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Synthesis and characterization of lactose and lactulose derived oligosaccharides by glucansucrase and trans-sialidase enzymes

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**Synthesis and characterization of
lactose and lactulose derived oligosaccharides by
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Phạm Thị Thu Hiền

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**Synthesis and characterization of
 lactose and lactulose derived oligosaccharides by
 glucansucrase and *trans*-sialidase enzymes**

PhD Thesis

to obtain the degree of PhD at the
 University of Groningen
 on the authority of the
 Rector Magnificus Prof. E. Sterken
 and in accordance with
 the decision by the College of Deans.

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Chapter 1

Introduction

Human gastrointestinal tract

The human digestive system is a complex series of organs and glands that processes food (Figure 1). After entering the mouth with physical breakdown by chewing, food continues its way through stomach and intestine where it is partly digested by human digestive enzymes, i.e. salivary enzymes, pancreatic enzymes and enzymes excreted in the small intestine. The undigested food ends up in the large intestine or colon where it is fermented by various microorganisms. The gut microbiome is the largest microbial community of the human body with approximately 1,000 bacterial species; most of the gut microbiome resides in the large intestine.¹ A healthy gut microbiome provides a barrier against colonization by pathogens through competition, assists the GI tract by degradation of complex nutrients providing energy and essential vitamins, contributes to lipid metabolism and lipid absorption by lowered pH as a result of short-chain fatty acids secretion, and stimulates the immune system.^{2,3}

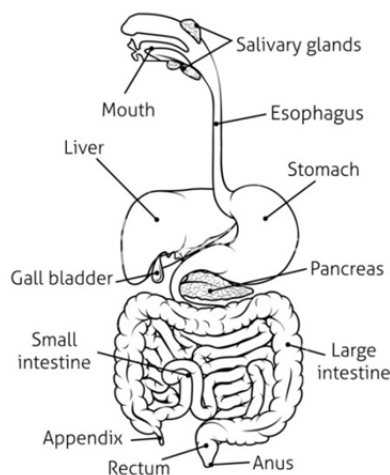


Figure 1: Scheme of the human digestive system (source: www.Storyblocks.com).

Human gut microbiota

The composition of the intestinal microbiota of infants is largely regulated by the diet.⁴ At birth the digestive tract of human is sterile and soon after becomes colonized by microbes originating from the mother's vagina and feces, as well as from the environment. The infant may be fed by breast milk or an alternative source like formula milk, resulting in different microbiota compositions. With breast-fed infants, gut microbiota composition is more dominated by bifidobacteria; in contrast, formula-feeding without added health beneficial oligosaccharides leads to the development of a gut microbiota with a more adult type of distribution.^{5,6,7} Breast-fed infants show significantly higher counts of *Bifidobacterium* and *Lactobacillus* and lower counts of *Enterobacteriaceae*, *Clostridium coccooides* group, *Staphylococcus* and *Bacteroides* compared with formula-fed infants.^{8,9,10} Bifidobacteria and lactobacilli are considered the most important health-beneficial bacteria for the human host, whereas staphylococci and clostridia are potentially pathogenic.¹¹

Human breast milk thus is an important source of oligosaccharides for the neonate's developing microbiota.¹² The intestinal microbiota is known to be very important for the development of the gut physiology and the immune system. Attempts have been made to mimic the intestinal microbiota of breast-fed infants by formula-feeding. The composition of the intestinal microbiota can be influenced either by administration of health-promoting bacteria, so-called probiotics, or the dietary ingredients, so called prebiotics.¹³ They have shown beneficial effects in infants' health, providing protection against infections,^{14,15,16} that can cause diarrhea,^{17,18} and necrotizing enterocolitis,¹⁹ as well as reducing atopic dermatitis.^{20,21,22} Probiotic bacteria, which are able to survive the gastrointestinal tract exert their biological activity by interaction with the surface of the small intestine, and colonize the colon. The most common probiotic bacteria that have been studied and used are

bifidobacteria or lactobacilli. However, to maintain colonization it is essential to keep them alive.²³ Upon ingestion they are confronted with physical and chemical barriers such as gastric acid, bile acids. Reaching the colon, they still have to compete for nutrients and colonization sites with the host's resident species. As a result, a small proportion of ingested probiotic bacteria successfully colonizes the colon.^{24,25,26} An alternative approach which partly overcomes the limitations of probiotics is the use of prebiotics.²⁷ Prebiotics are generally defined as "a substrate that is selectively utilized by host microorganisms conferring a health benefit".²⁸ Those substrates that are non-digestible during the passage through the small intestine without being absorbed or utilized, reach the colon, and stimulate selectively health promoting colonic bacteria.²⁹

Human milk oligosaccharides

The best known natural prebiotic compounds are oligosaccharides from human breast milk. Human milk oligosaccharides (*hMOS*) have been well studied and documented for their prebiotic, and particularly bifidogenic effects.^{30,31,32,33,34} In human milk, free oligosaccharides comprise the third most abundant component after lactose and fat, reaching levels of approximately 5 - 20 g L⁻¹.^{35,36} The concentration of *hMOS* is not constant over time, and tends to decrease during lactation.³⁷ Human milk oligosaccharides are built up from five monosaccharide building blocks; i.e. glucose (Glc), galactose (Gal), *N*-acetylglucosamine (GlcNAc), Fucose (Fuc), and *N*-acetylneuraminic acid (Neu5Ac). The structural composition of *hMOS* always starts with a lactose core at the reducing end. Lactose can be elongated with lacto-*N*-biose units (β -Gal-(1→3)-GlcNAc; Type 1) or lactosamine units (β -Gal-(1→4)-GlcNAc; Type 2) (Figure 2). The Type 1 core structure of *hMOS* can be further elongated in linear or branched form. Lactose and elongated lactose of *hMOS* can be further functionalized with fucose and/or sialic acid.³⁸

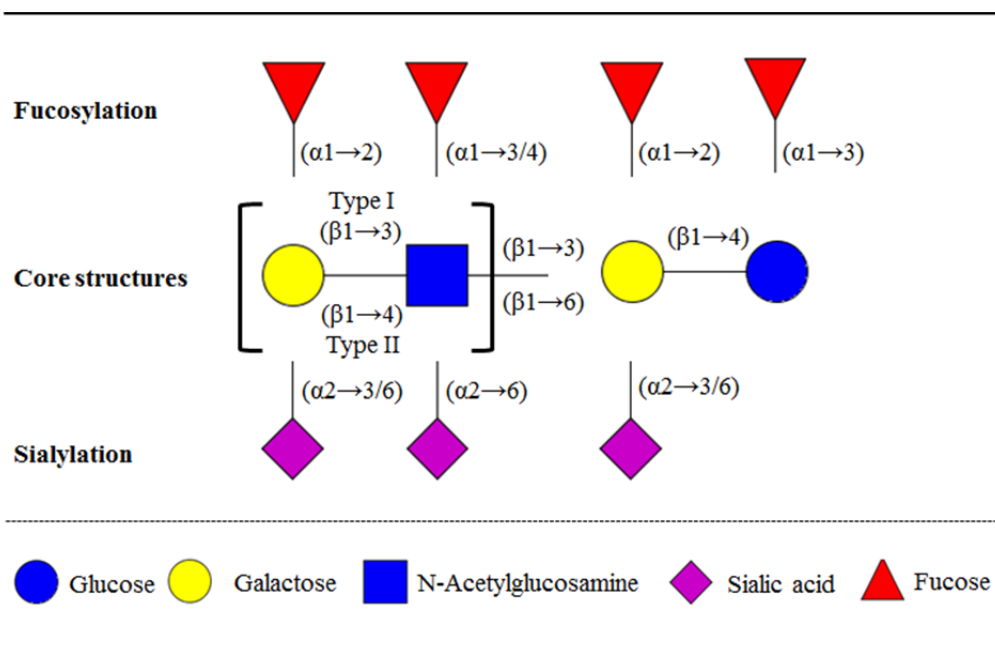


Figure 2: Schematic structures of *hMOS*.

An alternative source for *hMOS* in nature is currently not available. Milk of domesticated dairy animals does not match the large amount and high structural diversity of *hMOS*.³⁹ Content of milk oligosaccharides in human milk is 100 to 1000 fold higher than *MOS* in milk of most domesticated animals including cows, goats, sheep and pigs.^{40,41,42} Although these milks have a higher relative abundance of sialylated oligosaccharides (up to 90% of all *MOS*),⁴³ there are more acidic oligosaccharides in human milk in terms of mg mL^{-1} .⁴³ In bovine milk, which has been used as basis for infant formula milk, approximately 70 % of *MOS* are sialylated compared to 10 - 20 % in *hMOS*. But there are only approximately $0.03\text{--}0.06 \text{ g L}^{-1}$ free oligosaccharides in bovine milk compared to $5\text{--}20 \text{ g L}^{-1}$ in human milk, which amounts to $0.5\text{--}4 \text{ g L}^{-1}$ sialylated *hMOS*.^{44,45} Moreover, domesticated animal *MOS* contain not only Neu5Ac but also some of the non-human sialic acid derivative *N*-glycolylneuraminic acid (Neu5Gc). Nowadays, many babies have

limited access to human milk and receive infant formula as a replacement. Currently used bovine milk based infant formula lacks the abundance and complexity of oligosaccharides that human milk provides, and is enriched with synthetic prebiotics, which do not possess yet the advanced functionality of *h*MOS.³⁸ Synthesis of real *h*MOS or structurally/functionally effective *h*MOS mimics thus is highly interesting for application in infant formula.

Prebiotics

Non-digestible carbohydrates (NDCs) have received a lot of attention as candidates to apply in infant formula to mimic molecular size and prebiotic functions of *h*MOS.^{46,47,48,49,50} They are complex carbohydrates with a molecular size mostly ranging from 3 to 10 sugar moieties. There are several cases of very high DP up to 60 like inulin or very low down to 2 like lactulose.⁵¹ Their structural compositions contain sugars in α - or β -configuration, linear or branched chains that may play an essential role for their indigestibility in the upper parts of the intestine of the host.⁵² A number of saccharides has been explored for their prebiotic potential, the most well-known prebiotic is the mixture of 90% Galacto-oligosaccharides (GOS) and 10% fructo-oligosaccharides (FOS) which has been selected for use in infant formula milk to mimic the prebiotic effects of neutral human milk oligosaccharides.^{46,53} GOS comprise a mixture of galactosyl moieties linked with (β 1 \rightarrow 2), (β 1 \rightarrow 3), (β 1 \rightarrow 4), or (β 1 \rightarrow 6), with various sizes (mostly DP2 - DP5) (Figure 3).^{54,55} The composition of the GOS mixture highly depends on the source of the β -galactosidase used for their synthesis using lactose as acceptor substrate.^{54,56} There are currently several GOS products on the market such as Vivinal[®] GOS, Oligomate 55 and Bimuno.^{57,58,59} This group of oligosaccharides has been widely studied and shown to have stimulatory effects on the growth of probiotic bacteria to various extents.^{60,61,22} Another group of oligosaccharides that has attracted much commercial interest as prebiotics are FOS. These oligosaccharides can be obtained

from natural sources like chicory root derived inulin or synthesized enzymatically from sucrose by bacterial fructansucrase enzymes.^{62,77} Inulin normally consists of a sucrose core with one or more ($\beta 2 \rightarrow 1$) linked fructosyl unit elongations, but there is another type of FOS lacking the terminal glucose part of the sucrose. The degree of polymerization of FOS varies between 2 and 60 units (Figure 3).⁶³ The stimulatory effect toward bifidobacteria (Bifidogenic effects) of FOS have been widely studied.^{64,65}

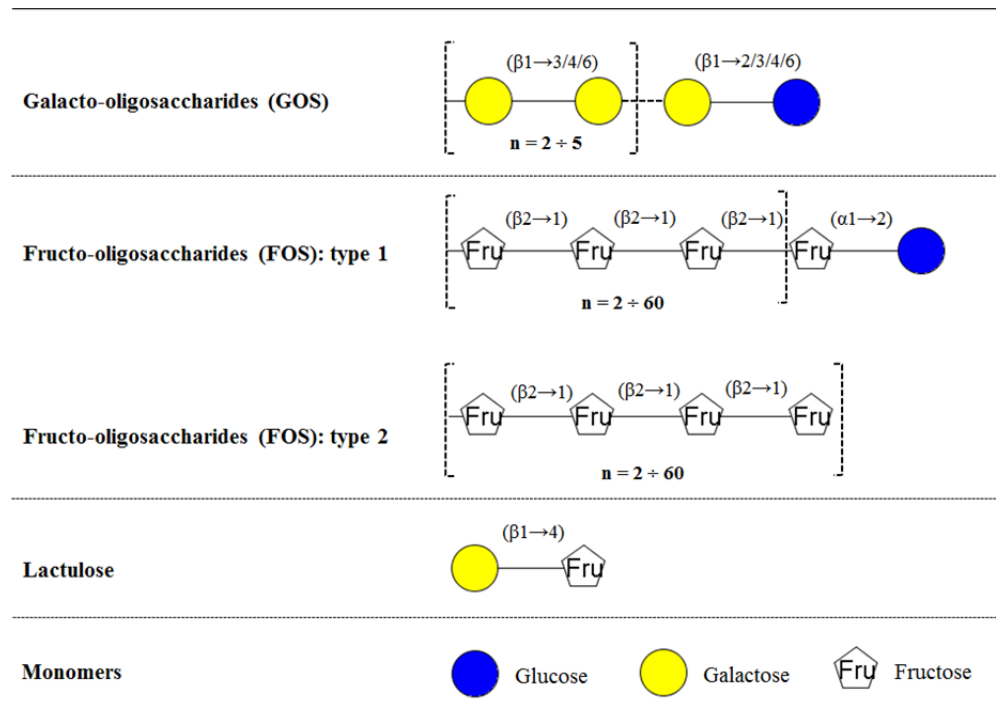


Figure 3: Schematic structures of GOS, FOS and Lactulose as examples of prebiotic compounds.

The most common disaccharide used as prebiotic is lactulose. This is a synthetic disaccharide in the form Gal($\beta 1 \rightarrow 4$)-Fru (Figure 3). Lactulose was shown to be resistant to digestion in the small intestine, and showed selective stimulation towards growth of lactobacilli and bifidobacteria.^{66,67,68,69} These are prebiotics with well-

known status supported by a significant number of human, double-blind and placebo controlled trials.^{70,71} However, there has been a growing search for new carbohydrates which could be considered as emerging prebiotics such as lactosucrose; isomalto-oligosaccharides; resistant starch; xylo-oligosaccharides, arabinoxylo-oligosaccharides and pectic-oligosaccharides.^{71,72,73,74,75,76} Where studied, most of these prebiotics however lack the pathogen exclusion and immune- and barrier modulating effects that *h*MOS possess.

Synthesis of Prebiotics and *h*MOS mimics

Prebiotics and *h*MOS mimics can be either chemically or enzymatically synthesized. However, chemical synthesis is cumbersome because it requires many synthetic steps and a lot of effort to get rid of side products.⁷⁷ The high selectivity and regio-specificity of enzymatic routes has advantages over the chemical approach.^{77,78} The microbial whole cell engineered biosynthetic routes, with outstanding features to scale-up for economic production, appeared to be the preferred choices to produce *h*MOS compounds like 2'-fucosyllactose (2'-FL).^{79,80} However, prebiotic synthesis using whole cell biosynthetic approaches requires a rigorous removal of the production strain before their application in infant food, and a clear proof that no genetically modified organisms remain is challenging. Application of isolated and highly specific enzymes for synthesis of oligosaccharides may simply overcome this obstacle. In addition, it is easier to control various incubation conditions, such as reaction conditions (enzyme/substrate concentrations) and environmental conditions (pH, temperature, metal ion), when using enzymes for synthesis of *h*MOS mimics compared to the whole-cell biocatalysts.⁸¹ From a practical viewpoint, glycosidase enzymes are the preferred choice, they are generally more available, and less expensive than glycosyl-transferases, and do not require expensive nucleotide-sugar donors.⁸² The choice of suitable substrates and highly active glycosidases clearly plays a key role in allowing the synthesis of 'tailor-made' *h*MOS mimics of high

interest for application in the food industry. Lactose is always at the reducing end of human milk oligosaccharides, this compound is considered as the initial substrate for *h*MOS synthesis.⁸³ Moreover, galactose is present in a high content in *h*MOS. Thus, lactose and lactose derivatives like GOS are potential candidates for *trans*-glycosylation to mimic *h*MOS.

Glucansucrase and *trans*-glycosylation

Glucansucrases belong to glycoside hydrolase family 70 (GH70) (<http://www.CAZy.org>) and are extracellular *trans*-glycosidases found in lactic acid bacteria.^{84,85} GH70 glucansucrases belong to the α -amylase superfamily based on amino acid sequence similarity and structure analogy.⁸⁶ They are structurally and mechanistically related to GH13 and GH77 enzymes.⁸⁷ To date, three-dimensional structures of four microbial glucansucrases were obtained by crystallization of the recombinantly produced and truncated forms of these proteins, including those from *Lactobacillus reuteri* 180,⁸⁸ *L. reuteri* 121,⁸⁹ *Streptococcus mutans*,⁹⁰ and *Leuconostoc mesenteroides* NRRL B-1299.⁹¹

The three-dimensional structures of truncated glucansucrases revealed that they exhibit a U-type shape and are organized into five domains (A, B, C, IV and V). All the domains except domain C are made up from discontinuous segments of the polypeptide. The catalytic domain adopts a $(\beta/\alpha)_8$ barrel fold and harbors a catalytic triad, which is composed of two aspartates and one glutamate.⁸⁷ An N-terminal domain of variable length and a C-terminal putative glucan-binding domain flank the central catalytic domain in these glucansucrase enzymes.⁸⁵

GH70 enzymes follow a double-displacement reaction mechanism, and possess 3 catalytic residues, D1025 (nucleophile), E1063 (acid/base) and D1136 (transition state stabilizing residue) (Gtf180- Δ N numbering). The reaction starts with cleavage of the (α 1 \rightarrow 2) bond of sucrose yielding a covalent glucosyl-enzyme intermediate.

This is followed by binding of the acceptor substrate and transfer of the covalently bound glucosyl residue to the acceptor molecule, forming a new glycosidic linkage.⁸⁸ The anomeric configuration of the donor is conserved in the product.^{92,93}

Glucansucrase enzymes in family GH70 transfer glucose from sucrose to the non-reducing end of oligosaccharides in a processive manner, retaining the α -regiospecificity.⁹⁴ Depending on the nature of the acceptor substrate, glucansucrase enzymes catalyze three types of reactions: hydrolysis of sucrose with water as acceptor, polymerization with growing α -glucan chains as acceptor, or *trans*-glycosylation with sucrose as donor substrate and other compounds as acceptor substrates (including oligosaccharides).⁸⁷ The glycosidic linkage type formed in the product is dependent on the acceptor substrate and the enzyme specificity. Glucansucrases are capable of producing α -glucans with various linkage types, namely dextran, containing mainly (α 1 \rightarrow 6) linkages; mutan, consisting predominantly of (α 1 \rightarrow 3) linkages; alternan, comprising alternating (α 1 \rightarrow 6) and (α 1 \rightarrow 3) linkages; and reuteran, containing (α 1 \rightarrow 4) and (α 1 \rightarrow 6) linkages.^{85,95} Only the branching glucansucrase Dsr-E from *Leuconostoc mesenteroides* NRRLB-1299 can introduce single (α 1 \rightarrow 2) glucosyl branches in a dextran backbone.^{96,97} Gtf180- Δ N produces an α -glucan with 69% (α 1 \rightarrow 6) and 31% (α 1 \rightarrow 3) glycosidic linkages while GtfA- Δ N produces an α -glucan with 58% (α 1 \rightarrow 4) and 42% (α 1 \rightarrow 6) glycosidic linkages.^{98,99}

These enzymes synthesize not only α -glucan polymers but also efficiently catalyze transfer of glucose moieties from sucrose as donor substrate to numerous hydroxyl-group containing molecules.^{100,101,102,103,104,105} In case of these small sugar acceptor substrates, low molecular mass oligosaccharides are synthesized with different types of linkage, size, branching, and physicochemical properties.¹⁰⁶ Maltose is considered to be the most effective acceptor substrate of glucansucrase enzymes, synthesizing various products (DP 3-6) such as panose or other isomaltooligosaccharides.^{107,108,109}

Other acceptor substrates that were studied include isomaltose, nigerose, methyl α -D-glucoside, 1,5-anhydro-D-glucitol, D-glucose, turanose, methyl β -D-glucoside, cellobiose, and L-sorbose.¹¹⁰

Lactose, raffinose, melibiose, D-galactose, and D-xylose are also used as acceptor substrate by these Gtf enzymes but only give a single glucosylated product each.¹¹⁰ More recently it was reported that dextransucrases from *Leuconostoc mesenteroides* and *Weissella confusa* also use lactose as their acceptor substrate synthesizing 2- α -D-glucopyranosyl-lactose.^{111,112} Beside carbohydrates, glucansucrase enzymes are also able to use non-carbohydrates as their acceptor substrates, i.e. L-ascorbic acid, luteolin, catechol and various phenolic compounds.^{82,101,103,104,113} Because of their diverse product structures in terms of α -glycosidic linkage types, molecular size, branching and physico-chemical properties, glucansucrases have attracted increasing interest for industrial applications in food, medicine, cosmetics etc.¹¹⁴

The most common application of α -glucans is the use as sweetening, stabilizing, viscosifying, emulsifying or water-binding agents in food as well as non-food industries.^{115,116,117,118} Moreover, α -glucans and oligosaccharides synthesized by glucansucrases have shown evidence of prebiotic properties, stimulating growth of beneficial intestinal bacteria such as *Bifidobacterium* and *Lactobacillus*.¹¹⁹ Isomalto-oligosaccharides (IMOs) are composed of glucose monomers linked by (α 1 \rightarrow 6) glucosidic linkages, and have been widely studied as potentially prebiotic.^{119,73,120} Another group of gluco-oligosaccharides, which are synthesized by glucansucrases from *Leuconostoc mesenteroides* using sucrose as donor substrate and maltose as acceptor substrate, also has potential stimulatory effects on gut bacteria.^{121,122} In another study, the addition of an α -glucan product to animal feed improved the weight gain of piglets and broilers.¹²³ A lactose-derived trisaccharide compound, i.e. 2-glucosyl-lactose, synthesized by *L. mesenteroides* dextransucrase using lactose as

acceptor substrate showed selective stimulatory effects on growth of *Bifidobacterium breve*.¹¹¹

Prebiotic effects of gluco-oligosaccharides were shown to be inversely dependent on the size of the oligosaccharides synthesized by alternansucrase and dextransucrase, with DP3 possessing the highest prebiotic potential towards bifidobacteria i.e. *B. bifidum*, *B. longum*, *B. angulatum*.^{124,125} Therefore, α -glucans and oligosaccharides synthesized by glucansucrases with a large variety of structures hold great potential for food applications, more particularly for prebiotic applications.

***Trans*-sialidase**

In human milk, lactose and *h*MOS backbones can be decorated with sialic acid to become acidic oligosaccharides.³⁸ There is increasing evidence for the functional effects of this group of oligosaccharides on human health.^{126,127,128,129} Sialylated oligosaccharides are able to prevent intestinal attachment of pathogens by acting as receptor analogs competing with epithelial ligands for bacterial binding.^{130,131,132,133} Binding of Cholera toxin was inhibited by 3'-sialyllactose.¹³⁴ An individual sialylated *h*MOS structure, disialyllacto-*N*-tetraose (DSLNT), contributes to the protective effects against one of the most common and fatal intestinal disorders in preterm infants, i.e. necrotizing enterocolitis (NEC).¹²⁹ Sialylated *h*MOS have also been indicated as important factors in brain development, sialic acids increase the production of gangliosides, which are important components of membrane receptors and cell surfaces of the nervous system.¹³⁵ The structure 3'-sialyllactose has been shown to induce the growth of various common probiotic bacteria including the infant gut-related *Bifidobacterium longum subsp. infantis*, *B. longum* 232, *B. infantis* 233, *B. infantis* 1497 and *B. lactis* HN019.¹³⁶ In view of their important functions, enzymatic synthesis of these acidic oligosaccharides for application in infant formula has attracted interest.

The *trans*-sialidases (EC 3.2.1.18) are glycosidases that naturally catalyze the transfer of sialyl residues from one sialo-glycan to the terminal Gal residue of another asialo-glycan.¹³⁷ In micro-organisms, these enzymes are virulence factors that enable spreading and infection of host cells.¹³⁸ *Trans*-sialidase was first identified in and isolated from *Trypanosoma* species. *Trans*-sialidase from *Trypanosoma cruzi* preferentially catalyzes the reversible transfer of ($\alpha 2 \rightarrow 3$)-linked sialic acids from donor glycans directly to terminal β -Gal-containing acceptor molecules, thereby giving rise to new ($\alpha 2 \rightarrow 3$) glycosidic linkages (Figure 4).^{139,140} When the acceptor substrate is absent, the enzyme acts as a hydrolase transferring sialic acid to water.¹³⁷ *Trans*-sialidase from *T. cruzi* (TcTS) has been best documented. The TcTS enzyme has been suggested to be involved in the mammalian host cell invasion and pathogenesis of *T. cruzi* leading to Chagas disease.^{137,141} In *T. cruzi*, surface sialylation to scavenge sialic acid plays a crucial role for their adhesion and invasion to the host cell.¹⁴² The recombinant TcTS enzyme catalyzes the transfer of sialic acid from donor to acceptor with retention of the configuration of the sialyl glycosidic linkages.¹⁴³ *Trans*-sialidase from *Trypanosoma* generally has a wide variety of acceptor substrate specificities, albeit that they favor oligosaccharides and glycoproteins.^{144,145,146} Recently, casein glycomacropeptide (GMP), an affordable source of sialic acid, was used in the synthesis of sialylated galacto-oligosaccharides.^{147,148} GMP is the soluble glycosylated casein residue produced by chymosin action on κ -casein during the cheese manufacturing process. The O-glycans on GMP comprise of Neu5Ac-containing components including major elements Neu5Ac($\alpha 2 \rightarrow 3$)-Gal($\beta 1 \rightarrow 3$)-GalNAc and Neu5Ac($\alpha 2 \rightarrow 3$)-Gal($\beta 1 \rightarrow 3$)-[Neu5Ac($\alpha 2 \rightarrow 6$)]GalNAc, which can be used as donor substrates.^{149,150} *Trans*-sialidase from *T. cruzi* is known to be specific for terminal β -galactosyl residues, any compounds possessing a terminal β -Gal residue can be used as acceptor substrate.¹⁵¹ However, it has been reported recently that sialylation of non-terminal β -Gal residues in GOS can also be catalyzed by

TcTS, provided that two Gal-residues are linked together with a ($\beta 1 \rightarrow 6$) linkage.^{136,152}

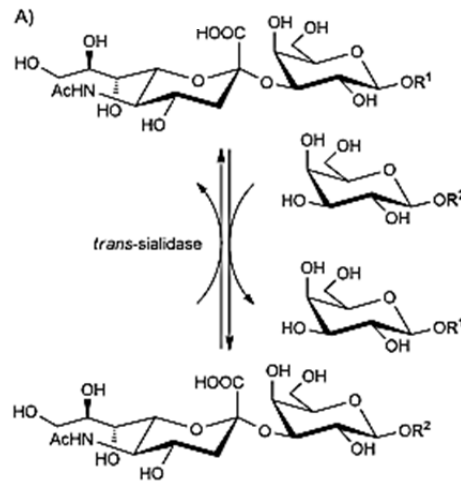


Figure 4: Reversible *trans*-glycosylation of ($\alpha 2 \rightarrow 3$)-linked *N*-acetylneuraminic acid between Neu5Ac-($\alpha 2 \rightarrow 3$)Gal-OR1 and Neu5Ac-($\alpha 2 \rightarrow 3$)Gal-OR2, catalyzed by trypanosomal *trans*-sialidases.¹³⁷

Outline of the thesis

Health beneficial oligosaccharides are of great interest for industry and society. Synthesis of prebiotic oligosaccharides are explored using a wide variety of methods. Enzymatic synthesis using cheap and available substrates and enzymes provides clear benefits for scale-up of the production. Glucansucrases are known as efficient catalysts for synthesis of α -glucans and other gluco-oligosaccharides. Relatively little is known about their ability to use lactose and galacto-oligosaccharides (GOS) as acceptor substrates. The aim of this PhD project was to provide more insights into the activity and product specificity of glucansucrases Gtf180- Δ N and GtfA- Δ N when acting on these acceptors with focus on product structural analysis and their possible selective stimulatory effects on growth of gut

bacteria. **Chapter 1** reviews the current literature and knowledge about health beneficial oligosaccharides including hMOS and the enzyme biocatalysts used, glucansucrase of *Lactobacillus reuteri* and *trans*-sialidase from *Trypanosoma cruzi*.

In **chapter 2**, we investigated the ability of the Gtf180- Δ N and GtfA- Δ N enzymes to use lactose as acceptor substrate for *trans*-glucosylation, using sucrose as donor substrate. The results showed that both enzymes synthesized similar transfer products with a degree of polymerization (DP) of 3 to 4, therefore called GL34 mixture. New linkage types were observed when using lactose as acceptor than observed in the α -glucan products from sucrose of these enzymes, i.e. (α 1 \rightarrow 2)/(α 1 \rightarrow 4) for Gtf180- Δ N and (α 1 \rightarrow 2)/(α 1 \rightarrow 3) for GtfA- Δ N. The Gtf180- Δ N enzyme was more efficient and produced also higher DP products than GtfA- Δ N. Further reaction and process engineering is required to optimize conversion and product yields.

The newly synthesized GL34 mixture may be of interest for the food industry, more particularly they may find application in infant foods, or in animal feed. We therefore studied its prebiotic potential (**chapter 3**) by analyzing the stimulatory effects of the GL34 mixture synthesized by Gtf180- Δ N on growth of selected gut bacteria, including lactobacilli, bifidobacteria and commensal bacteria. The mixture was also challenged with common carbohydrate degrading enzymes and showed resistance to most of the tested enzymes, including α -amylase from porcine pancreas. Bifidobacteria strains clearly grew better on the GL34 mixture than lactobacilli and commensal bacteria. Particularly *B. adolescentis* grew effectively on GL34.

When using lactose as acceptor substrate, the linkage specificity of these glucansucrases changed to also produce (α 1 \rightarrow 2)-linkages, which is totally new for these enzymes. Previous studies have shown that mutagenesis of residues in the glucansucrase active site pocket may change its linkage specificity.^{153,154} Therefore,

in **chapter 4**, we investigated the effects of mutational changes of different residues in the acceptor substrate binding subsites on the activity and specificity of Gtf180- Δ N when acting on lactose as acceptor substrate. The residues were selected based on *in silico* docking studies of lactose into the active site pocket of the crystal structure of Gtf180. Mutations in these residues, Q1140, W1065 and N1029, influenced the product spectra of the GL34 mixture. Four new DP4-DP5 structures were synthesized by mutant N1029G which favored synthesis of (α 1 \rightarrow 3) glycosidic linkages.

Chapters 2-4 demonstrated the ability of these glucansucrases to decorate galactose-containing compounds (lactose) and to introduce new linkage types, and indicated that the GL34 mixture has potential as prebiotic compounds. In an attempt to synthesize further *h*MOS mimics, **chapter 5** studied the glucosylation of model GOS with DP3 as acceptor substrates by Gtf180- Δ N and GtfA- Δ N. Both 4'-galactosyl-lactose (β 4'-GL) and 6'-galactosyl-lactose (β 6'-GL) were used by these enzymes and three new products were purified and structurally characterized. The third model GOS, 3'-galactosyl-lactose (β 3'-GL), was not used as an acceptor substrate by these enzymes.

With a final aim to synthesize *h*MOS mimics, in **chapter 6**, sialylation of the GL34 mixture was carried out using *trans*-sialidase from *Trypanosoma cruzi*. Compound F2 2-glc-lac was used as acceptor substrate by this TcTS to produce Neu5Ac-(α 2 \rightarrow 3)-2-glc-lac with a conversion degree of 47.6 %. This enzyme also sialylated at least five galactosylated-lactulose compounds (LGOS) structures and eleven Vivinal GOS DP3-4 compounds. Moreover, the results revealed a strong preference for terminal β -Gal residues to be sialylated. Only branched compounds with two non-reducing terminal β -Gal residues were di-sialylated. Our study showed that structures with a Gal(β 1 \rightarrow 3) terminal residue were more efficiently sialylated by TcTS.

Finally, in **chapter 7**, the results obtained in this research were summarized and discussed. The potential use of these newly synthesized oligosaccharides for food/feed products and their impact on future research towards hMOS mimics is reflected.

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Chapter 2

Structural characterization of glucosylated lactose derivatives synthesized by the *Lactobacillus reuteri* GtfA and Gtf180 glucansucrase enzymes

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ABSTRACT

Glucansucrase enzymes from lactic acid bacteria are receiving strong interest because of their wide range of gluco-oligosaccharide and polysaccharide products from sucrose, some of which have prebiotic potential. Glucansucrases GtfA and Gtf180 from *Lactobacillus reuteri* strains are known to convert sucrose into α -glucans with different types of linkages, but also to use other molecules as acceptor substrates. Here we report that incubation of (N-terminally truncated versions of) these enzymes with lactose plus sucrose resulted in synthesis of at least 5 glucosylated lactose products of a degree of polymerization (DP) of 3-4. Only glucansucrase Gtf180- Δ N also produced larger lactose-based oligosaccharides (up to DP9). Structural characterization of the glucosylated lactose products DP3-4 revealed glycosidic bonds other than (α 1 \rightarrow 4)/(α 1 \rightarrow 6) typical for GtfA- Δ N and (α 1 \rightarrow 3)/(α 1 \rightarrow 6) typical for Gtf180- Δ N: Both GtfA- Δ N and Gtf180- Δ N now introduced a glucosyl residue (α 1 \rightarrow 3)- or (α 1 \rightarrow 4)-linked to the non-reducing galactose unit of lactose. Both enzymes also were able to introduce a glucosyl residue (α 1 \rightarrow 2)-linked to the reducing glucose unit of lactose. These lactose derived oligosaccharides potentially are interesting prebiotic compounds.

INTRODUCTION

Glucansucrase enzymes (Gtfs) of glycoside hydrolase family 70 (GH70) are extracellular enzymes that only have been identified in lactic acid bacteria (LAB).¹ They catalyze three types of reactions, depending on the nature of the acceptor substrate: hydrolysis when water is used as acceptor substrate, polymerization when the growing glucan chain is used as acceptor, and transglycosylation when other compounds including oligosaccharides are used as acceptor.² The currently known diversity of glucansucrases is capable of synthesizing α -glucans with all the possible glycosidic linkage types [(α 1 \rightarrow 2), (α 1 \rightarrow 3), (α 1 \rightarrow 4) and (α 1 \rightarrow 6)]. They are classified into dextran-, mutan-, reuteran-, and alternansucrases based on the (dominant) linkage type(s) in their products.^{2,3,4,5} The catalytic mechanism of Gtfs is similar to that of the family GH13 enzymes, namely an α -retaining double displacement reaction.² The reaction starts with the cleavage of sucrose, resulting in the formation of a covalent β -glucosyl-enzyme intermediate. This is followed by transfer of the glucosyl moiety to an acceptor substrate with retention of the α -anomeric configuration. In case of acceptor reactions, the orientation of the bound acceptor substrate towards the reaction center determines the type of linkages formed in the transglycosylation products.² Gtfs are able to transfer glucose to a wide variety of acceptors, either non-glycan compounds or oligosaccharide compounds, mostly disaccharides or disaccharide derivatives.^{4,5} Maltose is a highly suitable acceptor substrate for Gtfs producing various products such as panose or other isomalto-oligosaccharides, while fructose is not a preferred acceptor for Gtfs.⁶ Use of lactose as acceptor substrate has been previously studied for the dextransucrases from *Leuconostoc mesenteroides* and *Weissella confusa*, and the only transfer product that has been structurally identified is 2- α -D-glucopyranosyl-lactose.^{7,8} The low cost of sucrose and lactose, combined with the broad acceptor substrate acceptance of glucansucrase enzymes, makes them useful tools in the synthesis of novel and potentially prebiotic oligosaccharides. This study explored

the ability of glucansucrase enzymes Gtf180- Δ N and GtfA- Δ N from *L. reuteri* strains 180 and 121, respectively, to decorate lactose as acceptor substrate, using sucrose as donor substrate. While Gtf180- Δ N of *L. reuteri* 180 converts sucrose into a dextran with 69 % (α 1 \rightarrow 6) linkages and 31 % (α 1 \rightarrow 3) linkages,⁹ GtfA- Δ N catalyzes the synthesis of a reuteran consisting of 58 % (α 1 \rightarrow 4) linkages and 42 % (α 1 \rightarrow 6) linkages.¹⁰ The transfer products synthesized by these two glucansucrases were structurally analyzed by high-pH anion-exchange chromatography (HPAEC), matrix-assisted laser-desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and 1D/2D $^1\text{H}/^{13}\text{C}$ nuclear magnetic resonance (NMR) spectroscopy (TOCSY, HSQC, ROESY). A total of five main structures were observed (DP3 and DP4) for both enzymes. Only in case of Gtf180- Δ N also longer oligosaccharides were observed.

MATERIALS AND METHODS

Glucansucrase enzymes

Escherichia coli BL21 (DE3) (Invitrogen) carrying plasmid pET15b with the *gtf180* and *gtfA* genes from *Lactobacillus reuteri* strains 180 and 121 was used for expression of the N-terminally truncated glucansucrase enzymes (Gtf180- Δ N and GtfA- Δ N). The expression and purification of these glucansucrases have been described previously.¹¹

Transglucosylation reaction

The total activity of Gtf180- Δ N or GtfA- Δ N was measured as initial rates by methods described previously by Van Geel-Schutten et al.¹² The products of the transglucosylation reaction were prepared by incubating a mixture of 0.5 M sucrose (donor) and 0.5 M lactose (acceptor) with 3 U mL⁻¹ glucansucrase (at 37 °C in 50 mM sodium acetate buffer with 0.1 mM CaCl₂ at pH 4.7. The reaction was stopped

after 24 h of incubation by heating at 100 °C for 10 min, followed by 400 times dilution of the inactivated sample with DMSO 95 % and analyzed by High-pH anion-exchange chromatography (HPAEC-PAD).

Isolation and purification of oligosaccharide products

The reactions were carried out in a volume of 100 mL with the conditions described in section 4.2. Afterwards the reaction mixtures were mixed with two volumes of cold ethanol 20 % and stored at 4 °C overnight to precipitate the polysaccharides. After centrifugation at 10,000 g for 10 min, the supernatant was applied to a rotatory vacuum evaporator to remove ethanol. The aqueous fraction was then absorbed onto a CarboGraph SPE column (Alltech, Breda, The Netherlands) using acetonitrile : water = 1:3 as eluent, followed by evaporation of acetonitrile under an N₂ stream before being freeze-dried. This was followed by fractionation HPAEC on a Dionex ICS-5000 workstation (Dionex, Amsterdam, the Netherlands), equipped with a CarboPac PA-1 column (250 x 9 mm; Dionex) and an ED40 pulsed amperometric detector (PAD). The gradient used for this fractionation is described in 4.4. The collected fractions were neutralized by acetic acid 20 % and then desalted using a CarboGraph SPE column as described earlier.

HPAEC-PAD

The profiles of the oligosaccharides products were analyzed by HPAEC-PAD on a Dionex ICS-3000 work station (Dionex, Amsterdam, the Netherlands) equipped with an ICS-3000 pulse amperometric detection (PAD) system and a CarboPac PA-1 column (250 x 4 mm; Dionex). The analytical separation was performed at a flow rate of 1.0 mL min⁻¹ using a complex gradient of effluents A (100 mM NaOH); B (600 mM NaOAc in 100 mM NaOH); C (Milli-Q water); and D: 50 mM NaOAc. The gradient started with 10 % A, 85 % C, and 5 % D in 25 min to 40 % A, 10 % C, and 50 % D, followed by a 35-min gradient to 75 % A, 25 % B, directly followed by

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5 min washing with 100 % B and reconditioning for 7 min with 10 % A, 85 % B, and 5 % D. External standards of lactose, glucose, fructose were used to calibrate for the corresponding sugars. For the determination of glucosylated lactose compounds with a degree of polymerization (DP) of 3, maltotriose was used as external standard.

MALDI-TOF mass spectrometry

Molecular mass of the compounds in the reaction mixture was determined by MALDI-TOF mass spectrometry on an AximaTM Performance mass spectrometer (Shimadzu Kratos Inc., Manchester, UK), equipped with a nitrogen laser (337 nm, 3 ns pulse width). Ion-gate cut-off was set to m/z 200 and sampling resolution was software-optimized for m/z 1500. Samples were prepared by mixing 1 μ L with 1 μ L aqueous 10 % 2,5-dihydroxybenzoic as matrix solution.

NMR spectroscopy

The structures of oligosaccharides of interest were elucidated by 1D and 2D ¹H NMR, and 2D ¹³C NMR. A Varian Inova 500 Spectrometer and 600 Spectrometer (NMR center, University of Groningen) were used at probe temperatures of 25 °C with acetone as internal standard (chemical shift of δ 2.225). The aliquot samples were exchanged twice with 600 μ L of 99.9%_{atom} D₂O (Cambridge Isotope Laboratories, Inc., Andover, MA) by freeze-drying, and then dissolved in 0.65 mL D₂O, containing internal acetone. In the 1D ¹H NMR experiments, the data was recorded at 8 k complex data points, and the HOD signal was suppressed using a WET1D pulse. In the 2D ¹H-¹H NMR COSY experiments, data was recorded at 4000 Hz for both directions at 4k complex data points in 256 increments. 2D ¹H-¹H NMR TOCSY data were recorded with 4000 Hz at 30, 60, 100 spinlock times in 200 increments. In the 2D ¹H-¹H NMR ROESY, spectra were recorded with 4800 Hz at a mixing time of 300 ms in 256 increments of 4000 complex data points.

MestReNova 5.9 (Mestrelabs Research SL, Santiago de Compostela, Spain) was used to process NMR spectra, using Whittaker Smoother baseline correction.

RESULTS

Transglucosylation of lactose

Initial reactions were performed with sucrose and lactose concentrations of 0.5 M (ratio of 1:1), at 37 °C and pH 4.7 during 24 h, which is the catalytic optimum of the Gtf180- Δ N and GtfA- Δ N enzymes for α -glucan synthesis from sucrose.¹² Blank reactions used only sucrose as both acceptor and donor substrate, mostly resulting in α -glucan synthesis. The HPAEC-PAD profiles of the oligosaccharide fractions of reactions with only sucrose (Figure 1, line a) showed only a few minor peaks (reflecting that mostly polymerization occurred), besides clear peaks for glucose and fructose. The profiles of the oligosaccharide fractions of incubations with sucrose plus lactose of GtfA- Δ N (Figure 1, line b) and Gtf180- Δ N (Figure 1, line c) showed similar profiles, with five significant novel peaks F1-F5, besides minor peaks eluting later which are expected to be higher DP oligosaccharides with lactose (DP5 – DP9).

Structural analysis of transglycosylation products

Five major glucosylation products corresponding to peaks F1-F5 (Figure 1) were isolated from the incubation mixture of Gtf180- Δ N for structural analysis by MALDI-TOF-MS and 1D/2D ¹H and ¹³C NMR spectroscopy. The purity and retention time of each fraction was confirmed by reinjection on an analytical CarboPac PA-1 (4 x 250 mm) column. The fragment size distribution of each fraction was determined by MALDI-TOF MS. The data showed that three major products corresponded to trisaccharides, as evidenced by a pseudo-molecular sodium adduct ion at m/z 527 (F1-F3) and two products were tetrasaccharides, as evidenced by a pseudo-molecular sodium adduct ion at m/z 689 (F4 and F5) (Figure

S1). Each product fraction was analyzed by 1D ^1H NMR, as well as 2D ^1H - ^1H and ^{13}C - ^1H NMR spectroscopy.

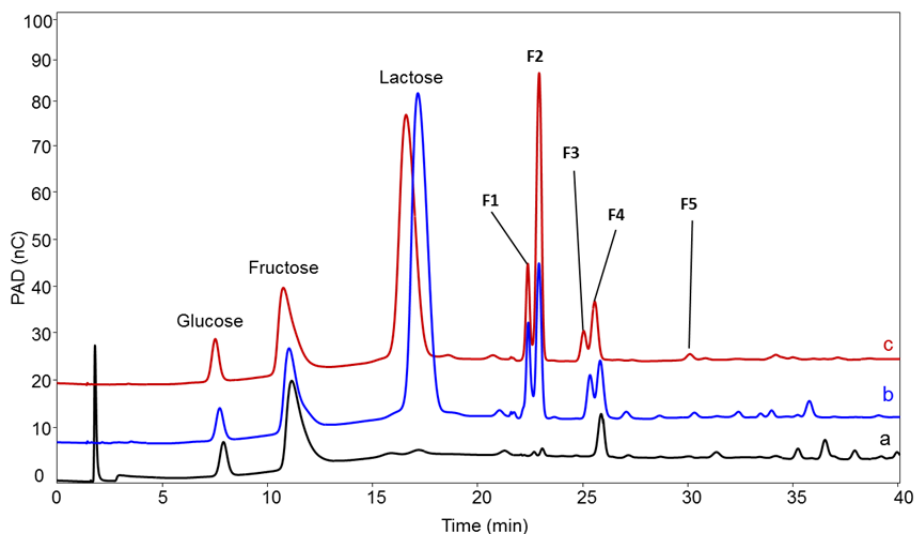


Figure 1: HPAEC-PAD chromatograms of the reaction product mixtures obtained with 3 U mL⁻¹ (a) GtfA- Δ N with 0.5 M sucrose; (b) GtfA- Δ N with 0.5 M sucrose and 0.5 M lactose; and (c) Gtf180- Δ N with 0.5 M sucrose and 0.5 M lactose. Reaction conditions: 24 h incubations at 37 °C and pH 4.7.

Mono-glycosylated lactose compounds

Fraction F1

Trisaccharide F1 includes 3 hexose residues, namely **A**, **B** (glucosyl and galactosyl residues from original lactose, respectively) and **C** (transferred glucosyl residue from sucrose) (Table 1). The 1D ^1H NMR spectrum of F1 displayed four anomeric ^1H signals at δ 5.225 (**A α** H-1, $^3J_{1,2}$ 3.79 Hz), 4.667 (**A β** H-1, $^3J_{1,2}$ 8.28 Hz), 4.510 (**B** H-1, $^3J_{1,2}$ 8.03 Hz) and 4.914 (**C** H-1 $^3J_{1,2}$ 4.49 Hz) (Figure S2Figure). All the ^1H and ^{13}C chemical shifts of these three residues were assigned by 2D ^1H - ^1H TOCSY and ^1H - ^{13}C HSQC spectra (Table 2). The data showed that resonances of non-anomeric protons of glucosyl residue **A α** and **A β** were not shifted compared to those

values of the glucosyl residue observed in lactose (Table 2). Residue **B**, however, showed significant downfield shifts for H-3 and H-4 at δ 3.75 ($\Delta\delta + 0.09$ ppm) and 4.027 ($\Delta\delta + 0.10$ ppm), respectively. The position of residue **B** C-4 at δ 78.4 ppm ($\Delta\delta + 8.4$ ppm), is indicative for substitution on the O4 of residue **B**. This is further supported by the 2D ROESY inter-residual cross-peak between **C** H-1 and **B** H-4 (Figure S2). Residue **C** showed a ^1H and ^{13}C chemical shift pattern fitting a terminal residue.¹³ Combining all data, the structure of trisaccharide compound F1 is determined to be $\alpha\text{-D-Glcp-(1}\rightarrow\text{4)-}\beta\text{-D-Galp-(1}\rightarrow\text{4)-D-Glcp}$ (Table 1).

Fraction F2

Trisaccharide F2 includes 3 hexose residues, namely **A**, **B** (glucosyl and galactosyl residues from original lactose, respectively) and **C** (transferred glucosyl residue from sucrose) (Table 1). The ^1H anomeric signals of fraction F2 were revealed by 500-MHz 1D ^1H NMR spectrum as following δ 5.433 (**A α** H-1, $^3J_{1,2}$ 3.49 Hz), δ 4.816 (**A β** H-1, $^3J_{1,2}$ 8.00 Hz), δ 4.465 (**B** H-1, $^3J_{1,2}$ 7.43 Hz), δ 5.355 (**D a** H-1, $^3J_{1,2}$ 3.82 Hz) and 5.094 (**D b** H-1, $^3J_{1,2}$ 3.75 Hz) (Figure S3). Using 2D ^1H - ^1H TOCSY and 2D ^1H - ^{13}C HSQC, all non-anomeric proton resonances were assigned (Table 2). The anomeric resonance value at δ 5.433 ppm of **A α** H-1 is the structural-reporter-group signal of the 2-substituted reducing $\alpha\text{-D-Glcp}$ unit.¹³ Additionally, strong downfield shifts were detected for **A α** H-2 at δ 3.68 ($\Delta\delta + 0.10$ ppm); **A α** C-2 at δ 80.0 ($\Delta\delta + 7.60$ ppm), **A β** H-2 at δ 3.41 ($\Delta\delta + 0.13$ ppm) and **A β** C-2 δ 80.0 ($\Delta\delta + 5.00$ ppm), confirming the substitution at O-2 of this residue. In residue **D**, **D a** H-5 and **D b** H-5 signals are shifted downfield to δ 4.084 ($\Delta\delta + 0.13$) and δ 3.98 ($\Delta\delta + 0.38$), respectively, compared to corresponding signals of the glucosyl residue of lactose, as an indicator for the $\alpha\text{-D-Glcp-(1}\rightarrow\text{2)}$ - unit.¹¹ This is further supported by ROESY inter-residual cross-peak between **D b** H-1 and **A α** H-2/ **D a** H-1 and **A β** H-2 (Figure S3). Meanwhile the set of chemical shifts values of residue **B** remained the same as those measured for lactose,¹⁴ indicating that no further substitution occurred

at this residue. Considering all data together, the structure of trisaccharide compound F2 is determined to be α -D-Glcp-(1 \rightarrow 2)-[β -D-Galp-(1 \rightarrow 4)-]D-Glcp (Table 1).

Table 1: Structures of the characterized oligosaccharide products F1-F5 of Gtf180- Δ N and GtfA- Δ N obtained with lactose and sucrose.

Nr.	Structures	Graphical presentation	Catalytic activity by
F1	α -D-Glcp-(1 \rightarrow 4)- β -D-Galp-(1 \rightarrow 4)-D-Glcp C1 \rightarrow 4B1 \rightarrow 4A		Gtf180- Δ N and GtfA- Δ N
F2	α -D-Glcp-(1 \rightarrow 2)-[β -D-Galp-(1 \rightarrow 4)-]D-Glcp D1 \rightarrow 2[B1 \rightarrow 4]A		
F3	α -D-Glcp-(1 \rightarrow 3)- β -D-Galp-(1 \rightarrow 4)-D-Glcp C1 \rightarrow 3B1 \rightarrow 4A		
F4	α -D-Glcp-(1 \rightarrow 4)- β -D-Galp-(1 \rightarrow 4)-[α -D-Glcp-(1 \rightarrow 2)-]D-Glcp C1 \rightarrow 4B1 \rightarrow 4[D1 \rightarrow 2]A		
F5	α -D-Glcp-(1 \rightarrow 3)- β -D-Galp-(1 \rightarrow 4)-[α -D-Glcp-(1 \rightarrow 2)-]D-Glcp C1 \rightarrow 3B1 \rightarrow 4[D1 \rightarrow 2]A		
Elongated glucosyl lactose derivatives			Gtf180- Δ N
Monomers			

Fraction F3

Trisaccharide F3 includes 3 hexose residues, namely **A**, **B** (glucosyl and galactosyl residues from original lactose, respectively) and **C** (transferred glucosyl residue from sucrose) (Table 1). The 1D ^1H NMR spectrum of fraction F3 found four anomeric signals at δ 5.225 (**A α** H-1, $^3J_{1,2}$ 3.89 Hz), δ 4.667 (**A β** H-1, $^3J_{1,2}$ 7.98 Hz), δ 4.525 (**B** H-1, $^3J_{1,2}$ 7.88 Hz) and δ 5.103 (**C** H-1, $^3J_{1,2}$ 3.53 Hz) (Figure S4). The non-anomeric proton resonances were assigned by using 2D ^1H - ^1H TOCSY and 2D ^1H - ^{13}C HSQC (Table 2). The data showed that the ^1H and ^{13}C NMR patterns of residue **A** match with those values of the glucosyl residue observed in lactose.¹⁴ Strong downfield shifts of residue **B** were detected for H-3 at δ 3.76 ($\Delta\delta$ + 0.10

ppm) and C-3 at δ 78.6 ($\Delta\delta + 4.90$ ppm), suggesting the occurrence of $\rightarrow 3$ -D-Galp. This is confirmed by the 2D ROESY inter-residual cross-peak between C H-1 and B H-3 (Figure S4). This substitution strongly influenced the chemical shift values of neighboring residue B H-4 (δ 4.161; $\Delta\delta + 0.24$ ppm). The chemical shift of C H-4 at δ 3.465 are indicative for the terminal residue.¹³ Combining all data together, the structure of disaccharide compound F3 was determined to be α -D-Glcp-(1 \rightarrow 3)- β -D-Galp-(1 \rightarrow 4)-D-Glcp (Table 1).

Di-glycosylated lactose compounds

Fraction F4

Tetrasaccharide F4 includes 4 hexose residues, namely A, B (glucosyl and galactosyl residues from original lactose, respectively), C and D (transferred glucosyl residues from sucrose) (Table 1). All NMR chemical shifts of compound F4 were assigned by 1D ^1H NMR, 2D ^1H - ^1H TOCSY NMR, 2D ^1H - ^{13}C HSQC NMR. The 1D ^1H NMR spectrum of fraction F4 detected six anomeric signals at δ 5.439 (A α H-1, $^3J_{1,2}$ 3.48 Hz), δ 4.818 (A β H-1, $^3J_{1,2}$ 7.90 Hz), δ 4.523 (B H-1, $^3J_{1,2}$ 7.73 Hz), δ 4.908 (C H-1, $^3J_{1,2}$ 7.53 Hz), δ 5.374 (D a H-1, $^3J_{1,2}$ 3.87 Hz) and δ 5.097 (D b H-1, $^3J_{1,2}$ 3.63 Hz)(Figure S5). The anomeric signal of A α H-1 at δ 5.439 ppm is indicative for the occurrence of a 2-substituted glucosyl residue.¹³ Moreover, the set of ^1H and ^{13}C chemical shifts of residue A matched very well with those values of this residue found in compound F2, reflecting the existence of an α -D-Glcp-(1 \rightarrow 2)-unit. This is further reflected by 2D ROESY inter-residual cross-peaks D b H-1/ A β H-2 (Figure S5). Residue D showed downfield shifts of D a H-5 and D b H-5 to δ 4.075 ($\Delta\delta + 0.13$) and δ 3.97 ($\Delta\delta + 0.37$), respectively, indicating the terminal residue at the branching side.¹¹ The resonances of B H-4 and C-4 at δ 4.019 ($\Delta\delta + 0.10$ ppm) and δ 78.1 ($\Delta\delta + 8.3$ ppm), respectively, showed considerable downfield shifts compared to those observed in lactose, suggesting the 4-substitution of the galactosyl residue. The 2D ROESY inter-residual cross-peaks C H-1/B H-4

confirmed the 4-substitution of residue **B**. The chemical shift pattern of residue **C** matched that of residue **C** in compound F1, fitting a terminal α -D-Glcp-(1 \rightarrow 4)-residue, linked to the Gal-residue **B**. Taking all data together, the structure of tetrasaccharide compound F4 was determined to be α -D-Glcp-(1 \rightarrow 4)- β -D-Galp-(1 \rightarrow 4)-[α -D-Glcp-(1 \rightarrow 2)-]D-Glcp, as illustrated in Table 1.

Fraction F5

Tetrasaccharide F5 includes 4 hexose residues, namely **A**, **B** (glucosyl and galactosyl residues from original lactose, respectively), **C** and **D** (transferred glucosyl residues). All NMR chemical shifts of compound F5 were assigned by 1D ^1H NMR, 2D ^1H - ^1H TOCSY NMR, 2D ^1H - ^{13}C HSQC NMR. The 1D ^1H NMR spectrum of fraction F5 found six anomeric signals at δ 5.437 (**A α** H-1, $^3J_{1,2}$ 3.48 Hz), δ 4.823 (**A β** H-1, $^3J_{1,2}$ 8.01 Hz), δ 4.525 (**B** H-1, $^3J_{1,2}$ 7.51 Hz), δ 5.103 (**C** H-1, $^3J_{1,2}$ 3.71 Hz), δ 5.355 (**D a** H-1, $^3J_{1,2}$ 3.82 Hz) and δ 5.095 (**D b** H-1, $^3J_{1,2}$ 3.48 Hz) (Figure S6). As discussed for compound F4, the anomeric signal of **A α** H-1 at δ 5.437 ppm is indicative for the occurrence of a 2-substituted glucosyl residue.¹³ The set of ^1H and ^{13}C chemical shifts of residue **A** matched very well with those values found for this residue in compound F2, indicating the existence of a \rightarrow 2/4)- α -D-Glcp, which is further reflected by 2D ROESY NMR inter-residual cross-peaks **D b** H-1/ **A β** H-2 (Figure). The resonances of residue **B** H-3 and C-3 have been shifted to δ 3.76 ($\Delta\delta$ + 0.10 ppm) and δ 78.6 ($\Delta\delta$ + 4.9 ppm) compared to those of the galactosyl residue found in lactose,¹² indicating the occurrence of a 3-substitution at residue **B**. This substitution was verified by 2D ^1H - ^1H ROESY NMR measurements (Figure S6), displaying inter-residual cross-peaks between **C** H-1 and **B** H-3. Moreover, the chemical shift patterns of residues **B** and **C** are nearly identical to those in compound 3, suggesting the same structural element. Combining all data, the structure of tetrasaccharide compound F5 was determined to be α -D-Glcp-(1 \rightarrow 3)- β -D-Galp-(1 \rightarrow 4)-[α -D-Glcp-(1 \rightarrow 2)-]D-Glcp, as illustrated in Table 1.

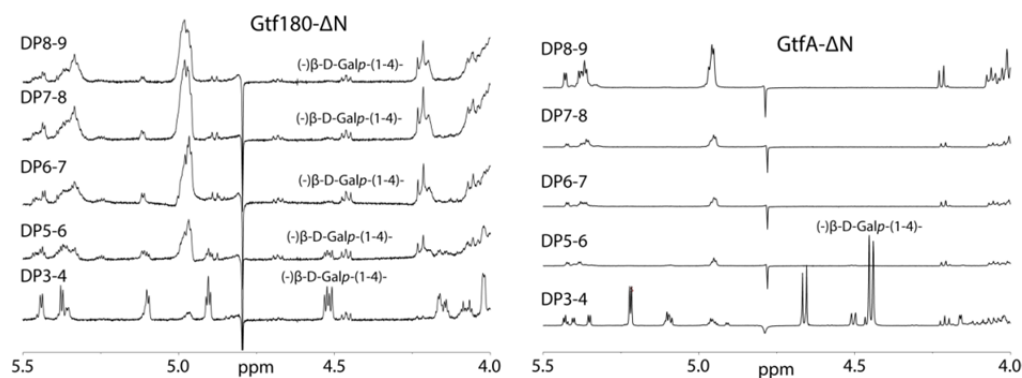


Figure 2: 500-MHz 1D ^1H NMR spectra of BioGel-P2 fractions (DP3 to DP9) of (a) the incubation of Gtf180- ΔN with sucrose and lactose and (b) the incubation of GtfA- ΔN with sucrose and lactose, at the same conditions. Reaction conditions: sucrose and lactose at 0.5 M; 3 U mL $^{-1}$ of enzyme; 24 h incubations at 37 °C and pH 4.7.

High DP transglucosylation products of lactose

The reaction mixtures of Gtf180- ΔN and GtfA- ΔN incubated with lactose as acceptor substrate and sucrose as donor substrate were subjected to precipitation with 20 % ethanol, followed by BioGel-P2 gel filtration (50 mL x 1.5 cm). The fraction size distribution in each pool was analyzed by MALDI-TOF-MS (Figure S7). Subpool 1 contained structures of 3 and 4 hexose units (DP3-4; m/z 527 and 689). Subpool 2 consisted of structures of 5 and 6 hexose units (DP5-6; m/z 851 and 1013). Subpool 3 contained structures with 6 and 7 hexose units (DP6-7; m/z 1013 and 1175). Subpool 4 consisted of structures of 7 and 8 hexose units (DP7-8; m/z 1175 and 1337). Finally, subpool 5 contained structures of 8 and 9 hexose units (m/z 1337 and 1499). These fractions were subjected to 1D ^1H NMR analysis. The 1D ^1H NMR spectra of all fractions synthesized by Gtf180- ΔN revealed anomeric signals at δ 4.523 ppm, δ 4.510 ppm and δ 4.465 ppm (Figure). These NMR resonances are indicative for a (-)- β -D-Galp-(1 \rightarrow 4)-,¹⁴ confirming the occurrence of a galactosyl residue in the fragment with various degrees of polymerization higher than 4. The data shows that Gtf180- ΔN elongated lactose with glucose units up to DP9 (m/z

1661). However, in case of GtfA- Δ N, only the 1D ^1H NMR spectrum of fraction DP3-4 showed the anomeric signals at δ 4.523 ppm, δ 4.510 ppm and δ 4.465 ppm, indicative for a (-) β -D-Galp-(1 \rightarrow 4)-, fitting with the occurrence of F1-F5. In the subpools with higher DP, only α -D-glucan related signals were observed. These data suggest that GtfA- Δ N elongated lactose with only one or two glucosyl residues.

DISCUSSION AND CONCLUSIONS

As previously reported for dextransucrases from *L. mesenteroides* and *W. confuse* the glucansucrases from *L. reuteri* strains 121 and 180 also exhibit the ability to decorate lactose with glucose.^{7,8} These dextransucrases apparently were only able to transfer a single glucose unit to lactose to form 2- α -D-glucopyranosyl-lactose (F2 in Table 1). At least five glucosylated lactose products with DP3 and DP4 were synthesized by GtfA- Δ N and Gtf180- Δ N and structurally characterized. In contrast to earlier findings that the linkage specificity of glucansucrases is conserved in oligosaccharide synthesis,^{15,16} new types of linkages were observed in the synthesized lactose glucosylation products. When using sucrose as donor and acceptor substrate, GtfA- Δ N synthesizes glucan with mainly (α 1 \rightarrow 4)/(α 1 \rightarrow 6) glucosidic linkages;¹⁰ with lactose as acceptor substrate this enzyme introduced (α 1 \rightarrow 4) but also (α 1 \rightarrow 3) and (α 1 \rightarrow 2) glucosidic linkages. Similarly, Gtf180- Δ N produces an α -glucan with 69 % (α 1 \rightarrow 6) and 31 % (α 1 \rightarrow 3) linkages from sucrose,⁹ but with lactose as acceptor substrate it synthesized (α 1 \rightarrow 3) but also (α 1 \rightarrow 2) and (α 1 \rightarrow 4) glucosidic linkages. A most interesting finding was that GtfA- Δ N and Gtf180- Δ N synthesized the same set of DP3 and DP4 oligosaccharides from lactose as acceptor (F1-F5, Table 1). Only glucansucrase Gtf180- Δ N, however, produced larger oligosaccharides with a lactose core, most likely elongating the F2 structure further from its non-reducing end. These unexpected results reflect the regiospecificity of these glucansucrases in binding the lactose acceptor substrate in

the active site. The structural features determining the product specificity of these Gtf enzymes acting on lactose as acceptor substrate remain to be elucidated.

In this study, the NMR spectral data of compound α -D-Glcp-(1 \rightarrow 2)-[β -D-Galp-(1 \rightarrow 4)-]D-Glcp (F2) are consistent with data obtained from previous studies.^{7,8} The full assignment of NMR spectra of two other trisaccharides (F1 and F3) and two tetrasaccharides (F4 and F5) are reported here for the first time. Lactose derivatives are interesting potential prebiotic compounds, especially those containing (α 1 \rightarrow 2)-linkages. These compounds are known to be highly resistant to the digestive enzymes in the human gut,^{17,18} and selectively stimulate the growth of health-beneficial microbiota.^{7,15} The studied glucansucrases are able to elongate α -D-Glcp-(1 \rightarrow 2)-[β -D-Galp-(1 \rightarrow 4)-]D-Glcp further with various types of linkages, such as α -D-Glcp-(1 \rightarrow 4)- β -D-Galp-(1 \rightarrow 4)-[α -D-Glcp-(1 \rightarrow 2)-]D-Glcp and α -D-Glcp-(1 \rightarrow 3)- β -D-Galp-(1 \rightarrow 4)-[α -D-Glcp-(1 \rightarrow 2)-]D-Glcp. Moreover, Gtf180- Δ N is able to produce glucosylated-lactose derivatives with a higher DP than 4. These results thus show that glucansucrases Gtf180- Δ N and GtfA- Δ N produce novel oligosaccharides (and putative prebiotic compounds) from cheap materials like lactose and sucrose. In our future research we will investigate the prebiotic properties of these glucosylated-lactose derivatives.

Acknowledgements

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Chapter 2

Table 2: ^1H and ^{13}C chemical shifts of the glucosylated lactose derivatives, measured at 25 °C in D_2O . Chemical shifts that are key in the structural determination are underlined.

	Lac		F1		F2		F3		F4		F5	
	^1H	^{13}C	^1H	^{13}C	^1H	^{13}C	^1H	^{13}C	^1H	^{13}C	^1H	^{13}C
A $_{\alpha}$ 1	5.222	92.8	5.225	92.7	5.433	90.0	5.225	92.8	5.439	90.0	5.437	90.2
A $_{\alpha}$ 2	3.58	72.4	3.57	72.4	<u>3.68</u>	<u>80.0</u>	3.57	72.4	<u>3.68</u>	<u>79.9</u>	3.69	79.4
A $_{\alpha}$ 3	3.83	72.5	3.82	72.5	3.94	70.5	3.82	72.5	3.94	72.8	3.96	72.8
A $_{\alpha}$ 4	3.66	79.8	3.66	79.5	3.72	79.9	3.66	79.8	3.73	79.5	3.72	78.6
A $_{\alpha}$ 5	3.95	71.2	3.950	71.2	4.00	70.5	3.95	71.2	3.99	70.9	4.00	70.9
A $_{\alpha}$ 6a	3.87	61.5	3.88	61.2	3.86	60.8	3.88	61.5	3.87	61.2	3.88	61.2
A $_{\alpha}$ 6b	3.84		3.84		3.65		3.84		3.84		3.84	
A $_{\beta}$ 1	4.662	96.9	4.667	97.1	4.816	96.7	4.667	96.9	4.818	97.1	4.823	97.0
A $_{\beta}$ 2	3.287	75.0	3.276	74.8	<u>3.414</u>	<u>80.0</u>	3.276	75.0	<u>3.408</u>	<u>79.2</u>	3.416	79.3
A $_{\beta}$ 3	3.63	75.4	3.63	75.5	3.72	75.8	3.63	75.4	3.73	74.1	3.72	74.0
A $_{\beta}$ 4	3.66	79.8	3.66	79.5	3.70	79.9	3.66	79.8	3.69	79.5	3.70	78.6
A $_{\beta}$ 5	3.60	75.8	3.60	76.1	3.60	76.4	3.60	75.8	3.59	75.8	3.60	75.8
A $_{\beta}$ 6a	3.95	61.6	3.95	61.2	3.95	60.8	3.95	61.6	3.84	61.2	3.97	61.2
A $_{\beta}$ 6b	3.80		3.80		3.80		3.80		3.974		3.80	
B1	4.447	104.4	4.510	104.0	4.465	103.6	4.525	103.9	4.523	104.4	4.525	103.9
B2	3.54	72.3	3.58	72.2	3.54	72.1	3.66	70.6	3.58	71.8	3.66	70.6
B3	3.66	73.7	3.75	72.8	3.66	73.2	<u>3.76</u>	<u>78.6</u>	3.76	72.8	<u>3.76</u>	<u>78.6</u>
B4	3.92	69.8	<u>4.027</u>	<u>78.2</u>	3.920	69.4	<u>4.161</u>	<u>66.1</u>	<u>4.019</u>	<u>78.1</u>	<u>4.161</u>	<u>66.1</u>
B5	3.72	76.4	3.78	76.1	3.96	75.9	3.71	76.0	3.67	76.4	3.71	76.0
B6a	3.80	62.2	3.80	62.2	3.77	61.7	3.80	62.2	3.83	61.5	3.80	62.2
B6b	3.75		3.74		3.70		3.75		3.78		3.75	
C1			4.914	101.1			5.103	96.7	4.908	101.0	5.103	96.7
C2			3.55	72.6			3.56	72.2	3.54	72.7	3.56	72.2
C3			3.75	73.9			3.80	79.8	3.75	73.9	3.80	79.8
C4			3.466	69.8			3.46	70.4	3.47	70.2	3.46	70.4
C5			<u>4.145</u>	72.9			3.96	72.8	<u>4.151</u>	72.8	3.96	72.8
C6a			3.80	61.2			3.84	61.2	3.82	61.2	3.84	61.2
C6b			3.75				3.78		3.74		3.78	
D $^{\alpha}$ 1					5.094	97.1			5.097	97.4	5.095	97.5
D $^{\alpha}$ 2					3.54	72.1			3.56	72.7	3.59	72.4
D $^{\alpha}$ 3					3.80	73.6			3.80	73.6	3.80	73.8
D $^{\alpha}$ 4					3.46	69.8			3.46	70.2	3.46	70.4
D $^{\alpha}$ 5					3.98	72.3			3.97	72.9	3.98	72.8
D $^{\alpha}$ 6a					3.88	60.8			3.81	61.2	3.88	61.2
D $^{\alpha}$ 6b					3.80				3.77		3.80	
D $^{\beta}$ 1					5.355	98.5			5.374	98.7	5.355	98.8
D $^{\beta}$ 2					3.53	72.1			3.54	72.7	3.53	72.4
D $^{\beta}$ 3					3.75	73.6			3.75	73.9	3.75	74.0
D $^{\beta}$ 4					3.47	69.8			3.47	70.2	3.47	70.4
D $^{\beta}$ 5					<u>4.084</u>	72.1			<u>4.075</u>	72.7	<u>4.089</u>	72.7
D $^{\beta}$ 6a					3.92	60.8			3.80	61.2	3.92	61.2
D $^{\beta}$ 6b					3.80				3.75		3.80	

Supplemental data

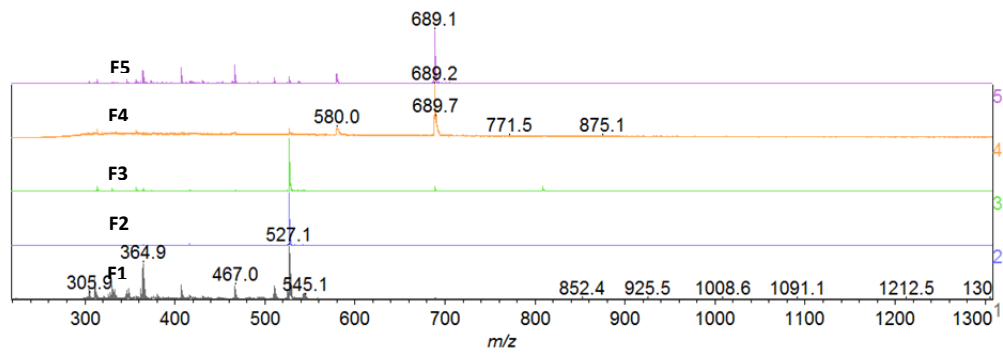


Figure S1: MALDI-TOF MS profile of the fractions (F1-F5) from incubation mixture of Gtf180- Δ N

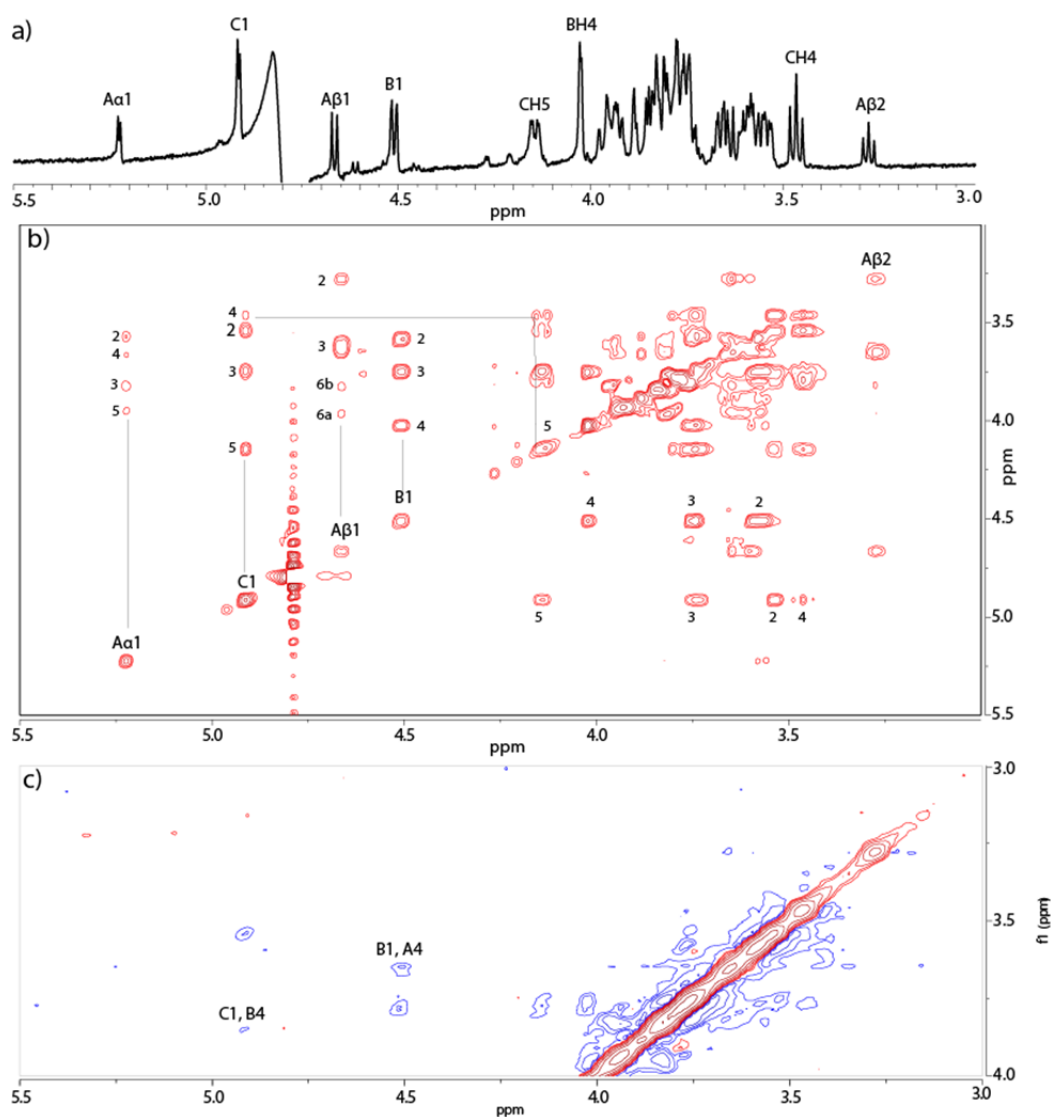


Figure S2: a) 500-MHz 1D ^1H NMR spectrum; b) 2D ^1H - ^1H TOCSY spectrum (mixing time 150 ms); and c) 2D ^1H - ^1H ROESY spectrum (mixing time 300 ms) of fraction F1, recorded at 25 $^\circ\text{C}$ in D_2O . In the TOCSY spectrum, anomeric signals are displayed on the diagonal; intra-residual correlations between well-resolved anomeric and ring-proton resonances are marked by numbers. In the ROESY spectrum, inter-residual couplings are indicated.

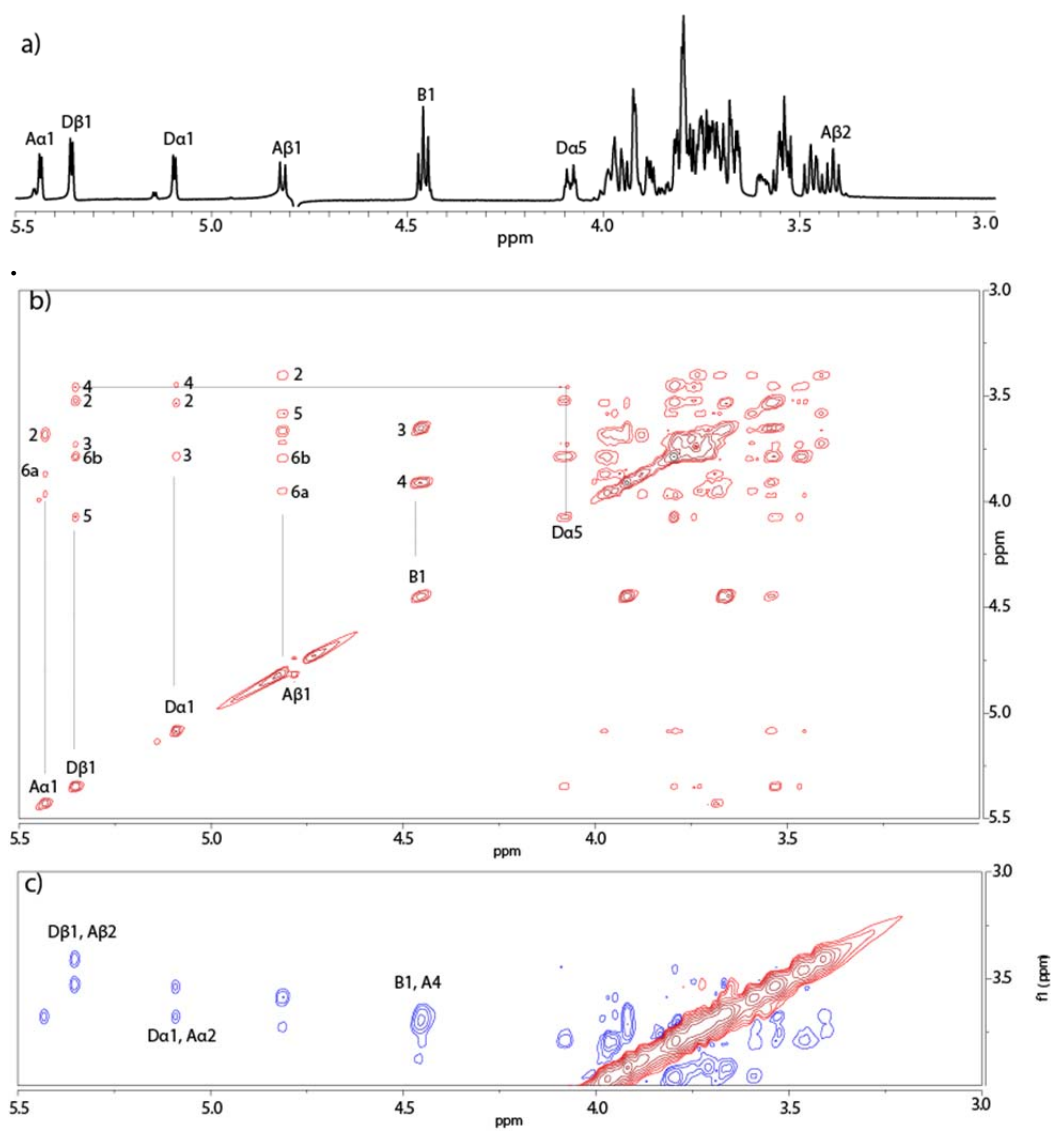


Figure S3: a) 500-MHz 1D ^1H NMR spectrum; b) 2D ^1H - ^1H TOCSY spectrum (mixing time 150 ms); and c) 2D ^1H - ^1H ROESY spectrum (mixing time 300 ms) of fraction F2, recorded at 25 $^\circ\text{C}$ in D_2O . In the TOCSY spectrum, anomeric signals are displayed on the diagonal; intra-residual correlations between well-resolved anomeric and ring-proton resonances are marked by numbers. In the ROESY spectrum, inter-residual couplings are indicated.

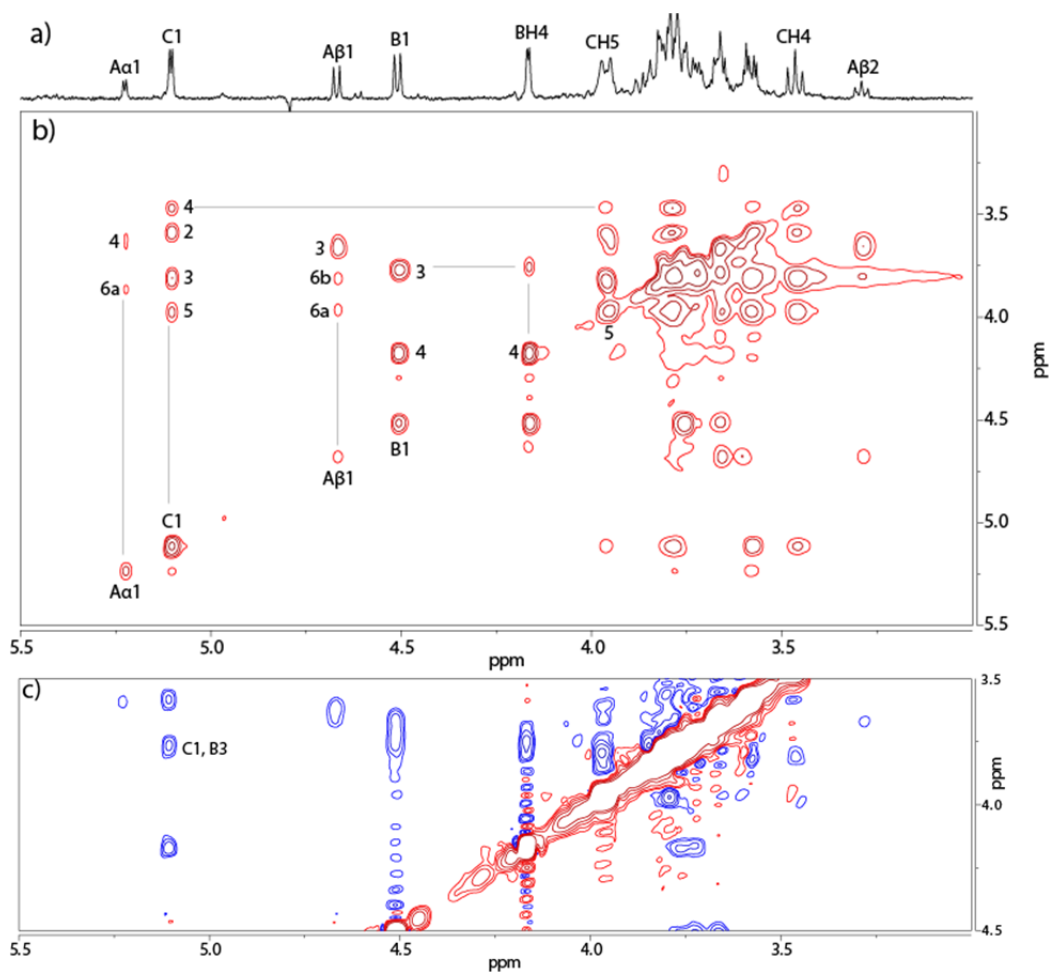


Figure S4: a) 500-MHz 1D ^1H NMR spectrum; b) 2D ^1H - ^1H TOCSY spectrum (mixing time 150 ms); and c) 2D ^1H - ^1H ROESY spectrum (mixing time 300 ms) of fraction F3, recorded at 25 $^\circ\text{C}$ in D_2O . In the TOCSY spectrum, anomeric signals are displayed on the diagonal; intra-residual correlations between well-resolved anomeric and ring-proton resonances are marked by numbers. In the ROESY spectrum, inter-residual couplings are indicated.

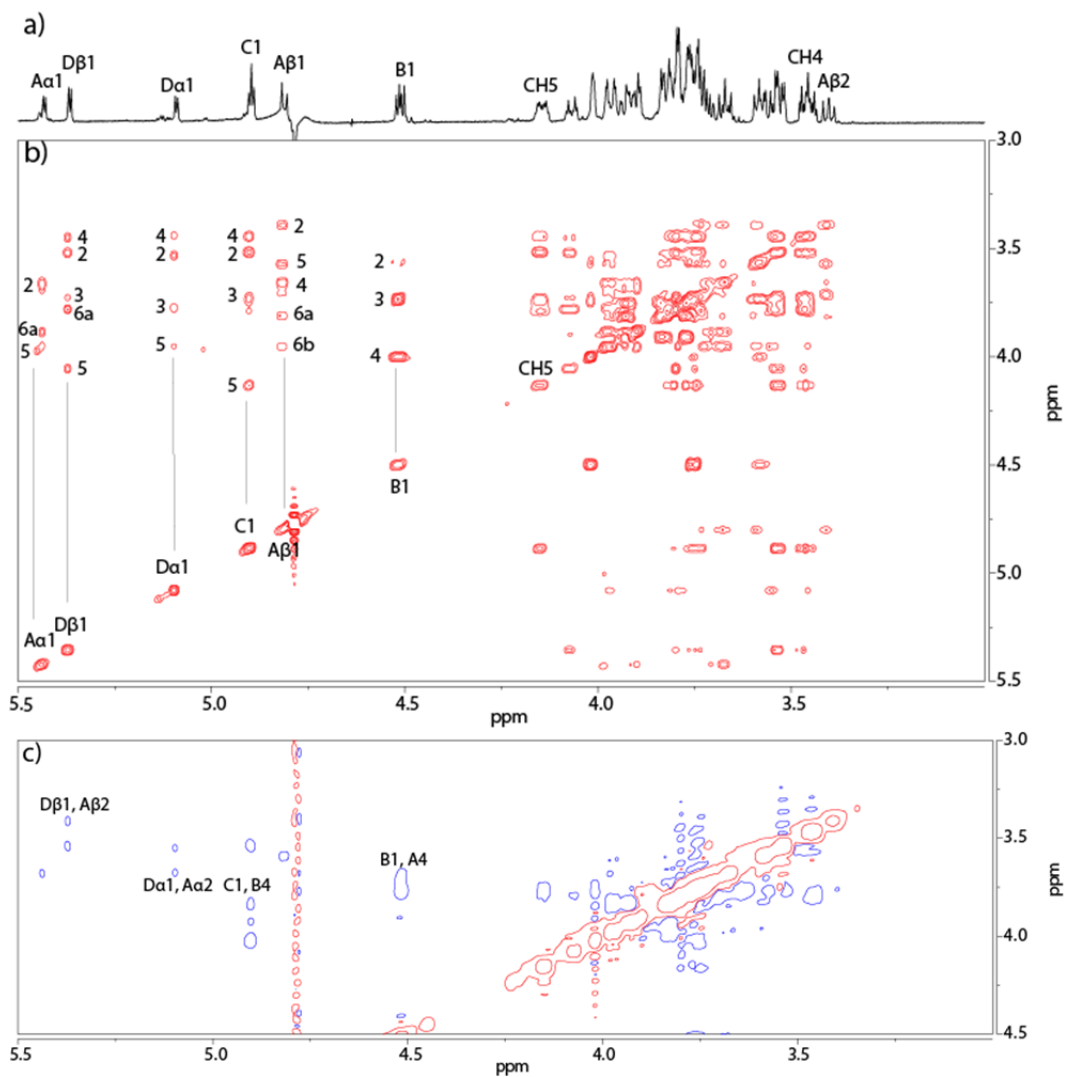


Figure S5: a) 500-MHz 1D ^1H NMR spectrum; b) 2D ^1H - ^1H TOCSY spectrum (mixing time 150 ms); and c) 2D ^1H - ^1H ROESY spectrum (mixing time 300 ms) of fraction F4, recorded at 25 $^\circ\text{C}$ in D_2O . In the TOCSY spectrum, anomeric signals are displayed on the diagonal; intra-residual correlations between well-resolved anomeric and ring-proton resonances are marked by numbers. In the ROESY spectrum, inter-residual couplings are indicated.

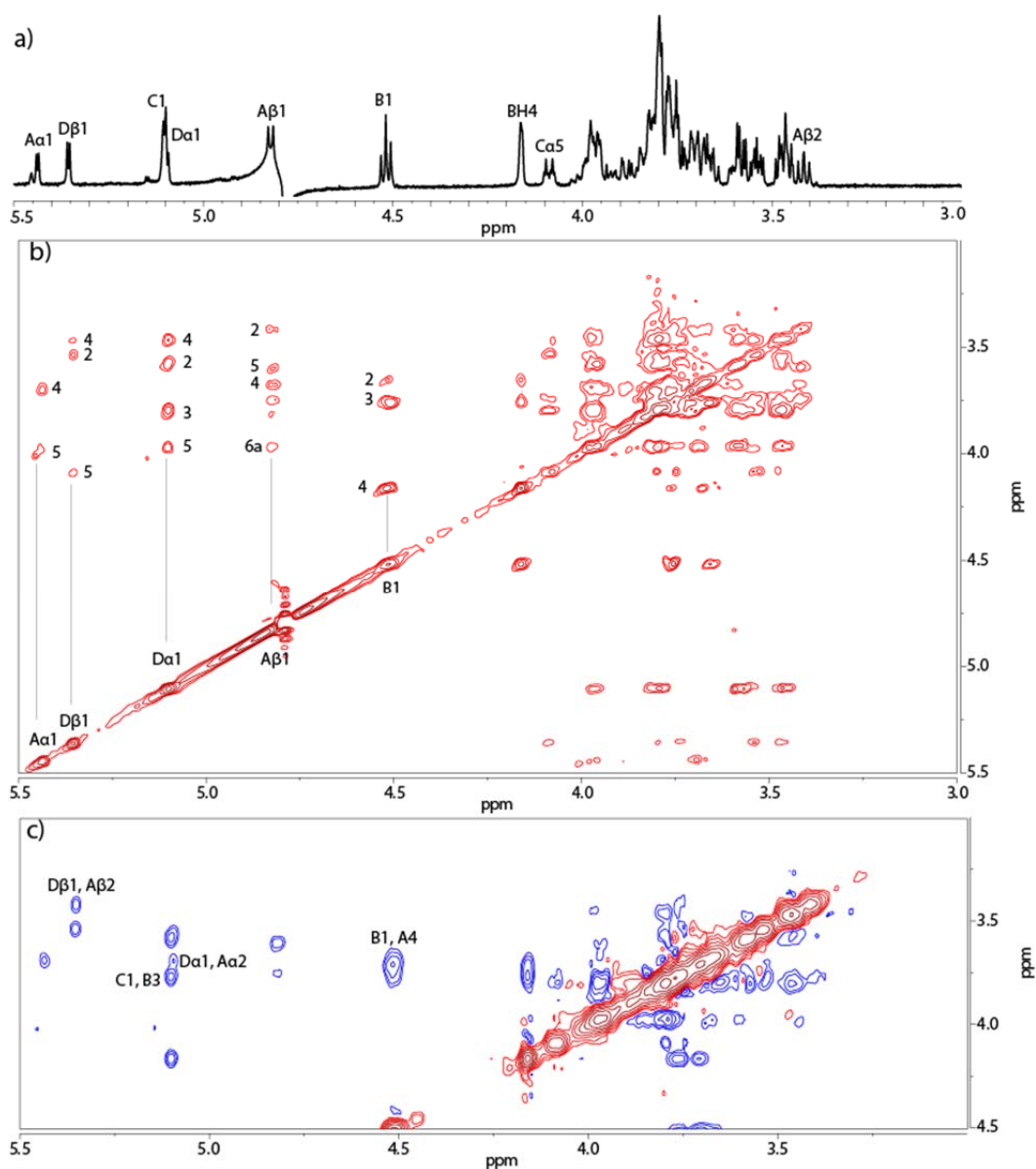


Figure S6: a) 500-MHz 1D ^1H NMR spectrum; b) 2D ^1H - ^1H TOCSY spectrum (mixing time 150 ms); and c) 2D ^1H - ^1H ROESY spectrum (mixing time 300 ms) of fraction F5, recorded at 25°C in D_2O . In the TOCSY spectrum, anomeric signals are displayed on the diagonal; the non-anomeric signals were track horizontally and vertically base on anomeric signals and displayed in numbers. In the ROESY, spectrum inter-residual couplings are indicated.

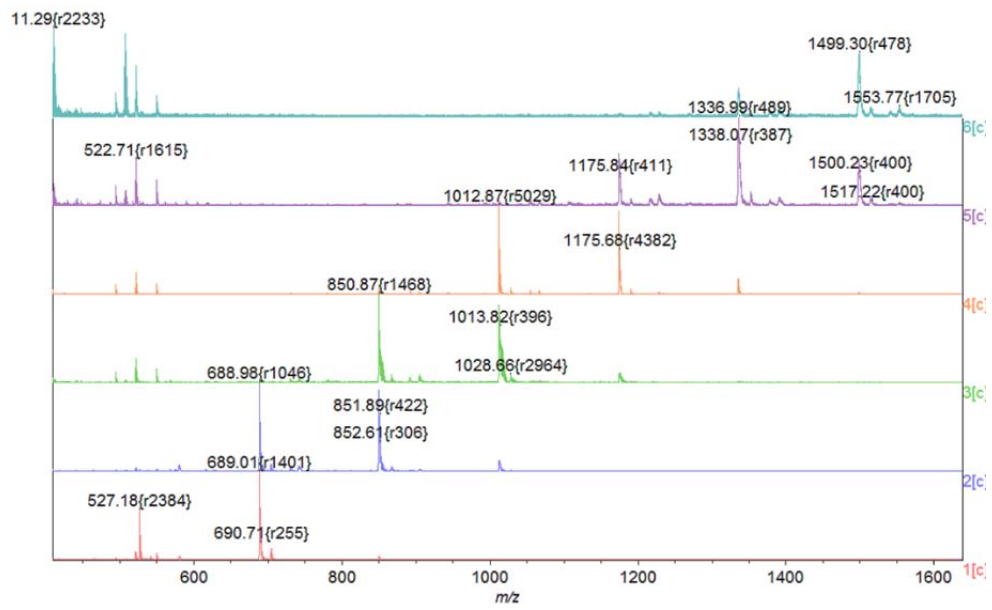


Figure S7: MALDI-TOF MS profile of the BioGel-P2 fractions from the incubation mixture of sucrose and lactose with Gtf180- Δ N

Chapter 3

Stimulatory effects of novel glucosylated lactose derivatives GL34 on growth of selected gut bacteria

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ABSTRACT

Previously we structurally characterized 5 Glucosylated Lactose derivatives (F1-F5) with a degree of polymerization (DP) of 3-4 (GL34), products of *Lactobacillus reuteri* glucansucrases, with lactose and sucrose as substrates. Here we show that these GL34 compounds are largely resistant to the hydrolytic activities of common carbohydrate degrading enzymes. Also the ability of single strains of gut bacteria, bifidobacteria, lactobacilli and commensal bacteria, to ferment the GL34 compounds was studied. Bifidobacteria clearly grew better on the GL34 mixture than lactobacilli and commensal bacteria. Lactobacilli and the commensal bacteria *Escherichia coli* Nissle and *Bacteroides thetaiotaomicron* only degraded the F2 compound α -D-Glcp-(1→2)-[β -D-Galp-(1→4)-]D-Glcp, constituting around 30 % w w⁻¹ of GL34. Bifidobacteria digested more than one compound from the GL34 mixture, varying with the specific strain tested. *Bifidobacterium adolescentis* was most effective, completely degrading four of the five GL34 compounds, leaving only one minor constituent. GL34 thus represents a novel oligosaccharide mixture with (potential) synbiotic properties toward *B. adolescentis*, synthesized from cheap and abundantly available lactose and sucrose.

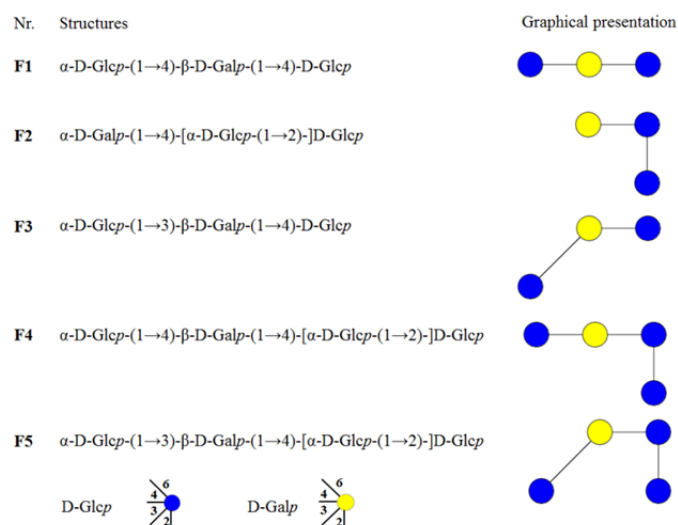
INTRODUCTION

The human gut microflora has drawn increasing attention in recent years. It constitutes a very interesting ecosystem that varies in density and functionality in the different gut compartments.¹ These complex ecosystems have a significant impact on host well-being.² Strongest interest is focused on understanding what factors cause variations in microbiota composition and how these gut bacteria modulate host health.³ Our work aims to stimulate the growth of health-promoting probiotic gut bacteria by using newly synthesized non-digestible carbohydrates, i.e. prebiotic compounds.

According to the latest definition, a prebiotic is “a substrate that is selectively utilized by host microorganisms conferring a health benefit”.⁴ Recently, part of the definition was disputed, since selective stimulation of health-promoting species seems not exclusively necessary to confer health benefits.^{5,6} Generally, prebiotics are carbohydrates that are not fully digested by the host. They are fermented by various commensal and health-beneficial gut bacteria, thus promoting their growth and activity which may confer health benefits upon the host.^{7,8} To date, the most well-known prebiotics, supported by good quality data, are human milk oligosaccharides (*h*MOS),⁹ GOS (β -galacto-oligosaccharides), FOS (β -fructo-oligosaccharides), inulin and lactulose.^{10,11} All of these prebiotics are also hydrolysed by brush border enzymes, but not completely.¹² Isomalto-/malto-polysaccharides (IMMP),^{13,14} xylo-oligosaccharides (XOS),¹⁵ resistant starch,¹⁶ and soy oligosaccharides also are (emerging) prebiotic oligosaccharides,¹⁷ although more data about their effects on gut health are still needed. Each of these prebiotic compounds may exert specific and selective effects on gut bacteria. The search for new and effective prebiotics combined with specific probiotics (synbiotics) is increasing rapidly.^{18,19}

Lactose-derived oligosaccharides attract much attention in view of their prebiotic potential. One example is GOS, which are synthesized from lactose by enzymatic

trans-galactosylation using β -galactosidases, achieving a degree of polymerization between 3 and 10.²⁰ This prebiotic has been widely studied and shown to stimulate probiotic bacteria to various extents.^{12,21,22} Another commercially available prebiotic in this group is lactosucrose which is hardly utilized by human digestive enzymes and has stimulatory effects on both lactobacilli and bifidobacteria.^{23,24} Also the selective bifidogenic effect of 4'-galactosyl-kojibiose, corresponding to compound F2 in our GL34 mixture,²⁵ on *Bifidobacterium breve* 26M2 has been reported.²⁴ These results indicate that there are clear perspectives to further develop and expand this group of lactose-derived prebiotic oligosaccharides.



Scheme 1: Structures of compounds F1-F5 from the mixture GL34.

We recently reported synthesis of a mixture of five novel lactose-derived oligosaccharides (F1-F5) using the *L. reuteri* glucansucrase enzymes Gtf180- Δ N and GtfA- Δ N.²⁵ Their structural characterization revealed the presence of various glycosidic linkages, (α 1 \rightarrow 2/3/4), with DP of 3 and 4 (Scheme 1).²⁵ Four out of these five structures were new and only F2 4'-galactosyl-kojibiose had been reported before. In this work, their resistance to degradation by common carbohydrate

degrading enzymes was studied by *in vitro* incubations. Also the growth of pure cultures of common gut bacteria, including commensal and probiotic strains, on these novel compounds was evaluated and compared with well-known prebiotic mixtures (GOS and FOS). This study provides information about the selective stimulatory effects of these compounds (and glycosidic linkage types) on growth of probiotic bacteria. The GL34 mixture particularly stimulated growth of *B. adolescentis*. This is also of interest from an industrial perspective, since these new oligosaccharides with very specific prebiotic effects are produced from low-cost lactose and sucrose, and may be an option for developing synbiotics.

MATERIALS AND METHODS

Bacterial strains, chemicals and reagents

Bacteroides thetaiotaomicron VPI-5482, *Lactobacillus acidophilus* ATCC 4356, *Bifidobacterium adolescentis* ATCC 15703, *Bifidobacterium longum* subsp. *infantis* ATCC 15697 were purchased from ATCC. *Bifidobacterium breve* DSM 20213 was obtained from the DSMZ culture collection. *Lactobacillus reuteri* strain 121 was obtained from TNO Quality of Life, Zeist, The Netherlands. *Lactobacillus casei* W56 was provided by Winclove Probiotics B.V. (Amsterdam). All reagents, chemicals or medium components were purchased from Sigma (Zwijndrecht, Netherlands), or as stated otherwise. The purified GOS mixture TS0903 (lacking glucose, galactose and lactose) was provided by FrieslandCampina Domo; its detailed GOS composition was published elsewhere.²⁶ The GOS/FOS mixture used is a 90/10 (w w⁻¹) mixture of the purified TS0903 GOS and long-chain Inulin (lcInulin, Frutafit TEX, provided by SENSUS, Roosendaal, The Netherlands), also serving as a control for the current prebiotic formula added to infant nutrition.²¹

***Lactobacillus* growth experiments**

Lactobacilli were pre-cultured in MRS-medium (Oxoid, Basingstoke, UK) anaerobically (or by using the GasPak system (Becton, Dickinson and Company, Sparks, USA)) under an N₂ atmosphere for up to 2 days at 37°C.²⁷ Then, 1 ml samples of the pre-cultures were harvested by centrifugation (2,500 x g, 2 min). The bacterial pellets were washed twice with sterile 10% NaCl and diluted 25-fold in 2x mMRS (modified MRS-medium that does not contain a carbon source for Lactobacilli).²⁸ In separate tubes, carbohydrates were dissolved with Milli-Q water to 10 mg ml⁻¹ and sterilized by filtration using 0.2 µm cellulose acetate filters (the GL34 mixture) or by autoclaving solutions (glucose). Cultures were inoculated by mixing 1:1 diluted bacterial suspensions with sterilized carbohydrate solutions in sterile microtiter plates to obtain an initial OD₆₀₀ of 0.01. Inoculation of microtiter plates was carried out in an anaerobic glove box (Sicco, Grünsfeld, Germany) with a constant nitrogen-flow, the microtiter plates sealed air tightly and transferred into a plate reader placed under constant N₂ flow. Glucose was used as positive control to compare growth of these lactobacilli on the mixture of GL34 compounds. Media without an added carbon source was used as negative control. Bacterial growth was followed at 37 °C by measuring the optical density at 600 nm (OD₆₀₀ nm) in 5 min intervals. OD values of the negative control samples (no carbohydrate added) were deducted while measuring their corresponding samples and positive controls.

***Bifidobacterium* and *B. thetaiotaomicron* growth experiments**

The *Bifidobacterium* strains were sub-cultured (from stocks stored at -80 °C) in 10 mL of *Bifidobacterium* medium (BM) supplemented with 1% glucose (One liter BM contained 10 g Trypticase peptone, 2.5 g yeast extract, 3 g tryptose, 3 g K₂HPO₄, 3 g KH₂PO₄, 2 g triammonium citrate, 0.3 g pyruvic acid, 1 ml Tween 80, 0.574 g MgSO₄ · 7H₂O, 0.12 g MnSO₄ · H₂O, and 0.034 g FeSO₄ · 7H₂O). After autoclaving, BM was supplemented with 0.05 % (wt vol⁻¹) filter-sterilized cysteine-HCl (Ryan et

al. 2006). *B. thetaiotaomicron* was cultured using a carbon-limited minimally defined medium of 100 mM KH_2PO_4 (pH 7.2), 15 mM NaCl, 8.5 mM $(\text{NH}_4)_2\text{SO}_4$, 4 mM L-cysteine, 1.9 M hematin, 200 M L-histidine, 100 nM MgCl_2 , 1.4 nM $\text{FeSO}_4 \cdot 7 \text{H}_2\text{O}$, 50 M CaCl_2 , 1 g mL^{-1} vitamin K3, 5 ng mL^{-1} vitamin B12, and individual carbon sources (0.5%, wt vol^{-1}).²⁹ Carbohydrates were prepared by dissolving in Milli-Q water to 10 mg mL^{-1} and sterilized by filtration using 0.2 μm cellulose acetate filters (the GL34 mixture, GOS, FOS) or by autoclaving solutions (lactose). Growth was carried out in fermentation tubes at 37 °C under anaerobic conditions maintained by GasPak EZ anaerobe container system (BD, New Jersey, US). Cell suspensions from overnight cultures were prepared in 3 mL of BM supplemented with different carbon source in a final concentration of 5 mg mL^{-1} . BM without an added carbon source was used as negative control. The initial OD_{600} of all incubations was adjusted to 0.01. OD_{600} nm measurements of the fermentation tubes were carried out manually at 1-h intervals and data used to generate growth curves. OD values of the negative control samples (no carbohydrate added) were deducted while measuring their corresponding samples and positive controls.

***E.coli* Nissle growth experiments**

E.coli Nissle was cultured using M9 medium as previously described, at 37 °C under aerobic conditions.³⁰ OD_{600} nm measurements of the fermentation tubes were carried out manually at 2-h intervals. OD values of the negative control samples (no carbohydrate added) were deducted while measuring their corresponding samples and positive controls.

Enzyme incubations

The GL34 mixture (1 mg mL^{-1}) was incubated for 24 h with different carbohydrate degrading enzymes (5 U mL^{-1}); α -amylase 1 (Porcine pancreas); α -amylase 2 (*Aspergillus oryzae*); α -glucosidase (Yeast); Iso-amylase (*Pseudomonas sp.*;

Pullulanase type 1 (*Klebsiella planticola*); β -galactosidase 1 (*Aspergillus oryzae*) and β -galactosidase 2 (*Kluyveromyces lactis*) (see Table S1 with detailed information).

Intracellular and extracellular activity essays

After growth with GL34 as their only carbon source, the three tested *Bifidobacterium* strains were harvested by centrifugation at 10,000 x g for 15 min at room temperature. Culture supernatants were sterilized using 0.45 μ m filters and concentrated 10 times by Amicon Ultra-4 centrifugal filter units (10,000 Da molecular weight cutoff, Millipore). The harvested cell pellets were washed twice with 0.1 M potassium phosphate buffer (pH 6.6) and then suspended in 1 mL of this buffer into 2.0-mL screw-cap microtubes containing 400 mg of 0.1-mm-diameter glass beads (Biospec Products). Cell disruptions were carried out by homogenization by a mini bead-beater (Biospec Products) at 4,200 rpm for six 1-min cycles with 40 sec cooling on ice in between. The cytoplasmic extracts were harvested by centrifugation at 10,000 x g for 5 min to remove cell wall fragments, and then concentrated to one-fifth of the initial volume using Amicon Ultra-4 units as above.

The concentrated cell free supernatants and cytoplasmic extracts (10 μ g protein for each) were incubated separately with 5 mg mL⁻¹ of the GL34 mixture. All reactions were performed in Milli-Q at 37 °C for 24 h. The progress of the reactions was followed by high-performance-anion-exchange chromatography (HPAEC).

High-pH Anion-Exchange Chromatography (HPAEC)

Samples were analyzed on an ICS-3000 workstation (Dionex, Amsterdam, the Netherlands) equipped with an ICS-3000 pulse amperometric detection (PAD) system and a CarboPac PA-1 column (250 x 2 mm; Dionex). The analytical separation was performed at a flow rate of 0.25 mL min⁻¹ using a complex gradient

of eluents A (100 mM NaOH); B (600 mM NaOAc in 100 mM NaOH); C (Milli-Q water); and D: 50 mM NaOAc. The gradient started with 10 % A, 85 % C, and 5 % D in 25 min to 40 % A, 10 % C, and 50 % D, followed by a 35-min gradient to 75 % A, 25 % B, directly followed by 5 min washing with 100 % B and reconditioning for 7 min with 10 % A, 85 % B, and 5 % D. External standards of lactose, glucose, fructose were used for calibration. For the determination of glucosylated lactose compounds with a degree of polymerization (DP) of 3, maltotriose was used as external standard.

Bioinformatic analysis

All protein sequences from *B. adolescentis* ATCC 15703, *B. longum* subsp. *infantis* ATCC 15697, *B. breve* DSM 20123 and *B. breve* UCC 2003 used in this study were extracted from the National Center for Biotechnology Information (NCBI) database. Database searches used the non-redundant sequence database accessible at the NCBI website (<http://www.ncbi.nlm.nih.gov>) using BLASTP and global align search. The BLASTP searches and multiple-sequence alignments were used to find similarity between the characterized glucosidases of *B. breve* UCC 2003 and annotated glucosidases encoded by the studied bifidobacterial strains. Annotation of Carbohydrate-active enzymes encoded by the genome sequences of *L. reuteri* 121 and *L. acidophilus* ATCC 4356 was carried out using dbCAN (<http://csbl.bmb.uga.edu/dbCAN>).

RESULTS

Enzymatic hydrolysis of compounds in the GL34 mixture

The GL34 mixture of five compounds was synthesized using glucansucrase Gtf180- Δ N, decorating lactose with one or two glucose units from sucrose as donor substrate, also introducing different types of linkages (Pham et al. 2017).²⁵ GL34

contains three DP3 compounds and two DP4 compounds, i.e. **F1** (4'-glc-lac): α -D-Glcp-(1 \rightarrow 4)- β -D-Galp-(1 \rightarrow 4)-D-Glcp; **F2** (2-glc-lac): α -D-Glcp-(1 \rightarrow 2)-[β -D-Galp-(1 \rightarrow 4)-]D-Glcp; **F3** (3'-glc-lac): α -D-Glcp-(1 \rightarrow 3)- β -D-Galp-(1 \rightarrow 4)-D-Glcp; **F4** (4',2-glc-lac): α -D-Glcp-(1 \rightarrow 4)- β -D-Galp-(1 \rightarrow 4)-[α -D-Glcp-(1 \rightarrow 2)-]D-Glcp and **F5** (3',2-glc-lac): α -D-Glcp-(1 \rightarrow 3)- β -D-Galp-(1 \rightarrow 4)-[α -D-Glcp-(1 \rightarrow 2)-]D-Glcp.²⁵ Four types of glycosidic linkages thus occur in this mixture, namely (α 1 \rightarrow 2), (α 1 \rightarrow 3), (α 1 \rightarrow 4), and (β 1 \rightarrow 4). Only the F2 2-glc-lac compound had been described before.³¹ The GL34 mixture also contains glucosyl residues linked (α 1 \rightarrow 3)/ (α 1 \rightarrow 4) to the galactosyl residue of the original lactose. In view of the novel composition of this mixture of glucosylated-lactose compounds we tested their resistance or sensitivity to hydrolysis with several commercially available enzymes. Following incubations with the porcine pancreas and *Aspergillus oryzae* α -amylases (Table S1), the HPAEC profiles at time 0 and 24 h showed no degradation of the GL34 compounds (Figure 1). Also various malto-oligosaccharide acting enzymes (α -glucosidase, iso-amylase and pullulanase, Table S1) were tested for their ability to hydrolyze GL34 compounds. However, after 24 h incubation, no (monomeric or dimeric) products were detected in the reaction mixtures with these three enzymes (Figure 1). None of these α -glucose cleaving enzymes thus was active on the GL34 compounds.

Subsequent incubation of the GL34 mixture with the β -galactosidase enzymes from *A. oryzae* and *Kluyveromyces lactis* (Table S1) however, did result in (some) hydrolysis. Fig. 1 shows that galactose and kojibiose (a glucose disaccharide with (α 1 \rightarrow 2)-linkage) were released during incubation with β -galactosidase, especially with the *A. oryzae* enzyme. Only the peak corresponding to F2 2-glc-lac disappeared, the only GL34 compound with a terminal galactosyl residue. We subsequently studied the utilization of these GL34 compounds for growth by (selected) common intestinal bacteria in more detail.

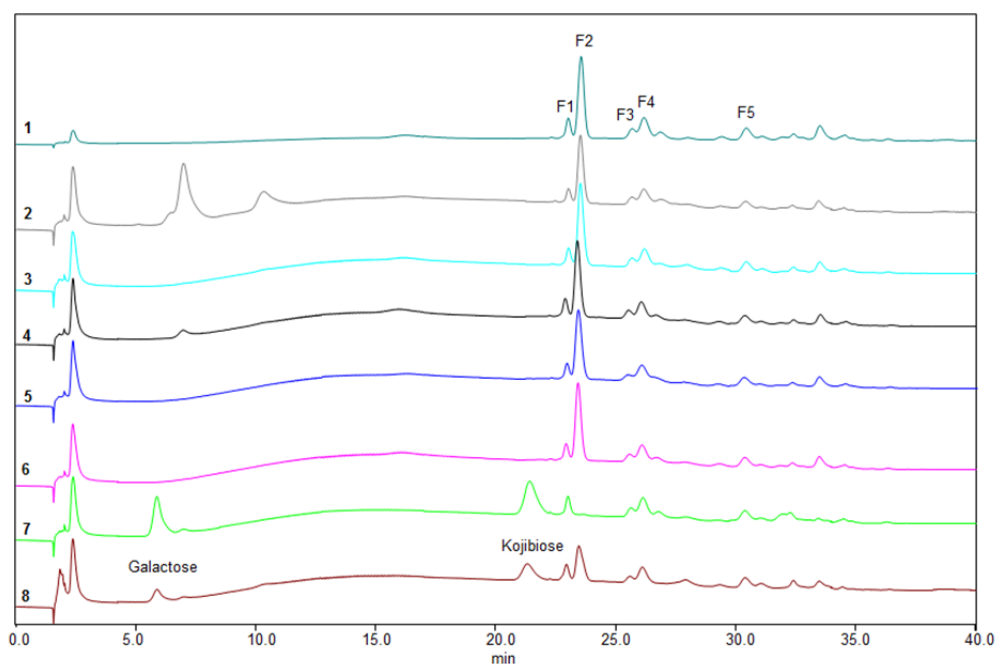


Figure 1: HPAEC profiles of oligosaccharides in 1) the GL34 mixture (1 mg mL^{-1} , blank) and the hydrolysis products after incubation of GL34 with 2) α -amylase from Porcine; 3) α -amylase from *A. oryzae*; 4) α -glucosidase from yeast; 5) iso-amylase from *Pseudomonas* sp.; 6) pullulanase type 1 from *K. planticola*; 7) β -galactosidase from *A. oryzae* and 8) β -galactosidase from *K. lactis*.

Growth of human gut bacteria on the GL34 mixture

Fermentation of GL34 compounds by probiotic Lactobacillus strains

In this study we tested *L. casei* W56, *L. acidophilus* ATCC 4356 and *L. reuteri* 121 and observed that all three strains showed limited growth on media with the GL34 mixture as the only carbon source (5 mg mL^{-1}): the final (relative) OD_{600} values reached were 3.8 %, 10.4 % and 26.5 %, respectively, compared to a 100 % control grown on glucose (Figure 2).

The GL34 mixture (5 mg mL^{-1}) showed different stimulatory effects on the growth of *B. breve* DSM 20123, *B. adolescentis* ATCC 15703 and *B. longum* subsp. *infantis* ATCC 15697. *B. adolescentis* grew very well on GL34, its final OD_{600} value

reached 80 % of that of a 100 % control growing on lactose, the purified TS0903 GOS mixture and the GOS/FOS mixture (Table 1). However, the final OD₆₀₀ values for growth of *B. breve* DSM 20123 and *B. longum* subsp. *infantis* ATCC 15697 on GL34 remained below 50% of the values for growth on lactose, the purified TS0903 GOS mixture and the GOS/FOS mixture (Table 1).

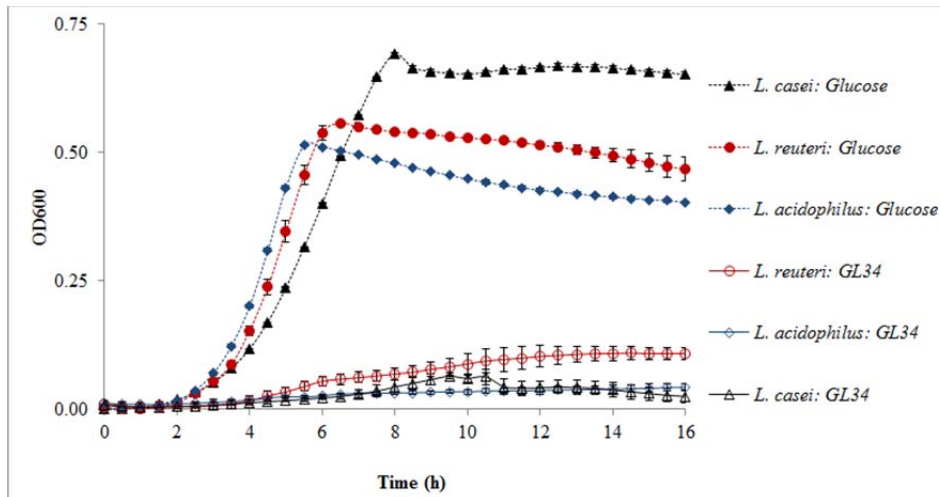


Figure 2: Growth of *L. casei* W56, *L. reuteri* 121 and *L. acidophilus* ATCC 4356 on GL34 compounds (5 mg mL⁻¹). Glucose (5 mg mL⁻¹) served as positive control; growth studies were carried out in triplicate.

Fermentation of GL34 compounds by probiotic Bifidobacterium strains

The tested bifidobacterial strains displayed two or more growth phases (Figure 3 and Figure S1). Some compounds in the GL34 mixture thus are more preferred growth substrates than others. *B. breve* and *B. longum* subsp. *infantis* grew more slowly on GL34 than *B. adolescentis*. They reached OD values around 0.70 after 24 h incubation compared to 12 h for *B. adolescentis*. Final maximal OD values were 0.86 for *B. breve* and 0.73 for *B. longum* subsp. *infantis* and 1.60 for *B. adolescentis*. The latter strain appeared to go through different lag phases, adapting to the

different carbon sources in GL34, reaching maximal OD after 36 h of incubation (Figure S1).

Table 1: Growth of *B. adolescentis* ATCC 15703, *B. longum* subsp. *infantis* ATCC 15697 and *B. breve* DSM 20123 on different carbon sources (5 mg mL⁻¹) for 36 h, and the final pH values.

Carbon sources	<i>Bifidobacterium adolescentis</i> ATCC 15703			<i>Bifidobacterium longum</i> subsp. <i>infantis</i> ATCC15697			<i>Bifidobacterium breve</i> DSM 20123		
	OD ₆₀₀	%	pH	OD ₆₀₀	%	pH	OD ₆₀₀	%	pH
Lactose	2.00 ± 0.00	100	5.0	2.00 ± 0.00	100	5.0	2.00 ± 0.00	100	5.0
GOS	2.00 ± 0.00	100	5.0	1.77 ± 0.02	89	5.2	1.64 ± 0.04	82	5.3
GOS/FOS	2.00 ± 0.00	100	4.9	1.79 ± 0.02	90	5.3	1.61 ± 0.01	81	5.2
GL34	1.60 ± 0.03	80	5.0	0.73 ± 0.03	37	5.3	0.86 ± 0.04	43	5.4

The experiments were carried out in triplicate, and the average values are shown.

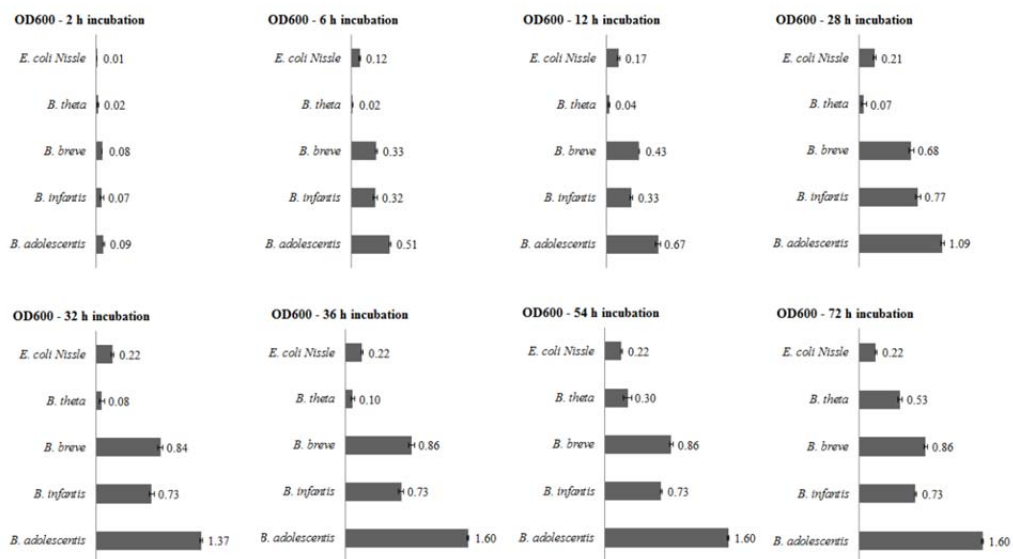


Figure 3: Growth of *B. adolescentis* ATCC 15703, *B. longum* subsp. *infantis* ATCC 15697, *B. breve* DSM 20123, *E. coli* Nissle, *B. theta* *taioamicon* in medium supplemented with 5 mg mL⁻¹ GL34 at different times of incubation (h). Growth experiments were carried out in triplicate.

Fermentation of GL34 compounds by commensal gut bacteria

Also the ability of two selected commensal bacteria to grow on the GL34 mixture was studied. *B. thetaiotaomicron* is a Gram-negative anaerobic bacterium found dominantly in human distal intestinal microbiota.³² *E.coli* Nissle represents ecologically important inhabitants of the human intestinal tract.³³ Growth of *E. coli* Nissle on the GL34 mixture, TS0903 GOS and a GOS/FOS mixture (5 mg mL^{-1}), was relatively minor with final OD_{600} values of $0.19 \div 0.35$ after 24 h of incubation, compared to growth on lactose and glucose with final OD_{600} values of 0.66 and 0.74, respectively (Figure 4-1). The final OD_{600} value of *B. thetaiotaomicron* was 0.53 after 72 h of incubation, but its growth displayed a pronounced lag-phase (Figure 4-2). This strain thus may fail to compete with other bacteria which have shorter lag-phases of growth with the GL34 mixture, such as the bifidobacteria tested and *E. coli* Nissle.

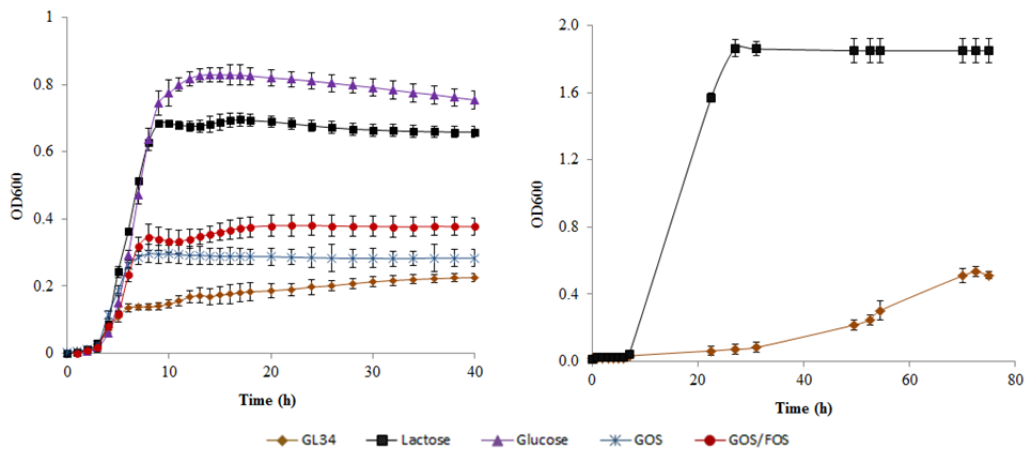


Figure 4: 1) Growth of *E. coli* Nissle on the GL34, TS0903 GOS and GOS/FOS mixtures (5 mg mL^{-1}); lactose and glucose (5 mg mL^{-1}) served as positive controls, and 2) Growth of *B. thetaiotaomicron* on the GL34 compounds (5 mg mL^{-1}); lactose (5 mg mL^{-1}) served as positive control. Growth studies were carried out in triplicate.

We subsequently identified the specific GL34 compounds utilized by these strains, and products derived, also aiming to elucidate which hydrolytic enzyme activities are involved, with emphasis on β -galactosidases and α -glucosidases.

Hydrolytic activity of commensal bacteria and lactobacilli on the GL34 mixture

The GL34 compounds can be visualized as individual peaks in HPAEC-PAD chromatograms. The compounds consumed by the tested bacteria were validated by peak disappearance at the corresponding retention time. HPAEC analysis of culture supernatants of the commensal bacteria grown on the GL34 mixture showed that the F2 peak corresponding to 2-glc-lac disappeared completely (Figure 5), indicating the selective and full utilization of only F2. Most likely this is based on β -galactosidase degradation of F2 2-glc-lac, followed by α -glucosidase degradation of the kojibiose formed, and finally consumption of the galactose and glucose formed for cellular growth. Only in case of *E. coli* Nissle degradation resulted in accumulation of kojibiose. This was not investigated any further. Besides, *B. thetaiotaomicron* was also able to degrade F4 4',2-glc-lac partially (Figure 5).

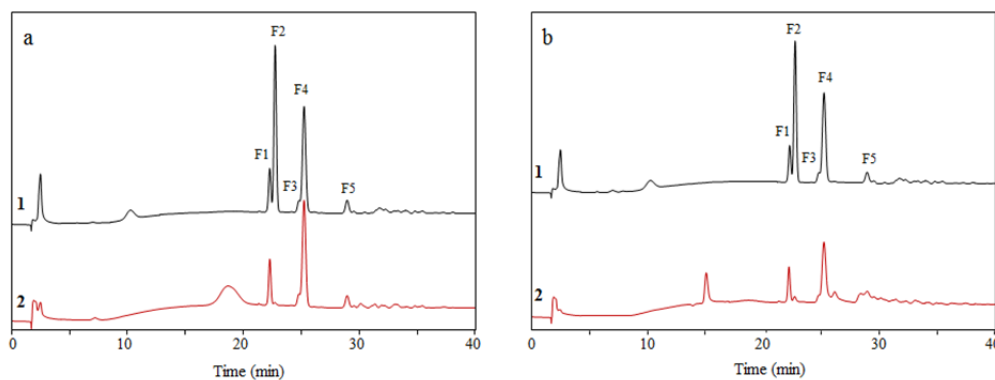


Figure 5: HPAEC profiles of oligosaccharides in **1**) the GL34 mixture (1 mg mL^{-1} , blank) and **2**) the hydrolysis products of GL34 fermentation by **a**) *E. coli* Nissle at 40 h and **b**) *B. thetaiotaomicron* at 72 h.

HPAEC profiles of *L. casei* W56 culture supernatants showed that none of the GL34 compounds were degraded (Figure 6), in line with the very limited growth observed (Figure 2). When searching the annotated genome sequence of this strain in the CAZy database (<http://www.cazy.org/b7858.html>) we did not find any (putative) β -galactosidase. This most likely explains the inability of *L. casei* W56 to hydrolyze the galactose (β 1 \rightarrow 4) linked to glucosyl residue in F2 2-glc-lac. Many putative α -glucosidases were found encoded in the genome sequence of this strain, however. In view of the results obtained, these enzymes apparently are inactive on the GL34 compounds, or the GL34 mixture is unable to induce their expression. This was not investigated further.

L. acidophilus ATCC 4356 and *L. reuteri* 121 strains only showed hydrolytic activity with F2 2-glc-lac in the GL34 mixture. *L. reuteri* 121 degraded this compound to a higher level than *L. acidophilus* ATCC 4356 (Figure 6). Annotation of Carbohydrate-active enzymes encoded by the genome sequence of *L. reuteri* 121 was carried out using dbCAN (<http://csbl.bmb.uga.edu/dbCAN>).³⁴ One putative β -galactosidase of family GH2 was detected (gene number: BJI45_06415), which may be responsible for hydrolysis of F2. β -Galactosidases of this GH2 family are known to hydrolyze a wide variety of β -(1 \rightarrow 2, 3, 4, or 6) GOS, including oligosaccharides with a degree of polymerization of 3 – 6.³⁵ Kojibiose released from the F2 compound by this strain remained in the medium without being degraded (completely). Only a single (predicted) extracellular α -glucosidase of family GH31 was encoded in the genome of *L. reuteri* 121 (gene number: BIJ45_02455).³⁴ This enzyme is apparently unable to effectively degrade kojibiose. Apparently, there is also no transporter expressed that can facilitate the uptake of kojibiose for intracellular degradation.

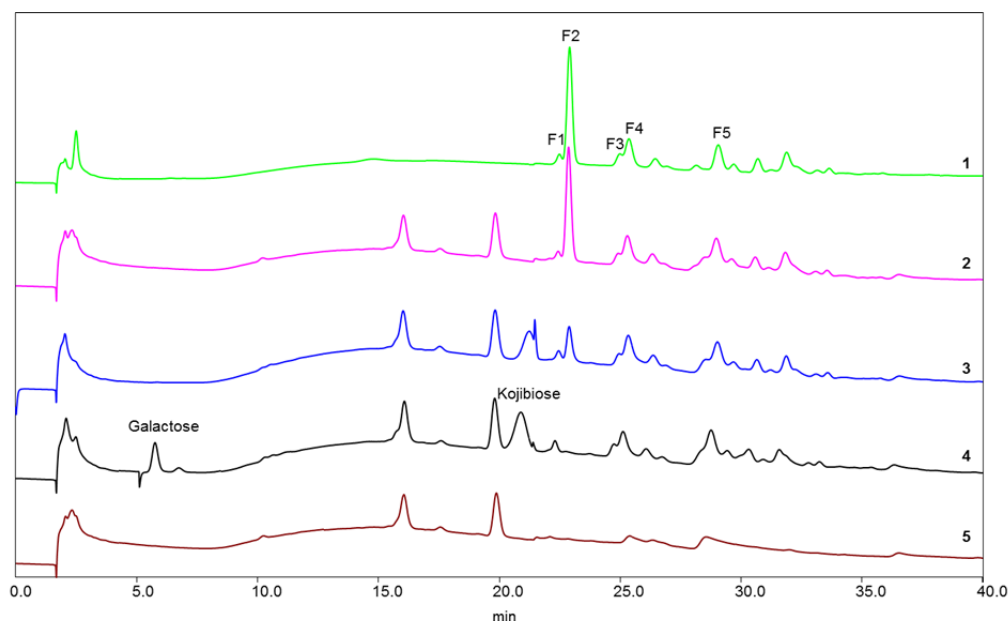


Figure 6: HPAEC profiles of oligosaccharides in 1) the GL34 mixture (1 mg mL^{-1} , blank) and the hydrolysis products of GL34 fermentation after 16 h of incubation with 2) *L. casei* W56; 3) *L. acidophilus* ATCC 4356; 4) *L. reuteri* 121 and 5) MRS medium (blank).

L. acidophilus ATCC 4356 is known to encode a β -galactosidase (LacZ, classified in the GH2 family).³⁵⁻³⁷ This LacZ enzyme thus may be responsible for degradation of the ($\beta 1 \rightarrow 4$)-linkage in F2 and release of kojibiose. Annotation of Carbohydrate-active enzymes encoded by the genome sequence of *L. acidophilus* ATCC 4356 was also carried out by dbCAN (<http://csbl.bmb.uga.edu/dbCAN>).³⁸ Ten putative α -glucosidase enzymes (Family GH13 and GH31) were annotated in the *L. acidophilus* ATCC 4356 genome. Agl3 is an α -glucosidase identified in *B. breve* UCC 2003, with a broad hydrolytic activity towards all possible α -glycosidic linkages, including the ($\alpha 1 \rightarrow 2$) linkage in sucrose and kojibiose.³⁹ However, a BLAST analysis of these putative glucosidases of *L. acidophilus* ATCC 4356 and Alg3 showed very low similarity in protein sequence (between 24 and 31 %) (Table S2). The observed accumulation of kojibiose in the growth medium of *L. acidophilus* ATCC 4356 (Figure 6) thus may be due to lack of an extracellular α -

glucosidase active on (α 1 \rightarrow 2) linkages. Furthermore, it is possible that the α -glucosidase enzymes of this strain are only intracellular enzymes and that a suitable transporter for kojibiose is absent.

Hydrolytic activity of the *Bifidobacterium* strains on the GL34 mixture

Typically, bifidobacteria have extracellular endohydrolases acting on glycosidic bonds of oligo- and polymeric substrates, yielding smaller products which are internalized by carbohydrate-specific (ABC-type) transporters. Further utilization may be carried out by cytoplasmic GHs such as α/β -glucosidases and α/β -galactosidases to produce monosaccharides which are used for growth.^{40,41} To try and identify the bifidobacterial enzymes responsible for utilization of GL34 compounds, cells grown in modified BM broth containing 5 mg mL⁻¹ GL34 were harvested at 36 h (Figure 3). Three fractions were prepared, namely growth culture supernatants, concentrated culture supernatants and cytoplasmic extracts (see Methods). GL34 was incubated with concentrated culture supernatants and cytoplasmic extracts to verify the presence or absence of extra- and/or intracellular enzyme activities involved in degradation.

***Bifidobacterium adolescentis* ATCC 15703**

B. adolescentis was able to utilize all GL34 compounds, and only 10 and 60 % of F4 4',2-glc-lac and F5 3',2-glc-lac remained, respectively (Figure 7-1). This also explains the relatively strong growth of this strain (Figure 3). None of the GL34 compounds were hydrolyzed by cell-free culture supernatants of this strain. Its cell extracts only hydrolyzed F1 4'-glc-lac, F2 2-glc-lac & F4 4',2-glc-lac compounds (Figure 7-1). Three α -glucosidases are annotated in the *B. adolescentis* ATCC 15703 genome sequence (<http://www.cazy.org>), namely AglB; AglA and BAD-0971. AglB exhibited a preference for hydrolyzing (α 1 \rightarrow 4) linkages in maltose (van den Broek et al. 2003),⁴² and most likely also is involved in cleavage of the (α 1 \rightarrow 4) linkages

present in the F1 and F4 compounds. AglA showed high hydrolytic activity only towards ($\alpha 1 \rightarrow 6$) linkages in isomaltotriose and minor activity towards ($\alpha 1 \rightarrow 1$) linkages in trehalose.⁴² None of these two enzymes are able to degrade ($\alpha 1 \rightarrow 3$) linkages occurring in F3 3'-glc-lac and F5 3',2-glc-lac. It thus remained possible that during growth the F3 and F5 were (partially) degraded by the BAD-0971 enzyme, the third *B. adolescentis* ATCC 15703 α -glucosidase.

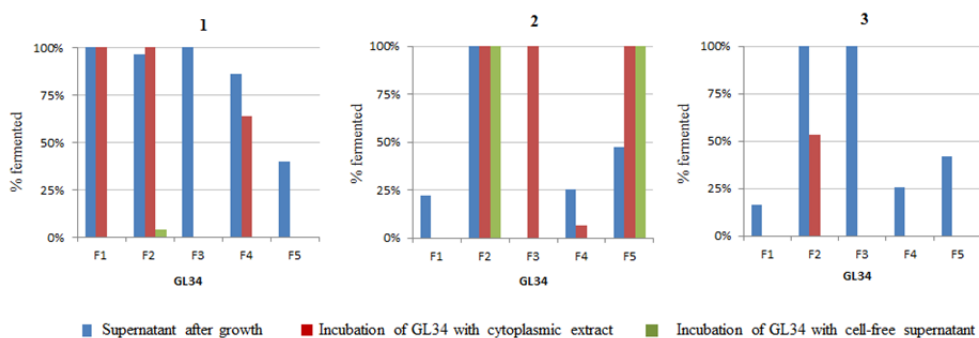


Figure 7: Consumption of GL34 compounds (%) during growth of 1) *B. adolescentis* ATCC 15703; 2) *B. longum* subsp. *infantis* ATCC 15697 and 3) *B. breve* DSM 20213, and after 24 h incubations of 5 mg mL⁻¹ GL34 with cytoplasmic extracts or cell-free supernatants (obtained after growth of these three strains on the GL34 mixture).

The BAD-0971 protein has been annotated as an α -1,4-glucosidase but has not been characterized yet.⁴³ A BLASTP analysis was carried out to compare its protein sequence with those of the characterized glucosidases of *B. adolescentis* ATCC 15703 (AglA and AglB) and *B. breve* UCC 2003 (Agl1; Agl2 and Agl3). Agl3 from *B. breve* UCC 2003, as mentioned above, has a broad hydrolytic specificity towards ($\alpha 1 \rightarrow 2$), ($\alpha 1 \rightarrow 3$), ($\alpha 1 \rightarrow 4$), ($\alpha 1 \rightarrow 5$) and ($\alpha 1 \rightarrow 6$) in kojibiose, turanose, maltose, leucrose and palatinose, respectively.⁴⁴ The two other α -glucosidases of this strain (Agl1 and Agl2) are more active in cleaving ($\alpha 1 \rightarrow 6$) linkages (in panose, isomaltose, isomaltotriose, and palatinose) and less efficient in cleaving ($\alpha 1 \rightarrow 3$) linkages in turanose and nigerose, ($\alpha 1 \rightarrow 4$) in maltulose and ($\alpha 1 \leftrightarrow 2$) in sucrose.⁴⁵ AglA and Agl3 exhibit 59 % and 73 % similarity/identity, respectively, to the BAD-

0971 protein of *B. adolescentis* (Table S3). The BAD-0971 enzyme may have a broad hydrolytic specificity similar to Agl3. However F3 3'-glc-lac and F5 3',2-glc-lac with (α 1 \rightarrow 3) linkages remained undigested in cell extracts. It cannot be excluded that the cell disruption process has weakened activity of BAD-0971 (annotated as an α -1,4-glucosidase), or other hitherto unidentified α -glucosidase enzymes.

***Bifidobacterium longum* subsp. *infantis* ATCC 15697**

The F2 2-glc-lac and F3 3'-glc-lac compounds were completely utilized during growth of *B. longum* subsp. *infantis*, and the F1 4'-glc-lac, F4 4',2-glc-lac and F5 3',2-glc-lac compounds only for 20-40% (Figure 7-2). Cell extracts only hydrolyzed F2 2-glc-lac, apparently involving (an) intracellular enzyme(s) (Figure 7-2). The F1 4'-glc-lac, F3 3'-glc-lac, F4 4',2-glc-lac and F5 3',2-glc-lac structures were not utilized by cell extracts of this strain, suggesting that it lacks α -glucosidase enzymes acting on glucose (α 1 \rightarrow 3)/(α 1 \rightarrow 4) linked to galactosyl residue. The complete use of the F3 3'-glc-lac compound during growth of *B. longum* subsp. *infantis* ATCC 15697 thus remained unexplained again. No extracellular activity of *B. longum* subsp. *infantis* toward the GL34 mixture was detected (Figure 7-2). Its limited activity on the GL34 mixture resulted in the lowest growth extent (37 %) compared to the other two bifidobacteria (Table 1). Various glucosidases have been annotated in the genome of *B. longum* subsp. *infantis* mainly with activity on (α 1 \rightarrow 4) and (α 1 \rightarrow 6) glucosidic linkages, namely α -1,4 glucosidase (locus tag BLIJ-0129); putative amylase (locus tag BLIJ-0286); putative iso-amylase (locus tag BLIJ-0286 and BLIJ-2315) and oligo-1,6-glucosidase (locus tag BLIJ-2526) (www.cazy.org/). However, BLAST analysis showed very low similarity in their protein sequence between the annotated glucosidases from this strain and Agl3 (Table S4). This most likely explains the poor growth of this strain on the GL34 mixture, and its limited intracellular activity on (α 1 \rightarrow 3)/(α 1 \rightarrow 4) glucosidic linkages in F1 4'-glc-lac, F3 3'-glc-lac, F4 4',2-glc-lac and F5 3',2-glc-lac.

***Bifidobacterium breve* DSM 20213**

In case of *B. breve*, four of the five GL34 compounds (F1 4'-glc-lac, F3 3'-glc-lac, F4 4',2-glc-lac and F5 3',2-glc-lac) (partly) remained unutilized in culture supernatants after growth (Figure 7-3). Amongst the three bifidobacteria tested only *B. breve* possessed extracellular activity on the mixture GL34: the F2 2-glc-lac and F5 3',2-glc-lac compounds were completely degraded by cell-free supernatants after 24 h incubation (Figure 7-3). The extracellular activity was also observed previously in different *B. breve* strains, however, on ($\alpha 1 \rightarrow 4$) and ($\alpha 1 \rightarrow 6$) glucosidic linkages present in starch, amylopectin and pullulan.⁴⁶ Nevertheless, cell extracts fully hydrolyzed F2 2-glc-lac, F3 3'-glc-lac and F5 3',2-glc-lac (Figure 7-3). Most likely *B. breve* is unable to transport F3 3'-glc-lac and F5 3',2-glc-lac into the cell. These data suggest that intracellular enzymes of this strain were able to cleave off the glucose units ($\alpha 1 \rightarrow 3$) linked to the galactosyl residues occurring in F3 3'-glc-lac and F5 3',2-glc-lac but not the glucose units ($\alpha 1 \rightarrow 4$) linked to the galactosyl residues in F1 4'-glc-lac and F4 4',2-glc-lac.

The putative α -glucosidases in the *B. breve* DSM 20213 genome, however, are annotated to act mainly on ($\alpha 1 \rightarrow 4$) and ($\alpha 1 \rightarrow 6$) glucosidic linkages; namely α -1,4 glucosidase (locus tag BBR-0095); putative amylase (locus tags BBR-0101; BBR-0825 and BBR-0257) and oligo-1,6-glucosidase (locus tags BBR-0484 and BBR-1863) (www.cazy.org/). This uncharacterized α -1,4 glucosidase (locus tag BBR-0095) was found to have 99 % similarity with Alg3 of *B. breve* UCC 2003 (Table S4). This annotated α -1,4 glucosidase thus also may be able to hydrolyze ($\alpha 1 \rightarrow 3$) linkage in F3 3'-glc-lac and F5 3',2-glc-lac. Structures of F1 4'-glc-lac and F4 4',2-glc-lac may be inaccessible to this α -glucosidase, or it is unable to cleave their ($\alpha 1 \rightarrow 4$) linkages, thus explaining their (very) limited degradation by *B. breve* DSM 20213.

The annotated β -galactosidases (family GH2) of these three *Bifidobacterium* strains are active on the F2 2-glc-lac compound, containing a galactosyl-moiety with a (β 1 \rightarrow 4) linkage to kojibiose. Following growth on GL34 as only carbon source, all three strains exhibited clear intracellular activity with F2 2-glc-lac. The data showed that individual *Bifidobacterium* strains have preference for degradation of glucosylated lactose compounds with (α 1 \rightarrow 3) and (α 1 \rightarrow 4) glucosidic linkages.

DISCUSSION

The GL34 compounds generally consist of a lactose molecule at the reducing end that is elongated with one or more glucose molecules involving different linkage types.²⁵ This combination of different monomers, i.e. glucose and galactose, with various linkage types ((α 1 \rightarrow 2), (α 1 \rightarrow 3), (α 1 \rightarrow 4) and (β 1 \rightarrow 4)) and different degrees of polymerization, increases the diversity of lactose-derived oligosaccharides available. The GL34 compounds were resistant to degradation by the α -amylases of porcine pancreas and *Aspergillus oryzae*. These are endo-acting enzymes (EC 3.2.1.1), degrading (α 1 \rightarrow 4) glucosidic linkages in polysaccharides such as starch or glycogen,⁴⁷ mainly yielding glucose and maltose. Also various MOS-acting enzymes were tested: The α -glucosidase enzyme of yeast used is known as an exo-acting enzyme, hydrolyzing (α 1 \rightarrow 4) glucosidic linkages but only at the terminal non-reducing (1 \rightarrow 4)-linked α -glucose residues of di- and oligosaccharides to release a single glucose unit.⁴⁸ Iso-amylase from *Pseudomonas* sp. is an (α 1 \rightarrow 4,6) debranching enzyme.⁴⁷ Pullulanase type I of *K. planticola* hydrolyzes (α 1 \rightarrow 6) glucosidic linkages in pullulan and starch.⁴⁹ Also these 3 enzymes failed to cleave any compounds in the GL34 mixture. Incubations with β -galactosidase enzymes however did result in hydrolysis, but only the F2 2-glc-lac molecule disappeared. This is explained by the ability of these enzymes to catalyze hydrolysis of β -glycosidic bonds between galactose and its organic moiety. The combined data thus

shows that the GL34 compounds are (largely) resistant to hydrolysis by these common carbohydrate degrading enzymes (Figure 1).

There is abundant clinical evidence for the important roles of *Bifidobacterium* and *Lactobacillus* species in the eco-physiology of the intestinal microbiota,^{50,51} and different strains of lactobacilli are marketed as commercial probiotics. Individual bifidobacteria are known to have specific substrate preferences.⁵² The GL34 mixture stimulated growth of bifidobacteria, but indeed to different extents. Only utilization of the F2 2-glc-lac compound has been studied previously and was shown to have a limited stimulatory effect on the growth of *B. breve* 26M2. This F2 compound did not stimulate growth of lactobacilli tested in our present study, as previously shown for *L. casei* LC-01.²⁴ However, we observed that F2 2-glc-lac stimulated growth of the probiotic bacteria *L. reuteri* 121, *B. adolescentis* ATCC 15703, *B. longum* subsp. *infantis* ATCC 15697, *B. breve* DSM 20213, and also of two commensal bacteria, *E. coli* Nissle and *B. thetaiotaomicron*, albeit to various extents. This F2 compound thus is less selective in comparison with the other compounds in the GL34 mixture. Both F1 4'-glc-lac and F4 4',2-glc-lac also stimulated growth of all three tested bifidobacteria, again to various extents. The presence of an (α 1 \rightarrow 3) linkage makes F3 3'-glc-lac more selective than F1 and F4: F3 was utilized by only two out of three studied *Bifidobacterium* strains, *B. adolescentis* ATCC 15703 and *B. breve* DSM 20213. Also F5 3',2-glc-lac with both (α 1 \rightarrow 2) and (α 1 \rightarrow 3) glucosidic linkages showed similar stimulatory effects on all three *Bifidobacterium* strains.

In conclusion, the GL34 mixture promotes growth of the tested bacteria to different extents. The bifidobacteria tested generally were better at degrading GL34 compounds than the lactobacilli and commensal bacteria. The stronger metabolic toolset of bifidobacteria in comparison with lactobacilli also has been observed when comparing their growth on human milk oligosaccharides and other prebiotic oligosaccharides as primary carbon source.^{28,44,53,54} The GL34 mixture thus showed

potential to shift microbiota composition by specifically stimulating growth of bifidobacteria, particularly *B. adolescentis*.

Four out of five compounds in this GL34 mixture exerted high and selective growth stimulatory effects towards health-beneficial probiotic bifidobacteria. The combination of monomer composition and linkage type clearly determines the fermentable properties of the GL34 compounds. Individual gut bacteria were able to utilize only specific compounds in the GL34 mixture. Synergistic activities between bacterial species thus are likely to be essential for the utilization of the whole GL34 mixture. In future work this will be studied in more detail e.g. by using faecal bacterial cultures. Only *B. adolescentis* was able to utilize almost all structures, providing a potential synbiotic combination.

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Supplemental data

Table S1: Carbohydrate acting enzymes used to analyze degradation of the GL34 compounds. The pH and temperature values used are shown.

Enzymes	Origins	Source	GH family	Reaction conditions	
				pH	Temperature °C
α -amylase 1	Porcine pancreas	Sigma-Aldrich	GH13	7.0	50
α -amylase 2	<i>Aspergillus oryzae</i>	Megazyme	GH13	5.0	50
α -glucosidase	Yeast	Megazyme	GH13	7.0	40
Iso-amylase	<i>Pseudomonas sp.</i>	Megazyme	GH13	4.0	40
Pullulanase type 1	<i>Klebsiella planticola</i>	Megazyme	GH13	5.0	40
β -galactosidase 1	<i>Aspergillus oryzae</i>	Megazyme	GH2	4.7	45
β -galactosidase 2	<i>Kluyveromyces lactis</i>	Megazyme	GH2	7.0	40

Table S2: Sequence similarity levels between Agl3 from *Bifidobacterium breve* UCC 2003 and putative α -glucosidases encoded in the *Lactobacillus acidophilus* ATCC 4356 genome.

NCBI accession number	Annotated GH family*	Query cover (%)	Identity level (%)
WP_003546237.1	GH31	35	23
WP_003548000.1	GH31	28	24
WP_003548741.1	GH31	21	23
WP_003549626.1	GH13	40	26
WP_003549917.1	GH13 GH31	28	30
WP_011254098.1	GH13 GH31	93	29
WP_011254225.1	GH13	46	26
WP_011254315.1	GH13	78	31
WP_011254601.1	GH13 GH31	96	28
WP_021721607.1	GH31	25	24

* Annotation of proteins from the genome sequence of *L. acidophilus* ATCC 4356 was carried out using dbCAN (<http://csbl.bmb.uga.edu/dbCAN>).

Table S3: Protein sequence similarity levels of BAD-0971 from *B. adolescentis* ATCC 15703 to annotated α -glucosidases in other bifidobacteria.

Protein name	Annotated or proven functions	Bacterial strain	NCBI accession number	Identity level (%)
AglA	α -1,6-glucosidase	<i>B. adolescentis</i> ATCC 15703	Query_123011	73
AglB	α -glucosidase	<i>B. adolescentis</i> ATCC 15703	Query_123011	73
Agl1	α -1,6-glucosidase	<i>B. breve</i> UCC 2003	Query_83439	30
Agl2	α -1,6-glucosidase	<i>B. breve</i> UCC 2003	Query_98509	32
Agl3	α -glucosidase	<i>B. breve</i> UCC 2003	Query_165455	59

Table S4: Similarity levels of GH13 glucosidase proteins from *B. longum* subsp. *infantis* ATCC 15697 and *B. breve* DSM 20123 to the annotated Alg3 from *B. breve* UCC 2003.

Bacterial strain	Locus tags	Annotated or proven functions	NCBI accession number	Identity level (%)
<i>B. infantis</i> ATCC 15697	BLIJ-0129	α -1,4-glucosidase	Query_130701	19
<i>B. infantis</i> ATCC 15697	BLIJ-0286	putative amylase	Query_56433	16
<i>B. infantis</i> ATCC 15697	BLIJ-1799	putative isoamylase	Query_179069	17
<i>B. infantis</i> ATCC 15697	BLIJ-2315	putative isoamylase	Query_117079	16
<i>B. infantis</i> ATCC 15697	BLIJ-2526	oligo-1,6-glucosidase	Query_140967	29
<i>B. breve</i> DSM 20123	<u>BBBR-0095</u>	<u>α-1,4-glucosidase</u>	<u>Query_5141</u>	<u>99</u>
<i>B. breve</i> DSM 20123	BBBR-0101	putative amylase	Query_163329	25
<i>B. breve</i> DSM 20123	BBBR-0257	putative amylase	Query_188975	17
<i>B. breve</i> DSM 20123	BBBR-0484	Oligo-1,6-glucosidase	Query_211369	29
<i>B. breve</i> DSM 20123	BBBR-0825	putative amylase	Query_229645	19
<i>B. breve</i> DSM 20123	BBBR-1863	Oligo-1,6-glucosidase	Query_8587	28

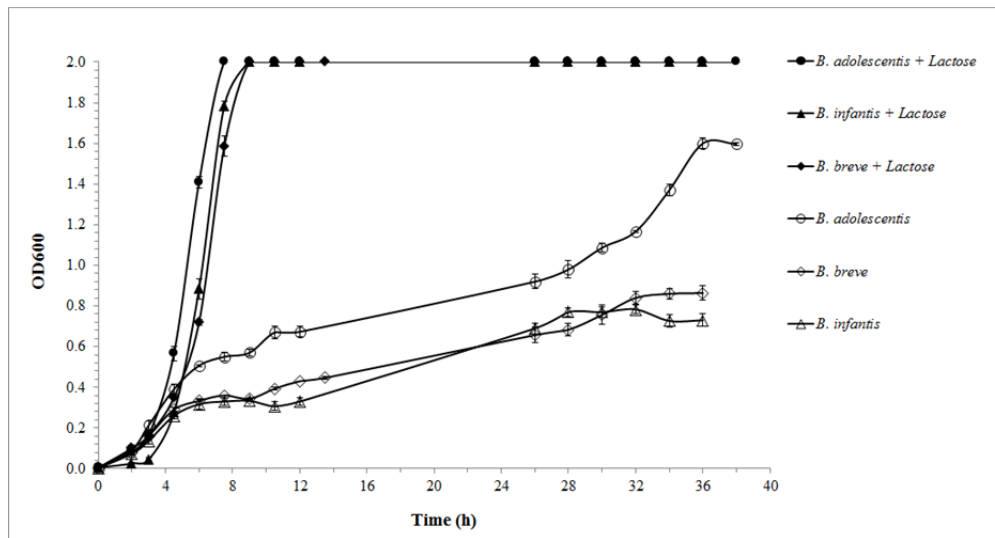


Figure S1: Growth of *B. adolescentis* ATCC 15703, *B. infantis* ATCC 15697 and *B. breve* DSM 20123 on GL34 compounds (5 mg mL^{-1}). Lactose (5 mg mL^{-1}) served as positive control, growth studies were carried out in triplicate.

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Mutational analysis of the role of the glucansucrase Gtf180- Δ N active site residues in product and linkage specificity with lactose as acceptor substrate

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ABSTRACT

The N-terminally truncated glucansucrase Gtf180 (Gtf180- Δ N) from *Lactobacillus reuteri* uses lactose as acceptor substrate to synthesize a mixture of 5 glucosylated lactose molecules (F1-F5) with a degree of polymerization (DP) of 3-4 (GL34) and with (α 1 \rightarrow 2)/(α 1 \rightarrow 3)/(α 1 \rightarrow 4)-glycosidic linkages. Mutagenesis of residues Q1140/W1065/N1029 significantly changed the GL34 F1-F5 product ratios. Q1140 mutations clearly decreased F3 3'-glc-lac with an (α 1 \rightarrow 3)-linkage and increased F4 4',2-glc-lac with (α 1 \rightarrow 4)/(α 1 \rightarrow 2)-linkages. Formation of F2 2-glc-lac with an (α 1 \rightarrow 2)-linkage and F4 was negatively affected in most W1065 and N1029 mutants, respectively. Mutant N1029G synthesized 4 new products with 1-2 additional (α 1 \rightarrow 3)-linked glucosyl moieties (2xDP4 and 2xDP5 compounds). The presence of sucrose plus lactose strongly reduced Gtf180- Δ N hydrolytic activity and increased transferase activity of Gtf180- Δ N and mutant N1029G, compared to activity with sucrose alone. N1029, W1065 and Q1140 thus are key determinants of Gtf180- Δ N linkage and product specificity in the acceptor reaction with lactose. Mutagenesis of key residues in Gtf180- Δ N thus may allow synthesis of tailor-made mixtures of lactose-derived oligosaccharides with potential applications as prebiotic compounds in food/feed, and in pharmacy/medicine.

INTRODUCTION

Glucansucrases (Gtfs) are extracellular transglycosidases found in lactic acid bacteria and belong to glycoside hydrolase family 70 (GH70).^{1,2,3} These enzymes synthesize α -glucan polymers from sucrose as acceptor and donor substrate, in a semi-processive manner.⁴ Glucansucrases have also been shown to efficiently catalyze transfer of glucose moieties from sucrose as donor substrate to numerous hydroxyl-group containing molecules, including maltose, isomaltose, catechol, primary alcohols (C₄, C₆ and C₈), and steviol based compounds.^{5,6,7,8,9,10} In case of small sugar acceptor molecules, low molecular mass oligosaccharides are synthesized differing in linkage type, size, branching degree, and physicochemical properties.^{1,3,10,11} These products are attracting strong interest for industrial applications as food or feed ingredients, and in pharmacy and medicine.^{12,13,14,15}

GH70 glucansucrases belong to the α -amylase superfamily, together with GH13 and GH77 enzymes,^{16,17} but they are much larger (~1600 - 1800 amino acid residues). An N-terminal domain of variable length and a C-terminal putative glucan-binding domain flank the central catalytic domain in these glucansucrase enzymes.¹ GH70 enzymes follow a double-displacement reaction mechanism, and possess 3 catalytic residues, D1025 (nucleophile), E1063 (acid/base) and D1136 (transition state stabilizing residue) (Gtf180- Δ N numbering). The reaction starts with cleavage of the (α 1 \rightarrow 2) bond of sucrose yielding a covalent glucosyl-enzyme intermediate. This is followed by binding of the acceptor substrate and transfer of the covalently bound glucosyl residue to the acceptor molecule, forming a new glycosidic linkage.¹⁸ The α -anomeric configuration of the donor is conserved in the product.^{19,20} The different glucansucrases characterized produce α -glucans with various types of linkages, depending on the orientation of the acceptor glucan towards the covalent glucosyl-enzyme intermediate. The glucansucrase linkage specificity, therefore, is determined by residues forming the acceptor-binding subsites.²¹ The glucansucrase binding

subsites for acceptor substrates are relatively open and involve several conserved sequence regions. Residues forming subsites +1 and +2 are crucial for acceptor recognition and orientation; their mutation resulted in altered ratios of glycosidic linkages in the synthesized α -glucans.^{22,23,24}

The Gtf180- Δ N glucansucrase from *Lactobacillus reuteri* 180 synthesizes an α -glucan with (α 1 \rightarrow 6) and (α 1 \rightarrow 3) glycosidic linkages.²⁵ Its three-dimensional structure has been elucidated,¹⁸ also with bound donor substrate (sucrose; PDB code 3HZ3) and with acceptor substrate (maltose; PDB code 3KLL), identifying amino acid residues in the donor and acceptor substrate binding subsites.¹⁸ The crystal structure of Gtf180- Δ N revealed five protein domains (A, B, C, IV and V); the active site is situated at the interface of catalytic domains A and B with a pocket-like cavity;²⁶ residues Q1140, N1411 and D1458 flanking subsite -1 prevent the presence of further donor subsites.¹⁸ The crystal structure of Gtf180- Δ N in complex with maltose revealed this acceptor substrate bound in subsites +1 and +2. At subsite +1, the highly conserved residue N1029 from domain A provides direct and indirect hydrogen bonds to the C3 and C4 hydroxyl groups at the non-reducing end of maltose.¹⁸ Residue W1065 has a hydrophobic stacking interaction with both the +1 and +2 glucosyl units of maltose, while the complex of Gtf180- Δ N D1025N with sucrose revealed a direct hydrogen bond of W1065 with the C1 hydroxyl group of the fructosyl moiety of sucrose.²¹

Our previous study showed that Gtf180- Δ N successfully catalyzes transglycosylation reactions from sucrose with lactose as the acceptor substrate.²⁷ Multiple glucose moieties were transferred to lactose to produce various glucosylated lactose derivatives. Five compounds F1-F5 of DP3-DP4 (GL34 mixture) were structurally characterized revealing (α 1 \rightarrow 2), (α 1 \rightarrow 3) and (α 1 \rightarrow 4)-linkages (Scheme 1).²⁷ Interestingly, Gtf180- Δ N introduced an (α 1 \rightarrow 2) linked Glc moiety at the reducing glucosyl unit of lactose. Such a glycosidic linkage specificity

had not been reported for this enzyme before. It remained unknown how the ($\alpha 1 \rightarrow 6$)/($\alpha 1 \rightarrow 3$) linkage specificity of Gtf180- Δ N was altered to ($\alpha 1 \rightarrow 2$)/($\alpha 1 \rightarrow 3$)/($\alpha 1 \rightarrow 4$) in the presence of lactose as an acceptor substrate. The branching sucrose enzyme Dsr-E from *Leuconostoc mesenteroides* NRRL B-1299 is one of the rare enzymes in the GH70 family that is able to introduce ($\alpha 1 \rightarrow 2$) branched linkages onto dextran backbones.^{28,29} Aiming to understand how the acceptor substrate lactose binds in the Gtf180- Δ N active site, and which amino acids are essential in binding lactose, we carried out docking experiments with lactose in a glucosyl-enzyme intermediate, using the crystal structure of *L. reuteri* 180 Gtf180- Δ N.¹⁸ Residues N1029, W1065 and Q1140 were found to be in close proximity of the acceptor substrate and may therefore be involved in the orientation of lactose in the acceptor subsite and influence the linkage type preference. To study this in more detail, mutants at these positions were biochemically characterized and evaluated for their lactose-derived product spectra, and linkage specificity. The results show that mutagenesis of key residues in Gtf180- Δ N may allow synthesis of tailor-made mixtures of lactose-derived oligosaccharides with various linkage types. Such mixtures of lactose-derived oligosaccharides have potential applications as prebiotic compounds in food and feed, and in pharmacy and medicine.

MATERIALS AND METHODS

Recombinant Gtf180- Δ N (mutants)

Mutation, expression and purification of the Gtf180- Δ N mutant enzymes used for this study has been described in detail by Meng et al.^{30,31}

Glucansucrase activity and kinetic analysis

Glucansucrase activity was quantified by measuring released glucose and fructose in the reaction with 100 mM sucrose at 37 °C in 25 mM sodium acetate buffer (pH 4.7)

with 1 mM CaCl₂, as described previously.³² Samples of 25 µL were taken at regular intervals of 1 min and glucosylation reactions were stopped immediately by addition of 5 µL 1 M NaOH. One glucansucrase activity unit (U) is defined as the amount of enzyme releasing 1 µmol of monosaccharide from sucrose per min. Release of glucose and fructose corresponds to hydrolysis activity and total activity, respectively, and the transglycosylation activity was calculated as the difference. Kinetic parameters (*V*_{max} and *K*_m) were determined using 10 different sucrose concentrations (ranging from 0.1 to 200 mM) with 200 mM lactose as acceptor substrate, and calculated by non-linear regression of the Michaelis-Menten equation with SigmaPlot. The effect of lactose concentration on initial activities of Gtf180-ΔN and mutants derived were determined using 8 different lactose concentrations (ranging from 0.1 to 150 mM) in the reactions with 150 mM sucrose at 37 °C in 25 mM sodium acetate buffer (pH 4.7) containing 1 mM CaCl₂.

Docking experiments

A model of the covalent glucosyl-enzyme intermediate of Gtf180-ΔN from *L. reuteri* strain 180 was used to dock lactose as an acceptor substrate, using the Vina-Carb of AutoDock Vina.^{18,33,34} Amino acid side chains were kept rigid. Seventy-seven poses were obtained, and these were evaluated in PyMOL (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC) based on their binding energy, proximity and orientation of the hydroxyl group with respect to the C1 atom of the covalent glucosyl moiety. The result was a collection of thirty-four poses in which the distance of the relevant lactose hydroxyl group to the C1 atom of the glucosyl-enzyme intermediate ranged between 3.0 and 4.6 Å; these poses were considered productive.

Mutant enzyme screening

Wild type and mutant Gtf180- Δ N enzymes (1 U mL^{-1} , total activity, with sucrose) were incubated with 0.5 M sucrose and 0.3 M lactose. Control incubations contained only 0.5 M sucrose with 1 U mL^{-1} of these enzymes. All reactions were performed at $37 \text{ }^\circ\text{C}$ in 25 mM sodium acetate buffer (pH 4.7) with 1 mM CaCl_2 for 24 h. The resulting reaction mixtures were subsequently subjected to HPAEC-PAD profiling for analysis.

Larger scale production and isolation of oligosaccharides

Larger scale reactions of 0.5 M sucrose and 0.3 M lactose with selected Gtf180- Δ N mutants were carried out in a volume of 100 mL with 1 U mL^{-1} (total activity) enzyme for 24 h. Two volumes of cold ethanol 20 % were added to the reaction mixtures and stored at $4 \text{ }^\circ\text{C}$ overnight to precipitate glucan polysaccharides. Full precipitation was promoted by centrifugation at 10,000 g for 10 min, the supernatant was applied to a rotatory vacuum evaporator to remove ethanol. The aqueous fraction was absorbed onto a CarboGraph SPE column (Alltech, Breda, The Netherlands) using acetonitrile : water = 1 : 3 as eluent, followed by evaporation of acetonitrile under an N_2 stream before being freeze-dried. Oligosaccharide mixtures were fractionated by HPAEC-PAD.

HPAEC-PAD analysis

Analytical scale HPAEC-PAD analyses were performed on a Dionex ICS-3000 work station (Dionex, Amsterdam, the Netherlands) equipped with an ICS-3000 pulse amperometric detection (PAD) system and a CarboPac PA-1 column (250 x 2 mm; Dionex). The analytical separation was performed at a flow rate of 0.25 mL min^{-1} using a complex gradient of effluents A (100 mM NaOH); B (600 mM NaOAc in 100 mM NaOH); C (Milli-Q water); and D: 50 mM NaOAc. The gradient started

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with 10 % A, 85 % C, and 5 % D in 25 min to 40 % A, 10 % C, and 50 % D, followed by a 35-min gradient to 75 % A, 25 % B, directly followed by 5 min washing with 100 % B and reconditioning for 7 min with 10 % A, 85 % B, and 5 % D. External standards of lactose, glucose, fructose were used to calibrate for the corresponding sugars. For determination of glucosylated lactose compounds with a degree of polymerization (DP) of 3, maltotriose was used as external standard.

Semi-preparative HPAEC-PAD samples were applied in a 4 mg mL⁻¹ concentration in 250 µL injections on an ICS-5000 system, equipped with an ICS-5000 PAD detector, using a CarboPac PA-1 column (250 x 9 mm; Dionex). Fractions were manually collected and immediately neutralized with 20% acetic acid, followed by desalting over a CarboGraph SPE column (Alltech, Breda, The Netherlands).

MALDI-TOF mass spectrometry

Molecular masses of the compounds in the reaction mixtures were determined by MALDI-TOF mass spectrometry on an AximaTM Performance mass spectrometer (Shimadzu Kratos Inc., Manchester, UK), equipped with a nitrogen laser (337 nm, 3 ns pulse width). Ion-gate cut-off was set to *m/z* 200 Da and sampling resolution was software-optimized for *m/z* 1500 Da. Samples were prepared by mixing 1 µL with 1 µL aqueous 10 mg mL⁻¹ 2,5-dihydroxybenzoic as matrix solution on the target plate.

NMR spectroscopy

The structures of oligosaccharides of interest were elucidated by 1D and 2D ¹H-¹H NMR, and 2D ¹H-¹³C NMR. A Varian Inova 500 Spectrometer (NMR center, University of Groningen) was used at probe temperatures of 25 °C. The aliquot samples were exchanged twice with 600 µL of 99.9%_{atom} D₂O (Cambridge Isotope Laboratories, Inc., Andover, MA) with intermediate freeze-drying, and then dissolved in 0.65 mL D₂O, containing internal acetone ($\delta^1\text{H}$ 2.225 ppm; $\delta^{13}\text{C}$ 31.08

ppm). In the 1D ^1H NMR experiments, the data was recorded at 16 k complex data points, and the HOD signal was suppressed using a WET1D pulse. In the 2D ^1H - ^1H NMR COSY experiments, data was recorded at 4800 Hz for both directions at 4k complex data points in 256 increments. 2D ^1H - ^1H NMR TOCSY data were recorded with 4000 Hz at 30, 60, 100, 150 and 200 ms spinlock times in 200 increments. In the 2D ^1H - ^1H NMR ROESY, spectra were recorded with 4800 Hz at a mixing time of 300 ms in 256 increments of 4000 complex data points. MestReNova 9.1.0 (Mestrelabs Research SL, Santiago de Compostela, Spain) was used to process NMR spectra, using Whittaker Smoother baseline correction.

RESULTS

Mutant Gtf180- ΔN enzymes and transglycosylation of lactose

The results of the docking experiments with lactose in a model of the glucosyl-enzyme intermediate of Gtf180- ΔN from *Lactobacillus reuteri* 180 showed that,¹⁸ within the selection of thirty-four productive poses, all three lactose transglycosylation types observed experimentally (F1-F3) were represented. In addition, some poses represented glycosylation types not identified experimentally. The highest number of poses favored ($\alpha 1 \rightarrow 3$)-elongation at the non-reducing end, followed by ($\alpha 1 \rightarrow 2$)-elongation at the reducing end, and then ($\alpha 1 \rightarrow 6$)-elongation at the reducing end (including the highest scoring pose) or ($\alpha 1 \rightarrow 4$)-elongation at the non-reducing end. The general picture emerging from the productive poses was that residues N1029, W1065 and Q1140 are in close proximity to, and are able to make hydrogen bond interactions (N1029, W1065, Q1140) or hydrophobic stacking interactions (W1065) with the acceptor substrate lactose. Figure 1 shows three poses corresponding to transglycosylation scenarios yielding compounds F1-F3.

A collection of 23 Gtf180- ΔN mutants with single amino acid residue changes at the N1029, W1065, Q1140 positions had been constructed previously,^{30,31} and was used

for analysis of transglycosylation reactions with lactose as acceptor substrate. All the reactions were carried out with 0.5 M sucrose and 0.3 M lactose, catalyzed by 1 U mL⁻¹ of the corresponding purified Gtf180-ΔN mutant or wild type enzymes for 24 h. The incubation mixtures were analyzed by HPAEC-PAD profiling for a semi-quantitative evaluation of the formation of the F1-F5 compounds. Mutations in these residues resulted in clear changes in the F1-F5 amounts synthesized. Figure 2 shows the percentages of the individual F1-F5 structures synthesized by the mutant enzymes compared to the 100 % values for the Gtf180-ΔN wild type. Each enzyme was used at 1 U mL⁻¹, but the total amount of the GL34 mixture synthesized was mostly less than by Gtf180-ΔN, except for mutants Q1140W, Q1140N and N1029T (Figure 2).

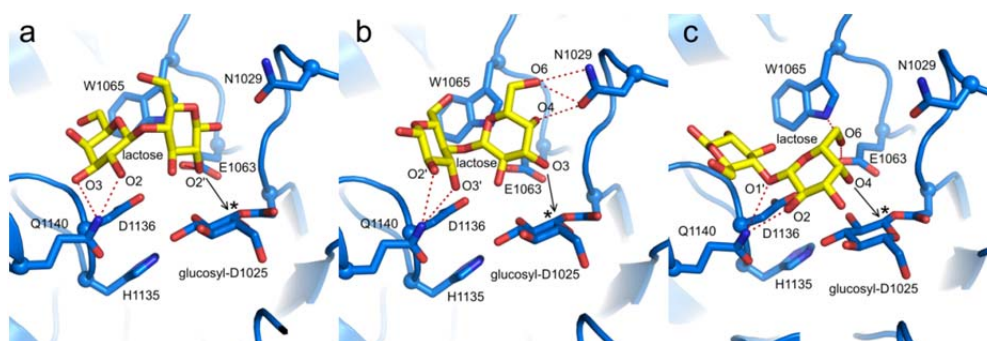


Figure 1: Views of the acceptor substrate lactose (yellow carbon atoms) docked in a glucosyl-enzyme intermediate position constructed using the crystal structure of *L. reuteri* 180 Gtf180-ΔN (PDB: 3KLL; Vujičić-Žagar et al., 2010). Three different poses are shown representing: a) (α1→2) transglycosylation at the reducing end, b) (α1→3) transglycosylation at the non-reducing end, and c) (α1→4) transglycosylation at the non-reducing end. Hydrogen bonds between amino acid residues and lactose are shown as red dotted lines; the arrow indicates the relevant hydroxyl group of lactose to attack the C1 atom of the glucosyl-enzyme intermediate (indicated with an asterisk).

Mutants of residue Q1140

HPAEC-PAD analysis of the Q1140 mutants showed a clear decrease of F3 with an (α1→3) linkage and an increase in F4 with (α1→4)/(α1→2) linkages in comparison

with the profiles of the wild type enzyme (Figure 2). Compound F5 with both ($\alpha 1 \rightarrow 2$) and ($\alpha 1 \rightarrow 3$) linkages remained relatively constant for all studied Q1140 mutants. Similarly, F1 with an ($\alpha 1 \rightarrow 4$) linked Glc was hardly affected by these mutations, except for mutants Q1140E and Q1140D, which showed a decrease in F1. Structure F2 with an ($\alpha 1 \rightarrow 2$) linked Glc was increased in mutants Q1140W and Q1140N, but was decreased in mutants Q1140H, Q1140E and Q1140D.

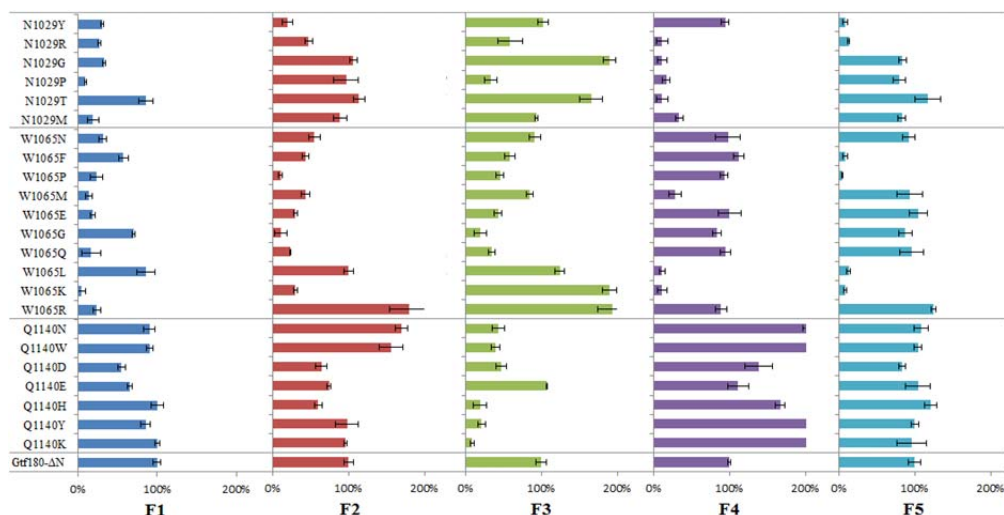


Figure 2: Effects of mutations in residues N1029, W1065 and Q1140 on the synthesis of structures F1-F5 in the GL34 mixtures, relative to wild-type Gtf180- Δ N (100%). Reactions were carried out with 0.5 M sucrose and 0.3 M lactose, catalyzed by 1 U mL⁻¹ of these enzymes at 37 °C for 24 h. The experiments were carried out in duplicate.

Mutants of residue W1065

Amino acid changes at W1065 exhibited more diverse effects on the glucosylated-lactose product profile (Figure 2). Most of the W1065 mutants displayed a decrease in all GL34 compounds. F2 with an ($\alpha 1 \rightarrow 2$) linked Glc and F4 with ($\alpha 1 \rightarrow 2$) and ($\alpha 1 \rightarrow 4$) linked Glc were most strongly affected (Figure 2). A decrease in F2 was found for most of the W1065 mutants, except for W1065L and W1065R. Three substitutions of Trp1065 with Arg, Lys and Asn resulted in a clear increase in the

amount of F3, with an (α 1 \rightarrow 3) linked Glc. In the reaction with maltose as acceptor substrate, the same mutants also synthesized increased levels of oligosaccharides with an (α 1 \rightarrow 3) linked Glc.³⁵ The positive charge of Arg and Lys may be an important determinant for favoring (α 1 \rightarrow 3) linkage formation. The amounts of F4 and F5 DP4 compounds were very minor in the profiles of mutants W1065L and W1065K (Figure 2).

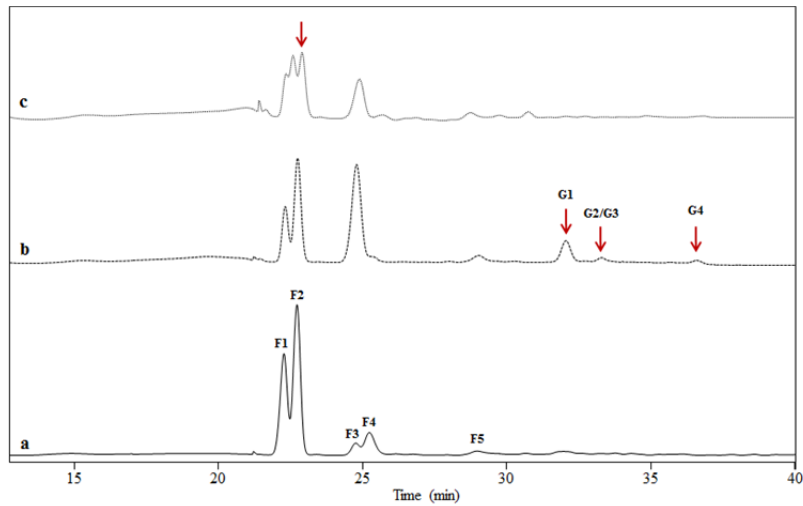


Figure 3: HPAEC-PAD profiles of lactose-derived oligosaccharide products in incubation mixtures with a) Gtf180- Δ N wild type and corresponding mutants b) N1029G and c) W1065M using 1 U mL⁻¹ (total activity) at 37 °C in 25 mM sodium acetate buffer (pH 4.7) with 1 mM CaCl₂ for 24 h. The red arrows indicate new peaks in the reaction mixture catalyzed by mutants of Gtf180- Δ N, identified as G1-G4.

Mutants of residue N1029

Mutants of N1029 showed a clear decrease in F4 with (α 1 \rightarrow 4) and (α 1 \rightarrow 2) linked Glc moieties; the most significant reduction was caused by replacing Asn1029 with Arg, Gly, Pro and Thr residues (Figure 2). Except for residue Arg, the three other amino acid residues are much smaller in size than Asn, which may result in significant changes in acceptor substrate binding by this glucansucrase. The relative

levels of F1, with an ($\alpha 1 \rightarrow 4$) linked Glc, were decreased in most of the N1029 mutants (Figure 2). Substitutions of Asn with Gly or Thr resulted in clear increases in the amount of F3, with an ($\alpha 1 \rightarrow 3$) linked Glc. N1029 mutagenesis thus has a clear effect on linkage specificity of lactose-derived oligosaccharides, with fewer ($\alpha 1 \rightarrow 4$) linkages and more ($\alpha 1 \rightarrow 3$) linkages.

Amongst all studied mutants, only the product profiles of N1029P, N1029G and W1065M with sucrose/lactose showed new peaks in comparison with that of Gtf180- Δ N wild type (Figure 3). The new peaks in the N1029P (not shown) and N1029G profiles appear at the same position in the HPAEC-PAD profile, but they were more intense in the N1029G profile. Mutant N1029G and W1065M were selected for further biochemical analysis. The new transglycosylated lactose products of N1029G were structurally characterized. The W1065M products were not characterized further.

Biochemical analysis of the glucansucrase reaction with sucrose and lactose

The N1029G and W1065M mutants were studied in comparison with Gtf180- Δ N wild type, in the reactions with lactose as acceptor substrate or with only sucrose. All tested (mutant) glucansucrases displayed Michaelis-Menten type kinetics for both the hydrolysis and transferase activities at sucrose concentrations between 0.1 and 200 mM (Figure 4). Mutations at the N1029 and W1065 positions clearly resulted in reduced activities. The K_m , k_{cat} and catalytic efficiency values were calculated accordingly (Table 1). The K_m values for sucrose in the Gtf180- Δ N wild type and the mutants N1029G and W1065M reactions with only sucrose were significantly lower than in the reactions with lactose plus sucrose. The presence of lactose molecules around the substrate binding site apparently results in a more limited access for sucrose and a reduced binding affinity of Gtf180- Δ N and its mutants for sucrose. The presence of sucrose plus lactose as acceptor substrate resulted in a strong reduction of hydrolytic activity for Gtf180- Δ N and an increase in

transferase activity for Gtf180- Δ N and mutant N1029G, compared to activity with sucrose alone. The k_{cat} value and hydrolytic efficiency k_{cat}/K_m of Gtf180- Δ N were suppressed strongly with lactose as acceptor substrate. However, the transferase efficacy k_{cat}/K_m of this enzyme with and without lactose was similar at about $52 \text{ s}^{-1} \text{ mM}^{-1}$. Analysis of the N1029G mutant showed that replacing Asn by Gly caused a clear decrease in activity (hydrolytic and total activity) in comparison with the wild type enzyme. Similar to Gtf180- Δ N wild type, the sucrose hydrolytic efficiency of this mutant is lower in the presence of lactose. Although the k_{cat} of N1029G with lactose plus sucrose is significantly higher than with only sucrose, 58.2 s^{-1} vs. 18.2 s^{-1} , the transferase efficacy k_{cat}/K_m values are not significantly different. Kinetic analysis of mutant W1065M showed that both its K_m and k_{cat} values were affected significantly, resulting in a very low catalytic efficiency (Table 1).

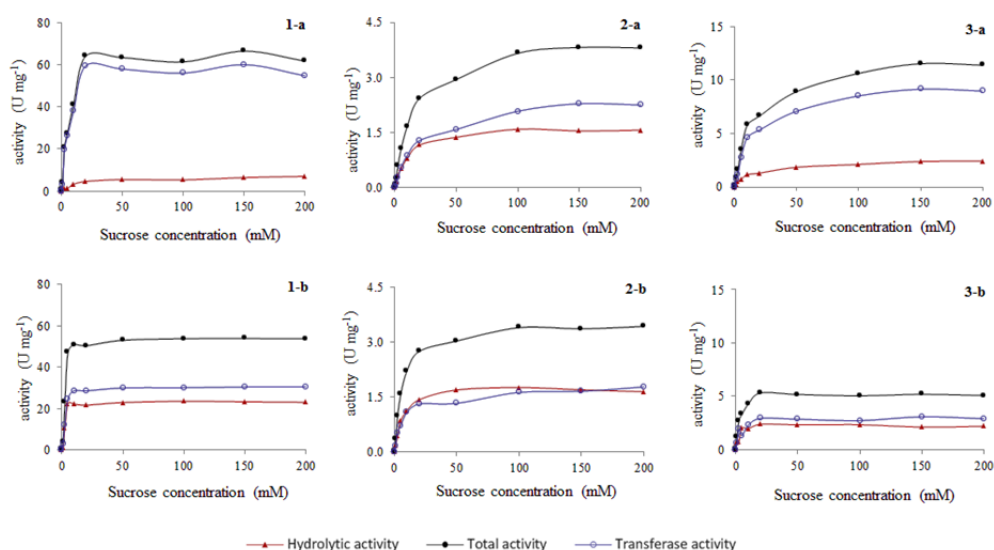


Figure 4: Influence of sucrose concentration on initial activities (hydrolytic, transferase and total activity) of different enzymes: **1)** Gtf180- Δ N; **2)** W1065M (and **3)** N1029G when using **(a)** sucrose as donor substrate and 200 mM lactose as acceptor substrate or **(b)** only sucrose as substrate; with 5 μg protein of corresponding enzymes at 37°C in 25 mM sodium acetate/1 mM CaCl_2 buffer, pH 4.7. Experiments were carried out in duplicate.

Structural characterization of transglycosylated products synthesized by Gtf180-ΔN N1029G

Mutant N1029G was used in a larger scale incubation with 0.5 M sucrose and 0.3 M lactose, followed by product fractionation by HPAEC-PAD on a semi-preparative column. Collected fractions were analyzed by MALDI-TOF mass spectrometry (data not shown). Fractions G1 and G2 each consisted of a major component with four hexose units (DP4; m/z 689). Fractions G3 and G4 each had a major component with five hexose units (DP5; m/z 851) (Figure S1). The structures of these compounds were elucidated by 1D ^1H NMR; 2D ^1H - ^1H NMR and ^1H - ^{13}C NMR.

Fraction G1

Compound G1 is composed of 4 hexose residues, namely **A**, **B** (glucosyl and galactosyl residues from original lactose, respectively) and **D** and **E** (transferred glucosyl residues from sucrose). The ^1H anomeric signals of fraction G1 were revealed by 500-MHz 1D ^1H NMR spectrum as following δ 5.447 (**A _{α}** H-1), δ 4.838 (**A _{β}** H-1), δ 4.469/4.452 (**B** H-1), δ 5.108 (**D ^{α}** H-1), δ 5.381 (**D ^{β}** H-1) and δ 5.358/5.349 (**E** H-1) (Figure 5). The splitting of the anomeric signals **B** H-1, **D** H-1 and **E** H-1 is influenced by the α/β configuration of the reducing residue **A**. All non-anomeric proton resonances were assigned by using 2D ^1H - ^1H TOCSY and 2D ^1H - ^{13}C HSQC (Table 2 and Figure S2-b). At the glucosyl residue **A**, strong downfield shifts were detected for **A _{α}** H-2 at δ 3.70 ($\Delta\delta + 0.12$ ppm); **A _{α}** C-2 at δ 79.3 ($\Delta\delta + 6.90$ ppm), **A _{β}** H-2 at δ 3.43 ($\Delta\delta + 0.14$ ppm) and **A _{β}** C-2 δ 78.9 ($\Delta\delta + 3.90$ ppm), suggesting the occurrence of substituted O-2 of this residue.³⁶ The 2D ROESY double inter-residual cross-peaks **D ^{α}** H-1/**A _{α}** H-2 and **D ^{β}** H-1/**A _{β}** H-2 confirmed this 2-substitution of residue **A** (Figure S2-c). The high anomeric resonance value of **A _{α}** H-1 at δ 5.447 ppm stems from this 2-substituted reducing α -D-Glcp unit.¹² Additionally, significant downfield shifts of **D ^{α}** H-3 at δ 3.93 ($\Delta\delta + 0.10$ ppm), **D ^{α}** C-3 at δ 80.6 ($\Delta\delta + 8.10$ ppm); **D ^{β}** H-3 at δ 3.89 ($\Delta\delta + 0.26$ ppm) and **D ^{β}** C-3 at δ

80.7 ($\Delta\delta + 5.30$ ppm) are indicative of a substitution at O-3 of this residue. This substitution is supported by the 2D ROESY inter-residual cross-peak between **E** H-1 and **D** ^{β} H-3 (Figure S2-c). The inter-residual interaction between **B** H-1 and **A** _{α} H-4 was also detected in the ROESY spectrum. Combining all data, the structure of tetrasaccharide compound G1 was determined to be α -D-Glcp-(1 \rightarrow 3)- α -D-Glcp-(1 \rightarrow 2)-[β -D-Galp-(1 \rightarrow 4)-] D-Glcp (Scheme 1).

Fraction G2

Compound G2 is composed of 4 hexose residues, including **A**, **B** (glucosyl and galactosyl residues from original lactose, respectively) and **C** and **E** (transferred glucosyl residues from sucrose). The ¹H NMR spectrum showed five anomeric signals at δ 5.225 (**A** _{α} H-1), δ 4.670 (**A** _{β} H-1), δ 4.511 (**B** H-1), δ 5.114 (**C** H-1) and δ 5.361 (**E** H-1) (Figure 5). Assignments of non-anomeric resonances of these residues were obtained by 2D ¹H-¹H TOCSY measurements (Table 2 and Figure S3-b). The chemical shifts of residue **A**, **B** and **C** of this fraction were found to be highly similar to those values of fraction F3 from the GL34 mixture.²⁷ The ROESY inter-residual cross peaks **C** H-1/**B** H-3 verified O-3 substitution occurring at residue **B** (Figure S3-c). Moreover, at residue **C**, the chemical shift of H-3 was shifted strongly to δ 3.93 ($\Delta\delta + 0.13$ ppm) in reference to that of the same residue from compound F3,²⁷ indicating the substitution at O-3 of this residue. This speculation was confirmed by the 2D ROESY inter-residual cross-peak between **E** H-1 and **C** H-3 (Figure S2-c). The 3-substitution at residue **B** and **C** has a strong influence on the chemical shift values residue **B** H-4 at δ 4.18 ($\Delta\delta + 0.26$ ppm). The ROESY spectrum also showed inter-residual correlations between **C** H-1 and **B** H-3 and between **B** H-1 and **A** H-4 (Figure S3-c). These data resulted in the identification of compound G2 as a tetrasaccharide with the structure α -D-Glcp-(1 \rightarrow 3)- α -D-Glcp-(1 \rightarrow 3)- β -D-Galp-(1 \rightarrow 4)- α -D-Glcp (Scheme 1).

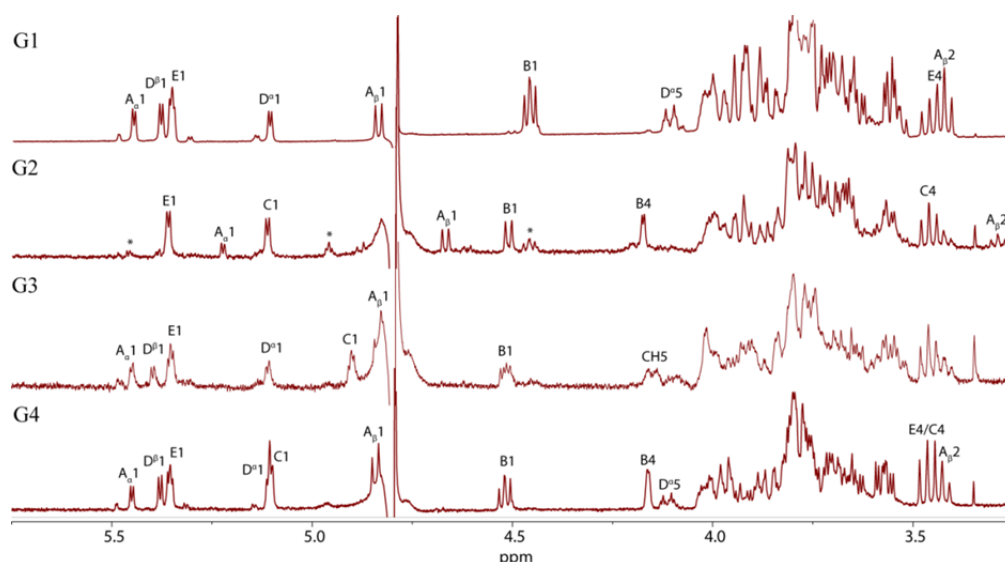


Figure 5: 500-MHz 1D ^1H NMR spectra of G1-G4 fractions from the reaction mixture with Gtf180- ΔN N1029G (see Figure S1), recorded at 25 °C in D_2O . Anomeric signals of each fraction were labelled according to the legends of corresponding structures indicated in Scheme 1.

Fraction G3

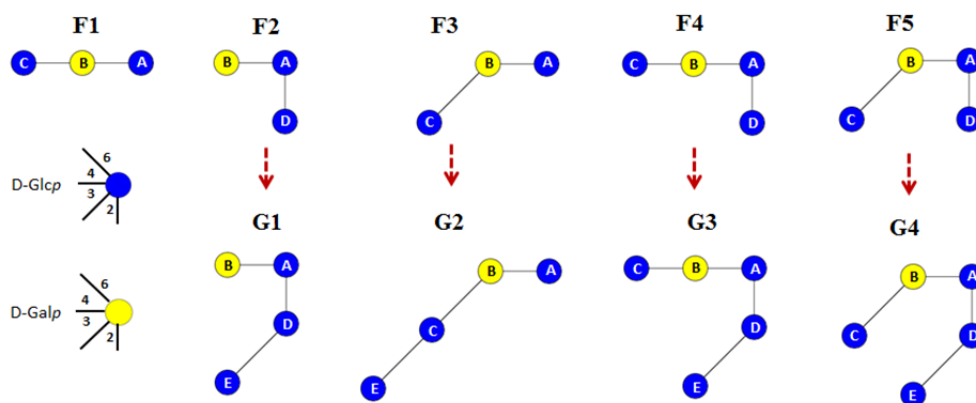
Compound G3 consists of five hexose residues: **A**, **B** (glucosyl and galactosyl residues from original lactose, respectively) and **C**, **D** and **E** (transferred glucosyl residues from sucrose). Seven anomeric signals of this compound were detected by 1D ^1H NMR to be at δ 5.450 (**A α** H-1), δ 4.828 (**A β** H-1), δ 4.525/4.508 (**B** H-1), δ 4.907/4.896 (**C** H-1), δ 5.110 (**D α** H-1), δ 5.399 (**D β** H-1) and δ 5.356/5.347 (**E** H-1) (Figure 5). Three other anomeric signals from this fraction G3 were also detected, marked * in the 1D ^1H NMR profile, they were too minor to be identified (Figure S4-a). The splitting of the anomeric signals **B** H-1, **D** H-1 and **E** H-1 was due to the influence of the α/β configuration of reduction residue **A**. All non-anomeric proton resonances were assigned by using 2D ^1H - ^1H TOCSY (Table 2 and Figure S4-b). The set of ^1H and ^{13}C chemical shifts of residue **A**, **B** and **C** correspond to the values of these residues occurring in compound F4 of the GL34 mixture.²⁷ The 2D ROESY

doubled inter-residual cross-peaks **C** H-1/**B** H-4 together with a strong downfield of residue **B** H-4 at δ 4.02 ($\Delta\delta + 0.10$ ppm) indicates the occurrence of an O-4 substitution at the residue **B** (compare structure F1 in Pham et al 2017).²⁷ Strong downfield shifts of the residue **A** with **A _{α}** H-2 at δ 3.69 ($\Delta\delta + 0.11$ ppm) and **A _{β}** H-2 at δ 3.42 ($\Delta\delta + 0.13$ ppm) were found as indications of substituted O-2 at this residue. This 2-substitution of residue **A** was verified by the 2D ROESY doubled inter-residual cross-peaks **D ^{β}** H-1/**A _{β}** H-2 (Figure S4-c). The resonances of residue **D ^{α}** H-3 and **D ^{β}** H-3 were shifted significantly to δ 3.94 ($\Delta\delta + 0.14$ ppm) and δ 3.90 ($\Delta\delta + 0.15$ ppm), respectively, compared to residue **D** of compound F4,²⁶ reflecting a $-(1\rightarrow3)-\alpha$ -D-Glcp- unit. The 2D ROESY inter-residual cross-peak between **E** H-1 and **D ^{β}** /**D ^{α}** H-3 supported this O-3 substitution (Figure S4-c). These data lead to the structure of this compound G3 as α -D-Glcp-(1 \rightarrow 3)- α -D-Glcp-(1 \rightarrow 2)-[Glcp-(1 \rightarrow 4)- β -D-Galp-(1 \rightarrow 4)-]D-Glcp (Scheme 1).

Fraction G4

Compound G4 consists of 5 hexose residues, namely **A**, **B** (glucosyl and galactosyl residues from original lactose, respectively) and **C**, **D** and **E** (transferred glucosyl residues from sucrose). The 1D ¹H NMR spectrum of fraction G4 showed seven anomeric signals at δ 5.450 (**A _{α}** H-1), δ 4.843 (**A _{β}** H-1), δ 4.527/4.512 (**B** H-1), δ 5.111/5.103 (**C** H-1), δ 5.102 (**D ^{α}** H-1), δ 5.379 (**D ^{β}** H-1) and δ 5.358/5.350 (**E** H-1,) (Figure 5). The splitting of the anomeric signal **B** H-1, **C** H-1, **D** H-1 and **E** H-1 is caused by α/β configuration of the reduction residue **A**. The other non-anomeric proton resonances were determined by 2D ¹H-¹H TOCSY and the carbon chemical shifts were correlated to ¹³C in the 2D ¹H-¹³C HSQC spectrum (Table 2 and Figure S5-c). The set of ¹H and ¹³C chemical shifts of residue **A**, **B** and **C** matched very well with the values of these residues in compound F5 of the GL34 mixture, although with slight shifts,²⁷ suggesting the occurrence of a 3-substituted galactosyl residue and 2-substituted glucosyl residue. The strong downfield shifts of residue **B** H-3 at 3.77 ($\Delta\delta + 0.11$ ppm), C-3 at 78.6 ($\Delta\delta + 4.9$ ppm) and the ROESY inter-

residual cross peaks **C** H-1/**B** H-3 verifies the α -D-Glcp-(1 \rightarrow 3)- linkage (Figure S5-c). Strong downfield shifts of residue **A** with **A _{α}** H-2 at δ 3.71 ($\Delta\delta + 0.13$ ppm); **A _{α}** C-2 at δ 79.6 ($\Delta\delta + 6.93$ ppm), **A _{β}** H-2 at δ 3.43 ($\Delta\delta + 0.14$ ppm) and **A _{β}** C-2 δ 79.0 ($\Delta\delta + 3.91$ ppm) were detected as indications for a substituted O-2 of residue **A**. This 2-substitution of residue **A** is confirmed by the 2D ROESY doubled inter-residual cross-peaks **D ^{β}** H-1/**A _{β}** H-2 and **D ^{α}** H-1/**A _{α}** H-2 (Figure S5-c). The resonances of residue **D ^{α}** H-3 and **D ^{β}** H-3 were revealed to shift significantly to be at δ 3.93 ($\Delta\delta + 0.13$ ppm) and δ 3.89 ($\Delta\delta + 0.14$ ppm), respectively, comparing to that residue of compound F5,²⁷ indicating the occurrence of O-3 substitution at residue **D**. This substitution is supported by the 2D ROESY inter-residual cross-peak between **E** H-1 and **D ^{β}** /**D ^{α}** H-3. These data determined the structure of G4 to be α -D-Glcp-(1 \rightarrow 3)- α -D-Glcp-(1 \rightarrow 2)-[α -D-Glcp-(1 \rightarrow 3)- β -D-Galp-(1 \rightarrow 4)-]D-Glcp (Scheme 1).



Scheme 1: Structures of F1-F5 and G1-G4. Red arrows reflect possible elongation of the corresponding compounds from the mixture GL34, F2-F5, to form G1-G4.

DISCUSSION AND CONCLUSIONS

Residues surrounding the glucanucrase active site have been subjected to several mutagenesis studies aiming to identify the structural determinants of product size and linkage specificity in these enzymes.^{21,23,30,31,37-39} The three residues (N1029,

W1065 and Q1140) targeted in this work have been studied before, but in reactions with different acceptor substrates. Notably, all three residues are fully conserved within glucansucrases, and they play an important role in the transglycosylation reaction. Residue N1029 in domain A of Gtf180- Δ N was previously shown to be critical for linkage specificity and activity. Meng et al reported the essential role of N1029 in acceptor substrate binding by Gtf180- Δ N.³⁰ Regarding α -glucan synthesis from sucrose, N1029 mutants tended to decrease the transglycosylation/hydrolysis ratio and to increase the relative amount of (α 1 \rightarrow 3) linkages in the products. This was attributed to the fact that N1029 is involved in the hydrogen bond network to bound acceptor substrates.^{18,30} In addition, residue W1065 appeared to be critical for the activity of this enzyme.³¹ The stacking interactions of this aromatic residue with acceptor substrates bound at subsites +1 and +2 were shown to be required for polysaccharide synthesis.³¹ Mutation of this W1065 also affected linkage specificity in polysaccharide and oligosaccharide synthesis in reactions using sucrose or sucrose (donor) plus maltose (acceptor) as substrates.^{18,21,31} Finally, the importance of residue Q1140 was shown in a study where Gtf180- Δ N was used to transglycosylate steviol glycosides.¹⁵

Also with lactose as an acceptor substrate, mutation of these amino acid residues (N1129, W1065 and Q1140) showed clear effects on the activity as well as the linkage specificity of Gtf180- Δ N. First, replacement of N1029 with Gly or Thr facilitated these Gtf180- Δ N mutants to synthesize (α 1 \rightarrow 3) glucosylated lactose derivatives (Figure 2). This resulted in a strong reduction in the synthesis of (α 1 \rightarrow 4) glucosidic linkages-containing compounds (F1 and F4) by mutant N1029G compared to the wild-type enzyme (Figure 2). Similar effects were observed in studies where maltose was used as acceptor substrate,³⁵ or even when non-carbohydrate compounds were used as acceptor substrate.⁶ The crystal structure of GTF180- Δ N in complex with maltose revealed that N1029 is involved in a hydrogen bond network with the non-reducing end glucosyl moiety of maltose in subsite +1,

making direct and indirect hydrogen bonds with its C3 and C4 hydroxyl groups.¹⁸ Mutant N1029G, when acting on lactose as acceptor substrate, added an (α 1 \rightarrow 3) linked Glc moiety to compounds F2-F5 of the GL34 mixture to synthesize G1-G4 (Scheme 1). However, at the incubation conditions tested, the amounts of the novel compounds G1-G4 were relatively low in comparison with F1-F5; optimization of these reactions clearly is required in order to increase the yield of this (α 1 \rightarrow 3) linkage containing lactose-derivative mixture.

Second, with most of the W1065 mutants we observed a decrease in the amounts of F1-F5 synthesized (Figure 2). This may be explained by a loss of the aromatic stacking interaction with the acceptor substrate lactose, similar to what has been observed in studies using sucrose or sucrose plus maltose.³¹ It is interesting to note that a wild-type glucansucrase is able to catalyze linear (α 1 \rightarrow 2)-glycosylation, albeit with a ‘non-natural’ acceptor substrate. The related and highly homologous branching sucrases also synthesize (α 1 \rightarrow 2) linkages, but only with dextran as acceptor substrate, and with a non-aromatic residue replacing the tryptophan at position 1065.^{18,29,40} Moreover, they only form (α 1 \rightarrow 2) branch points instead of linear (α 1 \rightarrow 2) linkages. W1065 mutants still synthesized products with (α 1 \rightarrow 2) linkages (F2, F4) although the amounts were decreased. Mutant W1065M was almost completely inactive, which is in agreement with an earlier study.³¹ Together, our results show that W1065 is essential for the transglycosylation of lactose.

Third, Q1140 mutant enzymes showed minor changes in their GL34 mixture profiles. Only compound F3 with an (α 1 \rightarrow 3) linked Glc moiety was clearly reduced. Residue Q1140, together with residues N1411 and D1458, lines the pocket-shaped cavity of Gtf180 in which the glucosyl moiety of sucrose binds (subsite -1), but is also near subsite +2.¹⁸ Mutations of Q1140 change the surface shape and/or local charge and thereby may affect the affinity for and/or orientation of bound acceptor molecules, explaining the observed changes in linkage specificity.

The results of the docking experiment of lactose in Gtf180- Δ N do not fully explain the transglycosylation types and the experimentally observed preferences, but do provide insights in how lactose may bind at acceptor binding subsites +1 and +2. Reflecting the variety in linkage types observed experimentally for lactose transglycosylation by wild-type Gtf180- Δ N, the set of obtained poses showed a large variation of orientations, which may be related to the fact that the acceptor binding region of Gtf180- Δ N is quite wide and open. The observation that product profiles of N1029, W1065 and Q1140 mutants are affected (with respect to wild-type) agrees with the fact that in the docking results, lactose interacts with these residues when bound for transglycosylation. Further insights in the linkage specificity determinants of Gtf180- Δ N acting on lactose as acceptor substrate may be provided by a crystal structure of Gtf180- Δ N in complex with lactose, but attempts to obtain such a complex have not been successful so far.

Recently we reported that the GL34 mixture synthesized by Gtf180- Δ N shows potential to shift microbiota composition: it specifically stimulated growth of bifidobacteria, particularly *B. adolescentis*.⁴¹ This novel GL34 oligosaccharide mixture, synthesized from cheap and abundantly available lactose and sucrose, thus (potentially) has synbiotic properties toward *B. adolescentis*. Glucansucrases are highly interesting glucosylating enzymes that are relatively easy to produce, highly active with sucrose as donor substrate, and with promising conversion degrees. Optimization of their *trans*-glucosylation reactions with galactose-containing compounds as acceptor substrates is needed to obtain higher yields of transfer products for further functional studies.

The current study identified three residues (N1029, W1065, Q1140) that likely play a role in determining linkage specificity regarding lactose transglycosylation. The fact that all three residues are fully conserved in glucansucrases, underpins their importance and expands the possibilities for understanding the synthesis of lactose-

derived oligosaccharides by glucansucrases (and their mutants). Ultimately, further tailoring and optimization of this synthesis can lead to desired products for further applications.

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Table 1: Kinetic analysis of the activities of Gtf180-ΔN and mutants derived using only sucrose or both sucrose and lactose as substrates.

Enzyme	Substrates	Hydrolytic activity			Transferase activity			Total activity		
		K_m mM	k_{cat} s^{-1}	k_{cat}/K_m $s^{-1} mM^{-1}$	K_m mM	k_{cat} s^{-1}	k_{cat}/K_m $s^{-1} mM^{-1}$	K_m mM	k_{cat} s^{-1}	k_{cat}/K_m $s^{-1} mM^{-1}$
Gtf180-ΔN	Lactose & Sucrose	13.3 ± 1.1	32.9 ± 0.2	2.5	5.6 ± 0.2	294.8 ± 3.2	52.6	6.0 ± 0.4	326.2 ± 4.2	54.4
	Sucrose	2.5 ± 0.3	118.1 ± 1.3	47.2	2.9 ± 0.2	153.3 ± 3.8	52.9	2.7 ± 0.2	271.4 ± 3.1	100.5
N1029G	Lactose & Sucrose	13.5 ± 0.3	15.2 ± 0.5	1.1	13.6 ± 0.1	58.2 ± 2.4	4.3	13.7 ± 1.3	73.3 ± 2.5	5.4
	Sucrose	2.6 ± 0.2	14.2 ± 0.6	5.6	3.0 ± 0.1	18.2 ± 1.8	6.1	2.7 ± 0.0	32.7 ± 1.2	12.1
W1065M	Lactose & Sucrose	10.4 ± 0.9	3.7 ± 0.6	0.4	19.3 ± 0.4	5.4 ± 0.3	0.3	15.1 ± 1.1	8.8 ± 0.4	0.6
	Sucrose	6.0 ± 0.7	3.9 ± 0.3	0.6	6.9 ± 0.8	3.7 ± 0.9	0.5	6.2 ± 0.6	7.5 ± 0.5	1.2

K_m values calculated for sucroses. Experiments were carried out in duplicate.

Table 2: ^1H and ^{13}C chemical shifts of the glucosylated lactose derivatives, measured at 25 °C in D_2O . Chemical shifts that are key in the structural determination are underlined.

	Lac		G1		G2		G3		G4	
	^1H	^{13}C	^1H	^{13}C	^1H	^{13}C	^1H	^{13}C	^1H	^{13}C
A _α 1	5.222	92.8	5.447	90.0	5.225	5.450	5.450	89.9		
A _α 2	3.58	72.4	<u>3.70</u>	<u>79.3</u>	3.59	<u>3.69</u>	<u>3.71</u>	<u>79.6</u>		
A _α 3	3.83	72.5	3.97	70.9	3.85	3.95	3.97	72.8		
A _α 4	3.66	79.8	<u>3.71</u>	<u>79.3</u>	<u>3.68</u>	3.70	<u>3.74</u>	<u>80.5</u>		
A _α 5	3.95	71.2	4.01	72.5	3.97	4.05	4.06	70.6		
A _α 6a	3.87	61.5	3.89	60.8			3.88	61.2		
A _α 6b	3.84									
A _β 1	4.662	96.9	4.838	96.7	4.670	4.828	4.843	97.0		
A _β 2	3.287	<u>75.0</u>	<u>3.43</u>	<u>78.9</u>	3.30	<u>3.42</u>	<u>3.43</u>	<u>79.0</u>		
A _β 3	3.63	75.4	3.73	76.3	3.63	3.74	3.76	73.8		
A _β 4	3.66	79.8	<u>3.68</u>	<u>79.3</u>	<u>3.66</u>	<u>3.68</u>	<u>3.69</u>	<u>79.5</u>		
A _β 5	3.60	75.8	3.60	75.3	3.61	3.60	3.61	75.5		
A _β 6a	3.95	61.6	3.96	60.9	3.96	3.83	3.94	61.2		
A _β 6b	3.80		3.80	60.9	3.81	3.97	3.80			
B1	4.447	104.4	4.469/4.452	103.6	4.511	4.525/4.508	4.527/4.512	104.1		
B2	3.54	72.3	3.55	72.0	3.68	3.59	3.70	70.8		
B3	3.66	73.7	3.67	72.3	<u>3.78</u>	3.76	<u>3.77</u>	<u>78.6</u>		
B4	3.92	69.8	3.92	69.5	<u>4.18</u>	<u>4.02</u>	<u>4.16</u>	66.0		
B5	3.72	76.4	3.94		3.72	3.68	3.69	75.9		
B6a	3.80	62.2	3.76	61.6	3.80	3.80	3.80	62.0		
B6b	3.75		3.71	61.6		3.70	3.74			
C1					5.114	4.907/4.896	5.111/5.103	96.7		
C2					3.68	3.54	3.58	72.3		
C3					<u>3.93</u>	3.75	3.78	74.1		
C4					3.66	3.47	3.46	70.2		
C5					4.00	<u>4.16</u>	4.02	72.7		
C6a					3.81	3.80	3.84	61.2		
C6b					3.78		3.76			
D ^α 1			5.108	97.2		5.110	5.102	96.7		
D ^α 2			3.64	75.1		3.65	3.59	74.5		
D ^α 3			<u>3.93</u>	<u>80.6</u>		<u>3.94</u>	<u>3.93</u>	<u>80.6</u>		
D ^α 4			3.49	70.4		3.48	3.46	70.3		
D ^α 5			<u>4.02</u>	72.4		4.00	3.96	72.6		
D ^α 6a			3.87	60.9		3.80	3.80	61.2		
D ^α 6b			3.80	60.9			3.76			
D ^β 1			5.381	98.8		5.399	5.379	98.6		
D ^β 2			3.53	72.1		3.64	3.64	71.0		
D ^β 3			<u>3.79</u>	<u>80.7</u>		<u>3.90</u>	<u>3.89</u>	<u>80.9</u>		
D ^β 4			3.47	70.3		3.46	3.47	70.2		
D ^β 5			<u>4.11</u>	72.1		<u>4.10</u>	<u>4.11</u>	72.1		
D ^β 6a			3.91	60.9		3.81	3.80	61.2		
D ^β 6b			3.80	60.9						
E1			5.358/5.349	100.0	5.361	5.356/5.347	5.358/5.350	100.2		
E2			3.54	72.1	3.57	3.57	3.57	72.4		

Chapter 4

E3			3.75	73.9	3.75	3.75	3.76	73.9
E4			3.45	70.2	3.48	3.45	3.44	70.4
E5			<u>4.02</u>	72.6	<u>4.02</u>	<u>4.02</u>	<u>4.02</u>	72.7
E6a			3.85	61.0	3.87	3.83	3.82	61.2
E6b			3.80	61.5	3.80			

Supplemental data

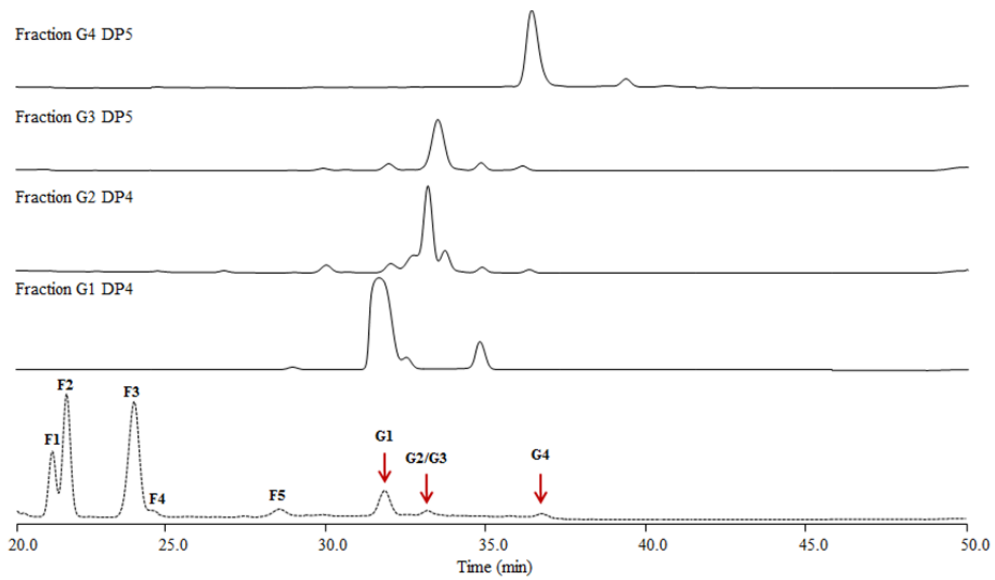


Figure S1. HPAEC-PAD profiles of fractions from the reaction mixture with mutant Gtf180- Δ N N1029G. Degree of polymerization (DP) is labelled on the corresponding fractions.

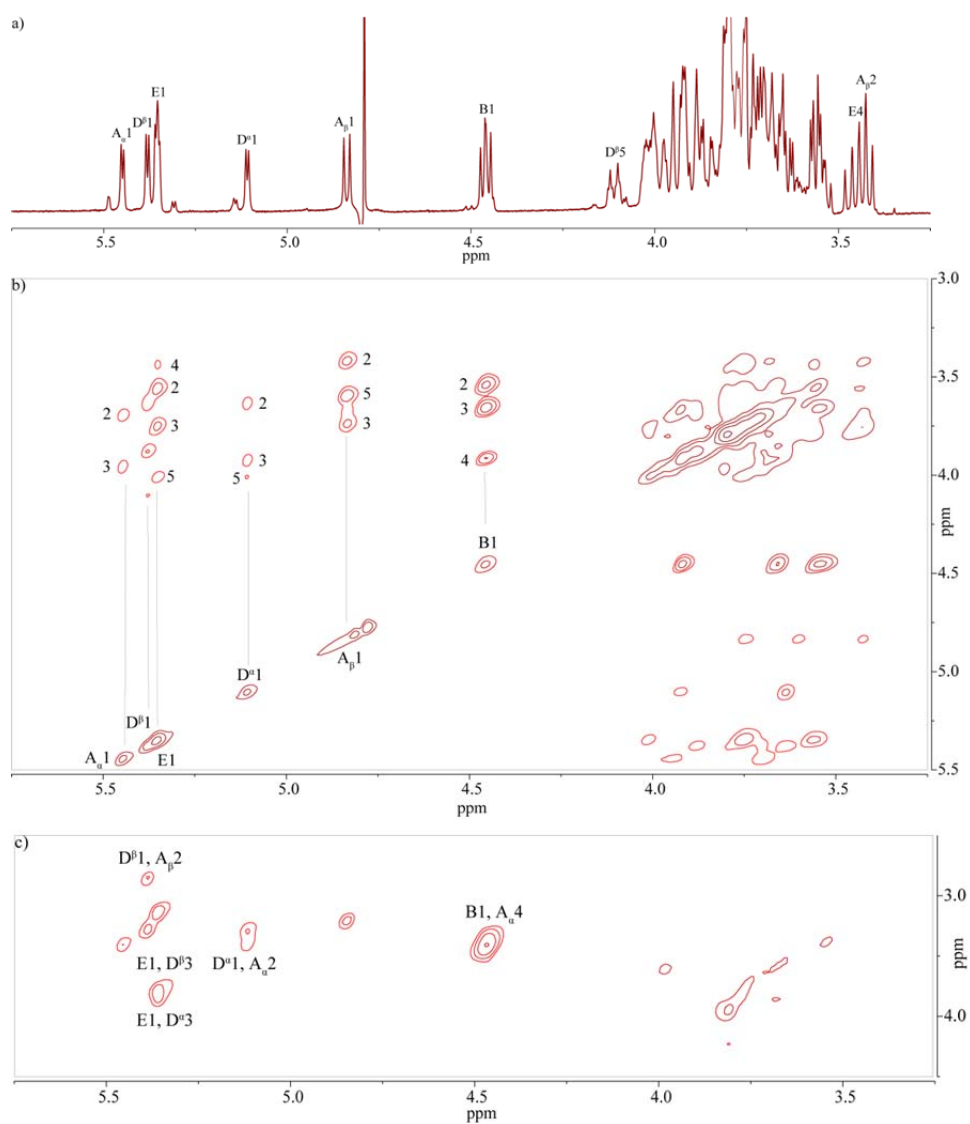


Figure S2: a) 500-MHz 1D ^1H NMR spectrum; b) 2D ^1H - ^1H TOCSY spectrum (mixing time 150 ms); and c) 2D ^1H - ^1H ROESY spectrum (mixing time 300 ms) of fraction G1, recorded at 25 °C in D_2O . In the TOCSY spectrum, anomeric signals are displayed on the diagonal; intra-residual correlations between well-resolved anomeric and ring-proton resonances are marked by numbers. In the ROESY spectrum, inter-residual couplings are indicated.

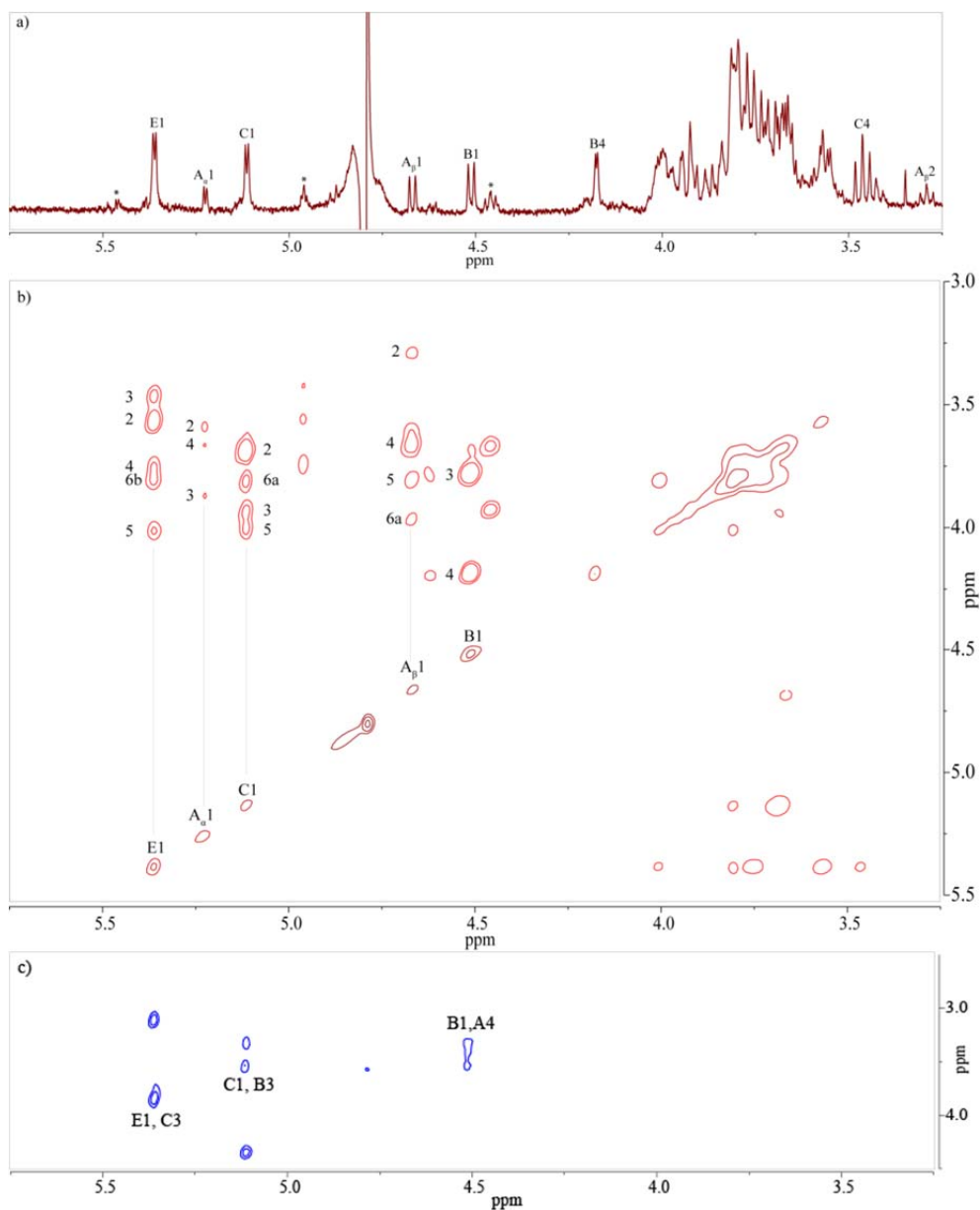


Figure S3: a) 500-MHz 1D ^1H NMR spectrum; b) 2D ^1H - ^1H TOCSY spectrum (mixing time 150 ms); and c) 2D ^1H - ^1H ROESY spectrum (mixing time 300 ms) of fraction G2, recorded at 25 °C in D_2O . In the TOCSY spectrum, anomeric signals are displayed on the diagonal; intra-residual correlations between well-resolved anomeric and ring-proton resonances are marked by numbers. In the ROESY spectrum, inter-residual couplings are indicated.

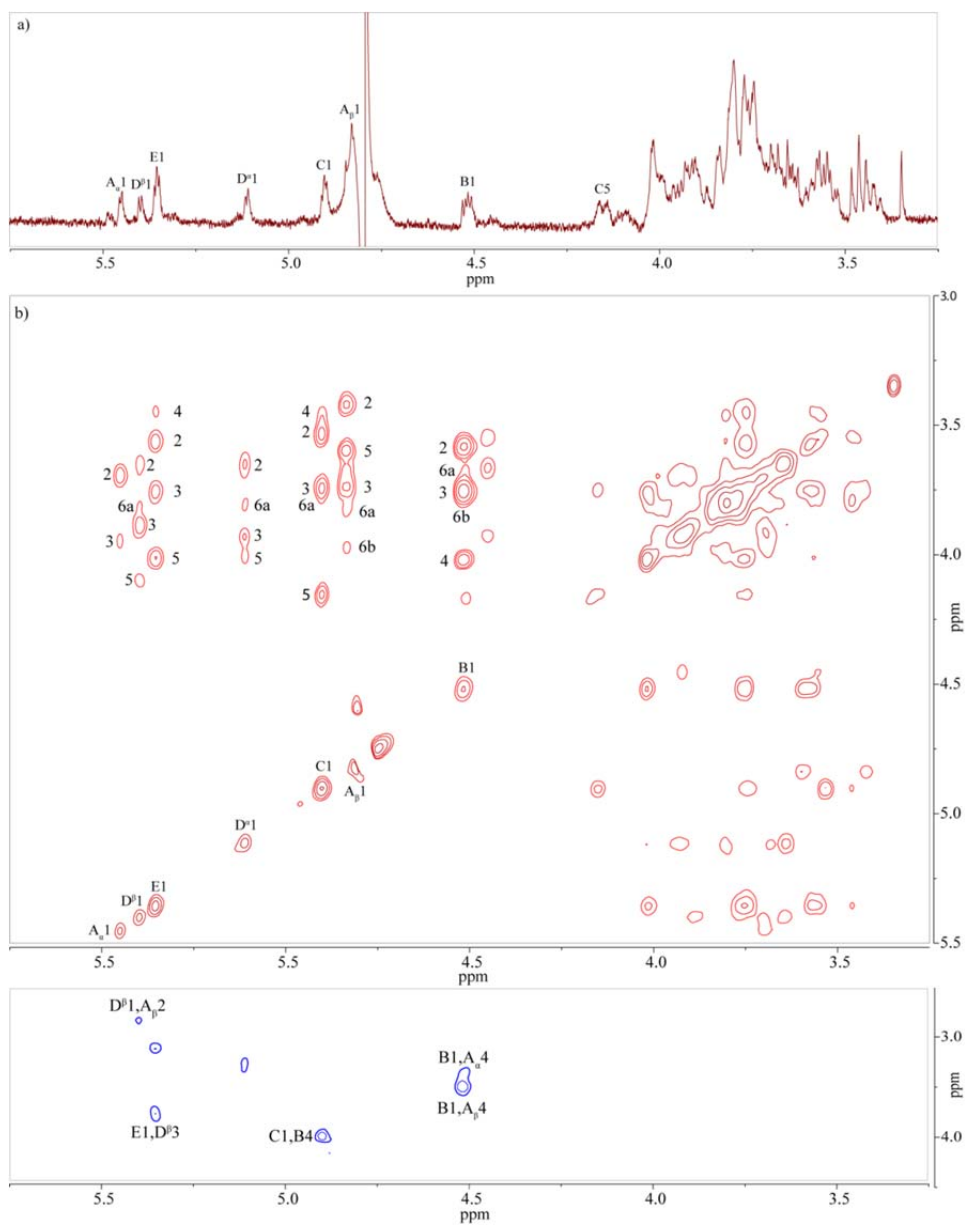


Figure S4: a) 500-MHz 1D ^1H NMR spectrum; b) 2D ^1H - ^1H TOCSY spectrum (mixing time 150 ms); and c) 2D ^1H - ^1H ROESY spectrum (mixing time 300 ms) of fraction G3, recorded at 25 °C in D_2O . In the TOCSY spectrum, anomeric signals are displayed on the diagonal; intra-residual correlations between well-resolved anomeric and ring-proton resonances are marked by numbers. In the ROESY spectrum, inter-residual couplings are indicated.

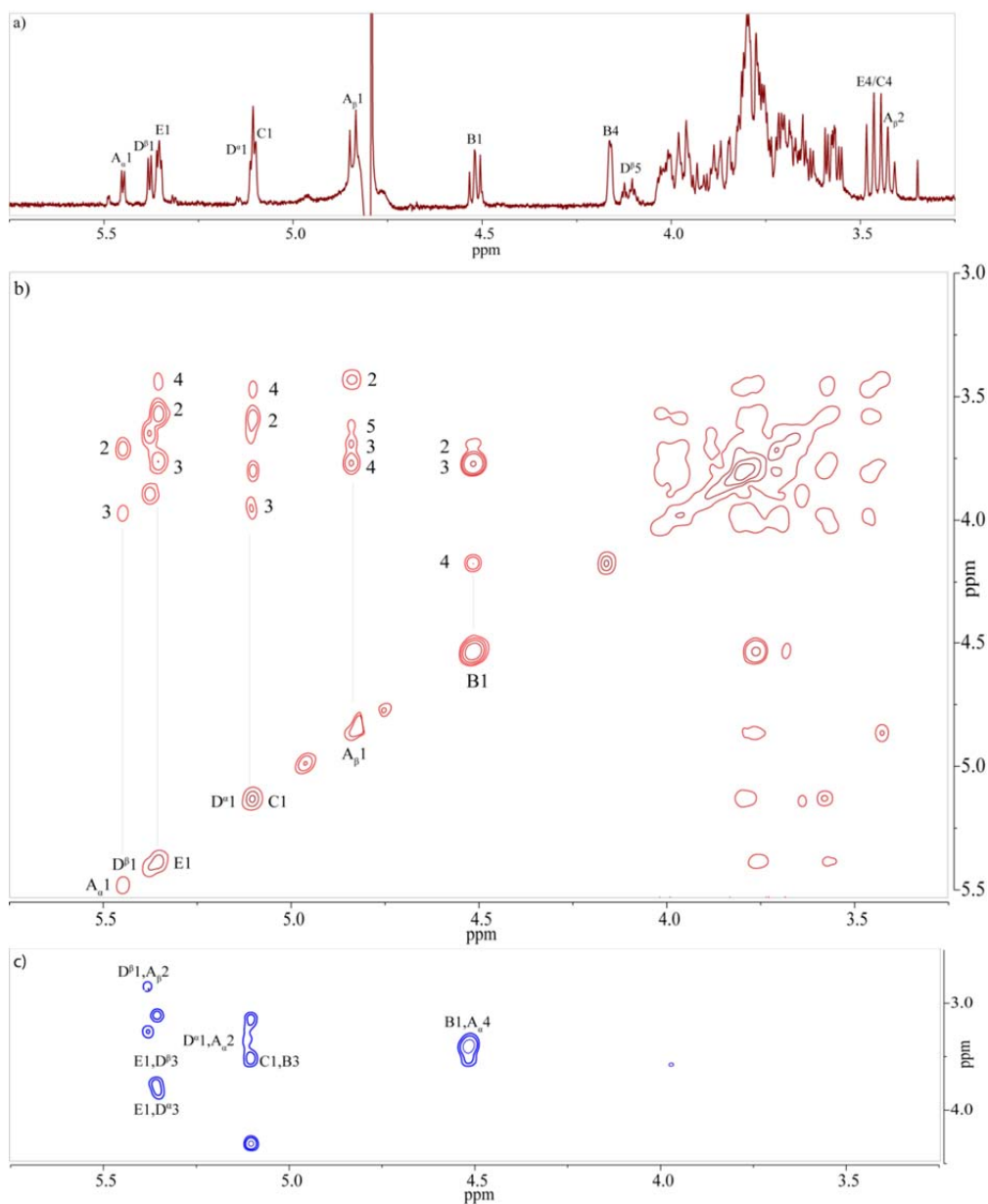


Figure S5: a) 500-MHz 1D ^1H NMR spectrum; b) 2D ^1H - ^1H TOCSY spectrum (mixing time 150 ms); and c) 2D ^1H - ^1H ROESY spectrum (mixing time 300 ms) of fraction G4, recorded at 25 °C in D_2O . In the TOCSY spectrum, anomeric signals are displayed on the diagonal; intra-residual correlations between well-resolved anomeric and ring-proton resonances are marked by numbers. In the ROESY spectrum, inter-residual couplings are indicated.

Chapter 5

Structural characterization of glucosylated GOS derivatives synthesized by the *Lactobacillus reuteri* GtfA and Gtf180 glucansucrase enzymes

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ABSTRACT

β -Galacto-oligosaccharides (GOS) are used commercially in infant nutrition, aiming to functionally replace human milk oligosaccharides (*h*MOS). Glucansucrases Gtf180- Δ N and GtfA- Δ N of *Lactobacillus reuteri* strains convert sucrose into α -glucans with (α 1 \rightarrow 6)/(α 1 \rightarrow 3) and (α 1 \rightarrow 4)/(α 1 \rightarrow 6) glucosidic linkages, respectively. Previously we reported that both glucansucrases glucosylate lactose, producing a minimum of 5 compounds (degree of polymerization 3-4) (GL34 mixture) with (α 1 \rightarrow 2/3/4) linkages. This GL34 mixture exhibited growth stimulatory effects on various probiotic bacteria. Aiming to obtain additional compounds mimicking *h*MOS in structure and function, we here studied glucosylation of 3 commercially available galactosyl-lactose GOS compounds. Both Gtf180- Δ N and GtfA- Δ N were unable to use 3'-galactosyl-lactose (β 3'-GL), but used sucrose to add a single glucose moiety to 4'-galactosyl-lactose (β 4'-GL) and 6'-galactosyl-lactose (β 6'-GL). β 6'-GL was elongated at its reducing glucosyl unit with an (α 1 \rightarrow 2)-linked moiety and at its non-reducing end with an (α 1 \rightarrow 4) linked moiety; β 4'-GL was only elongated at its reducing end with an (α 1 \rightarrow 2) linked moiety. Glucansucrases Gtf180- Δ N and GtfA- Δ N thus can be used to produce galactosyl-lactose-derived oligosaccharides containing (α 1 \rightarrow 2) and (α 1 \rightarrow 4) glucosidic linkages, potentially with valuable bioactive (prebiotic) properties.

INTRODUCTION

The beneficial effects on human and animal health of so called prebiotics, a class of bioactive oligosaccharides, are widely studied.¹⁻⁴ A prebiotic is defined as “a substrate that is selectively utilized by host microorganisms conferring a health benefit”.⁵ The most well-known prebiotics to date are human milk oligosaccharides (*hMOS*), β -galacto-oligosaccharides (GOS), β -fructo-oligosaccharides (FOS) and inulin, and lactulose.^{6,7,8} Novel non-digestible carbohydrates and prebiotic compounds are still high in demand for food and feed product applications.⁹ Recently, lactose-derived oligosaccharides have been at the focus of attention. The dominant members are GOS, with (β 1 \rightarrow 2), (β 1 \rightarrow 3), (β 1 \rightarrow 4), or (β 1 \rightarrow 6) linked galactosyl moieties, of various sizes (mostly DP2 - DP5). These prebiotic compounds stimulate growth of probiotic bacteria to various extents.^{7,10,11} Lactosucrose and lactulose are also well studied for their selective stimulatory effects on human gut bacteria.^{12,13}

Glucansucrases belong to glycoside hydrolase family 70 (GH70) (<http://www.CAZy.org>) and are extracellular *trans*-glycosidases found in lactic acid bacteria.^{14,15} Glucansucrase catalyzed reactions with sucrose follow an α -retaining double-displacement mechanism. Depending on the nature of the acceptor substrate, glucansucrase enzymes catalyze three types of reactions: hydrolysis of sucrose with water as acceptor, polymerization with growing α -glucan chains as acceptor, or *trans*-glycosylation with sucrose as donor substrate and other compounds as acceptor substrates (including oligosaccharides).¹⁶ Depending on their specificity, glucansucrases are capable of producing α -glucans with various linkage types, namely (α 1 \rightarrow 3), (α 1 \rightarrow 4) and (α 1 \rightarrow 6). Only the branching glucansucrase Dsr-E from *Leuconostoc mesenteroides* NRRLB-1299 can introduce single (α 1 \rightarrow 2) glucosyl branches in a dextran backbone.^{17,18} The *Lactobacillus reuteri* Gtf180- Δ N and GtfA- Δ N enzymes produce α -glucans with 69% (α 1 \rightarrow 6) and 31% (α 1 \rightarrow 3)

glycosidic linkages and 58% ($\alpha 1 \rightarrow 4$) and 42% ($\alpha 1 \rightarrow 6$) glycosidic linkages, respectively.^{19,20} Glucansucrases are known for their ability to use a wide variety of acceptor substrates including oligosaccharides and non-glycan compounds.^{21,22,23} One example is use of maltose as acceptor substrate resulting in synthesis of various longer oligosaccharides.^{15,24,25} Recently, lactose has attracted interest as acceptor substrate for glucansucrase enzymes. Dextransucrases from *Leuconostoc mesenteroides* and *Weissella confusa* added a single glucose moiety to lactose, involving an ($\alpha 1 \rightarrow 2$) linkage, to synthesize 2- α -D-glucopyranosyl-lactose.^{26,27} Lately, we reported that Gtf180- Δ N and GtfA- Δ N synthesize a mixture of five new glucosylated lactose derivatives (GL34), using sucrose as donor substrate and lactose as acceptor substrate.²⁸ A study of the potential prebiotic properties of GL34 revealed selective stimulatory effects on growth of various bifidobacteria. Structural analysis showed that these compounds are lactose elongated with one or two glucose moieties (DP3 - DP4) involving ($\alpha 1 \rightarrow 2$), ($\alpha 1 \rightarrow 3$), ($\alpha 1 \rightarrow 4$) glycosidic linkages.²⁹ With lactose as acceptor substrate, Gtf180- Δ N and GtfA- Δ N thus produced oligosaccharide compounds with new linkage types, ($\alpha 1 \rightarrow 2$) and ($\alpha 1 \rightarrow 4$) for Gtf180- Δ N and ($\alpha 1 \rightarrow 2$) and ($\alpha 1 \rightarrow 3$) for GtfA- Δ N, not observed for these glucansucrases before. The high resistance of this ($\alpha 1 \rightarrow 2$)-linkage type to human digestive enzymes and their selective stimulatory effects on probiotic bacteria make ($\alpha 1 \rightarrow 2$) linkage-containing oligosaccharides strong candidates for new non-digestible carbohydrates and prebiotic ingredients.³⁰

In view of these interesting findings, we aimed to further exploit the ability of glucansucrases Gtf180- Δ N and GtfA- Δ N to using the commercially available DP3 GOS structures 3'-galactosyl-lactose ($\beta 3'$ -GL), 4'-galactosyl-lactose ($\beta 4'$ -GL) and 6'-galactosyl-lactose ($\beta 6'$ -GL), as acceptor substrates to synthesize new (bioactive) oligosaccharides. The transfer products were structurally analyzed by high-performance anion-exchange chromatography (HPAEC), matrix-assisted laser-desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and

1D/2D $^1\text{H}/^{13}\text{C}$ nuclear magnetic resonance (NMR) spectroscopy (TOCSY, HSQC, ROESY). Three new DP4 structures were identified with ($\alpha 1 \rightarrow 2$) and ($\alpha 1 \rightarrow 4$) glycosidic linkages.

MATERIALS AND METHODS

Glucansucrase enzymes

Escherichia coli BL21 (DE3) (Invitrogen) carrying plasmid pET15b with the *gtf180* and *gtfA* genes from *Lactobacillus reuteri* strains 180 and 121 was used for expression of the N-terminally truncated glucansucrase enzymes (Gtf180- Δ N and GtfA- Δ N). The expression and purification of these glucansucrases have been described previously.^{22,31}

Trans-glycosylation reactions

The total activity of purified Gtf180- Δ N or GtfA- Δ N was measured as initial rates with sucrose by methods described previously by Van Geel-Schutten et al.³² The products of the *trans*-glycosylation reaction were prepared by incubating a mixture of 0.05 M sucrose (donor substrate) and 0.02 M GOS (acceptor substrate) with 3 U mL⁻¹ glucansucrase at 37 °C in 50 mM sodium acetate buffer with 0.1 mM CaCl₂ at pH 4.7. A volume of 10 μ L of the reaction mixtures was taken at 0 h, 5 h and 24 h and then mixed with 190 μ L DMSO. The diluted samples were analyzed by High-pH anion-exchange chromatography (HPAEC-PAD).

Isolation and purification of oligosaccharide products

The reactions were carried out in a volume of 20 mL under the conditions described in the previous section of *Trans*-glycosylation reactions. Afterwards the reaction mixtures were diluted with two volumes of cold ethanol 20 % and stored at 4 °C overnight to precipitate any polysaccharide material present. After centrifugation at

10,000 g for 10 min, the supernatants were applied to a rotatory vacuum evaporator to remove ethanol. The aqueous fractions were then absorbed onto a CarboGraph SPE column (GRACE, USA) using acetonitrile:water = 1:3 as eluent, followed by evaporation of acetonitrile under an N₂ stream before being freeze-dried. This was followed by fractionation HPAEC on a Dionex ICS-5000 workstation (Dionex, Amsterdam, the Netherlands), equipped with a CarboPac PA-1 column (250 x 9 mm; Dionex) and an ED40 pulsed amperometric detector (PAD). The collected fractions were neutralized by acetic acid 20 % and then desalted using a CarboGraph SPE column as described earlier.

HPAEC-PAD analysis

The profiles of the oligosaccharides products were analyzed by HPAEC-PAD on a Dionex ICS-3000 work station (Dionex, Amsterdam, the Netherlands) equipped with an ICS-3000 pulse amperometric detection (PAD) system and a CarboPac PA-1 column (250 x 4 mm; Dionex). The analytical separation was performed at a flow rate of 1.0 mL min⁻¹ using a complex gradient of effluents A (100 mM NaOH); B (600 mM NaOAc in 100 mM NaOH); C (Milli-Q water); and D (50 mM NaOAc). The gradient started with 10 % A, 85 % C, and 5 % D in 25 min to 40 % A, 10 % C, and 50 % D, followed by a 35-min gradient to 75 % A, 25 % B, directly followed by 5 min washing with 100 % B and reconditioning for 7 min with 10 % A, 85 % B, and 5 % D.

MALDI-TOF mass spectrometry

Molecular masses of the compounds in the reaction mixtures were determined by MALDI-TOF mass spectrometry on an AximaTM Performance mass spectrometer (Shimadzu Kratos Inc., Manchester, UK), equipped with a nitrogen laser (337 nm, 3 ns pulse width). Ion-gate cut-off was set to *m/z* 200 and sampling resolution was

software-optimized for m/z 1500. Samples were prepared by mixing 1 μL with 1 μL aqueous 10 % 2,5-dihydroxybenzoic as matrix solution.

NMR spectroscopy

The structures of oligosaccharides of interest were elucidated by 1D and 2D ^1H NMR, and 2D ^{13}C NMR. A Varian Inova 500 Spectrometer and 600 Spectrometer (NMR center, University of Groningen) were used at probe temperatures of 25 $^\circ\text{C}$ with acetone as internal standard (chemical shift of δ 2.225). The aliquot samples were exchanged twice with 600 μL of 99.9%_{atom} D_2O (Cambridge Isotope Laboratories, Inc., Andover, MA) by freeze-drying, and then dissolved in 0.65 mL D_2O , containing internal acetone. In the 1D ^1H NMR experiments, the data were recorded at 8 k complex data points, and the HOD signal was suppressed using a WET1D pulse. In the 2D ^1H - ^1H NMR COSY experiments, data were recorded at 4000 Hz for both directions at 4k complex data points in 256 increments. 2D ^1H - ^1H NMR TOCSY data were recorded with 4000 Hz at 30, 60, 100 spinlock times in 200 increments. In the 2D ^1H - ^1H NMR ROESY, spectra were recorded with 4800 Hz at a mixing time of 300 ms in 256 increments of 4000 complex data points. MestReNova 9.1.0 (Mestrelabs Research SL, Santiago de Compostela, Spain) was used to process NMR spectra, using Whittaker Smoother baseline correction.

RESULTS

***Trans*-glucosylation of GOS**

$\beta 3'$ -GL, $\beta 4'$ -GL and $\beta 6'$ -GL (0.02 M) were incubated with sucrose (0.05 M) plus the Gtf180- ΔN or GtfA- ΔN enzymes, at 37 $^\circ\text{C}$ and pH 4.7. Blank reactions minus β -GL compounds used sucrose as both acceptor and donor substrate, resulting in α -glucan synthesis. Incubation mixtures were sampled after 0 h, 5 h and 24 h, and subjected to HPAEC-PAD analysis (Figure 1). No *trans*-glucosylation products

were observed with β 3'-GL (data not shown). Gtf180- Δ N and GtfA- Δ N incubated with sucrose plus β 6'-GL yielded similar HPAEC-PAD profiles of oligosaccharides synthesized (Figure 1a and 1c), with three major peaks at retention times between 22-29 min. In case of Gtf180- Δ N, there are several minor peaks eluting later in time, which most likely are higher DP oligosaccharides (DP5 – DP9). The areas of the Gtf180- Δ N peaks at 23.0 min and 25.5 min, called GL1 and GL2 respectively, are much more significant than those from GtfA- Δ N. Regarding the glucosylation of β 4'-GL, one significant peak at retention time of 31.9 min, called GL3, was observed in HPAEC-PAD profiles of the reaction mixtures of both Gtf180- Δ N and GtfA- Δ N (Figure 1b and 1d). Especially with Gtf180- Δ N, there are several minor peaks that elute later than GL3. Similarly, the intensity of peak GL3 in the profile of Gtf180- Δ N is much more significant than that of GtfA- Δ N, especially after 5 h of incubation. The areas of the peaks GL1, GL2 and GL3, decreased upon prolonged incubation. This may be due to further glucansucrase catalyzed elongation reactions using these *trans*-glycosylation products as intermediate acceptor substrates. Further studies involving structural elucidation of the higher DP *trans*-glycosylation products are required to fully understand this. In case of Gtf180- Δ N, β 6'-GL and β 4'-GL were converted for 26 and 32% (Figure 1a,b; 24 h incubation time), estimated from their peak areas. Only limited amounts of β 6'-GL and β 4'-GL were available, and further optimizations of reaction conditions and product yields remain to be done.

Structural analysis of *trans*-glycosylation products

The three major glucosylation products corresponding to the peaks GL1, GL2 and GL3 of Gtf180- Δ N decorating β 6'-GL and β 4'-GL (Figure 11) were isolated from the incubation mixture for structural analysis by MALDI-TOF-MS and 1D/2D 1 H and 13 C NMR spectroscopy. The purity and retention time of each fraction was confirmed by reinjection on an analytical CarboPac PA-1 (4 x 250 mm) column. The

fragment size distribution of each fraction was determined by MALDI-TOF MS. The data showed that all three major products corresponded to tetrasaccharides, as evidenced by a pseudo-molecular sodium adduct ion at 689 m/z .

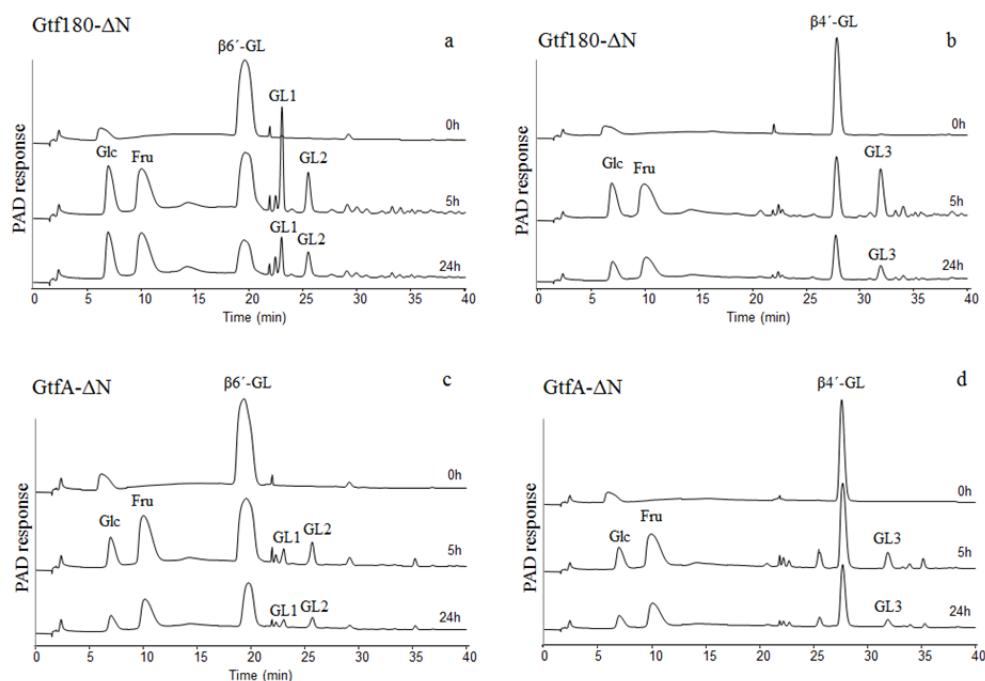


Figure 1: HPAEC-PAD chromatograms of the reaction product mixtures obtained with 3 U mL⁻¹ glucosucrases (Gtf180- Δ N and GtfA- Δ N) using a) β 6'-GL and b) β 4'-GL as acceptor substrate and sucrose as donor substrate, after various times of incubation at 37 °C and pH 4.7.

Glucosylated transfer products of β 6'-GL

Fraction GL1

Tetrasaccharide GL1 includes 4 hexose residues, namely **A**, **B** and **C** (one glucosyl and two galactosyl residues from the β 6'-GL substrate), and **D** (transferred glucosyl residue from sucrose) (Scheme 1). The 1D ¹H NMR spectrum of this fraction exhibited five anomeric signals at δ 5.222 (**A_α** H-1), δ 4.670 (**A_β** H-1), δ 4.468 (**B** H-

1), δ 4.55 (**C** H-1) and δ 4.927 (**D** H-1) (Figure 2). All non-anomeric proton resonances were assigned by using 2D ^1H - ^1H TOCSY and 2D ^1H - ^{13}C HSQC (Table 1 and Figure 2). Residue **A** showed the ^1H and ^{13}C pattern fitting with a reducing residue. Moreover, the set of chemical shifts of this residue matches very well with the corresponding residue from the structure $\beta 6'$ -GL.³³ These data show that there is no other substitution at residue **A** except for the 4-substitution with the galactosyl residue **B**. There are downfield shifts detected in residue **C** H-2 at δ 3.76 ($\Delta\delta + 0.09$ ppm), H-3 at δ 4.02 ($\Delta\delta + 0.05$ ppm), H-4 at 4.02 ($\Delta\delta + 0.05$ ppm) and H-5 at δ 3.78 ($\Delta\delta + 0.10$ ppm), which are indicative for substitution occurring at this residue. The residue **C** substitution was at C-4 as evidenced by the strong downfield shift to δ 77.9 ($\Delta\delta + 8.2$ ppm). This 4-substitution was supported by the ROESY inter-residual correlations between **D** H-1 and **C** H-4 (Figure 2). Other inter-residual interactions between **B** H-1 and **A** H-4 and between **C** H-1 and **B** H-6 were also detected in the ROESY spectrum. These data lead to identification of the GL1 structure as α -D-Glcp-(1 \rightarrow 4)-D-Galp-(1 \rightarrow 6)- β -D-Galp-(1 \rightarrow 4)-D-Glcp; **D1** \rightarrow **4C1** \rightarrow **6B1** \rightarrow **4A** (Scheme 1).

Fraction GL2

Tetrasaccharide GL2 includes 4 hexose residues, namely **A**, **B** and **C** (one glucosyl and two galactosyl residues from the $\beta 6'$ -GL substrate), and **D** (transferred glucosyl residue from sucrose) (Scheme 1). The 1D ^1H NMR of fraction GL2 showed anomeric peaks at δ 5.447 (**A _{α}** H-1), δ 4.572 (**A _{β}** H-1), δ 4.48 (**B** H-1), δ 4.448 (**C** H-1), δ 5.172 (**D ^{α}** H-1) and 5.428 (**D ^{β}** H-1) (Table 1). The anomeric signals **B** H-1 and **C** H-1 are split under influence of the reducing residue **A** anomeric configuration. This splitting is typically observed as a result of a 2-substitution of residue **A**.¹³ All other ^1H and ^{13}C chemical shifts of this structure were assigned from 2D NMR spectra (COSY, TOCSY, ROESY and HSQC) (Figure 3). Residue **A _{α}** and **A _{β}** H-1 at δ 5.447 and δ 4.527, respectively, are shifted very strongly compared with $\beta 6'$ -GL,

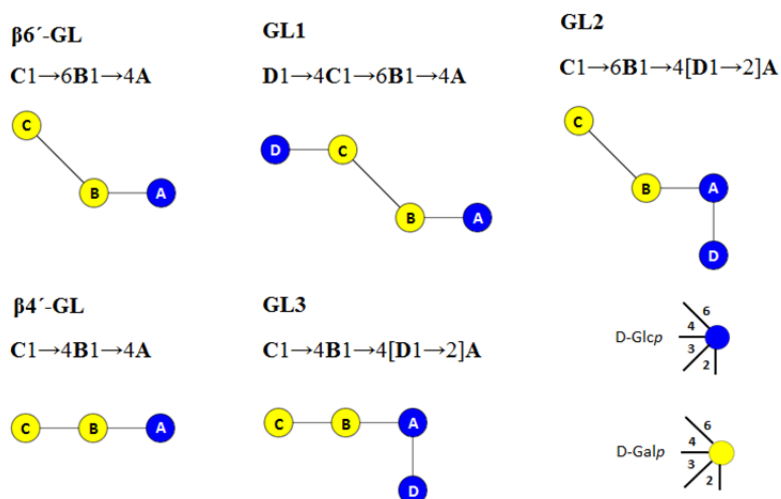
reflecting a substituted reducing α -D-Glcp residue at C-2.³⁴ Furthermore, the A_α H-2/C-2 at δ 3.70/80.0 ppm and A_β H-2/C-2 at δ 3.45/78.8 ppm indicates a 2-substituted residue. The 2-substitution of residue **A** is further confirmed by inter-residual ROESY correlations between D^α H-1/ A_α H-2 and D^β H-1/ A_β H-2 (Figure 3). Residue **D** showed the chemical shift pattern of a terminal α -D-Glcp residue with D^α / D^β H-4 at δ 3.45/3.46 and H-1 at δ 5.127/5.428.³⁴ Residue **C** showed H-2, H-3 and H-4 at δ 3.54, δ 3.66 and δ 3.92 matching with the terminal (1 \rightarrow 6)-linked galactosyl residue.³⁴ Moreover, ROESY inter-residual correlations were observed between **C** H-1 and **B** H-6 and between **B** H-1 and **A** H-4, confirming the β -D-Galp-(1 \rightarrow 6)- β -D-Galp-(1 \rightarrow 4)-D-Glcp-. These data resulted in identification of the GL2 structure as β -D-Galp-(1 \rightarrow 6)- β -D-Galp-(1 \rightarrow 4)-[α -D-Glcp-(1 \rightarrow 2)-]D-Glcp; C1 \rightarrow 6B1 \rightarrow 4[D1 \rightarrow 2]A (Scheme 1).

Glucosylated transfer product of β 4'-GL

Fraction GL3

Tetrasaccharide GL3 comprises of 4 hexose residues, namely **A**, **B** and **C** (one glucosyl and two galactosyl residues from β 4'-GL), and **D** (transferred glucosyl residue from sucrose) (Scheme 1). The 1D 1 H NMR spectrum of GL3 revealed six anomeric 1 H signals at δ 5.435 (A_α H-1), δ 4.817 (A_β H-1), δ 4.489 (**B** H-1), δ 4.594 (**C** H-1), δ 5.093 (D^α H-1) and δ 5.355 (D^β H-1) (Table 2). The splitting of the anomeric signals **B** H-1 and **C** H-1 is influenced by the α/β configuration of the reducing residue **A**. From 2D NMR spectra 1 H and 13 C chemical shifts were determined for all residues (Figure 4). The anomeric signals of residue **C** at δ 4.601/4.594 are indicative of a terminal β -D-Galp-(1 \rightarrow 4)- residue,³⁴ indicating that this residue had not become elongated. Whereas, residue A_α and A_β H-1 at δ 5.435 and δ 4.817, respectively, reflects a 2-substitution.³⁴ Also the A_α H-2/C-2 at δ 3.69/79.3 ppm and A_β H-2/C-2 at δ 3.41/79.0 ppm supports the occurrence of a 2-substitution. This (1 \rightarrow 2)-linked residue is further confirmed by inter-residual

ROESY correlations between D^{α} H-1 and A_{α} H-2 and between D^{β} H-1 and A_{β} H-2 (Figure 4). The chemical shift pattern of residue **D** reflects a terminal α -D-Glcp.³⁴ The signal at δ 4.497/4.483 of residue **B** fits with a $\rightarrow 4$ - β -D-Galp-(1 \rightarrow 4)-D-Glcp residue. The ROESY spectrum also showed inter-residual correlations between **C** H-1 and **B** H-4 and between **B** H-1 and **A** H-4. Combining all data, the structure of tetrasaccharide compound GL3 is determined as β -D-Galp-(1 \rightarrow 4)- β -D-Galp-(1 \rightarrow 4)-[α -D-Glcp-(1 \rightarrow 2)-]D-Glcp; C1 \rightarrow 4B1 \rightarrow 4[D1 \rightarrow 2]A (Scheme 1).



Scheme 1: Structures of the acceptor substrates $\beta 6'$ -GL and $\beta 4'$ -GL and the GL1-GL3 transfer products synthesized by Gtf180- Δ N and GtfA- Δ N using sucrose as donor substrate.

DISCUSSION AND CONCLUSIONS

Glucansucrases GtfA- Δ N and Gtf180- Δ N use sucrose to synthesize α -glucan polymers with ($\alpha 1\rightarrow 4$)/($\alpha 1\rightarrow 6$) and ($\alpha 1\rightarrow 6$)/($\alpha 1\rightarrow 3$) linkages, respectively.^{19,20} Their linkage specificity is maintained in the acceptor reaction with maltose and other carbohydrates, or non-glycans such as catechol.^{21,22,35,36} When acting on lactose as acceptor substrate, these two enzymes produced the same *trans*-glycosylation product mixture (GL34), with compounds F1 - F5 but introduced different linkage types, ($\alpha 1\rightarrow 2$)/($\alpha 1\rightarrow 4$) for Gtf180- Δ N and ($\alpha 1\rightarrow 2$)/($\alpha 1\rightarrow 3$) for

GtfA- Δ N.²⁸ Also in the present study, when using galactosyl-lactose GOS molecules as acceptor substrate, these two enzymes synthesized similar products. Both GtfA- Δ N and Gtf180- Δ N produced α -D-Glcp-(1 \rightarrow 4)-D-Galp-(1 \rightarrow 6)- β -D-Galp-(1 \rightarrow 4)-D-Glcp and β -D-Galp-(1 \rightarrow 6)- β -D-Galp-(1 \rightarrow 4)-[α -D-Glcp-(1 \rightarrow 2)]-D-Glcp (GL1 and GL2) when acting on β 6'-GL, and produced β -D-Galp-(1 \rightarrow 4)- β -D-Galp-(1 \rightarrow 4)-[α -D-Glcp-(1 \rightarrow 2)]-D-Glcp (GL3) when acting on β 4'-GL (Scheme 1). Gtf180- Δ N produced significantly more of these products than GtfA- Δ N (Figure 1). With β 6'-GL as acceptor substrate both enzymes introduced an (α 1 \rightarrow 4) linkage at the non-reducing end in the GL1 product, but this is not the case for β 4'-GL. Both glucansucrases added a Glc-(1 \rightarrow 4) residue on β -Gal-(1 \rightarrow 4)-Glc of lactose and β -Gal-(1 \rightarrow 6)-Gal- of β 6'-GL but not on β -Gal-(1 \rightarrow 4)- β -Gal of β 4'-GL. Different from lactose as acceptor substrate,²⁸ none of the Gal-(1 \rightarrow x)-Gal epitopes of β 6'-GL and β 4'-GL can be elongated with a Glc-(1 \rightarrow 3) residue. However, similar to the reaction with lactose,²⁸ with β 6'-GL and β 4'-GL as acceptor substrates, both enzymes introduced an (α 1 \rightarrow 2) linked glucose moiety at the reducing end in the GL2 and GL3 products, respectively (Scheme 1). Galactose-containing acceptor substrates thus appear to enforce changes in the glucoside linkage specificity of these two glucansucrases: Gtf180- Δ N and GtfA- Δ N favor the synthesis of (α 1 \rightarrow 2) linkage containing oligosaccharides when acting on galactose-containing acceptor compounds. Both Gtf180- Δ N and GtfA- Δ N were unable to use β 3'-GL as acceptor substrate. The presence of a (β 1 \rightarrow 3) linkage in this acceptor compound probably makes it inaccessible to the acceptor binding site of the studied glucansucrases. Docking experiments with the β 4'-GL and β 6'-GL substrates and a glucosyl-enzyme intermediate constructed using the crystal structure of *L. reuteri* 180 Gtf180- Δ N (PDB: 3KLL; Vujičić-Žagar et al., 2010) resulted in different poses and were inconclusive. Further insights in the linkage specificity determinants of Gtf180- Δ N acting on these GOS-DP3 acceptor substrates may be provided by a crystal structure

of Gtf180- Δ N in complex with lactose, but attempts to obtain such a complex have not been successful so far.

The acceptor substrates β 6'-GL and β 4'-GL used in this study are present in the well-known commercial prebiotic mixture Vivinal GOS.^{34,37} These two compounds previously showed selective stimulatory effects on growth of various beneficial bacteria including *Bifidobacterium breve*, *Bifidobacterium longum* subspecies *infantis*, *Bifidobacterium adolescentis*, *Bifidobacterium longum* subspecies *longum*, *Bifidobacterium lactis* and *Lactobacillus acidophilus*³⁸ and (M. Boger. et al. in preparation). However, the linear structure β 4'-GL also was readily digested by commensal bacteria like *Bacteroides thetaiotaomicron* and other bacteria encoding endo- β -galactanase GH53 enzymes.³⁹ Elongation of β 4'-GL with an (α 1 \rightarrow 2) linked glucose moiety may improve its resistance towards consumption by commensal bacteria, and may promote its non-digestible and prebiotic properties. The β 6'-GL compound was not digested by commensal bacteria and predicted to stimulate growth of beneficial gut bacteria in a similar manner as *h*MOS.³⁹ Glucosylation of β 6'-GL may even further enhance its selectivity and thus provides another *h*MOS mimicking compound. The potential stimulatory effects of these new GL1-GL3 DP4 compounds on growth of probiotic bacteria, and other functional properties, remain to be studied. Optimization of the reaction conditions, to enhance galactosyl-lactose conversion and product yields, are required to obtain sufficient amounts of the GL1-GL3 compounds for such functional studies. *Trans*-glucosylation of galactosyl-lactose compounds with glucansucrase enzymes is likely to further expand the already well-known prebiotic GOS status.

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TABLES AND FIGURES

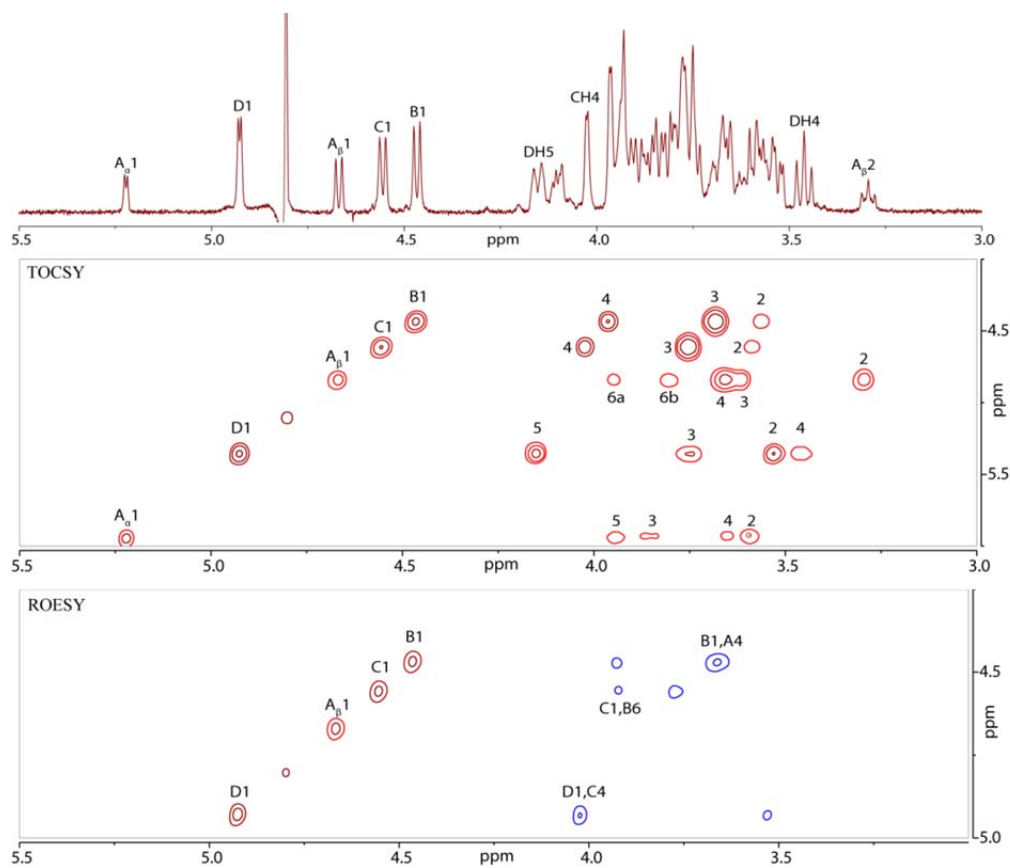


Figure 2: 500-MHz 1D ^1H NMR spectra of GL1 synthesized by Gtf180- ΔN from $\beta 6'$ -GL, recorded at 25 $^{\circ}\text{C}$ in D_2O . Anomeric signals of each fraction were labeled according to the legends of the corresponding structures indicated in Scheme 1. The ^1H and ^{13}C chemical shifts of GL1 are listed in Table 1.

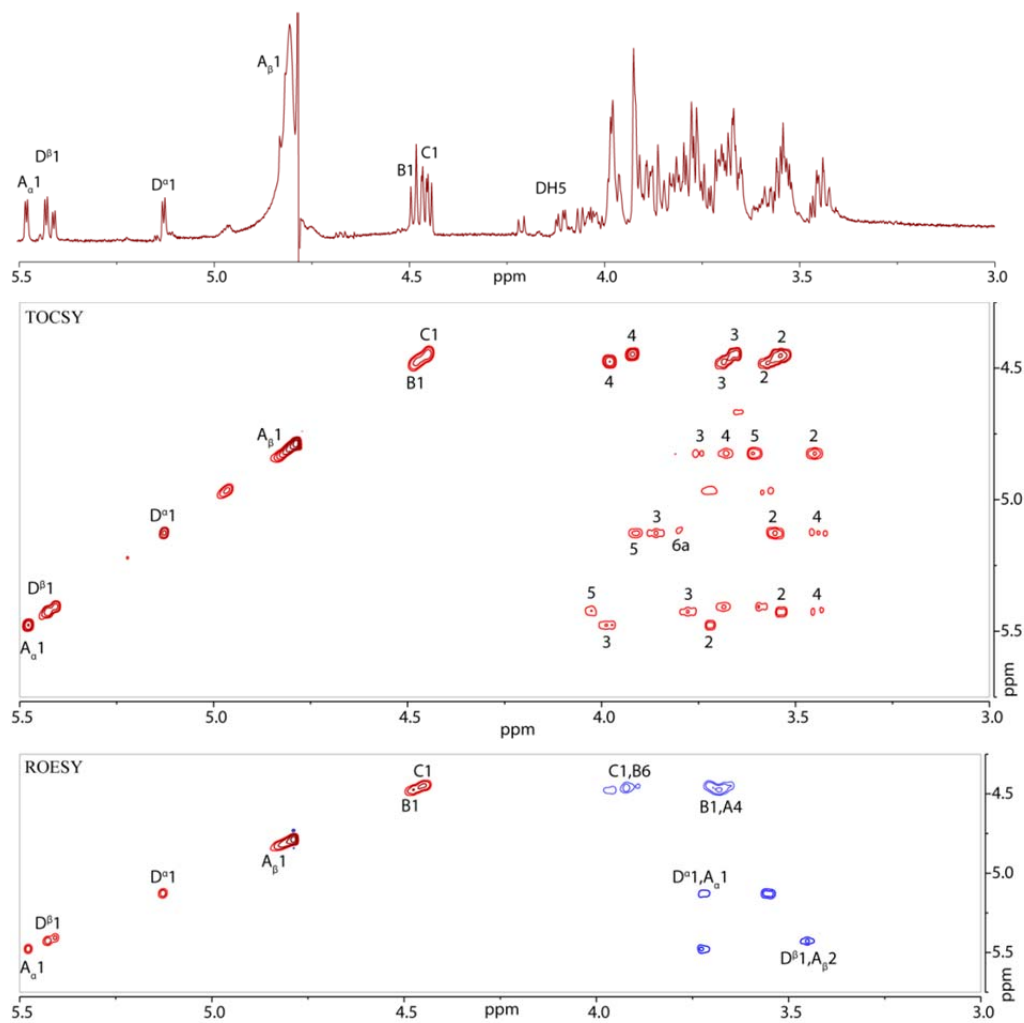


Figure 3. 500-MHz 1D ^1H NMR spectra of GL2 synthesized by Gtf180- ΔN from $\beta 6'$ -GL, recorded at 25 $^\circ\text{C}$ in D_2O . Anomeric signals of each fraction were labeled according to the legends of the corresponding structures indicated in Scheme 1. The ^1H and ^{13}C chemical shifts of GL2 are listed in Table 1.

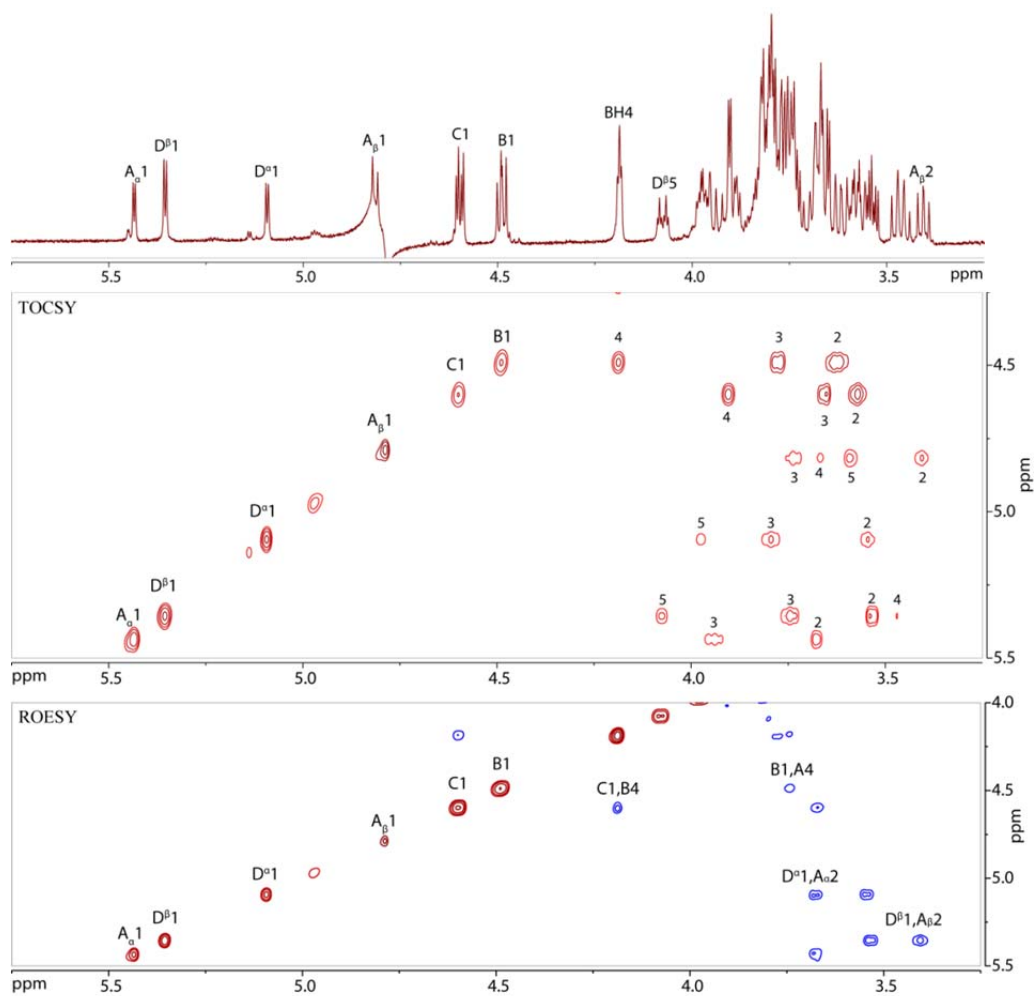


Figure 4. 500-MHz 1D ^1H NMR spectra of GL3 synthesized by Gtf180- ΔN from $\beta 4'$ -GL, recorded at 25 $^{\circ}\text{C}$ in D_2O . Anomeric signals of each fraction were labeled according to the legends of the corresponding structures indicated in Scheme 1. The ^1H and ^{13}C chemical shifts of GL3 are listed in Table 2.

Table 1: ^1H and ^{13}C chemical shifts of glucosylated- $\beta\text{6}^1\text{-GL}$ derivatives the GL1 and GL2, measured at 25 °C in D_2O . Chemical shifts that are key in the structural determination are underlined.

	$\beta\text{6}^1\text{-GL}$		GL1		GL2	
	^1H	^{13}C	^1H	^{13}C	^1H	^{13}C
A $_{\alpha}$ 1	5.223	93.1	5.222	92.5	5.447	89.6
A $_{\alpha}$ 2	3.57	72.1	3.60	71.7	<u>3.72</u>	<u>80.0</u>
A $_{\alpha}$ 3	3.83	72.8	3.85	72.8	3.99	71.0
A $_{\alpha}$ 4	<u>3.63</u>	<u>80.2</u>	<u>3.66</u>	<u>80.2</u>	<u>3.68</u>	<u>80.8</u>
A $_{\alpha}$ 5	3.95	71.1	3.96	69.5	3.98	69.2
A $_{\alpha}$ 6a	3.88	61.1	3.88	60.8	3.88	61.0
A $_{\alpha}$ 6b	3.84		3.84	61.0	3.84	61.2
A $_{\beta}$ 1	4.667	96.9	4.670	96.4	4.527	104.3
A $_{\beta}$ 2	3.294	74.9	3.296	74.5	<u>3.45</u>	<u>78.8</u>
A $_{\beta}$ 3	3.63	75.8	3.63	75.5	3.75	74.0
A $_{\beta}$ 4	<u>3.65</u>	<u>80.2</u>	<u>3.65</u>	<u>79.8</u>	<u>3.69</u>	<u>80.8</u>
A $_{\beta}$ 5	3.60	75.9	3.62	75.5	3.61	75.4
A $_{\beta}$ 6a	3.94	61.4	3.94	60.9	3.97	61.2
A $_{\beta}$ 6b	3.80		3.80	60.9	3.81	
B1	4.483	104.4	4.468	103.8	4.468/4.483	104.3
B2	3.53	72.0	3.56	72.2	3.57	71.8
B3	3.66	73.8	3.68	73.2	3.68	73.5
B4	3.94	69.7	3.96	69.4	3.98	69.3
B5	3.95	75.0	3.93	75.0	3.92	74.6
B6a	<u>4.079</u>	<u>70.3</u>	<u>4.10</u>	<u>69.8</u>	<u>4.06</u>	<u>69.8</u>
B6b	<u>3.93</u>		<u>3.94</u>		<u>3.90</u>	<u>69.8</u>
C1	4.460	104.4	<u>4.55</u>	104.1	4.441/4.454	104.3
C2	3.54	72.0	3.60	71.7	3.54	72.4
C3	3.67	73.8	3.76	73.2	3.66	73.4
C4	3.974	69.7	<u>4.02</u>	<u>77.9</u>	3.92	69.6
C5	3.68	76.3	3.78	76.0	3.70	75.9
C6a	3.81		3.81	60.8	3.81	61.2
C6b	3.76		3.79	60.8	3.77	61.8
D $^{\alpha}$ 1			4.927	100.8	5.127	96.5
D $^{\alpha}$ 2			3.54	72.8	3.55	71.6
D $^{\alpha}$ 3			3.75	72.0	3.86	<u>73.4</u>
D $^{\alpha}$ 4			3.46	69.8	3.45	70.3
D $^{\alpha}$ 5			4.152	72.7	3.96	72.3
D $^{\alpha}$ 6a					3.91	61.0

D ^α 6b						3.80	61.0
D ^β 1						5.428	98.3
D ^β 2						3.54	72.4
D ^β 3						<u>3.78</u>	<u>73.6</u>
D ^β 4						3.46	70.3
D ^β 5						4.03	60.9
D ^β 6a						3.82	
D ^β 6b							

Table 2: ¹H and ¹³C chemical shifts of the glucosylated-β4'-GL derivative GL3, measured at 25 °C in D₂O. Chemical shifts that are key in the structural determination are underlined.

	β4'-GL			GL3	
	¹ H	¹³ C		¹ H	¹³ C
A _α 1	5.225	92.9		5.435	90.1
A _α 2	3.58	<u>72.6</u>		<u>3.69</u>	<u>79.3</u>
A _α 3	3.83	72.6		3.94	70.7
A _α 4	3.65	79.5		<u>3.71</u>	<u>79.2</u>
A _α 5	3.95	71.1		3.95	70.7
A _α 6a	3.87	61.0			
A _α 6b	3.80				
A _β 1	4.664	96.9		4.817	96.8
A _β 2	3.281	74.9		<u>3.41</u>	<u>79.0</u>
A _β 3	3.65	75.3		3.74	75.4
A _β 4	3.65	79.5		<u>3.67</u>	<u>79.5</u>
A _β 5	3.60	76.0		3.59	75.5
A _β 6a	3.958	61.2		3.97	60.9
A _β 6b	3.80			3.81	
B1	4.486	104.0		4.483/4.497	103.8
B2	3.63	72.4		3.64	72.2
B3	3.77	73.9		3.78	73.8
B4	4.193	78.3		<u>4.192</u>	<u>78.0</u>
B5	3.74	75.5		3.74	75.4
B6a	3.80	61.9		3.82	61.57
B6b	3.11				
C1	4.603	105.2		4.594/4.601	105.1
C2	3.57	72.4		3.59	75.6
C3	3.66	74.0		3.66	72.3
C4	3.904	69.8		3.905	69.4

C5	3.71	76.1		3.69	76.1
C6a	3.82	61.6		3.81	61.0
C6b	3.80				
D ^{α} 1				5.093	97.4
D ^{α} 2				3.55	72.3
D ^{α} 3				3.80	73.7
D ^{α} 4				3.45	70.1
D ^{α} 5				3.98	72.5
D ^{α} 6a				3.89	60.7
D ^{α} 6b					
D ^{β} 1				5.355	98.7
D ^{β} 2				3.54	72.2
D ^{β} 3				3.75	73.7
D ^{β} 4				3.47	70.1
D ^{β} 5				4.077	72.3
D ^{β} 6a				3.91	60.9
D ^{β} 6b					

Chapter 6

Synthesis and characterization of sialylated lactose and lactulose derived oligosaccharides by *Trypanosoma cruzi* trans-sialidase

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ABSTRACT

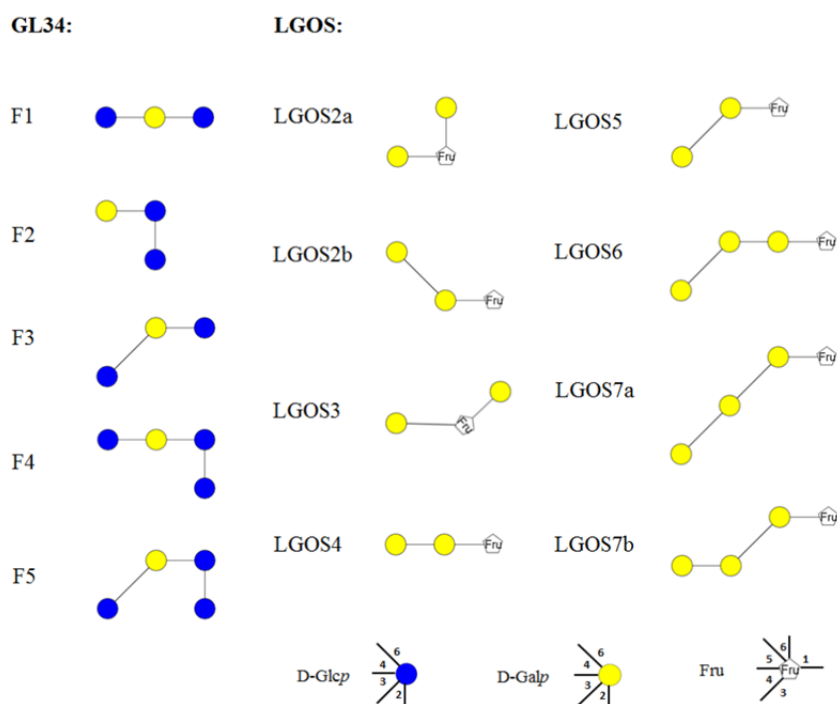
Sialylated oligosaccharides contribute 12.6-21.9 % of total free oligosaccharides in human milk (*hMOS*). These acidic *hMOS* possess prebiotic properties and display anti-adhesive effects against pathogenic bacteria. Only limited amounts of sialylated *hMOS* are currently available. The aim of our work is to enzymatically synthesize sialylated oligosaccharides mimicking *hMOS* functionality. In this study we tested mixtures of glucosylated-lactose (GL34), galactosylated-lactulose (LGOS) and galacto-oligosaccharide (Vivinal GOS) molecules, as *trans*-sialylation acceptor substrates. The recombinant *trans*-sialidase enzyme from *Trypanosoma cruzi* (TcTS) was used for enzymatic decoration, transferring (α 2 \rightarrow 3)-linked sialic acid from donor substrates to the non-reducing terminal β -galactopyranosyl units of these acceptor substrates. The GL34 F2 2-glc-lac compound with an accessible terminal galactosyl residue was sialylated efficiently (conversion degree of 47.6 %). TcTS also sialylated at least five LGOS structures and eleven Vivinal GOS DP3-4 compounds. Up to 52% of the LGOS acceptor substrate mixture was converted. These newly synthesized sialylated oligosaccharides are interesting as potential *hMOS*-mimics for applications in biomedical and functional food products

INTRODUCTION

In human milk, free oligosaccharides comprise the third most abundant component, after lactose and fat. Human milk oligosaccharides (*hMOS*) represent lactose molecules elongated with *N*-acetylglucosamine (GlcNAc), galactose (Gal), fucose (Fuc), and *N*-acetylneuraminic acid (Neu5Ac) with various glycosidic linkage types.¹ Sialic acid can be coupled to galactose residues in *hMOS* with ($\alpha 2 \rightarrow 3$) or ($\alpha 2 \rightarrow 6$) linkages and to GlcNAc with ($\alpha 2 \rightarrow 6$) linkages. These sialylated oligosaccharides, contribute 12.6-21.9 % of total *hMOS*.² There is increasing evidence for positive functional effects of this group of acidic oligosaccharides on human health.^{3,4,5} Specific *hMOS* structures, namely disialyllacto-*N*-tetraose and 2'-fucosyllactose, prevented and reduced necrotizing enterocolitis (NEC) in neonatal rats, thus may be used to prevent NEC in formula-fed infants.^{6,7} Preventive effects against NEC were also observed with a Sia-GOS mixture, particularly with disialylated GOS.^{7,8} 3'-Sialyllactose stimulates growth of various *Bifidobacterium* strains including the infant gut-related *Bifidobacterium longum* subsp. *infantis*.⁹ Sialylated oligosaccharides also prevent intestinal attachment of pathogens by acting as receptor analogs, competing with epithelial ligands for bacterial binding.^{10,11,12,13,14} Compared to human milk, free oligosaccharides in the milk of domesticated animals are much less abundant.¹⁵ Bovine milk, for instance, has only trace amounts of milk oligosaccharides.^{16,17} The natural scarcity of these highly bio-active sialylated oligosaccharides stimulated us to study the possible synthesis of mimics via *trans*-sialylation of β -galactose (β -Gal)-linked compounds in various oligosaccharide mixtures. One example is the Vivinal GOS mixture that is commercially used in infant nutrition.^{18,19}

Recently, we have reported the enzymatic synthesis of two novel oligosaccharide mixtures (GL34 and LGOS) and their structural characterization.^{20,21} GL34 is a mixture of five ($\alpha 1 \rightarrow 2/3/4$)-glucosylated lactose molecules, with a degree of

polymerization (DP) of 3-4, synthesized from sucrose as donor substrate by glucansucrases (Gtf180- Δ N and GtfA- Δ N) as biocatalysts (Scheme 1).²⁰ The GL34 mixture exhibited selective stimulatory effects on growth of various strains of lactobacilli and bifidobacteria.²² LGOS is a mixture of (β 1 \rightarrow 3/4/6)-galactosylated lactulose molecules, with one or two galactosyl moieties, synthesized from lactulose as donor and acceptor substrate by wild-type and mutant β -galactosidase enzymes from *Bacillus circulans* ATCC 31382 (Scheme 1).²¹ Previously, oligosaccharides derived from lactulose were shown to promote growth of bifidobacteria and to exert beneficial effects on the digestive tract.^{23,24,25,26}



Scheme 1: Schematic presentation of all structures in the GL34 glucosylated-lactose (lactose = galactosyl-glucose) and LGOS galactosylated-lactulose (lactulose = galactosyl-fructose) mixtures used.^{20,21}

In view of the potential functional properties of these novel GL34 and LGOS oligosaccharides we decided to try and further develop their structures to better mimic acidic *h*MOS. In this study, *trans*-sialidase from *Trypanosoma cruzi* (TcTS)²⁷ was employed for the *trans*-sialylation of oligosaccharides in the GL34 and LGOS mixtures. Amongst *trans*-sialidases (EC 3.2.1.18), *T. cruzi trans*-sialidase is one of the best studied enzymes.²⁷ It plays an important role in host cell invasion and pathogenicity of *T. cruzi* due to its ability to scavenge and transfer sialic acid to its extracellular mucins, thereby hiding from the host immune system.^{28,29}

TcTS catalyzes *trans*-sialylation reactions via a ping-pong mechanism,³⁰ which starts with formation of a stable sialo-enzyme intermediate through a covalent bond with the nucleophile Tyr342.³¹ This is followed by transfer of the sialic acid to a β -Gal-linked acceptor substrate involving a nucleophilic attack of the hydroxyl group at C3 of this β -Gal.³⁰ When a suitable β -Gal-linked acceptor is absent, this enzyme catalyzes a hydrolysis reaction and sialic acid is released.³² In case of TcTS, sialyl transfer is catalyzed with much greater efficiency than hydrolysis.³³ TcTS can use glycoproteins or oligosaccharides as acceptor substrates, but only uses compounds possessing sialic acid ($\alpha 2 \rightarrow 3$)-linked to a terminal β -Gal as donor substrates.³³ In previous work we have shown that TcTS catalyzes the transfer of sialic acid from κ -casein-derived glyco-macropptide (GMP) donor substrate to galacto-oligosaccharides (GOS).^{8,34} However, a detailed analysis of these mono-sialylated and di-sialylated GOS structures was not performed. GMP is a byproduct of cheese manufacturing and contains a high level of O-glycans which carry Neu5Ac, including mainly Neu5Ac($\alpha 2 \rightarrow 3$)-Gal($\beta 1 \rightarrow 3$)-GalNAc and Neu5Ac($\alpha 2 \rightarrow 3$)-Gal($\beta 1 \rightarrow 3$)-[Neu5Ac($\alpha 2 \rightarrow 6$)]GalNAc, which can be used as donor substrates.^{35,36}

In this study we used the GL34 and LGOS mixtures as acceptor substrates and GMP as donor substrate. The negatively charged products were fractionated using Dowex 1x8 Chloride. Furthermore, we characterized the sialylated GOS structures that were

synthesized in our previous work⁸ in more detail. The decorated GL34, GOS and LGOS structures were identified using High-pH anion-exchange chromatography (HPAEC), and one-dimensional ¹H nuclear magnetic resonance spectroscopy (1D ¹H NMR spectroscopy).

MATERIALS AND METHODS

Chemicals and materials

Bovine κ -casein-derived glyco-macropptide (GMP) was provided by the FrieslandCampina Innovation Center (Wageningen, The Netherlands). N-Acetylneuraminic acid (Neu5Ac), 2-O-(4-methylumbelliferyl)- α -N-acetylneuraminic acid (4MU-Neu5Ac), and N-acetylneuraminyl-(α 2 \rightarrow 3)lactose (3'-SL) were obtained from Carbosynth Ltd (Compton, UK). Neuraminidase from *Clostridium perfringens* was obtained from Roche (Germany). Synthesis of glucosylated-lactose compounds (GL34),²⁰ galactosylated-lactulose compounds (LGOS)²¹ and sialylated Vivinal GOS (DP3 and DP4) compounds⁸ has been reported previously.

TcTS expression and purification

Escherichia coli BL21 (DE3) (Invitrogen, Carlsbad, CA, USA) was used as a host for expression of the *trans*-sialidase from *Trypanosoma cruzi*. Precultures of *E. coli* BL21 (DE3) harboring pTrcTS611/243 were cultured overnight at 30 °C.^{37,34} Terrific broth (TrB) with 12 g Tryptone, 24 g yeast extract and 4 mL glycerol containing 100 μ g/mL ampicillin was used for inoculation with 1 % preculture at 30 °C, 200 rpm. Expression of the *trans*-sialidase was induced using 0.2 mM isopropyl β -D-thiogalactopyranoside (IPTG) when cell density reached A_{600} between 0.4 to 0.6. Cultivation was continued at 18 °C for 4 h. The cells were collected by centrifugation (10 min, 4 °C, 10,000 x g) and washed with 50 mM Tris-HCl buffer,

pH 8.0. Cell resuspension by B-Per Tris solution (Thermo Scientific, Pierce) was followed by incubation at room temperature for 30 min. The *trans*-sialidase enzyme was purified by HIS-Select® Nickel Affinity Gel (Sigma, USA). After 1.5 h of binding at 4°C on a rotary shaker, the bound protein was consecutively washed with Tris-HCl buffer (50 mM, pH 8.0) containing NaCl (0.3 M) and imidazole (5 and 30 mM) prior to its elution with 300 mM imidazole in the same buffer. Purified TcTS enzyme was washed and concentrated in buffer Tris-HCl pH 8.0 using Millipore filter 50k.

Enzymatic incubations

TcTS (5 µg mL⁻¹) was incubated with various concentrations of the GL34 mixture (average DP 3) and 67.5 mg mL⁻¹ GMP (corresponding to 5 mM (α2→3)-linked Neu5Ac)³⁴ in 50 mM sodium citrate buffer pH 5.0 at 25 °C for 24 h. Aliquots of 10 µL were diluted with 190 µL DMSO 95 % for HPAEC-PAD analysis. The reactions were stopped by heating at 65 °C for 10 min.

TcTS (5 µg mL⁻¹) was incubated with various concentrations of the LGOS mixture (average DP 3) and 67.5 mg mL⁻¹ GMP (corresponding to 5 mM (α2→3)-linked Neu5Ac)³⁴ in 50 mM sodium citrate buffer pH 5.0 at 25 °C. TcTS (5 µg mL⁻¹) was added to the incubation mixture at t = 0 h and after each 24 h of incubation. Aliquots of 20 µL were sampled after 24 h, 48 h and 72 h of incubation and mixed with 380 µL DMSO 95 % for analysis by HPAEC-PAD profiling. The reactions were stopped by heating at 65 °C for 10 min.

Isolation of negatively charged oligosaccharides by Dowex chromatography

Dowex 1x8 chloride (Cl⁻) (Sigma–Aldrich, Steinheim, Germany) was packed in an Econo-column 1.5 cm x 10 cm (Biorad) and activated with 10 column volumes (10 CV) of NaOH 2 M (at least 1 h contact time). Before injection of samples the column was equilibrated with water for 10 CV. Elution of the sialylated oligosaccharides compounds was performed at a flow-rate of 1 mL min⁻¹ with

MilliQ water (MQ) and ammonium bicarbonate as eluents. After injection, unbound compounds were removed from the column by washing with MQ for 3 CV. Monosialylated and di-sialylated oligosaccharides were eluted by 3 CV of 50 and 400 mM ammonium bicarbonate, respectively. An extra elution step with 500 mM ammonium bicarbonate was used to wash off all remaining sialylated structures. After elution, the column was regenerated with 1 M sodium formate for 10 CV and washed with water. The collected fractions were lyophilized.

Desialylation of sialylated oligosaccharides

Fractions of sialylated LGOS were treated with acetic acid (20 %) for 1 h at room temperature, followed by neutralization by 1 M NaOH. Desialylated fractions were desalted using Carbograph SPE columns.

Sialylated Vivinal GOS fractions of DP3 and DP4 were desialylated by incubation with 1 U mL⁻¹ Neuraminidase (Roche, Germany) in 0.1 M acetate buffer pH 5.0 at 37 °C for 24 h.

HPAEC-PAD Chromatography

Oligosaccharide mixtures were analyzed by HPAEC-PAD profiling on a Dionex ICS-3000 system (Thermo Scientific, Amsterdam, The Netherlands), equipped with a CarboPac PA-1 column (250 x 2 mm; Dionex), and detected by a pulsed amperometric detector (PAD). A gradient of 30 to 600 mM sodium acetate in 0.1 M NaOH (0.25 mL min⁻¹) was used for analytical separation of acidic oligosaccharides. Another complex gradient of eluents A (100 mM NaOH), B (600 mM NaOAc in 100 mM NaOH), C (MilliQ water), and D (50 mM NaOAc) was used for profiling neutral oligosaccharide mixtures as previously described.²⁰

NMR spectroscopy

Structures of the transferred compounds were determined by 1D ¹H NMR recorded at a probe temperature of 25 °C on a Varian Inova 500 Spectrometer (NMR center, University of Groningen). The samples were exchanged twice with D₂O (99.9 atom% D, Cambridge Isotope Laboratories, Inc., Andover, MA) with intermediate

lyophilization and then dissolved in 0.65 mL D₂O, containing acetone as internal standard ($\delta^1\text{H}$ 2.225 ppm). Data was recorded at 16 k complex data points, and the HOD signal was suppressed using a WET1D pulse (500 MHz spectra). MestReNova 9.1.0 (Mestrelabs Research SL, Santiago de Compostela, Spain) was used to process NMR spectra, using Whittaker Smoother baseline correction.

RESULTS AND DISCUSSION

Previously, *N*-acetylneuraminic acid (Neu5Ac) was determined to be a major component (>99 %) of the 3.6 % (w/w) sialic acid in GMP, in comparison with *N*-glycolylneuraminic acid (Neu5Gc).³⁴ A concentration of 67.5 mg mL⁻¹ of GMP, corresponding to 5 mM ($\alpha 2 \rightarrow 3$)-linked Neu5Ac, was used as donor substrate for the incubations in this study. At this fixed concentration of GMP as donor substrate, the concentrations of the acceptor substrates necessary to obtain their maximal conversion degree were determined. All the incubations were carried out in 50 mM sodium citrate buffer pH 5.0 at 25 °C, the optimal conditions for TcTS as previously reported.^{34,38}

Sialylation of GL34 by TcTS

The mixture GL34 (average DP3) was incubated at concentrations of 1 mM, 5 mM and 10 mM, with 67.5 mg mL⁻¹ GMP and TcTS (5 $\mu\text{g mL}^{-1}$) at 25 °C and pH 5.0 for 24 h. After incubation the HPAEC-PAD profiles showed a new peak eluting at a retention time of ~14.5 min, which is in the retention-area of negatively charged oligosaccharides in this gradient (Figure 1-2).⁸ In the HPAEC-PAD profile of neutral oligosaccharides, only the F2 compound peak had a significantly decreased area (Figure 1-1). These results suggested that F2 was used as an acceptor substrate for *trans*-sialylation by TcTS. The signals of Neu5Ac($\alpha 2 \rightarrow 3$) H-3e at δ 2.755 and H-3a at δ 1.795 were detected in the 1D ¹H NMR spectrum of the GL34 mixture after the *trans*-sialylation reaction (Figure S1). The presence of a new signal at δ

4.212 is fitting with the 3-substitution at the terminal galactosyl residue of F2 with Neu5Ac (Figure S1), confirming the synthesis of Neu5Ac(α 2 \rightarrow 3)Gal(β 1 \rightarrow 4)[Glc(α 1 \rightarrow 2)] Glc (Scheme 2). Based on the HPAEC-PAD responses, the maximal conversion of F2 into the corresponding sialylated-F2 was observed with 10 mM GL34 and calculated as 47.6%. The data shows that only F2 was used as an acceptor substrate for *trans*-sialylation by TcTS. In the GL34 mixture, F2 is the only compound with an accessible β -Gal residue at a non-reducing terminal position (Scheme 1). TcTS was shown to also glycosylate internal β -Gal residues, in specific structures, i.e. in a Gal(β 1 \rightarrow 6)Gal- epitope,^{27,30} but these are absent in GL34. F1 4'-glc-lac and F4 2,4'-glc-lac, the only other GL34 compounds with non-substituted OH-3 positions (but on the internal galactose residue; Scheme 1),²⁰ were not used as acceptor substrates.

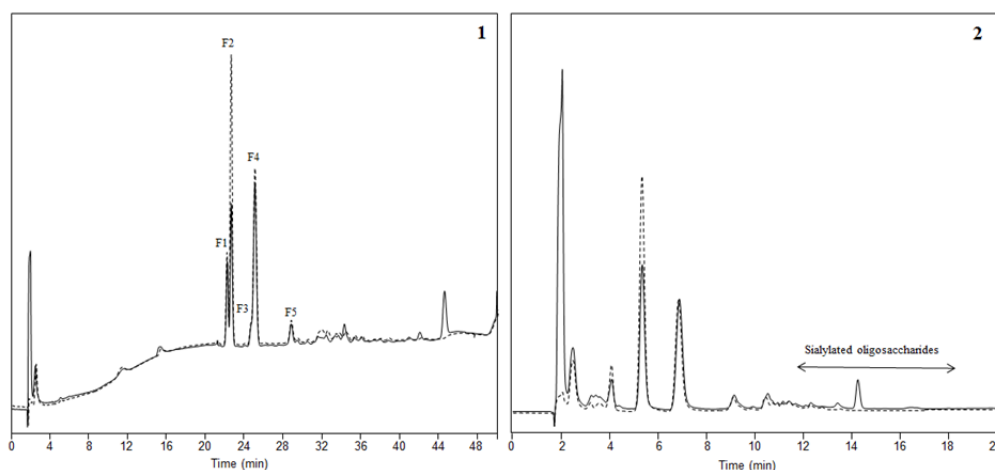
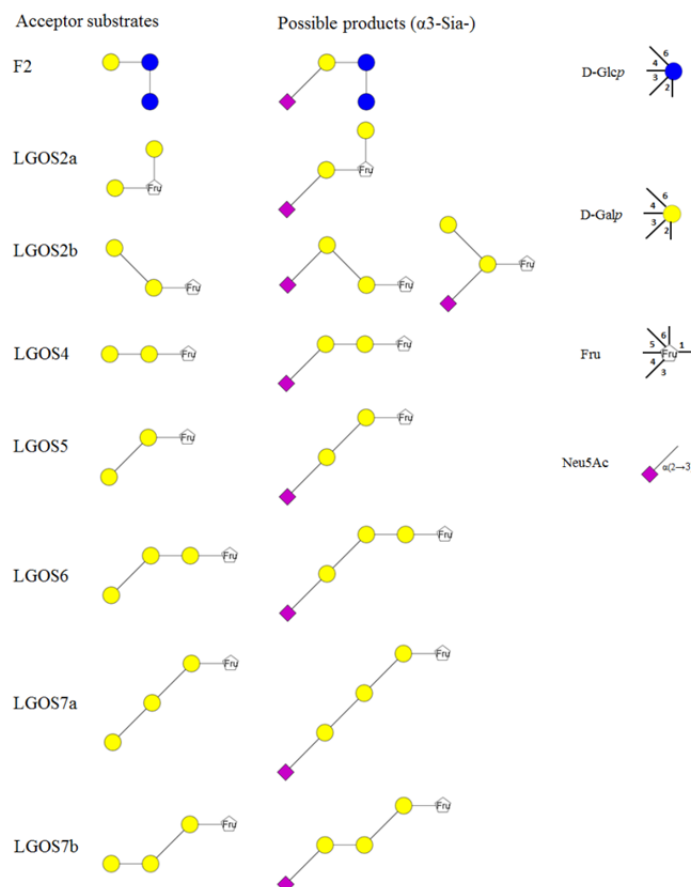


Figure 1: HPAEC-PAD profiles of compounds in the GL34/GMP/TcTS reaction mixture (incubation at 25 °C and pH 5.0) at t = 0 h (dotted line) and t = 24 h (solid line) using a CarboPac PA-1 column with gradient 1) for neutral oligosaccharides and 2) for acidic oligosaccharides.



Scheme 2: Schematic presentation of the ($\alpha 2 \rightarrow 3$)-sialylation product of F2 (GL34 mixture); and the possible structures of the 5-8 ($\alpha 2 \rightarrow 3$)-sialylation products of the LGOS mixture.

Sialylation of LGOS by TcTS

Various concentrations of the LGOS mixture (1 mM, 5 mM, 10 mM and 15 mM) were incubated with 67.5 mg mL^{-1} GMP as donor substrate and with TcTS ($5 \text{ } \mu\text{g mL}^{-1}$) at $25 \text{ } ^\circ\text{C}$ and pH 5.0. Because of the relatively low stability of this *trans*-sialidase,³⁸ extra TcTS ($5 \text{ } \mu\text{g mL}^{-1}$) was added to the incubation mixtures after every 24 h of incubation. The incubation experiments were followed over time, and the highest conversion degree of LGOS into sialylated LGOS was $\sim 52 \%$ after 48 h with

1 mM of the LGOS mixture (Figure 2). At this LGOS concentration the conversion degree increased significantly from 37.4 % to 52.0 % when the incubation lasted from 24 h to 48 h (Figure 2). In all cases the GMP-derived Neu5Ac(α 2 \rightarrow 3) as donor substrate was not completely utilized, with a maximal use of 80 % when incubated with 15 mM LGOS for 24 h. Enhanced conversion degrees were not observed when incubating other concentrations of the LGOS mixture longer than 24 h despite renewed addition of TcTS (Figures S2 – S4).

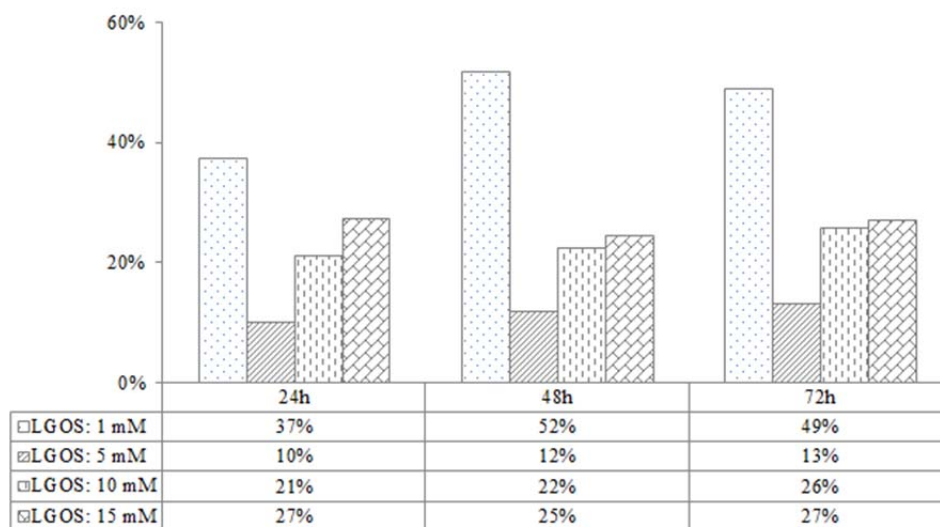


Figure 2: Conversion of the LGOS mixture compounds into sialylated oligosaccharides at different concentrations of LGOS and various incubation times, with renewed addition of TcTS after each 24 h. Data obtained from HPAEC-PAD responses (in duplicate).

The HPAEC-PAD profiles of the incubation mixtures with 1 mM LGOS showed development of several new peaks in time (Figure 3). These new peaks eluted at retention times between 12 - 22 min, indicating synthesis of a complex mixture of sialylated LGOS. The negatively charged (Sia-LGOS) oligosaccharides were separated from the neutral (LGOS) oligosaccharides by Dowex 1x8 (Cl⁻) chromatography and then re-analyzed by HPAEC-PAD profiling (Figure 4). The

neutral oligosaccharides in the unbound Dowex fraction eluted during the first 12 min in the HPAEC-PAD profile (Figure 4-1). The Dowex fraction that eluted with 50 mM ammonium bicarbonate (Sia-LGOS) eluted between 12 min and 18 min in the HPAEC-PAD profile (Figure 4-2), fitting with mono-sialylated structures.⁸ The Dowex fraction containing di-sialylated structures was relatively minor, limiting possibilities for further characterization.

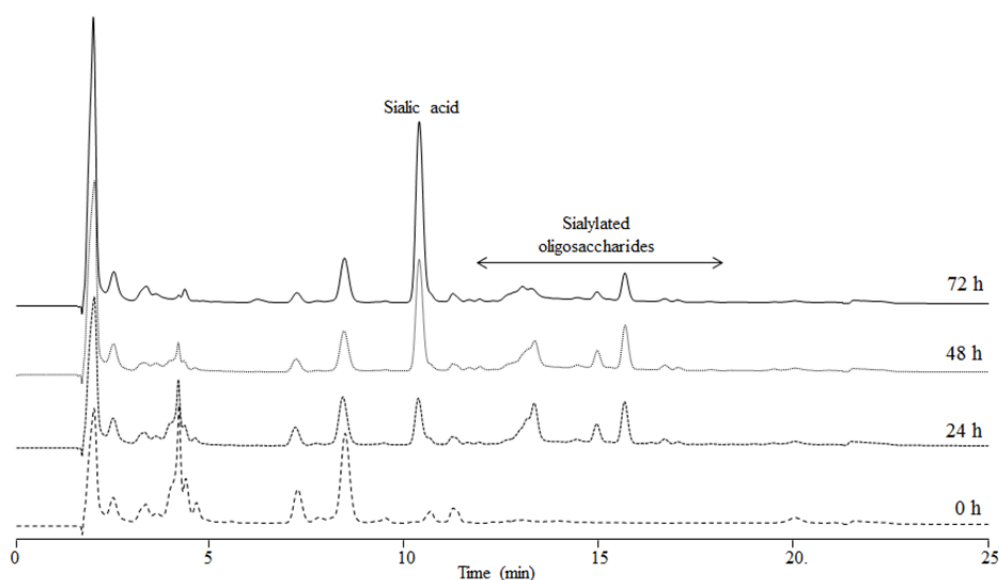


Figure 3: HPAEC-PAD profiles of compounds in the reaction mixtures of 1 mM LGOS/5 mM GMP-derived Neu5Ac(α 2 \rightarrow 3)/5 μ g mL⁻¹ TcTS, incubated at 25 °C and pH 5.0 for 0-72 h, with renewed addition of TcTs (5 μ g mL⁻¹) after each 24 h of incubation. Neutral LGOS and negatively charged Sia-LGOS eluted at 2-12 min and 12-18 min, respectively.

The 1D ¹H NMR spectrum (Figure S5) of the negatively charged fraction revealed signals at δ 2.760 and δ 1.803 which belong to the Neu5Ac H-3e and H-3a atoms, respectively, of Neu5Ac(α 2 \rightarrow 3) residues.³⁹ These NMR spectroscopy data confirmed the sialylation of LGOS by TcTS. To identify the compounds in the LGOS mixture that were decorated with Neu5Ac, desialylation of these sialylated-LGOS was carried out using 20 % acetic acid.

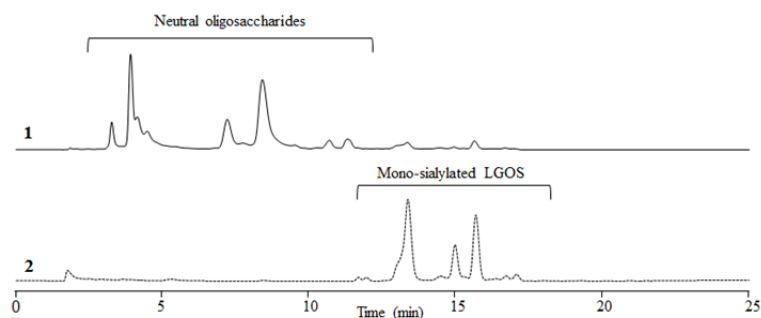


Figure 4: HPAEC-PAD profiles of the Dowex 1x8 (Cl⁻) chromatography fractions obtained by 1) MQ rinsing (neutral oligosaccharides), and by 2) elution with 50 mM ammonium bicarbonate (Sia-LGOS). The reaction mixture of 1 mM LGOS/5 mM GMP-derived Neu5Ac(α 2 \rightarrow 3)/TcTS (10 μ g mL⁻¹), incubated at 25 °C and pH 5.0 for 48 h, was used for Dowex chromatography.

Comparison of the HPAEC-PAD profiles of the desialylated fraction with that of the LGOS mixture, allowed identification of at least five structures that were mono-sialylated by TcTS, i.e. LGOS2a and/or 2b, LGOS4, LGOS5, LGOS6, LGOS7a and/or 7b (Figure 5). In the LGOS profile structures LGOS4 and LGOS5 were the major components, after sialylation and desialylation the HPAEC-PAD profile showed LGOS6 and LGOS7 to be the predominant structures. In the LGOS mixture LGOS6 is only a trace peak, but in the sialylated fraction LGOS6 is the major structure. This indicated that the Gal(β 1 \rightarrow 3)Gal(β 1 \rightarrow 4) epitope is very favorable for sialylation. The LGOS7 peak consisted of two structures, one with a Gal(β 1 \rightarrow 3)Gal(β 1 \rightarrow 3) epitope and one with a Gal(β 1 \rightarrow 4)Gal(β 1 \rightarrow 3) epitope. Although it is not possible to distinguish between the two structures, it is likely that structure LGOS7a, with a terminal Gal(β 1 \rightarrow 3) residue, is the mainly sialylated LGOS7 structure. This fits with previous results on galactosyl-lactose conversions, showing a much higher specificity constant (k_{cat}/k_M) for the transferase reaction to 3'-galactosyllactose than to 4'-galactosyllactose and 6'-galactosyllactose.³⁴ Closer inspection of the 1D ¹H NMR profile of the Sia-LGOS fractions revealed the B^f signals at δ 4.200 – 4.211, which originate from the LGOS4, LGOS5, LGOS6

and/or LGOS7 structures (slightly shifted). This provided evidence for the presence of the LGOS4, LGOS5, LGOS6 and/or LGOS7 compounds in the Sia-LGOS mixture. Moreover, the ^1H NMR spectrum of this mixture showed anomeric signals C-1 (slightly shifted) at δ 4.694, δ 4.650, δ 4.629 from the structures LGOS4-7.

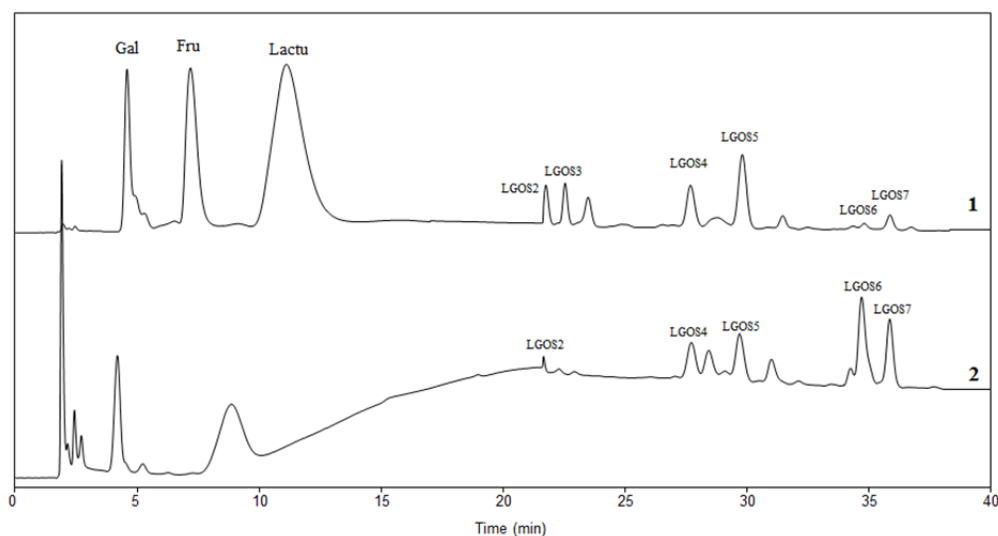


Figure 5: HPAEC-PAD profiles of compounds in 1) the LGOS mixture and the 2) Sia-LGOS fraction after being desialylated by acetic acid 20% treatment. Identified peaks are marked corresponding to the structures shown in Scheme 2. Peak 2 corresponds to the LGOS2a and/or LGOS2b structures, peak 7 corresponds to the LGOS7a and/or LGOS7b structures.

In LGOS5 ($\text{Gal}(\beta 1 \rightarrow 3)\text{Gal}(\beta 1 \rightarrow 4)\text{Fru}$) and LGOS7a ($\text{Gal}(\beta 1 \rightarrow 3)\text{Gal}(\beta 1 \rightarrow 3)\text{Gal}(\beta 1 \rightarrow 4)\text{Fru}$), the O-3 positions of the internal β -Gal residue are already substituted, only the terminal β -Gal residues of LGOS5 and LGOS7a are available for ($\alpha 2 \rightarrow 3$)-linked decoration with Neu5Ac to yield the corresponding mono-sialylated oligosaccharides: Neu5Ac($\alpha 2 \rightarrow 3$)Gal($\beta 1 \rightarrow 3$)Gal($\beta 1 \rightarrow 4$)Fru and Neu5Ac($\alpha 2 \rightarrow 3$)Gal($\beta 1 \rightarrow 3$)Gal($\beta 1 \rightarrow 3$)Gal($\beta 1 \rightarrow 4$)Fru (Scheme 2). The structure LGOS4 ($\text{Gal}(\beta 1 \rightarrow 4)\text{Gal}(\beta 1 \rightarrow 4)\text{Fru}$) was only mono-sialylated by TcTS although it

also possesses a non-substituted O-3 of the internal β -Gal residue. This was also observed for the similar structure β 4'-galactosyl-lactose of which only the terminal β -Gal residue was (α 2 \rightarrow 3)-substituted with Neu5Ac.³⁴ The structure LGOS2a with one terminal β -Gal residue and one 4-substituted internal β -Gal residue is most likely only mono-sialylated (Scheme 2). The di-sialylated LGOS fraction was too minor to be elucidated. In the LGOS mixture, only LGOS1, LGOS2a and LGOS3 with two terminal β -Gal residues, as well as LGOS2b with an internal β -Gal residue linked (β 1 \rightarrow 6) with a terminal β -Gal residue, are likely di-sialylated.³⁴

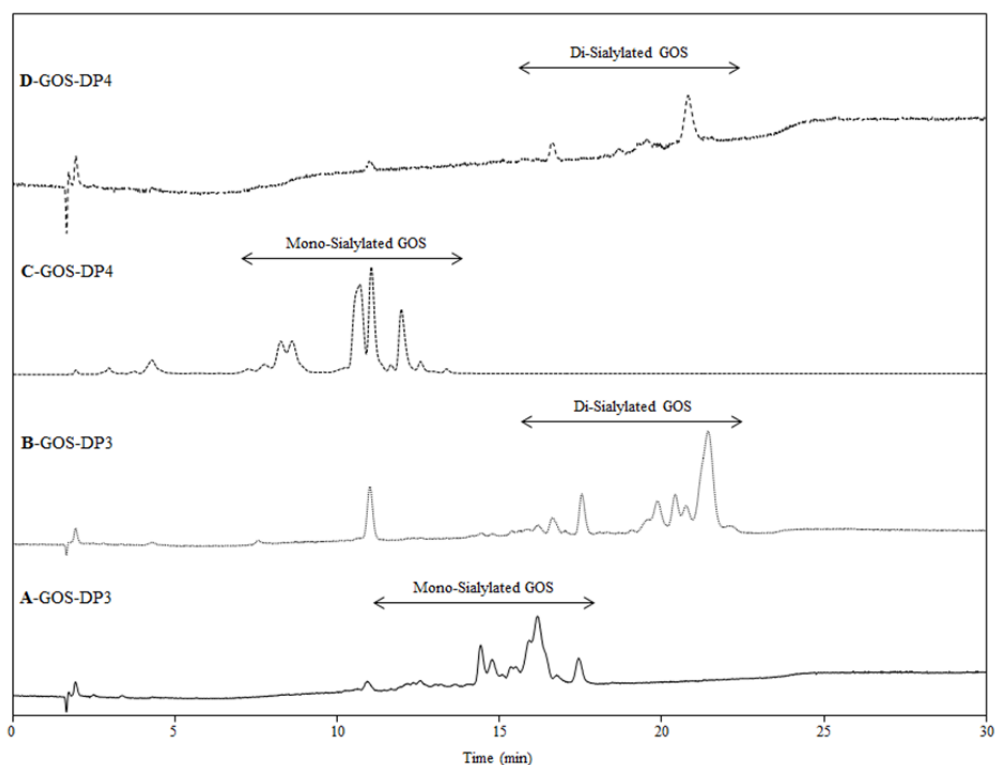


Figure 6: HPAEC-PAD profiles of the Sia-GOS DP3 (A,B) and DP4 (C,D) fractions. Mono- and di-Sialylated-GOS peaks are marked.

Sialylation of GOS by TcTs

In our previous work, the Vivinal GOS DP3 and DP4 fractions were sialylated using TcTS.⁸ These sialylated mixtures were applied onto a Resource Q anion exchange chromatography column to obtain the separate mono-sialylated- and di-sialylated-GOS fractions, depending on their negative charges.⁸ The HPAEC-PAD profiles of the sialylated GOS DP3 fraction showed the presence of multiple mono-Sia-GOS compounds at retention times between 11 - 17 min, and di-Sia-GOS compounds at retention times between 16 - 23 min (Figures 6A and 6B). Similarly, in the HPAEC-PAD profiles of the Sia-GOS DP4 fraction, the mono-Sia-GOS compounds eluted at retention times between 8 -13 min, and the di-Sia-GOS compounds eluted at retention times between 17 - 23 min (Figures 6C and 6D). The Sia-GOS mixtures were incubated with Neuraminidase from *Clostridium perfringens*, which prefers to hydrolyze ($\alpha 2 \rightarrow 3$)-linkages over ($\alpha 2 \rightarrow 6$)- and ($\alpha 2 \rightarrow 8$)-linked sialic acids, to remove the sialic acid groups attached to the GOS compounds. The HPAEC-PAD profiles of the desialylated fractions were compared with the Vivinal GOS mixture, which were previously annotated,^{40,41} in order to identify decorated structures (Figures 7-A1 and 7-B1). In the GOS DP3 fraction, at least five structures were mono-sialylated, namely GOS6a and/or 6b, GOS9, GOS10a and/or 10b, GOS11 and GOS12 (Figure 7-A2). At least three of these seven structures were also found in the di-Sia-GOS fraction, namely GOS6a and/or 6b, GOS9, GOS10a and/or 10b (Figure 7-A3). In the GOS DP4 fraction, the structures GOS14a and/or 14b, GOS15, GOS16, GOS17 and GOS18 were found to be mono-sialylated (Figure 7-B2) and the structures GOS14a, 14b, 15 and 16 were found to be di-sialylated (Figure 7-B3). The possible positions of sialic acid ($\alpha 2 \rightarrow 3$)-linked to these GOS compounds are presented in Scheme 3. As expected, the structures GOS6a; GOS9, GOS10a and/or 10b, GOS14a, GOS14b, GOS15a and/or 15b, GOS16a and/or 16b with two unsubstituted terminal β -Gal residues at O-3 position were either mono- or di-sialylated by TcTS.³⁴ The di-sialylation of the structure GOS6b (with an internal β -

Gal residue linked ($\beta 1 \rightarrow 6$) with a terminal β -Gal residue) by this *trans*-sialidase was already observed in a previous study (Scheme 3).³⁴ The linear Gal($\beta 1 \rightarrow 4$)Gal-epitope present in the structures GOS11, 16c, 17, 18a and 18b allowed only the terminal β -Gal residue to be sialylated by TcTS, resulting in only mono-sialylation for these types of structures (Scheme 3).

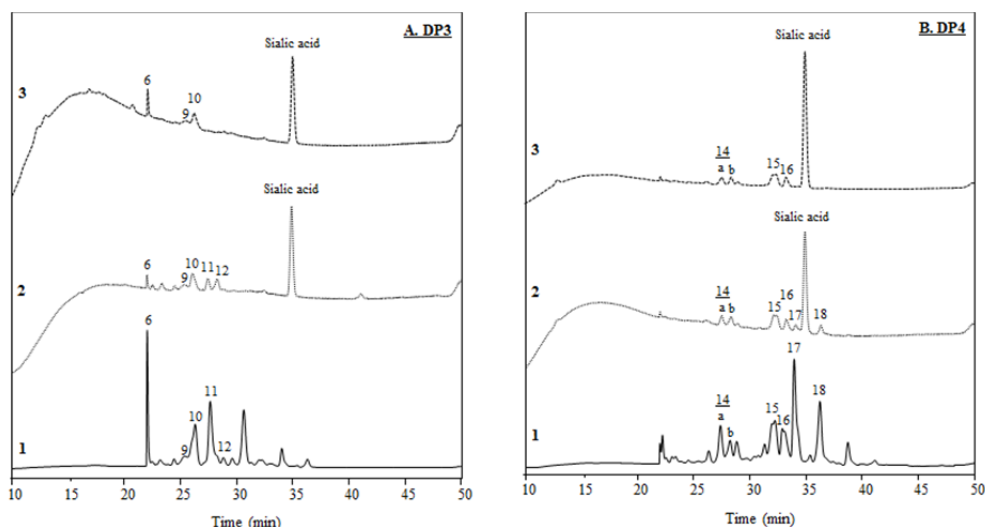
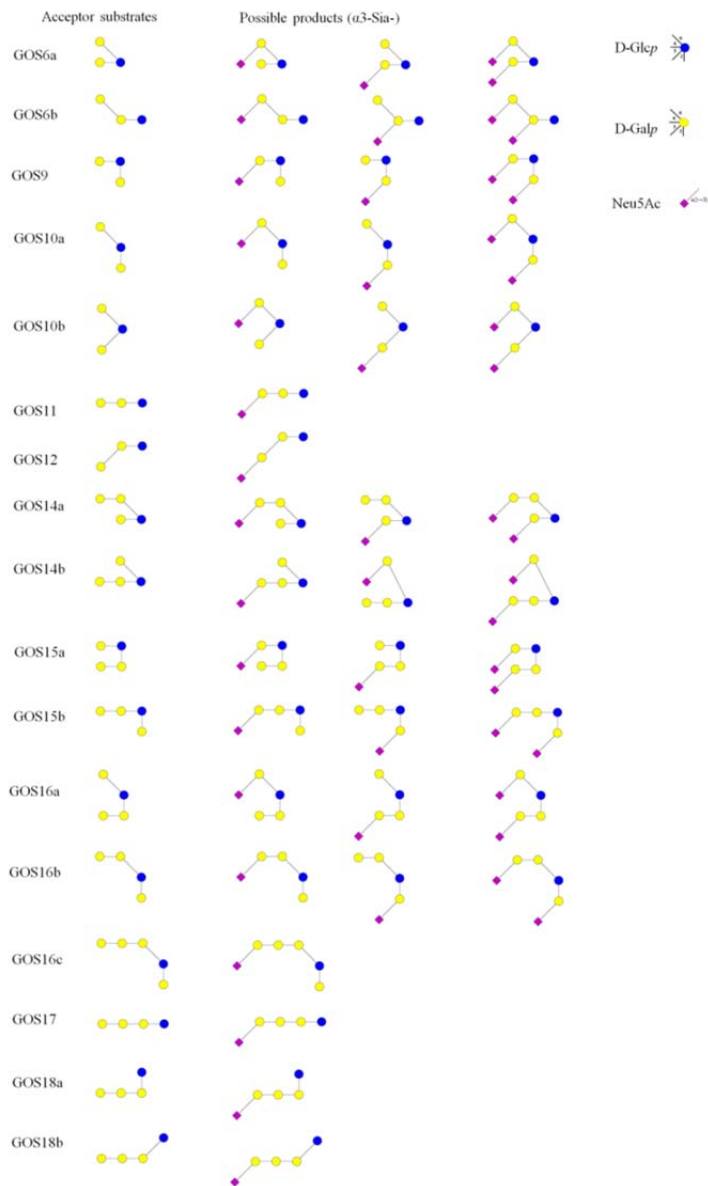


Figure 7: HPAEC-PAD profiles of compounds from Sialylated-GOS fractions A.DP3 and B.DP4: 1) the neutral GOS mixtures at corresponding DP; 2) the mono-Sia-GOS and 3) di-Sia-GOS fractions after being desialylated. Identified GOS peaks are marked with numbers as used by van Leeuwen et al,⁴⁰ corresponding with those in Scheme 3. Peak 6 corresponds to GOS6a and/or 6b; peak 10 corresponds to GOS10a and/or 10b; peak 15 corresponds to GOS15a and/or 15b; peak 16 corresponds to GOS16a and/or 16b and/or 16c; and peak 18 corresponds to GOS18a and/or 18b.

Close inspection of the HPAEC-PAD profiles (Figure 7A) showed in the Vivinal GOS DP3 fraction only trace amounts of GOS12 (3'-galactosyllactose) and a major peak for GOS11 (4'-galactosyllactose). After desialylation of the mono-sialylated DP3 pool, approximately equal amounts of GOS11 and GOS12 are observed. This fits with observations on LGOS and previous work, showing a higher specificity constant of TcTS towards 3'-galactosyllactose than to 4'-galactosyllactose.³⁴ Also, in the DP4 fraction (Figure 7B), the linear structures with terminal Gal($\beta 1 \rightarrow 4$)

residues GOS17 and GOS18 showed relatively low peaks, compared to the Vivinal GOS DP4 pool, whereas the branched structures were relatively increased.



Scheme 3: Schematic presentation of the possible structures of the 16-39 (α 2 \rightarrow 3)-sialylation products of the Vivinal GOS DP3 and DP4 fractions.

CONCLUSIONS

Trans-sialidase from *T. cruzi* was used to transfer sialic acid to oligosaccharides (DP3-4) in the GL34, LGOS and Vivinal GOS mixtures.⁸ Decorated structures were identified by HPAEC-PAD chromatography and NMR spectroscopy. As expected, various compounds in these mixtures with one or multiple accessible β -Gal-OH-3 groups were used as acceptor substrates by TcTS. The F2 (2-glc-lac) compound in the GL34 mixture was mono-sialylated yielding α 3Sia-2-glc-lac with a conversion degree of 47.6 %. TcTS was able to transfer sialic acid to at least five different compounds in the LGOS mixture with a conversion degree of up to 52 %. The conversion of galacto-oligosaccharides (GOS) with DP3-4 (3 mM GOS with 6 mM (α 2 \rightarrow 3)-linked Neu5Ac) into Sia-GOS by TcTS was clearly lower, at about 35 %, but obtained at different conditions.⁸ The optimal concentrations of the GL34 and LGOS mixtures for maximal conversion by TcTS ($10 \mu\text{g mL}^{-1}$) in the incubations with 5 mM (α 2 \rightarrow 3)-linked Neu5Ac (from GMP) were 10 mM and 1 mM, respectively. In fact, all structures the LGOS mixture possess terminal non-reducing β -Gal residue, only the F2 compound of the GL34 mixture has a terminal β -Gal residue. Previously, only lactulose was used as an acceptor substrate for a mutant *trans*-sialidase Tr13 from *T. rangeli*.⁴² The GOS mixture has been known to provide multiple C-3 hydroxyl groups and to be an easily accessible substrate for *trans*-sialidase including TcTS (acceptor) sites.^{9,8,43} Our study showed that in fact most of GOS structures of DP3 and DP4 from Vivinal GOS were sialylated by TcTS. Moreover, the results revealed a strong preference for terminal β -Gal residues to be sialylated. And only branched compounds with two non-reducing terminal β -Gal residues were di-sialylated. The only exception known so far is 6'-galactosyllactose, which is linear with a specific Gal(β 1 \rightarrow 6)Gal- epitope that could be di-sialylated by TcTS.³⁴ Moreover, our study showed that structures with a Gal(β 1 \rightarrow 3) terminal residue were more efficiently sialylated by TcTS.

In conclusion, the data shows that enzymatic synthesis of sialylated lactose and lactulose derived oligosaccharides, using the TcTS enzyme and (α 2 \rightarrow 3)-Neu5Ac from GMP as donor substrate, yields a highly interesting variety of sialylated oligosaccharides. This transfer of sialic acid as functional group is a first step in developing *h*MOS mimicking compounds. In future studies we aim to optimize their biosynthesis and to evaluate the potential use of these novel compounds for pathogen inhibition, and preventing NEC.

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Supplemental data

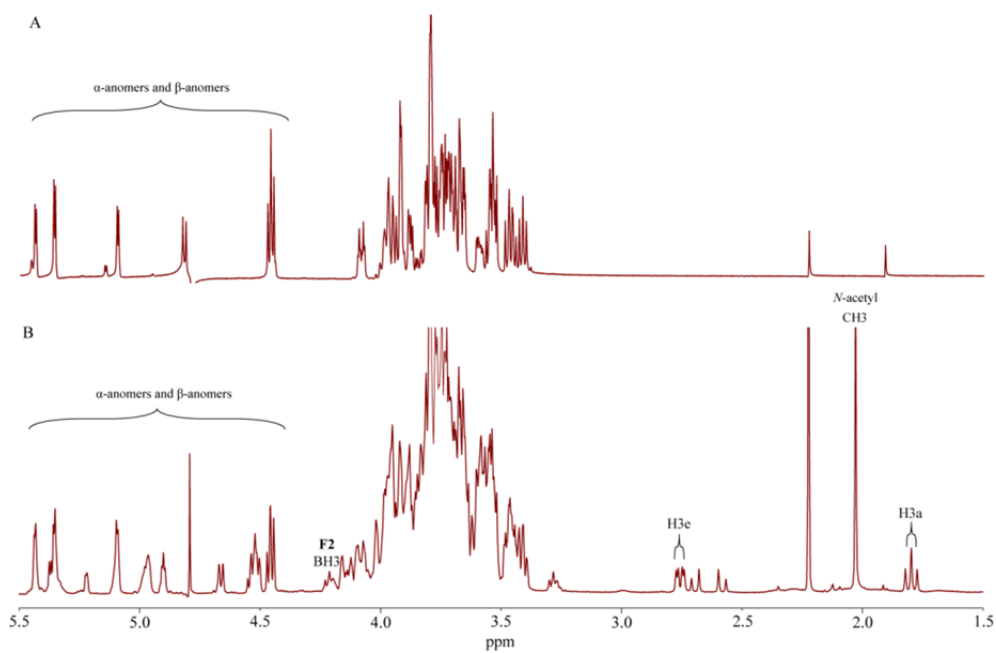


Figure S1: 500 MHz ^1H NMR spectra, recorded in D_2O at 25°C of A) structure F2 2-glc-lac and B) the GL34 mixture after *trans*-sialylation incubation of 10 mM GL34 plus 5 mM GMP-derived Neu5Ac($\alpha 2\rightarrow 3$) with TcTS ($10\ \mu\text{g mL}^{-1}$) at 25°C for 24 h.

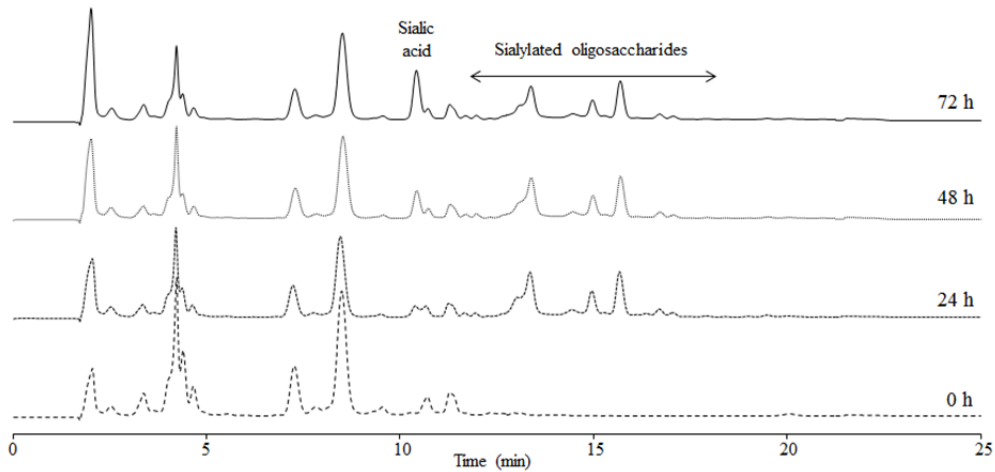


Figure S2: HPAEC-PAD profiles of compounds in the reaction mixtures of 5 mM LGOS/5mM GMP-derived Neu5Ac(α 2 \rightarrow 3)/5 μ g mL⁻¹ TcTS at 25 °C and pH 5.0 after various incubation times, and renewed addition of TcTS (5 μ g mL⁻¹) after each 24 h of incubation.

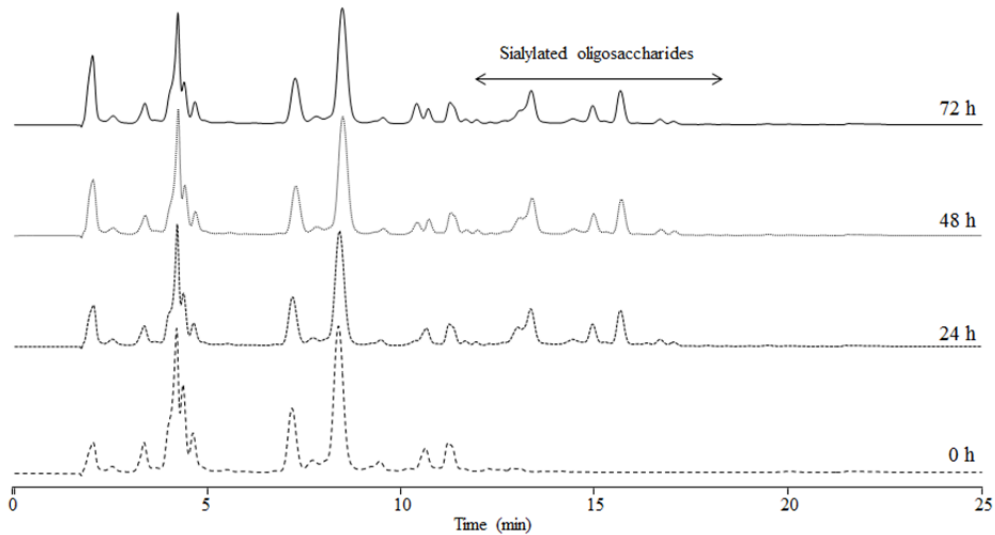


Figure S3: HPAEC-PAD profiles of compounds in the reaction mixtures of 10 mM LGOS/5mM GMP-derived Neu5Ac(α 2 \rightarrow 3)/5 μ g mL⁻¹ TcTS at 25 °C and pH 5.0 after various incubation times, and renewed addition of TcTS (5 μ g mL⁻¹) after each 24 h of incubation.

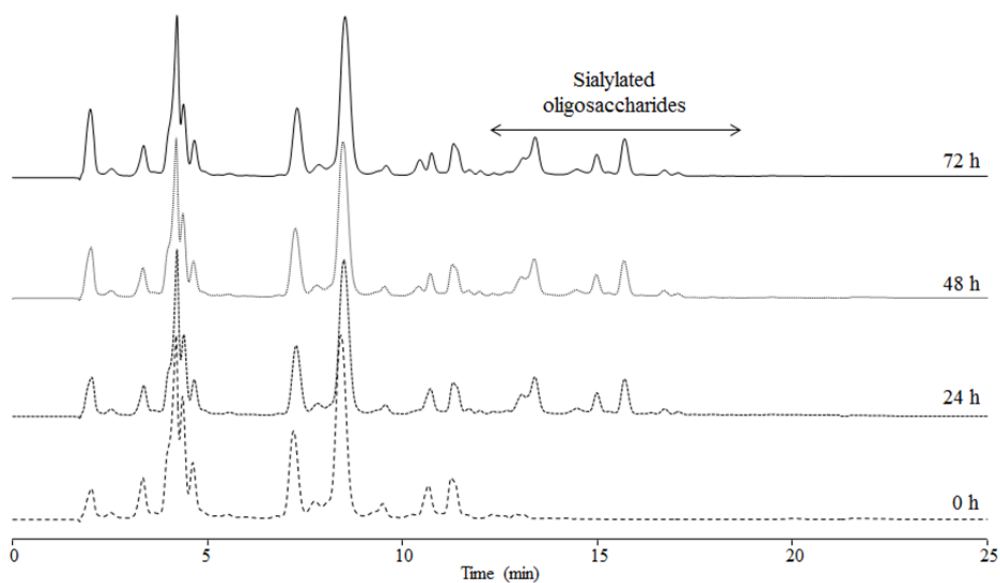


Figure S4: HPAEC-PAD profiles of compounds in the reaction mixtures of 15 mM LGOS/5mM GMP-derived Neu5Ac(α 2 \rightarrow 3)/5 μ g mL⁻¹ TcTS at 25 °C and pH 5.0 after various incubation times, and renewed addition of TcTS (5 μ g mL⁻¹) after each 24 h of incubation.

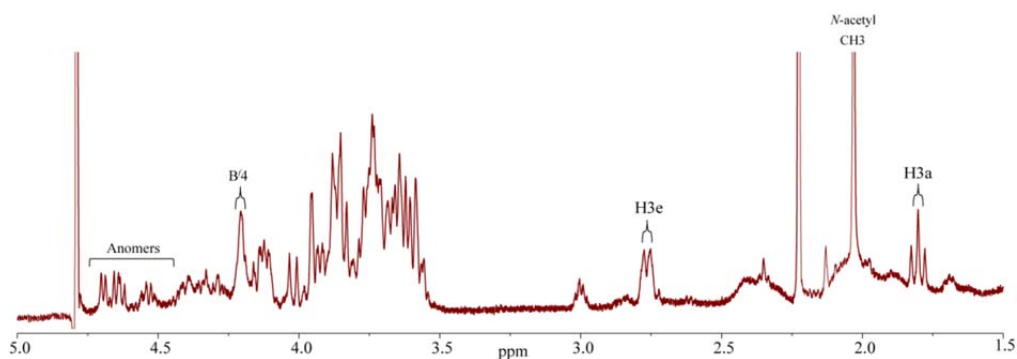


Figure S5: 500 MHz ¹H NMR spectra, recorded in D₂O at 25 °C of the Dowex 1x8 (Cl⁻) Sia-LGOS fraction eluted by 50 mM ammonium bicarbonate from the incubation of 1 mM LGOS plus 5 mM GMP-derived Neu5Ac(α 2 \rightarrow 3) with TcTS (10 μ g mL⁻¹) at 25 °C for 48 h.

Chapter 7

Summary and Perspectives

Human milk oligosaccharides (*hMOS*) have an essential role in infants' health by exerting prebiotic effects, leading to the growth of health-beneficial gut bacteria for the human host. In addition, *hMOS* also directly reduce pathogenic microbial infections by serving as antiadhesive antimicrobials, and they stimulate immune responses. Nowadays, many babies have limited access to human milk, and an alternative source for *hMOS* is currently not available in nature. Infant formula with the commercial prebiotics galacto-oligosaccharides (GOS) and fructo-oligosaccharides (FOS) added, lack the pathogen exclusion and immune- and barrier modulating effects exerted by *hMOS*. The development of efficient routes for the synthesis of *hMOS* or structurally/functionally effective *hMOS* mimics is thus highly demanded for application in infant formula. At present whole cell biosynthetic routes (*in vivo*) and single/multiple enzyme biocatalytic systems (*in vitro*) for synthesis of *hMOS* (mimics) are at the focus of attention (**Chapter 1**).

In this project we have used glucansucrases from *Lactobacillus reuteri* to synthesize mixtures of glucosylated lactose compounds from lactose or GOS as acceptor substrates (**Chapters 2 and 5**). The results showed that the normal linkage specificity of glucansucrase Gtf180- Δ N from *L. reuteri* became altered when acting on galactose-containing compounds. Mutational analysis of this enzyme was used (**Chapter 4**) to elucidate the roles of individual amino acid residues in its acceptor binding subsites in lactose binding. The growth stimulatory (prebiotic) effects of this newly synthesized oligosaccharide mixture on various gut bacteria was evaluated in **Chapter 3**. Finally, in **Chapter 6**, sialylated-oligosaccharides were synthesized using *trans*-sialidase from *Trypanosoma cruzi*, and their structures were characterized in detail.

***Trans*-glucosylation of lactose by the *Lactobacillus reuteri* GtfA- Δ N and Gtf180- Δ N glucansucrases**

Glucansucrases are well known for their ability to use a large variety of acceptor substrates to produce oligosaccharides with prebiotic potential.^{1,2,3} We investigated the ability of glucansucrase enzymes Gtf180- Δ N and GtfA- Δ N from *Lactobacillus reuteri* strains 180 and 121, respectively, to decorate the lactose acceptor substrate using sucrose as donor substrate. In these *trans*-glucosylation reactions, GtfA- Δ N and Gtf180- Δ N synthesized the same set of mono- and di-glucosylated lactose compounds (GL34) from lactose as acceptor substrate. Three mono-glucosylated lactose and two di-glucosylated lactose derivatives were isolated from the reaction mixtures and structurally identified as α -D-Glcp-(1 \rightarrow 4)- β -D-Galp-(1 \rightarrow 4)-D-Glcp (**F1**), α -D-Glcp-(1 \rightarrow 2)-[β -D-Galp-(1 \rightarrow 4)-]D-Glcp (**F2**), α -D-Glcp-(1 \rightarrow 3)- β -D-Galp-(1 \rightarrow 4)-D-Glcp (**F3**), α -D-Glcp-(1 \rightarrow 4)- β -D-Galp-(1 \rightarrow 4)-[α -D-Glcp-(1 \rightarrow 2)-]D-Glcp (**F4**) and α -D-Glcp-(1 \rightarrow 3)- β -D-Galp-(1 \rightarrow 4)-[α -D-Glcp-(1 \rightarrow 2)-]D-Glcp (**F5**). When using sucrose as donor and acceptor substrate, GtfA- Δ N synthesizes glucan with mainly (α 1 \rightarrow 4)/(α 1 \rightarrow 6) glucosidic linkages;⁴ with lactose as acceptor substrate this enzyme introduced (α 1 \rightarrow 4) but also (α 1 \rightarrow 2) and (α 1 \rightarrow 3) glucosidic linkages. Similarly, Gtf180- Δ N produces an α -glucan with 69% (α 1 \rightarrow 6) and 31% (α 1 \rightarrow 3) linkages from sucrose,⁵ but with lactose as acceptor substrate this enzyme synthesized (α 1 \rightarrow 2), (α 1 \rightarrow 3) and (α 1 \rightarrow 4) glucosidic linkages. The full assignment of the NMR spectra of three trisaccharides (**F1-F3**) and two tetrasaccharides (**F4** and **F5**) in the GL34 mixture were reported for the first time. Lactose derivatives are interesting potential prebiotic compounds, especially those containing (α 1 \rightarrow 2)-linkages. Such compounds and linkages are known to be highly resistant to the digestive enzymes in the human gut,⁶ and may selectively stimulate the growth of health-beneficial gut microbiota.²⁷ Therefore, in **Chapter 3**, we studied the growth stimulatory effects of the GL34 mixture on various gut bacteria.

Stimulatory effects of the GL34 mixture on growth of selected gut bacteria

A prebiotic is defined as “a substrate that is selectively utilized by host microorganisms conferring a health benefit”.⁸ The GL34 mixture meets the first requirement given that compounds **F1-F5** are resistant to common carbohydrate-active enzymes including α -amylases from various sources, α -glucosidase, isoamylase and pullulanase. Only **F2** (2-glc-lac) was partially consumed by microbial β -galactosidases. Three groups of gut bacteria including three *Bifidobacterium* strains, three *Lactobacillus* strains and two commensal bacterial strains were grown using GL34 as only carbon source. This mixture showed different stimulatory effects on the growth of *B. breve* DSM 20123, *B. adolescentis* ATCC 15703 and *B. infantis* ATCC 15697. Amongst those strains, *B. adolescentis* grew very well on the GL34 mixture, the final OD₆₀₀ value reached 80% of that on a 100% control growing on lactose and GOS. However, the final OD₆₀₀ of *B. breve* DSM 20123 and *B. infantis* ATCC 15697 on GL34 were below 50% of the values observed when these strains were grown on lactose, purified GOS mixture and GOS/FOS mixture. In case of the *Lactobacillus* strains, all three tested strains grew relatively weakly on the media with GL34 as the only carbon source, clearly unable to consume all compounds present, and the final OD₆₀₀ values of *L. casei* W56, *L. reuteri* 121 and *L. acidophilus* ATCC 4356 were only 3.8%, 26.5% and 10.4% respectively, compared to their 100% controls grown on glucose. Also the growth of commensal bacteria on these oligosaccharides in the GL34 mixture was studied. *Bacteroides thetaiotaomicron* and *Escherichia coli* Nissle showed slight and slow growth on the media using GL34 as only carbon source. The GL34 mixture thus promotes growth of the tested bacteria to different extents. The bifidobacteria tested generally were better at degrading GL34 compounds than the lactobacilli and commensal bacteria. Individual gut bacteria were able to utilize only specific compounds in the GL34 mixture. Synergistic activities between bacterial species may be essential for the *in vivo* utilization of the whole GL34 mixture. **F2** (2-glc-lac) stimulated growth of the

probiotic bacteria *L. reuteri* 121, *B. adolescentis* ATCC 15703, *B. longum* subsp. *infantis* ATCC 15697, *B. breve* DSM 20213, and also of two commensal bacteria, *E. coli* Nissle and *B. thetaiotaomicron*, albeit to various extents. This **F2** compound is less selective in comparison with the other compounds in the GL34 mixture. The compounds **F1** (4'-glc-lac), **F4** (4',2-glc-lac) and **F5** (3',2-glc-lac) stimulated growth of all three tested bifidobacteria, again to various extents. The more selective compound **F3** (3'-glc-lac) was utilized by only two out of three studied *Bifidobacterium* strains, *B. adolescentis* ATCC 15703 and *B. breve* DSM 20213. In conclusion, GL34 represents a novel oligosaccharide mixture with (potential) synbiotic properties toward *B. adolescentis*, synthesized from cheap and abundantly available lactose and sucrose.

Mutational analysis of the role of Gtf180-ΔN active site residues in product and linkage specificity with lactose as acceptor substrate

In **Chapter 2**, we observed that when acting on lactose as acceptor substrate glucansucrase Gtf180-ΔN introduced new linkage types $[(\alpha 1 \rightarrow 2)/(\alpha 1 \rightarrow 4)]$, compared to the normal linkage types $[(\alpha 1 \rightarrow 3)/(\alpha 1 \rightarrow 6)]$ when this enzyme acts on sucrose alone, or on other acceptor substrates studied. In **Chapter 4** docking experiments with lactose in a glucosyl-enzyme intermediate using the crystal structure of *L. reuteri* 180 Gtf180-ΔN were carried out in order to understand how the acceptor substrate lactose binds in the Gtf180-ΔN active site and which amino acids maybe essential in binding lactose. Three amino acid residues (Q1140, W1065 and N1029) were found to be in close proximity of the lactose acceptor substrate and may therefore be involved in the orientation of lactose in the acceptor subsite and influence the linkage type preference. Notably, all three residues are fully conserved within glucansucrases, and they are known to play an important role in the *trans*-glycosylation reaction.⁹ Mutagenesis of these residues resulted in significant changes in the GL34 **F1-F5** product ratios. Q1140 mutants showed a clear decrease

in **F3** with an ($\alpha 1 \rightarrow 3$) linkage and an increase in **F4** with ($\alpha 1 \rightarrow 4$)/($\alpha 1 \rightarrow 2$) linkages. Formation of **F2** with an ($\alpha 1 \rightarrow 2$) linkage and **F4** was negatively affected in most W1065 and N1029 mutants, respectively. Mutant N1029G, when acting on lactose as acceptor substrate, added an ($\alpha 1 \rightarrow 3$) linked Glc moiety to compounds **F2-F5** of the GL34 mixture to synthesize the new products **G1-G4**: α -D-Glcp-(1 \rightarrow 3)- α -D-Glcp-(1 \rightarrow 2)-[β -D-Galp-(1 \rightarrow 4)-]D-Glcp (**G1**), α -D-Glcp-(1 \rightarrow 3)- α -D-Glcp-(1 \rightarrow 3)- β -D-Galp-(1 \rightarrow 4)- α -D-Glcp (**G2**), α -D-Glcp-(1 \rightarrow 3)- α -D-Glcp-(1 \rightarrow 2)-[Glcp-(1 \rightarrow 4)- β -D-Galp-(1 \rightarrow 4)-]D-Glcp (**G3**) and α -D-Glcp-(1 \rightarrow 3)- α -D-Glcp-(1 \rightarrow 2)-[α -D-Glcp-(1 \rightarrow 3)- β -D-Galp-(1 \rightarrow 4)-]D-Glcp (**G4**). Mutant N1029G thus facilitated synthesis of new ($\alpha 1 \rightarrow 3$) glucosylated lactose derivatives. Similarly enhanced ($\alpha 1 \rightarrow 3$) elongating activity of N1029 mutants was observed in studies where maltose was used as acceptor substrate,¹⁰ or even when non-carbohydrate compounds were used as acceptor substrate.³ Kinetic analysis revealed that the presence of sucrose plus lactose as acceptor substrate resulted in a strong reduction of hydrolytic activity for Gtf180- Δ N and an increase in transferase activity for Gtf180- Δ N and mutant N1029G, compared to activity with sucrose alone. This study thus identified three residues (N1029, W1065, Q1140) that likely play a role in determining linkage specificity regarding lactose *trans*-glycosylation. Further insights in the linkage specificity determinants of Gtf180- Δ N acting on lactose as acceptor substrate may be provided by a crystal structure of Gtf180- Δ N in complex with lactose. Mutagenesis of key residues in Gtf180- Δ N is an effective strategy for synthesis of tailor-made mixtures of lactose-derived oligosaccharides with various linkage types, with potential applications as prebiotic compounds in food and feed, and in pharmacy and medicine.

***Trans*-glucosylation of GOS derivatives synthesized by the *Lactobacillus reuteri* GtfA- Δ N and Gtf180- Δ N glucansucrase enzymes**

GtfA- Δ N and Gtf180- Δ N showed altered linkage specificity when decorating lactose. This phenomenon was further investigated and exploited for *trans*-glucosylation of other galactose-containing compounds. In **Chapter 5**, three commercially available GOS structures with DP3, 3'-galactosyl-lactose (β 3'-GL), 4'-galactosyl-lactose (β 4'-GL) and 6'-galactosyl-lactose (β 6'-GL), were used as acceptor substrates for Gtf180- Δ N and GtfA- Δ N. Similar to acting on lactose as acceptor substrate, both GtfA- Δ N and Gtf180- Δ N synthesized the same transfer products when acting on these GOS DP3. These glucansucrases produced α -D-Glcp-(1 \rightarrow 4)-D-Galp-(1 \rightarrow 6)- β -D-Galp-(1 \rightarrow 4)-D-Glcp (**GL1**) and β -D-Galp-(1 \rightarrow 6)- β -D-Galp-(1 \rightarrow 4)-[α -D-Glcp-(1 \rightarrow 2)-]D-Glcp (**GL2**) when acting on β 6'-GL, and produced β -D-Galp-(1 \rightarrow 4)- β -D-Galp-(1 \rightarrow 4)-[α -D-Glcp-(1 \rightarrow 2)-]D-Glcp (**GL3**) when acting on β 4'-GL. However, Both Gtf180- Δ N and GtfA- Δ N were unable to use β 3'-GL as acceptor substrate. Both glucansucrases again introduced the (α 1 \rightarrow 2) linkage type when acting on β 6'-GL and β 4'-GL as acceptor substrates to produce **GL2** and **GL3**, respectively. Galactose-containing acceptor substrates thus appear to enforce changes in the glucoside linkage specificity of these two glucansucrases: Gtf180- Δ N and GtfA- Δ N favor the synthesis of (α 1 \rightarrow 2) linkage containing oligosaccharides when acting on galactose-containing acceptor compounds. The acceptor substrates β 6'-GL and β 4'-GL used in this study are present in the well-known commercial prebiotic mixture Vivinal GOS.^{11,12} Elongation of β 4'-GL and β 6'-GL with an (α 1 \rightarrow 2) linked glucose moiety may improve their selectivity, thus providing improved prebiotic compounds. *Trans*-glucosylation of galactosyl-lactose compounds with glucansucrase enzymes thus is likely to further expand their already well-known prebiotic GOS status.

***Trans*-sialylation of lactose- and lactulose- derived oligosaccharides by *Trypanosoma cruzi trans*-sialidase (TcTS)**

Human milk oligosaccharides contain 12.6-21.9 % sialylated oligosaccharides. Their positive functional effects on human health are widely studied and well-documented.^{15,16,17} With a final aim to synthesize mimics, in **Chapter 6**, TcTS was used to transfer sialic acid to mixtures of glucosylated-lactose (GL34), galactosylated-lactulose (LGOS) and galacto-oligosaccharide (Vivinal GOS) molecules as acceptor substrates. This enzyme preferentially catalyzes the reversible transfer of (α 2 \rightarrow 3)-linked sialic acids from donor glycans directly to terminal β -Gal-containing acceptor molecules.^{13,14} LGOS is a mixture of (β 1 \rightarrow 3/4/6)-galactosylated lactulose molecules, with one or two galactosyl moieties, synthesized from lactulose as donor and acceptor substrate by wild-type and mutant β -galactosidase enzymes from *Bacillus circulans* ATCC 31382.¹⁸ Decorated structures were identified by HPAEC-PAD chromatography and NMR spectroscopy. As expected, these mixtures with one or multiple accessible Gal-OH-3 groups were used as acceptor substrates by TcTS. In the GL34 mixture, structure **F2** (2-glc-lac) with an accessible β -Gal residue at a non-reducing terminal position was mono-sialylated yielding Neu5Ac(α 2 \rightarrow 3)Gal(β 1 \rightarrow 4)[Glc(α 1 \rightarrow 2)]Glc with a conversion degree of 47.6 %. TcTS was able to use at least five LGOS compounds as acceptor substrates; the maximal conversion degree was ~52 % at 1 mM of the LGOS mixture after incubation for 48 h. To date, only lactulose was reported to be used as an acceptor substrate for a mutant *trans*-sialidase Tr13 from *T. rangeli*.¹⁹ Most of the compounds in the Vivinal DP3-4 GOS mixture were sialylated by TcTS. The strong preference of TcTS for sialylation of terminal β -Gal residues was also observed in this study. Compounds with a Gal(β 1 \rightarrow 3) terminal residue are more efficiently sialylated by this enzyme.

Conclusions

Synthesis of new oligosaccharides by glucansucrase and *trans*-sialidase enzymes using various galactose-containing acceptor substrates was studied in this thesis. The structures of the newly synthesized oligosaccharides were elucidated in detail using HPAEC, MALDI-TOF MS and ¹H 1D/2D NMR and ¹³C 2D NMR. Glucansucrase enzymes showed a very interesting ability to use galactose-containing compounds as acceptor substrates. Despite their different linkage specificity with sucrose alone, Gtf180-ΔN and GtfA-ΔN produced identical transfer products when using lactose, β4'-GL and β6'-GL as acceptor substrates. When acting on these galactose-containing oligosaccharides, Gtf180-ΔN and GtfA-ΔN favored the synthesis of (α1→2) linkage containing products, which is not observed when these enzymes act on other acceptor substrates such as sucrose and maltose. *Trans*-glucosylation of galactosyl-lactose compounds with glucansucrase enzymes is likely to further expand their already well-known prebiotic GOS status. Mutational analysis revealed that three amino acid residues, namely N1029, W1065 and Q1140, play important roles in determining linkage specificity regarding lactose *trans*-glycosylation of Gtf180-ΔN. Mutagenesis of these residues caused significant changes in the preferred linkage types synthesized by Gtf180-ΔN, reflected in changed GL34 **F1-F5** product ratios.

The stimulatory effects of the GL34 mixture on growth of various groups of gut bacteria was studied in detail. Amongst the studied strains, *Bifidobacterium adolescentis* ATCC 15703 grew very well on the GL34 mixture, 80 % compared to the 100 % control growing on lactose. GL34 thus represents a novel oligosaccharide mixture with (potential) synbiotic properties toward *B. adolescentis*. Further investigation of their effects on growth of other probiotic bacteria may identify more synbiotic combinations with potential for application in the food/feed industry. Glucansucrases are interesting glucosylating enzymes that are relatively easy to

produce, highly active with sucrose as donor substrate, and with promising conversion degrees. Optimization of their *trans*-glucosylation reactions with galactose-containing compounds as acceptor substrates is needed to obtain higher yields of transfer products for further application as prebiotic compounds in food and feed, and in pharmacy and medicine.

Addition of sialic acid to prebiotic galactose-containing oligosaccharides is likely to diversify their functions towards human health other than prebiotic properties, as previously observed for sialylated *hMOS*.^{20,21,22} In the GL34 mixture, only the **F2** compound was decorated by TcTS to become **Sia-F2**. Further study using other *trans*-sialidase such as mutant *trans*-sialidase Tr13 from *T. rangeli*, which is able to add (α 2 \rightarrow 3)-linked Neu5Ac to glucosyl residues, may expand the range of sialylated products when using the GL34 mixture as acceptor substrates. This study showed that structures with a Gal(β 1 \rightarrow 3) terminal residue were more efficiently sialylated by TcTS. This finding would facilitate efficient synthesis of sialylated oligosaccharides in future studies.

The newly synthesized galactose-containing oligosaccharides and sialylated oligosaccharides hold strong potential for further applications in the food/feed industry. Glucansucrase and *trans*-sialidase thus are promising tools as biocatalysts for efficient synthesis of new oligosaccharides, which are diverse in glycosidic linkage types and molecular size.

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Samenvatting

Moedermelk oligosachariden (*hMOS*) hebben prebiotische eigenschappen en spelen een essentiële rol in de gezondheid van zuigelingen. Ze stimuleren de groei van gezonde darmbacteriën in de gastheer. Daarnaast verminderen *hMOS* infecties door te fungeren als anti-adhesieven tegen pathogene microben; ook stimuleren ze de immuunrespons. Veel baby's krijgen tegenwoordig geen moedermelk meer, of slechts voor een korte periode. Een alternatieve bron voor *hMOS* komt in de natuur niet voor. Een veelgebruikt alternatief is poedermelk voor zuigelingen met toegevoegde commerciële prebiotische galacto-oligosachariden (GOS) en fructo-oligosachariden (FOS), maar de anti-adhesieve en immuunsysteem stimulerende effecten ontbreken hierin. Momenteel ligt de focus in het onderzoek naar synthese van *hMOS* (vervangers) op “hele-cel biosynthese routes” en “biocatalytische systemen met één of meer enzymen” (**Hoofdstuk 1**).

In dit proefschrift wordt het mogelijke gebruik van glucansucrase enzymen uit *Lactobacillus reuteri* voor synthese van oligosacchariden onderzocht. Met deze enzymen zijn verschillende mengsels van geglycosyleerde-lactose derivaten geproduceerd met ofwel lactose, danwel GOS als acceptorsubstraat (**Hoofdstuk 2 en 5**). Tot onze verrassing veranderde de normale bindingtypespecificiteit van Gtf180- Δ N en GtfA- Δ N uit *L. reuteri* met galactose-bevattende acceptorsubstraten. Door gedetailleerde analyse van de producten gevormd door mutante enzymen konden we verschillende bijdragen van geselecteerde, individuele aminozuren op de bindingtypespecificiteit van het enzym identificeren (**Hoofdstuk 4**). De groeistimulerende effecten van het geglycosyleerde-lactose oligosaccharide mengsel zijn getest op verschillende darmbacteriën, en de resultaten hiervan zijn beschreven in **Hoofdstuk 3**. In **Hoofdstuk 6** is de enzymatische synthese van gesialyleerde

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oligosachariden met het *trans*-sialidase enzym uit *Trypanosoma cruzi* beschreven. De structuren van de gesialyleerde oligosachariden gemaakt uit geglycosyleerd-lactose en GOS uit lactose en lactulose als acceptor moleculen zijn in detail gekarakteriseerd.

***Trans*-glycosylering van lactose door *Lactobacillus reuteri* GtfA- Δ N en Gtf180- Δ N glucansucrase**

Van glucansucrases is bekend dat zij in staat zijn een grote variatie aan acceptor substraten te accommoderen voor de synthese van oligosachariden met prebiotisch potentieel.^{1,2,3} We hebben de glucansucrase enzymen Gtf180- Δ N en GtfA- Δ N uit *L. reuteri* stammen 180 en 121, respectievelijk, gebruikt om lactose als acceptorsubstraat te decoreren met glucose, met sucrose als donorsubstraat. GtfA- Δ N als Gtf180- Δ N synthetiseerden hetzelfde mengsel van enkel en dubbel geglycosyleerd lactose (GL34) moleculen met lactose als acceptorsubstraat. Drie enkelvoudig geglycosyleerd lactose and twee dubbel geglycosyleerd lactose derivaten zijn geïsoleerd en hun structuren zijn geïdentificeerd als α -D-Glcp-(1 \rightarrow 4)- β -D-Galp-(1 \rightarrow 4)-D-Glcp (**F1**), α -D-Glcp-(1 \rightarrow 2)-[β -D-Galp-(1 \rightarrow 4)-]D-Glcp (**F2**), α -D-Glcp-(1 \rightarrow 3)- β -D-Galp-(1 \rightarrow 4)-D-Glcp (**F3**), α -D-Glcp-(1 \rightarrow 4)- β -D-Galp-(1 \rightarrow 4)-[α -D-Glcp-(1 \rightarrow 2)-]D-Glcp (**F4**) en α -D-Glcp-(1 \rightarrow 3)- β -D-Galp-(1 \rightarrow 4)-[α -D-Glcp-(1 \rightarrow 2)-]D-Glcp (**F5**).

Met sucrose als donor- en acceptorsubstraat synthetiseert GtfA- Δ N een glucaan met voornamelijk (α 1 \rightarrow 4)/(α 1 \rightarrow 6) glycosidische bindingen;⁴ met lactose als acceptorsubstraat introduceert dit enzym echter niet alleen (α 1 \rightarrow 4), maar ook (α 1 \rightarrow 2) en (α 1 \rightarrow 3) glycosidische bindingen. Normaliter produceert Gtf180- Δ N een α -glucaan met 69% (α 1 \rightarrow 6) en 31% (α 1 \rightarrow 3) bindingen uit sucrose,⁵ maar wanneer lactose als acceptorsubstraat wordt gebruikt synthetiseert dit enzym (α 1 \rightarrow 2), (α 1 \rightarrow 3) en (α 1 \rightarrow 4) glycosidische bindingen. De volledige toekenning van NMR spectra van drie trisachariden (**F1-F3**) en twee tetrasachariden (**F4** en **F5**) in het

GL34 mengsel zijn hier voor het eerst beschreven. Lactose derivaten zijn interessante potentiële prebiotische verbindingen, vooral de structuren met ($\alpha 1 \rightarrow 2$)-bindingen. Dit soort verbindingen staan bekend om de hoge resistentie tegen spijsverteringsenzymen in de menselijke darm,⁶ en kunnen mogelijk selectief de groei bevorderen van gezondheidsbevorderende darmbacteriën.^{2,7} In **Hoofdstuk 3** zijn de groeistimulerende effecten van het GL34 mengsel getest op reincultures van verschillende darmbacteriën.

Stimulerende effecten van het GL34 mengsel op de groei van een selectie van darmbacteriën

Een prebioticum is gedefinieerd als: “Een substraat dat selectief wordt gebruikt door micro-organismen met een gezondheidsvoordeel als resultaat”.⁸ Het GL34 mengsel voldoet aan het eerste element, aangezien de verbindingen **F1-F5** resistent bleken te zijn tegen veel voorkomende koolhydraat-afbrekende enzymen, inclusief α -amylases van verschillende bronnen, α -glycosidase, iso-amylase en pullulanase. Alleen **F2** (2-glc-lac) werd gedeeltelijk afgebroken door microbiële β -galactosidase enzymen. Drie groepen van darmbacteriën, m.n. drie *Bifidobacterium* stammen, drie *Lactobacillus* stammen en twee commensalen zijn gegroeid met GL34 als enige koolstofbron.

Het GL34 mengsel toonde verschillen in stimulerende effecten op de groei van *B. breve* DSM 20123, *B. adolescentis* ATCC 15703 en *B. infantis* ATCC 15697. Van deze stammen groeide met name *B. adolescentis* uitzonderlijk goed op het GL34 mengsel, de uiteindelijke groei (optische dichtheid, OD₆₀₀) was 80% vergeleken met de 100% waarden bereikt in controle experimenten met groei op lactose en GOS. De uiteindelijke OD₆₀₀ waarden van *B. breve* DSM 20123 en *B. infantis* ATCC 15697 op GL34 bleven onder de 50% in vergelijking met de 100% waarden bereikt in controle experimenten op lactose, GOS en een GOS/FOS mengsel.

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Alle drie de geteste *Lactobacillus* stammen vertoonden een relatief beperkte groei op media met GL34 als enige koolstofbron. Deze lactobacilli waren duidelijk niet in staat om alle aanwezige GL34 te consumeren en de uiteindelijke OD₆₀₀ waarden van *L. casei* W56, *L. reuteri* 121 en *L. acidophilus* ATCC 4356 waren slechts 3.8%, 26.5% en 10.4% respectievelijk, in vergelijking met de 100% waarden in controle experimenten met glucose als koolstofbron. Ook de groei van commensaal bacteriën op de oligosachariden in het GL34 mengsel is onderzocht. *Bacteroides thetaiotaomicron* en *Escherichia coli* Nissle toonden een lichte en trage groei in media met GL34 als enige koolstofbron. Het GL34 mengsel bevordert dus de groei van de geteste bacteriën in verschillende mate. De geteste bifidobacteriën waren over het algemeen beter in staat de GL34 verbindingen af te breken dan lactobacillen en commensalen. Individuele darmbacteriën consumeerden specifieke verbindingen uit het GL34 mengsel. Synergetische activiteit tussen verschillende bacteriën kan essentieel zijn voor *in vivo* benutting van het gehele GL34 mengsel. Verbinding **F2** (2-glc-lac) stimuleerde (in verschillende mate) de groei van de probiotische bacteriën *L. reuteri* 121, *B. adolescentis* ATCC 15703, *B. longum* subsp. *infantis* ATCC 15697, *B. breve* DSM 20213, evenals de twee commensaal bacteriën, *E. coli* Nissle en *Bc. thetaiotaomicron*. Deze **F2** verbinding wordt dus minder selectief gebruikt dan de andere verbindingen in het GL34 mengsel. Verbindingen **F1** (4'-glc-lac), **F4** (4',2-glc-lac) en **F5** (3',2-glc-lac) stimuleerden (in verschillende mate) de groei van alle drie de geteste bifidobacteriën. Verbinding **F3** (3'-glc-lac) is nog meer selectief, en werd maar door twee van de drie bestudeerde *Bifidobacterium* stammen benut, nl. *B. adolescentis* ATCC 15703 en *B. breve* DSM 20213. Samenvattend, GL34 is een nieuw oligosacharide mengsel met (potentiële) synbiotische eigenschappen richting *B. adolescentis*, enzymatisch gesynthetiseerd uit goedkoop en ruim voorradig lactose en sucrose.

Mutatie analyse van de rol van Gtf180-ΔN actieve centrum aminozuren in product- en bindingstypespecificiteit met lactose als acceptorsubstraat

In **Hoofdstuk 2** hebben we gezien dat glucansucrase Gtf180-ΔN met lactose als acceptorsubstraat nieuwe typen glycosidische bindingen $[(\alpha 1 \rightarrow 2)/(\alpha 1 \rightarrow 4)]$ vormt, vergeleken met de normale bindingstypes $[(\alpha 1 \rightarrow 3)/(\alpha 1 \rightarrow 6)]$ wanneer dit enzym met alleen sucrose wordt geïncubeerd, of met andere tot nu toe bestudeerde acceptorsubstraten (bv maltose).

In **Chapter 4** zijn dockingexperimenten met lactose in de kristalstructuur van een glucosyl-enzym intermediair van Gtf180-ΔN uitgevoerd om te begrijpen hoe lactose bindt in het katalytische centrum van Gtf180-ΔN, en welke aminozuren mogelijk essentieel zijn in het binden van lactose. Drie aminozuur residuen (Q1140, W1065 en N1029) werden geïdentificeerd nabij het lactose acceptorsubstraat die mogelijk een rol spelen in de oriëntatie van lactose in de acceptor subsite en de bindingstypespecificiteit kunnen beïnvloeden.

Alle drie deze residuen zijn volledig geconserveerd in de glucansucrase enzymen en het is bekend dat ze een belangrijke rol spelen in de *trans*-glycosyleringsreactie.⁹ Mutagenese van deze residuen resulteerde in significante veranderingen in de gesynthetiseerde hoeveelheden van de **F1-F5** verbindingen in het GL34 mengsel. Mutaties van het Q1140 residue resulteerden in een duidelijke afname in **F3** met een $(\alpha 1 \rightarrow 3)$ binding en een toename in **F4** met $(\alpha 1 \rightarrow 4)/(\alpha 1 \rightarrow 2)$ bindingen. De synthese van **F2** met een $(\alpha 1 \rightarrow 2)$ binding en **F4** was lager in de meeste W1065 en N1029 mutanten, respectievelijk. Incubatie van mutant N1029G met lactose als acceptorsubstraat resulteerde in synthese van **F2-F5** verbindingen met een extra $(\alpha 1 \rightarrow 3)$ verknoopte Glc eenheid. De nieuwe producten **G1-G4** werden structureel gekarakteriseerd als α -D-Glcp-(1→3)- α -D-Glcp-(1→2)-[β -D-Galp-(1→4)-]D-Glcp (**G1**), α -D-Glcp-(1→3)- α -D-Glcp-(1→3)- β -D-Galp-(1→4)- α -D-Glcp (**G2**), α -D-Glcp-(1→3)- α -D-Glcp-(1→2)-[Glc-(1→4)- β -D-Galp-(1→4)-]D-Glcp (**G3**) and α -

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D-Glcp-(1→3)- α -D-Glcp-(1→2)-[α -D-Glcp-(1→3)- β -D-Galp-(1→4)-]D-Glcp (**G4**). Mutant N1029G faciliteerde dus de synthese van nieuwe (α 1→3) geglycosyleerde lactose derivaten. In eerdere studies met bijvoorbeeld maltose als acceptorsubstraat werd een vergelijkbare extra synthese van (α 1→3) bindingen door N1029 mutanten gezien,¹⁰ zelfs als niet-koolhydraat verbindingen gebruikt werden als acceptorsubstraat.³

Enzymkinetiek studies lieten zien dat, in vergelijking met de activiteit met alleen sucrose, de aanwezigheid van sucrose plus lactose als acceptorsubstraat resulteerde in een sterke reductie in de hydrolytische activiteit van Gtf180- Δ N en in een toename in transferase activiteit van Gtf180- Δ N en mutant N1029G. Dit is gunstig voor de synthese van lactose-gebaseerde oligosachariden.

Deze studie heeft dus drie Gtf180- Δ N residuen (N1029, W1065, Q1140) geïdentificeerd die een rol kunnen spelen in de bepaling van de bindingstypespecificiteit bij de *trans*-glycosylering van lactose. Verdere inzichten in factoren die van belang zijn voor de bindingstypespecificiteit van Gtf180- Δ N met lactose als acceptorsubstraat kunnen mogelijk verkregen worden uit een kristalstructuur van Gtf180- Δ N met een lactose gebonden in de actieve centrum. Ondanks de gedane pogingen is een dergelijke kristalstructuur nog niet beschikbaar. Na het verwerven van zulke detailinzichten wordt het in de toekomst wellicht mogelijk om door gerichte mutagenese van sleutelresiduen in Gtf180- Δ N de synthese van specifieke mengsels van lactose oligosachariden met verschillende bindingstypes te realiseren, met potentie voor toepassing als prebiotische verbindingen in voedsel en voeders, evenals in farmaceutische toepassingen.

***Trans*-glucosylering van GOS derivaten gesynthetiseerd door de *Lactobacillus reuteri* GtfA-ΔN en Gtf180-ΔN glucansucrase enzymen**

GtfA-ΔN en Gtf180-ΔN toonden gewijzigde bindingstypespecificiteit met lactose als acceptorsubstraat. Dit fenomeen is verder onderzocht in *trans*-glucosylering studies met andere galactose-bevattende verbindingen.

In **Hoofdstuk 5**, zijn drie commercieel verkrijgbare GOS structuren gebruikt als acceptorsubstraat voor Gtf180-ΔN en GtfA-ΔN, namelijk 3'-galactosyl-lactose (β3'-GL), 4'-galactosyl-lactose (β4'-GL) en 6'-galactosyl-lactose (β6'-GL). Zoals eerder gezien in de reacties met lactose synthetiseerden GtfA-ΔN en Gtf180-ΔN hetzelfde producten met deze GOS acceptorsubstraten.

Beide glucansucrases produceerden α-D-Glcp-(1→4)-D-Galp-(1→6)-β-D-Galp-(1→4)-D-Glcp (**GL1**) en β-D-Galp-(1→6)-β-D-Galp-(1→4)-[α-D-Glcp-(1→2)-]D-Glcp (**GL2**) uit β6'-GL als acceptorsubstraat, en β-D-Galp-(1→4)-β-D-Galp-(1→4)-[α-D-Glcp-(1→2)-]D-Glcp (**GL3**) uit β4'-GL als acceptorsubstraat. Geen van beide enzymen kon β3'-GL als acceptorsubstraat gebruiken. Beide glucansucrases introduceerden opnieuw een (α1→2) binding met zowel β6'-GL als β4'-GL als acceptorsubstraat, met als producten respectievelijk **GL2** en **GL3**.

Galactose-bevattende acceptorsubstraten blijken dus een veranderde bindingstypespecificiteit af te dwingen in beide geteste glucansucrasen: Gtf180-ΔN en GtfA-ΔN introduceren dan bij voorkeur een (α1→2) binding. De gebruikte acceptorsubstraten β4'-GL en β6'-GL zijn aanwezig in het welbekende commerciële prebiotische mengsel Vivinal GOS.^{11,12} Verlenging van β4'-GL en β6'-GL met een (α1→2) gebonden glucoseeenheid kan hun selectiviteit verbeteren, en dus een verbeterde prebiotische verbinding opleveren. *Trans*-glucosylering van galactosyl-lactose verbindingen met behulp van glucansucrase enzymen kan dan resulteren in een verbreding van de reeds welbekende prebiotische status van GOS.

***Trans*-sialylering van lactose en lactulose afgeleide oligosachariden door *trans*-sialidase uit *Trypanosoma cruzi* (TcTS)**

Moedermelk bevat 12.6-21.9% gesialyleerde oligosachariden. Hun positieve functionele effecten op gezondheid is breed bestudeerd en goed gedocumenteerd.^{15,16,17} Met als uiteindelijk doel om *h*MOS vervangers te synthetiseren hebben we TcTS gebruikt om siaalzuur over te zetten op mengsels van geglycosyleerd-lactose (GL34), gegalactosyleerd-lactulose (LGOS) en galacto-oligosacharide (Vivinal GOS) moleculen als acceptorsubstraten (**Hoofdstuk 6**). Dit TcTS enzym katalyseert bij voorkeur de omkeerbare overzetting van (α 2 \rightarrow 3)-gebonden siaalzuur van donor glycanen op β -Galactose-bevattende acceptorsubstraten.^{13,14} LGOS is een mengsel van (β 1 \rightarrow 3/4/6)-gegalycosyleerd lactulose moleculen, met één of twee galactose eenheden, gesynthetiseerd uit lactulose als donor- en acceptorsubstraat door wild-type en mutante β -galactosidase enzymen van *Bacillus circulans* ATCC 31382.¹⁸

De gevormde producten zijn geanalyseerd met behulp van HPAEC-PAD chromatografie en NMR spectroscopie. Alle drie geteste mengsels hebben één of meerdere toegankelijke Gal-OH-3 groepen en werden gebruikt als acceptorsubstraat door TcTS.

In het GL34 mengsel werd structuur **F2** (2-glc-lac), met een toegankelijke β -Gal residue op een niet-reducerend uiteinde, enkelvoudig gesialyleerd tot Neu5Ac(α 2 \rightarrow 3)Gal(β 1 \rightarrow 4)[Glc(α 1 \rightarrow 2)]Glc met een conversie tot 47.6 %. Alleen lactulose was bekend als acceptorsubstraat voor een mutant *trans*-sialidase Tr13 uit *Trypanosoma rangeli*.¹⁹ TcTS was in staat om tenminste vijf LGOS verbindingen als acceptorsubstraat te gebruiken; de maximale conversie was ~52 % bij 1 mM LGOS mengsel na een incubatie van 48 uur. De meeste verbindingen in het Vivinal GOS DP3-4 mengsel werden gesialyleerd door TcTS. TcTS heeft een sterke voorkeur voor het sialyleren van eindstandige β -Gal residuen. Verbindingen met een

eindstandige Gal(β 1 \rightarrow 3) eenheid werden efficiënter gesialyleerd door dit enzym dan andere eindstandige Gal-eenheden.

Conclusies

In dit proefschrift is de synthese bestudeerd van nieuwe oligosachariden door glucansucrase en *trans*-sialidase enzymen gebruikmakend van verschillende galactose-bevattende acceptorsubstraten. De structuren van de nieuw gesynthetiseerde oligosachariden werden in detail opgehelderd met behulp van HPAEC, MALDI-TOF MS en ^1H 1D/2D NMR en ^{13}C 2D NMR.

De glucansucrase enzymen Gtf180- Δ N en GtfA- Δ N tonen een zeer interessante eigenschap wanneer ze galactose-bevattende verbindingen gebruiken als acceptorsubstraat. Gtf180- Δ N en GtfA- Δ N hebben duidelijk verschillende bindingstypespecificiteiten in reacties met alleen sucrose, wat resulteert in synthese van verschillende oligosaccharideproducten. In incubaties met lactose, β 4'-GL en β 6'-GL als acceptorsubstraten, en sucrose als donorsubstraat, maken ze echter identieke producten. Met deze galactose-bevattende oligosachariden als acceptorsubstraat hebben Gtf180- Δ N en GtfA- Δ N een sterke voorkeur voor de synthese van (α 1 \rightarrow 2) verknoopte producten, wat niet waargenomen wordt met andere acceptorsubstraten zoals maltose. *Trans*-glucosylering van galactosyl-lactose verbindingen met glucansucrase enzymen levert een breder pallet aan structuren en kan mogelijk het prebiotische potentiël van GOS verder uitbreiden. Mutantenstudies toonden aan dat drie aminozuur residuen, namelijk N1029, W1065 en Q1140, een belangrijke rol spelen in het bepalen van de bindingstypespecificiteit van Gtf180- Δ N met betrekking tot *trans*-glycosylering van lactose. Mutagenese van deze residuen leverde significante veranderingen in de voorkeursbindingstypes gesynthetiseerd door Gtf180- Δ N, resulterend in veranderde GL34 **F1-F5** product ratio's.

Samenvatting

De stimulerende effecten van het GL34 mengsel op groei van verschillende groepen van darmbacteriën werd gedetailleerd bestudeerd. Van de bestudeerde stammen groeide *Bifidobacterium adolescentis* ATCC 15703 zeer goed op het GL34 mengsel, 80% groei vergeleken met de 100% controle groei op lactose. GL34 is een nieuw oligosacharide mengsel met (potentieel) synbiotische eigenschappen richting *B. adolescentis*. Verder onderzoek naar de effecten op groei van andere probiotische bacteriën kan nog meer synbiotische combinaties aantonen met potentiële toepassingen in de voedsel en voeder industrie.

Glucansucrases zijn interessante glucosylerende enzymen die relatief eenvoudig te produceren zijn, met hoge activiteit op sucrose als donorsubstraat en met veelbelovende conversieniveaus met verschillende acceptorsubstraten. Optimalisatie van hun *trans*-glucosyleringsreacties met galactose-bevattende verbindingen als acceptorsubstraat is echter nog nodig om een hogere productopbrengst te realiseren, voor verdere functionele studies als prebiotische verbindingen in voedsel, voeders en in farmaceutische toepassingen.

Toevoeging van siaalzuur aan prebiotische, galactose-bevattende oligosachariden resulteert niet in verbetering van prebiotische eigenschappen, maar brengt waarschijnlijk meer functionaliteit richting gezondheidseffecten op mensen, zoals voorheen waargenomen voor siaalzuur-bevattende *hMOS*.^{20,21,22} In het GL34 mengsel werd alleen verbinding **F2** gesialyleerd door TcTS. Verdere studies, waarbij ook gebruik gemaakt wordt van bijvoorbeeld mutant *trans*-sialidase Tr13 van *T. rangelii* welke in staat is om (α 2→3)-gebonden Neu5Ac aan glucosyl residuen toe te voegen, kan het aantal gesialyleerde producten vergroten wanneer gebruik wordt gemaakt van het GL34 mengsel als acceptorsubstraat.

Onze studies hebben aangetoond dat substraten met een eindstandig Gal(β 1→3) residu effectiever gesialyleerd worden door TcTS. Op basis van deze resultaten kan

mogelijk een efficiëntere synthese van gesialyleerde oligosachariden gerealiseerd worden in toekomstige studies.

De nieuw gesynthetiseerde glucose-bevattende oligosachariden en gesialyleerde oligosachariden hebben potentie voor toepassingen in de voedingsmiddelen en diervoeder industrie. De gebruikte glucansucrase en *trans*-sialidase enzymen zijn dus veelbelovende gereedschappen voor gerichte synthese van nieuwe oligosachariden, met een variatie aan glycosidische bindingstypes en molecuul grootte.

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Curriculum Vitae



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