Synthesis and characterization of lactose and lactulose derived oligosaccharides by glucansucrase and trans-sialidase enzymes
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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-ΔN 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-ΔN and Gtf180-ΔN glucansucrases

Glucansucrases are well known for their ability to use a large variety of acceptor substrates to produce oligosaccharides with prebiotic potential. We investigated the ability of glucansucrase enzymes Gtf180-ΔN and GtfA-ΔN 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-ΔN and Gtf180-ΔN 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-Glc-(1→4)-β-D-Galp-(1→4)-D-Glc (F1), α-D-Glc-(1→2)-[β-D-Galp-(1→4)]D-Glc (F2), α-D-Glc-(1→3)-β-D-Galp-(1→4)-D-Glc (F3), α-D-Glc-(1→4)-β-D-Galp-(1→4)-[α-D-Glc-(1→2)-D-Glc (F4) and α-D-Glc-(1→3)-β-D-Galp-(1→4)-[α-D-Glc-(1→2)-D-Glc (F5). When using sucrose as donor and acceptor substrate, GtfA-ΔN synthesizes glucan with mainly (α1→4)/(α1→6) glucosidic linkages; with lactose as acceptor substrate this enzyme introduced (α1→4) but also (α1→2) and (α1→3) glucosidic linkages. Similarly, Gtf180-ΔN produces an α-glucan with 69% (α1→6) and 31% (α1→3) linkages from sucrose, but with lactose as acceptor substrate this enzyme synthesized (α1→2), (α1→3) and (α1→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 (α1→2)-linkages. Such compounds and linkages are known to be highly resistant to the digestive enzymes in the human gut, and may selectively stimulate the growth of health-beneficial gut microbiota. 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 \textbf{F1-F5} are resistant to common carbohydrate-active enzymes including \(\alpha\)-amylases from various sources, \(\alpha\)-glucosidase, isomaltase and pullulanase. Only \textbf{F2} (2-glc-lac) was partially consumed by microbial \(\beta\)-galactosidases. Three groups of gut bacteria including three \textit{Bifidobacterium} strains, three \textit{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 \textit{B. breve} DSM 20123, \textit{B. adolescentis} ATCC 15703 and \textit{B. infantis} ATCC 15697. Amongst those strains, \textit{B. adolescentis} grew very well on the GL34 mixture, the final OD\(_{600}\) value reached 80% of that on a 100% control growing on lactose and GOS. However, the final OD\(_{600}\) of \textit{B. breve} DSM 20123 and \textit{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 \textit{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 OD\(_{600}\) values of \textit{L. casei} W56, \textit{L. reuteri} 121 and \textit{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. \textit{Bacteroides thetaiotaomicron} and \textit{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 \textit{in vivo} utilization of the whole GL34 mixture. \textbf{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 [(α1→2)/(α1→4)], compared to the normal linkage types [(α1→3)/(α1→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. 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 (α1→3) linkage and an increase in F4 with (α1→4)/(α1→2) linkages. Formation of F2 with an (α1→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 (α1→3) linked Glc moiety to compounds F2-F5 of the GL34 mixture to synthesize the new products G1-G4: α-D-Glc-p-(1→3)-α-D-Glc-p-(1→2)-[β-D-Galp-(1→4)]D-Glc-p (G1), α-D-Glc-p-(1→3)-α-D-Glc-p-(1→2)-β-D-Galp-(1→4)-α-D-Glc-p (G2), α-D-Glc-p-(1→3)-α-D-Glc-p-(1→2)-[α-D-Glc-p-(1→4)-β-D-Galp-(1→4)-]D-Glc-p (G3) and α-D-Glc-p-(1→3)-α-D-Glc-p-(1→2)-[α-D-Glc-p-(1→3)-β-D-Galp-(1→4)-]D-Glc-p (G4). Mutant N1029G thus facilitated synthesis of new (α1→3) glucosylated lactose derivatives. Similarly enhanced (α1→3) elongating activity of N1029 mutants was observed in studies where maltose was used as acceptor substrate, or even when non-carbohydrate compounds were used as acceptor substrate. Kinetic analysis revealed that the presence of sucrose plus lactose as acceptor substrate resulted in a strong reduction of hydrolytic activity for Gtf180-ΔN and an increase in transferase activity for Gtf180-ΔN 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-ΔN acting on lactose as acceptor substrate may be provided by a crystal structure of Gtf180-ΔN in complex with lactose. Mutagenesis of key residues in Gtf180-ΔN 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.
Chapter 7

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

GtfA-ΔN and Gtf180-ΔN 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 (β3´-GL), 4´-galactosyl-lactose (β4´-GL) and 6´-galactosyl-lactose (β6´-GL), were used as acceptor substrates for Gtf180-ΔN and GtfA-ΔN. Similar to acting on lactose as acceptor substrate, both GtfA-ΔN and Gtf180-ΔN synthesized the same transfer products when acting on these GOS DP3. These glucansucrases produced α-D-Glc-(1→4)-D-Galp-(1→6)-β-D-Galp-(1→4)-D-Glc (GL1) and β-D-Galp-(1→6)-β-D-Galp-(1→4)-[α-D-Glc-(1→2)-]D-Glc (GL2) when acting on β6´-GL, and produced β-D-Galp-(1→4)-β-D-Galp-(1→4)-[α-D-Glc-(1→2)-]D-Glc (GL3) when acting on β4´-GL. However, Both Gtf180-ΔN and GtfA-ΔN were unable to use β3´-GL as acceptor substrate. Both glucansucrases again introduced the (α1→2) linkage type when acting on β6´-GL and β4´-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-ΔN and GtfA-ΔN favor the synthesis of (α1→2) linkage containing oligosaccharides when acting on galactose-containing acceptor compounds. The acceptor substrates β6´-GL and β4´-GL used in this study are present in the well-known commercial prebiotic mixture Vivinal GOS. Elongation of β4´-GL and β6´-GL with an (α1→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.\textsuperscript{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 (\(\alpha_2 \rightarrow 3\))-linked sialic acids from donor glycans directly to terminal \(\beta\)-Gal-containing acceptor molecules.\textsuperscript{13,14} LGOS is a mixture of (\(\beta_1 \rightarrow 3/4/6\))-galactosylated lactulose molecules, with one or two galactosyl moieties, synthesized from lactulose as donor and acceptor substrate by wild-type and mutant \(\beta\)-galactosidase enzymes from \textit{Bacillus circulans} ATCC 31382.\textsuperscript{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 \(\beta\)-Gal residue at a non-reducing terminal position was mono-sialylated yielding Neu5Ac(\(\alpha_2 \rightarrow 3\))Gal(\(\beta_1 \rightarrow 4\))[Glc(\(\alpha_1 \rightarrow 2\))]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 \(\sim 52\%\) 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 \textit{trans}-sialidase Tr13 from \textit{T. rangeli}.\textsuperscript{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 \(\beta\)-Gal residues was also observed in this study. Compounds with a Gal(\(\beta_1 \rightarrow 3\)) 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 \(^1\)H 1D/2D NMR and \(^{13}\)C 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. 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 (α2→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(β1→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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