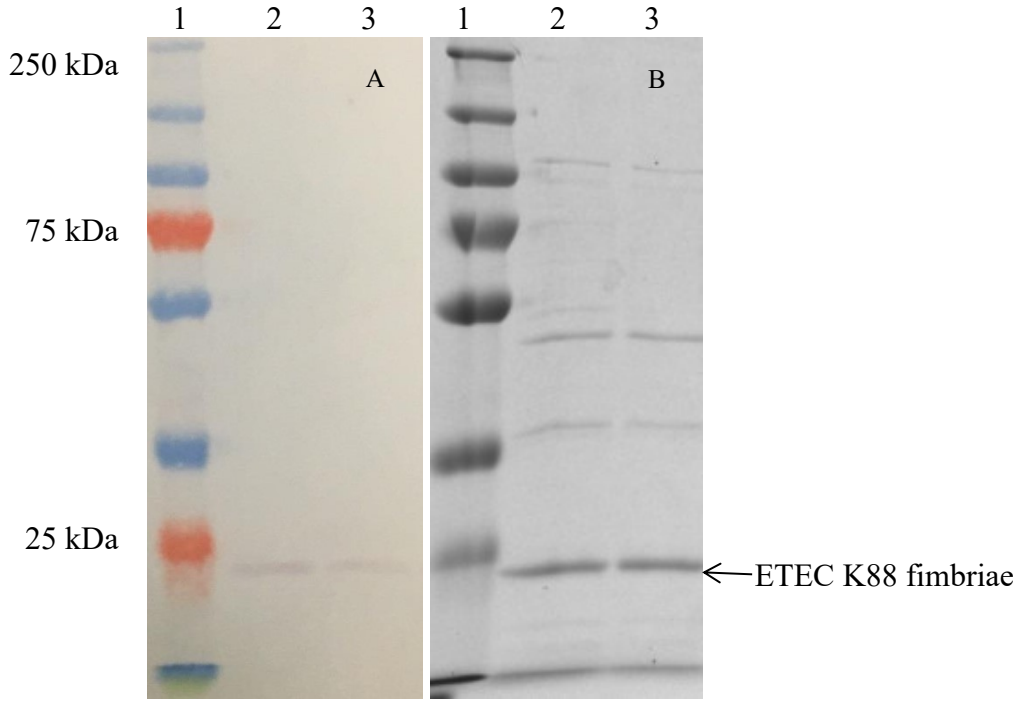

Figure 3.6. Panel A: Western blot of K88 fimbriae purified from ETEC ECL 13975. The molecular weight marker was applied on lane 1 and two independent extractions were applied on lane 2 and 3. Panel B: SDS-page of K88 fimbriae purified form ETEC ECL 13975. The molecular weight marker was applied on lane 1 and two independent extractions were applied on lane 2 and 3.

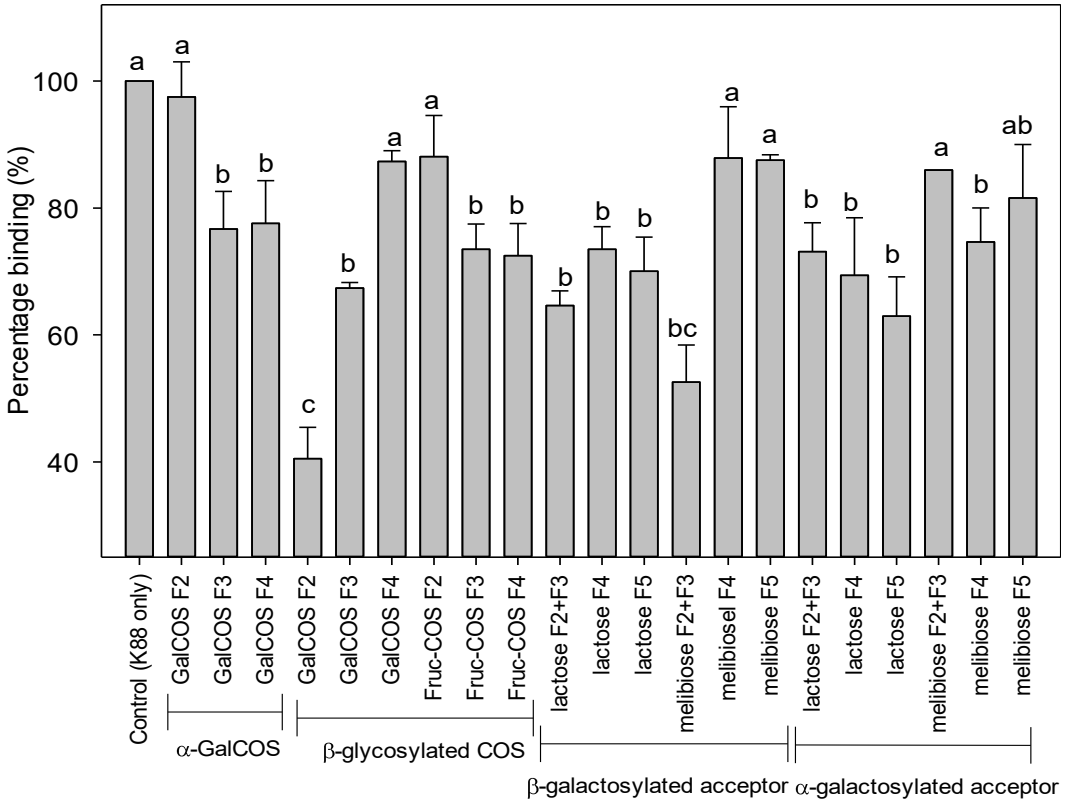

Figure 3.7. Reduction of binding of $K88_{ac}$ fimbriae purified from ETEC ECL 13975 (O149, virotype STb:LT:EAST1:F4) to porcine erythrocytes by galactosylated or fructosylated chitosan oligosaccharides, or by α-, β-, or α/β-linked galacto-oligosaccharides. The carbohydrate concentration was 10g/L. ELISA 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 ($P< 0.05$).

3.4. 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 3.8. Lactose and melibiose are known to be suitable acceptor carbohydrates for β- as well as α-galactosidases (Lu et al., 2009; Wang et al., 2014) but the synthesis of fructosylated COS with levansucrase was not previously reported. The synthesis of β-GalCOS by β-galactosidase was previously confirmed by MS/MS and LC-MS/MS analysis of products (Black et al., 2014; Yan et al., 2017). The present study used cation-exchange separation, SEC fractionation, HPACE-PAD analysis and the evaluation of the biological activity as indication the formation of α-GalCOS and β-FrucCOS formation. The structural analysis of α-GalCOS and β-FrucCOS, however, remains subject to future investigations. Taken together, the present study demonstrates that the use of retaining glycosyl hydrolases (Gänzle, 2012) with reciprocal acceptor / donor pairs is a suitable tool to synthesize oligosaccharide libraries for studies aiming to elucidate structure/function relationships.

Hemagglutination is a relatively simple assay to quantify ETEC binding to host cells (Coddens et al., 2011; Moonens et al., 2015; Sun, Gänzle, & Wu, 2017), and results obtained with the hemagglutination assay have been verified *in vivo* (Chen et al., 2014).

The assay is sensitive, however, to the presence of salts introduced by oligosaccharide purification and concentration, necessitating the use of buffers composed of volatile components for oligosaccharide fractionation to allow removal of buffer components by freeze-drying (Sun et al., 2017; Yan et al., 2017). Moreover, COS are reducing carbohydrates containing primary amines; the formation of Maillard products that interfere with hemagglutination is observed even in the temperature range of 20 – 50°C (Hrynets, Ndagijimana, & Betti, 2015; Yan et al., 2017). The present study therefore complemented the hemagglutination assay with the quantification of the binding of ETEC and purified K88 fimbriae to porcine erythrocytes by ELISA. The three assays provided consistent results with exception of the determination of the activity of α-GalCOS and β-FrucCOS (Table 3.1, Fig. 3.4 and Fig. 3.7). The discrepancies may relate to non-K88 binding molecules, or to Maillard reaction products that were present in low molecular weight fractions prepared from α-GalCOS and β-FrucCOS. Owing to longer incubation times for the enzymatic reaction, discoloration caused by the non-enzymatic browning reaction was more pronounced in reactions with α-galactosidase and levansucrase. Fractionation with cation exchange chromatography and SEC to obtain high molecular weight oligosaccharide fractions, however, removed interferences. The use of multiple assays to determine pathogen binding including the use of purified $K88_{ac}$ fimbriae, however, demonstrated that the activity of oligosaccharide fractions directly relates to binding to the lectin domain of K88 fimbriae (Moonens et al., 2015).

K88 fimbriae of *E. coli* occur in three variants, $K88_{ab}$, $K88_{ac}$, and $K88_{ad}$, which have overlapping yet distinct receptor specificities (Coddens et al., 2011; Guinée & Jansen, 1979; Moonens et al., 2015). β-Galactosylated melibiose and lactose with DP > 6, β-

GalCOS with DP > 8 prevented binding of ETEC K88 as well as purified $K88_{ac}$ fimbriae to porcine erythrocytes (Table 3.2, Fig. 3.5 and Fig. 3.7). Fructosylation of COS or α-galactosylation of lactose and melibiose did not greatly enhance increase the anti-adhesive activity of acceptor carbohydrates. β-Linked galactosyl moieties were thus promoting anti-adhesive activity; the presence of multiple *N*-glucosamine moieties at the reducing end also increased activity. Our results conform to prior studies demonstrating that β-linked galactosidase and /or *N*-acetylglucosamine residues are important for receptor binding of all three variants of K88 fimbriae, including the most common type $K88_{ac}$ (Coddens et al., 2011; Grange, Mouricout, Levery, Francis, & Erickson, 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 synthesized 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 favors bacterial adherence with multiple fimbriae per cell (Nizet et al., 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 & 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 et al., 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 et al., 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, Casettari, Ciandrini, Illum, & Baffone, 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.

Acknowledgements.

The Alberta Livestock and Meat Agency is acknowledged for funding (2014R045R). Michael Gänzle acknowledges support from by the Canada Research Chairs Program.

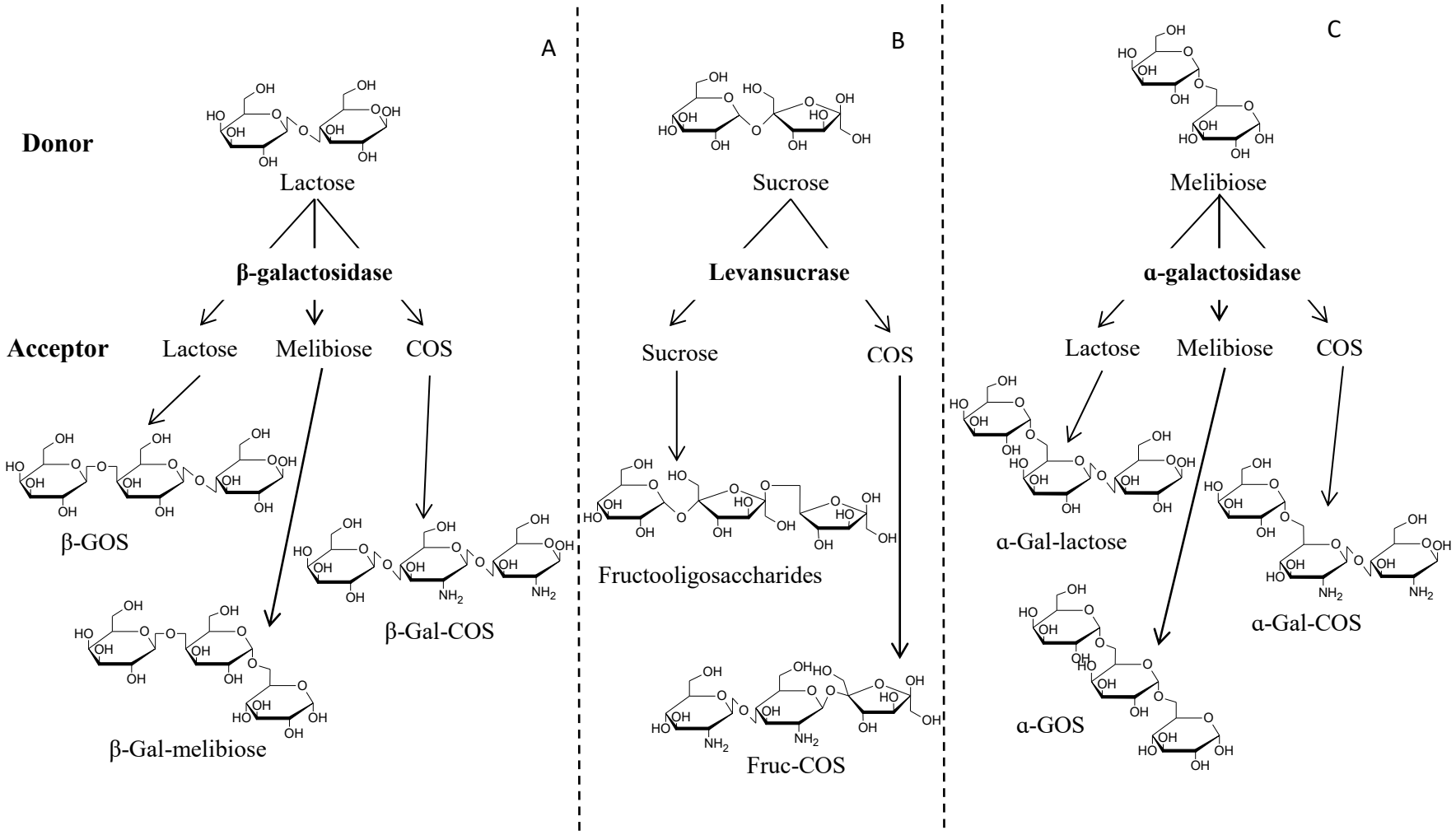
A
B
C
Donor
Lactose
β-galactosidase
Acceptor
Lactose
Melibiose
COS
β-GOS
β-Gal-COS
β-Gal-melibiose
Sucrose
Levansucrase
Sucrose
COS
Fructooligosaccharides
Fruc-COS
Melibiose
α-galactosidase
Lactose
Melibiose
COS
α-Gal-lactose
α-Gal-COS
α-GOS

Figure 3.8. 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).

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Chapter 4. Scale-up of the production of galactosylated chitosan-oligosaccharides for animal trials

4.1 Introduction

Human milk oligosaccharides (HMOs) are a group of complex oligosaccharides in human milk and more than 150 different types have been identified (Bode et al., 2016). HMOs promoted the growth of bifidobacteria, such as *Bifidobacterium longum* ssp. *infantis, B. breve* and *B. longum* (Sela & Mills, 2010; Lewis et al., 2015; Katayama, 2016); suppressed inflammation (Newburg, Ko, Leone, & Nanthakumar, 2016; Yu, Nanthakumar, & Newburg, 2016) and modulated the immune system (Comstock et al., 2017). Moreover, HMOs prevented pathogen infections by blocking the adherence of pathogens such as *Escherichia coli*, *Campylobacter jejuni,* and *Salmonella* to host cell surface (Manthey, Autran, Eckmann, & Bode, 2014; Morrow et al., 2004; Coppa et al., 2006).

Oligosaccharides from other sources, such as GOS, xylooligosaccharides, fructo-oligosaccharides, isomalto-oligosaccharides, chito-oligosaccharides and other hetero-oligosaccharides, exhibited similar beneficial effects as HMOs (Chen & Gänzle, 2017; Gänzle, 2012; Mano et al., 2018). Those oligosaccharides could modulate gut microbiota composition, promote short-chain fatty acid production, and prevent pathogen adhesion (Davis, Martinez, Walter, Goin, & Hutkins, 2011; L. Wang et al., 2017; Shoaf, Mulvey, Armstrong, & Hutkins, 2006).

Oligosaccharides can be produced by hydrolysis of polysaccharides. For example, fructo-oligosaccharides and xylo-oligosaccharides are produced from chemical or enzymatic hydrolysis of inulin and xylan, respectively (Mano et al., 2018; Moura, Macagnan, & Silva, 2015).

Another alternative way for oligosaccharides production is through transglycosylation reactions by retaining glycosyl hydrolase with high concentration of starting sugars, such as lactose and sucrose (Gänzle, 2012). β-Galactosidase synthesizes GOS from lactose. The degree of polymerization and linkage types of synthesized GOS are highly depended on the source of β-galactosidase (Chen & Gänzle, 2017; Gänzle, 2012). β-Galactosidase from *Kluvyeromyces lactis,* is a widely used enzyme in industries and forms β-(1→6) linked GOS with the degree of polymerization ranging from 2-4 (Chen & Gänzle, 2017). While other β-galactosidase produced GOS with β-(1→3), β-(1→4) or β-(1→6) linkage types and various chain length (Chen & Gänzle, 2017). Genetic modifications were also reported to alter the enzyme specificities. Lu (2015) reported an increased transgalactosylation activity on phenolic compounds after conducting a site-directed mutagenesis on β-galactosidase from *Lactobacillus bulgaricus.* Diverse oligosaccharides can be produced with different retaining glycosyl hydrolases or with different acceptor sugars by the same retaining glycosyl hydrolase. Mannose, fructose, fucose, and chitosan-oligosaccharides (COS) have been reported to be suitable acceptors for β-galactosidase to produce hetero-oligosaccharides (Black et al., 2014; Gänzle, 2012; Yan, Hu, Simpson, & Gänzle, 2017; Chen & Gänzle, 2017).

Synthesized oligosaccharides have been reported to have multiple beneficial effects on host health (Chen & Gänzle, 2017; Gänzle, 2012). Previous studies demonstrated the formation of β-galactosylated COS (β-GalCOS) from lactose and COS with β-galactosidase (Black et al., 2014; Yan et al., 2017). Synthesized β-GalCOS prevented enterotoxigenic *E.coli* (ETEC) K88 adherence to porcine erythrocytes *in vitro* and the anti-adhesive effects increased with increased molecular weight (Yan et al., 2017).

However, the anti-adhesive effects of galactosylated COS against ETEC K88 *in vivo* remain unknown. Therefore, this study aimed to achieve a scale-up production for β-GalCOS to facilitate the animal trials on the anti-adhesive effects of galactosylated COS against ETEC K88. To determine the effects of linkage types on the anti-adhesive activities *in vivo*, two enzymes, β-galactosidase and α-galactosidase, were included for the production of β-GalCOS and α-galactosylated COS (α-GalCOS), respectively. The validation of scale-up production was conducted with three different reaction volumes. Scale-up purification and separation method was developed.

4.2 Materials and Methods

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

Crude cell extracts of β-galactosidase and α-galactosidase were prepared as described in chapter 3. In brief, *Lactococcus lactis* MG 1363 expressing the LacLM type β-galactosidase of *Lactobacillus plantarum* (Schwab, Sørensen, & Gänzle, 2010) were cultivated in M17 broth supplemented with 5% glucose and 5mg/L erythromycin (mM17) and incubated anaerobically at 30ºC overnight. *L. lactis* MG 1363 harbouring the α-galactosidase of *Lb. reuteri* 100-23, Aga23 (Wang, Black, Curtis, & Gänzle, 2014) was cultivated in mM17 with 0.0274% $MnSO_4$ and incubated anaerobically at 37ºC for 24h. Crude cell extracts were obtained by centrifugation after cells disruption as described (Black et al., 2014; Wang et al., 2014). Protein content for both crude cellular extracts was determined with Bradford protein assay. Enzymatic activities of β-galactosidase and α-galactosidase were measured with *o*-nitrophenyl-*β*-galactoside and 4-nitrophenyl-*α*-D-galactopyranoside respectively (Schwab, Lee, Sørensen, & Gänzle, 2011; Wang et al., 2014).

4.2.2. Transgalactosylation reactions scale up

α-/β-Galactosylated oligosaccharides were previously synthesized in a 1mL reaction volume as described in Chapter 3. Briefly, β-galactosylated oligosaccharides were synthesized using 180g/L lactose and 180g/L COS (Glycobio Company, Dalian, P.R. China) at 45ºC for 16h. α-Galactosylated oligosaccharides were produced with 180g/L melibiose and 180g/L COS at 37ºC for 24h. The scale-up reactions were conducted for both enzymes with same concentrations of starting materials and enzyme actives but with different reaction volume, 1mL, 10mL, and 25mL. All the reactions were terminated by addition of perchloric acid to a final concentration of 3.5% (v/v).

4.2.3. Oligosaccharides purification and fractionation

β-GalCOS and α-GalCOS were purified by cation exchange chromatography using CM Sepharose® (Sigma) column (30mL resin, 2.5cm x 30cm). The column was pre-equilibrated with 90mL of 50mM ammonium acetate buffer (pH 4.5). Sugar mixtures were diluted with 50mM ammonium acetate buffer (pH 4.5) to a concentration of 20g/L and loaded onto the column. Charged oligosaccharides, β-GalCOS and α-GalCOS, were eluted with 0.2% triethylamine. The eluted fractions were referred as "elution' and fractions collected before elution step were referred as "flow-through (FT)". The fractions were lyophilized prior to size exclusion separation and HPAEC-PAD analysis.

Lyophilized β-GalCOS elution and α-GalCOS elution were separated by G-25 size exclusion column (GE Healthcare, 65mL resin, 2.5cm x 65mL) with 0.2M ammonium acetate. Mobile phase 0.2M ammonium acetate was delivered at a flow rate of 1mL/min by Econo Gradient Pump (Model 7319001, Bio-rad, CA). Glucose (Fisher), lactose (Sigma), and raffinose (Sigma) were used as external standards. Two fractions were

collected for each sample, and the collected samples were lyophilized prior to HPAEC-PAD analysis and anti-adhesive activity test. The fractions containing large molecular weight of β-GalCOS and α-GalCOS were referred as "β-GalCOS" and "α-GalCOS", respectively.

4.2.4. Production of exopolysaccharides

Exopolysaccharides (EPS) produced by *Lb. reuteri* TMW1.656 were used as positive control in the swine small intestine segment perfusion model challenged with ETEC K88. The anti-adhesive effect of EPS has been demonstrated in previous study with the same animal model (Chen, Woodward, Zijlstra, & Gänzle, 2014). EPS was produced as previously described (Chen, Woodward, Zijlstra, & Gänzle, 2014; Wang, Gänzle, & Schwab, 2010) with some modification. In brief, *Lb. reuteri* TMW1.656 were grown overnight in sucrose-mMRS broth at 37ºC. Culture supernatant was collected by centrifugation, mixed with 2-volumes of chilled ethanol. Culture-ethanol mixtures were left at 4ºC overnight for EPS precipitation. The precipitated EPS were collected by centrifugation and dialyzed with a molecular weight cutoff of 12,000 to 14,000 (Spectra/Por 4 membrane tubing; Spectrum Laboratories Inc., Rancho Dominguez, CA, USA) at 4ºC for 2 days. The retentate was lyophilized prior to use in animals. The anti-adhesive activity of EPS was tested *in vitro* with a sugar concentration of 10g/L by hemagglutination assay.

4.2.5. High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD).

Oligosaccharides were diluted to 1g/L prior to analysis on a HPAEC-PAD ICS-3000 system (Dionex, Oakville, Canada). Samples (10μL) were loaded on a CarboPac PA-20

column using water (A), 200mM NaOH (B), and 1M sodium acetate (C) as eluent at flow rate of 0.25mL/min with following gradient: 0min 6% B, 20min100% B.

4.2.6 Determination of the anti-adhesive activity against ETEC K88 of fractionated oligosaccharides with hemagglutination assay and Enzyme-linked immunosorbent assay (ELISA)

The anti-adhesive activity against ETEC K88 of collected oligosaccharides was analyzed by hemagglutination assay and ELISA assay. The lyophilized oligosaccharides were dissolved in phosphate buffer saline (PBS) (pH 7.2) prior to bioassays. Hemagglutination assay was performed with the porcine ETEC ECL 13795 expressing $K88_{ac}$ fimbriae (Yan et al., 2017). In brief, *E. coli* cells were washed with 1ml PBS after overnight cultivated on Minca agar. *E.coli* cell suspensions were diluted 2-fold in V-bottom 96-well polystyrene microtiter plates (Corning, Fisher Scientific, CA), followed by the addition of PBS or oligosaccharide fractions. Oligosaccharides were used at 10g/L, and in two-fold serial dilutions to the exhaustion of biological activity except for EPS. Porcine red blood cells (Innovative Research, Novi, MI, USA) were added into the plate, and the plate was incubated at 4ºC overnight before visual inspection of hemagglutination.

ELISA assay was conducted as described previously (Yan et al., 2017) with some modifications. In brief, 96-well high bind microtiter plate (Corning, Fisher Scientific, CA) was coated overnight with 100µL of 5% (v/v) porcine red blood cells (Innovative Research, Novi, MI, USA) and blocked with 200µL of 3% (w/v) bovine serum albumin for 2h at 4ºC. Oligosaccharides were at 10g/L. The same volume of ETEC was mixed with PBS or oligosaccharides; 100µL of the mixture was added into plates and incubated for 30min at 4ºC. The wells were then treated with mouse anti *E.coli* K88A antibody

(Biorad, USA) followed by a goat anti-mouse IgG (H+L) secondary antibody conjugated to horseradish peroxidase (Invitrogen, Fisher Scientific, CA) for 1h. The assay was developed by the addition of TMB-ELISA substrates and terminated with 2M sulphuric acid. The absorbance at 450 nm was measured on a microplate reader (Thermo Scientific, CA).

4.3. Results

4.3.1. Scale-up production of oligosaccharides with β-galactosidase and α-galactosidase

The formation of oligosaccharides by different reaction volume, 1mL 10mL, and 25 ml, were analyzed by HPAEC-PAD (Figure 4.1). A similar pattern of oligosaccharides in each tested reaction volume was observed, which indicated the small influence of reaction volumes on the transgalactosylation reactions of β-galactosidase with lactose as galactosyl donor and COS as acceptor sugars (Fig 4.1A). Several synthesized oligosaccharides were labeled with "*", only the retention time and peak area were compared due to the lack of standards as an indication of transgalactosylation reaction. Compared to 1mL preparation, 25mL preparation had similar production level of galactosylated oligosaccharides. Similar oligosaccharides separation pattern was also observed with α-galactosidase with melibiose as galactosyl donor and COS as acceptor (Fig 4.1B), which indicated low influence for different volumes on the transgalactosylation reaction

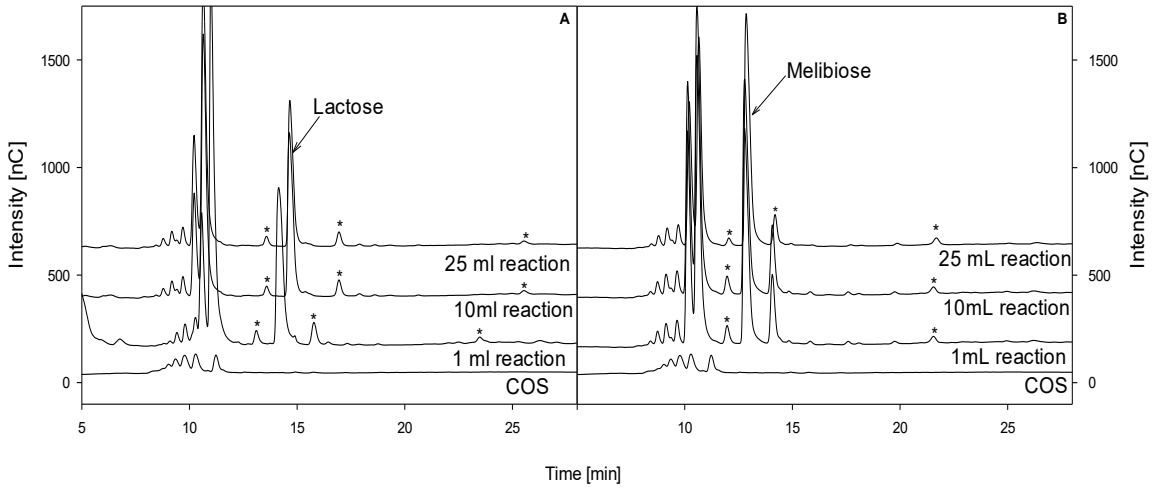

Figure 4.1. HPAEC-PAD results of transgalactosylation reaction of β-galactosidase with lactose and COS using reaction volumes of 1mL, 10mL and 25mL (A); HPAEC-PAD results of transgalactosylation reaction of α-galactosidase with melibiose and COS using different reaction volumes of 1mL, 10mL and 25mL (B). "*" represents the examples of synthesized oligosaccharides. All chromatography are based on two independent reactions.

4.3.2. Fraction of oligosaccharides by cation exchange chromatography and SEC

Enzymatic reaction mixtures were initially separated by cation exchange chromatography in order to obtain charged oligosaccharides namely COS and α-/β-galactosylated COS. CM Sepharose® (Sigma) is weak cation exchange resin. Non-charged sugars including glucose, galactose, lactose, and GOS were washed out in the flow-through fraction (Fig 4.2A and 4.2B). The charged oligosaccharides were bounded to the CM resin, and eluted with 0.2% triethylamine (Fig 4.2C and 4.2D). The column packed with CM Sepharose® (Sigma) resin was able to achieve comparable separation of charged oligosaccharides and

other compounds in the reaction mixture as reported in Yan et al. (2017). However, there were a trace amount of lactose and melibiose in elution fractions, and those were further removed by SEC fractionation with the molecular weight cut off of DP 2 (Fig 4.2E and Fig 4.2F).

4.3.3. Hemagglutination and ELISA assay on the anti-adhesive activity against ETEC K88

EPS had anti-adhesive activity at 10g/L when tested with hemagglutination assay, which matched with previous reports (Chen et al., 2014; Wang, Gänzle, & Schwab, 2010). Enzymatic reaction mixtures of β-/α-galactosidase, fractions collected with cation exchange separation and fractions obtained from SEC fractionation were tested for anti-adhesion activity against ETEC K88. Samples were prepared to 10g/L and diluted to exhaustion of the anti-adhesive activity. Enzymatic reaction mixtures exhibited strongest anti-adhesive activity, followed by β-GalCOS and α-GalCOS (Table 4.1). The anti-adhesive activity of β-GalCOS and α-GalCOS was also confirmed with ELISA targeting K88 fimbriae (Fig 4.3). Consistent with hemagglutination assay, β-GalCOS and α-GalCOS after cation exchange separation and SEC fractionation prevented ETEC K88 adherence on porcine red blood cells (Fig 4.3). The anti-adhesive effects matched with previous observation for the reaction mixture and Gal-COS elution in hemagglutination assay and ELISA assay when compared with 1mL preparation (Table 4.1 and Figure 4.3). Two fractions were collected in the 25mL preparation with G-25 resin, while five fractions were collected in the 1mL preparation with Superdex peptide column. Thus, the anti-adhesive activities of oligosaccharides after SEC fractionation were not compared between 1mL and 25mL preparation.

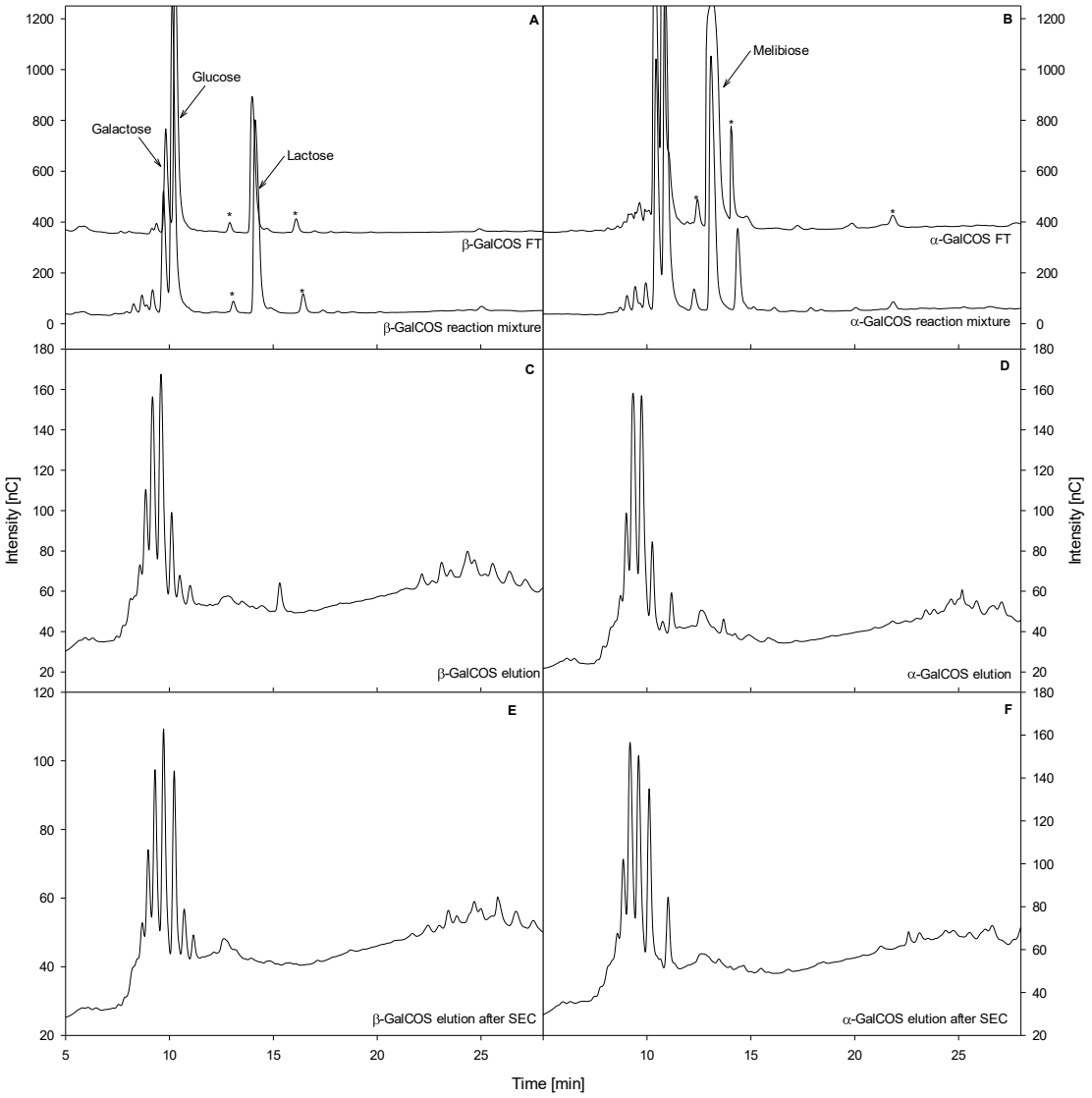

Figure 4.2. HPAEC-PAD diagrams of galactosylated oligosaccharides fractions collected with cation exchange and SEC chromatography. Panel A. β-GalCOS FT; Panel B: α-GalCOS FT; Panel C. β-GalCOS elution; Panel D: α-GalCOS elution; Panel E. β-GalCOS F1; Panel F. α-GalCOS F1. All chromatography are based on three independent reactions.

Table 4.1. Inhibition of erythrocyte agglutination by ETEC K88 with oligosaccharides synthesized with 1mL preparation and 25mL preparation.

Oligosaccharides	**Minimum concentration for erythrocyte agglutination with ETEC (g/L)**	
	25mL preparation	1mL preparation (Data from Chapter 2 & 3)
COS	> 10	>10
β-GalCOS reaction mixture	0.63 ± 0.0	0.69 ± 0.00
β-GalCOS elution	3.13 ± 1.4	2.92 ± 0.48
β-GalCOS	1.35 ± 0.6	ND
α-GalCOS reaction mixture	0.83 ± 0.3	0.63 ± 0.0
α-GalCOS elution	1.56 ± 0.5	1.25 ± 0.0
α-GalCOS	1.75 ± 0.6	ND

ND: Not determined

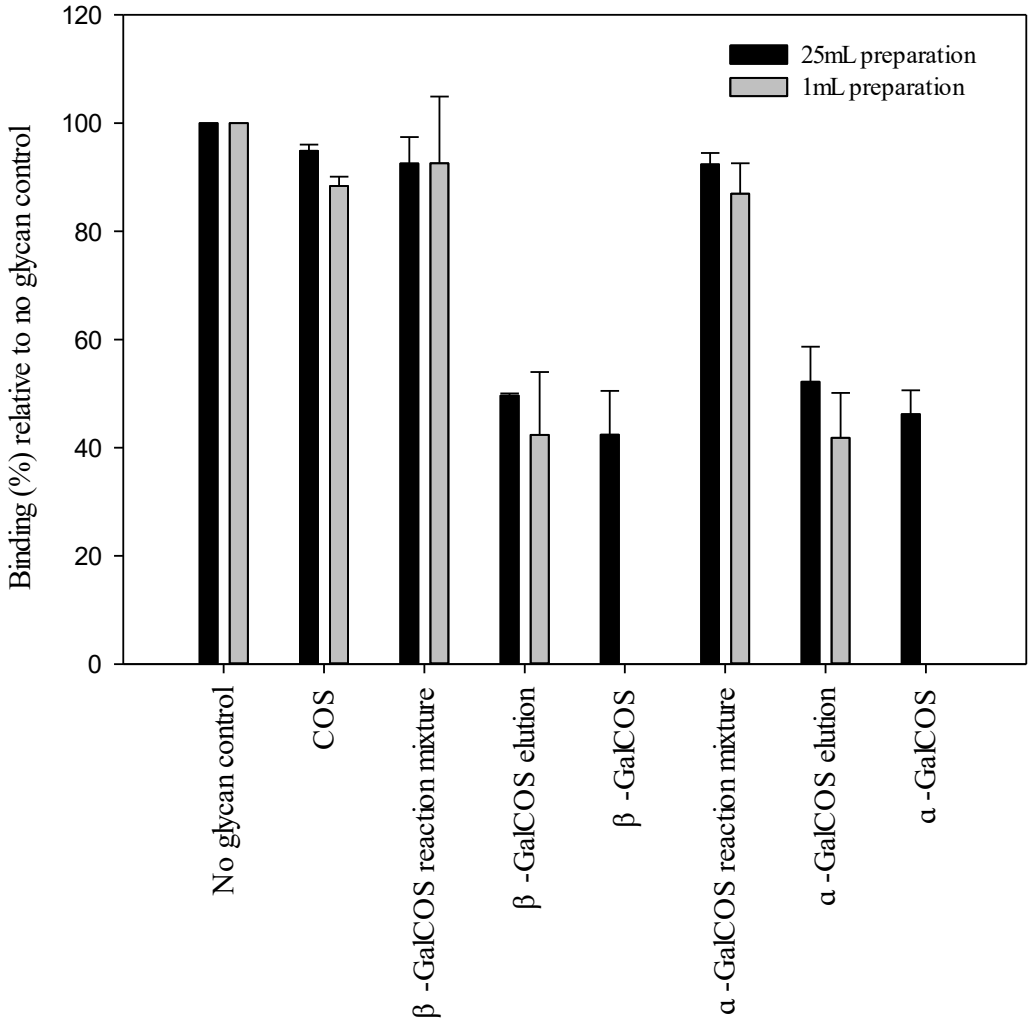

Figure 4.3. Quantification of ETEC K88 ECL 13975 (O149, virotype STb:LT:EAST1:F4) binding to porcine erythrocytes with ELISA targeting K88 antibodies for different reaction scales, 1mL(grey) and 25mL(black). ETEC were incubated with erythrocytes without addition of glycans (no glycan control) or with addition of 10 g/L of tested carbohydrates as indicated on x-axis. Results are shown as means ± standard deviation of three independent assays.

4.4. Discussion

This study synthesized β-/α-galactosylated COS using lactose and melibiose as galactosyl donors in the acceptor reactions with β-galactosidase and α-galactosidase, respectively. COS were used as acceptor sugars in reactions. The formation of β-GalCOS from lactose was verified by MS/MS and LC-MS in a 1mL preparation (Black et al., 2014; Yan et al., 2017). Previous results indicated the formation of α-GalCOS by separation, fractionation, and bioassay determination; however, the structure of α-GalCOS remains unclear (Chapter 3). In this study, oligosaccharides synthesized by both enzymes using different scales of reaction were analyzed by HPACE-PAD as a validation for scale-up production. Similar separation patterns and comparable peak areas for the synthesized oligosaccharides were observed at the same retention time among three tested reaction volumes (1mL, 10mL, and 25mL). This indicated a relatively small influence of reaction volume on the production of galactosylated oligosaccharide when using β-/α-galactosidase. The key concern for scale-up of the β-/α-GalCOS process described in this study is the appropriate mixing of the starting sugar materials and crude cell extracts to ensure even distribution of sugars and enzymes.

β-/α-Galactosylated COS were purified from the reaction mixture by cation exchange chromatography and further fractionated by SEC in order to remove monosaccharides and disaccharides. HPAEC-PAD results for each step of purification and separation showed the successful removal of glucose, galactose, lactose, melibiose, and GOS from galactosylated COS. However, structural analysis of galactosylated COS needs further investigation by mass spectrometry. Purified galactosylated COS exhibited comparable anti-adhesive activity against ETEC K88 as observed in previous studies in the

hemagglutination assay and the ELISA assay (Yan et al., 2017, Chapter 3, Table 4.1 and Fig 4.3).

The large-scale production of oligosaccharides from lactose has been achieved with the combination of free or immobilized β-galactosidase from different bacterial sources in various bioreactors (Nath et al., 2016; Mano et al., 2017). The yield of synthesized oligosaccharides ranged from 10% to 50% depending on the initial lactose concentration (Nath et al., 2016). One of the challenges for large-scale production of oligosaccharides by β-galactosidase with lactose is to remove glucose, galactose, and lactose. Iindustrial approaches currently apply SEC columns or ultrafiltration membrane systems to remove those undesired sugars or salts (Torres, Gonçalves, Maria do Pilar F, Teixeira, & Rodrigues, 2010). Preparative columns are widely applied in industrial purification process (Rathore & Velayudhan, 2002). In theory, according to the van Deemter equation, the effects of Eddy diffusion and longitudinal diffusion are small in the industrial production process because of high flow rates; mass transfer become the dominant factor for separation efficiency (theoretical plates) of the column (Rathore & Velayudhan, 2002) If the particle diameters of the packing resins, the length of the column, and the flow rate all remain constant, scale-up of the column can be achieved by increasing the column diameter (Rathore & Velayudhan, 2002). However, large scale production in industries is far more complicated. For example, industrial production intends to obtain the maximum solute capacity in the column separation step. This can be achieved through increasing mass transfer rate, increasing pressure drop over the column, and providing large mass transfer surface (Rathore & Velayudhan, 2002). However, in order to separate the products from impurities, smaller interfacial area and minimum

pressure drop over the column are preferred (Rathore & Velayudhan, 2002) These aspects need to be considered and balanced during production scale-up. The two separation methods, cation exchange chromatography and size exclusion chromatography, used in this study also have potential challenges during scale-up. Column overload can occur during cation exchange purification because of limited resin binding capacity and a large feed loading volume. Overload problem can be avoided by decreasing the loading amounts or increasing the length of columns (Rathore & Velayudhan, 2002). The latter is not favoured by industrial production and also poses challenges in finding suitable columns (Rathore & Velayudhan, 2002). For size exclusion chromatography, the physical stability of gel resin limits the maximum bed height and column diameters; the use of several smaller columns in tandem instead of one massive column is more common in industrial applications (Rathore & Velayudhan, 2002).

In conclusion, the present study showed scale-up production of galactosylated COS with increased reaction volume and self-packed columns. Two columns, cation exchange chromatography and SEC, are capable of scaling up for large amount purification Synthesized GalCOS inhibited ETEC K88 adherence to porcine erythrocytes. Moreover, the use of lactose and COS as starting materials provided an alternative approach for the valorization of dairy and seafood industry by-products.

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Chapter 5. General discussion

β-Galactosidase belongs to glycosyl hydrolase family, and it is also a retaining glycosyl hydrolase as it maintains stereochemistry of the substrate in enzymatic reactions (Gänzle, 2012). β-Galactosidase transfers galactosyl moieties from lactose to suitable acceptor sugars to synthesize oligosaccharides, examples of synthesized oligosaccharides including GOS, fructo-oligosaccharides, and lactosucrose (Gänzle, 2012). Oligosaccharides that are produced by β-galactosidase are widely applied in the food and pharmaceutical industries (Chen & Gänzle, 2017; Gänzle, 2012). The therapeutic use of oligosaccharides as carbohydrate receptor decoys to prevent diarrhea is an emerging application. Oligosaccharides decoys are recognized by pathogens and block pathogens adherence to host cells surface, and thus prevent pathogen infections (Sharon, 2006). The anti-adhesive effects of oligosaccharides are highly dependent on their structures, including the composition, the degree of polymerization, and the linkage type (Sharon, 2006). This thesis research aimed to demonstrate the structure and function relationship of enzymatically produced oligosaccharides and their ability to prevent ETEC K88 from adherence to porcine erythrocytes. ETEC K88 is of concern to the swine industry because it causes diarrhea in piglets, which can result in substantial mortality and subsequent economic loss. Current preventative and therapeutic approaches used in the swine industry are dependent on the use of antibiotics such as penicillin, tetracycline, and ceftiofur (Barton, 2014). However, usage of antibiotics leads to the development of antibiotic resistance in the microbiota of the swine gut, and antibiotic-resistant organisms may later transfer to human (Gresse et al., 2017). This raises concerns around animal and human health. Moreover, the antibiotic ban in animal production, such as ban on the usage of antibiotics as growth promoter, force industries to find alternatives to antibiotics,

for instance using receptor glycan analogs to prevent pathogens infections (Castanon, 2007). Previous work has shown that such an approach has promise. For example, chitooligosaccharides and galacto-oligosaccharides successfully served as carbohydrate decoys and prevented EPEC adhesion to human epithelial cells (Quintero-Villegas et al., 2013; Shoaf, Mulvey, Armstrong, & Hutkins, 2006; Gänzle, 2012). Quintero-Villegas (2013) reported the inhibitory effects of oligosaccharides against EPEC. However, the effects of oligosaccharides on swine ETEC remains unknown. Therefore, this thesis investigated the anti-adhesive effects of galactosylated COS against swine ETEC.

5.1. Synthesis of galactosylated chitosan-oligosaccharides (COS)

The hypothesis that COS can be galactosylated by β-galactosidase using lactose as a donor of galactosyl moieties was demonstrated in Chapter 2. Transgalactosylation reactions were conducted with lactose and COS using crude cell extracts containing heterologously expressed β-galactosidase (Chapter 2). The result was the synthesis of galactosylated COS (β-GalCOS) (Chapter 2). Previous studies have demonstrated the formation of GOS, galactosylated *N*-acetylglucosamine, and other hetero-oligosaccharides using lactose and crude cell extracts containing β-galactosidase (Schwab, Sørensen, & Gänzle, 2010; Schwab, Lee, Sørensen, & Gänzle, 2011; Black et al., 2014). The results of this study support previous observations that galactose moieties from lactose can be transferred by β-galactosidase to suitable acceptors, in this case, COS (Chapter 2). To further investigate β-GalCOS, monosaccharides (glucose and galactose), lactose, and synthesized GOS were removed from the reaction mixture by a two-step separation. LC-MS provided information on the formation and the chemical formula of β-GalCOS, and the number of transferred galactose moieties varied from 1 to 4 (Chapter 2).

Multiple structural related oligosaccharides in each fraction prevented further investigation on detailed structural information by mass spectrometry. Moreover, transgalactosylation by β-galactosidase forms different linkage types, such as β-(1→4), β-(1→3) and β-(1→6) linkages (Chen & Gänzle, 2017; Gänzle, 2012). Previous research identified the formation of β-(1→4) linkages in galactosylated chitinbiose or chitintriose when using lactose as a galactosyl donor (Black et al., 2014). However, the linkage type between transferred galactose moieties and COS need further investigation.

5.2. Anti-adhesive effects of β-GalCOS against ETEC K88 *in vitro*

Anti-adhesive effects of β-GalCOS against ETEC K88 were confirmed *in vitro* with porcine erythrocytes in Chapter 2. β-GalCOS F2 with highest molecular weight ranging from DP 8 and higher, exhibited the greatest anti-adhesive activity against ETEC K88 in two bioassays, a hemagglutination assay and an ELISA assay. The anti-adhesive activities of β-GalCOS against ETEC K88 decreased with decreased molecular weight. Inconsistent results were observed for acceptor reaction mixtures in the hemagglutination assay and ELISA assay (Chapter 2). This may be because of impurities contained in the β-galactosidase-containing crude cell extracts. Only β-galactosidase in crude cell extracts was quantified, other components, such as cytoplasm materials, remained unknown. The unknown compounds might interfere with the hemagglutination assay; however, the reaction mixture from GOS control samples did not interfere with the hemagglutination assay. Other impurities might have been generated from non-enzymatic browning reaction from COS. COS are composed of *N*-glucosamine and *N*-acetyl-glucosamine (Black et al., 2014). *N*-glucosamine has similar, relevant structural characteristics when compared to glucose, with a key difference being the location of a primary amine group

(C2 position), and as such it could undergo the Maillard reaction at 37ºC, 40ºC, and 60ºC (Hrynets, Ndagijimana, & Betti, 2015; Hong & Betti, 2016). The temperature used for the acceptor reactions in this study was 45ºC, at which COS likely underwent the Maillard reaction. Visible browning of reaction mixtures was observed, and an increased UV absorption was recorded when monitoring the reactions for 16h at 1h intervals (data not shown). The increased UV-absorption at 340-360nm is consistent with reported observations as an indication for the occurrence of Maillard reactions by glucosamine (Hrynets et al., 2015). Hrynets et al. (2015) reported the production of two major α-dicarbonyl compounds, glucosone and 3-deoxyglucosone, by glucosamine in the Maillard reactions. Other compounds, such as diacetyl, hydroxypyruvaldehyde, and methylglyoxal, were also identified as Maillard reaction products but they presented in a lower amount (Hrynets et al., 2015),

Taken together, various impurities existed in the reaction mixtures and interfered with bioassays. In order to better understand glycan functionalities, purification and separation were necessary steps. Two different bioassay methods were used to confirm anti-adhesive effects of β-GalCOS against ETEC K88. The use of two bioassays eliminated potential false positive results obtained with single bioassay. The anti-adhesive activity against ETEC K88 of EPS was demonstrated by use of hemagglutination assay, and the anti-adhesion activity matched the results for piglets in a subsequent *ex vivo* experiment (Chen, Woodward, Zijlstra, & Gänzle, 2014; Wang, Black, Curtis, & Gänzle, 2014). This suggests a possible application of β-GalCOS in future animal studies, including swine, for the prevention of ETEC K88 adhesion and corresponding disease.

5.3. Structure and function relationship of β-/α-oligosaccharides as receptor analogs to prevent ETEC K88 adhesion

The anti-adhesive activities of β-/α-galactosylated oligosaccharides against ETEC K88 adherence to porcine erythrocytes were investigated in Chapter 3, in order to have a better understanding of the structure and function relationship of oligosaccharides and ETEC K88 adhesion.

Fimbriae of ETEC K88 mediate adherence to porcine cell surfaces, a process which relies on high specificity for carbohydrate receptors on the porcine cell surface. To investigate the necessity of the presence of β-linkages in the mediation of adhesion, β-galactosidase and α-galactosidase were included in acceptor reactions with lactose and melibiose (Chapter 3). β-Linked oligosaccharides exhibited higher anti-adhesion activity compared to α-linked oligosaccharides, which suggested the preference of K88 fimbriae for binding to β-linked oligosaccharides (Chapter 3). Using an ELISA assay and purified K88 fimbriae, in addition to a hemagglutination assay and ELISA with ETEC cells, to assess the anti-adhesion activity provided direct evidence for the blockage of K88 fimbriae on ETEC cells (Chapter 3). Oligosaccharides synthesized with β-galactosidase and levansucrase were examined to investigate the importance of galactose at the non-reducing end (Chapter 3). Galactosylation, preferably β-galactosylation, was important for recognition between K88 and complementary carbohydrate receptors (Chapter 3). This observation matched with proposed recognition carbohydrate sequence of the K88 adhesin (Grange, Mouricout, Levery, Francis, & Erickson, 2002; Jin, Marquardt, Baidoo, & Frohlich, 2000; Moonens et al., 2015; Trevisi et al., 2017).

ETEC K88 fimbriae are encoded by the *fae* gene operon (Moonens et al., 2015). Based on the difference in the binding domain *faeG,* three variants have been identified for K88 fimbriae: $K88_{ab}$, $K88_{ac}$, and $K88_{ad}$ (Moonens et al., 2015; Zhang, Fang, & Francis, 2009). Genetic differences in these three variants result in differences in amino acid composition of the binding protein FaeG (Zhang, et al., 2009). Amino acids from positions 125 to 163 were indicated as the binding region on FaeG, however, the specific binding sites within the binding region on FaeG for each variant were different (Zhang et al., 2009). Amino acid residues from positions 140-145 and 151-156 were responsible for $K88_{ab}$ binding to sugar receptors; and positions 147-160 were identified as the binding site for $K88_{ac}$ (Xia et al., 2015). Moreover, the amino acid sequence 150-152 and 166-170 played an important role in the interactions between $K88_{ad}$ and the terminal galactose moiety in lactose (Moonens et al., 2015). Those differences in FaeG among three variants resulted in overlapping yet different binding specificities of each variant. All three K88 variants were able to bind glycoprotein and glycolipids, however, $K88_{ac}$ and $K88_{ab}$ preferred binding to glycoprotein, while $K88_{ad}$ favoured interactions with glycolipids (Jin & Zhao, 2000). Glycoproteins with molecular weight of 210 kDa and 240 kDa isolated from swine brush borders were identified as receptors for K88ac fimbriae (Erickson, Willgohs, Mcfarland, Benfield, & Francis, 1992). Jin (2000) purified an 80 kDa glycoprotein from 14-day-old piglets, and this glycoprotein specifically bound to $K88_{ac}$ adhesin. GM_3 [NeuAc-α-(2→3)-Gal-β-(1→4)-Glc-β-(1→1)Cer] bonds to K88ac only; while other compounds such as Lc_4 [Gal-β-(1→3)GlcNAc-β-(1→3)-Gal-β-(1→4)-Glc-β-(1→1)Cer] and Lc_3 [GlcNAc-β-(1→3)-Gal-β-(1→4)-Glc-β-(1→1)Cer] bond to two variants ($K88_{ac}$ and $K88_{ad}$) and all three variants, respectively (Grange et al., 2002). Another $K88_{ac}$-

specific receptor porcine aminopeptidase N was recently identified. The interactions between $K88_{ac}$ fimbriae with porcine aminopeptidase N were associated with sialic acids on its carbohydrate moieties (Melkebeek et al., 2012). A 76 kDa porcine serum transferrin was recognized as a $K88_{ab}$ receptor, and terminal *N*-acetylglucosamine residues were essential for $K88_{ab}$ binding activity in this case (Grange et al., 2002). Moreover, the addition of β-Gal, preferably β-(1→4)-Gal, enhanced the binding ability of all three variants (Grange et al., 2002). β-Linked *N*-acetylhexosamine either at the non-reducing end or linked to a terminal galactose moiety at the non-reducing end was the core structure for K88 fimbriae recognition (Grange et al., 2002). Terminal β-galactose located at the non-reducing end was reported to be a necessary component in recognition of K88 with carbohydrate receptors (Grange et al., 2002; Jin et al., 2000; Moonens et al., 2015; Trevisi et al., 2017).

Taken together, oligosaccharides containing galactose, *N*-acetylglucosamine, and sialic acid were important in ETEC K88 adherence. β-GalCOS produced in the present thesis conformed with previous observation that terminal β-galactose moieties at the non-reducing end were essential in K88 fimbriae adherence to sugar receptors on swine cell surface. Galactosylation of COS produced oligosaccharides containing galactose, *N*-glucosamine, and *N*-acetylglucosamine, which matched with the proposed core structure of K88 fimbriae receptors (Chapter 2; Grange et al., 2002). β-GalCOS produced in this work exhibited inhibitory effects against ETEC K88 adherence to porcine erythrocytes (Chapter 2). Moreover, β-GalCOS was synthesized with food grade materials (β-galactosidase, lactose, and COS), thus β-GalCOS could be a potential feed additive for swine industries to prevent piglets' diarrhea with a relatively low cost because lactose

and COS are by-products from dairy and seafood industries. Structural modification of β-GalCOS, such as acetylation, fucosylation, and sialidation, can produce oligosaccharides mimicking HMOs, which may prevent different pathogens.

5.4 Other anti-adhesive compounds

Besides oligosaccharides, other compounds, such as polyphenols, casein glycomacropeptides, and egg white proteins were also reported to have anti-adhesive effects. Cranberry extract prevented uropathogenic *E. coli* adherence to bladder epithelial cells and vaginal epithelial cells (Gupta et al., 2007; Howell et al., 2010; Sun, Gänzle, & Wu, 2017). The anti-adhesive effects of cranberry extracts were due to the presence of A-type proanthocyanidins, and the anti-adhesive effects exhibited a dose-depended manner within the range of 5 to 75μg/mL (Gupta et al., 2007; Howell et al., 2005; Howell et al., 2010). Cranberry extracts also reduced the adherence of *H.pylori* to human gastric mucus and human erythrocytes (Burger et al., 2000). The mechanism of anti-adhesive effects of polyphenols is poorly described and more research needs to be conducted. Casein glycomacropeptides (CGMP) reduced the adherence of EHEC, EPEC, and ETEC including ETEC K88 adherence to cultured cell lines (Feeney, Ryan, Kilcoyne, Joshi, & Hickey, 2017; González-Ortiz et al., 2014; Rhoades et al., 2005). The anti-adhesive effects of CGMP might due to the glycosylation portions especially those containing *N*-acetylneuraminic acid, *N*-acetylgalactosamine, and galactose residues (Rhoades et al., 2005). Moreover, CGMP inhibited the NF-κB signaling pathway which promoted the integrity of Caco-2 cells (Feeney et al., 2017). Anti-adhesive effect of CGMP is hypothesized due to the presence of sugar residues and direct interactions with bacteria cells; however, the detailed mechanism of anti-adhesive effects of CGMP still remains

unclear (Feeney et al., 2017). Glycopeptides obtained from egg white had anti-adhesive effects against ETEC K88 with the minimum inhibitory concentrations of 0.03g/L, and the anti-adhesive effects were proposed because of the existence of terminal galactose (Sun et al., 2017).

5.5. Swine as suitable animal models

Investigations of using glycan analogs to prevent human pathogens have been limited to *in vitro* experiments with cultured human epithelial cell lines, such as HEp-2 cell lines or other cultured human epithelial cells, because of host-specificity of the pathogens (Chen & Gänzle, 2017; Shoaf et al., 2006). Swine animal models with porcine specific pathogens allow various studies ranging from *in vitro* tests with erythrocytes and cultured cell lines, to *in vivo* studies and feeding trials (Wang, Gänzle, & Schwab, 2010; Chen et al., 2014; Yang, Zhao, Le, Zijlstra, & Gänzle, 2015). Swine are omnivorous mammals and have a high degree of similarity to humans with respect to the gastrointestinal tract, including aspects of anatomy, physiology, and biochemistry (Guilloteau, Zabielski, Hammon, & Metges, 2010). Piglets also have similar intestinal development such as bacteria colonization (Puiman & Stoll, 2008).

ETEC strains expressing fimbriae such as CFA/I, CS1, CS2, and CFA/III, can cause infections resulting in diarrheal diseases of varying severity in humans (Qadri, Svennerholm, Faruque, & Sack, 2005). Human ETEC pathogens with various colonization factors had distinct specificity for receptors on intestinal cells. More than 20 different colonization factors, including fimbriae and non-fimbrial proteins, have been identified in human ETEC strains, with CFA/I being the most common colonization factors for diarrhea disease (Qadri et al., 2005). CfaB was demonstrated as the adhesin

protein for CFA/I fimbriae, and was found to bind to receptors such as human erythrocyte sialyglycoprotein, NeuAc-GM2, and glycosphingolipids (Klemm & Schembri, 2000; Pereira & Giugliano, 2013). Different from CFA/I fimbriae, CFA/II bond to monosialotetrahexosylganglioside and another colonization factor CS6 recognized glycosphingolipid sulphatide on human epithelial cells (Pereira & Giugliano, 2013). However, the binding specificities of other human ETEC fimbriae remain unclear, and assessments of using carbohydrate decoys to prevent human ETEC has been limited to *in vitro* studies (Pereira & Giugliano, 2013; Qadri et al., 2005). Swine model allows investigation of the protective effects of oligosaccharides against pathogens as a proof of concept study for the potential human application.

5.6. Enterohemorrhagic *E.coli* (EHEC)

Beside human ETEC, other *E.coli* strains also cause illness in humans, such as enterohemorrhagic *E.coli* (EHEC). EHEC can cause diarrhea, and in some cases cause hemorrhagic colitis, hemorrhagic uremic syndrome, and even death (McWilliams & Torres, 2014). The pathogenesis of EHEC is due to various virulence factors, including Shiga toxin (Stx) and adhesins (McWilliams & Torres, 2014).

Stx is an AB_5 type toxin, containing one A subunit and five identical B subunits (Flagler, Mahajan, Kulkarni, Iyer, & Weiss, 2010). Subunit A damages protein synthesis by cleaving adenine form 28S ribosomal RNA, while subunit B is important in adherence to cell surfaces and delivery of subunit A (Flagler et al., 2010). Two Stx, Stx1 and Stx2, were identified based on differences in amino acid sequences; seven subtypes of Stx2 (Stx2a-Stx2g) have been reported (Flagler et al., 2010). Different receptor-binding specificity was observed in Stx1, Stx2, and subtypes. Globotriaosylceramide (Gb3) was

identified as the binding receptor for both Stx1 and Stx2; however, Stx1 appears to exhibit a higher affinity to Gb3 compared to Stx2 (Karmali, 2004; Flagler et al., 2010). The binding of Stx to receptors was highly dependent on the structure and composition of the carbohydrate portion of receptors. Pk trisaccharide (Gal-α-(1→4)-Gal-β-(1→4)-Glc) was identified as a receptor for Stx 1 (Yosief, Iyer, & Weiss, 2013). Stx2a and Stx2c favored the terminal *N*-acetylgalactosamine which differed from Stx1, Stx2b, and Stx2d (Yosief et al., 2013); Human milk oligosaccharides, such as 2'-fucosyllactose, lacto-*N*-tetraose, and lacto-*N*-fucopentaose, can also be recognized by Stx1 and Stx2 (El-Hawiet, Kitova, & Klassen, 2015).

Bacterial adhesin intimin, encoded by *eae* genes on the locus of enterocyte effacement (LEE) island, was identified as adhesion protein for EHEC (McWilliams & Torres, 2014). The specificities of EHEC adherence were dependent on 27 different intimin variants, such as α-, β-, and γ-intimin (McWilliams & Torres, 2014). Deletion of the γ-intimin coding gene *eae* in EHEC O157: H7 reduced its binding ability to human intestinal cells *in vitro* (Fitzhenry et al., 2002). Site-directed mutagenesis on α-intimin decreased adherence activity (Reece et al., 2001). Besides intimin, diverse proteins were identified in EHEC which contributed to cell adhesion and colonization, such as long polar fimbriae, F9 fimbriae, flagella, and Efa 1 adhesin (McWilliams & Torres, 2014; Shoaf-Sweeney & Hutkins, 2008). Pectin-oligosaccharides decreased the adherence of *E.coli* O157: H7 to human epithelial cells by competitively binding to the Gb3 receptor *in vitro*, and the anti-adhesive effects depended on the molecular weight and deesterfication of pectin-oligosaccharides (Di et al., 2017). In order to prevent Stx and EHEC adherence,

various oligosaccharide decoys are necessary due to overlapping yet different binding mechanisms.

5.7. Future directions of this study

Feeding trial: β-GalCOS are potential carbohydrate decoys to prevent pathogen adhesion. This study determined the anti-adhesive activity of enzymatically produced α-/β-oligosaccharides (GalCOS and GOS) *in vitro.* An quantification method to detect attached bacteria or fimbriae adhesin on porcine erythrocytes with an ELISA assay was developed (Chapter 2 and Chapter 3). However, the *in vitro* ELISA assay cannot evaluate the influence of anti-adhesive oligosaccharides in the GI tract in animals. *In vivo* studies and feeding trials are necessary to elucidate the anti-adhesive activity of oligosaccharides against ETEC as well as the influence of those tested oligosaccharides on host gut microbiota.

Structure identification: The anti-adhesive activity of oligosaccharide fractions, instead of single purified oligosaccharides, was evaluated in this study. To fully understand the anti-adhesive property of β-GalCOS against ETEC K88, a series of purification steps should be conducted in order to obtain single oligosaccharides for structural determination.

5.8 Conclusion and significance

In conclusion, this research explored and demonstrated the structure and function relationships of β-galactosidase-produced oligosaccharides in preventing ETEC K88 adherence to porcine erythrocytes. The potential application of oligosaccharides as anti-adhesive agents in swine industries can provide an alternative to antibiotics. Moreover,

GalCOS match the composition of human milk oligosaccharides, which prevent adhesion of multiple pathogens.

The effects of dietary fiber and oligosaccharides in the modulation of gut microbiota composition are based on the two following aspects. Oligosaccharides change the composition and diversity of gut microbiota by acting as energy sources for bacterial fermentation and modulating SCFAs production. Oligosaccharides also shape gut microbiota by targeting pathogens and toxins adherence to host cell surfaces, which is highly dependent on the structure of oligosaccharides. Different types of oligosaccharides are necessary for preventing multiple pathogens from colonization and proliferating and for improving the function of beneficial gut microbiota to improve human health. Thus, the long-term aim of this thesis is to provide therapeutic options for infants and adults necessitate design of novel oligosaccharides targeting lectins of human pathogenic organisms.

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Appendix

Appendix A.

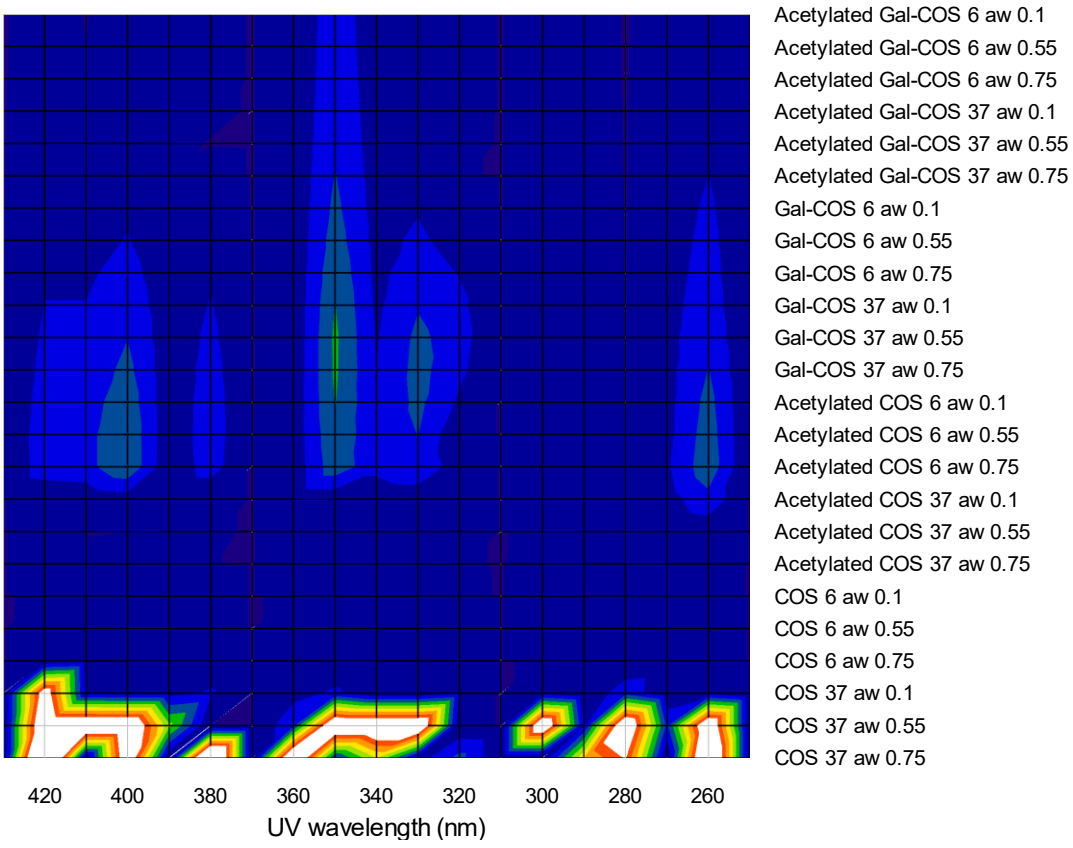


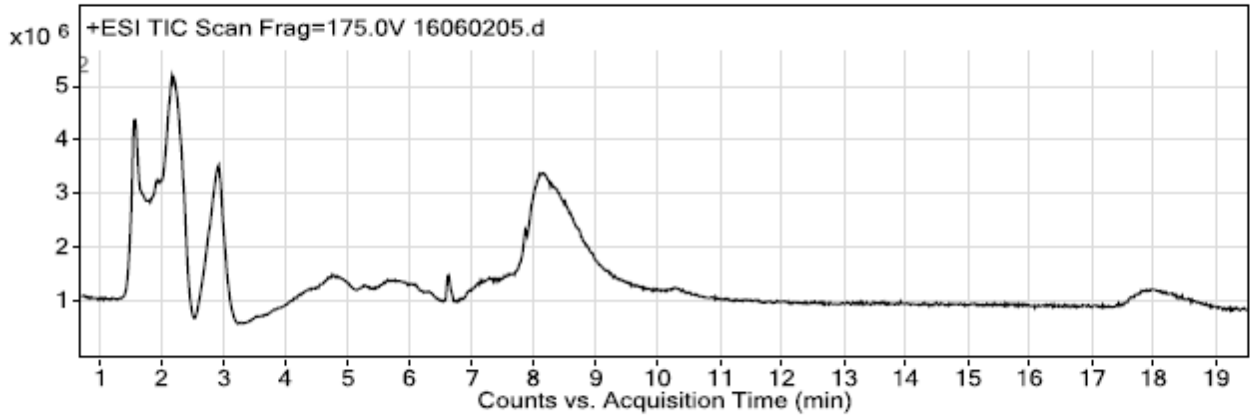
Figure A1. UV−vis absorbance difference for COS, acetylated COS, Gal-COS elution, and acetylated Gal-COS elution after 7 days storage with 2 storage temperatures (37 and 6 °C) and 3 water activities (a_w 0.75, 0.55, 0.1). The lighter the color indicate the greater the UV−vis absorption difference.

Qualitative Compound Report

Data File	16060205.d	**Sample Name**	Gal-COS Elution F3
Sample Type	Sample	**Position**	P1-F5
Instrument Name	oaTOF6220	**User Name**	
Acq Method	LC_GlycanPAC_AXH1_UV_pos_20min.m	**Acquired Time**	6/2/2016 12:09:36 PM
IRM Calibration Status	Success	**DA Method**	LCMS_QTOF_HR_Yalu.m
Comment	Y. Yan, M. Gaenzle, AFNS		

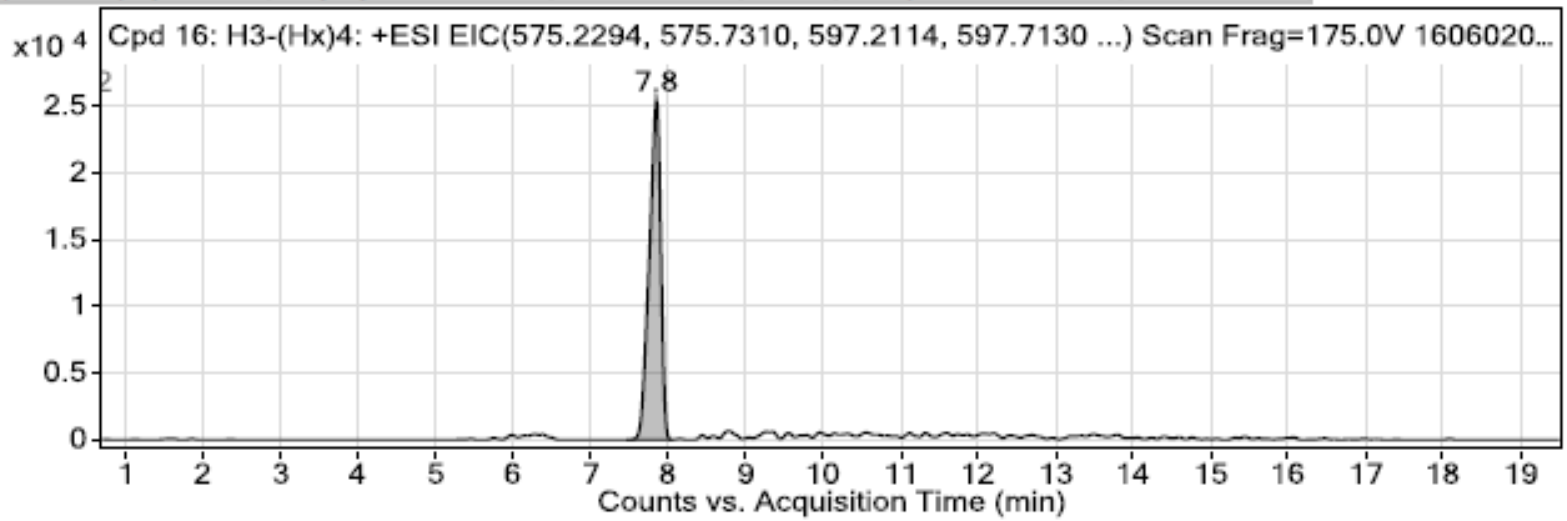
Sample Group **Info.**

Fragmentor Voltage 175 **Collision Energy** 0 **Ionization Mode** ESI



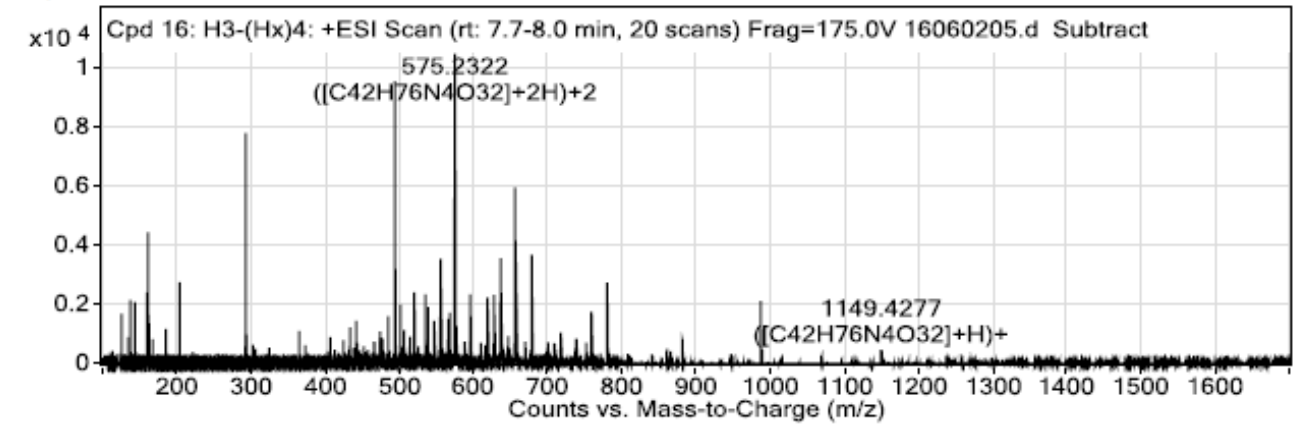
Fragmentor Voltage **Collision Energy** **Ionization Mode**

Compound Label	Name	*m/z*	RT	Algorithm	Mass
Cpd 16: H3-(Hx)4	H3-(Hx)4	575.2322	7.8	Find By Formula	1148.4479

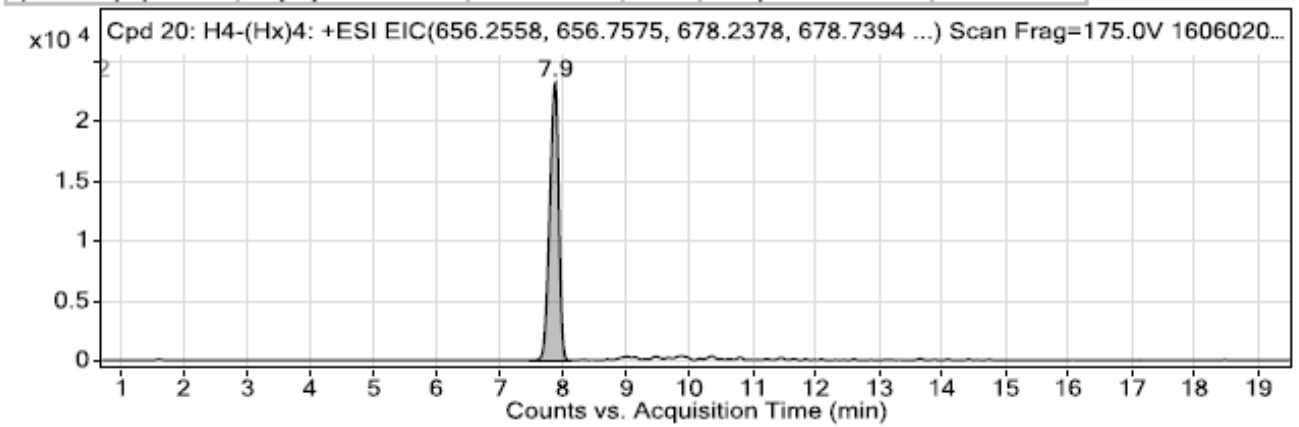


Qualitative Compound Report

MS Spectrum

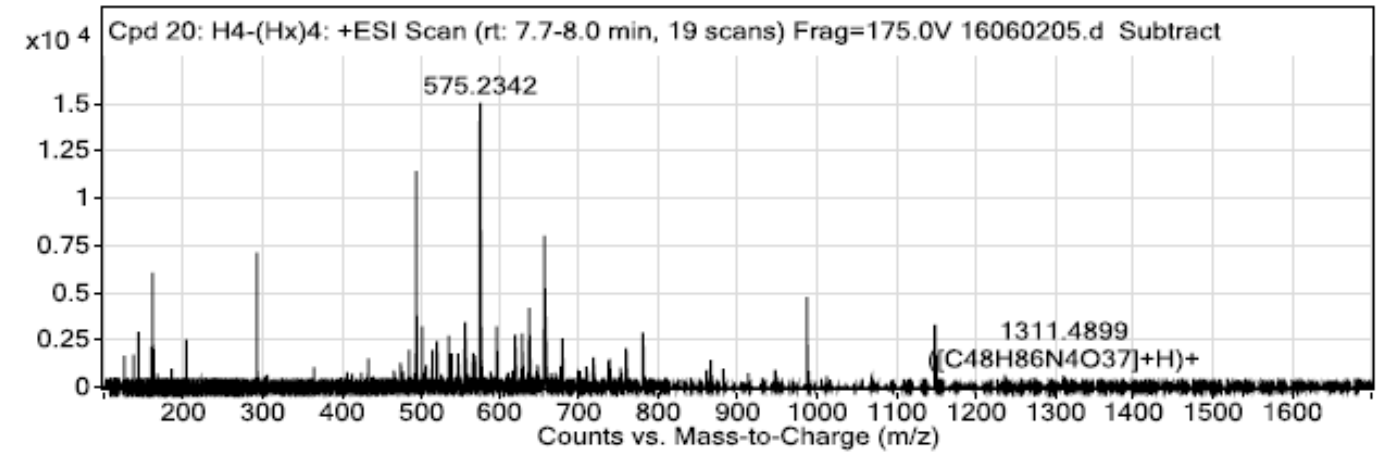


Compound Label	Name	*m/z*	RT	Algorithm	Mass
Cpd 20: H4-(Hx)4	H4-(Hx)4	656.2578	7.9	Find By Formula	1310.5067

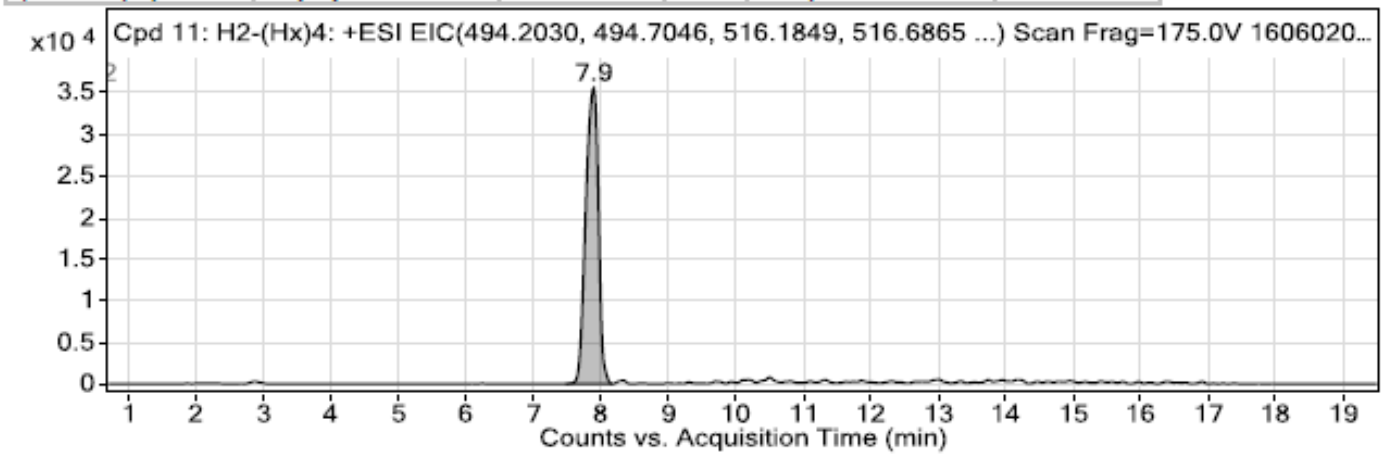


Qualitative Compound Report

MS Spectrum

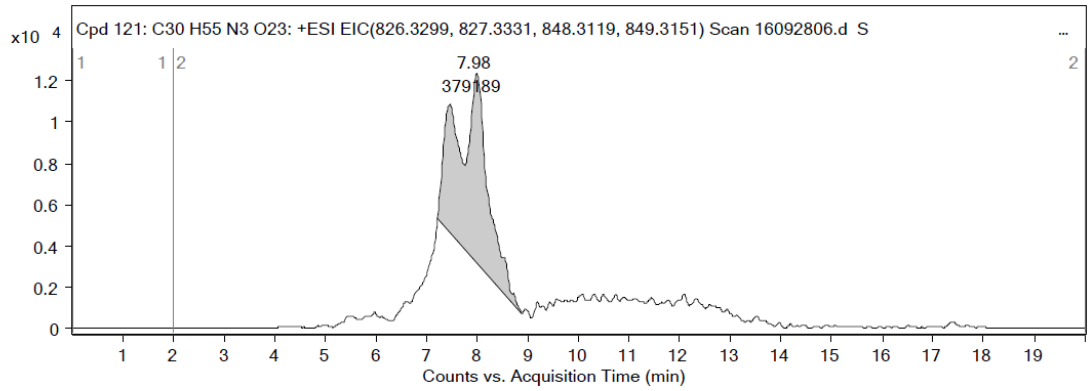


Compound Label	Name	*m/z*	RT	Algorithm	Mass
Cpd 11: H2-(Hx)4	H2-(Hx)4	987.3985	7.9	Find By Formula	986.3933

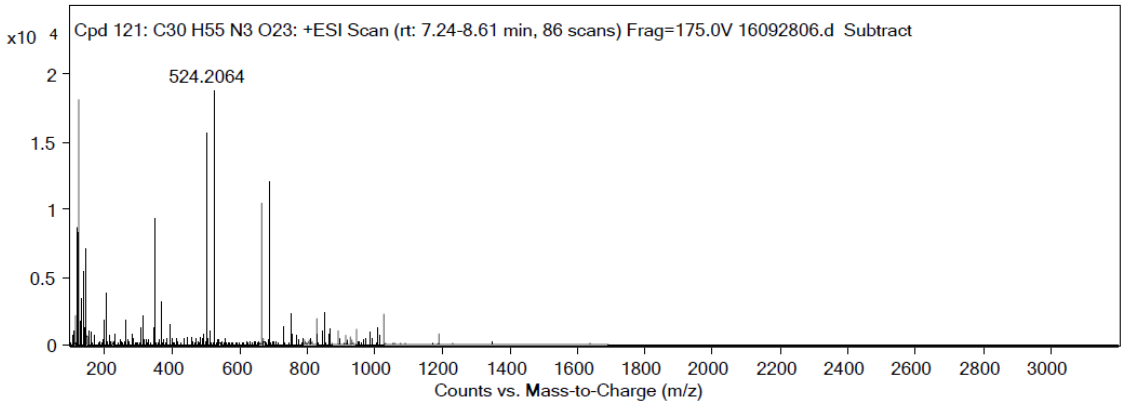


Compound Label	m/z	RT	Algorithm	Mass
Cpd 121: C30 H55 N3 O23	848.3139	7.98	Find By Formula	825.3237

Compound Chromatograms



MS Spectrum



MS Zoomed Spectrum