Structure and Function Relationships of Exopolysaccharides Produced by Lactic Acid Bacteria

by

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Abstract

Lactic acid bacteria produce glycans that may be applied in food and pharmaceutical industries as prebiotics, food additives, to prevent pathogen adhesion, or to modulate the host immune system. The recent identification of the structure of galacto-oligosaccharides (GOS) preparations demonstrates that their functionalities are dependent on the chemical structures. This research aimed to investigate the relation between the structures of exopolysaccharides (EPS) produced by lactic acid bacteria and their functions in food and health applications.

Enterotoxigenic *Escherichia coli* (ETEC) is a major cause of secretory diarrhea in piglets; ETEC colonizes to the intestinal mucosa by fimbriae and produces diarrheal toxins. Anti-adhesive properties of the bacterial glycans reuteran and levan and of the commercial glycans dextran and inulin were determined using a small intestinal segment perfusion (SISP) model. Quantitative PCR identified *E. coli* as the dominant organism in infected segments. The presence of autochthonous ETEC K88 was revealed by qPCR. Bacterial EPS significantly decreased adherent ETEC K88; however, this effect was not attributed to reuteran but to bacterial extracts produced by the reuteransucrase negative strain *L. reuteri* TMW1.656Δ*gtfA*. The enzymatic digestion of the anti-adhesive compound by DNase, RNase, lysozyme and mutanolysin, and the screening of heteropolysaccharides gene cluster implied that heteropolysaccharides produced by *L. reuteri* was a candidate for the anti-adhesive activity.

EPS produced by lactic acid bacteria improve the texture and shelf life of bread. The effect of EPS on bread quality depends on the properties of EPS and EPS-producing strains. The construction of a heterologous expression system of dextransucrase and reuteransucrase, and site-directed mutagenesis of glucansucrases allowed the *ex situ* production of pure reuterans and dextran. Linkage type and molecular weight of enzymatically-produced glucans were determined

by ^{1}H-NMR and asymmetric flow-field-flow fractionation. *L. reuteri* TMW1.656 and *L. reuteri* TMW1.656Δ*gtfA* served as fermentation strains to remove confounding effects of bacterial metabolites. Bacterial and enzymatically produced reuterans had comparable effects on bread volume and crumb hardness. Reuteran with higher portion of α-(1→4) linkages and lower molecular weight was as efficient as dextran in enhancing wheat bread volume and texture. Overall, this study established a valuable model to elucidate structure-function relationships of glucans in baking applications.

In general, this is the first study to demonstrate structure and function relationships of bacterial and enzymatically-produced reuterans in bread baking application. The structural determinants of bacterial EPS that prevent ETEC K88 adhesion needs to be confirmed with further study.

Preface

This thesis is an original work by Xiaoyan Chen.

Chapter 2 is a literature review which has been accepted as Chen, X. Y. and Gänzle M. G., "Lactose and lactose-derived oligosaccharides: more than prebiotics?" *International Dairy Journal*, in press. I was responsible for compiling the relevant information regarding the functionalities and structures of lactose-derived oligosaccharides and writing the manuscript with Dr. Gänzle.

Chapter 3 has been published as Chen, X. Y., Woodward, A., Zijlstra, R. T., and Gänzle, M. G. (2014), "Exopolysaccharides synthesized by *Lactobacillus reuteri* protect against enterotoxigenic *Escherichia coli* in piglets" *Applied and Environmental Microbiology*, *80*, 5752-5760. Dr. Woodward was responsible for handling the animal. I was responsible for preparing exopolysaccharides, collecting and analysing samples, and writing the draft of the manuscript. Drs. Woodward, Zijlstra, Gänzle contributed to the hypothesis development, experimental design, manuscript composition and revision.

Chapter 4 has been published as Chen, X. Y. and Gänzle, M. G. (2016), "Site directed mutagenesis of dextransucrase DsrM from *Weissella cibaria*: Transformation to a reuteransucrase?" *Journal of Agricultural and Food Chemistry*, *64*, 6848-6855. I was responsible for conducting the experiments and writing the manuscript. Dr. Gänzle contributed to the hypothesis development, manuscript composition and revision.

Chapter 5 contains experimental work performed by myself under the supervision of Dr. Gänzle.

Chapter 6 has been published as Chen, X. Y., Levy, C., and Gänzle, M. G. (2016), "Structure-function relationships of bacterial and enzymatically produced reuterans and dextran in

sourdough bread baking application" *International Journal of Food Microbiology*, *239*, 95-102. I was responsible for conducting the experiments with Clemens Levy and writing the manuscript. Part of the results was presented in the Bachelor thesis of Clemens Levy. Dr. Gänzle contributed to the hypothesis development, manuscript composition and revision.

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List of Abbreviations

AACC	American Association of Cereal Chemists
AF4	Asymmetrical flow field-flow-fractionation
DP	Degree of polymerization
DSS	4,4-dimethyl-4-silapentane-1-sulfonic acid
EPS	Exopolysaccharides
ETEC	Enterotoxigenic *Escherichia coli*
FISH	Fluorescent *in situ* hybridization
FODMAP	Fermentable oligosaccharides, disaccharides, monosaccharides and polyols
GH	Glycosyl hydrolase family
GlcNAc	N-acetylglucosamine
GOS	Galacto-oligosaccharides
HMO	Human milk oligosaccharides
HPAEC-PAD	High performance anion exchange chromatography with pulsed amperometric detection
HRM-qPCR	High Resolution Melting-qPCR
LAB	Lactic acid bacteria
LacNAc	N-acetyllactosamine
LB	Luria-Bertani
LT	Heat labile toxin
MALS	Multi-angle light scattering
mMRS	Modified DeMan-Rogosa-Sharpe

NaAc	Sodium acetate
Neu5Ac	N-acetylneuraminic acid
NMR	Nuclear magnetic resonance
PBS	Phosphate-buffered saline
pNP-Fuc	*para*-nitrophenyl-fucose
ppm	parts per million
qPCR	quantitative Polymers Chain Reaction
RI	Refractive index
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEC	Size exclusion chromatography
SISP	Small intestinal segment perfusion
TPA	Texture profile analysis

1. Introduction

1.1. Overview of exopolysaccharides

Bacterial exopolysaccharides (EPS) are long-chain polysaccharides that are classified based on their compositions. Heteropolysaccharides are composed of 2 - 8 different carbohydrate moieties while homopolysaccharides are composed of only one constituting monosaccharide (Korakli & Vogel, 2006). EPS are biosynthesized by intracellular glycosyltransferases or extracellular glycosyl hydrolases. Most homopolysaccharides are synthesized by glycosyl hydrolases of glucansucrase and fructansucrase, heteropolysaccharides and some homopolysaccharides are synthesized by glycosyltransferases (Badel, Bernardi, & Michaud, 2011). Glucan-/fructan-sucrases synthesize EPS in large amounts using the energy generated from the cleavage of sucrose osidic bond (Korakli & Vogel, 2006). Intracellular glycosyltransferases synthesize EPS in small amount from activated sugar nucleotides (Vuyst & Degeest, 1999). Heteropolysaccharides are synthesized by a cluster of genes encoding proteins that produce UDP-sugar precursors, assemble the repeating unit, or polymerize and export the repeating unit (Badel et al., 2011). Due to diverse structures, bacterial EPS with different physico-chemical and biological properties are commercially applied in the food, cosmetics and pharmaceuical industries (Freitas, Alves, & Reis, 2011). The bacterial EPS widely applied in the global hydrocolloids market is the heteropolysaccharide xanthan that is produced by *Xanthomonas campestris* (Freitas et al., 2011). The homopolysaccharide cellulose is the biomaterial for making artificial blood vessels (Chawla, Bajaj, Survase, & Singhal, 2009). Dextran produced by *Leuconostoc mesenteroides*, is hydrolyzed to smaller fragments (70 kDa) to be used as gel filtration compounds and blood plasma substitutes (Monsan et al., 2001). EPS naturally produced by lactic acid bacteria (LAB) improve yoghurt texture, which can eliminate or reduce the use of

texture improvers, such as stabilizers or milk solids (fat, proteins and sugars) (Zannini, Waters, Coffey, & Arendt, 2015).

Food-related *Lactobacillaceae* and *Leuconostocaceae* have little or no potential to cause disease in humans and animals, EPS produced by these LAB strains are thus attracting industrial attentions. LAB-produced EPS benefit the host as prebiotics or through immunomodulation, antioxidant, antitumour or anti-atherosclerotic activities (Patten & Laws, 2015). However, some EPS are also regarded as virulence factors. For example, mutan produced by *Streptococcus mutans* is a causative factor in dental caries, as mutan is a main component of cariogenic biofilms (Klein, Hwang, Santos, Campanella, & Koo, 2015). There are around 30 species of lactobacilli that have been well characterized as EPS producers, including *Lactobacillus reuteri* (Badel et al., 2011; Tieking & Gänzle, 2005).

Homopolysaccharides produced by *L. reuteri* have potential to be used in commercial applications. Inulin, the β-(2,1) fructan produced by *L. reuteri* 121, was demonstrated as a prebiotic (Badel et al., 2011). Levan, the β-(2,6) fructan produced by *L. reuteri* 100-23, was reported to increase Treg cell proportion to modulate host immune system (Sims et al., 2011). Reuteran produced by *L. reuteri* TMW 1.106, was a component of the polysaccharides matrix in bacterial biofilm formation, which facilitated the colonization of beneficial lactobacilli in murine gut (Walter, Schwab, Loach, Gänzle, & Tannock, 2008). Reuteran or levan produced by *L. reuteri* TMW1.656 or *L. reuteri* LTH5794 prevented enterotoxigenic *Escherichia coli* adhering to erythrocyte cells (Wang, Gänzle, & Schwab, 2010). Dextran and reuteran were potential hydrocolloid alternatives in bread baking applications to improve bread volume and delay bread staling (Galle, Schwab, Dal Bello, Coffey, Gänzle, et al., 2012a, 2012b). Commercial exploitation of glycans produced by LAB in food and health applications requires information on

mechanisms and structure-function relationships of the glycans. The structure and function relationships of reuteran are not well studied. Galacto-oligosaccharides (GOS) produced by glycosyl hydrolase (β-galactosidase) of LAB, have been well characterized with respect to structure, mechanisms of activity, and potential applications. Structure and function relationships of GOS in potential commercial applications will be described in detail in the following review. The challenges and opportunities of glycans produced from LAB will be discussed using GOS as an example.

1.2. Hypothesis and objectives:

Hypotheses:

- Reuteran but not dextran prevents adhesion of enterotoxigenic *Escherichia coli* K88 to swine epithelial cells;
- Reuteran and dextran improve dough hydration, bread volume and texture;
- Technological and therapeutic functionalities of reuteran are dependent on the molecular weight and the linkage type.

Objectives:

- Review the literature on health benefits of GOS to benchmark work on reuteran against current concepts in functional oligosaccharides. Galacto-oligosaccharides were chosen as well-characterized glycans that are produced by glycosyl hydrolases from LAB (Chapter 2).
- To detect ETEC K88 adhesion in the piglet small intestinal segment perfusion model, and to determine the effect of bacterial EPS to prevent ETEC K88 adhesion *ex vivo* (Chapter 3).
- Construction of heterologous expression system of reuteransucrase and dextransucrase from *L. reuteri* TMW1.656 and *Weissella cibaria* 10M, and site-direct mutagenesis of both

glucansucrases towards the alteration of linkage type and molecular weight of reuteran and dextran (Chapter 4).

- Investigation of the structural determinants that relate to the ability of reuteran and dextran to prevent adhesion of ETEC K88 (Chapter 5).
- Investigation of the structural determinants that relate to the ability of reuteran and dextran to improve the texture of bread (Chapter 6).

1.3. References

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2. Lactose and lactose-derived oligosaccharides: more than prebiotics

2.1. Introduction

The disaccharide lactose occurs almost exclusively in the milk of mammals (Gänzle, Haase, & Jelen, 2008; Jelen, 1979). Because lactose is the major component of cheese whey; the main routes of lactose valorization rely on the purification for pharmaceutical applications or the chemical or enzymatic conversion to lactose derivatives with nutraceutical properties (Gänzle et al., 2008). Commercial lactose derivatives include galacto-oligosaccharides (GOS), lactitol, lactulose, and lactosucrose. Application development for hetero-oligosaccharides derived from lactose is currently emerging (Díez-Municio, Herrero, Olano, & Moreno, 2014; Gänzle, 2012).

The term GOS is used for β-linked oligosaccharides with a degree of polymerization (DP) of 2 to 9 that are composed of galactose and may contain one glucose unit, typically at the reducing end (Dixon, 1982; Gänzle, 2012; van Leeuwen, Kuipers, Dijkhuizen, & Kamerling, 2016). In keeping with IUPAC nomenclature, the term GOS is used to include disaccharides, however, lactose is typically excluded because it is digestible in human infants. GOS are produced by β-galactosidase (β-Gal)-catalyzed transgalactosylation with lactose as glycosyl-acceptor and -donor (Gänzle, 2012; Gosling, Stevens, Barber, Kentish, & Gras, 2010). Hetero-oligosaccharides are obtained by transgalactosylation of carbohydrates other than lactose, or by transglycosylation of lactose with enzymes other than β-Gal (reviewed by Gänzle, 2012).

GOS are low caloric and non-cariogenic, non-digestible, and prevent attachment of some pathogens to intestinal cells (Gänzle, 2012). Application development of GOS was based on human milk oligosaccharides (HMO) as the conceptual template. HMO modulate infant microbiota based on their prebiotic activity, they also prevent the adhesion of pathogens,\ and stimulate the immune systems (Bode, 2012). The structure of GOS is less complex and less

diverse when compared to HMO, however, GOS are used in infant formula to mimic the functions of HMO (Barile & Rastall, 2013).

Production, structure, and applications of GOS and other lactose derivatives have been studied for more than 4 decades; several reviews cover enzyme selection and process engineering to increase the yield of GOS (Gänzle, 2012; Gosling et al., 2010), the development of lactose derivatives (Gänzle, 2012), and prebiotic properties of GOS (Macfarlane, Steed, & Macfarlane, 2008). The structure of most compounds in commercial or experimental GOS preparations, however, has been elucidated only recently (van Leeuwen, Kuipers, Dijkhuizen, & Kamerling, 2014; van Leeuwen et al., 2016); these recent data allow a novel perspective on structure-function relationships of GOS. Moreover, current discussions on the definition of prebiotics necessitate a revision of the prebiotic activity of GOS (Bindels, Delzenne, Cani, & Walter, 2015). The increasing number of studies related to the production of hetero-oligosaccharides from lactose (Díez-Municio, Herrero, et al., 2014) also allows to produce structural and functional analogues of HMO. This review aims to summarize recent development and concepts on composition and functional properties of lactose and lactose-derived oligosaccharides.

2.2. GOS-intolerance or GOS as prebiotics?

Lactose digestion in humans relies on the brush border lactase, which is is specific for the β(1→4) linked lactose and cellobiose and thus differs from microbial β-galactosidases that also hydrolyze other GOS (Hooton, Lentle, Monro, Wickham, & Simpron, 2015; Mantei et al., 1988; Schwab & Gänzle, 2011). Lactase activity decreases with age and approximately 70% of human adults do not digest lactose; lactase activity and the ability to digest lactose persists in 30% of human adults (Corgneau et al., 2015). Undigested GOS and lactose are fermented by large

intestinal microbiota (Venema, 2012); vigorous fermentation of GOS and lactose results in formation of gas and microbial metabolites that cause intestinal discomfort, bloating and flatulence, and osmotic diarrhea (Venema, 2012). Adverse effects are observed after consumption of more than 10 – 15 g of lactose or GOS per day (Corgneau et al., 2015; Macfarlane et al., 2008; Venema, 2012). Tolerance of lactose and GOS corresponds well to the maximum tolerated dose of other non-digestible oligosaccharides, which was reported as 0.3 g / kg body weight (Oku & Nakamura, 2009). Tolerance of lactose and GOS can be increased by gradual adaptation of the intestinal microbiome (Corgneau et al., 2015; Davis, Martínez, Walter, & Hutkins, 2010). The effects of lactose and GOS fermentation are considered beneficial to host health if diarrhea is avoided (Corgneau et al., 2015; Macfarlane et al., 2008; Venema, 2012).

GOS and lactose were described as (conditional) prebiotics that exert health benefits through selective stimulation of intestinal bifidobacteria and lactobacilli (Macfarlane et al., 2008; Szilagyi, 2004; Venema, 2012). Previous definitions of the term “prebiotic”, however, were recently questioned or modified (Bindels et al., 2015; Louis, Flint, & Michel, 2016). Comprehensive analysis of intestinal microbiota through high-throughput sequencing of 16S rRNA gene fragments demonstrated that GOS consumption increased the intestinal abundance not only of *Bifidobacterium* but also of other *Firmicutes* and *Fusobacterium;* this effect varies strongly among individuals (Davis, Martínez, Walter, Goin, & Hutkins, 2011; Louis et al., 2016; Monteagudo-Mera et al., 2016). Moreover, prebiotic health benefits relate to the function rather than the composition of intestinal microbiota. Independent of selective stimulation of specific members of intestinal microbiota, lactose and GOS are metabolized to short chain fatty acids (Bruno-Barcena & Azcarate-Peril, 2015; Venema, 2012), which are major mediators of physiological benefits of dietary fibre and non-digestible oligosaccharides (Bruno-Barcena &

Azcarate-Peril, 2015; Mudgil & Barak, 2013). Acid production in the large intestine modulates the composition of gut microbiota by decreasing the intestinal pH, and may protect against intestinal pathogens (Fukuda et al., 2011). Moreover, short chain fatty acids and particularly butyrate are a main energy source for the colonic mucosa, and have anti-inflammatory properties (Bindels et al., 2015; Bruno-Barcena & Azcarate-Peril, 2015; Venema, 2012; Vinolo et al., 2011). Accordingly, the new definition of prebiotics no longer requires "specific stimulation" of bacterial taxs but emphasizes that health benefits are derived through microbial metabolism (Bindels et al., 2015; Louis et al., 2016). This definition not only includes GOS and β-fructans but generally includes non–digestible oligosaccharides and dietary fibre (Bindels et al., 2015).

It is noteworthy that GOS are not included in the definition of dietary fibre in the U.S., and that health claims for prebiotic carbohydrates including GOS are not approved in the U.S., Canada, or the European Union (Anonymous, 2016; EFSA Panel on Dietetic Products, Nutrition and Allergies, 2011 and 2014). The discussion related to prebiotic GOS, lactose intolerance, and intestinal health is further confounded by the suggestion that diets low in fermentable oligosaccharides, disaccharides, monosaccharides and polyols (FODMAPs) relieve symptoms of the irritable bowel syndrome (Gibson & Shepherd, 2005). GOS are included in the FODMAPs although the effect of GOS on the irritable bowel syndrome and intestinal barrier properties may be opposite to the effect ascribed to FODMAPs (Akbari et al., 2015, 2016; Alizadeh et al., 2016; Gibson & Shepherd, 2005). A reduction of the FODMAP intake over the long term also decreases gut bifidobacteria (Staudacher et al., 2012). A low FODMAP diet might be a short-term strategy to relief the symptoms but is not recommended as a long-term treatment option (Barrett, 2013; Halmos et al., 2015; Tuck, Muir, Barrett, & Gibson, 2014).

Adverse and health-promoting properties of dietary carbohydrates that are described with the terms “dietary fibre”, “prebiotics”, “lactose intolerance” and “FODMAP” are based on overlapping concepts and mechanisms. The recognition of adverse effects of lactose and GOS or the adjusted definition of the term prebiotic do not challenge the evidences for beneficial health effects of GOS and other non-digestible oligosaccharides. However, further application development of GOS and other lactose derivatives requires detailed functionality description based on structure identification of GOS or novel hetero-oligosaccharides.

2.3. Composition of GOS synthesized by βGal

Microbial βGal are found in the glycoside hydrolase (GH) families GH1, GH2, GH35, and GH42 (Gänzle, 2012; Gosling et al., 2010). Commercial GOS production uses of βGal from *Kluyveromyces lactis, Bacillus circulans* (Vivinal GOS), *Bifidobacterium bifidum* (Bimuno), *Aspergillus oryzae* and *Streptococcus thermophilus* (oligomate55) (Torres, Gonçalves, Teixeira, & Rodrigues, 2010). Table 1 summarizes the composition of GOS produced by commercial βGal preparations and three experimental and purified βGal. Different βGal differ in their substrate specificity and the corresponding spectrum of GOS (Table 1). Some commercial βGal preparations contain several isoforms of βGal differing in catalytic activities and specificities, for instance, βGal from *Bc. circulans* and bifidobacteria (Arreola et al., 2014; Goulas, Goulas, Tzortzis, & Gibson, 2009; Warmerdam, Paudel, Jia, Boom, & Janssen, 2013). In commercial GOS, oligosaccharides including lactose with DP2 to DP4 constitute >90% of the total solids (van Leeuwen et al., 2016). *Bc. circulans* βGal preparation generates predominantly β-(1→4) linked GOS with a high DP while *K. lactis* and GH2 LacLM type βGal produce mainly β-(1→6) linked GOS with DP 2 – 4 (Table 1). The GH2 LacZ type βGal synthesize mainly β-(1→6) and β-(1→3) linked GOS; GOS from *A. oryzae* βGal include β-(1→6), β-(1→3) and β-(1→4) linked

GOS (Table 1). The ratio of GOS with different DP and linkage types also depends on the reaction conditions. For example, Gal-β(1→4)-Gal-β(1→4)-Glc was converted to Gal-β(1→3)-Gal-β(1→4)-Glc during conversion of lactose with βGal from *A. oryzae* (Carević et al., 2016). Similarly, the main product Gal-β(1→4)-Gal-β(1→4)-Glc was decreased after 70 h while the concentration of β(1→3 or 6)-Gal-β(1→4)-Glc increased (Rodriguez-Colinas, Poveda, Jimenez-Barbero, Ballesteros, & Plou, 2012).

Table 2.1 Composition and yield of GOS produced by β-galactosidases using lactose as glycosyl donor and -acceptor

Structure	Transgalactosylation of lactose reaction mixture yield (g/100g GOS)							
	Aspergillus oryzae[a]	*Aspergillus aculeatus*[b]	*Kluyveromyces lactis*[c]	*Bacillus circulans*[d]	*LacZ* type[e]	*LacIM* type[f]	*Bifidobacterium breve*[g]
	16% - 30% of total sugars	11% of total sugars	26% - 34% of total sugars	15% of total sugars	29% of total sugars[*]	25% - 30% of total sugars[#]	19% - 33% of total sugars
	4% - 10% of total sugars	3% of total sugars	14% - 16% of total sugars	1% of total sugars	12% of total sugars[*]	12% - 14% of total sugars[#]	18% - 25% of total sugars
	30% - 59% of total sugars	76% of total sugars	7% - 49% of total sugars	43% of total sugars	10% of total sugars	15% - 22% of total sugars	5% - 30% of total sugars
	0.2-12	**26**	**10-15**	-	**2-15**	**14-19**	1-6
	1-18	**11**	**17-25**	1	**34-39**	**22-27**	**45-50**
	3	-	-	-	-	-	
	2	-	-	-	1-6	2	4-6
	7	-	-	-	6-8	1-3	2-8
	32-77	**35**	**34-48**	1	**19-25**	**22-37**	2-5
	5-7	6	1	**54**	-	-	1-4
	4-9	7	2	-	**11-15**	5-6	**19-32**
	-	-	-	4	-	-	
	5-6	3	4	-	-	-	
	-	-	-	**26**	-	-	
	-	-	-	4	-	-	

a, GOS are synthesized by commercial β-galactosidase from *A. oryzae* with 400 g/L lactose in buffer solution (Urrutia et al., 2013) or in UF-skimmed milk permeate at 40 ^{o}C (Frenzel, Zerge,

Clawin-Rädecker, & Lorenzen, 2015). GOS mixture yield of these two reactions was combined by authors of this review and presented in a range.

b, GOS are synthesized by commercial β-galactosidase from *A. aculeatus* with 400 g/L lactose in UF-skimmed milk permeate at 60 ^{o}C (Frenzel et al., 2015)

c, GOS are synthesized by commercial β-galactosidase from *K. lactis* with 400 g/L lactose in buffer solution (Rodriguez-Colinas et al., 2011) or in UF-skimmed milk permeate at 40 ^{o}C (Frenzel et al., 2015). GOS mixture yield of these two reactions was combined by authors of this review and presented in a range.

d, GOS are synthesized by commercial β-galactosidase from *B. circulans* with 400 g/L lactose in UF-skimmed milk permeate at 40 ^{o}C (Frenzel et al., 2015)

e, GOS are synthesized by heterologous expressed *LacZ* type β-galactosidases from *S. salivarius* subsp. *thermophilus* DSM 20259 (Geiger et al., *in press*) or *L. delbrueckii* subsp. *bulgaricus* DSM 20081 (Nguyen et al., 2012) with 205 g/L lactose in buffer solution at 30 ^{o}C or 37 ^{o}C. GOS mixture yield of these two β-galactosidases was combined by authors of this review and presented in a range.

f, GOS are synthesized by *LacLM* type β-galactosidases from *L. sakei* Lb790 (Iqbal et al., 2011) or *L. plantarum* WCFS1 (Iqbal, Nguyen, Nguyen, Maischberger, & Haltrich, 2010) or *L. reuteri L103* (Splechtna et al., 2006) with 205 g/L or 215 g/L lactose in diluted whey permeate or buffer solution at 30 ^{o}C or 37 ^{o}C. GOS mixture yield of these three β-galactosidases was combined by authors of this review and presented in a range.

g, GOS are synthesized by heterologous expressed β-galactosidases from *Bifidobacterium breve* DSM 20213 (Arreola et al., 2014) with 200 g/L lactose in buffer solution at 30 ^{o}C. GOS mixture yield of the β-galactosidases was combined by authors of this review and presented in a range.

[*] No separated glucose and galactose yield data is available for β-galactosidase from *L. reuteri L103*. The glucose and galactose yield was combined as 42% of total sugar that displayed in the original publication (Splechtna et al., 2006).

[#] No separated glucose and galactose yield data is available for β-galactosidase from *S. salivarius* subsp. *thermophilus* DSM 20259. The glucose and galactose yield was combined as 40% of total sugar that displayed in the original publication (Geiger et al., n.d.).

The number in bold font represented for the main GOS with a yield amount more than 10% of total GOS

2.4. Hetero-oligosaccharides synthesized by glycoside hydrolases

HMO represent 1-2% (w/v) of total human milk and play a crucial role in the development of infants (Bode, 2012; Smilowitz, Lebrilla, Mills, German, & Freeman, 2014). Most HMO contain lactose at the reducing end and are elongated with galactose and N-acetyglucosamine units to form linear or branched oligosaccharides (Smilowitz et al., 2014). HMO can be further fucosylated with α-(1→2)/(1→3)/(1→4) linkages and/or sialylated with α-(2→3)/(2→6) linkages (Bode, 2012). Variation of monomer composition, linkage type and degree of polymerization generates more than 1000 structures of HMO. HMO structure, particularly the structures of fucosylated HMO, varies among individuals and is related to the Lewis blood type of mothers (Venema, 2012). The most prevalent HMO structures are summarized by Bode (2012) and Kunz, Rudloff, Baier, Klein, & Strobel (2000).

HMO are specifically metabolized by *Bifidobacterium longum* ssp. *infantis*, however, their benefit to infant health is not limited to their bifidogenic effect. HMO prevent the attachment of pathogens to the intestinal mucosa, stimulate the immune system, and provide sialic acid as an essential nutrient for infants (Bode, 2012). Because structure and function of HMO differ substantially from GOS, recent efforts to synthesize HMO-like structures involve transglycosylation with lactose as donor or acceptor as outlined below.

2.4.1. Hetero-oligosaccharides synthesized by βGal with lactose as galactosyl-donor

Transgalactosylation by β-galactosidases from lactose to other acceptor sugars such as fructose, glucose, N-acetylglucosamine, chitosan, sucrose and sucralose yields a variety of hetero-oligosaccharides (Table 2). Lactulose (Gal-β(1→4)-Fru) is produced by chemical isomerization or enzymatic synthesis, and is applied in treatment of hepatic encephalopathy and as prebiotic (Panesar & Kumari, 2011). Lactulose production by transgalactosylation of fructose with βGal also generates allolactulose (Gal-β(1→6)-Fru) and GOS (Shen et al., 2012) (Table 2). The yield of lactulose depends on the ratio of lactose:fructose (Guerrero, Vera, Plou, & Illanes, 2011). Lactulose and allolactulose also act as galactosyl-acceptors to yield fructosyl-GOS (Guerrero, Vera, Conejeros, & Illanes, 2015). *Bc. circulans* βGal only synthesized low amount of fructosyl-GOS (Guerrero et al., 2015), the mixture contained oligosaccharides up to DP 4, including Gal-β(1→6)-lactulose and Gal-β(1→4)-lactulose (Corzo-Martínez, Copoví, Olano, Moreno, & Montilla, 2013). βGal from *K. lactis* and *K. marxianus* predominantly synthesized trisaccharides (Cardelle-Cobas, Corzo, Martínez-Villaluenga, Olano, & Villamiel, 2011; Guerrero et al., 2015). Gal-β(1→6)-lactulose and Gal-β(1→1)-lactulose were the main trisaccharides produced by βGal from *K. lactis* and *K. marxianus* (Guerrero et al., 2015).

Lactosucrose (Gal-β(1→4)-sucrose), a non-reducing trisaccharide produced from transgalactosylation of sucrose or transfructosylation of lactose (described in section 3.2), is commercially available as a low-calorie sweetener and prebiotic. Commercial βGal from *Bc. circulans* generates lactosucrose and several by-products from lactose and sucrose (Table 2). Lactosucrose is hydrolyzed during the reaction, or elongated to produce Gal-β(1→4)-lactosucrose. The lactosucrose analogue Gal-β(1→3)-sucrose, however, increased over time. Reaction time is thus important to control the yield of lactosucrose. A yield of 146 g transgalactosylated products /L was obtained with βGal from *Bc. circulans* after 4 h of reaction and a molar lactose to sucrose ratio of 1:1 (Li et al., 2009).

The core structures of HMO including lacto-N-biose (Gal-β(1→3)-GlcNAc) or N-acetyllactosamine (Gal-β(1→4)-GlcNAc, LacNAc) (Bode, 2012) were synthesized by transgalactosylation of GlcNAc with microbial βGal (Arreola et al., 2016; Bayón, Cortés, Berenguer, & Hernáiz, 2013; Black et al., 2012; Bridiau & Maugard, 2011; Schwab, Lee, Sørensen, & Gänzle, 2011) or by trans(N-acetyl)glucosaminylation (described in Section 4.2). LacNAc is the main product of transglycosylation of GlcNAc with βGal from *Bc. circulans* (Table 2); The LacNAc homologues from DP2 to DP4 are produced as by-products. The glycosidic bonds formed by *Bc. circulans* βGal are dependent on the enzyme concentration. βGal at 140 U mL^{-1} preferably synthesized LacNAc (15 g L^{-1}) as the main product but higher levels of βGal directed the reaction towards formation of allo-LacNAc (Bridiau & Maugard, 2011). Unlike β-(1→4) linked LacNAc, the β-(1→3) linked lacto-N-biose is not commonly synthesized as main product. Microbial βGal favour formation of β-(1→6) and/or β(1→4) linkages (Arreola et al., 2016); however, recombinant βGal-3 from *Bc. circulans* forms lacto-N-biose as the main product (Bayón et al., 2013).

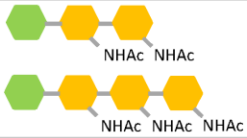
Chitin-oligosaccharides [$(GlcNAc)_n$ or $(GlcN)_n$] are also suitable acceptor carbohydrates for microbial βGal to form galactosylated GlcNAc- or GlcN oligosaccharides (Black et al., 2014). Biosynthesis of lacto-N-neotetraose (Gal-β(1→4)-GlcNAc-β(1→3)-Gal-β(1→4)-Glc) was achieved with a di-enzyme system using chitinbiose and lactose as substrates. GlcNAc-β(1→3)-Gal-β(1→4)-Glc, generated by transferring a GlcNAc moiety to lactose by βN-acetylhexosaminidase, was further galactosylated by *Bc. circulans* βGal (Zeuner, Nyffenegger, Mikkelsen, & Meyer, 2016). Alternative chemo-enzymatic routes for production of lacto-N-biose do not employ lactose as substrate (Yu et al., 2010; Li & Kim, 2014).

βGal-catalysed transgalactosylation of other acceptor sugars including mannose, galactosamine, fucose and sialic acid also generates hetero-oligosaccharides (Table 2) (Arreola et al., 2016; Schwab et al., 2011). Sucralose, an artificial sweetener derived from sucrose with several hydroxyl group substituted by chlorine, is also transgalactosylated by βGal; the reaction generated 41% Gal-β(1→6)-sucralose (Lu et al., 2012). This new product may combine properties of a sweetener and a prebiotic (Lu et al., 2012). UDP-activated sugars with a DP ranging from 2 to 4 were synthesized by *Bc. circulans* βGal and UDP-glucose, UDP-GlcNAc or UDP-GalNAc as acceptor sugars (Kamerke, Pattky, Huhn, & Elling, 2012, 2013). All transgalactosylation reactions with lactose as donor accumulate glucose in the reaction mixture. Isomerization after transgalactosylation effectively converts glucose and GOS to fructose, tagatose, lactulose and a variety of lactulose derived oligosaccharides (Cardelle-Cobas, Corzo, Villamiel, & Olano, 2008; Padilla et al., 2015).

Optimization of transgalactosylation of acceptor sugars is comparable to GOS optimization; reaction temperature, time, pH, substrate concentration and water activity influence the product yield and composition (Guerrero et al., 2015). An additional parameter for synthesizing hetero-

oligosaccharides and minimizing GOS formation is the ratio of galactosyl-donor to –acceptor. A maximum yield of lactulose, 0.28 g lactulose / g lactose, was obtained with a molar lactose to fructose ratio of 1:8 (Guerrero et al., 2011) but the highest yield of galactosylated GlcNAc was obtained when the ratio of GlcNAc to lactose was 1:1 (Guerrero et al., 2015). The affinity of different microbial βGal for acceptor sugars also influences the yield of hetero-oligosaccharides. βGal from *Lactobacillus* and *Bifidobacterium* prefer glucose and GlcNAc over lactose as galactosyl-acceptors; GalNAc and fucose are weak acceptors (Arreola et al., 2016). βGal from *K. lactis*, *A. aculeatus*, *A. niger*, *A. oryzae* preferentially galactosylate lactulose over lactose while βGal from *Bc. circulans* prefers lactose as galactosyl-acceptor (Guerrero et al., 2015). In conclusion, the molar ratio of galactosyl-acceptor to -donor and the choice of acceptor sugar and enzyme influence the yield hetero-oligosaccharides.

Table 2.2 Hetero-GOS synthesized using β-galactosidases and lactose or lactulose as donor sugar

Enzyme source	donor	acceptor	HOS structure
ß-galactosidase			
A. oryzae, *K. lactis*, *P. furiosus*[1]	lactose	fructose	
A. oryzae, *A. niger*, *A. aculeatus*, *B. circulans*[2] *K. lactis*[2, 3] *K. marxianus*[3]	lactose lactulose	lactulose	
B. circulans[4]	lactose	UDP-glucose UDP-glucosamine	
L. plantarum[5]	lactose	chitinbiose chitintriose	
K. lactis, *B. circulans*[6] *L. bulgaricus*, *B. breve*[7]	lactose	GlcNAc	
B. circulans[8]	lactose	sucrose	
L. bulgaricus[9]	lactose	sucralose	 Sucrose α or β..
ß-galactosidase + isomerization			
K. lactis[10, 11] *K. marxianus*[11]	lactose		

1, (H. Wang, Yang, Hua, Zhao, & Zhang, 2013); 2, (Guerrero, Vera, Conejeros, & Illanes, 2015); 3, (Padilla et al., 2012); 4, (Kamerke, Pattky, Huhn, & Elling, 2012); 5, (Black et al., 2014); 6, (Bridiau & Maugard, 2011); 7, (Arreola et al., 2016); 8, (Wei Li et al., 2009); 9, (Lu et al., 2012); 10, (Cardelle-Cobas, Corzo, Villamiel, & Olano, 2008; Padilla et al., 2015); 11, (Padilla et al., 2015).

[#] proposed structure based on literature content.

2.4.2. Hetero-oligosaccharides synthesized with lactose as acceptor

Lactose is a suitable acceptor carbohydrate for various glycoside hydrolases, allowing the synthesis of hetero-oligosaccharides by transglycosylation with lactose acceptor. An overview of the diversity of oligosaccharides produced by this enzymatic route is provided in Table 3.

Acidic HMO are sialylated with N-acetylneuraminic acid (Neu5Ac) to form 3'/6' linked oligosaccharides (ten Bruggencate, Bovee-Oudenhoven, Feitsma, van Hoffen, & Schoterman, 2014). Sialic acid is a group of neuraminic acid with N- or O-substitutions. Sialic acid substrates include the bovine κ-casein-derived glycomacropeptide which contains mainly Neu5Ac (Wang et al., 2015; Wilbrink et al., 2014), glycoproteins from bovine blood plasma (45% Neu5Ac, 55% Neu5Gc) (Wilbrink et al., 2015) and fetal calf serum fetuin (Lee, Shin, & Kim, 2002). Neu5Ac is more suitable for food applications than Neu5Gc as the latter has been linked to immune problems in humans (Padler-Karavani et al., 2008). Sialidase and trans-sialidase transfer sialic acid from donor carbohydrates to lactose or GOS. Trans-sialidase is homologous to sialidase but exhibits increased transferase activity (Paris et al., 2005). Trans-sialidases are mostly found in *Trypanosoma* spp., the trans-sialidase from *Trypanosoma cruzi* belongs to GH33. Neu5Ac or Neu5Gc are transferred as α-(2→3)linked monomers to terminal galactosyl unit of lactose or GOS (Wilbrink et al., 2014, 2015), or to internal galactosyl units of Gal-β(1→6)-lactose (Wilbrink et al., 2014). Disialylation of GOS is also catalyzed (Wilbrink et al., 2015). Shorter GOS are better acceptors than longer GOS (Holck et al., 2014). Trans-sialidase produced 40 mg 3'-sialylactose / g lactose with a high transfer efficiency from the donor glycoside when lactose was present in large excess (Holck et al., 2014; Wilbrink et al., 2014).

Transfructosylation of lactose by fructansucrase yields lactosucrose. β-Fructofuranosidase of *Microbacterium saccharophilum* K-I belongs to GH68 and is homologous to levansucrases

(Tonozuka et al., 2012). This enzyme acted as hydrolase when sucrose is the sole substrate but synthesize lactosucrose when lactose is present (Tonozuka et al., 2012). Levansucrase and inulosucrase are also GH68 enzymes and produce β-(2→6) or β-(2→1) linkages, respectively. Microbial levansucrases also produce lactosucrose with lactose as acceptor; a yield of 224 g lactosucrose L^{-1} was obtained with levansucrase of *L. mesenteroides* (Li et al., 2015). Inulosucrase prefers lactosucrose over lactose as acceptor and transfructosylates lactosucrose to yield β-(2→1) linked lactosyl-oligofructosides (Díez-Municio et al., 2015).

Glucansucrases belong to GH70; enzymes were characterized in the genera *Leuconostoc*, *Lactobacillus*, *Streptococcus* and *Weissella*. Dextransucrases from *Leuconostoc* and *Weissella* synthesize α-(1→6) linked polymers from sucrose; the reaction is shifted to oligosaccharide synthesis in presence of suitable acceptor sugars. Transglucosylation of lactose by dextransucrase yields Glc-α(1→2)-lactose as main product (Díez-Municio et al., 2012; Shi et al., 2016). Glc-α(1→2)-lactose was applied for kojibiose synthesis (Díez-Municio, Montilla, Moreno, & Herrero, 2014), the α(1→2) linkage is not digested in the human intestinal tract (García-Cayuela et al., 2014). In order to decrease dextran production and increase the yield of acceptor products, high concentration of lactose and sucrose (ratio at 1) were applied (Díez-Municio et al., 2012).

The backbone of HMO is usually fucosylated with α-(1→2), α-(1→3) or α-(1→4) linkages and 2'-fucosyllactose (Fuc-α(1→2)-lactose) is the main fucosylated sugar in HMOs. Fucosylated HMO analogues are synthesized with GH29 α-fucosidases. Transfucosylation of lactose or LacNAc from *para*-nitrophenyl-fucose (pNP-Fuc) by α-fucosidase from *Thermotoga maritime* yielded α-(1→2), α-(1→3) or α-(1→6) fucosylated HMO analogues (Osanjo et al., 2007). *Alcaligenes* sp. α-fucosidase synthesized Fuc-α(1→3)-lactose or Fuc-α(1→3)-LacNAc with

lactose or LacNAc as acceptor sugar in reactions with a high acceptor to donor ratio (around 33:1) (Murata, Morimoto, Zeng, Watanabe, & Usui, 1999). Similar reactions catalyzed by porcine liver α-fucosidase yielded α-(1→2), α-(1→3) and α-(1→6) linked fucosyl-lactose and fucosyl-LacNAc (Murata et al., 1999). Several novel α-fucosidases were identified in a soil-derived metagenomic library and used to synthesize 2'-fucosyllactose from pNP-Fuc and lactose (Lezyk et al., 2016). pNP-Fuc is a preferred fucosyl-donor but transfucosylation from this donor releases the toxic *para*-nitrophenol. A natural fucose-containing xyloglucan was used as alternative fucosyl donor to synthesize 2'-fucosyllactose with a yield of up to 3.6% (Lezyk et al., 2016).

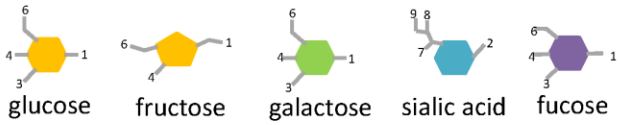
Bacterial N-acetylglucosaminidases belong to glycosyide hydrolase family GH20 are found in *Aspergillus oryzae*, *Nocardia orientalis* and some soil bacteria (Nyffenegger et al., 2015; Matsuo et al., 2003; Murata, Tashiro, Itoh, & Usui, 1997). N-acetylglucosaminidases transfer GlcNAc residues onto lactose and usually synthesize GlcNAc-β(1→3)-Lac and GlcNAc-β(1→6)-Lac from pNP-GlcNAc with low yield (Matsuo et al., 2003). The N-acetylglucosaminidases HEX1 and HEX2 originated from soil-derived metagenomic library, however, use chitin-oligosaccharides with DP2-4 to synthesize GlcNAc-β(1→3)-Lac (Nyffenegger et al., 2015). Moreover, the N-acetylglucosaminidases HEX1 and HEX2 also use glucose, galactose, sucrose and maltose as acceptor sugars (Nyffenegger et al., 2015).

Sialidases, glucansucrases, fructansucrases, α-fucosidases and N-acetylglucosaminidases are retaining glycoside hydrolases with transglycosylation activity to transfer corresponding sugar moieties to acceptor sugars such as lactose, to generate a large variety of hetero-oligosaccharides, which include several core structures of HMO, HMO-analogues. However, most of these glycoside hydrolases with weak transferase activities hindered a large-scaled synthesis of hetero-oligosaccharides. Site-directed engineering of trans-sialidase from *Trypanosoma rangeli* and

α-fucosidase from *Thermotoga maritime* increased the efficiency of transglycosylation by these glycoside hydrolases (Osanjo et al., 2007; Saumonneau et al., 2016; Zeuner, Luo, Nyffenegger, Aumala, Mikkelsen, & Meyer, 2014). This provides a strategy to increase transferase activities of other glycoside hydrolases by mutating the amino acids located at hydrolysis-related subsites.

Table 2.3 Hetero-GOS synthesized glycosyl hydrolases and lactose or GOS as acceptor sugar

Enzyme source	donor	acceptor	HOS structure
trans-sialidase			
Trypanosoma cruzi (TcTS)[1, 2, 3]	cGMP	lactose	
		3'Gal-lactose	
	cGMP	4'Gal-lactose	
		6'Gal-lactose	
	cGMP/BPG*	GOS	
Pasteurella multocida[4]	cGMP	lactose	
glucansucrase			
Leuconostoc mesenteroides[6] *Weissella. confusa*[7]	sucrose	lactose/cheese whey permeate	
fructansucrase			
L. mesenteroides[8, 9]; *B. methylotrophicus*[10]	sucrose	lactose	
B. subtilis + *L. gasseri*[11]			
fucosidase			
Alcaligenes sp.[12]	fucose-pNP^	lactose	
		lacNAc	



α linkage

β(1→1/3/4/6) linkage

*cGMP, Bovine κ-casein-derived glycomacropeptide; BPG, Bovine blood plasma glycoprotein

^fucose-pNP, *p*-nitrophenyl α-L-fucopyranoside

1, (Holck et al., 2014); 2, (Wilbrink et al., 2014); 3, (Wilbrink et al., 2015); 4, (Guo et al., 2014); 5, (Wang et al., 2015); 6, (Díez-Municio et al., 2012; Shi et al., 2016); 7, (Shi et al., 2016); 8, (Díez-Municio et al., 2012); 9, (Li et al., 2015; Wu, Zhang, Mu, Miao, & Jiang, 2015); 10, (Wu et al., 2015); 11, (Díez-Municio et al., 2015); 12, (Murata, Morimoto, Zeng, Watanabe, & Usui, 1999)

2.5. Immune modulation by GOS and hetero-oligosaccharides

The immunomodulation effects of GOS are indirect and mediated by shifting the composition of gut microbiota and their metabolites (Oozeer et al., 2013), or are mediated through direct interaction of oligosaccharides with the gut-associated immune system. Evidence for immunological effects of GOS was provided by a single report of GOS triggered IgE-mediated anaphylaxis in Asian atopic patients (Chiang et al., 2012). The direct effect of GOS on the gut immune system and the intestinal barrier function, however, were mostly determined *in vitro*.

GOS are TLR4 ligands of immune cells such as intestinal epithelial cells, macrophages and dendritic cells (Lehmann et al., 2015; Ortega-González et al., 2014; Searle et al., 2012). GOS activate the TLR4-NFκB pathway, which also responds to LPS activation with production of pro-inflammatory cytokines. Stimulation of the TLR4-NFκB by GOS produces a comparable patterns of cytokines but is less efficient than stimulation by LPS, which may contribute to gut homeostasis rather than an inflammatory response (Ortega-González et al., 2014). Activation of the TLR4-NFκB by GOS is likely to induce naïve T cell develop to regulatory Treg cell (Lehmann et al., 2015; Verheijden et al., 2016). GOS increase immune tolerance in a dose and DP dependent manner (Lehmann et al., 2015; Searle et al., 2012; Vendrig, Coffeng, & Fink-Gremmels, 2013). *In vitro* experiments indicated GOS that higher than DP 3 were the primary stimulants to murine macrophages (Searle et al., 2012). Similar to GOS, the hetero-oligosaccharides may also stimulate the immune system directly (Vendrig et al., 2013). 3'-Sialyllactose modulated the immune response by activating PGlyRP3 receptor on Caco-2 cell (non-TLR4 cell) (Zenhom et al., 2011).

GOS strengthen the integrity of the intestinal barrier. GOS increased the expression of mucin and enterocyte-associated sucrase in the small intestine (Leforestier et al., 2009). Administration

of GOS was also effective in preventing deoxynivalenol induced impairment of the epithelial barrier (Akbari et al., 2015, 2016). Modulation of intestinal barrier junction by GOS could be partially due to a direct stimulation of goblet cells (Bhatia et al., 2015), however, the mechanism is not well-defined.

Table 2.4. *In vivo* studies of identified therapeutic oligosaccharides that prevent or reduce pathogen adherence by the adhesin-receptor interaction

Toxin/fimbriae	host/ experimental host	Receptor	Inhibitory Glycans	Source
		E. coli		
Heat stable toxin a (STa)	Human/infants	Guanyl cyclase C[a]	2'-fucosyllactose[b]	Human milk
K99 Fimbriae	Pig, cattle/calve	Mucin glycopeptides[c]	glycoprotein glycans[d]	Bovine plasma
K88 Fimbriae	Pig/piglet	sialoglycoproteins (IMTGP-1/2); Intestinal transferrin (GP74); neolactotetraosylceramide	Casein glycomacropeptide[e)]	Milk
Shiga-like toxin (Stx2, Stx2d)	Human/Mice	Globotriaosylceramide (Gb_3)[f]	Gal-α(1→4)-Gal-β(1→4)-Glc[g]	Synthetic
		Campylobacter jejuni		
Fibronectin - binding protein (CadF, FlpA)	Human/mice and ex vivo human intestinal mucosal cells	Fibronectin[h]	fucosylated human milk oligosaccharide[i]	Human Milk
		Vibrio cholerae		
Cholera toxin	Human/ex vivo rabbit intestinal loop	Ganglioside G_{m1}[j]	3'-Sialyllactose[k]	Human milk
		Clostridium botulinum		
Neurotoxin A	Human/mice	Glycoprotein 2[l]	IPTG[*], Lactulose[m]	Synthetic
		Salmonella enterica		
Type 1 fimbriae FimH	A variety of mammals such as humans, cattle, poultry/mice	Glycoprotein[n]	Sialyloligosaccharides, asialo-oligosaccharides, sialylglycopeptide[o]	Chicken egg yolk
		Listeria monocytogenes		
Listeria adhesion protein (LAP)	Human, rabbit/Guinea pig	Stress response protein Hsp60[p]	Xylooligosaccharides, GOS[q]	enzymatic synthesis

[*]IPTG, isopropyl-β-D-thiogalacto-pyranoside

a, Giannella & Mann, 2003; b, Morrow, Ruiz-Palacios, Jiang, & Newburg, 2005); c (Lindahl & Carlstedt, 1990); d (Mouricout, Petit, Carias, & Julien, 1990); e (Moonens et al., 2015); f

(Karmali, 2004); g (Mulvey et al., 2003); h (Flanagan, Neal-McKinney, Dhillon, Miller, & Konkel, 2009); i (Ruiz-Palacios, Cervantes, Ramos, Chavez-Munguia, & Newburg, 2003); j (Pan & Charych, 1997); k (Idota, Kawakami, Murakami, & Sugawara, 1995); l (Matsumura et al., 2015); m (Lee et al., 2013, 2015); n (Grzymajło et al., 2013); o (Sugita-Konishi et al., 2002); p (Burkholder & Bhunia, 2010); q (Ebersbach et al., 2010)

2.6. Prevention of pathogen adhesion by GOS and lactose-derivatives

Intoxication or infection by intestinal pathogens is generally initiated by binding of toxins or pathogens to specific carbohydrate signatures on the surface of the intestinal mucosa (Shoaf-Sweeney & Hutkins, 2009; Kulkarni, Weiss, & Iyer, 2010). Glycan recognition by pathogens or toxins is highly specific for specific pathogens, and also mediates specificity for the host species. For example, due to the specific interaction of bacterial glycan binding proteins and host receptors, enterotoxigenic *Escherichia coli* are host specific and infect different hosts with different fimbriae (Grange, Mouricout, Levery, Francis, & Erickson, 2002; Li, Poole, Rasulova, McVeigh, Savarino, & Xia, 2007). Likewise, the species- and tissue specificity of the Shiga-toxins is determined by the recognition of globotriaosylceramide on the cellular surface (Johannes & Römer, 2010). Shiga-toxin Stx2e also recognises globotetraosylceramide and is the only toxin variant known to cause disease in swine (Johannes & Römer, 2010). Human milk oligosaccharides are structurally similar to glycan receptors that mediate pathogen adhesion; competitive inhibition of bacterial glycan binding proteins by HMO or analogous oligosaccharides prevents infection or intoxication (Martin-Sosa, Martin & Hueso, 2002; Shoaf-Sweeney & Hutkins, 2009, Hickey, 2012). The constant dietary intake of oligosaccharides prevents the establishment of infection or colonization; the large diversity of oligosaccharides structure provides protection against a broad range of pathogens and toxins (Hickey, 2012). The

use of dietary glycans as receptor analogues may also prevent bacterial infections. A large diversity of natural or synthetic oligosaccharides including GOS were evaluated with respect to their ability to prevent pathogen adhesion *in vitro* (Shoaf-Sweeney & Hutkins, 2009; Jin & Zhao, 2000; Sinclair, de Slegte, Gibson, & Rastall, 2009); however, only few studies provide confirmation of a protective effect *in vivo*. Because the recognition of host glycans by bacterial pathogens is typically specific for the host species, the use of animal models for validation of glycans for human therapy is inherently challenging. Moreover, some pathogens express multiple alternative adhesins. For example, *Salmonella enterica* adheres with fimbriae-type, pilli-type, auto-transporter adhesins (Wagner & Hensel, 2011) and the use of a single oligosaccharide may not suffice to prevent adhesion to the intestinal mucosa.

Several *in vivo* studies nevertheless provide proof of concept that dietary oligosaccharides can be effective tools to prevent infection or intoxication (Table 4). A majority of the successful studies employed human milk oligosaccharides or lactose-derived oligosaccharides (Table 4). However, many of the *in vivo* studies that reported prevention of infection by dietary oligosaccharides did not confirm the mechanism of action. Therefore, protective effect of oligosaccharides may result from binding to glycan-binding domains of bacterial adhesins or toxins, from modulation of intestinal microbiota, or from immunomodulating effect of oligosaccharides (Laparra, Hernandez-Hernandez, Moreno, & Sanz, 2013). Egg yolk derived sialyloligosaccharides inhibited the binding of *S. enterica* but did not influence the production of TNF-α by macrophages (Sugita-Konishi et al., 2002). Convincing evidence for the use of oligosaccharides as receptor decoys with high specificities was provided for the use of lactulose or IPTG to prevent intoxication with the *Clostridium botulinum* neurotoxins (Lee et al., 2013, 2015). The host- and tissue specificity of botulinum neurotoxins is mediated by glycan-lectin

interactions. The botulinum neurotoxin is secreted in a complex with heme agglutinins and non-heme-non-toxic proteins. The first step in toxin activity is binding of the heme agglutinins to the intestinal mucosa via glycan recognition; this binding step contributes to the specificity of the individual toxin types for the host species (Fujinaga, Sugawara, & Matsumura, 2013). Lactulose binds to the glycan-binding domain of the heme agglutinin complex and prevents toxin binding and internalization *in vitro* and *in vivo* (Lee et al., 2013, 2015).

2.7. Conclusion

GOS synthesized from transgalactosylation of lactose by βGal are available as a mixture of glucose, galactose, lactose, and GOS. GOS produced by different enzymes differ in their composition. Enzymatic synthesis of oligosaccharides yields structurally diverse hetero-oligosaccharides by transferring galactose from lactose to suitable acceptor carbohydrates such as fucose and GlcNAc, or by transferring sialyl-, glucosyl-, fructosyl-, or fucosyl-units to lactose. Hetero-oligosaccharides produced by transglycosylation include components of human milk oligosaccharides. The characterization of commercial and experimental GOS and other lactose-derived oligosaccharides increasingly allows the elucidation of structure function relationships, and the development of new applications.

Application development of GOS, lactulose and lactosucrose has focused on prebiotic effects. However, prebiotic effects do not differentiate GOS from other non-digestible oligosaccharides. Moreover, a majority of humans is lactose intolerant; in these individuals, lactose exerts comparable prebiotic effects. GOS and other lactose-derived oligosaccharides also exhibit potent biological activities that are highly specific to the oligosaccharide structure. These activities include the binding to glycan-binding proteins of pathogens or glycan-binding domains or toxins, and direct immunomodulation. These activities were not only demonstrated for analogues of

human milk oligosaccharides, fucosyllactose and sialyllactose, but also for other lactose-derived oligosaccharides.

2.8. References

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3. Exopolysaccharides synthesised by *Lactobacillus reuteri* protect against enterotoxigenic *Escherichia coli* in piglets

3.1. Introduction

Enterotoxigenic *E. coli* (ETEC) infects humans and animals. ETEC infection is a major cause of traveler's and infant diarrhea (DuPont, 1995; Qadri, Svennerholm, Faruque, & Sack, 2005). ETEC also infects young farm animals such as calves and piglets (Foster & Smith, 2009), leading to economic losses due to mortality, morbidity, decreased growth rate, and cost of medication (Fairbrother, Nadeau, & Gyles, 2005). ETEC colonizes the small intestine by fimbriae that attach to intestinal epithelial glycoprotein receptors (Kulkarni, Weiss, & Iyer, 2010). The subsequent secretion of heat-stable and/or heat labile enterotoxins leads to a disruption of electrolyte balance and diarrhea (Dubreuil, 2012; Ondrackova, Alexa, Matiasovic, Volf, & Faldyna, 2012; Zhou, Liu, Jiang, Yu, & Zhang, 2012). Adhesion to mucosal tissue is host specific. Human ETEC strains adhere with fimbriae termed colonization factor antigen I and coli surface antigens 1 to 6 (Levine, Giron, & Noriega, 1994). Porcine strains of ETEC adhere with K88 (F4) and F18 fimbriae; strains expressing K88 and F18 fimbriae account for 93% of ETEC infections in piglets (Frydendahl, 2002; Grange, Parrish, & Erickson, 2006).

Antimicrobial growth promoters or therapeutic antibiotics are widely used to prevent or treat diarrhea of farm animals (Docic & Bilkei, 2003). The use of antibiotics, however, supports the development of antibiotic resistance (Docic & Bilkei, 2003). This resistance leads to more severe postweaning syndromes (Casewell, Friis, Marco, McMullin, & Phillips, 2003) and contributes to the transfer of antibiotic resistance to human pathogens (Casewell et al., 2003). Therefore, alternatives are needed to control ETEC in swine. The multitude of colonization factors expressed by ETEC complicates vaccine development (Nataro & Kaper, 1998). Feed

supplementation with egg yolk antibodies from chickens immunized with K88 or F18 adhesins, dietary organic acids, phage therapy, or the use probiotics may allow the control of ETEC in pig production (Joerger, 2003). The inhibition of bacterial adherence via receptor analogs of epithelial glycoprotein is a promising method to prevent ETEC infection (Shoaf-Sweeney & Hutkins, 2008). Inulin and fructo-oligosaccharides reduced the adherence of enteropathogenic *E. coli* to Caco-2 and Hep-2 tissue culture cells (Shoaf-Sweeney, Mulvey, Armstrong, & Hutkins, 2006). Polysaccharides from natural fermented green olive brines reduced the adhesion of ETEC expressing K88 fimbriae (ETEC K88) to IPEC-J2 cell-lines (González-Ortiz, Hermes, Jiménez-Díaz, Pérez, & Martín-Orúe, 2013). Reuteran and levan produced by *Lactobacillus reuteri* strains TMW1.656 and LTH5794 prevented hemagglutination by ETEC K88 (Wang, Gänzle, & Schwab, 2010). However, only a few *in vivo* studies indicated that selected glycans decrease illness incidence in piglets. For example, dietary mannan oligosaccharides promoted the growth of nursery piglets in three farms (Rozeboom et al., 2005), and chitooligosaccharide increased growth of weaning piglets and decreased the incidence of diarrhea (Liu et al., 2008). The limited number of animal studies, however, hampers practical application of dietary glycans to prevent pathogen adhesion. Moreover, prevention of pathogen adhesion has not been demonstrated as the mechanism that was responsible for the beneficial effects observed *in vivo*.

It was therefore the aim of this study to determine whether reuteran and levan, exopolysaccharides (EPSs) that prevented adhesion of ETEC to porcine erythrocytes (Wang et al., 2010), also reduce pathogen adhesion and ETEC-induced diarrhea in swine. The piglet small intestinal segment perfusion (SISP) model was used to quantify adhesion of ETEC as well as ETEC induced fluid accumulation. The SISP model has been developed to quantify the fluid- and electrolyte loss caused by infection with diarrheal pathogens (Kuller et al., 2007; Nabuurs,

Hoogendoorn, Van Zijderveld, & Van der Klis, 1993; Van der Meulen & Jansman, 2010). The model may also be suitable for investigation of the potential role of dietary glycan in the prevention of pathogen adhesion *in vivo*. The SISP model maintains the blood supply of the intestinal segments and allows sampling over time, and each animal can be used as its own control. To confirm that the antidiarrheal effects are attributable to the prevention of pathogen adhesion, microbiota in the intestinal segments were characterized by quantitative PCR (qPCR), high-resolution melting curve qPCR (HRM-qPCR), and fluorescent *in situ* hybridization (FISH).

3.2. Materials and methods

3.2.1 Strains and culture conditions

Two ETEC strains carrying the K88 antigen were obtained from the *Escherichia coli* Laboratory at the University of Montréal. *E. coli* ECL13795 (O149, virotype STb:LT:EAST1:F4) and ECL13998 (O149, virotype STa:STb:LT:EAST1:F4:Paa) were cultivated on Minca agar (Guinée, Veldkamp, & Jansen, 1977) at 37 °C overnight. Strain identity was confirmed by PCR targeting the genes for K88 fimbriae, heat-labile (LT) and heat stable (ST) toxins. Cell suspensions from Minca agar were characterized with respect to the optical density at 600 nm (OD_{600}) and cell counts to establish that an OD_{600} of 1.2 corresponds to a cell count of 10^9 CFU/mL for both strains. Exopolysaccharide producing *L. reuteri* strains TMW1.656 and LTH5794 were cultivated overnight at 37 °C on modified DeMan-Rogosa-Sharpe (mMRS) agar containing 100 g/L sucrose as the sole carbon source. Single colonies were subcultured in 10 mL sucrose-mMRS broth for inoculation of 1 L sucrose-mMRS broth and incubation overnight.

3.2.2 Purification of exopolysaccharides

Reuteran produced by *L. reuteri* TMW1.656 and levan produced by *L. reuteri* LTH5794 were harvested via ethanol precipitation and purified by dialysis and hot phenol extraction as described previously (Wang et al., 2010). In brief, the culture supernatant from 1 L of cultures grown overnight in sucrose-mMRS was obtained by centrifugation and 2 volumes of chilled ethanol were added to the supernatant. Exopolysaccharides were precipitated overnight at 4 °C, harvested by centrifugation, and dialyzed by using membrane tubing with a molecular weight cut off of 12,000 to 14,000 (Spectra/Por 2 membrane tubing, Spectrum Laboratories Inc., Rancho Dominguez, CA, USA) at 4 °C for 3 days. The retentate was freeze dried, redissolved in Milli-Q water to a concentration of 20 g/L, and mixed with an equal volume of UltraPure buffer-saturated phenol (Invitrogen, Burlington, ON, Canada). The mixture was incubated in a water bath at 70 °C for 70 min, cooled on ice for at least 30 min, and centrifuged at 3,000 × g for 20 min at 4 °C. The aqueous layer was collected and dialyzed as described above for 4 days to remove phenol. Samples were freeze-dried and kept at -20 °C. Dextran from *Leuconostoc mesenteroides* and inulin from chicory were obtained from Sigma-Aldrich (Oakville, ON, Canada).

3.2.3 Hemagglutination

Cultures of *E. coli* ECL13795 and ECL13998 grown overnight on Minca agar (Guinée et al., 1977) were washed with 1.5 mL of phosphate-buffered saline (PBS, pH 7.2). Hemagglutination tests were conducted as described previously (Wang et al., 2010). Twenty-five microliter ETEC suspensions were diluted 2-fold in V-bottom 96-well polystyrene microtiter plates (Corning). The same volume of PBS or PBS containing EPS at concentration of 10 g/L was added, and the suspensions were incubated for 5 min with gentle shaking. Erythrocytes were harvested by washing porcine whole blood (Innovative Research, Novi, USA) three times in PBS and

suspended in PBS at 5% (vol/vol). Twenty-five microliters of the 5% erythrocyte suspension was applied to each well. Microtiter plates were mixed gently and visually inspected after 2 h of incubation at 4 °C. Triplicate independent replicates were conducted.

3.2.4 Animals and surgical procedure

All animal procedures were conducted in accordance with the guidelines of the Canadian Council on Animal Care, and were approved by the Animal Care and Use Committee: Livestock of the University of Alberta. Six crossbred weanling gilts (Duroc × Large White/Landrace F1, aged 5 – 6 wk, 10.2 ± 0.7 kg BW) with no history of gastrointestinal disturbances were randomly selected for use in the SISP model (Nabuurs et al., 1993). Pigs were group housed and provided with free access to a commercial starter diet until 16 h before surgery, when pigs were isolated and fasted overnight.

Prior to the surgical procedure, pigs were tranquillized. Once a sufficient depth of anesthesia was reached, pigs were intubated and remained under general anesthesia (4% v/v isoflurane; Abbott Animal Health, Abbott Park, IL, U.S.A.) and oxygen (5 - 10 mL/kg/min L O_2 per min) for the duration of the experiment. The surgical site was cleaned, a midline incision was created, and the underlying mesentery was removed to expose the intestine. An incision was made across the intestine without cutting the mesentery; a second incision was made 20 cm distal to the first incision, again keeping the mesentery intact. This 20-cm section of intestine served as the first segment; nine additional 20-cm segments were created along the median 50% of the jejunum. Between each pair of odd and even segment, a 2-cm segment was created for later measurement of intestinal circumference. Inflow tubes and outflow tubes were attached to each intestinal segment. Inflow tubes were connected to 100-mL syringes containing the assigned treatments, and outflow tubes were connected to drainage bottles for collection of net fluid losses.

3.2.5. Experimental design and sample collection

Standardized cell suspensions of *E. coli* ECL13795 were obtained by washing cultures grown overnight on Minca plates with PBS; the OD_{600} of cell suspensions was adjusted to 1.2. Five milliliter of the ETEC cell suspension was introduced into the lumen of odd segments and 5 mL PBS were introduced into even segments as pair-matching segments. Fifteen minutes after injection, perfusion into segments was conducted with 64 mL PBS (pH = 6.6) containing reuteran, levan, dextran, or inulin at 10 g/L each or saline (control) for 8 h. Glycans were allotted to 4 pairs (reuteran, levan, dextran or inulin) of ETEC-infected and control segments in a 4-by-4 Latin square to ensure each glycan was rotated in different segment-pairs among four piglets. Nonabsorbed fluid from each segment was collected into the drainage bottles. At the end of experiment, the pig was killed, segments were opened longitudinally, and tissue samples were collected for analysis of morphology and ETEC counts. Morphological samples with an area of 0.5-1 cm^2 were transferred into a chilled Carnoy solution (ethanol-acetic acid-chloroform, 6: 6: 1) immediately and stored at 4 °C for later assessment. Mucosal scrapings samples for DNA extraction were obtained by scraping the surface area of intestinal segments (8 to 9 by 4 to 5 cm) with a glass slide. Scrapings samples were flash frozen in liquid nitrogen immediately after collection and stored at -80 °C. Prior to DNA extraction, samples were thawed, weighted, and suspended in 1 mL TN150 buffer. Outflow fluid samples were stored at -20 °C.

3.2.6. Genomic DNA extraction and quantitative PCR analysis of intestinal bacteria

Bacterial DNA was extracted from mucosal scraping and outflow fluid samples by using a DNA Stool Mini Kit and a DNeasy Blood & Tissue Kit (Qiagen, Inc., Valencia, CA, USA), respectively. DNA concentration and quality were checked on a NanoDrop ND-2000 spectrophotometer system (Fisher Scientific, Wilmington, DE, USA). Quantitative PCR was

performed on a 7500 Fast real-time PCR System (Applied Biosystems, Foster City, CA, USA) with primers targeting the eubacterial 16S rRNA genes, or genes coding for K88 fimbriae and LT (Table 3.1). To generate standard curves for quantification of genes, the respective genes were amplified from genomic DNA of *E. coli* with the same primer pair, purified, and the concentration was determined by UV-spectroscopy. Tenfold serial dilutions of the amplicons were used to generate standard curves for absolute quantification. Standard curves for quantification of eubacteria and *E. coli* were generated with amplicons that were obtained from mucosal and outflow fluid DNA. DNA isolated from mucosal and outflow samples was diluted to a concentration of 50 mg/L and analyzed in duplicate in MicroAmp Fast Optical 96-well reaction plates capped with MicroAmp Optical Adhesive Film (Applied Biosystems). The qPCR mixtures contained 12.5 µL QuantiFast SYBR Green mixture (Qiagen), 0.4 mM primer, 2 µL of template DNA and sterile Milli-Q water to final volume of 25 µL. The cycling programme included an initial denaturation step at 95 °C for 5 min followed by 40 cycles of denaturation at 95 °C for 15 s, primer annealing at optimal temperatures (Table 3.1) for 30 or 45 s, and extension at 72 °C for 30 s. Melting curves were obtained after 40 cycles. Melting curves and agarose gel separation of amplicons were used to verify the specific amplification of target genes.

3.2.7 DNA sequencing

PCR amplicons obtained with primers LTf and LTr were purified from agarose gels and sequenced by Sanger sequencing (Macrogen, Rockville, MD). Sequences were compared to each other with the Align Sequences tool of the NCBI blastn suite (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

3.2.8. Qualitative analysis of intestinal microbiota by high-resolution melting curve analysis (HRM-qPCR)

High resolution melting curve analysis were performed on a Rotor-Gene Q instrument (Qiagen) by using Type-it HRM PCR master mix (Qiagen) containing EvaGreen™ fluorescent intercalating dye. Two pairs of 16S rRNA universal primers were used on DNA samples extracted from mucosal scraping and outflow fluid samples to detect adhering and luminal microbiota. The reaction mixtures contained 5 µL Type-it HRM PCR master mix, 0.7 mM primer, 1 µL of template DNA diluted to 10 mg/L, and sterile Milli-Q water to a final volume of 10 µL. Reaction conditions included an activation step at 95 °C for 5 min, followed by 45 cycles of 95 °C for 10 s, primer annealing (Table 3.1) for 30 sec, and elongation at 72 °C for 10 s. HRM analysis was carried out over the temperature range from 70 °C to 95 °C, increasing at 0.1 °C each step with waiting for 90 s under premelt conditions on the first step and a 2 -s hold at each increment. Data analysis was performed by using the Rotor-Gene Q Software version 2.0.3, and figures were plotted with SigmaPlot 11.0 (Systat Software, Inc., Chicago, IL, USA).

3.2.9. Quantification of adhering enterotoxigenic *E. coli* by fluorescent *in situ* hybridisation (FISH)

Intestine biopsies (0.5-1 cm^2) were fixed in Carnoy solution at 4 °C for at least two days, followed by washing of biopsy in fresh Carnoy solution for 1 h at 4 °C; 70% ethanol for 1 h at room temperature, and 100% ethanol for 1 h at room temperature and two washes in xylene for 1 h at room temperature. The fixed intestine biopsies specimens were embedded in paraffin (Fisher Scientific, Ottawa, Canada) by using Thermoscientific Histostar embedding machine (Fisher Scientific), sectioned at 4.0 µm by using a Leica RM 2135 sliding microtome (Leica Microsystems, Concord, Canada) and mounted on Superfrost Plus Positively Charged slides (Fisher Scientific). The sections were deparaffinized by passing through xylene (Leica Microsystems) (three times, 4 min each) and 100% ethanol (three times, 4 min each), followed

by drying in an oven for 10 min. Lysozyme (Sigma-Aldrich) was applied to the tissue in an additional permeation step to facilitate detection of gram-positive bacteria. DNA probes (Table 3.1) were synthesised by Integrated DNA Technologies (IDT, Coralville, USA) with fluorescent probes linked to the 5' end of oligonucleotides. Probes (50 µL of 0.1 nM probe hybridization buffer) were added to each slide, and slides were covered with coverslip. After hybridization for 50 min at 55 °C, ProLong Gold Antifade Reagent with 4',6-diamidion-2-phenylindole (DAPI) (Invitrogen) was added to the tissue after removal of the coverslip by washing slides with autoclaved Milli-Q water. The sections were examined with an Axio Imager M1m microscope (Carl Zeiss Inc., Thornwood, USA) and images were captured at a X40 magnification with an AxioCam M1m camera and AxioVision version 4.8.2.0 (Carl Zeiss Inc.). Enumeration was conducted on six fields per section by persons blind to sample coding. Bacterial cells were counted when a signal was observed with Cy5 (LT) and Alexa Fluor 546 (Alexa546) (total bacteria) but not with 6-carboxyfluorescein (6FAM) (nonsense probe) (Table. 3.1).

3.2.10. Statistical analysis

Differences in bacterial populations quantified by qPCR and ETEC enumeration by FISH between treatments of same animal (n = 5 or 6) were analyzed using PROC MIXED in SAS Version 9.2 (SAS Institute Inc., Cary, NC, USA). The model included the fixed effects of segment, ETEC treatment, glycan treatment, and the interaction of ETEC with glycan treatment as well as the random effect of pig. Mean separation was performed by using LSMEANS in SAS Version 9.2 (SAS Institute Inc.). Results are expressed as means ± standard error of the means. Significant differences are reported with a 5% probability of error (P<0.05), and trends are reported with a 10% probability of error (P < 0.10).

3.2.11. Nucleotide sequence accession numbers

The GenBank accession numers for the sequences reported here are KJ416139 and KJ412974.

Table 3.1. Primers and probes used in this study

Target gene	Oligonucleotide sequence (5' –3')	Product size (bp)	Tm (°C)	Reference
Total bacteria (Total B)	BACTf: AGAGTTTGATCMTGGCTCAG	919	58	a
	BACTr: CCGTCAATTCATTTGAGTTT			
	DomainBf: CGGYCCAGACTCCTACGGG	200	63	b
	DomainBr: TTACCGCGGCTGCTGGCAC			
	Eub338: Alex546-GCTGCCTCCCGTAGGAGT		55	c
Escherichia coli (*E. coli*)	UspAf: CCGATACGCTGCCAATCAGT	884	65	d
	UspAr: ACGCAGACCGTAGGCCAGAT			
ETEC K88 fimbriae (K88)	K88f: GCACATGCCTGGATGACTGGTG	439	63	e
	K88r: CGTCCGCAGAAGTAACCCCACCT			
	Cy3-GCACATGCCTGGATGACTGGTG		55	
Heat labile toxin (LT)	LTf: CCGTGCTGACTCTAGACCCCCA	480	65	f
	LTr: CCTGCTAATCTGTAACCATCCTCTGC			
	Cy5- CCGTGCTGACTCTAGACCCCCA		55	
Non specific binding	Non338: 6FAM-ACTCCTACGGGAGGCAGC		55	g

a, (Weisburg, Barns, Pelletier, & Lane, 1991); b, (Lee, Zo, & Kim, 1996); c, (Amann et al., 1990); d, (Chen & Griffiths, 1998); e, (Setia, Bhandari, House, Nyachoti, & Krause, 2009); f, (Kotlowski, Bernstein, Sepehri, & Krause, 2007); g, (Wallner, Amann, & Beisker, 1993)

3.3. Results

3.3.1. Anti-adhesive properties of reuteran and levan *in vitro*

The anti-adhesive property of exopolysaccharides form *L. reuteri* were validate by a hemagglutination assay (Wang et al., 2010). Dextran and inulin were used for comparison to reuteran and levan, respectively, because they have the same monosaccharide composition as reuteran and levan respectively, but differ in their linkage type. Hemagglutination of porcine

erythrocytes by both ETEC strains was prevented by reuteran and to a lesser extent by levan (Table 3.2). Dextran and inulin did not affect hemagglutination. Equivalent results were obtained with the two ETEC K88 strains; strain ECL13795 was selected for subsequent *in vivo*

Table 3.2. Inhibition of erythrocyte agglutination by two ETEC K88 strains with bacterial exopolysaccharides and structurally related polysaccharides at a concentration of 3.3 g/L.

EPSs and their sources		Agglutination[a]
L. reuteri TMW1.656	Reuteran	++
L. reuteri LTH5794	Levan	+
Commercial	Dextran	-
Commercial	Inulin	-

++, strong agglutination observed; +, agglutination observed; -, agglutination not observed

[a], stronger anti-adhesive activity leads to agglutination of erythrocyte at higher ETEC concentration; *E. coli* ECL13795 (O149, virotype STb:LT:EAST1:F4) and ECL13998 (O149, virotype STa:STb:LT:EAST1:F4:Paa) were used for detection

experimentation.

3.3.2. Reduction of net fluid absorption

Anti-adhesive properties of **r**euteran and levan with the controls dextran and inulin were evaluated *in vivo* in the SISP model. The net fluid loss was used as the primary outcome to document the antidiarrheal effect of glycans. The reduction of net fluid absorption is plotted as the difference between each infected segment and its paired non-infected segment (Figure 3.1). All glycans decreased ($P < 0.05$) the net fluid loss caused by ETEC infection compared to saline (Figure 3.1).

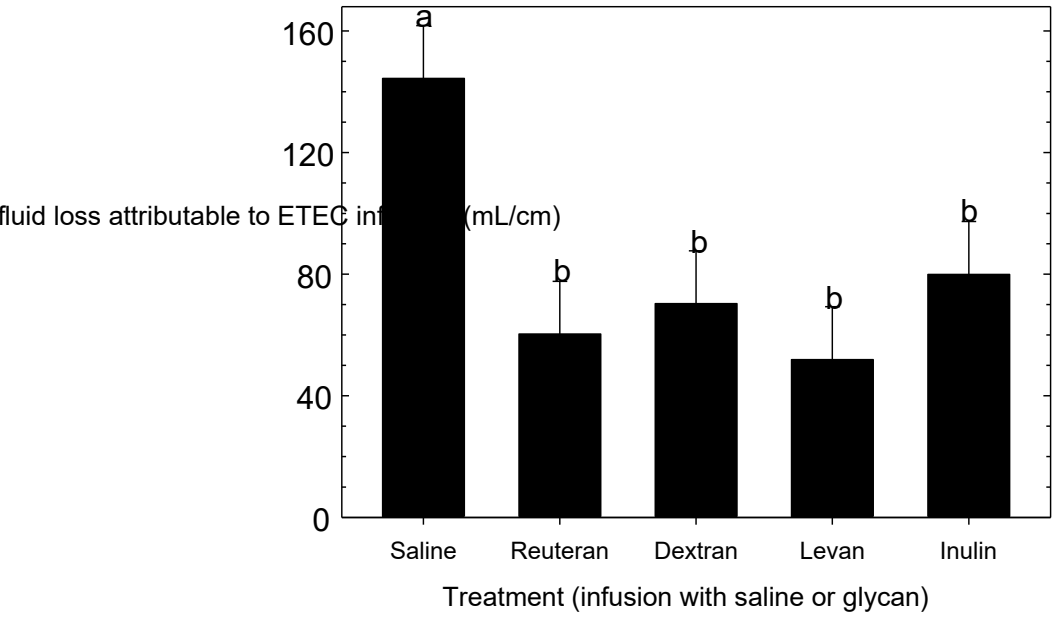

Figure 3.1. Reduction of net fluid absorption in piglet jejunal segments challenged with ETEC K88 or PBS, and perfused with saline (control), reuteran, dextran, levan or inulin (10 g/L) for 8 hours. Each bar represented the mean difference of the net fluid loss ETEC in challenged segments and the net fluid loss of the paired PBS perfused segment, n = 6. Mean values with unlike letters are significantly different, $P<0.05$.

3.3.3. Quantification of bacteria in jejunal outflow fluid by qPCR

To determine whether the ETEC strain was infective in the SISP model, rRNA from eubacteria and *E. coli* and genes coding for K88 fimbriae and LT in outflow fluid were quantified by qPCR (Figure 3.2). Gene copy numbers for eubacteria in fluid from infected segments ranged from 10^8 to 10^9 gene copies per mL. Copy numbers of *E. coli* rRNA and genes coding for K88 fimbriae

and LT were not different from copy numbers for the total bacterial rRNA. In noninfected segments, total bacteria ranged from 10^7 to 10^8 gene copies per mL. *E. coli* accounted for 1 to 10% of the total bacteria, and ETEC K88 accounted for 1-10% of *E. coli* cells (Figure 3.2). Levels of eubacterial rRNA genes in infected segments were at least 10-fold higher than those in the paired noninfected segments, indicating that ETEC K88 accounted for >90% of bacterial cell counts in infected segments. Glycan infusion did not significantly influence the number of ETEC bacteria in the outflow fluid.

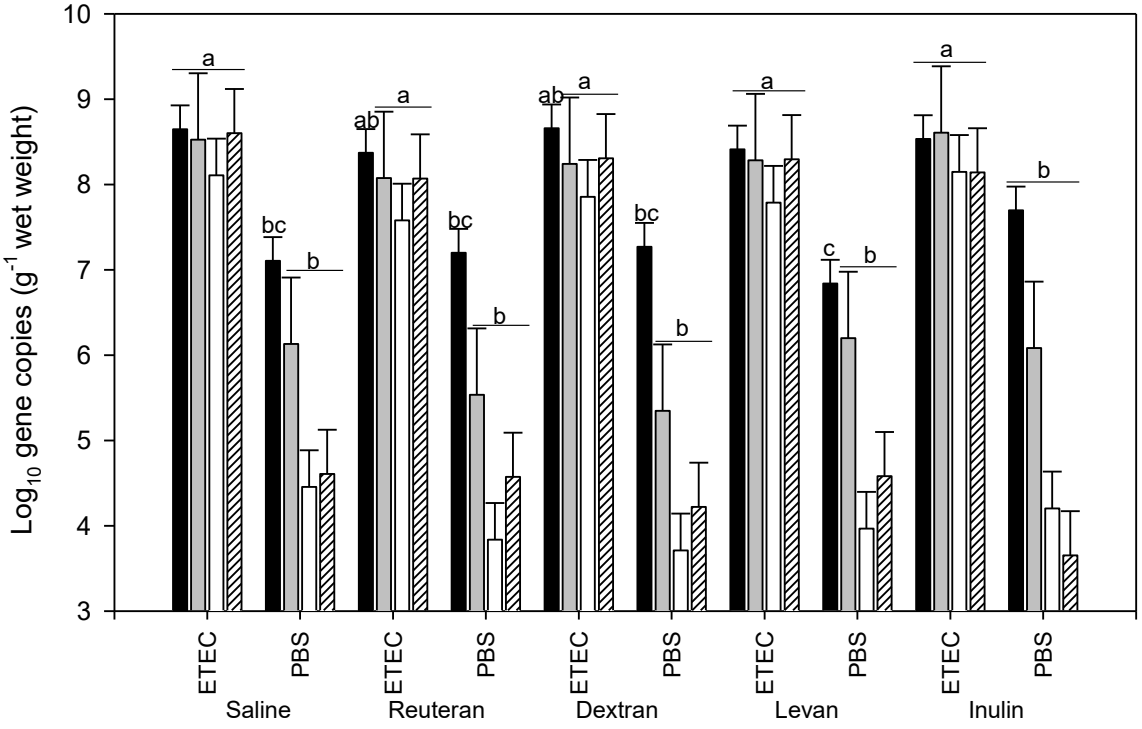

Figure 3.2. Quantitative PCR analysis of bacterial populations in outflow fluid samples collected from piglet jejunal segments challenged with ETEC K88 or PBS, and perfused with saline (control), reuteran, dextran, levan or inulin (10 g/L) for 8 hours. Black bars represent total bacterial numbers, grey bars represent *E. coli,* white bars represent gene copy numbers for K88 fimbriae; hatched bars represent gene copy numbers for LT. Data are shown as mean ± standard

error of the mean, n = 5. Mean values obtained with the same primer pairs with unlike letters were significantly different, $P<0.05$. K88 = K88 fimbriae, LT = heat labile toxin of ETEC.

3.3.4. Analysis of jejunal luminal microbiota by HRM-qPCR

The predominance of ETEC in the lumen of infected segments was assessed by a second method, HRM-qPCR, with primers targeting eubacterial rDNA (DomainB primer pair). In infected segments, only one melting peak was observed (Figure 3.3A), and the melting temperature of this peak matched the melting temperature of *E. coli* ECL13795, confirming the predominance of ETEC in infected segments. For noninfected segments, several peaks were observed (Figure 3.3B). HRM-pPCR analysis with the Bact primer pair provided equivalent results (see Figure S3.1 in the supplemental material). HRM-qPCR analysis of piglets 2, 4, 5, and 6 with the same primer pairs also gave comparable results (data not shown). Glycan infusion did not significantly influence the composition of the intestinal microbiota in the outflow fluid of infected segments.

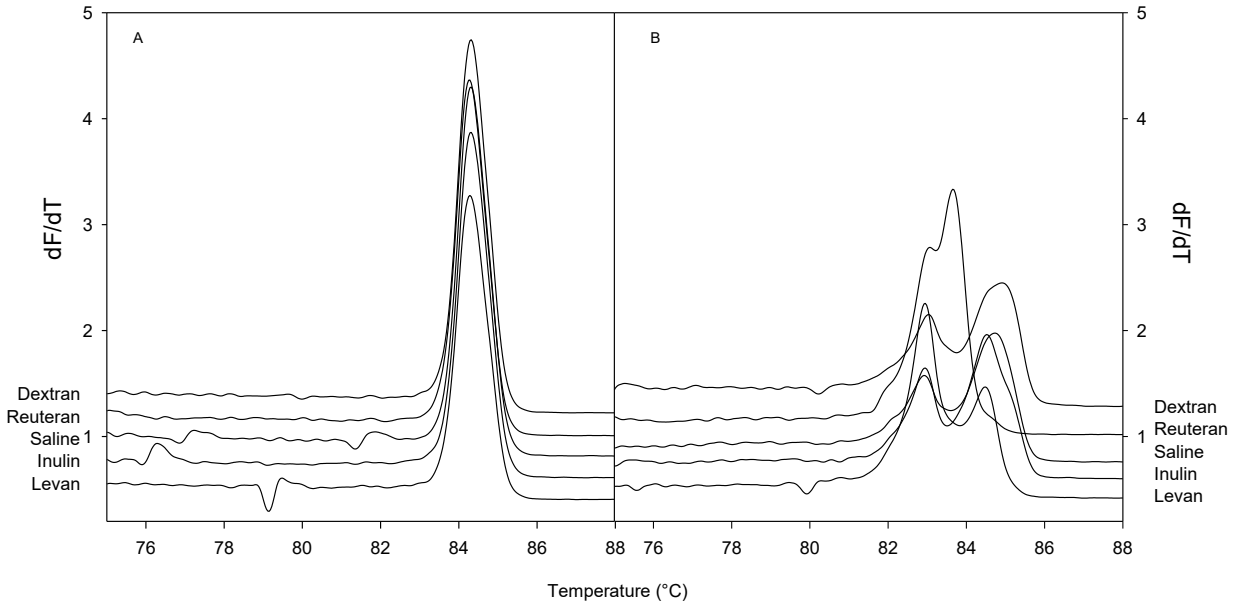

Figure 3.3. Pig 3 luminal microbiota melt peak profiles determined by primer pair DomainB for ETEC K88 challenged (A) and non-ETEC K88 challenged (B) piglet jejunal segments after 8 hours glycans or saline perfusion. Similar profiles were obtained for all 5 animals analysed (data not shown).

3.3.5. Quantification of bacterial populations in mucosal scrapings by qPCR

ETEC infection is initiated by attachment to mucosa, followed by the production of toxins that cause electrolyte imbalance and fluid loss. Therefore, bacteria in mucosal scraping samples were analysed by qPCR to quantify attached ETEC K88. ETEC was detected in mucosal scraping samples form noninfected segments (Figure 3.4), indicating the presence of autochthonous ETEC K88 in the piglets used for experiments. The number of ETEC K88 cells in noninfected segments accounted for 1 to 10% of the number of ETEC cells in infected segments. In outflow fluid, the number of ETEC bacteria in noninfected segments was <0.1% of the number of ETEC bacteria in infected segments, suggesting that the autochthonous ETEC bacteria were mucosa associated. HRM-qPCR analysis of LT amplcons demonstrated that different strains of ETEC

were present in infected segments (melting temperature of 81.6 ºC) and noninfected segments (melting temperature of 85.4 ºC). LT amplicons were purified from agarose gels and sequenced, The sequences of LTs from ETEC K88 strain ECL 13795 and the LT amplicon obtained from noninfected segments of piglets were 99% identical over 481bp.

A trend toward a reduced number of adhering ETEC K88 bacteria was observed for segments infused with ETEC and reuteran (P = 0.06) (Figure 3.4). Inulin tended to reduce gene copy numbers for K88 fimbriae (P = 0.07) but not gene copy numbers for LT in ETEC-infused segments (Figure 3.4).

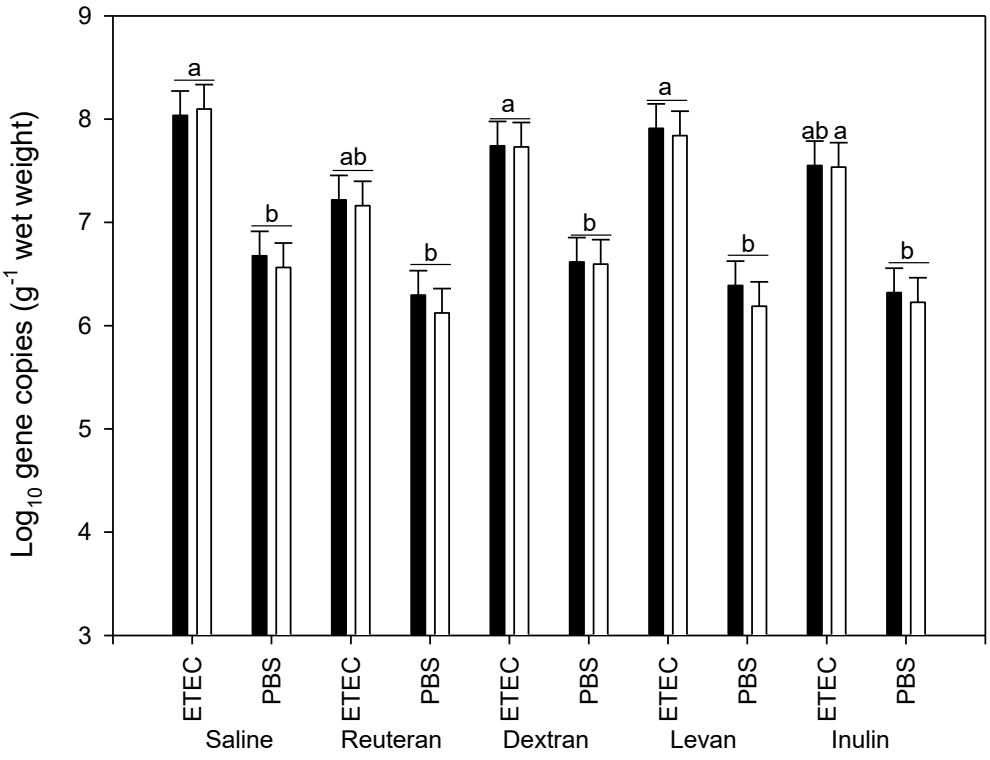

Figure 3.4. Quantitative PCR analysis of gene coding for K88 (■) and LT (□) in mucosal scraping samples collected from piglet jejunal segments that were challenged with ETEC K88 or PBS and perfused with saline (control), reuteran, dextran, levan or inulin (10 g/L) for 8 hours.

Data are shown as means ± standard error of the mean, n = 5. Mean values (obtained with the same primer pairs) with unlike letters were significantly different, $P<0.05$. (K88 = K88 fimbriae, LT = heat labile toxin)

3.3.6 Analysis of jejunal mucosal microbiota by HRM-qPCR

Mucosal microbiota were analysed by HRM-qPCR with the same primers employed for analysis of luminal microbiota. The predominance of *E. coli* in ETEC K88-challenged segments was confirmed by profiles of DomainB amplicons (see Figure S3.2A in the supplemental material). HRM-qPCR of noninfected segments indicated the presence of bacteria other than *E. coli*, as a different melting temperature from the temperature of *E. coli* was observed to be the main melting peak in non-infected segments (see Figure S3.2B in the supplemental material).

3.3.7. Fluorescent *in situ* hybridization

DNA isolated from mucosal scrapings samples was likely contaminated by luminal, non-adhering ETEC K88, thus obscuring reduced pathogen adhesion in presence of glycans. Evaluation of biopsy samples by FISH eliminated the contamination by luminal bacteria and visually revealed the colonization of ETEC K88 to epithelial cells. Cells were counted when their DNA hybridised to the LT and total bacterial probes but not to the non-sense probe Non338 (Figure 3.5). FISH analysis confirmed that ETEC K88 attached to the porcine mucosa (Figure 3.6). FISH analysis also confirmed colonization with autochthonous ETEC K88. The number of adhering ETEC cells in noninfected segments was generally > 30% of the number of ETECs in infected segments (Figure 3.6). Autochthonous ETEC K88 was thus primarily mucosa associated. Reuteran produced by *L. reuteri* TMW1.656 significantly reduced ETEC colonization of epithelial cells compared to saline. Levan from *L. reuteri* LTH5794 as well as

inulin and dextran did not reduce adhesion of ETEC. In the inulin perfusion group, no difference was observed between infected and noninfected segments but both segments had a relatively high number of ETEC cells of around 1400/mm^2.

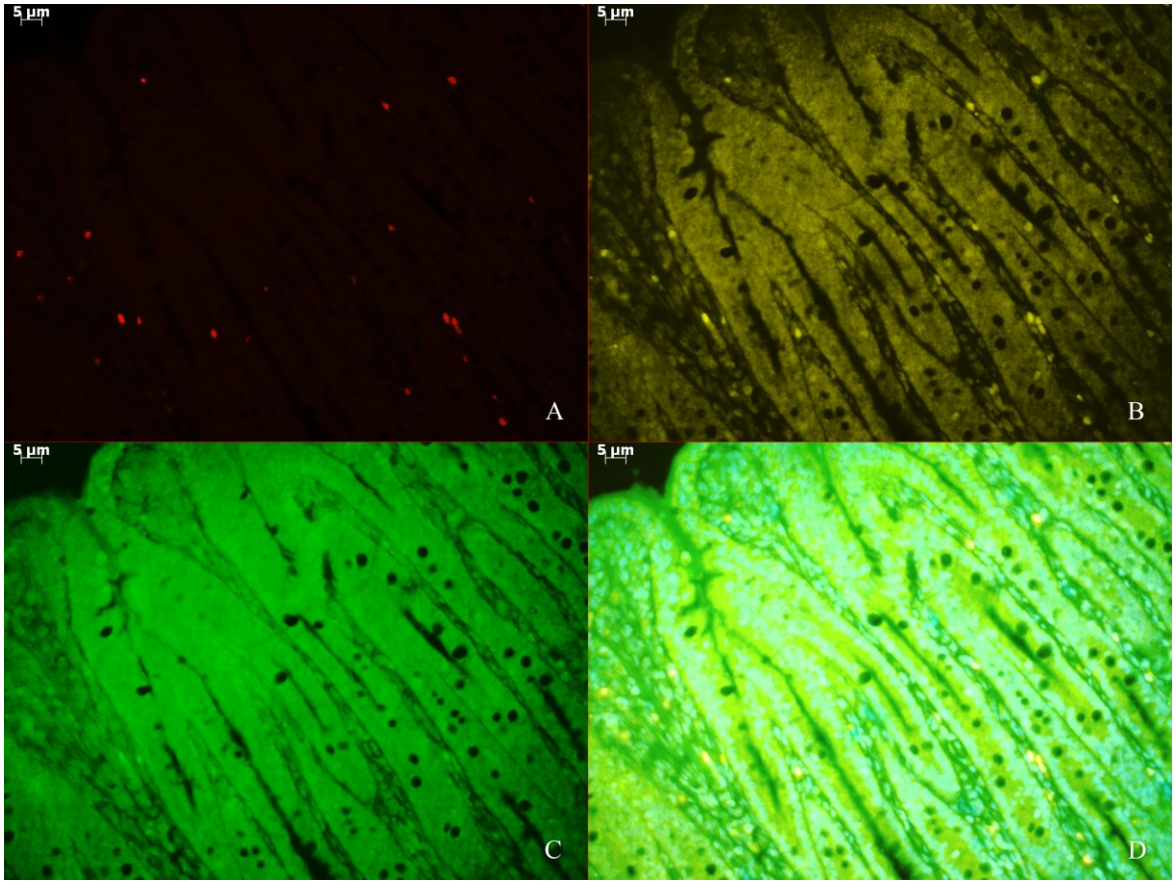

Figure 3.5. Fluorescent *in situ* hybridization localization of attached ETEC K88 and total bacteria of carnoy-fixed and paraffin embedded SISP intestine biopsy. 5'Cy5-LT (red), 5'6FAM-Eub338 (yellow) and 5'Alex546-Non338 (green) indicated the number and location of ETEC (A), all bacteria (B) and non-specific binding (C) respectively. DAPI stained nuclei and fluorescent from three probes were merged (D).

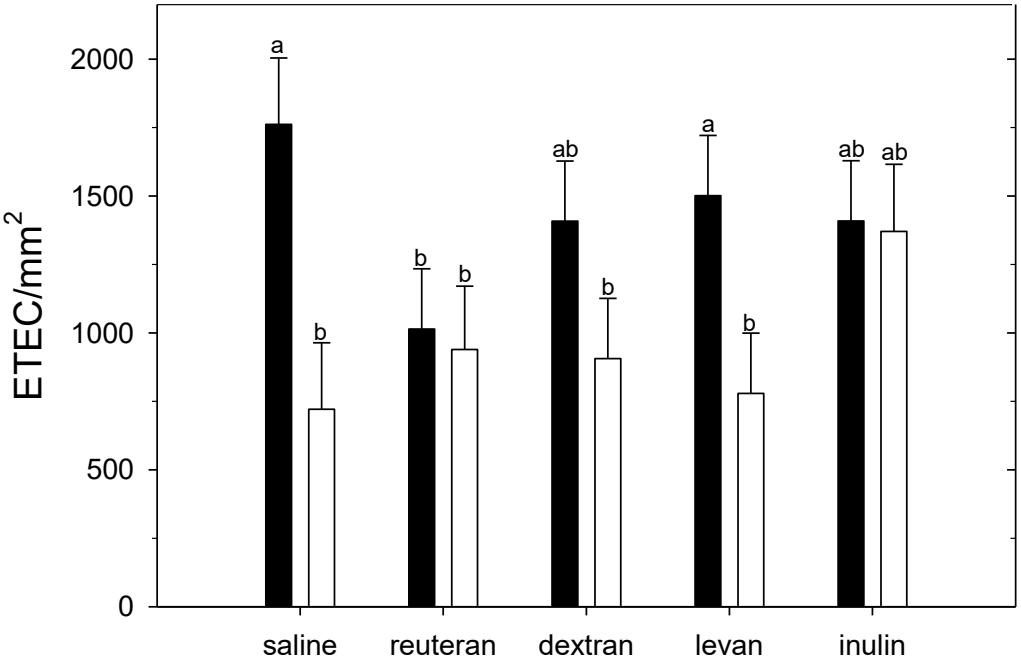

Figure 3.6. Mucosa ETEC blind enumeration of carnoy fixed and paraffin embedded SISP intestine biopsy (■, ETEC infected; □, non ETEC infected). Each bar represented mean ETEC number per square millimeter of biopsy ± standard error of the mean, n = 6. Mean values with unlike letters were significantly different, $P<0.05$.

3.4. Discussion

Post-weaning diarrhea in piglets accounts for considerable economic losses. (Fairbrother et al., 2005). ETEC is the main cause of postweaning diarrhea in piglets (Grange et al., 2006). The use of glycans to prevent pathogen adhesion may inhibit colonization of the swine intestine by ETEC; however, *in vivo* confirmation of the efficacy of glycans and their mode of action remains scarce. The SISP model was originally developed to determine the net absorption of fluid and electrolytes in the small intestine of newly weaned pigs (Nabuurs et al., 1993), but its use was later extended to evaluate the protective effects of food additives (Kiarie, Krause, & Nyachoti,

2008), nonstarch polysaccharides (Kiarie, Slominski, & Nyachoti, 2010), and plant proteins (Van der Meulen & Jansman, 2010) in ETEC-induced diarrhea. The SISP model also allowed determination of the early gene expression response of the pig small intestinal mucosa to infection with ETEC (Niewold, Kerstens, van der Meulen, Smits, & Hulst, 2005) and *Salmonella* (Niewold et al., 2007). Most studies employing SISP, however, focused on analysis of physiological and immune responses of the host, including fluid loss, electrolyte secretion, and the expression of cytokines (Kiarie, Krause, et al., 2008, 2008; Takanashi et al., 2013; Van der Meulen & Jansman, 2010). Very few studies provided a detailed bacteriological analysis of the model; previous studies using the SISP model reported only cell counts of ETEC in the lumen (Bruins et al., 2006; Kiarie, Slominski, Krause, & Nyachoti, 2008; Kiarie et al., 2010). To determine whether glycans prevent pathogen adhesion, we focused on the quantification of mucosa-adherent ETEC. Luminal and mucosal microbiota of outflow fluid and mucosal scraping samples were characterized by qPCR and HRM-qPCR. All analyses demonstrated that ETEC ECL13795 infected the SISP model and accounted for >90% of the total microbiota in the infected segments. However, q-PCR and FISH also demonstrated the presence of an autochthonous ETEC K88. HRM-qPCR and sequencing excluded the possibility that ETEC K88 in noninfected segments results from contamination with the experimental strain ETEC ECL13795.

All glycans in this study reduced the ETEC-induced net fluid loss compared to saline. The analyses of intestinal microbiota in infected segments provided no indication that infusion with glycans altered luminal microbiota; however, in addition to the interference with pathogen adhesion, competitive exclusion or pathogen aggregation may account for the reduced fluid loss (Korakli & Vogel, 2006; Shoaf-Sweeney & Hutkins, 2008). The presence of autochthonous

ETEC reduced the sensitivity of the SISP model to detect the activity of anti-adhesive glycans. In infected segments, autochthonous ETEC accounted for up to 40% of ETEC K88 adhering to the mucosa, and glycans may not be effective in detaching already adhering cells. Nevertheless, FISH and qPCR analyses consistently demonstrated reduced adhesion of ETEC K88 to reuteran produced by *L. reuteri* TMW1.656. Because both *in vitro* data (Wang et al., 2010; this study) and the SISP *in vivo* model (this study) indicate the ability of reuteran to prevent ETEC adhesion in swine, this glycan is a promising candidate for further evaluation in feeding trials. *In vivo* data provided no indication of reduced ETEC colonization in segments perfused with levan, inulin, or dextran.

K88 fimbriae recognize specific carbohydrate structures expressed on host cell glycoprotein; terminal GlcNAc, GalNAc, and galactose moieties of enterocyte receptors interact with K88 fimbriae (Anderson, Whitehead, & Kim, 1980). Some dietary carbohydrates prevent pathogen adhesion, possibly by acting as receptor analogs that saturate the glycan-binding domains of bacterial lectins and thus prevent binding to host glycoproteins. For example, polysaccharides extracted from green olive fermentation brines prevented adhesion of ETEC K88 to IPEC-J2 cells (González-Ortiz et al., 2013) but not to intestinal mucus (González-Ortiz et al., 2014). Glycans evaluated *in vivo* by using the SISP model were often classified only as ethanol-soluble and ethanol-insoluble fractions derived from hydrolysis of soybean and canola meal (Kiarie, Slominski, et al., 2008) or crops (Kiarie et al., 2010). In our study, the structure and composition of well-defined microbial or commercial polysaccharides were tested in an ETEC-challenged SISP model at the concentration of 10 g/L. The composition and linkage type of EPSs was reported to be important determinants for their activity in preventing pathogen adhesion (Korakli & Vogel, 2006; Wang et al., 2010). The active reuteran is an α-(1→4) and α-(1→6)-linked

glucan while the predominantly α-(1→6)-linked dextran has little or no activity (Wang et al., 2010; this study). Likewise, the β-(2→6) linked levan prevents ETEC adhesion, but the β-(2→1) inulin is less active (Wang et al., 2010). Our results indicate that the linkage type influences anti-adhesive property of polysaccharides. However, the compounds also differ in their molecular weights (dextran > reuteran; levan > inulin), and differences in the molecular weight or the degree of branching of the glycans (Korakli & Vogel, 2006) may additionally account for their different activities.

L. reuteri is recognized as an autochthonous member of the intestinal microbiota of vertebrates (Walter, 2008; Mitsuoka, 1992). The evolution of *L. reuteri* resulted in phylogenetically distinct lineages that are adapted to their vertebrate host (Frese et al., 2011). Although *L. reuteri* has been termed a gut symbiont, the benefit of *L. reuteri* colonization to the host is poorly understood (Frese et al., 2013; Heavens et al., 2011). *L. reuteri* TMW1.656 is closely related to the rodent-lineage strains *L. reuteri* LTH2584 and TMW1.106 (Michael G Gänzle & Vogel, 2003; Su, Oh, Walter, & Gänzle, 2012). However, *L. reuteri* also colonize the upper intestinal tract of swine (Leser et al., 2002; Walter, 2008). *L. reuteri* produces exopolysaccharides *in vivo* to form the polysaccharide matrix of bacterial biofilms on nonsecretory epithelia of the upper intestine (M. G. Gänzle & Schwab, 2009; Walter, 2008). Reuteran formed by *L. reuteri* in the upper intestine of piglets may thus prevent adhesion of pathogens in the distal intestinal tract, including the jejunum. This protective mechanism provides an identifiable benefit to the host and justifies the promotion of *L. reuteri* from “gut commensal” (Sims et al., 2011) to “gut symbiont” (Frese et al., 2013).

In conclusion, the *in vivo* SISP model used in this study confirmed the anti-adhesive properties of reuteran that were previously observed *in vitro* (Wang et al., 2010). Reuteran perfusion

reduced not only ETEC-induced net fluid loss but also the adhesion of ETEC K88 to the intestinal mucosa. Reuteran may be a suitable feed additive to lessen the impact of ETEC-induced diarrhea in piglets. Comparable to the commercial production of dextran from *Leuconostoc mesenteroides,* the conversion of sucrose to fructans or glucans by lactobacilli is achieved with high yields (Korakli, Pavlovic, Gänzle, & Vogel, 2003; Korakli & Vogel, 2006), facilitating commercial applications of reuteran as a feed additive to prevent ETEC infections and post-weaning diarrhea in piglets.

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3.6. Supplementary materials

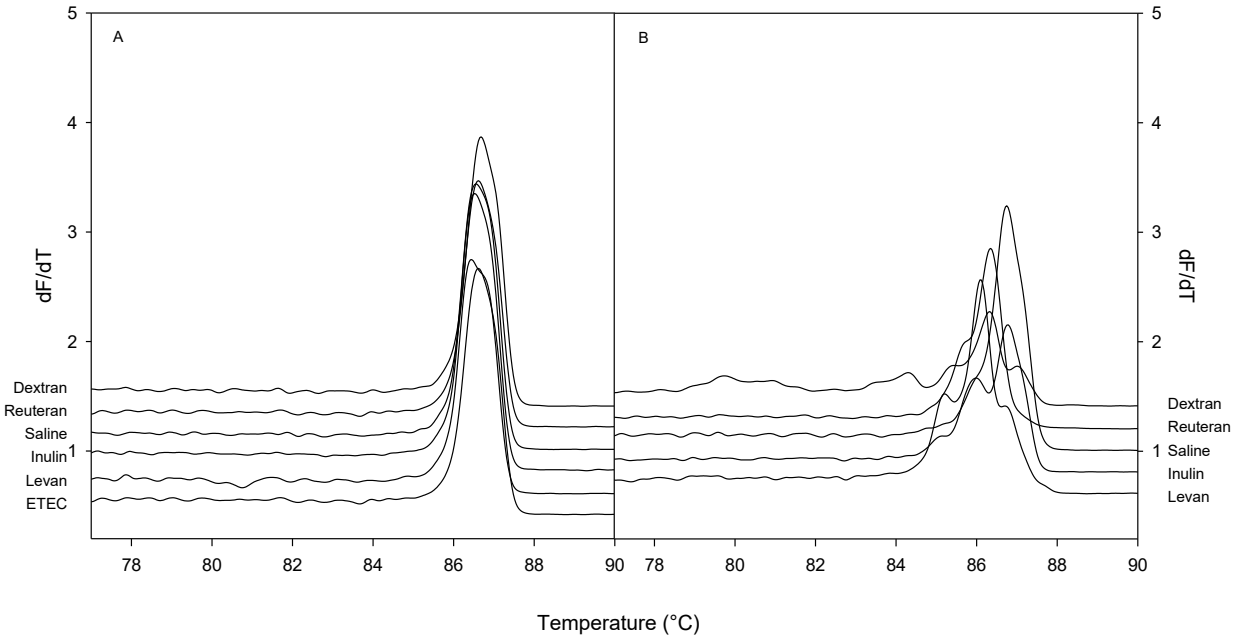


Figure S3.1. Luminal microbiota melt peak profiles determined by primer pair Bact for ETEC K88 challenged (A) and non-ETEC K88 challenged (B) piglet jejunal segments after 8 hours glycans or saline perfusion.

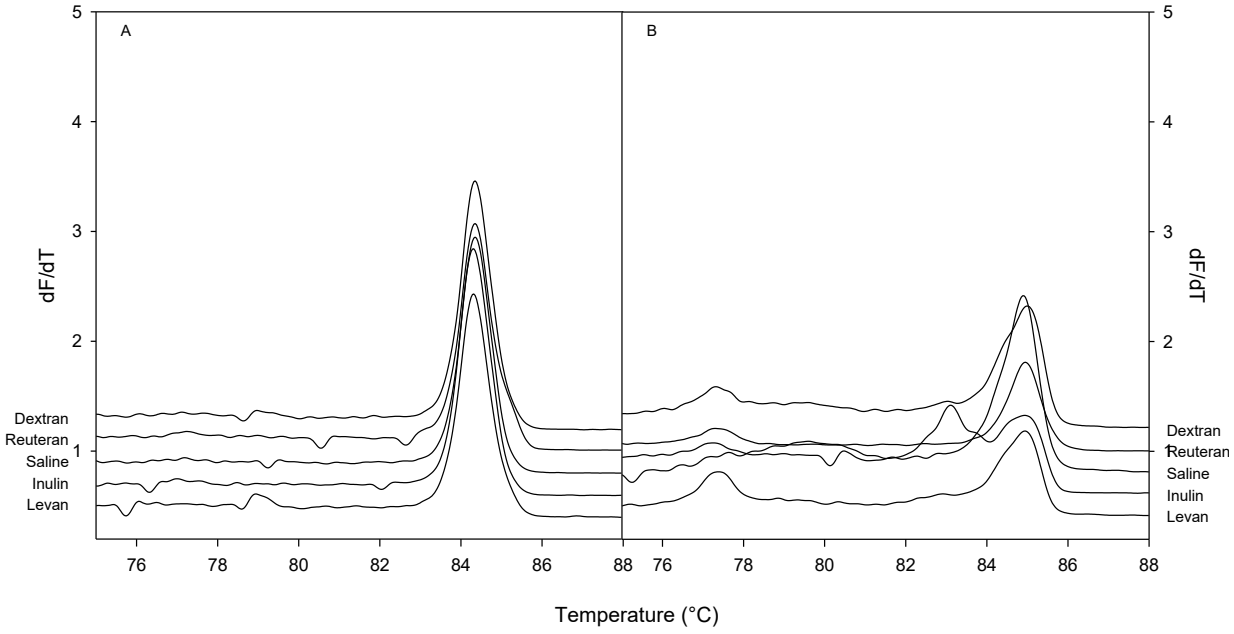


Figure S3.2. Mucosal microbiota melt peak profiles determined by primer pair DomainB for ETEC K88 challenged (A) and non-ETEC K88 challenged (B) piglet jejunal segments after 8 hours glycans or saline perfusion.

4. Site directed mutagenesis of dextransucrase DsrM from *Weissella cibaria*: transformation to a reuteransucrase?

4.1. Introduction

Lactic acid bacteria in the genera of *Lactobacillus*, *Weissella*, *Leuconostoc* and *Streptococcus* produce glucans and gluco-oligosaccharides from sucrose by extracellular glucansucrases (Leemhuis et al., 2013). Gluco-oligosaccharides such as isomalto-oligosaccharides are commercially applied as prebiotics (Hu, Ketabi, Buchko, & Gänzle, 2013; Yen, Tseng, Kuo, Lee, & Chen, 2011). Polymeric glucans, such as dextran, are used as food hydrocolloids (Galle et al., 2012; Katina et al., 2009). Beneficial technological or functional effects of glucans relate to their linkage type and molecular weight (Hu et al., 2013; Shi et al., 2016).

Glucans are categorized based on their linkage type: predominantly α-(1→6) linked dextran is synthesized by dextransucrases from *Leuconostoc, Streptococcus* and *Weissella*; α-(1→3) and α-(1→6) linked alternan is synthesized by some strains of *Leuconostoc* and *Streptococcus*; predominantly α-(1→3) linked mutan is synthesized by *Streptococcus*; and predominantly α-(1→4) linked reuteran is synthesized by reuteransucrases from *L. reuteri* (Leemhuis et al., 2013). Glucansucrases are GH70 family enzymes in the α-amylase superfamily and contain an amylase-type catalytic $(\alpha/\beta)_8$-barrel (Hijum, Kralj, Ozimek, Dijkhuizen, & van Geel-Schutten, 2006; Korakli & Vogel, 2006). Glucansucrases are retaining glycosyl hydrolases that transfer the glucosyl unit from sucrose to water, acceptor sugars, or a growing glucan chain (Hijum et al., 2006; Korakli & Vogel, 2006). Reuteransucrases and dextransucrases typically have an average molecular weight of 160 kDa and contain a signal peptide that directs protein export from the cytoplasm, an N-terminal variable region with unknown function, a region with the conserved

catalytic residues of nucleophilic Asp, acid/base Glu and transition state stabilizer Asp, and a C terminal region which may be involved in glucan binding (Hijum et al., 2006).

The retaining glycosyl hydrolases form a glycosyl-enzyme intermediate. The orientation of acceptor sugars binding to glucansucrases determines the linkage type of their glucan products (Ito et al., 2011; Kang, Kimura, & Kim, 2011; Leemhuis, Pijning, Dobruchowska, Dijkstra, & Dijkhuizen, 2012; Meng, Pijning, Dobruchowska, Gerwig, & Dijkhuizen, 2015). The analysis of three dimensional structure, acceptor docking studies, and site-directed mutagenesis of glucansucrases demonstrated that residues forming acceptor-binding sites, especially subsites +1 and +2 in domain A and B, are potential modulators of glycosidic bond specificities of glucansucrases (Ito et al., 2011; Kang et al., 2011; Leemhuis et al., 2012; Meng et al., 2015). Substitution of the acceptor-binding subsite residues P1026V:I1029V and N1134S:N1135E:S1136V in GtfA of *L. reuteri* 121 resulted in enzymes that synthesize glucans with a lower proportion of α-(1→4) linkages (Kralj, van Geel-Schutten, Faber, van der Maarel, & Dijkhuizen, 2005). Likewise, the parallel substitution of V1027P:S1137N:A1139S in Gtf180 from *L. reuteri* 180 increased the proportion of α-(1→4) linkages (van Leeuwen, Kralj, Gerwig, Dijkhuizen, & Kamerling, 2008). Modification of the same conserved residues P and I, however, decreased the activity of the dextransucrase of *Leuconostoc mesenteroides* B-1299CB4 without changing the structure of the dextran (Kang et al., 2011). The conserved catalytic core domains containing acceptor-binding sites are of the similar size. However N-variable and C-terminal regions vary between dextransucrase and reuteransucrases (Kang, Oh, & Kim, 2009; Kralj, van Geel-Schutten, van der Maarel, & Dijkhuizen, 2004; Leemhuis et al., 2013). Generally, dextransucrases have a shorter N-variable region with partial glucan binding domain, and a

longer C-terminal region when compared to reuteransucrases (Kralj, van Geel-Schutten, van der Maarel, et al., 2004).

Dextran-forming *Weissella* spp. were applied in sourdough fermentations to increase bread volume and to delay staling (Galle et al., 2012; Galle, Schwab, Arendt, & Gänzle, 2010). In contrast to other heterofermentative lactic acid bacteria, *Weissella* spp. do not use fructose as electron acceptor and sucrose addition thus does not increase acetate production (Galle, Schwab, Arendt, & Gänzle, 2010; Zheng, Ruan, Sun, & Gänzle, 2015). However, the relationship between the sequence of dextransucrases of *W. cibaria* (Kang et al., 2009) and the functionality of the respective polymers has not been explored. This study therefore aimed to compare glucansucrases from *L. reuteri* and *W. cibaria* in their structural similarity and compatibility of parallel acceptor-binding site residues, in order to facilitate the further application of glucansucrases in the production of functional glucan and gluco-oligosaccharides.

4.2. Material and methods

4.2.1. Bacteria, plasmids, media and growth condition

W. cibaria 10M was cultivated anaerobically at 30^{o}C in mMRS medium. *Escherichia coli* TOP10 (Invitrogen, Toronto, ON, Canada) harboring pUC19 plasmid (Thermo scientific, Burlington, ON, Canada) was cultivated aerobically at 37 ^{o}C in LB medium (BD, Mississauga, ON, Canada) containing 0.05 g/L ampicillin. *E. coli* BL21 Star (DE3) (Invitrogen) harboring pET-28a^{+} (Novagen, Etobicoke, ON, Canada) was cultivated aerobically at 37 ^{o}C in LB medium containing 0.05 g/L kanamycin. *E. coli* were grown with agitation (200 rpm) in LB broth.

4.2.2. General molecular techniques

Bacterial DNA was isolated with DNeasy Blood & Tissue Kit (Qiagen, Toronto, ON, Canada). Bacterial plasmid DNA was isolated with QIAprep Spin Miniprep Kit (Qiagen). DNA was amplified by PCR using PfuUltra High-Fidelity DNA polymerase (Agilent Technologies). DNA fragments were purified from agarose gel using MinElute Gel Extraction Kit (Qiagen). Cloning, *E. coli* transformation, DNA manipulations and agarose gel electrophoreses were performed as described (Green & Sambrook, 2012). Restriction endonuclease (Thermo Scientific, Ottawa, Canada) digestion and DNA ligation with T4 ligase (Thermo Scientific) were performed following procedures provided by the suppliers. Chromosomal and plasmid DNA were sequenced by Macrogen (Macrogen, Rockville, MD, USA). Nucleotide and amino acid sequences analysis was performed using DNAMAN software (Lynnon Biosoft, San Ramon, CA, USA).

4.2.3. Amino acid sequence alignment and phylogenetic tree generation

Glucansucrase amino acid sequences of *L. fermentum* kg3, *W. cibaria* CMU, *W. cibaria* 10M, *W. confusa* LBAE C39-2, *Leuconostoc mesenteroides* B-1299CB4, *L. parabuchneri* 33, *Streptococcus mutans* GS-5, *L. reuteri* ML1, *L. reuteri* 180, *L. reuteri* ATCC55730, *L. reuteri* 121 and *L. reuteri* TMW1.656 (accession number AAU08008, ACK38203, AND77013, CCF30682, ABF85832, AAU08006, AAA88588, AAU08004, AAU08001, AAX97502, AAU08015, WP051675362, respectively) with different glycosidic bond preferences were aligned by using Clustal W software (Larkin et al., 2007). Mega6 (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013) was used to generate condensed phylogenetic tree of glucansucrases using neighbor-joining method.

4.2.4. Prediction of the domains architecture of DsrM and GtfA

The molecular domain architectures of DsrM and GtfA were assigned with the analysis of amino acid sequences of DsrM and GtfA through InterProScan (http://www.ebi.ac.uk/interpro/), and the alignment with Wccab3-Rdsr (Shukla et al., 2016) (AKE50934) or GtfA from *L. reuteri* 121 (4AMC_A), respectively. The final domain boundaries of DsrM and GtfA were predicted according to the structure of Wccab3-Rdsr or GtfA from *L. reuteri* 121, respectively (Figure S4.1 of the supplementary material). (Leemhuis et al., 2013; Shukla et al., 2016)

4.2.5. Glucansucrase cloning, and site-directed mutagenesis

The reuteransucrase *gtfA* in *L. reuteri* TMW1.656 was identified in the genome of that strain by nucleotide blast with *gtfA* from *L. reuteri* TMW1.106 (EF189716) as query sequence. GtfA from *L. reuteri* TMW1.656 is identical to GtfA from *L. reuteri* TMW1.106. For construction of the expression plasmid, *gtfA* without the N-terminal variable region was cloned into two fragments with a site silent mutation to inactive NcoI restriction site and ligated into pUC19 plasmid. pUC19-GtfA-ΔN-2571 and GtfA-ΔN-616 were ligated after the digestion at BamHI and PaeI sites to allow the generation of pUC19-GtfA-ΔN. pE-GtfA-ΔN was constructed by ligating GtfA-ΔN with pET28a$^+$ plasmid at NcoI and NotI sites after digestion (Table 6.1). To simplify further mutagenesis, pUC18-gtfA1500 was constructed by ligating pUC18 plasmid and 1500 bp catalytic domain of *gtfA* at PstI and KpnI sites (Table 6.1). To facilitate the ligation of N terminal truncated dextransucrase DNA sequence *dsrM*-Δ*N* in pET-28a$^+$ at NcoI and NotI restriction sites, *dsrM*-Δ*N* sequence was amplified as two fragments by using megaprimer to inactive the NcoI restriction site (Table 4.1). The upstream and downstream fragments of *dsrM*-Δ*N* were cloned into pUC19 and then ligated together at restriction site of Bsp1407I (Table 4.1). The construction of expression vector was achieved by ligating *dsrM*-Δ*N* with pET28a$^+$ at NcoI and NotI. Sequencing was performed to ensure the identity of *dsrM*-Δ*N*. To simplify site-directed

mutagenesis, pUC19-*dsrM*-2100 was constructed by digesting and ligating 2100bp of *dsrM*-Δ*S* fragment into a pUC19 vector at EcoRI, PaeI sites. Site-directed mutagenesis of desired mutation sites was achieved by using QuikChange II Site Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA). The generation of new restriction enzyme cutting site (Table 4.1) facilitated the screening of desired mutants. Nucleotide sequencing was performed to ensure desired mutation sites were altered. pUC19-*dsrM*-2100-V583P:V586I, pUC19-*dsrM*-2100-S693N:E694N:V695S and pUC19-*dsrM*-2100-S622N (Table 4.1) were exchanged in pUC19-*dsrM*-Δ*S* to generate the corresponding site-directed mutant and finally ligated in the expression vector of pET-28a^{+}. The sequence of mutant pUC18-gtfA1500 was ligated into pUC19-GtfA-ΔN after digested by PstI and FspI endonucleases. The whole sequence of the mutated GtfA-ΔN was ligated into pET28a^{+} plasmid to generate GtfA-ΔN-V1024P:V1027I, GtfA-ΔN-S1135N:A1137S, GtfA-ΔN-S1062N and GtfA-ΔN-V1024P:V1027I:S1135N:A1137S.

4.2.6. Heterologous expression and purification of glucansucrases

DsrM-ΔN, DsrM-ΔS, GtfA-ΔN and their site-directed mutagenesis derivatives were expressed in *E. coli* and purified as described (Kang, Oh, & Kim, 2009; Kralj et al., 2011) The purity of dextransucrases or reuteransucrases was verified by SDS-PAGE (Bio-Rad, Mississauga, ON, Canada).

4.2.7. Recombinant enzyme characterization

Protein concentration was determined using protein assay reagent (Bio-Rad) with bovine serum albumin as a standard. Enzyme activities of reuteransucrase GtfA-ΔN and mutant enzymes were evaluated essentially as described (Kralj, van Geel-Schutten, van der Maarel, & Dijkhuizen, 2004). In brief, enzyme activities were determined in 25 mM sodium acetate buffer (pH = 4.7)

containing 1 mM $CaCl_2$, 100 mM sucrose, and 1 μM enzyme. Samples were taken at 5 min intervals and the enzymatic reaction was stopped at 95 ˚C for 10 mins. Enzyme activity of DsrM-ΔN, DsrM-ΔS and its mutant derivatives were determined with the similar method with minor modifications. In brief, enzyme activity of 1 μM purified dextransucrases was determined at 30 ^{o}C in 20 mM NaAc buffer containing 1 mM $CaCl_2$ at pH 5.2. Samples were taken over 30 min at 5 min intervals and inactivated at 95^{o}C; the reaction time was extended to 1 h for enzymes that displayed low activity. The concentration of glucose and fructose was determined enzymatically (Glucose and Fructose Assay Kit, Sigma-Aldrich, Oakville, ON, Canada). The amount of free glucose represents hydrolysis activity and the amount of fructose represents the total enzyme activity. Transferase activity was calculated as difference between total and hydrolysis activity. One unit of enzyme activity was defined as the release of 1 μmol of monosaccharides per min. The detection limit was defined as no detectable fructose after 2 h enzymatic reaction. All the enzyme activities were measured in duplicates or triplicates.

4.2.8. Analysis of acceptor reaction products

Oligosaccharides were synthesized by an acceptor reaction of 1 μM of DsrM-ΔN, DsrM-ΔS, GtfA-ΔN or their mutant derivatives incubated with 500 mM sucrose and 500mM maltose in reaction buffer for 24 h. Samples were analyzed by HPAEC-PAD with a Carbopac PA20 column coupled to an ED40 chemical detector (Dionex, Oakville, Canada) (Galle et al., 2010). Fructose, glucose, sucrose, maltose, maltotriose and panose were identified by external standards (Sigma Aldrich).

4.2.9. Glucan production and purification

To purify enzymatically-produced glucans, 50 nM purified DsrM-ΔN, DsrM-ΔS, GtfA-ΔN or their mutant derivatives was incubated with 500 mM sucrose in 25 mM sodium acetate buffer (pH = 4.7) containing 1 mM $CaCl_2$ for 2 days. Reuteran and dextran were harvested via 2 volumes ethanol precipitation and purified by dialysis. The retentate was freeze-dried and stored at -20 ˚C. To purify glucans produced by *L. reuteri* TMW1.656, the strain was cultured in sucrose mMRS broth for 16 h and subcultured overnight in 1 L sucrose-mMRS broth. Reuteran was harvested via 2 volumes ethanol precipitation and purified by dialysis as described (Chen, Woodward, Zijlstra, & Gänzle, 2014). The retentate was freeze-dried and stored at -20˚C.

4.2.10. Characterization of glucan produced from recombinant enzymes

One-dimensional ^{1}H-NMR spectra were recorded on an Agilent/Varian Inova three-channel 400 MHz spectrometer at the University of Alberta NMR facility. All spectra were recorded at 353K with Z-gradient probe. EPS samples were dissolved in 99.97% D_2O. Chemical shifts were expressed in parts per million (ppm) by reference to internal standard 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS). The proportion of α-(1→4) and α-(1→6) glucosidic linkages was determined at 5.3 and 5.0 ppm, respectively (Kralj et al., 2005).

Molar mass determination of glucan was performed with asymmetrical flow field-flow-fractionation (AF4) coupled to multi-angle light scattering (MALS) and RI detectors (Postnova, Salt Lake City, UT, USA). The regenerated cellulose membrane (Postnova) of the accumulation wall had a molecular weight cut off of 10 kDa. Glucan samples were dissolved to 1 g/L in 10mM NaCl and injected onto the channel at a flow rate of 0.2 mL/min and a cross flow of 1 mL/min for 6 mins. After injection, the cross flow rate remained constant for 2 min, decreased exponentially to 0.1 mL min over 10 mins, and was then maintained at 0.1 mL/min for 10 mins.

The molar mass was calculated MALS signals and RI signal by AF 2000 software (Postnova). A value of 0.146 ml/g was employed as refractive index increment (dn/dc) (Vilaplana & Gilbert, 2010). Poly-styrolsulphonate standards and BSA were used for calibration of detectors.

4.2.11. Statistical analysis

Differences of dextransucrases and reuteransucrases enzyme activity (n = 2 or 3) were analyzed using PROC GLM of SAS Version 9.4 (SAS Institute Inc, USA). Mean separation was performed by using multiple range test. Results were expressed as least square mean ± standard deviation. Significant differences are reported with a 5% probability of error ($P < 0.05$).

Table 4.1 Primers and plasmids

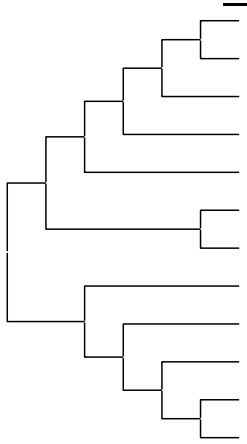
Wild type / Mutation	plasmid	Primer pair (5'-3')	Restriction site
GtfA-ΔN-2571	pUC19	For ATTCGAGCTCCCATGGGCACTATTAACG Rev: TGAAGGATCCATAGCAACCCCAGT	SacI NcoI Inactivation of NcoI
GtfA-ΔN-616	pUC19	For TCAACTGGGGTTGCTATGGATCCTT Rev ACATGCATGCGGCCGCTAGTTTTTTCTGA	Inactivation of NcoI PaeI NotI
GtfA-ΔN	pET-28a$^+$		NcoI NotI
GtfA-1500	pUC18		PstI KpnI
DsrM-ΔS	pUC19 / pET-28a$^+$	For GCCGGGATCCGATACTGTATTACCAAGTGAA Rev ATTCTAGAGCGGCCGCAATCGTCACCAACGTA	BamHI XbaI NotI
GtfA-ΔN V1024P:V1027I	pUC18-GtfA-1500 pUC19/ pET-28a$^+$	For CGAGTAGATGCGCCGGATAACATCGATGCAGATC Rev GATCTGCATCGATGTTATCCGGCGCATCTACTCG	Inactivation of AclI
GtfA-ΔN S1135N:A1137S	pUC18-GtfA-1500 pUC19/ pET-28a$^+$	For CCGTGCGCATGATAATAATTCACAAGATC Rev GATCTTGTGAATTATTATCATGCGCACGG	FspI
GtfA-ΔN V1024P:V1027I: S1135N:A1137S	pUC18-GtfA-1500 (V1024P:V1027I) pUC19/ pET-28a$^+$	For CCGTGCGCATGATAATAATTCACAAGATC Rev GATCTTGTGAATTATTATCATGCGCACGG	FspI
L. reuteri TMW1.656Δ*gtfA*	pUC18/ pJRS233	For-up CGCAAGCTTAATGATTTACAACCGGGTC Rev-up ATTCGAGCTCCCTTGGAATTGAATACC GCTA For-down AACTGCAGTACTTATCTGGTCATAGCCTAG Rev-down CTAGTCTAGACTCCACGGAATGATACACC	HindIII PstI PstI XbaI
DsrM-ΔN	pUC19/ pET-28a$^+$	For-mega CGCGGGACAATGGCACGCTTT Rev-mega CTGGCCGCCATTGCACCCTTA For ATTC**GAGCTCCCATGG**CCACAATTGCAAAC Rev CTAG**TCTAGAGCGGCCGC**AATCGTCACCAC	NcoI inactivation NcoI inactivation SacI NcoI XbaI, NotI
DsrM-2100	pUC19		EcoRI, PaeI
DsrM-V583P:V586I	pUC19-DsrM-2100/ pUC19/ pET-28a+	For CGGATTGATGCGCCGGAT**AATATT**GATGCTGATT Rev AATCAGCATC**AATATT**ATCCGGCGCATCAATCCG	SspI
DsrM- S693N:E694N:V695S	pUC19-DsrM-2100/ pUC19/ pET-28a+	For TGTGCG**TGCGCA**TGATAATAATAGCCAAACAGTCATTG Rev CAATGACTGTTTGGCTATTATTATCA**TGCGCAC**GCACA	FspI
DsrM-S622N	pUC19-DsrM-2100/ pUC19/ pET-28a+	For CTTTCAATT**CTCGAG**GATTGGAACCATAACGATC Rev GATCGTTATGGTTCCAATC**CTCGAG**AATTGAAAG	XhoI
GtfA-S1062N	pUC18-GtfA-1500* pUC19/ pET-28a$^+$	For GAAGATTGGAACCACGC**GGATCC**GGA	BamHI

Plasmids and oligonucleotides used for *gtfA* and *dsrM* cloning, site-directed mutagenesis of *gtfA* and *L. reuteri* TMW1.656Δ*gtfA* knock out. Underlined nucleotides stand for silent mutation or site-directed mutation.

4.3. Results

4.3.1.Glucansucrase amino acid sequence alignment and amino acid substitutions

Twelve glucansucrases from lactic acid bacteria that synthesize different glycosidic bonds were aligned in order to identify the acceptor-binding sites that may relate to the linkage specificity of glucansucrases (Figure 4.1 and Figure S4.1 of the supplementary material). Site-directed mutagenesis of S622N was chosen because N622 is present in α-(1→4) reuteran producing *L. reuteri* ATCC55730 and *L. reuteri* 121 (Figure 4.1). The same mutagenesis of S1062N was also employed in GtfA-ΔN which synthesizes low proportion of α-(1→4) linkages (Figure 4.1). To compare the role of amino acid substitutions in the acceptor-binding site in phylogenetically distinct enzymes DsrM-ΔS and GtfA-ΔN (Figure 4.1), the mutation of V1024P:V1027I, S1135N:A1137S of GtfA-ΔN were also substituted in DsrM-ΔS as DsrM-V583P:V586I and DsrM-S693N:E694N:V695S.



Accession #	Strain	Main linkage type*	Nucleophile	General acid/base	Transition-state stabilizer
AAU08008	*L. fermentum* kg3	α-(1→6)/α-(1→3)	560 RIDAVDNVDAD	605 LSILEDWSHND	678 FVRAHDSEVQT
AND77013	*W. cibaria* 10M	α-(1→6)	579 RIDAVDNVDAD	615 LSILEDWSHND	687 FVRAHDSEVQT
ACK38203	*W. cibaria* CMU	α-(1→6)	579 RIDAVDNVDAD	615 LSILEDWSHND	687 FVRAHDSEVQT
CCF30682	*W. confusa* LBAE C39-2	α-(1→6)/α-(1→3)	519 RVDAVDNVDAD	555 LSILEDWSHND	626 FVRAHDSEVQT
ABF85832	*Lc mesenteroides* B-1299CB4	α-(1→6)	528 RVDAVDNVDAD	564 LSILEDWSHND	635 FVRAHDSEVQT
AAU08006	*L. parabuchneri* 33	α-(1→6)	500 RVDAVDNVDAD	536 LSILEDWSNND	599 FIRAHDSEVQT
AAA88588	*Streptococcus mutans* GS-5	α-(1→3)/α-(1→6)	463 RVDAVDNVDAD	499 LSILEAWSDND	579 FIRAHDSEVQD
AAU08004	*L. reuteri* ML1	α-(1→6)/α-(1→3)	1023 RVDAVDNVDAD	1059 INILEDWGGQD	1131 FIRAHDNGSQD
AAU08001	*L. reuteri* 180	α-(1→6)/α-(1→3)/α-(1→4)	1023 RVNAVDNVDVD	1059 INILEDWGWDD	1131 FVRAHDSNAQD
AAX97502	*L. reuteri* ATCC55730	α-(1→4)	1022 RVDAPDNIDAD	1057 INILEDWNSSD	1128 FIRAHDNNSQD
AAU08015	*L. reuteri* 121	α-(1→4)/α-(1→6)	1022 RVDAPDNIDAD	1057 INILEDWNHAD	1128 FVRAHDNNSQD
WP051675362	*L. reuteri* TMW1.656	α-(1→6)/α-(1→4)	1020 RVDAPDNVDAD	1055 LNILEDWSHAD	1129 FIRAHDSNAQD

Figure 4.1 Phylogenetic tree of glucansucrase amino acid sequences of *L. fermentum* kg3 (AAU08008) (Kralj, van Geel-Schutten, Dondorff, Kirsanovs, van der Maarel, & Dijkhuizen, 2004), *W. cibaria* CMU (ACK38203) (Kang et al., 2009), *W. cibaria* 10M (AND77013), *W. confusa* LBAE C39-2 (CCF30682) (Amari et al., 2012), *Leuconostoc mesenteroides* B-1299CB4 (ABF85832) (Kang et al., 2011), *L. parabuchneri* 33 (AAU08006) (Kralj et al., 2004), *Streptococcus mutans* GS-5 (AAA88588) (Shiroza., Ueda, & Kuramitsu, 1987), *L. reuteri* ML1 (AAU08004) (Kralj et al., 2004), *L. reuteri* 180 (AAU08001) (Kralj et al., 2004), *L. reuteri* ATCC55730 (AAX97502) (Kralj., Stripling, Sanders, van Geel-Schutten, & Dijkhuizen, 2005), *L. reuteri* 121 (AAU08015) (Kralj, van Geel-Schutten, et al., 2005) and *L. reuteri* TMW1.656 (WP051675362). The amino acids in nuclophile, general acid/base and transition-state stabilizer regions of glucansucrases with different linkage type preferences are shown. Dark grey background, identical residue; light grey background, highly conserved residue. Target amino acids that been mutated in this study are underlined.

* The different linkage types formed by the same enzyme are listed in the order of their abundance

4.3.2. Cloning and characterization of signal peptide or N terminal truncated glucansucrase

To determine whether acceptor-binding site residues substitutions in the catalytic core domain of *W. cibaria* dextransucrase alter the molecular weight and the linkage type of dextran, DsrM was cloned in *E. coli* and subjected to site-specific mutagenesis. Initially, the enzyme was cloned as DsrM-ΔN with truncated N-variable region and partial removal of glucan binding- and cell wall binding repeats, and as DsrM-ΔS with maintained the N-variable region (Figure S4.1 of the supplementary material). The purity of DsrM-ΔN and DsrM-ΔS was verified by SDS-PAGE (data not shown). The molecular weight of DsrM-ΔN was 130 KDa, which was smaller than DsrM-ΔS (160 KDa). Remarkably, the enzyme activity of DsrM-ΔN was below the detection limit and EPS production by DsrM-ΔN was not detected. However, DsrM-ΔN retained the ability to produce oligosaccharides in the acceptor reaction with 500 mM maltose and the oligosaccharide profile was similar to that produced by DsrM-ΔS (Figure S4.2 of the supplementary material). Thus, the N-variable region with glucan binding and cell wall binding repeats are required for glucan binding but not for linkage type specificity of DsrM. The role of the N-variable region for DsrM activity contrasts the high enzyme activity of the N-terminally truncated reuteransucrase GtfA-ΔN.

4.3.3. Biochemical characterization of reuteransucrase and dextransucrase mutants with analogous amino acid substitutions

To investigate parallel substitutions of amino acids in DsrM-ΔS and GtfA-ΔN, enzyme activity of DsrM-V583P:V586I or GtfA-V1024P:V1027I, DsrM-S693N:E694N:V695S or GtfA-S1135N:A1137S, and S622N or S1062N in DsrM-ΔS or GtfA-ΔN were determined (Figure 4.2). The purity of the enzymes was verified by SDS-PAGE (data not shown). To facilitate the

comparison among DsrM-ΔS and GtfA-ΔN derivatives, total activity and transferase activity of each mutant enzyme was compared to the corresponding wild-type enzymes. The substitutions of DsrM-V583P:V586I and GtfA-V1024P:V1027I did not reduce enzyme activity relative to the respective wild type enzymes. However, the substitutions of DsrM-S693N:E694N:V695S and GtfA-S1135N:A1137S significantly decreased transferase activity of both mutants. Moreover, a decreased enzyme activity was observed in DsrM-S693N:E694N:V695S but not in the corresponding mutant of GtfA-ΔN. The amino acid substitution S622N (S1062N) also differentially affected the activity of DsrM-ΔS and GtfA-ΔN. A dramatically reduced total activity and transferase activity was observed in DsrM-S622N but not in GtfA-S1062N. Thus, DsrM-ΔS and GtfA-ΔN responded differently to the analogous amino acid substitutes S693N:E694N:V695S (S1135N:A1137S in GtfA) and S622N (S1062N in GtfA).

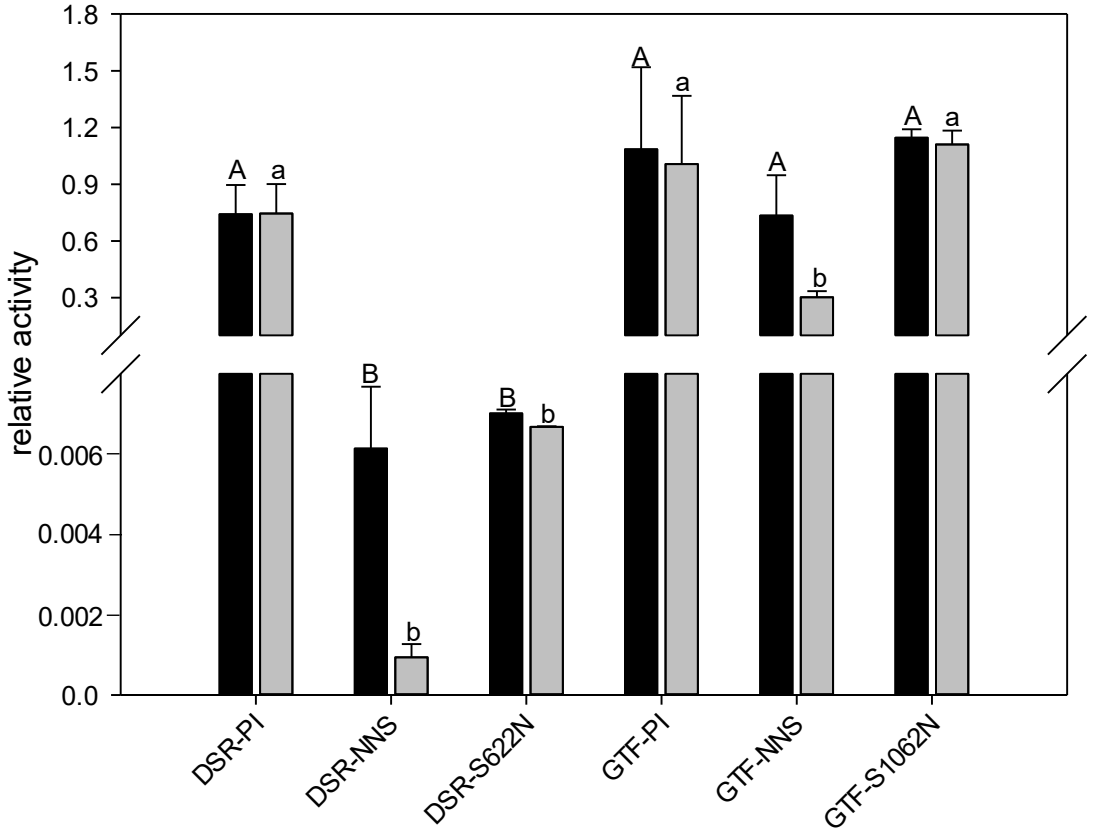


Figure 4.2 Enzymatic activity of mutant derivatives of dextransucrase DsrM-ΔS and reuteransucrase GtfA-ΔN. DSR-PI = DsrM-V583P:V586I; DSR-NNS = DsrM-S693N:E694N:V695S; DSR-S622N = DsrM-S622N; GTF-PI = GTF-V1024P:V1027I; GTF-NS = GtfA-S1135N:A1137S; GTF-S1062N = GtfA-S1062N. Mutant derivatives were incubated in sodium acetate buffer, with 500 mM sucrose. Relative total activity (■) and relative transferase activity (■) are shown as least square means ± standard deviation of duplicate independent experiments. The relative total activity was calculated as: mutant total activity / WT total activity; the relative transferase activity was calculated as: mutant transferase activity / WT transferase activity. Mean values at same color with unlike letters were significantly different, *P*

< 0.05. The detection limit was 0.0001 and corresponds to no detectable change of fructose concentration after 2 h incubation.

4.3.4. Glucan linkage type and molar mass distribution

The linkage type and the molar mass of glucans produced by DsrM-ΔS and GtfA-ΔN and the mutant derivatives were evaluated to investigate the catalytic preference of both enzymes with analogous amino acid substitutions (Table 4.2, Figure 4.3). Dextran and reuteran produced from DsrM-ΔS and GtfA-ΔN consisted of 98% and 84% α-(1→6) linkage, respectively (Table 6.2). DsrM-S693N:E694N:V695S produced dextranNNS with 52% α-(1→4) and 48% α-(1→6) and a molar mass of 36 MDa; the lowest molar mass observed among dextrans (Table 4.2, Figure 4.3). The effect of the mutation in DsrM was similar to the analogous modification in GtfA; reuteranNS produced by GtfA-S1135N:A1137S had an elevated proportion of α-(1→4) linkage (39%), decreased α-(1→6) linkage (61%) and lowest molar mass of 12 MDa among other reuterans (Table 6.2). V583P:V586I and S622N substitutions in DsrM-ΔS did not change linkage type and molar mass of dextranPI and dextranS622N (Table 4.2). However, the increased α-(1→4) linkage type was observed in reuteranPI to 25% (Table 6.2) and reuteranS1062N to 30% (Table 4.2), respectively. Remarkably, the molar mass of reuteranS1062N was increased to 102 MDa compared to 57MDa of reuteran from GtfA-ΔN (Table 4.2, Figure 4.3).

Table 4.2 Linkage type and molar mass of dextrans and reuterans produced from WT or mutant glucansucrases

	Chemical shift (%)		Mw	Mn (10^6 Da)	Mz
	α-(1→4)	α-(1→6)			
dextran	2*	98*	118	96	153
dextranPI	3	97	112	97	128
dextranNNS	52	48	36	30	156
dextranS622N	3	97	111	96	134
reuteran	14*	86*	57	42	76
reuteranS1062N	30	70	102	80	260

400 MHz ^{1}H NMR recorded at 300 K in D_2O and AF4 molar masses analysis of the glucans produced by DsrM-ΔS and GtfA-ΔN and their mutant derivatives. Displayed are the anomeric signals at ~4.96 ppm [α-(1→6) linkages], ~5.33 ppm [α-(1→4) linkages]. The molar mass data is displayed as mean number of two independent measurements.

* ^{1}H NMR result was taken from figure 6.2.

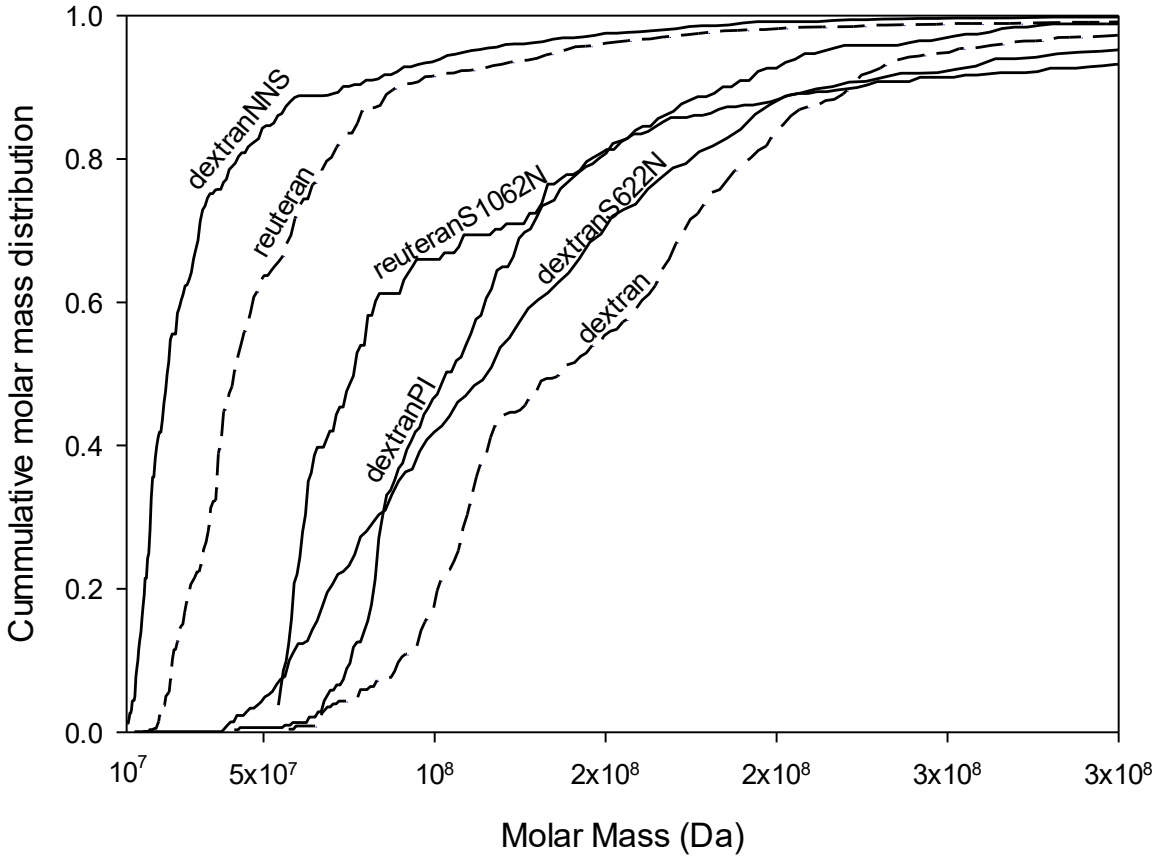


Figure 4.3 Cumulative molar mass distribution of dextrans and reuterans as determined by AF4 coupled with multi angle laser scattering detector. Shown is the molar mass distribution from 10^7 to 10^8 Da of dextrans and reuterans produced by WT enzymes (dotted line) and mutant enzymes (solid line).

4.3.5. Oligosaccharides synthesis with either maltose or glucose as acceptor sugar

Analysis of the catalytic specificity of DsrM-ΔS and GtfA-ΔN and their mutant derivatives was complemented by evaluation of the acceptor reaction with maltose or glucose (Figures 4.4 and 4.5). DsrM-ΔS and most mutant derivatives produced a α-(1→6) linked panose series with DP4 to DP8 with the presence of maltose as acceptor sugar. In contrast, DsrM-S693N:E694N:V695S did not produce panose series oligosaccharides but produced several oligosaccharides that were absent in the oligosaccharide profile generated by DsrM-ΔS (Figure 4.4). Remarkably, the oligosaccharides profile generated by DsrM-S693N:E694N:V695S was highly similar to the profile generated by GtfA-S1135N:A1137S (Figure 6.2), indicated a comparable preference for formation of glucosidic bonds other than α-(1→6). Maltose and maltotriose were synthesized by GtfA-ΔN and GtfA-S1062N with glucose as acceptor sugar but were absent in oligosaccharides produced by DsrM-ΔS and DsrM-S622N, indicating a higher preference for synthesis of α-(1→4) glycosidic bonds of GtfA enzymes (Figure 4.5). Moreover, GtfA-S1062N tended to produce oligosaccharides with higher DP value (after 25 min) than GtfA-ΔN. This trend was not observed with DsrM-S622N (Figure 4.5).

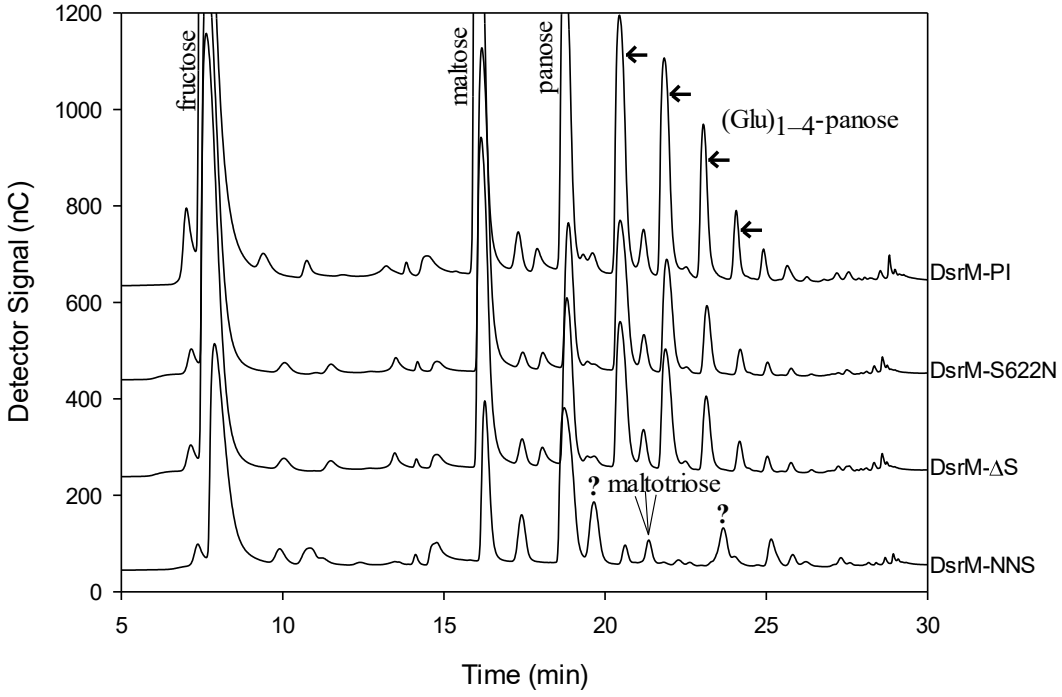


Figure 4.4 HPAED-PAD analysis of oligosaccharides produced with maltose as acceptor by DsrM-ΔS WT and mutant derivatives. Chromatographic traces are offset by 200nC. DsrM-ΔS = oligosaccharides produced by DsrM-ΔS; DsrM-PI = oligosaccharides produced by DsrM-V583P:V586I; DsrM-S622N = oligosaccharides produced by DsrM-S622N; DsrM-NNS = oligosaccharides produced by DsrM-S693N:E694N:V695S. Oligosaccharides identified by external standards are indicated in the figure; "?" indicates unknown oligosaccharides. $(Glu)_{1-4}$-panose indicates the panose series with one to four α(1→6) linked glucose units

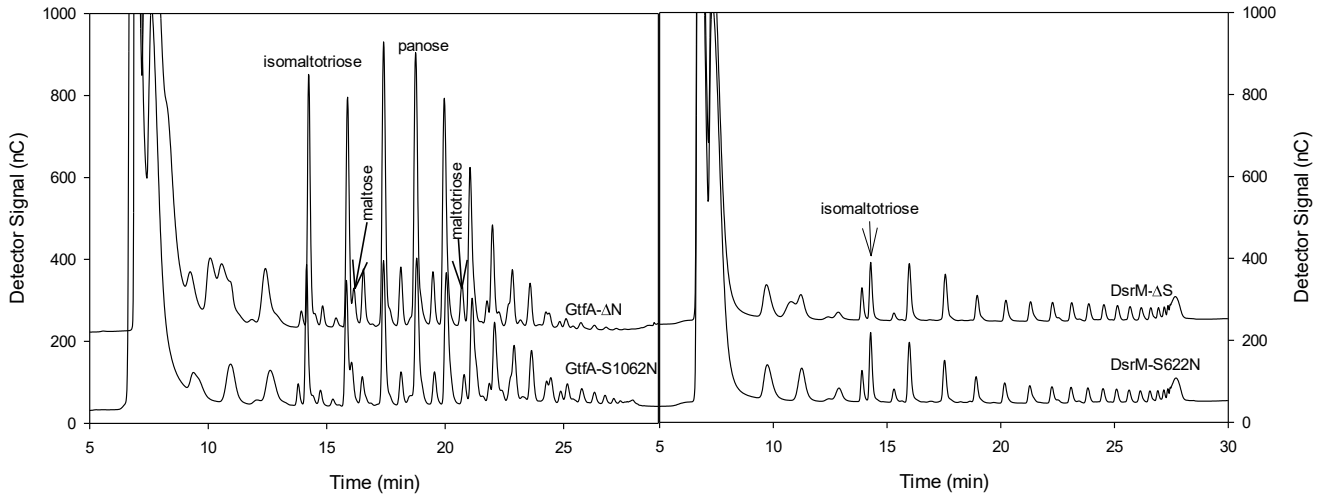


Figure 4.5 HPAED-PAD analysis of oligosaccharides produced with glucose as acceptor by glucansucrases WT and mutant derivative. Chromatographic traces are offset by 200nC. DsrM-ΔS = oligosaccharides produced by DsrM-ΔS; DsrM-S622N = oligosaccharides produced by DsrM-S622N; GtfA-ΔN = oligosaccharides produced by GtfA-ΔN; GtfA-S1062N = oligosaccharides produced by GtfA-S1062N. Oligosaccharides identified by external standards are indicated in the figure.

4.4 Discussion

Recent studies achieved the molecular modification of reuteransucrase towards the production reuteran with different proportion of α-(1→4) (Chen, Levy, & Gänzle, 2016; Kralj, van Geel-Schutten, et al., 2005; Leemhuis et al., 2013; van Leeuwen et al., 2008). However, the modification of the phylogenetically distinct dextransucrases from *Weissella* spp. has not been carried out. Dextran produced by DsrM from *W. cibaria* 10M showed superior performance in baking applications when compared to reuteran produced by GtfA from *L. reuteri* TMW1.656,

and both linkage type and molecular weight are related to the technological functionality in baking applications (Chen et al., 2016; Galle et al., 2012; Katina et al., 2009;). To the best of authors' knowledge, this is the first mutagenesis study of dextransucrase from a *Weissella* strain to alter linkage type and molecular weight of dextran.

Glucansucrases are composed of N and C terminal domains, the catalytic core that consist of A, B and C domains, and two domains IV and V that are adjacent to the catalytic core (Leemhuis et al., 2013; Shukla et al., 2016). The acceptor binding sites are mostly located in domain A and partially distributed in domain B (Leemhuis et al., 2013). Structural analysis and acceptor docking studies of reuteransucrases indicated that residues of +1 and +2 acceptor binding sites are related to acceptor binding, and potentially influence the preference for the glycosidic bond by orientating the position of the acceptor sugar (Leemhuis, Pijning, Dobruchowska, Dijkstra, & Dijkhuizen, 2012). Mutations of +1 subsite neighbor residues in GTF180 resulted in a derivative enzyme producing glucans with altered linkage type and branching degree (Meng et al., 2015). The +2 subsite residues N1134, N1135 and S1136 in GTFA121 and the corresponding residues in dextransucrases are linked to a high abundance of α-(1→4) linkages (Kang et al., 2011; Kralj, van Geel-Schutten, et al., 2005; van Leeuwen et al., 2008). It was also shown that +2 subsite residue W1065 is essential for GTF180 activity and has a stacking interaction with acceptor sugar (Leemhuis et al., 2013). The mutation of W1065 in GTF180 (Meng et al., 2015) and W491 in GTF-I (Tsumori, Minami, & Kuramitsu, 1997) inactivated the enzymes. Multiple sequence alignment revealed N1062, adjacent to the +2 subsite residue W1061 of GtfA, is conserved in reuteransucrases of *L. reuteri* 121 and *L. reuteri* ATCC55730, which produce glucans with a high proportion of α-(1→4) linkages (Figure 4.1). The substitution of S1062N increased the preference of GtfA for formation of α-(1→4) linkages from 14% to 30%. Moreover, GtfA-

S1062N also increased the molecular weight of reuteran. It was suggested that the +2 subsite residue Trp relates to production of long chain and linear glucans; DSR-E CD2 from *Lc. mesenteroides* NRRL B-1299, which only produce branched glucan, is not conserved at this position (Leemhuis et al., 2013). The increased molecular weight of reuteranS1062N may be related to the role of residue S1062 in determining chain length and degree of branching.

Due to the structural difference of reuteransucrase and dextransucrase, the same amino acid substitutions functioned differently in different glucansucrases (Kang et al., 2011; van Leeuwen et al., 2008). This study therefore compared dextransucrase and reuteransucrase activity and specificity after analogous amino acid substitutions. Reuteransucrase GtfA is 47% identical to dextransucrase DsrM; sequence divergence is observed mainly in the N-terminal sequences. An overview on the effect of analogous amino acid substitutions in reuteransucrase and dextransucrase on the molecular weight and the linkage type of the resulting glucans is shown in Figure 4.6. The relative enzyme activity of DsrM-V583P:V586I and GtfA-V1024P:V1027I was comparable but only GtfA-V1024P:V1027I produced a glucan with increased proportion of α-(1→4) linkages. GtfA-S1135N:A1137S and DsrM-S693N:E694N:V695S exhibited a comparable preference for the linkage type as the respective wild type enzymes, but only DsrM-S693N:E694N:V695S exhibited a strongly decreased enzyme activity. DsrM-S622N lost most of the enzyme activity without changing glucan linkage type while GtfA-S1062N displayed an increased preference to formation of α-(1→4) linkages. Moreover, the N variable region largely varies among glucansucrases (Leemhuis et al., 2013). Reuteransucrases usually contain a longer N variable region around 700 amino acid residues than dextransucrases with around 150 residues (Leemhuis et al., 2013). The activity and specificity of glucansucrases from *L. reuteri* and *Lc. mesenteroides* NRRL B-1299 were not dependent on the N-variable region (Kang et al.,

2011; Kralj, van Geel-Schutten, van der Maarel, et al., 2004). However, partial removal of glucan binding repeats by truncation of the N variable region (Shukla et al., 2016) of DsrM from *W. cibaria* reduced enzyme activity and glucan production without influencing the linkage type. Taking together, reuteransucrase and dextransucrase differ substantially in their structure. Dextransucrase activity is easily abolished when the amino acids located around transition-state stabilizer are substituted to the corresponding residues of reuteransucrase.

Dextransucrase produce prebiotic isomalto-oligosaccharides by transferring glucosyl units to acceptor sugars such as maltose, isomaltose and glucose. Maltose is reported as an efficient acceptor sugar whereas glucose is a weak acceptor. However, our results indicate that glucansucrases differing in their specificity also show different affinities to carbohydrate acceptors. GtfA produced higher amount of isomaltotriose but shorter chain oligosaccharides compared to DsrM when glucose was presented as acceptor sugar. Isomalto-oligosaccharides with different DP, including the panose series of oligosaccharides, stimulated the growth of lactobacilli and bifidobacteria differently (Hu et al., 2013). Lactobacilli preferentially utilize di- and trisaccharides while bifidobacteria preferentially metabolize oligosaccharides with a higher DP (Hu et al., 2013). Remarkably, most lactobacilli harbor enzymes degrading α-(1→6)-linked oligosaccharides whereas some strains of *L. reuteri* lack enzymes hydrolyzing α-(1→4) linked oligosaccharides other than maltose (Gänzle & Follador, 2012). Isomalto-oligosaccharides structure and DP are depending on glucansucrase specificity and substrate concentration. Different ratio of sucrose and maltose concentration altered the acceptor reaction of isomalto-oligosaccharides production towards higher or lower DP values (Shi et al., 2016). DsrM-S693N:E694N:V695S did not produce high DP panose-series oligosaccharides in the acceptor reaction with maltose formation, matching previous observations with the analogous mutant

GtfA-S1135N:A1137S (Chen et al., 2016). Both results are in agreement with the fact that NNS next to transition state stabilizer residue is not in favor of α-(1→6) panose generation (Kralj, van Geel-Schutten, et al., 2005). Different enzymes thus produce oligosaccharides with different structure and DP, which may allow oligosaccharides for targeted prebiotic applications.

Dextransucrases also glycosylate non-carbohydrate molecules with hydroxyl groups, including phenolic and alcoholic compounds (Tsumori et al., 1997; Yoon, Fulton, & Robyt, 2010). Mutagenesis of +1 and +2 subsite residues stopped glucan synthesis but enhanced the glycosylation of phenolic and alcoholic compounds (Devlamynck, Poele, Meng, van Leeuwen, & Dijkhuizen, 2016). GtfA-S1135N:A1137S and DsrM-S693N:E694N:V695S with decreased transferase activity have thus potential for use in glycosylation of non-carbohydrate acceptor compounds.

The mutagenesis of dextransucrase to produce glucans differing in molecular weight and linkage type may allow a broadened exploration of dextran applications in food production, and of the role of soluble and insoluble glucans in biofilm formation by lactic acid bacteria. For example, the use of reuteran and dextran as additives, or as product of fermentation, improves bread quality, and this effect is closely linked to the structure and the size of the glucans (Chen et al., 2016). This study expands the toolset to study structure - function relationships of glucan in baking applications. Likewise soluble and insoluble glucans are essential for biofilm formation of *Streptococcus mutans* and *L. reuteri* in the upper intestinal tract of humans and animals (Koo, Xiao, Klein, & Jeon, 2010; Frese et al., 2013). Biofilm formation by lactic acid bacteria also protect the organisms from varies environmental stress, such as ethanol and acid, compared to planktonic strains (Kubota, Senda, Tokuda, Uchiyama, & Nomura, 2009). The linkage type of glucans, particularly the ratio of α-(1→3) and α-(1→6) linkages, determines their solubility and

thus their ecological role in biofilm formation, aggregation, and stress resistance (Koo et al., 2010).

In conclusion, mutagenesis of a dextransucrase from *W. cibaria* demonstrated that structural determinants for activity only partially overlap with the reuteransucrases. DsrM but not GtfA activity depends on the N-variable region. The amino acid substitution of S622N in DsrM (S1062N in GtfA) influenced both the linkage type and the molecular weight of glucan synthesized by GtfA derivatives but not in glucans from DsrM derivatives. This study thus expands the toolset for structure and function relationships of glucans and their technological, nutritional, or ecological role. Further exploration of structure function relationships of *Weissella* spp. will be facilitated by information on the three dimensional structure and acceptor docking studies.

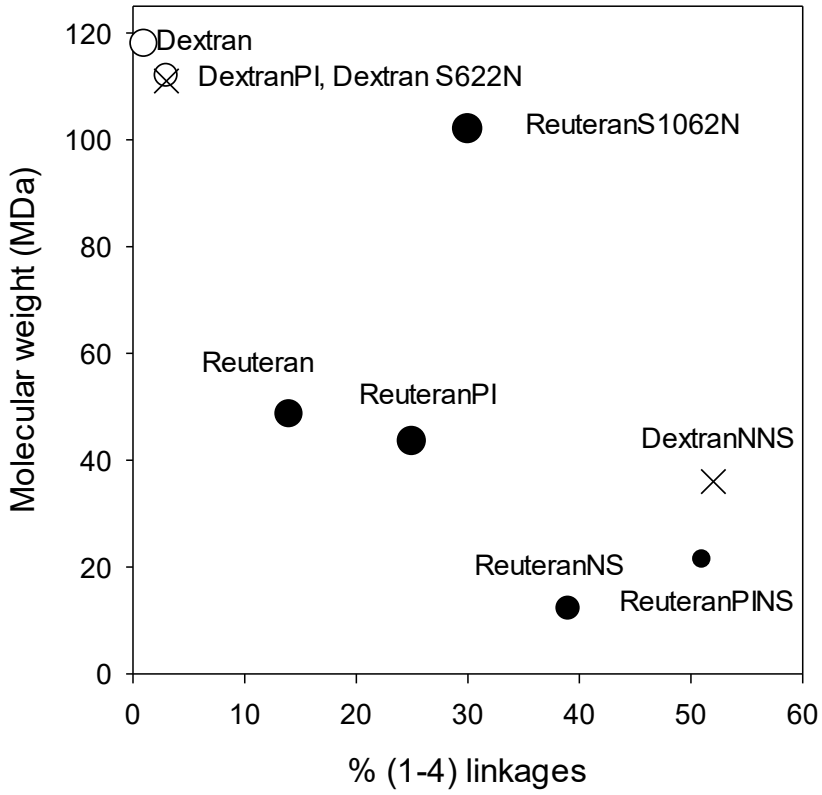


Figure 4.6 Comparison of the effect of analogous mutations on the activity of DsrM-ΔS and GtfA-ΔN and their derivative enzymes, and the linkage type and the molecular weight of glucans produced by these enzymes. The symbol area is directly proportional to the activity of mutant enzymes relative to the wild type enzyme; glucans are represented by cross-hairs if the enzyme activity was less than 1% of the activity of the corresponding wild type enzyme. See Table 4.2, Figure 4.2, and Table 6.2 for details.

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4.6 Supplementary materials

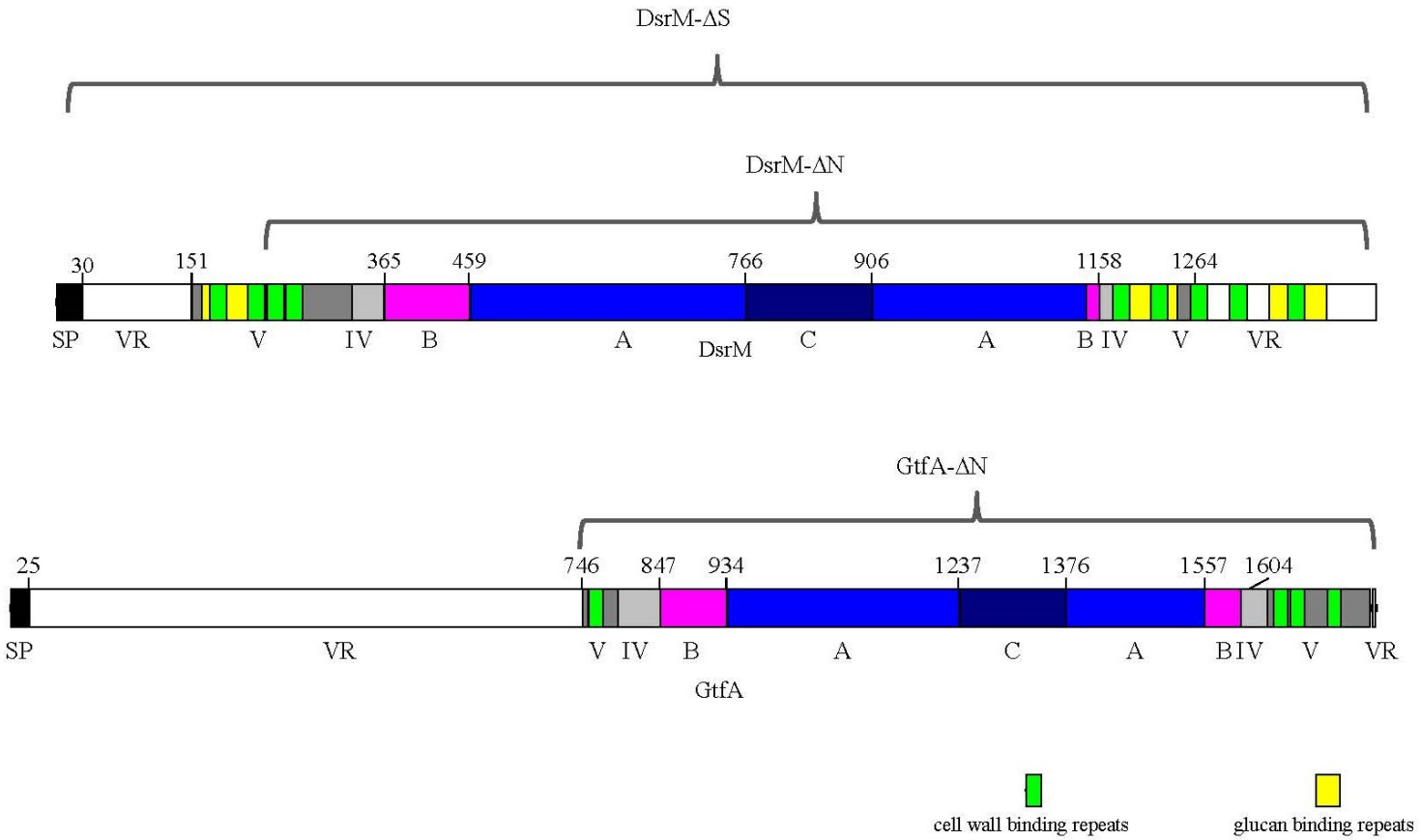


Figure S4.1 DsrM and GtfA amino acid sequence structure and assigned domains that obtained by InterProScan analysis and high similar glucansucrase alignment. From left to right are domains of signal peptide (SP); N terminal variable region (VR); domain V (V); domain IV (IV); domain B (B); domain A (A); domain C (C); domain A (A); domain B (B); domain IV (IV); domain V (V) and C terminal variable region (VR). The region that used to construct DsrM-ΔS, DsrM-ΔN and GtfA-ΔN are bracketed.

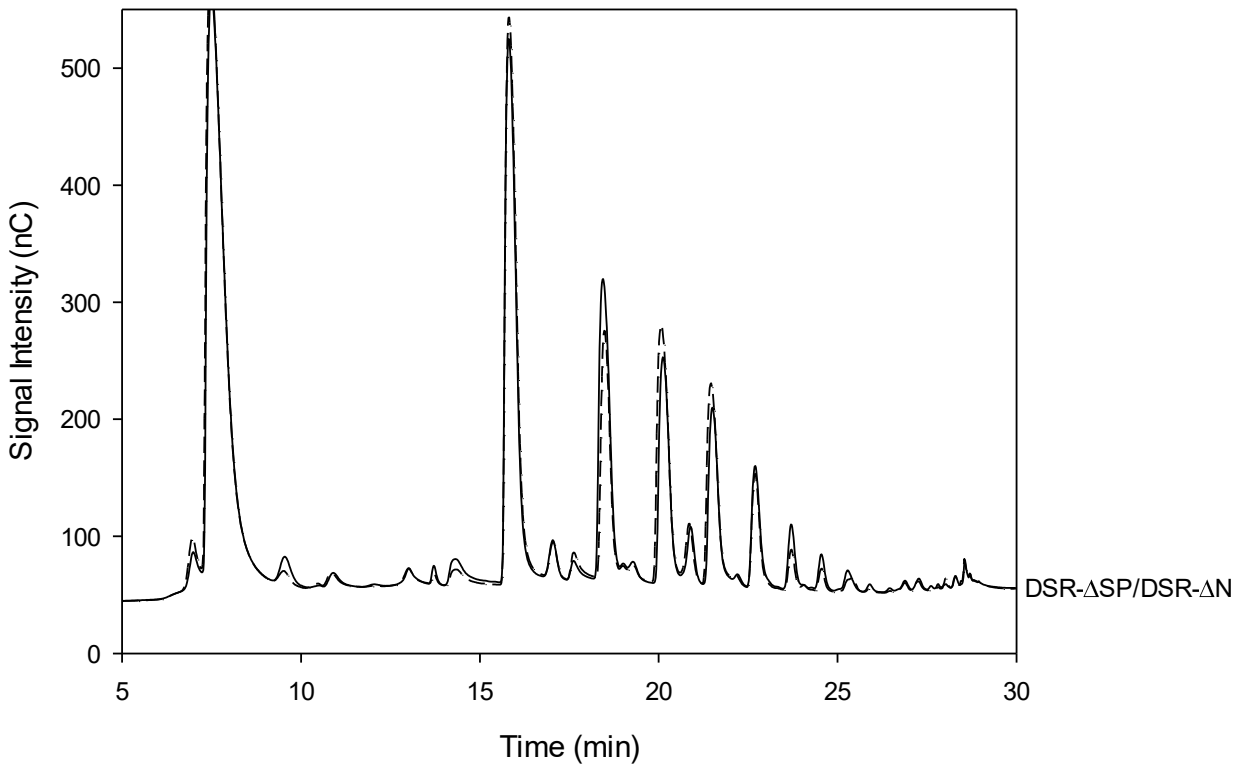


Figure S4.2 HPAED-PAD analysis of oligosaccharides produced by DsrM-ΔS (solid line) and DsrM-ΔN (dashed line) with 500mM maltose as acceptor sugar after 24h incubation.

5. Structure and function relationships of bacterial Exopolysaccharides in preventing enterotoxigenic *Escherichia coli* K88 adhesion

5.1. Introduction

Enterotoxigenic *Escherichia coli* (ETEC) infection is a common cause of watery diarrhea in animals and humans (Kaper, Nataro, & Mobley, 2004). It is the major problem of weanling piglets in the swine industry, leading to economic losses due to significant death and morbidity (Fairbrother, Nadeau, & Gyles, 2005). ETEC can be taken up by consuming contaminated water and food, then it colonizes intestines and secrete enterotoxins which lead to watery diarrhea (Nagy & Fekete, 2005). The initial colonization of ETEC through fimbriae adhering to enterocyte brush border receptors is a crucial step in pathogenesis of ETEC (Kulkarni, Weiss, & Iyer, 2010). ETEC possessing different fimbriae colonization factors infect different hosts (Gaastra & Svennerholm, 1996). ETEC K88 is the major infectious serotype in swine industry (Nagy & Fekete, 1998). K88 (F4) fimbriae are catalogued as F4ab, F4ac and F4ad based on their antigenic specificities. The K88 fimbriae variants recognize different host receptors including brush border glycoproteins and glycolipids. Mucin-type sialoglycoproteins (IMTGP-1 and IMTGP-2) are F4ab and F4ac receptors, while transferrin (GP74) is specifically recognized by F4ab, and glycosphingolipid (IGLad) is identified as F4ad receptor (Jin & Zhao, 2000). Based on the nature of glycoproteins and glycolipids, the carbohydrate moieties of these receptors are important in the recognition between ETEC fimbriae and brush border receptors.

The pathogenic mechanism of ETEC provides a new treatment approach that uses receptor analogs to prevent ETEC attachment (Shoaf-Sweeney & Hutkins, 2008) in order to replace the use of antimicrobial growth promoters or antibiotics. Dietary glycans that share structural similarities with brush border receptors are potential anti-adhesive agents of ETEC. Bacterial

reuteran produced from *Lactobacillus reuteri* TWM1.656 was able to reduce the number of ETEC K88 colonizing piglet jejunum epithelial cells (Chapter 3). Reuteran or dextran originated from *L. reuteri* DSM 17938 or *L. reuteri* L26 inhibited ETEC adhesion to IPEC-1 cells (Kšonžeková et al., 2016). The anti-adhesive activities of bacteria polysaccharides extracts were demonstrated in these studies. Due to the structural-dependent recognition mechanism between fimbriae antigens and brush border receptors, anti-adhesive effects of bacteria polysaccharides might be related to their structures (Jin & Zhao, 2000). The lack of clarification of glycan structural specificities in existing studies, limits the potential applications of glycans as pathogen anti-adhesive agents. The previous study suggested that levan produced by *L. reuteri* LTH5794 did not exert the same anti-adhesive effect compared to reuteran produced from *L. reuteri* TMW1.656 (Chapter 3). However, reuteran and levan differ greatly in the aspects of composition, linkage type, and molecular weight. To control structure diversity of the tested polysaccharides, heterologous-expressed wild-type and site-directed mutated reuteransucrases described in section 4.2.5 were applied to produce reuterans with different linkage type and molecular weight. This study aimed to explore structure and function relationships of reuterans as receptor analogs in preventing ETEC K88 adhesion.

5.2. Material and methods

5.2.1. Bacteria, plasmids, media and growth condition

L. reuteri TMW1.656, *L. reuteri* TMW1.112, *L. reuteri* LTH5448, *L. reuteri* LTH2584, *L. reuteri* 100-23, *L. reuteri* DSM20016 and *L. plantarum* TMW1.460 were cultivated anaerobically at 37 ^{o}C in mMRS medium with or without sucrose supplementation. *Weissella cibaria* 10M, *L. brevis* TMW1.465, *L. spicheri* LP38, *L. hammesii* DSR16381, *L. fermentum* ATCC14931 were cultivated anaerobically at 30 ^{o}C in mMRS medium. ETEC strain *E. coli*

ECL13795 (O149, virotype STb:LT:EAST1:F4) was cultivated aerobically at 37 °C on minca agar (Guinée, Veldkamp, & Jansen, 1977). *E. coli* BL21 Star (DE3) (Invitrogen, Toronto, ON, Canada) harbouring pE-GtfA-ΔN, pE-GtfA-V1024P:V1027I, pE-GtfA-S1135N:A1137S or pE-GtfA- V1024P:V1027I:S1135N:A1137S (section 4.2.5) were cultivated aerobically at 37 °C in LB broth containing 0.05 g/L kanamycin with agitation (200 rpm).

5.2.2. In frame deletion of *gtfA* from *L. reuteri* TMW1.656

The conserved catalytic domain of *gtfA* was truncated according to a deletion strategy described previously (Su, Schlicht, & Gänzle, 2011). In brief, an upstream 1494 bp fragment and a downstream 1132 bp fragment were amplified from genomic DNA of *L. reuteri* TMW1.656 (Table 5.1) and ligated together into pJRS233 at PstI and XbaI sites. The first and second crossover were selected as described (Su et al., 2011). The genotype of *L. reuteri* TMW1.656Δ*gtfA* was confirmed by using PCR and DNA sequencing (KU363983). The EPS producing character of *L. reuteri* TMW1.656Δ*gtfA* was determined by using AF4 followed the same protocol as described in section 4.2.10.

5.2.3. EPS production and purification

Bacterial reuteran or dextran was produced by *L. reuteri* TMW1.656 or *W. cibaria* 10M in the presence of 100 g/L sucrose. Bacterial extracts were produced by *L. reuteri* TMW1.656, *L. reuteri* TMW1.656ΔgtfA, *L. reuteri* TMW1.112, *L. reuteri* LTH5448, *L. reuteri* LTH2584, *L. reuteri* 100-23, *L. reuteri* DSM20016, *L. plantarum* TMW1.460, *L. brevis* TMW1.465, *L. spicheri* LP38, *L. hammesii* DSR16381, and *L. fermentum* ATCC14931 without the addition of sucrose. Purified reuteran, dextran, bacterial extracts or mMRS was harvest via ethanol precipitation, dialysis, hot phenol extraction and lyophilization as described in section 3.2.2. Reuteran, reuteranPI, reuteranNS or reuteranPINS was produced by heterologous expressed

reuteransucrases with 100 mM sucrose in NaAc reaction buffer for 2 days as described in section 4.2.7. Enzymatically produced reuterans were harvested via ethanol precipitation, dialysis and lyophilization.

5.2.4. Enzyme digestion

Bacterial extracts were digested by 8,000 U/g Lysozyme (Sigma-Aldrich, Oakville, ON, Canada), 5,000 U/g mutanolysin (Sigma-Aldrich), 300 U/g DNase (Thermo Scientific, Burlington, ON, Canada) or 300 U/g RNase (Thermo Scientific) at 37 ^{o}C for 1 h. After enzymatic treatment, samples were harvested via hot phenol extraction, dialysis and lyophilisation.

5.2.5. Hemagglutination assay

An overnight culture of *E. coli* ECL13795 was washed with 1 mL of 150 mM phosphate buffer (pH = 7.2). Hemagglutination assay was conducted as described in section 3.2.3. Reuteran, dextran, mMRS, or bacterial extracts produced from *L. reuteri* TMW1.656, *W. cibaria* 10M, mMRS or *L. reuteri* TMW1.656ΔgtfA were applied at 10 g/L or 1 g/L in the assay. Bacterial extracts produced from all of the lactobacilli strains before and after enzymatic digestion were tested at 10 g/L in the assay. The hemagglutination assay was conducted in duplicate or triplicate.

5.2.6. Bacterial extracts structure characterization

To determine the molecular size of the unknown compound extracted from lactobacilli, the purified bacterial extracts and mMRS were detected by size exclusion chromatography (SEC) coupled with superdex 200 column (GE Healthcare Life Sciences, Mississauga, ON, Canada) as described (Galle et al., 2010). One-dimensional ^{1}H-NMR spectra of the purified bacterial extracts that produced from *L. reuteri* DSM20016, *L. reuteri* LTH5448 and *L. fermentum* ATCC14931 were recorded on Agilent/Varian VNMRS four-channel 600 MHz spectrometer at

the University of Alberta NMR facility. The purified bacterial extracts that produced from *L. reuteri* TMW1.656, *L. reuteri* LTH5448, *L. reuteri* DSM20016 and *L. brevis* TMW1.465 were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) for structural characterization at the University of Alberta Mass Spectrometry Facility. The bacterial extracts were analyzed in carbohydrate matrix (2,5-dihydroxybenzoic acid) and protein matrix (sinapinic acid) over the range of 20 kDa to 220 kDa.

5.2.7. Genomic DNA sequence alignment and putative heteropolysaccharides cluster identification

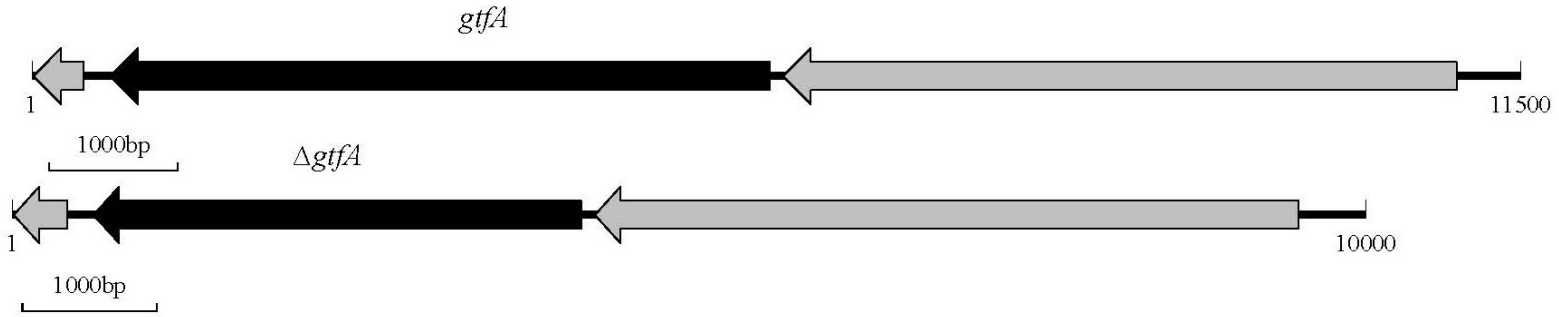
The genome sequences of *L. reuteri* TMW1.656, *L. reuteri* TMW1.112, *L. reuteri* LTH5448, *L. reuteri* LTH2584, *L. reuteri* 100-23, *L. reuteri* DSM20016, *L. brevis* TMW1.465, *L. spicheri* LP38, *L. hammesii* DSR16381, and *L. fermentum* ATCC14931 were downloaded from NCBI (http://www.ncbi.nlm.nih.gov/) and aligned by MAUVE using the progressive mauve alignment option. The genes for undecaprenyl-phosphate galactose phosphotransferase and flippase were searched against the annotated genome sequences to identify potential heteropolysaccharides biosynthesis clusters.

Table 5.1 Primers and plasmids

	plasmid	Primer pair (5'-3')	Restriction site
L. reuteri TMW1.656Δ*gtfA*	pUC18/ pJRS233	For-up CGCAAGCTTAATGATTTACAACCGGGTC	HindIII
		Rev-up ATTCGAGCTCCCTTGGAATTGAATACC GCTA	PstI
		For-down AACTGCAGTACTTATCTGGTCATAGCCTAG	PstI
		Rev-down CTAGTCTAGACTCCACGGAATGATACACC	XbaI

Plasmids and oligonucleotides used for *gtfA* and *dsrM* cloning, site-directed mutagenesis of *gtfA* and *L. reuteri* TMW1.656Δ*gtfA* knock out. Underlined nucleotides stand for silent mutation or site-directed mutation.

Figure 5.1 Scheme of in frame deletion of *gtfA* gene from *L. reuteri* TMW 1.656 chromosomal DNA. Reuteransucrase function was disrupted with catalytic domain deletion



5.3. Results and discussion

5.3.1. In frame deletion of *gtfA* in *L. reuteri* TMW1.656

To obtain an EPS-negative derivative of *L. reuteri* TMW1.656, *gftA* was disrupted by deletion of the section corresponding to the GtfA catalytic domain by double crossover mutagenesis. The disruption of *gtfA* in *L. reuteri* TMW1.656Δ*gtfA* was verified by sequencing (Figure 5.1). A comparison of polysaccharides in culture supernatants of *L. reuteri* TMW1.656 and *L. reuteri* TMW1.656Δ*gtfA* was performed by AF4. *L. reuteri* TMW1.656 but not *L. reuteri* TMW1.656Δ*gtfA* produced a high molecular weight polysaccharide (Figure 5.2), confirming that the phenotype *L. reuteri* TMW1.656Δ*gtfA* matches the genotype.

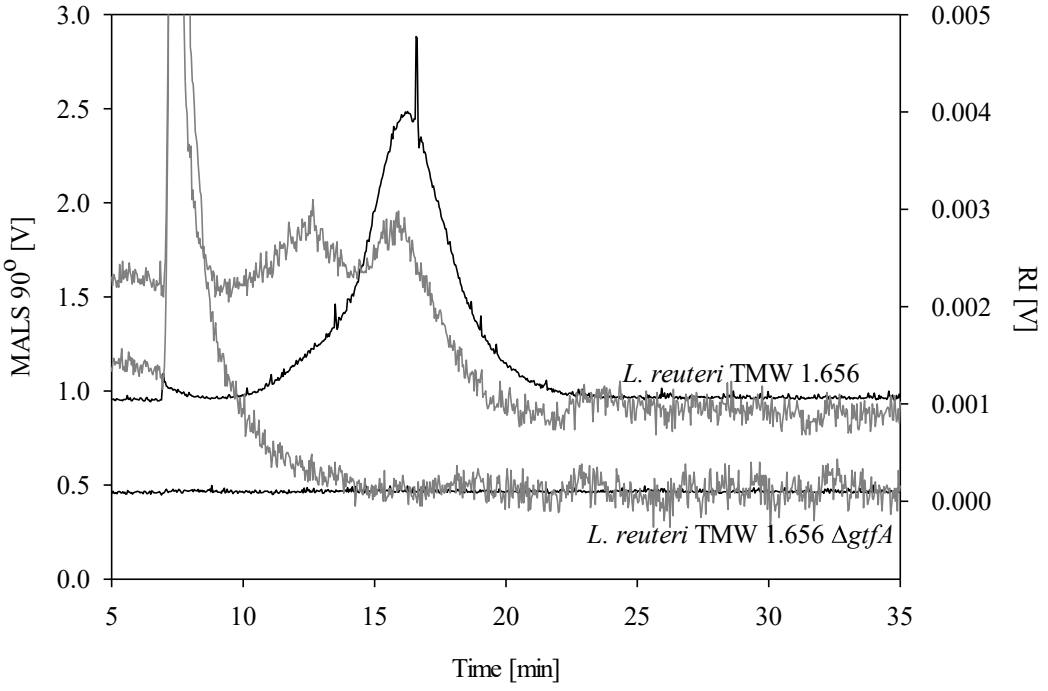


Figure 5.2 Analysis of polysaccharides purified from culture supernatant of *L. reuteri* TMW1.656 and *L. reuteri* TMW1.656Δ*gtfA* by AF4 coupled with multi angle laser scattering detector. Shown are signals from the light scattering at 90° angle (black line) and RI (grey line)

5.3.2. Structure and function relationships of bacterial and enzymatically produced EPS in preventing ETEC K88 adhesion

To explore the structure and function relationships of reuteran to prevent ETEC K88 adhesion *in vitro* (Wang, Gänzle, & Schwab, 2010), the enzymatically produced reuterans with different structures were tested. Reuteran, reuteranPI, reuteranNS, and reuteranPINS produced by GtfA, GtfA-PI, GtfA-NS and GtfA-PINS showed increased portion of α-(1→4) linkage type and different molecular weight (Figure 4.6). Compared to the positive control of bacterial reuteran produced by *L. reuteri* TMW1.656 and the negative control of mMRS, all of the enzymatically-produced reuteran, however, did not prevent ETEC K88 adhering to erythrocytes (Table 5.2). The enzymatically-produced reuterans were pure homopolysaccharides, while the bacterial reuterans extracted from *L. reuteri* TMW1.656 fermentation were homopolysaccharides with impurities. To determine if the anti-adhesive effect was conferred by bacterial reuteran, reuteransucrase gene knockout mutant was generated as *L. reuteri* TMW1.656ΔgtfA. Interestingly, bacteria extracts produced from the knockout mutant and wild-type strains showed the same anti-adhesive effect (Table 5.2). Previous studies indicated reuteran extracts produced by *L. reuteri* strains prevented ETEC adhesion (Kšonžeková et al., 2016; Wang et al., 2010). However, feeding fermentations of *L. reuteri* TMW1.656 decreased swine intestinal ETEC K88 load with or without the production of reuteran (Yang, Galle, Le, Zijlstra, & Gänzle, 2015). Therefore, the ETEC K88 anti-adhesive property is not conferred by reuteran but by the compounds purified along with reuteran. This result indicated bacterial homopolysaccharides purification method does not guarantee 100% pure compound. Therefore, validation by knockout of the functional genes is essential to characterize the functionalities of the bacterial products.

Table 5.2 Inhibition erythrocyte agglutination of ETEC K88 tested against bacterial EPS and enzymatically produced reuteran

EPS source	Sucrose addition	10 g/L	1 g/L
L. reuteri TMW1.656	+	+	N.D
L. reuteri TMW1.656	-	+	+
Heterologous expressed GtfA	/	-	N.D
Heterologous expressed GtfA-PI	/	-	N.D
Heterologous expressed GtfA-NS	/	-	N.D
Heterologous expressed GtfA-PINS	/	-	N.D
L. reuteri TMW1.656 ΔgtfA	+	N.D	+
L. reuteri TMW1.656 ΔgtfA	-	+	+
W. cibaria 10M	+	-	N.D
mMRS	-	-	-

- / No hemagglutination effect. Polysaccharides disabled the agglutination of blood cells at higher counts of ETEC than control.
- + Polysaccharides enabled the agglutination of blood cells at ETEC counts 2-8 fold higher than the control.
- ++. Polysaccharides enabled the agglutination of blood cells at counts of ETEC more than 8 fold higher than control.
- ND = not detected.

5.3.3. Screening of anti-adhesive compound among lactobacilli strains

To screen the anti-adhesive compounds produced by lactobacilli strains, six *L. reuteri* strains and five other lactobacilli strains with different phylogenetic distance (Zheng, Ruan, Sun, & Gänzle, 2015) were chosen (Table 5.3). All of the bacterial extracts produced from strains of *L. reuteri* showed anti-adhesive properties compared to other lactobacilli extracts and mMRS extracts, indicating that the anti-adhesive effect of bacterial extracts followed a species-specific manner (Table 5.3). *L. reuteri* TMW1.656, *L. reuteri* LTH2584, *L. reuteri* TMW1.112 and *L. reuteri* LTH5448 are sourdough isolates, while *L. reuteri* 100-23 is a rodent isolate and *L. reuteri* DSM20016 is a human isolate (Zheng, Zhao, Lin, & Gänzle, 2015). The sourdough isolates

belong to rodent linage III, and all of vertebrate isolates (Zheng, Zhao, et al., 2015) displayed ETEC K88 anti-adhesive effects. The host beneficial effects displayed by all of the *L. reuteri* strains (Table 5.3) suggested that *L. reuteri* strains could be intestinal probiotics.

To identify the anti-adhesive compounds, the bacterial extracts were treated with different enzymes to digest any known compounds that may exist in the extracts. EPS purification steps, including chill ethanol precipitation, ultracentrifugation, dialysis and hot-phenol protein extraction, might leave residues of water soluble and high molecular weight bacterial DNA, RNA, and cell wall materials. DNase, RNase, mutanolysin and lysozyme were thus applied to digest the bacterial extracts. The bacterial extracts of *L. reuteri* strains exhibited the anti-adhesive effects after digestion (Table 5.3). *L. reuteri* DNA, RNA and peptidoglycan were excluded from the list of potential anti-adhesive compounds.

Table 5.3 Inhibition erythrocyte agglutination of ETEC K88 tested against lactobacilli EPS after enzyme treatment

EPS source	Treatment					
	No treatment	**Mutanolysin**	**Lysozyme**	**DNase**	**DNase + lysozyme**	**RNase**
L. reuteri LTH5448	+/-	-	-	-	-	+/-
L. reuteri TMW1.656	+	+	+	+	+	+
L. reuteri LTH2584	+	+	+	+	+	+
L. reuteri TMW1.112	++	++	++	++	++	++
L. reuteri 100-23	+	+	+	+	+	+
L. reuteri DSM20016	++	++	++	++	++	++
L. spicheri Lp38	-	-	-	N.D	N.D	N.D
L. brevis TMW1.465	-	-	-	N.D	N.D	N.D
L. hammesii DSM16381	-	-	-	N.D	N.D	N.D
L. fermentum ATCC14931	-	-	-	N.D	N.D	N.D
L. plantarum TMW1.460	-	-	-	N.D	N.D	N.D
mMRS	-	-	-	N.D	N.D	N.D

- / No hemagglutination effect. Polysaccharides disabled the agglutination of blood cells at higher ETEC counts than control.
- +/- Results varies among repeats, both agglutination and anti-agglutination were observed.
- + Polysaccharides enabled the agglutination of blood cells at ETEC counts 2-8 fold higher than control.
- ++. Polysaccharides enabled the agglutination of blood cells at ETEC counts more than 8 fold higher than control.
- ND = not detected.

5.3.4. Structure characterization of the anti-adhesive compounds

To explore the structure specificity of the potential anti-adhesive compounds, bacterial extracts were analyzed by using SEC, ^{1}H-NMR and MALDI-TOF. Lactobacilli extracts (represented by extracts produced from *L. plantarum* and *L. brevis*) showed different patterns in SEC analysis

when compared to extracts produced from strains of *L. reuteri* and mMRS, which indicated different compositions between lactobacilli- and *L. reuteri*-produced extracts (Figure 5.3). Three distinct peaks of extracts from *L. reuteri* had a different retention time compared to mMRS (Figure 5.3), which suggested the components of *L. reuteri*-produced extracts were different from mMRS extracts in molecule size. Peptide bond and aromatic amino acids have UV absorption at UV_{200nm} or UV_{280nm}, respectively. The components of bacterial extracts were confirmed to have different chemical properties when detected using UV (Figure 5.4). A protein-like structure was found in the second component in all the bacterial and mMRS extracts (Figure 5.4). Bacterial extracts were also scanned over 20-220 kDa on MALDI-TOF in a carbohydrate or protein matrix to detect any potential carbohydrates or proteins (appendix A). Detectable carbohydrate signals with 162 Da intervals in the range of 1-10 kDa of all the bacterial and mMRS extracts indicated the existence of carbohydrate which was composed of a series of hexose sugars (appendix A). The carbohydrate compound characterized by MADI-TOF had a low MW, which suggested it corresponded to the third peak (lowest MW) in SEC chromatograph. The ^{1}H-NMR detection indicated the bacterial extracts were composed of high MW carbohydrates with protein background (appendix B). The anti-adhesive compound might be a high MW carbohydrate but the structure was not confirmed.

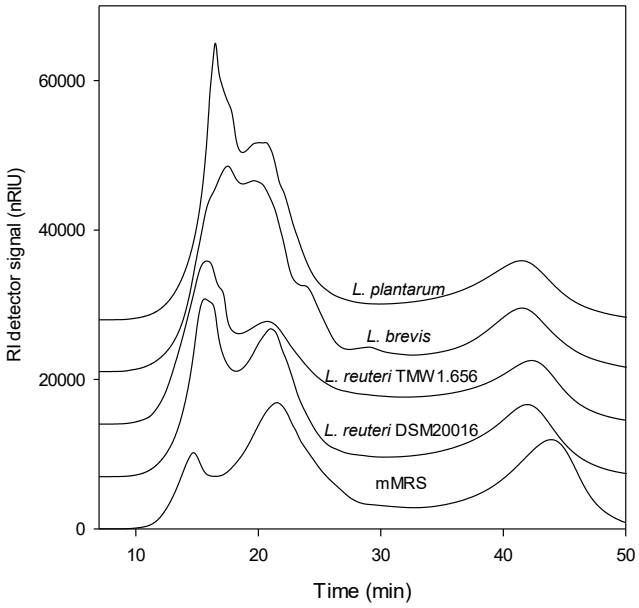


Figure 5.3 Results of size exclusion chromatography analysis of mMRS extracts or bacterial extracts produced from *L. reuteri* TMW1.656, *L. reuteri* DSM20016, *L. plantarum* TMW1.460 and *L. brevis* TMW1.465. The signals were collected from a refractive index (RI) detector

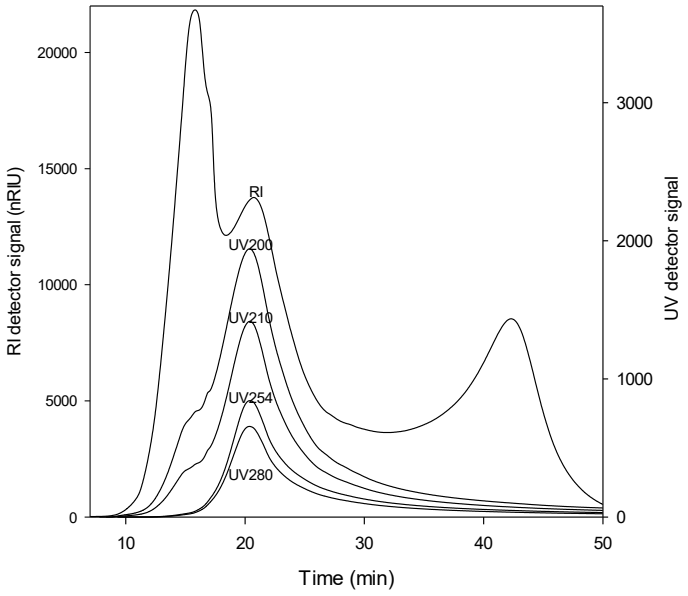


Figure 5.4 Results of size exclusion chromatography analysis of bacterial extracts produced by *L. reuteri* TMW1.656. The signals were collected from a UV detector at different wavelengths and a RI detector.

5.3.5. Identification of potential heteropolysaccharides biosynthesis gene clusters

Heteropolysaccharides or capsular polysaccharides are bacterial cell surface macromolecules involved in cell recognition (Lebeer, Vanderleyden, & De Keersmaecker, 2010). N-acetylglucosamine, N-acetylgalactosamine and galactose, which are components of heteropolysaccharides (Vuyst & Degeest, 1999), are also involved in the interaction between ETEC K88 fimbriae and host receptors (Jin & Zhao, 2000). Therefore, heteropolysaccharides produced by *L. reuteri* may play a role in preventing ETEC K88 adhesion.

The genomes of six strains of *L. reuteri* were searched for genes potentially related to heteropolysaccharides biosynthesis. Heteropolysaccharide biosynthesis gene clusters include four groups of genes, encoding proteins that synthesize sugar nucleotide precursors, regulate polysaccharides production and polymerization, assemble the repeating unit and export the polysaccharides (Badel, Bernardi, & Michaud, 2011). Among all the heteropolysaccharides biosynthesis proteins, polysaccharides transporter flippase and undecaprenyl-phosphate galactosephosphotransferase (priming glycosyltransferase) are the key proteins to identify potential heteropolysaccharides biosynthesis gene clusters (Lebeer et al., 2009). Two potential polysaccharides biosynthesis gene clusters were identified in strains of *L. reuteri* (Figure 5.5). However, flippase and priming glycosyltransferase are also involved in the biosynthesis of other cell wall-related polysaccharides, such as peptidoglycan and teichoic acids (Chapot-Chartier & Kulakauskas, 2014). This screening method may thus result in the overlap of gene clusters that are responsible for the synthesis of other cell-wall polysaccharides. However, the targeted gene clusters are not likely to be responsible for teichoic acid biosynthesis because of the absence of the key enzyme of glycerol glycerophosphotransferase (Figure 5.5) (Chapot-Chartier & Kulakauskas, 2014). Peptidoglycan is a fundamental component of bacterial cell wall, and its

composition and structure share high similarities among different strains (Chapot-Chartier & Kulakauskas, 2014). The high diversity of the targeted gene clusters among six strains of *L. reuteri* suggested that they were less likely to be associated with peptidoglycan synthesis (Figure 5.5). The recognized gene clusters are potential heteropolysaccharide biosynthesis gene clusters. The putative heteropolysaccharide gene cluster that present in *L. reuteri* TMW1.656, *L. reuteri* TMW1.112, *L. reuteri* 100-23 and *L. reuteri* LTH5448 is missing in *L. reuteri* LTH2584 (Figure 5.5a). The other putative heteropolysaccharides gene cluster in *L. reuteri* LTH2584 showed high diversity among the six *L. reuteri* strains (Figure 5.5b). The result indicated THE heteropolysaccharides were potentially produced by these *L. reuteri* strains. However, further confirmation of the heteropolysaccharides gene cluster and identification of the anti-adhesive property requires the knockout of glycosyltransferase genes.

A

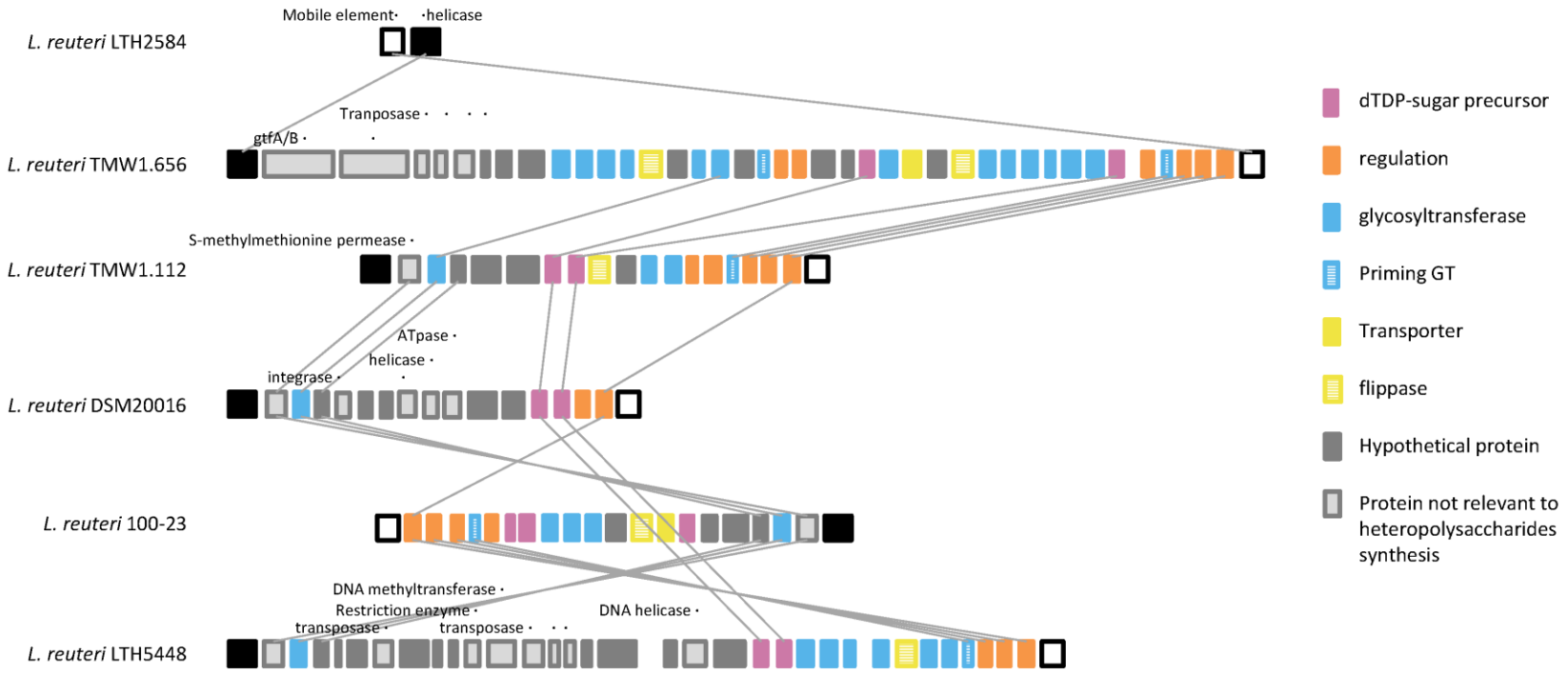


Figure 5.5 Comparison of the putative heteropolysaccharides biosynthesis gene clusters of six *L. reuteri* strains. Genes with deduced functions to synthesis sugar nucleotide precursors ( ), regulate polysaccharides production ( ), assemble repeating unit ( )and export polysaccharides ( ) are illustrated in different color.

B

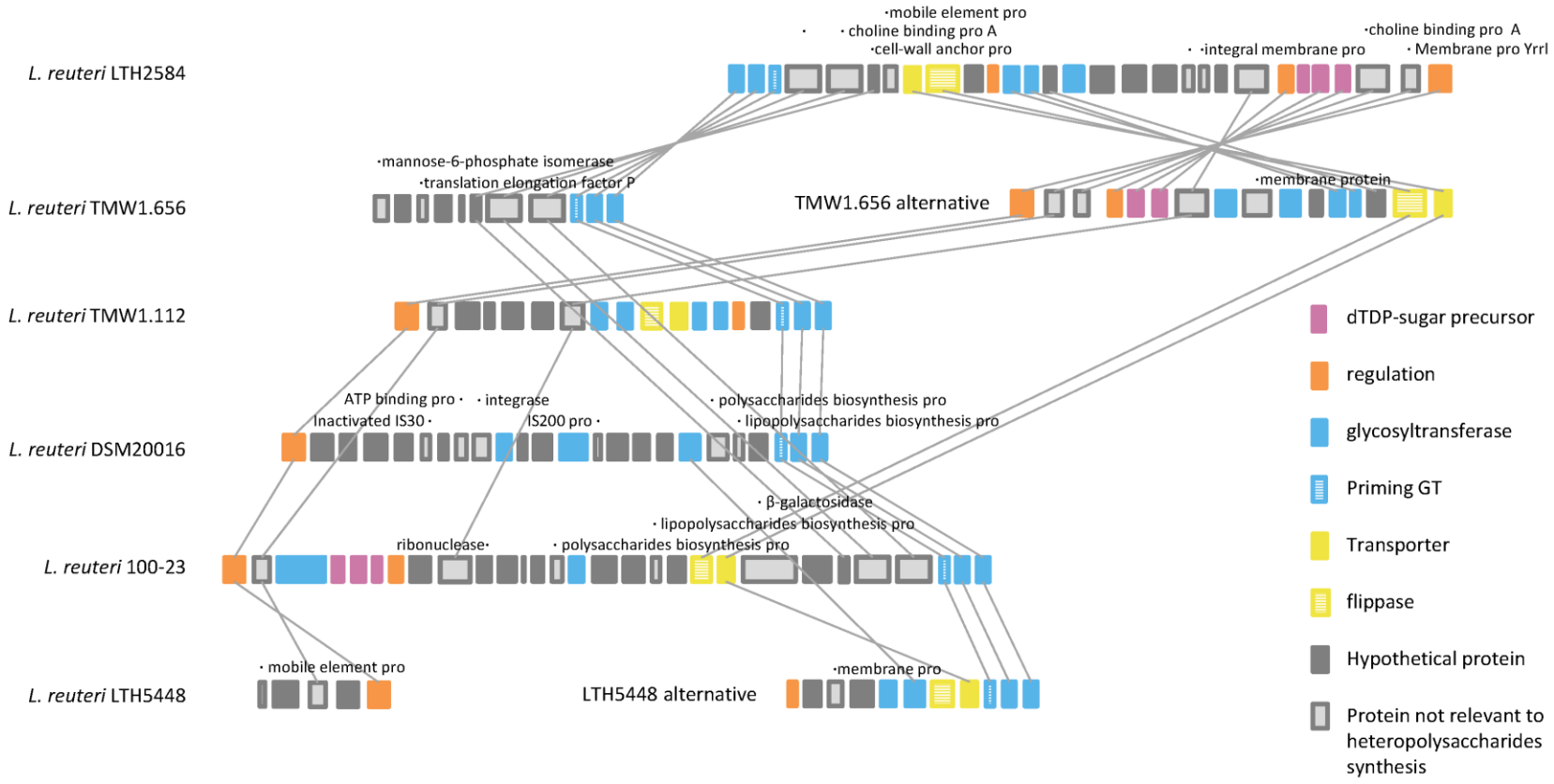


Figure 5.5 Comparison of the putative heteropolysaccharides biosynthesis gene clusters of six *L. reuteri* strains. Genes with deduced functions to synthesis sugar nucleotide precursors ( ), regulate polysaccharides production ( ), assemble repeating unit ( )and export polysaccharides ( ) are illustrated in different color.

5.4. Conclusion

This study indicated bacterial homopolysaccharides purified in previous studies were mixtures with impurities such as cell wall components. With reuteransucrase knockout mutant strain *L. reuteri* TMW1.656ΔgtfA and heterologous expressed reuteransucrases, the ETEC K88 anti-adhesive effect of reuteran was rejected in this work. Structure and function relationships of reuteran in preventing ETEC adhesion were not demonstrated. By structural characterization of bacterial extracts using SEC, ^{1}H-NMR and MALDI-TOF, the anti-adhesive compound was proposed to be a high molecular weight carbohydrate. The potential heteropolysaccharides biosynthesis gene clusters in strains of *L. reuteri* suggested a direction for further investigating of anti-adhesive compounds.

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6. Structure-function relationships of bacterial and enzymatically produced reuterans and dextran in sourdough bread baking application

6.1. Introduction

Sourdough improves texture, flavour, shelf life, and nutritional properties of baked and steamed bread (Gänzle, Vermeulen, & Vogel, 2007; Liu et al., 2016). These beneficial effects are related to the metabolism of lactic acid bacteria (LAB) during sourdough fermentation. Exopolysaccharides (EPS) produced by LAB increase bread volume, decrease bread firmness, and function as prebiotics (Galle, Schwab, Arendt, & Gänzle, 2010; Jakob, Steger, & Vogel, 2012; Kaditzky et al., 2008; Katina et al., 2009; Tieking & Gänzle, 2005). Because of their beneficial effects on bread quality, EPS from LAB may replace or reduce the use of hydrocolloids as bread improvers (Galle & Arendt, 2014).

Most EPS produced in sourdough fermentation are high molecular weight polymers composed of glucose (glucan) or fructose (fructan). Among these EPS, α-(1→6) linked dextrans have been regarded as the most promising bread improvers. Dextran from *Weissella cibaria* 10M decreased firmness and improved freshness of sorghum bread (Galle et al., 2012a); however, levan produced by *Lactobacillus reuteri* in the same bread formula showed no effect on bread quality. Reuteran, an α-(1→4) and α-(1→6) linked glucan produced by *L. reuteri*, and dextran from *W. cibaria* MG1 also exhibited differential effect on wheat bread quality (Galle et al., 2012a). It remains unclear, however, whether differences relate to functional differences of the EPS, or to other differences between the EPS producing strains. For example, *L. reuteri* but not *W. cibaria* produce high levels of acetate in presence of sucrose (Schwab, Mastrangelo, Corsetti, & Gänzle, 2008). Thus, different acidification levels may confound the beneficial impact of *in situ* produced EPS. To assess the effect of EPS that differ in linkage type and molecular weight on

sourdough bread quality, it is necessary to eliminate the differential impact of EPS producing strains on bread quality as confounding factor.

Reuteran and dextran are synthesized by the glycoside hydrolase family 70 (GH70) enzymes reuteransucrase and dextransucrase, respectively. Glucansucrases catalyze the alternative reactions sucrose hydrolysis, EPS synthesis, and oligosaccharide synthesis when suitable acceptor sugars are present (Korakli & Vogel, 2006). Glucansucrases harbor of four distinct domains, a signal peptide, an N variable region, a catalytic domain and a C-terminal domain (Monchois, Willemot, & Monsan, 1999). Sequence alignments, site directed mutagenesis and three-dimensional structure analysis identified the catalytic sites of glucansucrases, namely the catalytic nucleophile, the acid/base catalyst, and the transition-state stabilizer (van Hijum, Kralj, Ozimek, Dijkhuizen, & van Geel-Schutten, 2006). Linkage specificity is determined by several catalytic residues. The amino acids V1024, V1027 in nucleophile region, and S1135, N1136, A1137 in the transition-state stabilizer region (GtfA of *L. reuteri* TMW1.656 numbering) determined the linkage specificity of glucansucrases (Kralj, van Geel-Schutten, Faber, van der Maarel, & Dijkhuizen, 2005; van Leeuwen, Kralj, Gerwig, Dijkhuizen, & Kamerling, 2008). Knowledge on the amino acids that determine glucan structure thus allows the generation of mutant glucansucrases producing glucans differing in linkage type and / or molecular weight.

This study aimed to investigate structure-function relationships of reuteran produced from *L. reuteri* TMW1.656 and dextran produced from *W. cibaria* 10M in baking application. The primary structure of reuteransucrase was modified to produce reuterans with different linkage types and molar masses, and the effect of these EPS on bread quality was determined. Moreover, the effect of *in situ* produced reuteran on bread quality was compared to the effect of reuteran used as additive.

6.2. Material and methods

6.2.1. Bacteria, plasmids, media and growth condition

L. reuteri TMW1.656 was cultivated anaerobically at 37 ˚C in modified DeMan-Rogosa-Sharpe (mMRS) medium (Stolz, Vogel, & Hammes, 1995) containing either 10 g/L maltose, 5 g/L glucose and 5 g/L fructose, or 100 g/L sucrose. *W. cibaria* 10M was cultivated anaerobically at 30 ˚C in the same medium. *E. coli* strains TOP10 (Invitrogen, Toronto, ON, Canada), *E. coli* XL1 Blue (Agilent Technologies, Santa Clara, CA, USA) containing plasmids pUC18, pUC19 (Thermo scientific, Burlington, ON, Canada) were cultivated aerobically at 37 ˚C in LB (BD, Mississauga, ON, Canada) medium with 50 mg/L ampicillin. *E. coli* strain BL21 Star (DE3) (Invitrogen) with plasmid pET28a^{+} (Novagen, Etobicoke, ON, Canada) was cultivated aerobically at 37 ˚C in LB broth with 50 mg/L kanamycin (Invitrogen) for the purpose of expression *dsrM*, *gtfA* and derived mutant genes.

6.2.2. Heterologous expression and purification of glucansucrases

DsrM-ΔS, GtfA-ΔN and its mutant derivatives (Generated in section 4.2.4) were expressed and purified as described previously (Kralj et al., 2011). The purity of glucansucrases was determined by SDS-PAGE (Bio-Rad, Mississauga, ON, Canada).

6.2.3. Recombinant enzyme characterization

Protein concentration and enzyme activity of GtfA-ΔN, DsrM-ΔN and DsrM-ΔS, and their mutants were measured as described in section 4.2.6. Protein concentration was determined using protein assay reagent (Bio-Rad) with bovine serum albumin as standard. The concentration of glucose and fructose was determined enzymatically (Glucose and Fructose Assay Kit, Sigma-Aldrich, Oakville, ON, Canada). The amount of free glucose represents hydrolysis activity and

the amount of fructose represents the total enzyme activity. Transferase activity was calculated as difference between total and hydrolysis activity. One unit of enzyme activity was defined as the release of 1 µmol of monosaccharides per min.

6.2.4. Analysis of acceptor reaction products

Oligosaccharides were synthesized by an acceptor reaction of 1 µM of GtfA-ΔN or mutant derivatives incubated with 500 mM sucrose and 500mM maltose in reaction buffer for 24 h. Samples were analyzed by HPAEC-PAD with a Carbopac PA20 column coupled to an ED40 chemical detector (Dionex, Oakville, Canada) (Galle et al., 2010). Fructose, glucose, sucrose, maltose, maltotriose and panose were identified using external standards (Sigma Aldrich).

6.2.5. Glucan production and purification

To purify enzymatically-produced glucans, 50 nM purified DsrM-ΔS, GtfA-ΔN and derivative mutant enzymes were incubated with 500 mM sucrose in 25 mM sodium acetate buffer (pH = 4.7) containing 1 mM $CaCl_2$ for 2 days. Reuteran and dextran were harvested via 2 volume ethanol precipitation and purified by dialysis. The retentate was freeze dried and stored at -20 ˚C. To purify glucans produced by *L. reuteri* TMW1.656, the strain was cultured in sucrose mMRS broth for 16 h and subcultured overnight in 1 L sucrose-mMRS broth. Reuteran was harvested via 2 volume ethanol precipitation and purified by dialysis as described (Chen, Woodward, Zijlstra, & Gänzle, 2014). The retentate was freeze dried and stored at -20˚C.

6.2.6. Characterization of EPS

One-dimensional ^{1}H-NMR spectra were recorded on an Agilent/Varian Inova three-channel 400 MHz spectrometer at the University of Alberta NMR facility. All spectra were recorded at 353K with Z-gradient probe. EPS samples were dissolved in 99.97% D_2O. Chemical shifts were

expressed in parts per million (ppm) by reference to internal standard 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS). The proportion of α-(1→4) and α-(1→6) glucosidic linkages was determined at 5.3 and 5.0 ppm, respectively (Kralj et al., 2005).

Molar mass determination of glucan was performed with asymmetrical flow field-flow-fractionation (AF4) coupled to multi-angle light scattering (MALS) and RI detectors (Postnova, Salt Lake City, UT, USA). The regenerated cellulose membrane (Postnova) of the accumulation wall had a molecular weight cut off of 10 kDa. Glucan samples were dissolved to 10 g/L in 10 mM NaCl and injected onto the channel at a flow rate of 0.2 mL/min and a cross flow of 1 mL/min for 6 mins. After injection, the cross flow rate remained constant for 2 min, decreased exponentially to 0.1 mL min over 10 mins, and was then maintained at 0.1 mL/min for 10 mins. The molar mass was calculated MALS signals and RI signal by AF 2000 software (Postnova). A value of 0.146 mL/g was employed as refractive index increment (dn/dc) (Vilaplana & Gilbert, 2010). Poly-styrolsulphonate standards and BSA were used for calibration of detectors.

6.2.7. Sourdough fermentation

L. reuteri TMW1.656 or *L. reuteri* TMW1.656Δ*gtfA* were incubated at 37 ˚C overnight in mMRS broth and subcultured with 1% inoculum in 10% sweet wort with 15% sucrose addition (Zhao, Kinner, Wismer, & Gänzle, 2014). Sourdoughs with a dough yield of 200 [100 x (wheat flour (15% sucrose) + malt solution) / wheat flour] were fermented for 24 h (Galle et al., 2012b). Cell counts and pH were determined at 0 h and 24 h of fermentation. After 24 h fermentation, organic acids and EPS were quantified as described (Galle et al., 2010). EPS formation was analyzed and quantified by size exclusion chromatography using a Superdex 200 column (GE Healthcare Life Sciences, Mississauga, ON, Canada). Fermented sourdough was directly used for baking at a dosage level of 10%.

6.2.8. Bread baking

White wheat flour with 13% protein and whole rye flour with 10% protein were purchased from local supermarket. Bread dough was prepared with 55% (w/w flour base) tap water; 10% (w/w flour base) sourdough; 2% (w/w flour base) dry yeast; 1.5% (w/w flour base) table salt; 0%, 0.09% or 0.19 % (w/w flour base) glucan produced from GtfA-ΔN and its mutant derivatives, and the rest was made up by wheat flour or wheat flour (80%) and rye flour (20%). To keep a constant water/flour ratio, flour and water present in sourdough replaced equal amount of flour and water for control dough (Galle et al., 2012b). Control bread was prepared by replacing sourdough with equal amount of commercial flour and tap water.

Dough ingredients were blended with a C-dough hook kneader for 2 min at low speed (level 2) and 3 min at high speed (level 5) in a KitchenAid mixer (Mississauga, ON, Canada). After a dough rest at 30°C for 75 min, dough was scaled to 230 g, shaped, and placed in baking pans greased with sunflower oil. Dough was proofed at 30°C for 20 min before baking at 177°C for 25 min in a preheated convection oven (Bakers pride, Allen, Texas, USA). Loaves were removed from pans after baking and cooled for at least 2 h at room temperature before further analysis. Breads were stored in sealed plastic boxes at room temperature. Two to six independent repetitions on different baking days were performed for each type of bread.

6.2.9. Evaluation of bread characteristics

Bread volume was measured according to the AACC method 10-05 (AACC International, 2001) with four loaves for each bread type. Bread hardness was measured by texture profile analysis (TPA) according to the AACC method 74-09 (AACC International, 1995). TPA was performed using a TA.XT Express Enhanced texture analyser (Stable Micro System, Godalming, Surrey, UK) equipped with a 2 kg load cell and an aluminum plunger with a diameter of 12.7 mm.

Measurements were performed with a test speed of 2.0 mm/s and a trigger force of 20 g. Slices were compressed to 50% of their original height. For TPA, loaves were cut into 20 mm thick slices. Two slices were cut from the centre of the bread and each slice was measured 3 times at different sites. Hardness was measured after 0, 2, 5, and 8 d of storage, or after 0, 5, and 7 d of storage.

6.2.10. Statistical analysis

Differences of glucans effect on bread volume and hardness (n = 2, 4 or 6) were analyzed using PROC MIXED of SAS Version 9.4 (SAS Institute Inc, USA). Model included the fixed effect of glucan treatment and random effect of baking day. Mean separation was performed by using multiple range test. Results were expressed as least square mean ± standard error of the means. Significant differences are reported with a 5% probability of error ($P < 0.05$).

6.3. Results

6.3.1. Biochemical characterization of wild-type glucansucrase and derived mutants

DsrM- ΔS, GtfA-ΔN and derived mutants were expressed in *E. coli*. The purity and molecular weight of recombinant enzymes were verified by SDS-PAGE (data not shown). No background activity was detected from *E. coli* with the empty pET28a$^+$ plasmid itself (data not shown). GtfA-ΔN and mutants enzymes had a molecular weight of 118 KDa. The molecular weight of DsrM-ΔS was 160 kDa. Total enzyme activity and hydrolysis activity of GtfA-ΔN and derived mutants were determined with 500 mM sucrose. GtfA-ΔN-V1024P:V1027I and GtfA-ΔN-S1135N:A1137S yielded the same total activity but a lower ratio of transferase activity to hydrolysis activity compared to the wild-type GtfA-ΔN. GtfA-ΔN-V1024P:V1027I:S1135N:A1137S showed a significant decreased total activity and relative

transferase activity compared to GtfA-ΔN (Figure S6.1 of the supplementary material). The difference of enzyme activity among wild-type and mutant reuteransucrase indicated that site-directed mutations of the catalytic domain decreased total activity and transferase activity of the reuteransucrase GtfA.

6.3.2. Glucan linkage type and molar mass determination

Glucan structure and size may relate to their effects in bread quality; therefore, the linkage type and the molar mass of enzymatically-produced dextran, reuteran and derived mutant reuterans were evaluated (Table 6.1). ^{1}H NMR analysis of EPS produced by DsrM-ΔS, GtfA-ΔN and mutant derivatives indicated that DsrM-ΔS produced dextran with mainly α-(1→6) linkages. GtfA produced reuteran with 14% α-(1→4) and 86% α-(1→6) linkages, in keeping with the reuteran produced from GtfA from *L. reuteri* TMW1.106 (Kaditzky et al., 2008). Reuteran synthesized from GtfA-ΔN-V1024P:V1027I (reuteranPI) showed an elevated level of α-(1→4) linkages compared to wild type reuteran. Reuteran from GtfA-ΔN-S1135N:A1137S (reuteranNS) and GtfA-ΔN-V1024P:V1027I:S1135N:A1137S (reuteranPINS) also had an increased content of α-(1→4) linkages (Table 6.1).

The molar mass distribution of bacterial and enzymatically produced glucan was determined by AF4 (Table 6.1 and Figure 6.1). Dextran produced with DsrM-ΔS had the highest molar mass. The molar mass of the reuteran produced enzymatically with GtfA-ΔN, except for reuteranNS, was higher than the molar mass of reuteran produced by fermentation with *L. reuteri* TMW1.656. ReuteranPI and ReuteranPINS had a molecular weight similar to that of enzymatically produced reuteran.

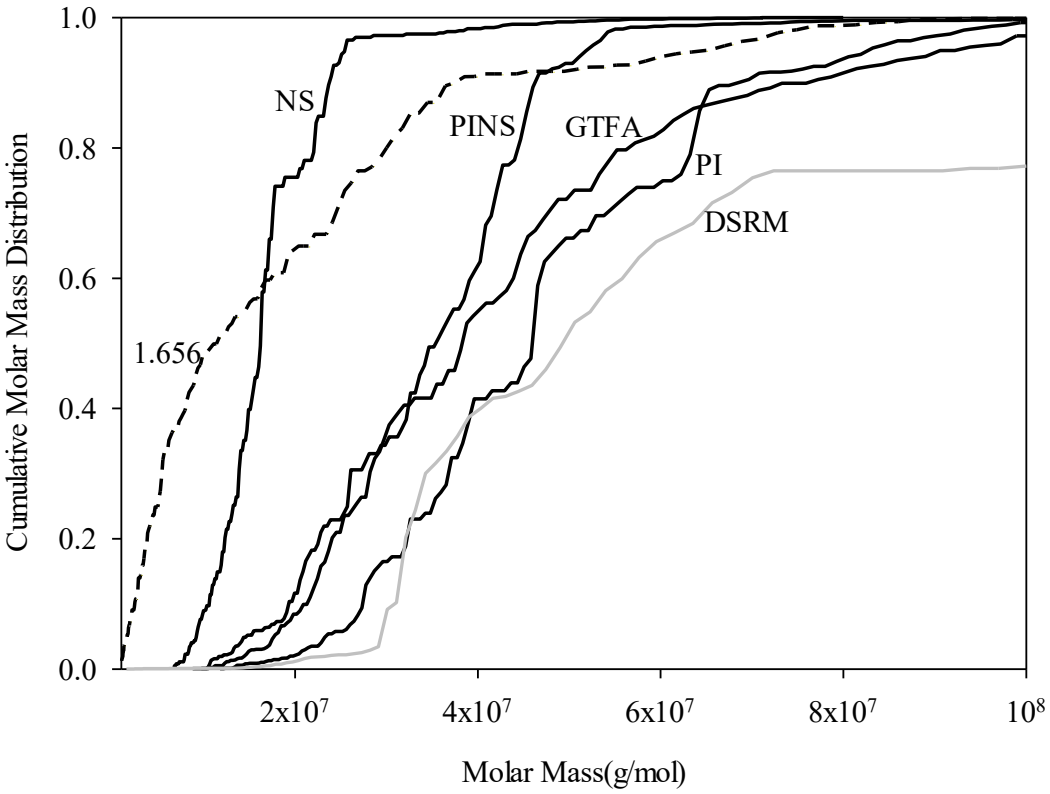

Figure 6.1 Cumulative molar mass distribution of glucans as determined by AF4 coupled with multi angle laser scattering detector. Shown is the cumulative molar mass distribution of reuteran produced from *L. reuteri* TMW1.656 (black dotted line) or enzymatically produced reuterans (black solid lines) and dextran (grey solid line). 1.656 = reuteran produced from *L. reuteri* TMW1.656; NS = reuteran produced from GtfA-ΔN-S1135N:A1137S; PINS = reuteran produced from GtfA-ΔN-V1024P:V1027I:S1135N:A1137S; PI = reuteran produced from GtfA-ΔN-V1024P:V1027I; GtfA = reuteran produced from GtfA-ΔN; DsrM = dextran produced from DsrM-ΔS

Table 6.1 Chemical properties of enzymatically produced reuterans and dextran

Enzyme used for synthesis	Chemical shift %			Mw	Mn	Mz
				(10^6 g·mol^{-1})		
	α-(1→3)	α-(1→4)	α-(1→6)			
GtfA-ΔN	/	14	86	48.6	33.9	99.8
V1024P:V1027I	/	25	75	43.5	36.9	50.6
S1135N:A1137S	/	39	61	12.2	9.6	13.8
V1024P:V1027I:S1135N:A1137S	/	51	49	21.4	20.2	22.7
DsrM-ΔS	≤2*	≤2*	98	82.9	47.3	192

400 MHz ^{1}H NMR recorded at 300 K in D_2O and AF4 molar masses analysis of the glucans produced by purified wild-type GtfA-ΔN and DsrM-ΔS enzymes and derived mutants. Displayed are the anomeric signals at ~4.96 ppm [α-(1→6) linkages], ~5.33 ppm [α-(1→4) linkages] and ~5.29 ppm [α-(1→3) linkages]

* Dextran produced from DsrM-ΔSP with trace amount (no more than 2%) of α-(1→3) and α-(1→4) linkages

6.3.3. Oligosaccharides synthesis with 500 mM maltose as acceptor sugar

Analysis of enzyme properties was complemented by determination of oligosaccharide synthesis. All reuteransucrases synthesized panose. GtfA-ΔN and GtfA-ΔN-V1024P:V1027I also yielded the α-(1→6) linked panose series of oligosaccharides with DP4 to DP8. GtfA-ΔN-S1135N:A1137S and GtfA-ΔN-V1024P:V1027I:S1135N:A1137S, however, produced several new oligosaccharides that could not be identified, indicating a glucosidic bond preference other than α-(1→6), in accordance with NMR result (Table 6.1). GtfA-ΔN-V1024P:V1027I:S1135N:A1137S produced maltotriose, in keeping with the preference for synthesis of α-(1→4) linkages (Figure 6.2).

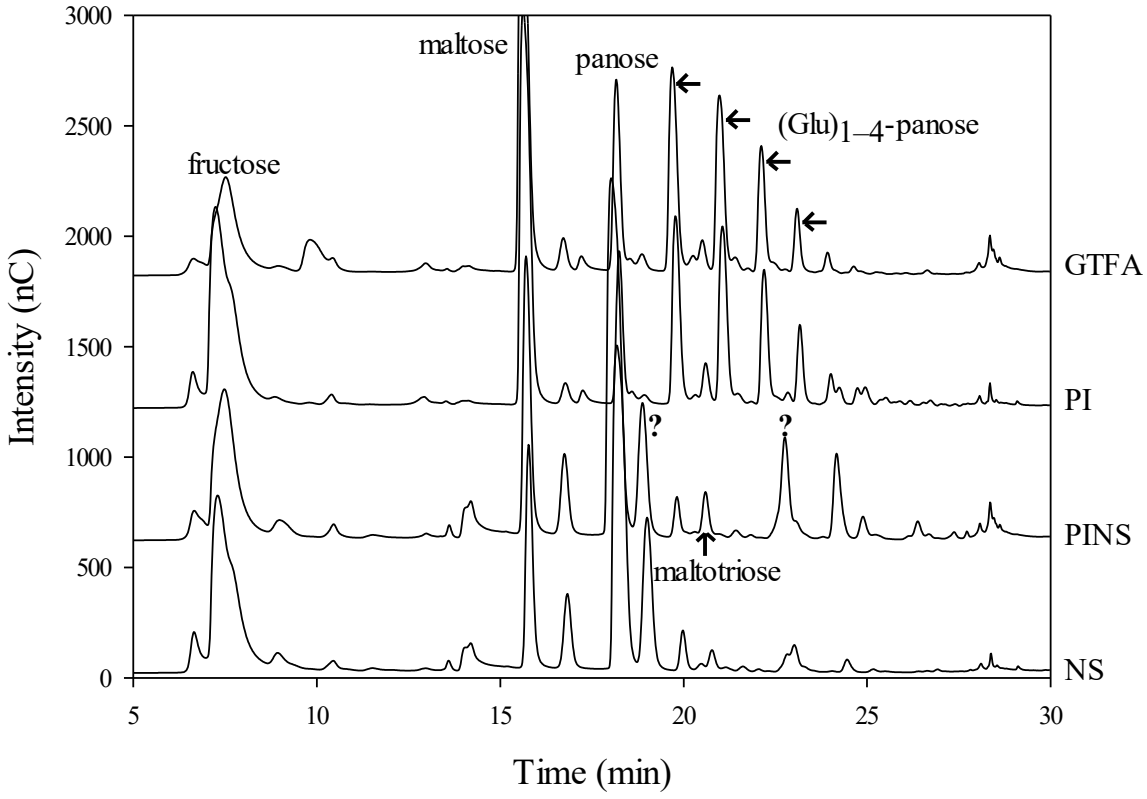

Figure 6.2 HPAED-PAD analysis of oligosaccharides products produced from acceptor reaction of GtfA-ΔN and derived mutants. Oligosaccharides were formed by incubation of 2µM GtfA-ΔN enzyme with 500 mM sucrose and 500 mM maltose for 24 h. Chromatographic traces are offset by 600nC. GtfA = oligosaccharides produced from GtfA-ΔN; PI = oligosaccharides produced from GtfA-ΔN-V1024P:V1027I; PINS = oligosaccharides produced from GtfA-ΔN-V1024P:V1027I:S1135N:A1137S; NS = oligosaccharides produced from GtfA-ΔN-S1135N:A1137S. Oligosaccharides identified by external standards are indicated; "?" indicates unknown oligosaccharides.

6.3.4. Sourdough fermentation and EPS quantification

After 24 h fermentation of *L. reuteri* TMW1.656 and TMW1.656Δ*gtfA* with 15% sucrose, a similar pH of 3.4 ± 0.1 and 3.3 ± 0.1 was observed. A comparable organic acid profile was

observed in sourdough fermented with *L. reuteri* TMW1.656 and *L. reuteri* TMW1.656ΔgtfA; the concentration of lactate (116.4 ± 3.5 mM and 107.0 ± 20.5 mM), acetate (45.8 ± 1.2 mM and 34.0 ± 5.5 mM) and ethanol (7.6 ± 0.2 and 16.5 ± 3.8 mM) were similar. The results indicate *L. reuteri* TMW1.656 and *L. reuteri* TMW1.656ΔgtfA have similar organic acid production behavior.

Quantification of bacterial glucan production in sourdough was performed to guide the addition of enzymatically produced glucan to bread. Purification of water soluble polysaccharides from sourdough fermented with *L. reuteri* TMW1.656 and TMW1.656Δ*gtfA*, followed by acid hydrolysis, confirmed that the wild type strain but not the mutant strain produced EPS composed of glucose. Quantification of glucan by size exclusion chromatography, and by quantification of glucose after hydrolysis revealed that *L. reuteri* TMW1.656 produced 15.8 ± 4.2 g reuteran/kg sourdough dry weight. For the subsequent studies, enzymatically produced glucans were added at two levels corresponding to 9.0 or 19 g/kg sourdough dry weight.

6.3.5. Impact of bacterial and enzymatically produced glucans on sourdough bread volume and texture

To compare the influence of *in situ* produced reuteran and enzymatically produced reuteran used as additive, bread quality was evaluated by the determination of loaf volume and crumb hardness. Enzymatically produced glucans were added at 0.09 or 0.19% to the bread dough. EPS was added to bread dough produced with 10% sourdough fermented with the EPS-negative *L. reuteri* TMW1.656Δ*gtfA* to account for metabolic properties of the strain. Sourdough bread was baked with two types of flours, 100% white wheat flour, or with addition of 20% whole rye flour to weaken the gluten structure. Bread volume and crumb hardness of wheat breads produced with

sourdough and EPS addition are shown in Table 6.2; bread characteristics of 80% wheat and 20% rye bread produced with sourdough and EPS addition are shown in Table 6.3.

Wheat sourdough bread had a higher bread volume when compared to control bread (Table 6.2), except for the bread with 0.09% reuteran addition. The loaf volume of bread with 0.19% reuteran addition and *L. reuteri* TMW1.656Δ*gtfA* sourdough was not different from the bread baked with *L. reuteri* TMW1.656 sourdough. However, volume enhancement by 0.19% reuteran was accompanied by uneven pore formation in bread (Figure 6.4). The hardness of the crumb of bread produced with *L. reuteri* TMW1.656 or with *L. reuteri* TMW1.656Δ*gtfA* and addition of 0.19% reuteran remained comparable throughout 5 days of storage (Table 6.2); bread produced with *L. reuteri* TMW1.656Δ*gtfA* and addition of 0.09% reuteran exhibited a higher crumb hardness. *In situ* produced and *ex situ* added reuteran thus had comparable effects on the volume and crumb hardness of wheat sourdough bread.

The loaf volume of 100% wheat sourdough bread was not increased by the addition of dextran when compared to *L. reuteri* TMW1.656Δ*gtfA* sourdough bread (Table 6.2). The volume of bread produced with 80% wheat flour and 20% rye flour, however, increased after addition of 0.09% dextran (Table 6.3). Remarkably, 0.09% addition of reuteran decreased the volume of sourdough bread when compared to sourdough bread produced with *L. reuteri* TMW1.656Δ*gtfA* (Table 6.3). Thus, weakening of gluten structure promoted EPS impact on sourdough bread volume. Hardness measurement on both 100% wheat and 20% rye sourdough bread revealed addition of dextran decreased crumb hardness and reduced bread staling (Table 6.2 and 6.3).

Reuterans produced from reuteransucrase mutants differed in their linkage type and molar mass (Table 6.1). To compare the effect of EPS structure on bread volume and hardness, 0.19%

reuteran, reuteranPI, reuteranNS, and reuteranPINS were added to sourdough bread produced with *L. reuteri* TMW1.656Δ*gtfA*. Remarkably, sourdough bread produced with reuteranNS and reuteranPINS were higher in volume when compared to bread produced with reuteran addition (Table 6.2), demonstrating that reuteran structure impacts bread volume. The effect of reuteranNS and reuteranPINS on bread volume was comparable to the effect of dextran (Table 6.2). Addition of reuteranNS and reuteranPINS, however, resulted in uneven pore formation, particularly the formation of large pores under crust, an effect that was also observed with reuteran but not with dextran (Figure 6.3). The crumb hardness of sourdough bread produced with reuteranNS was comparable to dextran and lower than the crumb hardness of bread produced with reuteranPINS or reuteran (Table 6.2). Thus, reuteran linkage type and molar mass both contributed to bread quality improvement.

Table 6.2 Effect of added EPS on the impact of wheat sourdough bread volume and crumb hardness. Bread was produced with 10% wheat sourdough containing 15% sucrose and fermented with *L. reuteri* TMW1.656, or with 10% sourdough containing 15% sucrose and fermented with *L. reuteri* TMW1. 656Δ*gtfA*.

L. reuteri strain used for fermentation	Glucan added to bread dough	Volume [mL/g]	Hardness day 0 [gf]	Hardness day 5 [gf]	Hardness day 7 [gf]	Hardness day 8 [gf]
TMW1.656	no addition*	3.02 ± 0.08^{b}	$251 \pm 25^{c,d}$	$775 \pm 30^{c,d,e}$	ND	921 ± 37^{c}
TMW1.656Δ*gtfA*	0.09% reuteran*	2.86 ± 0.07^{c}	$297 \pm 18^{b,c}$	896 ± 25^{b}	ND	1110 ± 32^{b}
TMW1.656Δ*gtfA*	0.19% reuteran	3.03 ± 0.08^{b}	$329 \pm 25^{a,b}$	827 ± 31^{c}	1170 ± 20^{a}	ND
TMW1.656Δ*gtfA*	0.09% dextran	$3.04 \pm 0.08^{a,b}$	224 ± 25^{d}	$659 \pm 30^{g,h}$	ND	765 ± 37^{d}
TMW1.656Δ*gtfA*	0.19% dextran	$3.08 \pm 0.08^{a,b}$	$273 \pm 25^{b,c,d}$	$698 \pm 31^{e,f,g}$	806 ± 20^{c}	ND
TMW1.656Δ*gtfA*	0.19% reuteran-PI	$3.06 \pm 0.08^{a,b}$	$272 \pm 25^{b,c,d}$	$736 \pm 31^{d,e,f}$	907 ± 20^{b}	ND
TMW1.656Δ*gtfA*	0.19% reuteran-NS	3.18 ± 0.08^{a}	231 ± 25^{d}	$684 \pm 31^{f,g}$	754 ± 20^{c}	ND
TMW1.656Δ*gtfA*	0.19% reuteran-PINS	3.19 ± 0.08^{a}	$277 \pm 25^{b,c,d}$	600 ± 31^{h}	867 ± 20^{b}	ND
TMW1.656Δ*gtfA*	no addition	2.99 ± 0.07^{b}	$289 \pm 15^{b,c}$	$784 \pm 23^{c,d}$	897 ± 20^{b}	908 ± 32^{c}
No sourdough	no addition	2.80 ± 0.08^{c}	375 ± 25^{a}	1050 ± 30^{a}	ND	1250 ± 37^{a}

Data are shown as least square means ± standard error, n = 2, 4 or 6. Mean values in the same column with unlike letters are significantly different, $P < 0.05$.

*Sourdough fermented with *L. reuteri* TMW1.656 contained 15.8 ± 4.2 g reuteran/kg sourdough dry weight. Sourdough fermented with *L. reuteri* TMW1.656Δ*gtfA* contained no EPS

Table 6.3 Effect of added EPS on the impact of bread volume and crumb hardness of 20% rye sourdough. Bread was produced with 70% wheat flour, 20% rye flour, and 10% wheat sourdough containing 15% sucrose and fermented with *L. reuteri* TMW1.656Δ*gtfA,* and addition of 0.09% enzymatically produced EPS.

Glucan added to bread dough	Volume [mL/g]	Hardness day 0 [gf]	Hardness day 2 [gf]	Hardness day 5 [gf]	Hardness day 8 [gf]
Reuteran	2.47 ± 0.03^{c}	457 ± 8.46^{a}	888 ± 13^{a}	1150 ± 31^{a}	1410 ± 12^{a}
Dextran	2.59 ± 0.03^{a}	382 ± 8.46^{c}	717 ± 13^{c}	853 ± 31^{b}	1140 ± 12^{c}
no EPS	2.53 ± 0.03^{b}	423 ± 8.46^{b}	757 ± 13^{b}	1056 ± 31^{a}	1250 ± 12^{b}

Data are shown as least square means ± standard error, n = 2. Mean values with unlike letters in the same column were significantly different, $P < 0.05$

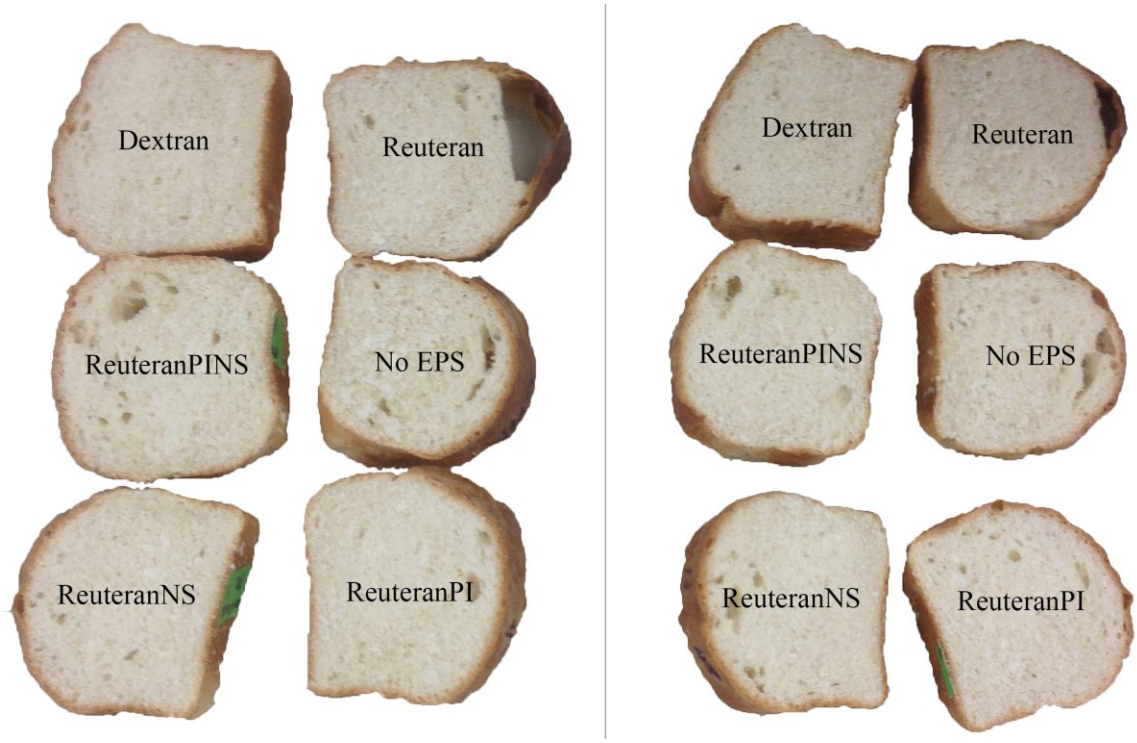

Figure 6.3 Crumb structure of wheat sourdough bread produced with 10% sourdough fermented with *L. reuteri* TMW1.656Δ*gtfA* and addition of 0.19% dextran, reuteran, reuteranPI, reuteranNS, or reuteranPINS. Pictures are representatives of 50% of all breads baked with 0.19% EPS addition.

6.4. Discussion

EPS production during sourdough fermentation may beneficially affect bread quality. Dextran production by *W. cibaria* in sourdough increased bread volume and reduced staling (Galle et al., 2012b; Katina et al., 2009). A beneficial effect of reuteran, however, was not demonstrated, possibly because acetate formation by *L. reuteri* in presence of sucrose compensated any beneficial effects of reuteran (Galle et al., 2012b). Likewise, beneficial effects of levan from *L. sanfranciscensis* were obscured by increased acetate formation, which reduced bread volume (Kaditzky, Seitter, Hertel, & Vogel, 2007). Most heterofermentative lactobacilli preferentially

metabolize fructose as electron acceptor, which results in the formation of mannitol and acetate (Galle et al., 2010; Korakli, Rossmann, Gänzle, & Vogel, 2001; Zheng, Ruan, Sun, & Gänzle, 2015). However, the absence of mannitol dehydrogenase in most strains of *Weissella* spp prevents excessive acidification even when sucrose is present (Galle et al., 2010). The differential effect of sucrose on metabolism of heterofermentative lactobacilli and *Weissella* confounds the comparison of EPS with different composition and structure. The present study added enzymatically produced reuterans and dextran to sourdough fermented with the EPS-negative *L. reuteri* TMW1.656Δ*gtfA*, thus eliminating confounding factors related to bacterial metabolism.

The application of glucan in bread can be achieved either by *in situ* production with starter culture or *ex situ* addition of the same compound. It was suggested that *in situ* produced EPS is superior to addition of *ex situ* produced EPS with respect to its effect on bread quality (Brandt, Roth, & Hammes, 2003; Tieking & Gänzle, 2005). The use of the levansucrase mutant strain *L. sanfranciscensis* LTH2590, however, indicated that *in situ* produced levan was not as effective as addition of *ex situ* produced levan (Kaditzky et al., 2007). In *L. sanfranciscensis*, the deletion of levansucrase activity also abolishes sucrose metabolism and thus reduces acetate formation (Tieking & Gänzle, 2005); any effects related to levan formation are thus confounded by the different acetate concentrations. *L. reuteri* TMW1.656Δ*gtfA* retains sucrose phosphorylase as second sucrose metabolic enzyme (Teixeira et al., 2012) and deletion of glucansucrases in *L. reuteri* has thus only a minor effect on sucrose metabolism and acetate formation ((Schwab, Walter, Tannock, Vogel, & Gänzle, 2007; Walter, Schwab, Loach, Gänzle, & Tannock, 2008). Accordingly, lactate production by *L. reuteri* TMW1.656Δ*gtfA* was comparable to *L. reuteri* TMW1.656. *L. reuteri* TMW1.656Δ*gtfA* formed slightly less acetate and more ethanol, which

may relate to the observation that metabolic turnover by extracellular glycansucrases is faster than metabolism by intracellular enzymes (Teixeira et al., 2012). Nevertheless, the cell counts, the pH, and organic acid levels in bread dough produced with *L. reuteri* TMW1.656 were comparable to bread dough produced with *L. reuteri* TMW1.656Δ*gtfA*, eliminating the confounding effect of acetate formation. This improved experimental setup demonstrated that the effect of *in situ* produced reuteran on bread volume and crumb hardness was similar to the effect of addition of reuteran.

This study compared bread produced with 100% wheat flour to bread produced with 20% rye flour. EPS effects on bread quality were more pronounced in the bread with rye addition. This appears to contrast previous studies reporting beneficial effects of EPS in wheat bread (Galle et al., 2012a). Galle et al. (2012a), however, used wheat flour with a protein content of 8-10% while the protein content of the wheat flour used in the present study was 13%. Both studies thus consistently indicate that EPS effects are more pronounced when a strong gluten network is absent. Remarkably, rye bran was recently shown to be an excellent substrate to boost dextran formation by *W. cibaria* (Kajala et al., 2015). Therefore, dextran-enriched rye or rye bran sourdoughs may serve as functional ingredient in bread production to improve sensory and nutritional properties without compromising texture and volume.

Only few previous studies compared the effect of linkage type, molecular weight, and branching on the technological functionality of glucans in baking. It was suggested that dextran with a linear chain structure may be more effective in increasing bread volume when compared to dextran with a similar molar mass but more branching (Lacaze, Wick, & Cappelle, 2007). Moreover, reuteran produced from *L. reuteri* TMW1.106 with 19 % α-(1→4) linkage was more efficient in increasing bread volume than dextran produced from *L. curvatus* TMW 1.624 with 8-

9 % α-(1→4) linkage (Jakob et al., 2012). Different from previous studies, the use of *L. reuteri* TMW1.656Δ*gtfA* in combination with addition of EPS from mutant reuteransucrases provided an unprecedented opportunity to study structure-function relationships of EPS. Reuterans from mutant enzymes with increased α-(1→4) linkage (25% to 51%) were more effective in enhancing wheat bread volume than wild type reuteran with 14% α-(1→4) linkages. Moreover, the effect of reuteranNS with 39% α-(1→4) linkage and the lowest molecular weight of 12.2 MDa was comparable to the effect of dextran with a molecular weight of 82.9 MDa. The present study thus demonstrates that size as well as structure of glucans relate to their effects on bread quality. Remarkably, specific effects of EPS structure extended to the crumb porosity. Addition of reuteran, reuteranNS and reuteranPINS resulted in an uneven pore distribution while reuteranPI and dextran improved bread texture without leading to formation of large pores (Table 6.2 and Figure 6.3). The effect of hydrocolloids on crumb porosity is poorly documented in the literature. It was previously reported that high concentrations of pectin increased crumb porosity when compared to addition of low levels of pectin because pectin was not able to stabilize gas cells in the dough and leaded to the coalescence of the cells (Lazaridou, Duta, Papageorgiou, Belc, & Biliaderis, 2007).

In conclusion, this study investigated the effect of glucans on sourdough bread quality. EPS was added to bread produced with sucrose metabolizing *L. reuteri* TMW1656Δ*gtfA*, thus eliminating confounding effects of (sucrose) metabolism during sourdough fermentation on bread quality. In wheat bread, *in situ* produced reuteran and addition of enzymatically produced reuteran exerted comparable effects on bread quality. Effects of dextran on bread volume and texture were superior to reuteran. Site-directed mutagenesis of reuteransucrase generated reuterans which differed in the linkage type and the molar mass. Noticeably, reuteranNS was as efficient as

dextran in bread quality improvement. Therefore, a structure function relation is suggested high percentage of α-(1→4) linkage and low MW of reuteran promoted the improvement of bread volume and softness. Our experiment allowed a fast screening of suitable EPS on bread quality enhancement in baking application.

6.5. References

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6.6. Supplementary materials

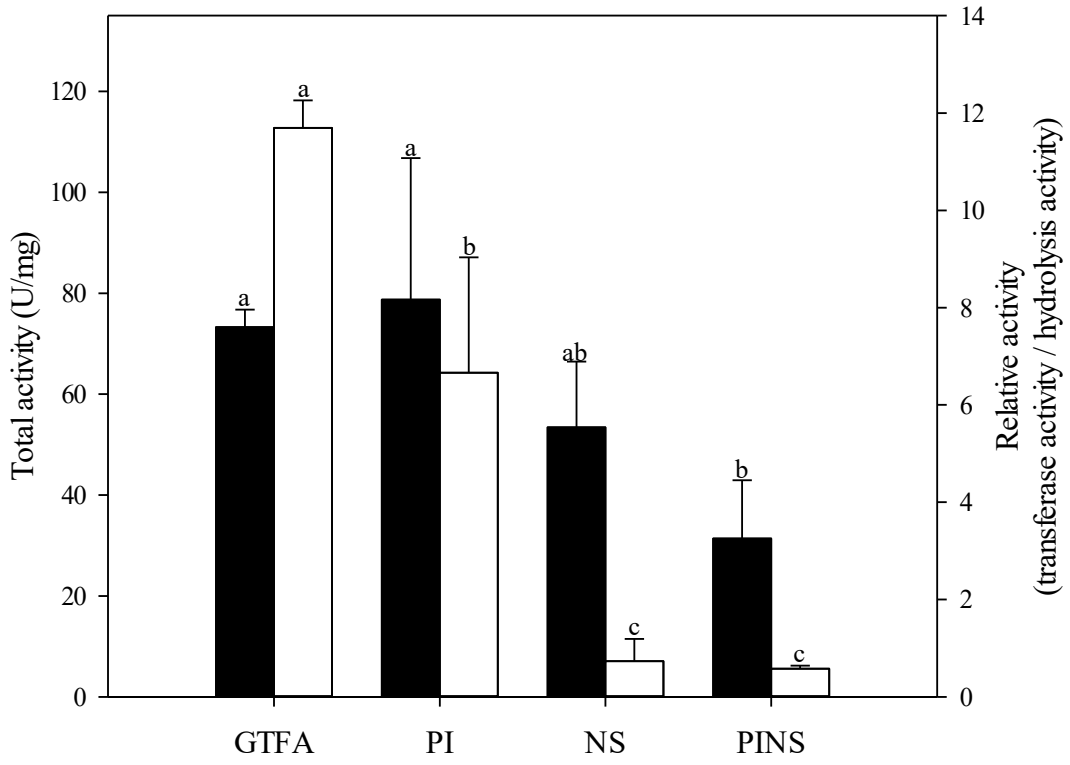

Figure S6.1 Enzyme activity measurement of 2µM reuteransucrase GtfA and its mutated derivatives incubated at 40 °C in 25 mM sodium acetate buffer, pH 4.7, with 500 mM sucrose. Total activity (■) and relative activity (□) are shown as least square means ± standard error, n = 3. Mean values (same colored column) with unlike letters were significantly different, $P < 0.05$. PI = GtfA-ΔN-V1024P:V1027I; NS = GtfA-ΔN-S1135N:A1137S; PINS = GtfA-ΔN-V1024P:V1027I:S1135N:A1137S

7. General discussion

Bacterial glycans, such as galacto-oligosaccharides (GOS) and exopolysaccharides (EPS), are commercially applied in food, pharmaceutical and cosmetic industries (Freitas, Alves, & Reis, 2011). Unlike other bacterial glycans, glycans produced from food grade lactic acid bacteria (LAB) do not require separation from the biomass of producing organisms. Lactic acid bacteria-produced GOS have prebiotic effects, pathogen anti-adhesion properties and immunomodulation activities (Chapter 2). Galacto-oligosaccharides functionalities are closely related to their structures, including linkage type, chain length and composition (Chapter 2). Bacterial EPS, produced from inexpensive sucrose in large quantities by glycosyl hydrolases or from intracellular sugar nucleotides in a limited amount by glycosyltransferases, can serve as prebiotics, anti-pathogen agents and hydrocolloids (Patten & Laws, 2015). This thesis research aimed to demonstrate structure and function relationships of LAB-produced EPS, in order to facilitate their commercial applications in food and pharmaceutical industries.

7.1. Structure and function relationships of lactobacilli EPS in bread baking applications

The hypotheses that "reuteran and dextran improve bread quality" and "technological functionality of reuteran is dependent on the molecular weight and the linkage type" have been both proven in Chapter 6.

In the bread baking industry, hydrocolloids are commercially applied as a bread improver. Improved gas and water binding abilities of bread dough are achieved with the addition of hydrocolloids, which are important to increase bread volume and delay bread staling. EPS produced by lactic acid bacteria are potential hydrocolloid alternatives (Galle & Arendt, 2014). Dextran produced from *Weissella cibaria* was superior to reuteran or fructan produced from *Lactobacillus reuteri* for improving bread shelf life (Galle et al., 2012b) and increasing bread

volume (Galle et al., 2012a). However, EPS structure and function relationships were not well established for bread baking in previous studies. The amount and variety of other metabolites from EPS producing strains also influence bread qualities (Galle et al., 2012a). To eliminate the confounding effects of metabolites from different strains, the heterologous expression system of reuteransucrase and dextransucrase were generated to produce pure EPS (Chapter 6). In order to facilitate the comparison of produced EPS (*in situ*) and added EPS (*ex situ*) on the impact of bread quality, the reuteransucrase isogenic knockout mutant *L. reuteri* TMW1.656Δ*gtfA* was generated and served as the fermentation strain (Chapter 6). Sucrose utilization of *L. sanfranciscensis* was abolished by knocking out the levansucrase gene, which resulted in the production of low amounts of organic acids (Tieking and Gänzle, 2005). Therefore, dough fermented by the levansucrase knockout mutant and the wildtype of *L. sanfranciscensis* LTH2590 were acidified differently in the presence of sucrose, which changed bread qualities (Kaditzky, Seitter, Hertel, & Vogel, 2007). For *L. reuteri*, sucrose phosphorylase is an alternative sucrose metabolic enzyme when reuteransucrase is absent (Teixeira, McNeill, & Gänzle, 2012). The mutant strain *L. reuteri* TMW1.656Δ*gtfA* was able to generate comparable amounts of organic acids when compared to the wild-type strain of *L. reuteri* TMW1.656 in the presence of sucrose (Chapter 6). To compare the effects of reuterans and/or dextran with different structures to improve bread texture and volume, reuteransucrase and dextransucrase were mutated to exhibit different linkage type and molecular weight catalytic specificities (Chapter 6). Glucan linkage type and molecular weight that specifically improved bread volume and texture were identified. This is the first research that established the structure and function relationships of LAB-produced EPS in bread baking applications (Chapter 6). The research results will facilitate

the utilization of LAB-produced EPS as hydrocolloids replacements in the food industry. For example, EPS with a desired structure can be applied to improve gluten-free bread quality.

7.2. Structure and function relationships of lactobacilli EPS as receptor analogues to prevent pathogen adhesion

The hypothesis of "reuteran but not dextran prevents adhesion of enterotoxigenic *Escherichia coli* (ETEC) K88 to swine epithelial cells" was rejected in Chapter 5. The hypothesis of "therapeutic functionality of reuteran is dependent on the molecular weight and the linkage type" is rejected in Chapter 5.

ETEC is a major cause of after-weaning diarrhea in piglets, which leads to economic loss in the swine industry. ETEC fimbriae attach to epithelial carbohydrate receptors to initiate ETEC infection (Jin & Zhao, 2000). Bacterial glycans with similar structure to carbohydrate receptors are able to competitively bind ETEC fimbriae and thus prevent ETEC attachment. Compared to commercial dextran and inulin, bacterial reuteran and levan produced by *L. reuteri* are anti-adhesive agents that prevent ETEC K88 adherence (Wang, Gänzle, & Schwab, 2010). Reuteran that was extracted from *L. reuteri* TMW1.656 fermentation products reduced the load of ETEC K88 on epithelial cells in a swine small intestine segment perfusion model (Chapter 3). However, bacterial extracts produced from the non-reuteran producing strain *L. reuteri* TMW1.656Δ*gtfA* prevented ETEC K88 adhesion. Meanwhile, enzymatically produced-reuterans did not exhibit the anti-adhesive activity to ETEC K88 adhesion. The results indicated reuteran did not prevent pathogen adhesion (Chapter 5). Therefore, the structure and function relationships of reuteran as anti-adhesive agent were not demonstrated in this study.

Current bacterial EPS purification methods, including the steps of ethanol precipitation, ultracentrifugation, dialysis and protein removal, do not guarantee the yield of pure EPS without

other cell wall materials. A high molecular weight, water-soluble compound extracted and purified along with reuteran, which was specifically derived from *L. reuteri* displayed anti-adhesive activity (Chapter 5). The virulence factors of pathogens, such as fimbriae and toxins, require specific epithelial carbohydrate receptors to initiate the infection (Chapter 2). Interactions between ETEC K88 fimbriae and carbohydrate receptors involve terminal galactose, *N*-acetylglucosamine and *N*-acetylgalactosamine (Jin & Zhao, 2000). Lactobacilli heteropolysaccharides contain monosaccharides, monosaccharide derivatives and substitutes as repeating units, including glucose, galactose and *N*-acetylglucosamine (Vuyst & Degeest, 1999). Therefore, the heteropolysaccharides produced by *L. reuteri* strains are potential receptor analogues because of the structural similarities to host carbohydrate receptors. However, the structures of heteropolysaccharides from *L. reuteri* were not determined in this study.

Taken together, the structure and function relationships of lactobacilli EPS as receptor analogues were not demonstrated in this study. Moreover, knockout of functional genes was an essential verification step to study bacterial glycans functionalities.

7.3. Do probiotics need EPS?

Feeding fermentation products of *L. reuteri* reduced intestinal ETEC K88 load in weanling piglets (Yang, Galle, Le, Zijlstra, & Gänzle, 2015). This health benefit justified the "promotion" *L. reuteri* from commensal bacteria to probiotics (Yang et al., 2015). Reuteran was not an active compound to prevent ETEC K88 adhesion to erythrocytes (Chapter 5). However, reuteran protects *L. reuteri* from acid/dry stress (Kaditzky et al., 2008) and helps *L. reuteri* strains colonize to the gastrointestinal tract. Reuteran is the component of polysaccharides matrix in a biofilm formed by *L. reuteri* TMW1.106 (Walter, Schwab, Loach, Gänzle, & Tannock, 2008). Biofilm formation contributes to *L. reuteri* colonization (Walter et al., 2008) and might protect

probiotics from gastrointestinal stress (Caggianiello, Kleerebezem, & Spano, 2016). In addition to the protection effects of homopolysaccharides to probiotics, lactobacilli biofilm and bacterial EPS (heteropolysaccharides in most cases) are associated with host-microbe interaction to modulate host immune system and benefit host health (Caggianiello et al., 2016; Rieu et al., 2014; Yasuda, Serata, & Sako, 2008).

It remains matter of debate whether probiotic activity is strain-specific or species-specific (Hill et al., 2014). This study suggested the beneficial effect of *L. reuteri* was specific at the species level (Chapter 5). However, a sufficient number of strains of *L. reuteri* are needed to demonstrate that the beneficial effect is species-wide. Moreover, the mechanisms underlying the probiotic effects to confer different health benefits have different specificities. For example, probiotic immunomodulation effects tend to follow a strain-specific manner (Baarlen et al., 2011) while metabolites dependent probiotic effects seems to be species specific (Kumar et al., 2013).

7.4. Limitations and future directions of this study

EPS are potential receptor analogues to prevent pathogen adhesion. This study detected the anti-adhesive activity of bacterial and/or enzymatically-produced EPS *in vitro* and/or *ex vivo*. An accurate quantification method to detect attached bacteria in a piglet small intestine segment perfusion model was developed (Chapter 3). This model will facilitate further exploration of utilizing anti-adhesive compounds to prevent ETEC infection in piglets in the swine industry. However, the small intestine segment perfusion model is an *ex vivo* model that cannot represent the real digestive system of a living animal. Feeding functional compounds to ETEC infected piglets is necessary to elucidate the anti-adhesive property of the compounds in the future.

The reuteran purified from *L. reuteri* fermentation prevented ETEC K88 adherence in the piglet small intestine segment perfusion model (Chapter 3) which was in accordance with the reuteran

in vitro results (Wang, Gänzle, & Schwab, 2010). However, the conclusion was later on revised as the purified bacterial extracts but not reuteran from *L. reuteri* displayed the ETEC K88 anti-adhesive effect (Chapter 5). Regarding the functionality investigation of any bacterial extract, this study suggested using functional gene knockout mutants and pure compounds produced from enzymes as the first step to identify functionalities. To fully understand if *L. reuteri*-produced heteropolysaccharides prevent ETEC adherence, a series of glycosyltransferases isogenic knockout mutants should be created.

Lactic acid bacteria-produced EPS are candidates to be commercially applied in the food industry. However, it was doubted if the technological functionality was related to EPS structure or not. This research explored and demonstrated the structure and function relationships of LAB-produced EPS in a bread baking application. This study proposed a structural-based method for rapid screening of EPS and EPS producing strains in commercial applications. The heterologous expression system of dextransucrase and reuteransucrase, and site-directed mutated glucansucrases allow further exploration of reuterans and dextrans in other applications.

7.5. References

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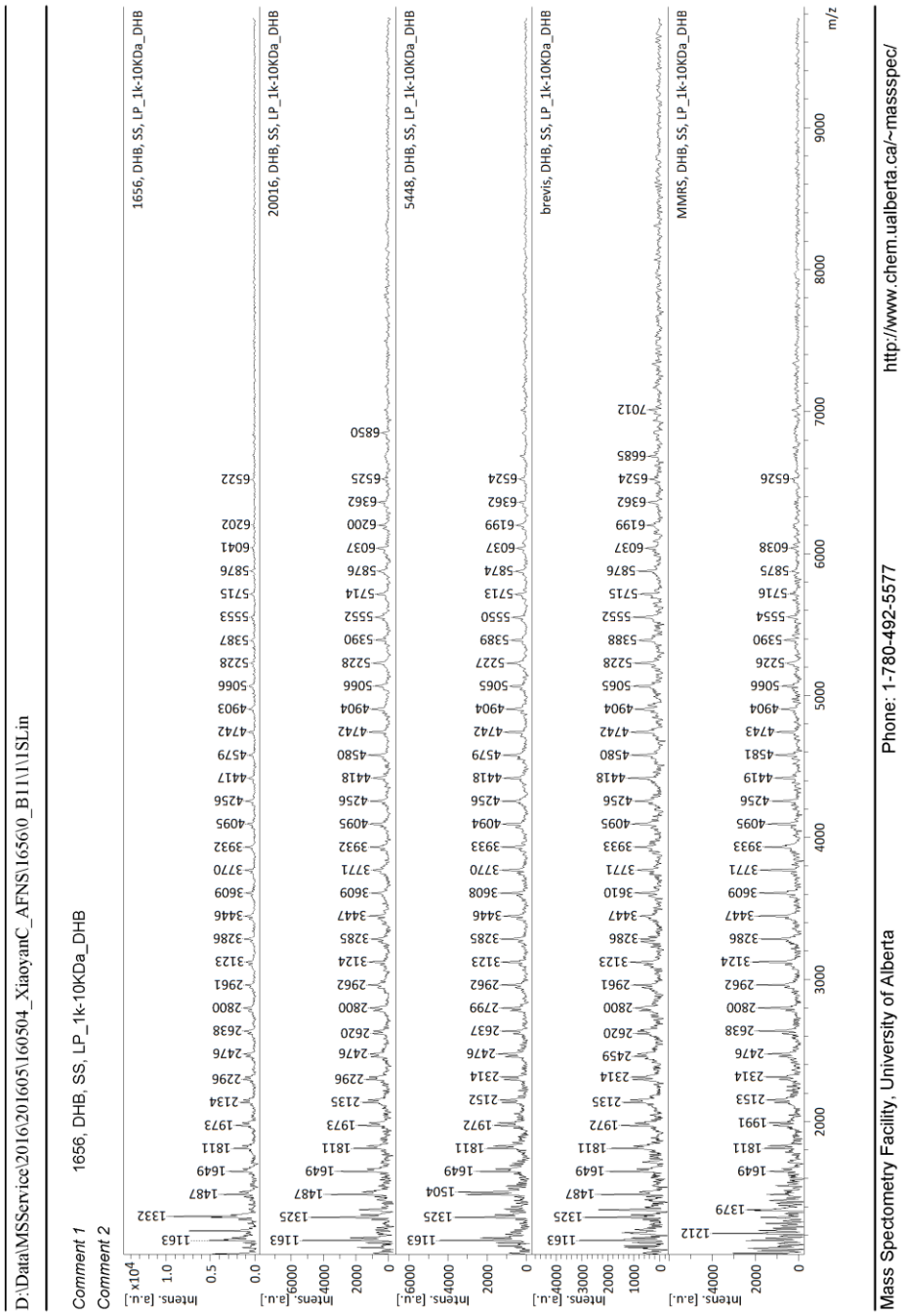
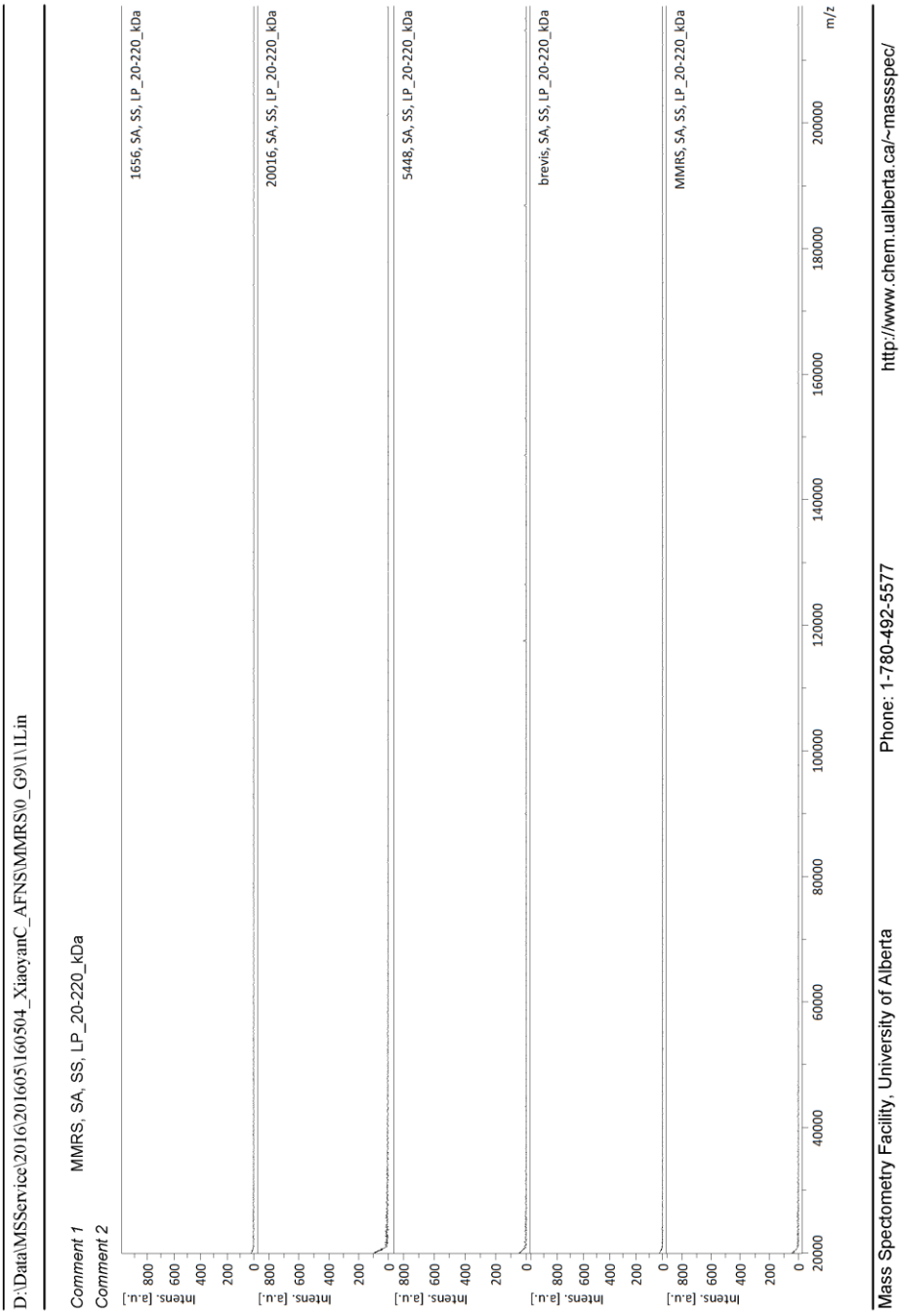
Appendix A

Bacterial extracts detected by MALDI-TOF over the range of 20-220 kDa in carbohydrate matrix (DHB)

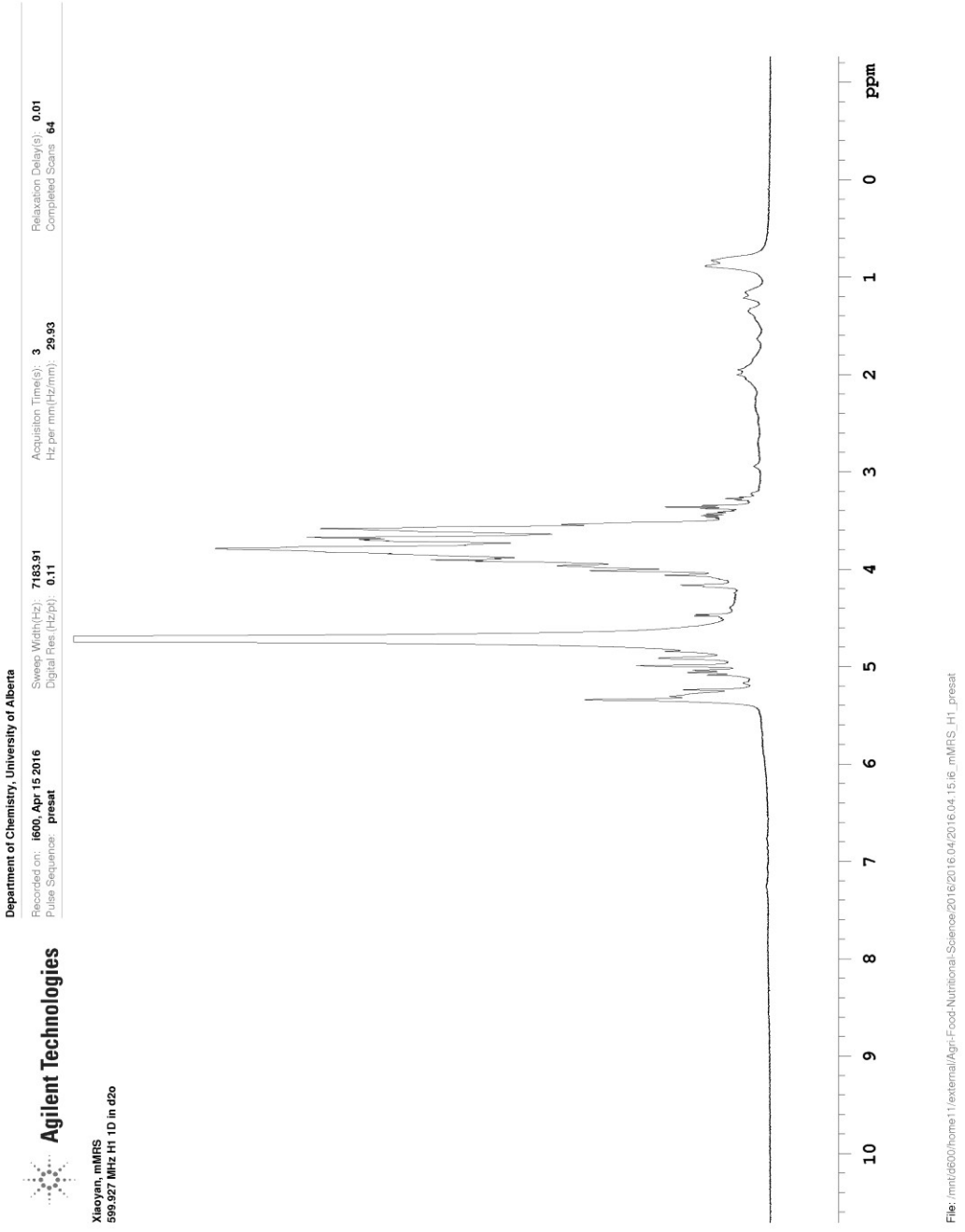


Bacterial extracts detected by MALDI-TOF over the range of 20-220 kDa in protein matrix (SA)

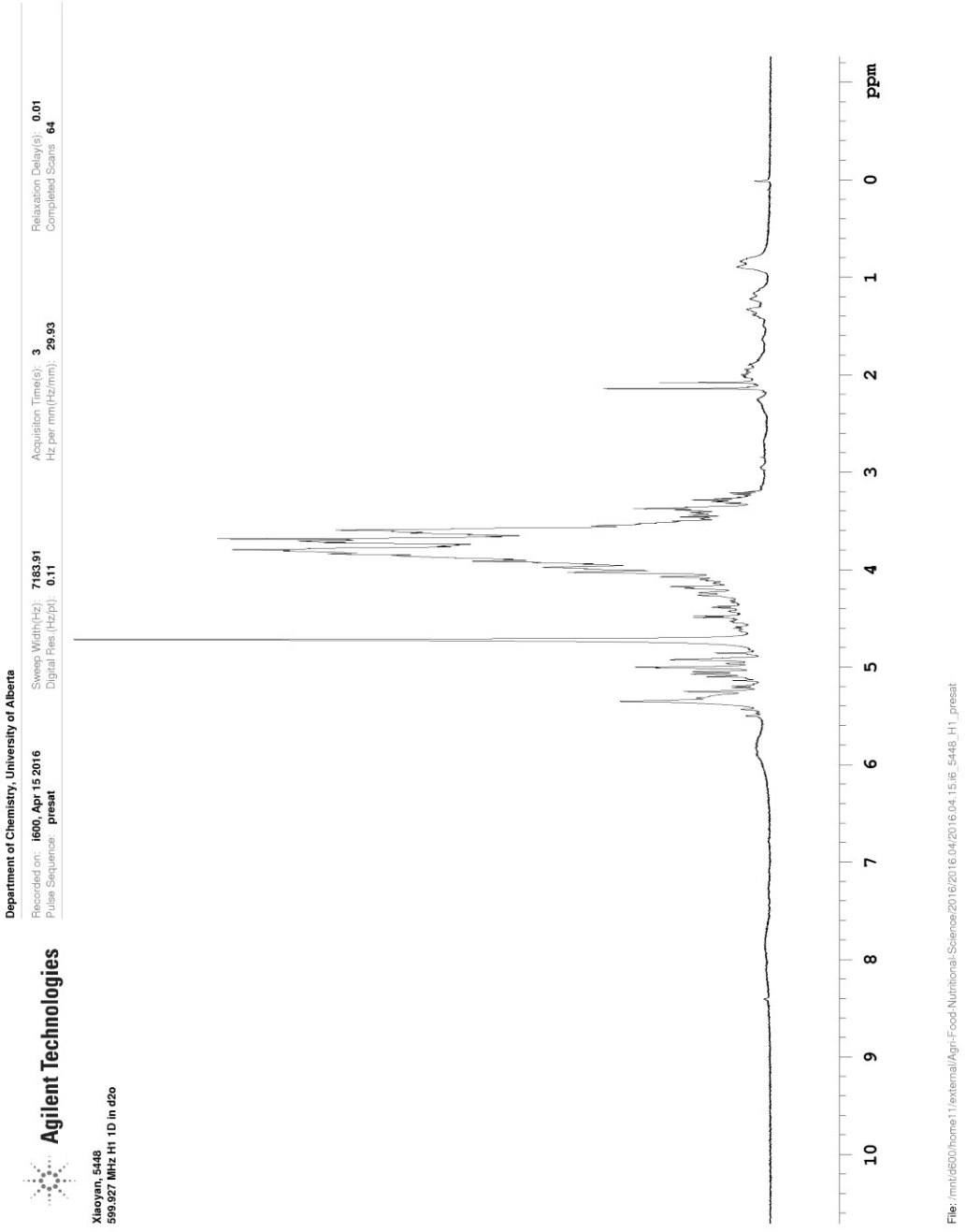


Appendix B

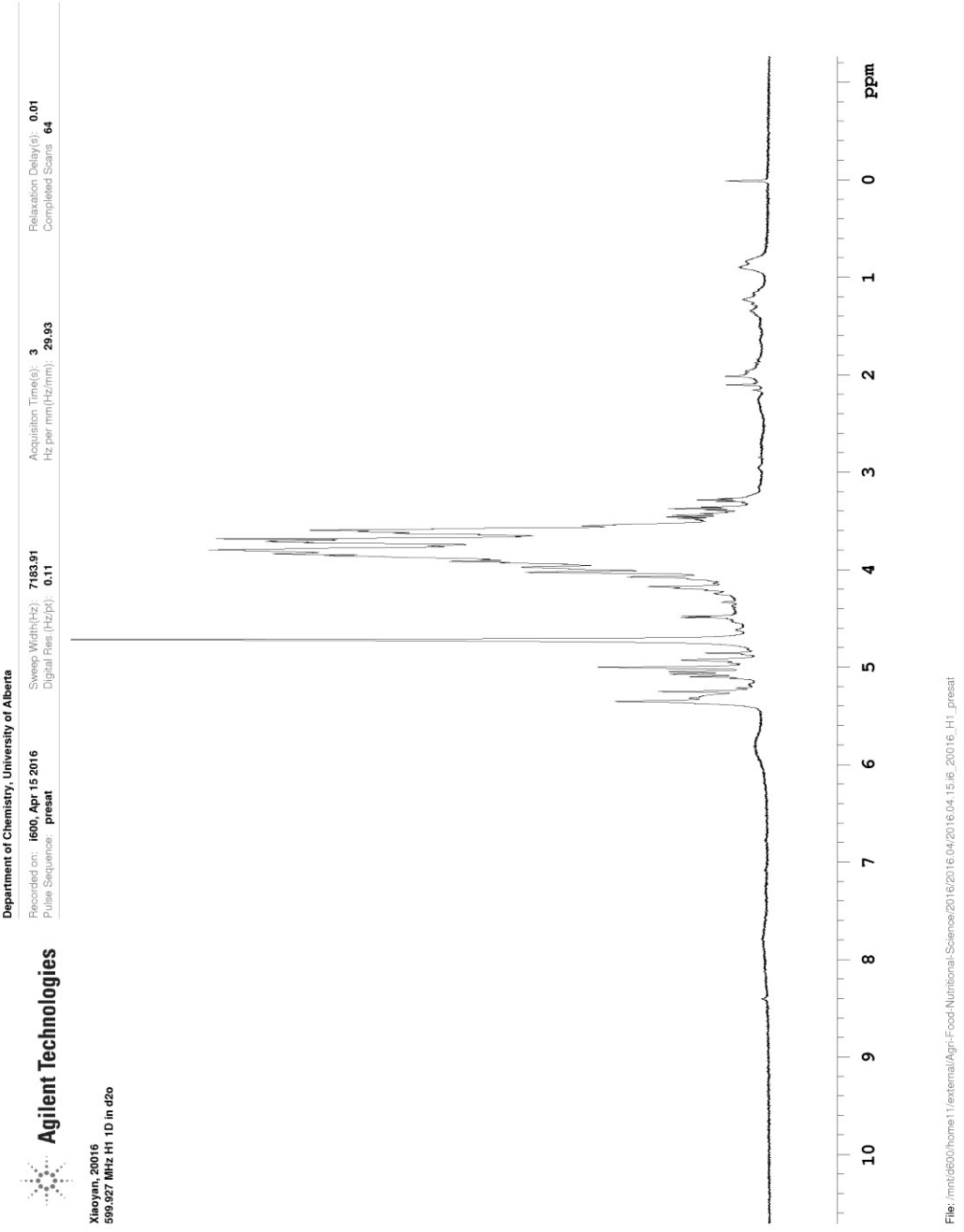
One-dimensional ^{1}H-NMR spectra of mMRS extracts



One-dimensional ^{1}H-NMR spectra of the bacterial extracts produced from *L. reuteri* LTH5448



One-dimensional ^{1}H-NMR spectra of the bacterial extracts produced from *L. reuteri* DSM20016



One-dimensional ^{1}H-NMR spectra of the bacterial extracts produced from *L. fermentum* ATCC14931