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Structure-function relationships of (prebiotic) carbohydrates and their selective consumption by probiotic bacteria

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Structure-function relationships of (prebiotic) carbohydrates and their selective consumption by probiotic bacteria

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Structure-function relationships of (prebiotic) carbohydrates and their selective consumption by probiotic bacteria

PhD thesis

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Chapter 1

Pre- and Probiotics: Representatives, composition and functions

Introduction

Microorganisms are fastidiously growing cells with a high adaptability to their environment; they ubiquitously colonize any ecosystem on planet Earth. They are found in all three domains of life (Bacteria, Archaea, and Eukarya). They comprise complex microbial communities that dynamically interact with their surroundings. Microbes are key players in many global processes, such as Carbon (re)cycling, Nitrogen fixation, decomposition and fermentation of foods. Microorganisms infiltrate their surroundings and may exert both negative and positive effects. On the negative side, pathogenic bacteria are a main cause of mammalian diseases. They have successfully been fought against by antibiotics, although in the last decades with decreasing efficiency (1). On the positive side, microorganisms occupy key positions in many element cycles that are responsible for the global energy turnover. The interaction of a microorganism with its host is referred to as symbiosis, ‘a term that is used to describe the outcome of an interaction between a bacterium and its host’ (2). As humans carry an enormous diversity of microbes in their gut, skin, lungs, they also represent a microbial community that shows the traits of a symbiotic relation. From an evolutionary perspective, there is evidence that the ‘human microbiota’ and the human species co-evolved (3). In the context of mammalian microbiota, the human microbiota closest relates to that of non-human primates, while the composition is further distant from that in other mammals and vertebrates (3).

Carbohydrates are bio(macro-) molecules that through glycosidic linkages of their monomers (monosaccharides) form very stable connections (4). While other biomacromolecules such as proteins or DNA comprise only one basic linkage type, carbohydrates can form diverse linkages on any available position (hydroxyl groups) of another molecule. Carbohydrate structures thus essentially contribute in developing biological diversity and complexity. Glycans are often found on cell-surfaces where they may be involved in cell-surface communication processes. Besides, the relatively high resistance of the internal linkages of carbohydrate polymers (4) assigned them a function as nutrient storage. Carbohydrates such as starch, sucrose, plant cell wall polymers, are commonly part of the diet of organisms that acquire their energy from hexose-based metabolism. From evolutionary and nutritional perspectives, differences in carbohydrate content of diet of mammals correlate with their gut microbiota composition (5). Meals consumed by Western human populations increasingly lack complex carbohydrates such as dietary fibers that are indigestible by human

enzymes. Such dietary fibers are readily degraded by our gut microbiota. The absence of dietary fibers lowers human gut microbiota diversity (6). Indeed, Western and Non-Western human populations differ in their gut microbiota populations (6). Diet of Western human populations is nowadays high in (simple) sugar, fat and meat, while Non-Western populations consume fiber rich diets. Thus, carbohydrates are a major factor that shape human gut microbiota diversity. The selective effects of indigestible carbohydrates on growth of (human) gut bacteria, and their metabolism, are the topic of analysis in this PhD thesis.

Human microbiota

The human body harbors roughly an equal number of bacterial cells and human cells (7). Altogether these bacteria are called the human microbiota, one of the most complex microbial communities. The microbiota is distinguished by the different anatomic parts where it is found: skin, oral, vaginal or gut microbiota. The gut microbiota, found in the human gastrointestinal (GI) tract, harbors the biggest community. Within the GI tract, each compartment varies in physicochemical parameters and harbors a different sub-community. Bacterial counts are the lowest in the small intestine. This part is mostly colonized by facultative anaerobes; Lactobacillaceae and Enterobacteriaceae are the dominant bacterial families (Fig. 1A) (8). These bacteria may still compete with the host for easily digestible nutrients such as simple sugars. In addition, the proximal site of the small intestine harbors the bile duct secreting bile acids into the tract which may act bactericidal to certain species and therefore clearly limit bacterial growth. The caecum and colon harbor a much richer microbial population than the small intestine. Here typical saccharolytic strict anaerobes are the dominant inhabitants. In humans, the dominant bacterial families are Bacteroidaceae and Clostridiaceae (Fig. 1A). Various factors were identified that shape the composition. For instance, the colon wall folds over itself and thereby creates subregions that in mice were colonized with a different microbiota than in the lumen (9). Throughout the GI tract, goblet cells that are specialized epithelial cells secrete mucus that forms in the small intestine a single layer and in the colon two layers, divided as outer and inner mucus layer. The inner mucus layer is essentially sterile while the outer is colonized by bacteria with mucus degrading capabilities (10). These bacteria are often found in the phyla Actinobacteria and Proteobacteria (11). Another factor influencing microbiota composition is lifetime. In early life, the gut microbiota first has to become

established. Different factors, such as the delivery process (12), intake of breast milk (13), shape the microbiota which first comprises mostly facultative anaerobes that reduce the redox potential of the gut and thereby facilitate entrance of obligate anaerobes. In adult life the gut microbiota is relatively stable developed, although different enterotypes can be distinguished (14). During aging, changes occur that include a decrease in diversity and a reduction in the metabolic activity of the gut microbiota (15). It was further shown that diet (including dietary supplements) as well as host genetics can determine gut microbiota composition (16, 17).

Research has shown that the human gut microbiota strongly is associated with human well-being. These functions include digesting food (18), stimulating cell growth, strengthening the immune system, preventing allergies and diseases, and impacting emotion (19). On a metabolic level, members of the gut microbiota produce a diversity of bioactive compounds, such as short-chain fatty acids (SCFAs) which are strongly associated with modulation of the intestinal barrier and inflammation (20). SCFAs that are majorly produced are acetate, propionate, and butyrate (21). Dysbiosis of the gut microbiota, i.e. a decrease in abundance and diversity of commensal bacteria, correlates with multiple diseases, increasingly also at non-distal sites (22). This was shown for atherosclerosis, metabolic disorders, asthma, and autism spectrum disorders (19). In some cases, causality of gut dysbiosis was proven for the following diseases: human obesity (23), kwashiorkor (24), childhood asthma (25), massive weight loss after bariatric surgery (26), and the insulin-resistant state of third-trimester pregnancy (27).

In summary, there is clear evidence for the important roles of commensal bacterial cells on the well-being of the human body. This has prompted application of single gut bacterial strains or mixtures of strains in clinical interventions to treat diseases that are associated with gut microbiota dysbiosis (19) (Fig. 1B).

Fig. 1 **A** Dominant bacterial inhabitants (family level) in the various parts of the human gastrointestinal tract. In- or decrease in physicochemical parameters along the longitudinal axis of the gut is shown at the bottom. Adopted from (10). **B** Use of gut commensals in therapeutic interventions for different human disorders and diseases. Adopted from (19).

Probiotics

The intriguing discovery that (gut) bacteria are strongly involved in human well-being and disease has prompted the application of particular strains as clinical treatments in case of disturbance of human health. These bacteria are referred to by the term probiotics. The beneficial effects that certain bacterial strains exert on human health date back to fermented food products. Metchnikoff assigned this effect first to lactobacilli typically present in fermented foods, consumption

of which was leading to shifts in the intestinal microbiota (28). In 1974, Parker defined probiotics in a manner as it is used today, namely as: “organisms and substances which contribute to intestinal microbial balance” (29). In 1989, Fuller strengthened the definition by emphasizing that probiotics need to be administered as live organisms, stating: “A live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance”(30). In 2001, an expert panel of the Food and Agriculture Organization of the United Nations (FAO) and the WHO broadened the concept including sites outside the human or animal intestine: “live microorganisms which when administered in adequate amounts confer a health benefit on the host” (31). This definition remained valid till it was updated in 2013 by an international expert panel, and written in a grammatically more correct way: “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host” (Table 1). The concept thus applies to live microorganisms that in various ways affect human or animal health. The probiotic microorganisms that are used in human are found in the following genera: *Lactobacillus*, *Bifidobacterium*, and *Lactococcus*, *Streptococcus*, *Enterococcus*. Certain strains of *Bacillus* and *Saccharomyces* are also used (32). Recently, new-generation probiotics are being studied, including strains that through genetic engineering may deliver specific health effects (33).

A robust dataset can be found in literature based on clinical trials that demonstrates the health benefits of probiotics in various aspects. These trials were conducted in case of e.g. infantile colic, atopic eczema, inflammatory bowel diseases, antibiotic-associated diarrhea and *Clostridium difficile* infection, necrotizing enterocolitis, lactose intolerance, reduction of blood cholesterol, infectious diseases of the upper respiratory tract and prevention of colorectal cancer (32, 34). Table 2 gives an overview of probiotic strains used in this thesis and their application as studied so far.

The ways that probiotics act differ, and their effects on gastrointestinal health and the immune system may have far reaching effects (Table 2). The mechanisms that probiotics use to exert their health effects have been established as follows: (1) Antagonism through the production of antimicrobial substances; (2) competition with pathogens for adhesion to the epithelium and for nutrients; (3) immunomodulation of the host; (4) inhibition of bacterial toxin production (32, 35). Using strain designation is important, as certain activities may be unique to a given strain. These strain specific properties comprise antimicrobial activities, neurological, immunological or endocrinological effects, and the

production of specific bioactive substances (35). However, the 2014 published definition of probiotics lists the increasingly accepted assumption that certain activities occur more widespread among species, or even genera. The widespread mechanisms are competitive exclusion of pathogens and colonization resistance, normalization of altered microbiota, production of SCFAs with increased turnover of enterocytes, regulation of intestinal transit (35).

In recent years, activity of the gut microbiota also has become associated with more distant sites of the human body, such as the brain referred to as gut-brain axis (36). These more recent discoveries raise the promising concept that microorganisms in future may find broader applications in ‘microbe-based’ therapies.

Table 1 Current definition of pre-, pro- and synbiotics.

Probiotics	live microorganisms that, when administered in adequate amounts, confer a health benefit on the host	(35)
Prebiotics	substrates that are selectively utilized by host microorganisms conferring a health benefit	(37)
Synbiotics	combinations of pre- and probiotics	

Table 2 Commercial probiotic strains used in this PhD study and examples for demonstrated health benefits. *Part of a mixture of probiotic strains.

Strains	Subjects or model	Outcomes	Reference(s)
<i>L. acidophilus</i> W37	participants with mood disorder*, type 2 diabetes mellitus patients*, dendritic cells	↓ reactivity to sad mood, (38-41) ↓ insulin resistance, ↑ TLR2, 3	
<i>L. casei</i> W56	Caco-2, participants with mood disorder*, Caco-2	↓ <i>Salmonella</i> -induced IL-8 release, ↓ reactivity to sad mood, ↑ intestinal barrier	(38, 40, 42)
<i>L. paracasei</i> W20*	Participants taking 7 days, amoxicillin	↑ Enterococci, ↓ diarrhea-like bowel movements	(43)
<i>L. salivarius</i> W57*	Human mononuclear cells	↓ IL-4, IL-5, IL-13; ↑ IFN-gamma, IL-10	(44)
<i>E. faecium</i> W54*	exhaustive aerobic exercise in trained athletes	↓ post-exercise tryptophan	(45)
<i>B. lactis</i> W51	peripheral blood mononuclear cells, Caco-2, exhaustive aerobic exercise in trained athletes*	↑ IL-10; ↑ IL-10, ↓ IL-4, IL-5, IL-13; ↑ intestinal barrier; ↓ post-exercise tryptophan	(45-47)
<i>B. lactis</i> W52	Caco-2, Pregnant women with functional constipation*, 3-month old children at risk to develop eczema*, participants with mood disorder*, type 2 diabetes mellitus patients*	↓ <i>Salmonella</i> -induced IL-8; ↓ constipation during pregnancy; ↑ fecal short-chain fatty acids; ↓ reactivity to sad mood, ↓ insulin resistance	(38, 39, 42, 48, 49)

Lactobacilli. Lactobacilli are Gram-positive bacteria that produce as major product of bacterial fermentation lactic acid. They form the largest genus among lactic acid bacteria. Three groups are distinguished: obligately homofermentative, facultatively heterofermentative, obligately heterofermentative strains. Lactobacilli are found in fermented foods which are frequently consumed by humans. Thereby, they are often part of the transient microbiota of the human gastrointestinal (GI) tract passing without permanent colonization. To a low extent, some species colonize the human gut with the following examples: *L. gasseri*, *L. reuteri*, *L. crispatus*, *L. salivarius*, and *L. ruminis* (50). Other species that are found to a fluctuating extent in the human gut are: *L. acidophilus*, *L. fermentum*, *L. casei*, *L. rhamnosus*, *L. johnsonii*, *L. plantarum*, *L. brevis*, *L. delbrueckii*, *L. curvatus*, and *L. sakei* (50). The female vaginal tract comprises a microbiota that is particularly rich in lactobacilli (51). In fecal microbiota, lactobacilli constitute only between 0.01% and 0.6% of total bacterial counts (52).

Lactobacilli have been characterized by a number of factors that closely relate to adaption to their host. For instance, lactobacilli are present in the stomach which is characterized by low pH that bacterial inhabitants need to successfully cope with. Lactobacilli encode genes that enhance their membrane integrity and activate pathways for removal of excess acid and bile (53). Adaptation is also observed towards the nutritional environment. In the human GI tract bacteria compete for dietary carbohydrates that are not ingested by the human body. Complexity of nutrients increases towards the distal sites of the GI tract, where typical saccharolytic bacteria are found (discussed further in the next paragraph). In contrast, lactobacilli encode transport-based pathways that are important in uptake of simple sugars still available in the proximal site of the GI tract. Known transport systems that are involved in sugar utilization by lactobacilli will be further discussed below.

Lactobacilli exert numerous health-promoting effects that are a strong basis for their use as probiotics. Those include synthesis of vitamins and lactic acid, competitive exclusion of pathogens, antimicrobial properties and a strong interaction with host immune cells through cell-surface components such as lipopolysaccharides, peptidoglycans, and lipoproteins.

Bifidobacteria. Bifidobacteria are Gram-positive obligately anaerobic bacteria that were first isolated from feces of breast-feed infants by Tissier 1899 (54). After birth, they are among the first bacteria to colonize the human gut (55). They are heterofermentative and produce as end-products of fermentation

often lactic acid such as LAB. Phylogenetically they do however belong to actinobacteria and therefore are not counted as LAB (56, 57). Use of bifidobacteria is directly linked to numerous health effects that successfully have been proven in treatment of colorectal cancer, diarrhea, necrotizing enterocolitis, inflammatory bowel disease (58). Other examples are their beneficial effects on colon regularity and successful exclusion of pathogens. Therefore, bifidobacteria are among the prominent members of (commercially) applied probiotics. The 'human' group of bifidobacteria comprises strains from the following species: *Bifidobacterium pseudocatenulatum*, *Bifidobacterium catenulatum*, *Bifidobacterium adolescentis*, *Bifidobacterium longum*, *Bifidobacterium breve*, *Bifidobacterium angulatum* and *Bifidobacterium dentium* (56).

Bifidobacteria form the dominant part of the bacterial population in breast-fed healthy infants, while in adults they comprise between 3–6% of the gut microbiota (55, 59). Successful persistence in the human gut requires a couple of features such as metabolic abilities, evasion of the host adaptive immune system and colonization of the host through specific appendages (58). An important factor in colonizing the human gut is successful competition for nutrients. In early stage of human development, glycan of mucus and human milk oligosaccharides (HMOs) are the dominant nutrients bacteria compete for. Bifidobacteria are saccharolytic. On average more than 14% of bifidobacterial encoded genes are involved in carbohydrate metabolism (60). Among bifidobacterial species, *B. longum* subsp. *infantis* and *B. bifidum* were shown to effectively degrade lactose-N-tetraose, comprising HMOs (61, 62). Compared to all known bifidobacterial species, only few species represent strains that by themselves are able to degrade HMO components. However, bifidobacterial strains benefit through cross-feeding on HMO-degradation products, e.g. sialic acid and fucose (63). In fact, various studies have shown that carbohydrate resource sharing is common among bifidobacteria clearly indicating symbiotic interactions of this genus (64). Taken together, bifidobacteria function as ecological specialists or generalists (65), utilizing either a narrow or broad range of nutrients, traits from generalists clearly contributing to their success as colonizers of the human gut. The saccharolytic capabilities of bifidobacteria include also other glycans that for instance are part of the human diet and escape digestion by human enzymes (58, 66). These include prebiotic oligo- and polysaccharides such as fructooligosaccharides (FOS), galactooligosaccharides (GOS), xylooligosaccharides (XOS), inulin, or arabinoxylan, as discussed below.

Prebiotics

Another nowadays widely accepted dietary intervention to restore and benefit human (gastrointestinal) health is reflected by prebiotics (Table 1). When introduced in 1995, Gibson defined a prebiotic as “a nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health” (67). Compounds fulfilling this definition were found in fructooligosaccharides (FOS), inulin and galactooligosaccharides (GOS) (Table 3, Fig. 2). In 2004, Gibson et al. updated their definition stating, prebiotics are “selectively fermented ingredients that allow specific changes, both in the composition and/or activity in the gastrointestinal microflora that confer benefits upon host wellbeing and health” (68). The term then remained unchanged for some time; however, in 2007 Roberfroid updated the definition by mentioning inulin and GOS as the only two substrates entirely fulfilling the definition as prebiotic (69). In 2008, a scientific panel of the Food and Agricultural Organization (FAO) of the United Nations (UN) defined prebiotic as “a non-viable food component that confers a health benefit on the host associated with modulation of the microbiota” including the evolution of the concept to extraintestinal sites. The concept was redefined two years later by Gibson emphasizing again prebiotics as “**selectively** fermented food ingredients”. By this time, dietary carbohydrates were still listed as the only compounds fulfilling the prebiotic definition, including FOS, inulin, GOS and lactulose. In recent years, a number of other (candidate) prebiotics emerged. Also noncarbohydrates, for instance polyphenols and vitamins, became listed as prebiotic candidates, but dietary carbohydrates remained the group with the highest number of prebiotics (70). In 2016 a new definition of the prebiotic concept was published reflecting recent developments, stating as follows: “a prebiotic is a substrate that is selectively utilized by host microorganisms conferring a health benefit” (37). The prebiotic concept thus applies to compounds, sometimes also referred to as chemicals, that selectively are utilized by members of the human microbiota and that stimulate bacterial metabolic activity which is essential in establishing the health effect. A current review lists fructans and galactans as widely accepted prebiotics including a number of adjusted preparations, e.g. inulin and oligofructose for fructans, while the number of candidate prebiotics remains growing (71, 72).

The main targets of prebiotics for bacterial stimulation are *Lactobacillus* and *Bifidobacterium* (73), although newer studies report that species like

Faecalibacterium prausnitzii and *Akkermansia muciniphila* may benefit to some extent from prebiotics (74, 75). It remains largely unknown how novel candidate prebiotics stimulate bacterial growth *in vivo* and *in vitro*. The selective effect of prebiotics towards certain gut bacteria is studied using culture-based techniques (76). This allows a very clear identification whether a given bacterial species responds to prebiotics or not. However, the increasing number of full genome sequences available allows nowadays, in addition to culture-techniques, identification of (potential) metabolic capabilities. This provides increasing knowledge about the mechanisms of prebiotic degradation by *Lactobacillus* and *Bifidobacterium* (77).

Prebiotics exert numerous proven health effects that have been determined in chronic and acute disease states of gastrointestinal and metabolic health. Prebiotics were successfully studied with irritable bowel syndrome (IBS) and functional constipation; bowel habit and general gut health in infants; traveler's diarrhea; allergy; inflammatory bowel disease (IBD); hepatic encephalopathy; infections and vaccine response; immune function in elderly; necrotizing enterocolitis in preterm infants; urogenital health; skin health; bone health; absorption of calcium and other minerals; overweight and obesity; type 2 diabetes mellitus and metabolic syndrome (37, 78). As a result of bacterial fermentation in the colon, prebiotic carbohydrates are metabolized into SCFAs mostly (79). These compounds were shown, also when administered directly, to play key roles in establishing the health effects associated with prebiotics (80). It should be noticed, that bacterial fermentation of carbohydrates also leads to production of metabolites such as pyruvate, ethanol, succinate. Also the production of gases like H₂, CO₂, CH₄ and H₂S can occur during this process (81).

Fructooligosaccharides (FOS) and inulin. FOS and inulin comprise the most well-known and by far most extensively studied group of prebiotic carbohydrates. They are comprised by mostly fructose units linked with $\beta(2\rightarrow1)$ glycosidic linkages and in inulin to minor extent $\beta(2\rightarrow6)$ linkages. For both, FOS and inulin, synthesis starts with sucrose, serving as donor for fructose and acceptor for adding fructose. The first product of this synthesis with a $\beta(2\rightarrow1)$ linkage is a trisaccharide, called 1-kestose (GF2). Homologous elongation products are nystose (GF3) and the pentasaccharide fructofuranosyl nystose (GF4). FOS and inulin are further grouped into fructans which also comprise the $\beta(2\rightarrow6)$ linked levan. Based on origin, the following terms are used as follows: Fructooligosaccharides refer to $\beta(2\rightarrow1)$ linked oligosaccharides synthesized

with fungal or bacterial enzymes (fructosyltransferase, FTF; Glycoside hydrolase family 32 and 68, www.cazy.org) (82). Inulin is obtained through hot water extraction from plants e.g. chicory root comprising compounds with DP between 2 and 60 (83). Oligofructose (OF) is received from enzymatic hydrolysis of inulin using inulinase from *Aspergillus* sp. (84). The resulting products mostly lack terminal sucrose residues as a result of inulinase hydrolysis.

A number of FOS producing enzymes has nowadays been described. In fungi, these enzymes are found extra- and intracellularly, e.g. in *Aspergillus* and *Penicillium* species. Bacterial fructosyltransferases have been characterized in strains of *Bacillus macerans*, *Lactobacillus reuteri*, *Lactobacillus johnsonii* (85) and *Zymomonas mobilis*. These enzymes, when industrially applied, can produce FOS with a product yield ranging between 55% and 60% of initial sucrose concentration. Synthesis is carried out in a two-step process that produces the microbial enzyme first and then incubates the purified enzyme with sucrose (also called submerged fermentation) (86). The DP of the resulting products is important for its transition through the gastrointestinal tract. Short-chain FOS are fermented in the proximal sites of the bowel, while fractions with higher DP, prominently present in inulin, reach the colon (87).

Inulin is typically found in plants like *Helianthus tuberosus* (Jerusalem artichoke), *Cichorium intybus* (chicory), *Dahlia pinnata* (dahlia) and *Polymnia sonchifolia* (yacon) (88). There, inulin is synthesized as part of an extended sucrose metabolism and serves as storage polymer. In plants the reaction is catalyzed by two or more enzymes, the two most important, a sucrose:sucrose 1-fructosyltransferase, transferring a fructose residue from one sucrose molecule to another, and a fructan:fructan 1-fructosyltransferase, transferring fructose residues from 1-kestose or larger fructans to sucrose and other fructans. Importantly, the structural composition of inulin depends on different factors such as species, growing conditions, harvesting and storage time (89). These factors strongly influence the structure, e.g. inulin DP in chicory roots (88).

A number of studies have targeted the influence of structural parameters of FOS/inulin on growth of gut related bacterial strains. It was found that bacteria often use different fractions of FOS/inulin, but hardly any strains were able to consume FOS/inulin completely. Cross-feeding events that may contribute to complete FOS/inulin consumption have been reported in some cases, e.g. *Bacteroides thetaiotaomicron*/bifidobacteria, involving SCFAs as cross-feeding

molecules (113). Little is known about cross-feeding on intermediate degradation products that may occur during bacterial FOS/inulin utilization.

Galactooligosaccharides (GOS). Galactooligosaccharides are synthesized from lactose using β -galactosidase enzymes from bacteria, fungi and yeast (90). These enzymes are glycoside hydrolases catalyzing hydrolysis of terminal β -galactose residues and are annotated in GH families 1, 2, 35 and 42. Besides hydrolysis, transglycosylation occurs transferring galactose from lactose onto available acceptor molecules, such as lactose or previously formed GOS molecules, yielding oligosaccharides with DP ranging from 2 to 8 (Table 3). GOS are widely applied in (infant) nutritional products. In recent years, various microbial β -galactosidase enzymes have been identified and characterized and this has prompted development of GOS mixtures with varying DP and glycosidic linkage profiles. Commercially applied enzymes are from *Bacillus circulans*, *Kluyveromyces lactis* and *Aspergillus oryzae*. The product profiles of GOS obtained from these enzymes depend strongly on the origin of the β -galactosidase and structural differences observed in the 3-dimensional structures of β -galactosidases (91). On average industrial synthesis using microbial enzymes yields products with GOS content up to 55% and lactose and monosaccharides as byproducts.

Recently, our research group made efforts for the complete structural characterization of (commercial) GOS mixtures covering >99% of the products (92, 93). Before, complete assessment was limited to identity of GOS isomers at the DP level (94). Our structural characterization work has shown that GOS mixtures differ in their distribution of DP and in the presence of the following glycosidic linkages: $\beta(1\rightarrow3)$, $\beta(1\rightarrow4)$ and $\beta(1\rightarrow6)$. Selective utilization of GOS molecules, separated at the level of DP, has been shown for probiotic bacteria, however, a study that includes glycosidic linkages is still missing. The impact of both parameters on selective utilization by, and growth of, probiotic bacteria has not been studied so far.

IsoMalto-/Maltopolysaccharides (IMMPs). IMMPs are a new class of dietary fiber obtained through enzymatic modification of starch by the GtfB enzyme. GtfB is a 4,6- α -glucanotransferase identified in *L. reuteri* 121 and uses starch and maltodextrins cleaving $\alpha(1\rightarrow4)$ linkages and adding the released glucose moieties consecutively with $\alpha(1\rightarrow6)$ linkages at the non-reducing ends of the starch molecule (Fig. 2) (95). The enzyme comprises a subfamily in glycoside hydrolase (GH) family 70. Incubation of the enzyme with maltooligosaccharides (DP 2–7) yielded linear IMMPs with DP up to 35 and

more (96) (Table 3). Conversion of a broad range of maltodextrins and starches by the GtfB enzymes yielded IMMPs with % $\alpha(1\rightarrow6)$ ranging from 7–91% (97). GtfB only converts amylose and starch/amylopectin pretreated with debranching enzymes allowing introduction of higher % $\alpha(1\rightarrow6)$ (98). This showed that through substrate selection, in- or excluding debranching enzymes, reaction time or GtfB enzyme concentration the % $\alpha(1\rightarrow6)$ in the resulting products can be controlled. IMMPs are dietary fibers that to a lesser extent are degraded by small intestine enzymes as compared to the initial starch products (96). In fermentation experiments using human fecal samples the microbiota composition in time shifted towards beneficial bifidobacteria and lactobacilli, qualifying IMMPs as potential prebiotics (99). Compared to FOS/inulin and GOS compounds, these IMMPs are not yet commercialized and their prebiotic potential remained to be studied.

Table 3 Known structural parameters of the (potential) prebiotic carbohydrates studied in this PhD thesis. *D-Glcp residues at reducing ends in GOS and as part of terminal sucrose residues in FOS/inulin

	Monosaccharide	Glycosidic linkage	Degree of polymerization
FOS/inulin	D-Fruc + D-Glcp*	$\beta(1\rightarrow2)$	2–60
IMMP	D-Glcp	Variable $\alpha(1\rightarrow6)$, $\alpha(1\rightarrow4)$	Variable, >50
GOS	D-Galp + D-Glcp*	majorly $\beta(1\rightarrow4)$, also $\beta(1\rightarrow2)$, $\beta(1\rightarrow3)$, $\beta(1\rightarrow6)$	2–8

Fig. 2 Structural composition, donor substrate and natural source of prebiotics (shown for inulin) or donor substrates for synthesis of prebiotics (shown for GOS and IMMP).

Metabolism of prebiotic carbohydrates in lactobacilli and bifidobacteria

The popularity of the prebiotic concept, providing novel opportunities to sustain human health in times of rapidly decreasing effectiveness of classical antibiotics, has prompted production of a growing number of carbohydrate substrates with varying and often unknown precise structural composition. While for substrates such as FOS and GOS the prebiotic effect is already well established, for candidate prebiotics such as IMMP their effects are still not completely understood (99). Often, identifying the complete structural composition of a given prebiotic is a challenge. Also the question how prebiotics and probiotic bacteria synergistically interact remains to be answered. A number of studies have shown how certain probiotic strains degrade prebiotic carbohydrates. These studies often used single strains to show (i) whether strains actually grow on prebiotics, by determining OD600nm, and (ii) demonstrating substrate utilization by TLC, HPLC and/or HPAEC-PAD techniques. Additionally, transcriptomic, genomic and biochemical studies have identified important genes/pathways in *Lactobacillus/Bifidobacterium* strains highlighting individual strain capabilities to degrade prebiotic carbohydrates.

In *L. acidophilus* NCFM a FOS degradation operon has been identified (*msm*). This operon encoded genes for a LacI family transcriptional regulator (*msmR*), an ABC transporter (*msmEFGK*), a β -fructofuranosidase (β -FFase) (*bfrA*) and a sucrose phosphorylase, the latter two part of family GH32 (Fig. 3A) (100). In *L. plantarum* WCFS1 another FOS utilization gene cluster was found, encoding a sucrose phosphoenolpyruvate-dependent phosphotransferase system (PTS) and a cytoplasmic β -FFase (Fig. 3A) (101). This pathway was selective for uptake of FOS comprising GF_n molecules (with a terminal sucrose residue). *L. paracasei* 1195 encoded an additional FOS utilization operon (*fosRABCDXE*) including a cell wall-anchored β -FFase (*fosE*) and a fructose PTS transporter (*fosABCDX*) (Fig. 3A) (102). In bifidobacteria, FOS degradation was identified in *B. adolescentis* G1, *B. breve* UCC2003, *B. lactis* DSM 10140T and *B. longum* NCC2705 (Fig. 3B). These strains encoded a sucrose permease (*cscB*) and an intracellular β -FFase (*cscA*) (103–105). It was shown that CscA selectively degrades $\beta(2\rightarrow1)$ linkages in GF_n-type molecules but not in FF_m-type molecules.

GOS utilization systems also were identified in *L. acidophilus* and *L. plantarum*. These bacteria encoded LacS permeases leading to intracellular uptake of lactose/GOS substrates. Gene clusters in these strains further comprised intracellular β -galactosidases from GH families 2 and 42 (106). *L. gasseri* lacks these gene clusters and instead encodes lactose PTS and phospho- β -galactosidase for lactose utilization (107). Regarding bifidobacteria, extracellular GH53-endogalactanases (*galA*) were proven to degrade GOS in *B. longum* NCC2705 and *B. breve* UCC2003. GalA was part of the galactan operon *galCDEGRA* in *B. breve* UCC2003 encoding also an ABC transporter (GalCDE) and an intracellular GH42 β -galactosidase (GalG). Other bifidobacteria, e.g. *B. lactis* BI-04, encoded also ABC transporters and an intracellular GH42 β -galactosidase, but lack extracellular GH53 galactanases (Fig. 3B) (108).

A Lactobacillus **B Bifidobacterium**

Fig. 3 Catabolic pathways identified in *Lactobacillus/Bifidobacterium* strains involved in degradation of (prebiotic) carbohydrates. **(A)** *Lactobacillus* strains. FOS/inulin degradation occurred in *L. acidophilus* NCFM by an ABC transporter and intracellular β -fructofuranosidase (β -FF), in *L. plantarum* WCFS 1 by sucrose PTS/intracellular β -FF and in *L. paracasei* 1195 by extracellular β -FF/fructose PTS. GOS is taken up by LacS (*L. acidophilus*) or LacY (*L. plantarum*) permeases and degraded by intracellular β -galactosidases. *L. acidophilus* expresses an ABC transporter in order to take up isomaltomaltotriooligosaccharides that are further degraded intracellularly by various family GH13 enzymes such as glucan-1,6- α -glucosidase (*LaGH13_31*; G16G). **(B)** FOS/inulin metabolism in bifidobacteria involves ABC transporters (*B. lactis* BI-04) or MFS

permeases (*B. longum* NCC2705) for take up and degradation in the cytoplasm. Their chain-length specificity limits the ability of bifidobacteria to use the entire spectrum of inulin substrates. GOS utilization involves ABC transporters (*B. lactis* BI-04) and in some cases extracellular family GH53 enzymes (*B. breve* UCC2003). α -Glucans are internalized by ABC transporters that also take up galactosides (*B. lactis* BI-04) or broken down extracellularly by type II amylopullulanases (*B. breve* UCC2003).

When starch-type of substrates are considered, lactobacilli are poorly equipped to utilize these polymeric substrates. In *L. acidophilus* NCFM, this involves an ABC transporter that utilizes malto- and isomaltooligosaccharides (109). In terms of enzymatic capabilities, lactobacilli often encode a high number of intracellular family GH13 enzymes that are typically involved in metabolism of α -glucans. These enzymes for instance confer ability of *L. acidophilus* to degrade isomaltooligosaccharides $>DP\ 2$ involving a glucan-1,6- α -glucosidase (*LaGH13_31*; G16G). In bifidobacteria, metabolism of isomaltooligosaccharides is linked to α -1,6-galactosides (e.g., raffinose and stachyose) and involves an ABC transporter that is expressed in the presence of both types of substrates (109). Additionally, bifidobacteria are able to extracellularly degrade starch. This feature also was observed in *B. breve*, *Bifidobacterium pseudolongum*, and *Bifidobacterium thermophilum* (110).

Knowledge of these pathways provides a firm basis for studying the selective utilization of (prebiotic) carbohydrates by probiotic bacteria.

Bacterial cross-feeding on prebiotic substrates. As stated above, gut bacteria employ strain specific metabolic pathways to degrade prebiotic carbohydrates. In practice, they colonize as part of a microbial community where resource sharing is commonly observed, as well as competing for nutrients among different members. Well-established cross-feeding events have for instance been proven with bifidobacteria and prebiotic inulin. While inulin generally is believed to be bifidogenic, implying the enrichment of the *Bifidobacterium* genus by prebiotic inulin, culture experiments with single *Bifidobacterium* strains showed that by themselves they only use a limited part of the compounds present in the inulin, and none of the strains was able to use inulin completely (111). When searching gut bacterial members with extracellular enzyme activities for degradation of prebiotic inulin, suitable strains were found in the genus of *Bacteroides* spp., *Lactobacillus* spp., *Roseburia* spp., *Eubacterium rectale* and *Faecalibacterium prausnitzii* (112). During co-culturing of bifidobacteria with *Bacteroides thetaiotaomicron* LMG 11262 employing an extracellular fructofuranosidase to degrade inulin,

bifidobacteria dominated these cultures while they were unable themselves to grow on longer fractions of inulin (113). Such interspecies interactions among gut bacteria may be a pure commensal trait or as shown for bifidobacteria also have beneficial traits as the end-products of fermentation of inulin fractions by bifidobacteria cross-feed other butyrate producing gut bacterial members (114). In fact, cross-feeding on SCFAs was commonly observed among gut bacteria, while cross-feeding on extracellular carbohydrate degradation products is only shown for a few examples especially in bifidobacteria.

Scope of the thesis

The gut microbiota constitutes a highly complex ecosystem. The physiology and catabolic potential of individual bacterial strains is relevant, but the final contribution in substrate conversion also depends on the number of cells present in such a mixed culture. As outlined above, bacterial strains employing extracellular enzymes to hydrolyze the (oligomeric, polymeric) substrate generally are able to transport hydrolysis products into the cell via membrane transport systems, for further degradation intracellularly. In mixed cultures there will be competition for such hydrolysis products. This may result in (un)desirable synergistic effects. In this PhD thesis project we have focused on analysis of growth of pure cultures of probiotic bacteria on selected prebiotic carbohydrates. Following the results obtained from single culture experiments, we also observed a few examples of cross-feeding between these bacteria in mixed-cultures. The synergistic interactions between prebiotics and probiotic bacteria are still poorly understood. A scientific definition for synbiotics has been proposed, but an overall accepted definition is still lacking in literature. In this thesis, we therefore aimed to investigate the synergistic effects between (commercial) prebiotic carbohydrates and (commercial) probiotic bacterial strains by first characterizing the structural composition of prebiotic carbohydrates in detail, second studying their effects on growth of probiotic bacteria using anaerobic cultivation experiments and finally identifying the transporter systems and glycoside hydrolases that probiotic bacteria may use to degrade prebiotic carbohydrates, by analyzing the available genome sequences. The combined dataset provides a clear understanding about the selective consumption of these prebiotic carbohydrates by the probiotic bacteria studied. In future work the results may provide a firm basis for the design of synbiotics important in food/feed and medicine/pharmacy.

In **Chapter 1** a general overview of current knowledge on (potential) prebiotic carbohydrates and their composition is provided. Additionally, probiotic bacteria are described and their influence on human well-being and health is outlined. In a final paragraph, carbohydrate transporters and glycoside hydrolases (GHs) are introduced and an overview presented of those GHs potentially involved in bacterial metabolism with the prebiotic carbohydrates studied here.

In **Chapter 2** the selective consumption and cross-feeding potential of FOS/inulin prebiotics was investigated among selected probiotic gut bacteria. A detailed structural analysis of commercial $\beta(2\rightarrow1)$ fructans was conducted and growth with probiotic commercial strains of lactobacilli and bifidobacteria studied. The gene encoding an extracellular GH32 inulinase enzyme from *Lactobacillus paracasei* W20 was cloned, expressed and the enzyme biochemically characterized. Its potential role in cross-feeding among probiotic strains was studied in defined mixed cultures. This revealed different cross-feeding events between probiotic lactobacilli and based on genomic annotations also bifidobacteria when strains are grown on prebiotic FOS/inulin. The results were sustained by differential annotation of proteins of the fructose/sucrose/FOS pathways in bacterial genomes. The results provide an example showing how cross-feeding among probiotic lactobacilli involving prebiotic FOS/inulin may occur *in vivo*.

In **Chapter 3** the three-dimensional structure of the extracellular family GH53 endo-galactanase from the human gut commensal *Bacteroides thetaiotaomicron* is presented (BTGH53). This BTGH53 structure is the second from bacterial origin and differs in lacking an important loop in the substrate binding site. The BTGH53 enzyme digested GOS and galactan down to galactose and DP 2 GOS, which was not observed with other known bacterial GH53 galactanases. We hypothesize that the domain that is absent in BTGH53 is responsible for this difference. Various *Lactobacillus* strains incubated under anaerobic conditions only grew to a limited extent on two GOS preparations tested. In contrast these strains grew well on the BTGH53 GOS products. The differences in *Lactobacillus* growth correlated with selective consumption of GOS molecules mostly with DP 2, yielding good growth for the BTGH53 digested GOS and limited growth for the pure GOS. This demonstrated the potential role of the extracellular BTGH53 enzyme in cross-feeding on GOS substrates among members of the human gut microbiota and its potential application in synergistic synbiotics based on GOS.

In **Chapter 4** the structural identity of GOS molecules selectively utilized by various lactic acid bacteria and *Bifidobacterium* strains is presented. The strains clearly differed in ability to consume GOS molecules depending on their degree of polymerization and on glycosidic linkages present. Each strain grew on an individual set of the 40 known structures of the prebiotic GOS mixture. LAB strains mostly consumed DP 2–3 molecules including lactose, $\beta(1\rightarrow4)$ linked, and DP 2 molecules with $\beta(1\rightarrow2)$, $\beta(1\rightarrow3)$ and $\beta(1\rightarrow6)$ linkages. Bifidobacteria consumed these molecules as well and with variable extent the DP 3–6 GOS. This differential ability correlated with presence of known pathways for lactose/galactan utilization annotated in bacterial genome sequences. LAB strains mostly encoded proteins for lactose utilization pathways, while bifidobacteria additionally comprised GOS/galactan utilization systems. The strains highly differed in number of genes for the studied pathways. These results are a firm basis for studying selective utilization of individual GOS molecules with a given structure by probiotic bacteria.

Chapter 5 reports on the synthesis of exo-polysaccharides by *L. reuteri* strains encoding 4,6- α -glucanotransferases. Previously, we characterized the 4,6- α -glucanotransferase (GtfB) from *L. reuteri* 121 biochemically using starch and maltodextrins as substrates to introduce $\alpha(1\rightarrow6)$ linkages at non-reducing ends. These products were called Isomalto-/Maltopolysaccharides (IMMPs). Here we tested various *L. reuteri* strains encoding 4,6- α -glucanotransferase genes. These strains synthesized homoexopolysaccharides from various starch and maltodextrin substrates that showed an increase in % $\alpha(1\rightarrow6)$ similar to what was observed with the purified GtfB enzyme. In terms of size, the polysaccharides synthesized by the strains were larger than the IMMPs produced by GtfB. Several of these purified exopolysaccharides were tested in growth experiments with probiotic bifidobacteria. This showed that some bifidobacterial strains were able to grow up to 100% on *L. reuteri* produced exopolysaccharides and further indicate the prebiotic potential of these bacterial exopolysaccharides.

Chapter 6 provides an overview of the most important research findings of this work as well as a discussion in context of the existing literature.

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Chapter 2

Cross-feeding among probiotic bacterial strains on prebiotic inulin involves the extracellular *exo*-inulinase of *Lactobacillus paracasei* strain W20

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Abstract

Probiotic bacteria isolated from the human gut employ specific metabolic pathways to degrade dietary carbohydrates which go beyond capabilities of their host. Here we report an analysis of the abilities of individual commercial probiotic strains to degrade prebiotic (inulin type) fructans. We have performed a structural analysis of the commercial fructose-oligosaccharides/inulin samples used in this study. These $\beta(2\rightarrow1)$ fructans are comprised of two types, differing in termination by either a glucose (GF) or a fructose residue (FF), with a broad variation in degree of polymerization (DP). Growth of individual probiotic bacteria on short-chain (sc)Inulin (Frutafit® CLR), a $\beta(2\rightarrow1)$ fructan (DP 2–40), was studied. Growth of *Lactobacillus salivarius* W57 and other probiotic bacteria on scInulin was relatively poor with only fractions of DP 3 and DP 5 utilized, reflecting uptake via specific transport systems followed by intracellular metabolism. *Lactobacillus paracasei* W20 completely used all scInulin components employing an extracellular *exo*-inulinase enzyme (glycoside hydrolase family GH32, *LpGH32*; also found in other strains of this species); the purified enzyme converted high DP compounds into fructose, sucrose, 1-kestose and F2 (inulobiose). Co-cultivation of *L. salivarius* W57 and *L. paracasei* W20 on scInulin resulted in cross-feeding of the former by the latter, supported by this extracellular *exo*-inulinase. The extent of cross-feeding depended on the type of fructan, i.e. GF type (clearly stimulating) versus FF-type (relatively low stimulus), and on fructan chain-length, since relatively low DP $\beta(2\rightarrow1)$ fructans contain a relatively high content of GF-type molecules, thus resulting in higher concentrations of GF-type DP 2 to 3 degradation products. The results provide an example of how *in vivo* cross-feeding on prebiotic $\beta(2\rightarrow1)$ fructans may occur amongst probiotic lactobacilli.

Importance

The human gut microbial community is associated strongly with host physiology and human diseases. This observation has prompted research on pre- and probiotics, two concepts allowing specific changes in composition of the human gut microbiota that result in beneficial effects for the host. Here we show how fructooligosaccharide/inulin prebiotics are fermented by commercial probiotic bacterial strains involving specific sets of enzymes and transporters. Cross-feeding strains such as *L. paracasei* W20 thus may act as keystone strains in degradation of prebiotic inulin in the human gut, and this strain/*exo*-inulinase combination may be used in commercial *Lactobacillus*/inulin synbiotics.

Introduction

Probiotics are live microorganisms that, when administered in adequate amounts, confer a health benefit to the host (1). Probiotic bacteria are typically found in the genera *Bifidobacterium* and *Lactobacillus*, however some strains of *Enterococcus* and *Escherichia* also exhibit interesting probiotic properties. Providing bacteria with prebiotic compounds allows for the selective stimulation and enrichment of desirable bacterial strains important for sustaining human health. Prebiotics are defined as substrates that are selectively utilized by host microorganisms conferring a health benefit (2). A prominent class of prebiotics is constituted by dietary non-digestible carbohydrates. These non-digestible carbohydrates escape from being metabolized by human digestive enzymes and are therefore available as carbon and energy source to bacteria residing in the lower gastrointestinal tract (3).

There are several classes of prebiotic carbohydrates, including arabinoxylanooligosaccharides (AXOS), maltooligosaccharides (MOS), galactooligosaccharides (GOS), and fructooligosaccharides (FOS, such as inulin and oligofructose (OF)) (4). Fructose-based prebiotics are the most well studied non-digestible carbohydrates considered for their prebiotic effects. These include the $\beta(2\rightarrow1)$ fructans derived by enzyme synthesis from sucrose, and inulin or oligofructose obtained from plant sources. $\beta(2\rightarrow1)$ Fructans exhibit structural differences in both their degree of polymerization (DP) which may vary between DP 2 to 60, and the constituent terminal non-reducing end sugar, being glucose for GF-type fructans, and fructose for FF-type fructans (5, 6).

Bacteria that metabolize prebiotics and other non-digestible carbohydrates produce short-chain fatty acids (SCFA), such as acetate, propionate and butyrate. These biologically important compounds mediate crucial health effects in the human body at the molecular level (7). Prebiotics thus serve as selective nutritional sources for probiotic bacteria, and the two independently defined dietary concepts of pre- and probiotics may synergistically come to action in the human gut.

Probiotic bacteria may benefit selectively from the presence of prebiotic carbohydrates through their set of Carbohydrate Active Enzymes (CAZymes, www.cazy.org), which include glycoside hydrolases (GHs), allowing carbohydrate utilization as carbon and energy sources (8). GHs encoded in bacterial genomes often outnumber the number of human GH genes by several folds (9). Bacterial species strongly vary in the set of GH enzymes encoded.

Different carbohydrates thus are fermented by different bacterial species, resulting in selective growth stimulation of species that benefit from a certain carbohydrate structure. Modulation of the human gut microbiota by prebiotic carbohydrates thus may occur at the species level (10). This provides clear opportunities to supply combinations of probiotic strains and prebiotic compounds with beneficial effects. In practice, however, pre- and probiotic treatments are often still given independently from each other. It still remains largely unknown how the increased beneficial effects of novel probiotic bacterial strains can be potentiated through addition of selected prebiotics. Further studies into the physiology and growth of probiotic bacteria on prebiotic oligosaccharides are therefore clearly needed.

Utilization of certain $\beta(2\rightarrow1)$ fructans has been investigated with various strains from the genera *Lactobacillus* and *Bifidobacterium* (11–14). These studies mostly focused on the selective utilization of fructans with a certain length per strain. For instance, *Bifidobacterium longum* IPLA20021–20022, *Bifidobacterium animalis* IPLA20031–20032, *B. animalis* IPLA20020 and *B. animalis* Bb12 grew better on scFOS Actilight 950P (low DP), than on inulin from dahlia tubers (high DP) (11). A study of oligofructose and inulin utilization by 18 *Bifidobacterium* strains from 10 different species revealed 4 groups, reflecting their individual catabolic abilities, but none of the strains consumed inulin completely (12). The ability to utilize specific fructan components differed even amongst strains from the same *Lactobacillus* or *Bifidobacterium* species (13, 14). No single species used FOS/inulin completely. Besides direct stimulation of probiotic bacteria by prebiotic substrates, they also may indirectly benefit from prebiotic substrates via cross-feeding mediated through smaller carbohydrate degradation products or by SCFA as end-products of bacterial fermentation. While the structural identity of SCFA cross-feeding other bacteria often is clear (15–17), little is known about the structures of the extracellular degradation products of prebiotic fructans that may accumulate through bacterial GH enzyme activity. Elucidation of such structural features is essential for the further unravelling of the cross-feeding mechanisms that may occur amongst human gut microbiota, and will allow determination of the ecological roles that individual members play and show how members contribute to the gut microbiota as a whole. In particular, some bacteria have been assigned a role as keystone species, present at relatively low abundance but exerting a strong, stabilizing influence on the community (18). Only a single study currently reports such a role for a *Lactobacillus*, *Lactobacillus reuteri* DSM 17938 (19).

For the human isolate *L. paracasei* subsp. *paracasei* 8700:2 beneficial effects of this strain towards other members of the gut microbiota have been observed due to its extracellular breakdown of inulin-type fructan, but the extent of this effect on other strains has not been investigated in detail (17, 20).

In this study we aimed to identify which factors influence FOS/inulin degradation by pure cultures and defined co-cultures of probiotic *Lactobacillus* strains. The results show that an extracellular family GH32 enzyme employed by *Lactobacillus paracasei* W20 increases the availability of suitable FOS carbon and energy substrates to *Lactobacillus salivarius* W57 in co-cultures. To our knowledge, it has not been shown before that an extracellular GH32 enzyme can mediate cross-feeding propensity to another bacterium. These stimulatory properties of *L. paracasei* towards another gut bacterium offer possibilities for multispecies symbiotic combinations.

Materials and Methods

Bacterial strains and growth conditions. The commercial probiotic bacterial strains *Lactobacillus paracasei* subsp. *paracasei* W20 (*L. paracasei* W20), *Lactobacillus acidophilus* W37, *Lactobacillus salivarius* W57, *Lactobacillus casei* W56, *Enterococcus faecium* W54 and *Pediococcus acidilactici* W143, *Bifidobacterium animalis* subsp. *lactis* W51 (*B. lactis* W51), *Bifidobacterium animalis* subsp. *lactis* W52 (*B. lactis* W52), *Bifidobacterium animalis* subsp. *lactis* W53 (*B. lactis* W53) were provided by Winclove Probiotics B.V. (Amsterdam, The Netherlands). All strains belonging to lactic acid bacteria (LAB) were maintained in de Man-Rogosa-Sharpe (MRS) broth (Oxoid, Basingstoke, UK) containing 2% glucose as carbon source. Liquid cultures (5 ml) were inoculated in anaerobic culture glass tubes (Boom, Meppel, The Netherlands), anaerobic conditions provided using the Hungate system with nitrogen (37) and incubated at 37°C. For permanent storage, fresh 200 µl aliquots of the cultures were diluted with sterile glycerol to 15% (v/v) in 1.5 ml tubes and the tubes maintained at -80°C. Purity of the cultures was frequently checked by spreading aliquots on agar plates prepared with MRS and Luria-Bertani (LB) medium, respectively, and by light microscopy. For growth experiments with $\beta(2\rightarrow1)$ fructans modified MRS-medium (mMRS) was used with LAB strains prepared according to (38). In brief, 1 L mMRS contained: peptone 10 g; granulated yeast extract 2.5 g; tryptose 3 g; Tween80 1 g; K₂HPO₄ 3 g; KH₂PO₄ 3 g; ammonium citrate 2 g; pyruvic acid sodium salt 0.2 g;

MgSO₄·7H₂O 0.575 g; MnSO₄·H₂O 0.12 g; FeSO₄·7H₂O 0.034 g. Components were dissolved in bidest. H₂O by bringing the medium to boil, subsequently 0.5 g/l cysteine·HCl was added and the pH adjusted to 6.8. The medium was sterilized by autoclaving (15 min, 121°C).

Bifidobacterial strains were maintained in *Bifidobacterium* medium (BM) that was prepared according to (39). 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·H₂O, 0.12 g MnSO₄·H₂O in 1 l distilled water), 5 g NaCl. After autoclaving BM was supplemented with 0.05% (w/v) filter-sterilized cysteine-HCl, and strains were grown at 37 °C under anaerobic conditions maintained by 100% CO₂.

Carbohydrates. Native inulin (Frutafit® HD; inulin DP2-DP60 ≥ 90% w/w; average chain length 11), long-chain inulin (lcInulin, Frutafit® TEXT; inulin ≥ 99,5% w/w; average chain length 22), short-chain inulin (scInulin, Frutafit® CLR; inulin/oligofructose ≥ 85% w/w; average chain length 7) and oligofructose (Frutalose® OFP; oligofructose ≥ 92 ± 2% w/w; average chain length 4) were provided by SENSUS (Roosendaal, The Netherlands). 1-Kestose (≥ 98%) and nystose (≥ 98%) were purchased from CarboSynth (Compton, UK). FOS P1 (oligofructose DP2-DP8 ≥ 93% w/w), Inulin P2 (inulin DP 2-DP60 ≥ 90% w/w; average chain length 10), scFOS P6 (oligofructose DP3-DP5 ≥ 95% w/w) were obtained from Winclove Probiotics B.V.. Levan was produced by sucrose conversion with *Bacillus subtilis* as described (40). Carbohydrates were dissolved to 1% (w/v) in Milli-Q water and filter-sterilized using 0.2 µm cellulose acetate membrane filters (VWR International, Radnor, PA). lcInulin was dissolved and sterilized by autoclaving. DPs and average chain lengths were added as indicated by suppliers.

Growth experiments. Growth experiments with LAB strains were carried out using 96-well microtiter plates (Greiner Bio-one, Frickenhausen, Germany). Prior to inoculation of the plates, overnight grown cultures of bacterial strains were harvested by centrifugation (2,300 x g, 2 min) and diluted 25-fold in 2x mMRS. Separately, carbohydrate solutions were added in triplicate to wells of plates, while glucose and bidest water served as positive and negative controls, respectively. Subsequently, diluted bacterial suspensions and plates containing carbohydrate solutions were transferred into an anaerobic glove box (Bohlander, Grünsfeld, Germany) with constant nitrogen flow, and medium inoculated with 2% (v/v) of bacteria (total volume of cultures per well 160–200

μl) to provide anaerobic conditions in microtiterplates. Then, the plate was sealed with an airtight, transparent seal (Simport, Beloeil, Canada) and placed into a plate-reader (at 37°C) for monitoring OD600nm in 5 min intervals with continuous shaking at medium speed. The plate-reader was installed inside AtmosBag (Sigma-Aldrich, Schnelldorf, Germany), constantly flushed with nitrogen throughout OD600nm-measurements. For growing *L. salivarius* W57 in the presence of the GH32 enzyme from *L. paracasei* W20 (*LpGH32*) 10 μl of the recombinantly produced and purified enzyme were added per well, yielding a final concentration of 0.5 μg/ml. The co-culture experiments were inoculated with 1% (v/v) of an overnight-culture of *L. salivarius* W57 and *L. paracasei* W20 of each strain. To determine cfu/ml, bacterial cultures were harvested at 18 h (*L. paracasei* W20 single cultures and *L. paracasei* + *L. salivarius* co-cultures) and 8 h (*L. salivarius* W57 single cultures), respectively. Harvested cultures were diluted to appropriate levels and spread on MRS-Agar (MRS-medium supplemented with 1.5% (w/v) Agar). Subsequently plates were incubated anaerobically in jars using the GasPak EZ container system (BD, Sparks, MD). Plates incubated with both strains were kept at 18°C (growth of *L. paracasei* W20 only) and 37°C (growth of both strains).

Bifidobacterial strains were grown in 3 ml cultures in BM supplemented with 0.5% (w/v) of carbohydrates using anaerobic glass tubes maintained anaerobic with 100% CO₂ at 37°C. BM with and without 5 mg/ml glucose was used as positive and negative control, respectively. Growth was followed in time by measuring OD600nm manually directly in anaerobic glass tubes using the cell density meter WPA CO 8000 (Biochrom, Cambridge, UK).

Structural analysis of fructo-oligosaccharides. FOS composition was analyzed using thin layer chromatography (TLC) as reported previously (41). In brief, samples of 1 μl were spotted up to 3 times on TLC sheets (Silica gel 60 F254; Merck, Darmstadt, Germany) that were developed with n-butanol : ethanol : water = 5 : 5 : 3 (v/v). Bands were visualized by urea staining. FOS composition was determined by high-pH anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) by diluting samples to appropriate concentration in 80% (v/v) dimethylsulfoxide. Samples were analyzed on a CarboPac PA1 column (4 mm x 250 mm; Dionex, Sunnyvale, CA) using a linear gradient (buffer A = 0.1 M NaOH, buffer B = 0.6 M NaOAc in 0.1 M NaOH): 0 min, 0% B; 10 min, 22% B; 25 min, 40% B; 25.1 min, 100% B. Glucose, fructose, 1-kestose and nystose were used as standards to determine monosaccharides and short oligosaccharides (42). Inulin synthesized by the InuJ

enzyme (DP range 2–16; inulin polymer) was used to annotate any larger oligosaccharides (41).

Detection of extracellular enzyme activity in bacterial culture supernatants. After 24 h of growth on FOS, cultures of probiotic bacteria were centrifuged (10,000 x g, 15 min). Subsequently, supernatants were filtered using 0.45 µm cellulose acetate filters (VWR International) and mixed 1:1 with 10 mg/ml scInulin in 1.5 ml reaction tubes. Incubation was allowed for 72 h at 37°C before analyzing (changes in the) scInulin composition by TLC and HPAEC-PAD. Culture supernatants from growth on glucose served as negative controls.

Cloning, overexpression and purification of truncated GH32 enzyme from *L. paracasei* W20. Genomic DNA isolation from *L. paracasei* W20 was performed using GenElute™ Bacterial Genomic DNA kit (Sigma-Aldrich). Genomic DNA (10 ng) was used as template in PCRs to amplify the gene encoding GH32 enzyme from *L. paracasei* W20. Primers used in PCRs were
LPGH32-Fwd 5'-
CAGGGACCCGGTCCATACCGAAACCAGTATCACTACTCAAGTAGC-3'
and
LPGH32-Rev 5'-
CGAGGAGAAGCCCGGTTAGTTCCAAATTGAAGTAATTGGATTGATAG
TTAAGTCGC-3'. The underlined complementary overhangs were used for joining inserts with vector pET15b which was modified to allow for ligation independent cloning (LIC) of the amplified DNA (43). Phire Hot Start II DNA Polymerase (Thermo Scientific, Waltham, MA) was used for amplification in MJ Mini™ Personal Thermal Cycler (BioRad, Hercules, CA). Reactions contained 0.05% (v/v) dimethylsulfoxide. PCR mixtures yielding sufficient amplification of the desired gene were cleaned up using illustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare, Buckinghamshire, UK). Purified PCR products were then cloned into pET15b following LIC procedures. In brief, T4 DNA polymerase treatment of insert and vector was allowed for 60 min at room temperature (RT), followed by inactivation of the enzyme (20 min, 75°C). T4 DNA polymerase treated vector and insert were ligated for 15 min at RT, EDTA added to 10 mM final concentration and the ligation mixture incubated for another 5 min at RT. Subsequently, the ligation mixture was transformed to *E. coli* TOP10 competent cells and positive clones checked by isolating plasmid DNA followed by digestions with NcoI/XhoI. For overexpression of the recombinant protein, plasmid DNA containing the desired gene was transformed to *E. coli* BL21 Star (DE3) and pre-cultures of transformed *E. coli* BL21 Star (DE3) grown overnight in 5 ml LB-medium

containing 50 µg/ml ampicillin. Subsequently, expression cultures of 250 ml LB containing 100 µg/ml ampicillin were inoculated with 1% (v/v) of the pre-cultures. Flasks were put into incubator shakers agitating with 210 rpm at 37°C and growth allowed until OD_{600 nm} reached 0.5. Then, cultures were put on ice and expression started by adding 0.1 mM IPTG. Expression was continued in incubator shakers at 18°C and 160 rpm for 16 h. Bacterial pellets were collected by centrifugation (10 min, 3,500 x g), resuspended in 20 ml 20 mM Tris HCl pH 8.0 supplemented with 1 mM CaCl₂, 5 mM β-mercaptoethanol and 4 mM imidazole and sonicated 10 rounds for 15 s using Soniprep 150 Plus (MSE, Lower Sydenham, UK). Between every sonication step the suspension was put on ice for 30 s. Afterwards, the cell-free extract was centrifuged (15,000 x g, 20 min) and subjected to HisTrap™ HP 1 ml column (GE Healthcare) connected to a peristaltic pump for protein purification. The column was equilibrated with 1.5 ml buffer A (20 mM Tris HCl pH 8.0 supplemented with 500 mM NaCl, 3 mM CaCl₂ and 3 mM MgCl₂), eluted with 1.5 ml of elution buffer (containing in addition 500 mM imidazole), and again equilibrated with 1.5 ml buffer A. Then, the protein sample was applied to the column, washed with 5 column volumes of buffer A and elution performed with a gradient of imidazole 10–500 mM by eluting with 1.5 ml of each concentration of imidazole. Protein content and purity of collected fractions was analyzed by running samples on SDS-PAGE using 12% (v/v) polyacrylamide gels. Subsequently, elution fractions with highest content of purified protein were combined and dialyzed over night against 100 mM sodium acetate buffer pH 5.4 as described previously (41).

Analyzing substrate specificity of GH32 enzyme. Substrates (10 mg/ml) were incubated with excess and minimal amounts of *LpGH32* using 100 mM citric acid buffer at pH 4.0 and pH 5.0. Reaction volumes (100 µl) were supplemented with 0.01% (w/v) NaN₃ to prevent microbial growth and incubations were carried out at 37°C for 40 h. Product formation through GH32 enzyme activity was checked by TLC and HPAEC-PAD.

Statistical analysis. All growth experiments are biological triplicates; the OD_{600nm} values are expressed as average. Medium without bacterial inoculation was used to obtain blank values during OD measurements. Differences in the growth results between *L. salivarius* W57 and *L. paracasei* W20 in single and co-culture were assessed by running non-parametric unpaired t-tests between the results of each strain obtained in single and co-culture. Tests yielding p-values <0.05 were considered as significantly different.

Nucleotide accession numbers. The putative fructose und fructan utilization gene clusters of *L. paracasei* W20 were assigned the following accession numbers: fructose utilization, MH035726 and fructan utilization, MH047828. The putative fructose and sucrose utilization gene clusters of *L. salivarius* W57 were assigned the following accession numbers: fructose utilization, MH047826 and sucrose utilization, MH047827.

Results

Structural analysis of commercial $\beta(2\rightarrow1)$ fructans. An increasing number of $\beta(2\rightarrow1)$ fructans is commercially available. We first analyzed the structural composition of 9 commercial $\beta(2\rightarrow1)$ fructans (Table 1). Two types of compounds were found (6): fructan chains with a terminal sucrose unit on one end (GF-type), or consisting of $\beta(2\rightarrow1)$ linked fructosyl residues only (FF-type). GF-type compounds were present in all products analyzed, while FF-type compounds were found only in 5 out of 9 (Table 1). When we compared intensities of peaks obtained by HPAEC-PAD analysis (data not shown), peak responses for FF-type compounds were the highest in Fructose OFP and FOS P1. These compounds were classified as oligofructoses. For Frutafit HD, Frutafit CLR and Inulin P2, signal responses for FF-type compounds were lower, while the number of GF-type compounds found in chromatograms increased. These fructans were thus classified as inulin-type $\beta(2\rightarrow1)$ fructans. Highest diversity in structural composition overall was found in the degree of polymerization (Table 1). Frutafit HD and Inulin P2 offered the broadest range of fructan compounds (DP 2–60), while Fructose OFP, FOS P1 and scFOS P6 had a limited range (DP 2–7). Most preparations still contained some glucose and fructose monosaccharides (ranging between 0.5 and 8.2% relative peak height) and sucrose (constituting 2–15% relative peak height). The 1-kestose and nystose samples were pure and comprised only these specific DP 3 and DP 4 compounds, respectively. This comparative structural analysis of 9 commercial fructans provided a firm basis for the subsequent study of the utilization of specific compounds by probiotic bacteria.

Growth of selected probiotic bacteria on short-chain Inulin. Various probiotic bacteria were tested for their ability to grow on scInulin (Frutafit® CLR). This prebiotic has been derived from inulin and comprises over 50 individual compounds with varying DP (see below), of both GF- and FF-type $\beta(2\rightarrow1)$ fructans (Table 1). Frutafit® CLR thus shows all the structural

variations that are unique to prebiotic $\beta(2\rightarrow1)$ fructans, allowing analysis of their effects on growth of individual probiotic bacteria. Various lactobacilli and bifidobacteria strains, plus *Enterococcus faecium* and *Pediococcus acidilactici*, were tested. The growth responses of individual strains with scInulin varied strongly, e.g. in the length of the lag phase and in the final OD reached (Fig. 1A–C). All strains grew well on glucose (positive control) reaching final (100%) OD values at stationary phase of approximately 1.0 in microtiter plates and 2.0 in Hungate tubes, set as a relative OD value of 1.00. When incubated with scInulin, most strains reached a stationary phase relative OD around 0.4, indicating that scInulin compounds were supporting growth of these strains only partly compared to the positive control (Fig. 1C).

Table 1 Composition of commercial prebiotic $\beta(2\rightarrow1)$ fructans obtained by comparative HPAEC-PAD analysis. GF- and FF-type compounds (DP, degree of polymerization) found in commercial $\beta(2\rightarrow1)$ fructans.

Commercial name	Supplier	Description	GF-type (DP)	FF-type (DP)
Frutafit HD	Sensus BV	native inulin	2–60	2–14
Frutafit CLR	Sensus BV	scInulin	2–40	2–14
Frutafit TEX	Sensus BV	lcInulin	10–60	
Frutalose OFP	Sensus BV	oligofructose	2–7	2–7
FOS P1	Winclove BV	oligofructose	2–7	2–7
Inulin P2	Winclove BV	native inulin	2–60	2–14
scFOS P6	Winclove BV	scFOS	2–6	
Nystose	Carbosynth	GF3	4	
1-Kestose	Carbosynth	GF2	3

Fig. 1 Anaerobic growth of various probiotic bacterial strains with 5 mg/ml prebiotic scInulin. **A** Lactic acid bacterial strains were grown in 160–200 μ l aliquots in microtiter plates under constant N_2 -flow, and growth was followed in time using a microtiterplate reader. **B** *Bifidobacterium* strains were grown as 3 ml cultures in Hungate tubes under a CO_2 -atmosphere, and growth was followed by measuring OD600nm manually at 4 and 5 different time points, respectively. **C** Relative OD600nm at stationary phase (using maximum OD600nm of positive controls on glucose to normalize OD600 nm obtained with scInulin). All values shown are means of $n = 3$ biological replicates.

Carbohydrate analysis of scInulin consumption. Following bacterial growth, culture supernatants were analyzed for the presence of any remaining components from the scInulin mixture. Carbohydrate analysis revealed four patterns: (1) *L. salivarius* W57, *E. faecium* W54 and *P. acidilactici* W143 specifically used only fructose, GF (sucrose) and GF2 (1-kestose) (Fig. 2A). (2) Bifidobacteria utilized scInulin compounds with the following preference: F2>F3>F4 and GF>GF2>GF3>GF4, but only the F2 (β -D-Frup-(2 $\rightarrow$ 1)-D-Fru) and F3 (β -D-Frup-(2 $\rightarrow$ 1)- β -D-Frup-(2 $\rightarrow$ 1)-D-Fru) compounds were completely used at 24 h for *B. lactis* W51 and *B. lactis* W53, and at 36 h for *B. lactis* W52 (Fig. 2B). (3) *L. acidophilus* W37 utilized short-chain compounds of the FF- and GF-type. (4) *L. paracasei* W20 utilized all GF- and FF-type compounds present in scInulin. These results clearly highlight that probiotic bacteria have diverse substrate specificities for utilization of β (2 $\rightarrow$ 1) fructans.

Utilization of β (2 $\rightarrow$ 1) fructans by *L. paracasei* W20. *L. paracasei* W20 was clearly exceptional by consuming all GF- and FF-type compounds present in scInulin. To analyze its fructan metabolism in more detail, we grew *L. paracasei* W20 on scInulin and GF3 (both GF-type fructans) and Frutalose oligofructose (FF-type fructan). Analysis of changes in fructan composition over time revealed preferential degradation of fructose and all scInulin compounds larger than DP 3 in culture supernatants harvested at the mid-exponential phase from *L. paracasei* W20 growth (Fig. 1A *L. paracasei*, t = 12 h), while sucrose (GF) and 1-kestose (GF2) were present in relatively large amounts (Fig. 3A). Also degradation of the pure GF3 compound nystose by *L. paracasei* W20 first resulted in release of 1-kestose (not shown) in the supernatant followed by sucrose GF (Fig. 3C). Oligofructose degradation resulted in time in formation of GF and GF2 and of the disaccharide F2 (Fig. 3B). All compounds had been consumed completely in stationary phase samples (not shown). These results show that *L. paracasei* W20 degrades β (2 $\rightarrow$ 1) fructans in a sequential manner leading to temporary increase of GF and GF2 in case of GF-type fructans and F2 in case of FF-type fructans.

Fig. 2 HPAEC-PAD analysis of FOS/inulin components (DP2–DP20 range shown) in culture supernatants of probiotic bacterial strains grown on scInulin (Frutafit® CLR) (see Fig. 1). Peaks were identified using inulin (DP2–16, polymer) as standard **A** Selected probiotic lactic acid bacteria, grown for 18 h. **B** *B. lactis* W51 and *B. lactis* W53, grown for 24 h, and *B. lactis* W52, grown for 36 h. mMRS: modified MRS-medium. Arrows indicate peaks that completely disappeared and therefore show compounds that were completely utilized by strains.

Fig. 3 Temporary accumulation of breakdown products in culture supernatants of *L. paracasei* strain W20 growing on different prebiotic $\beta(2\rightarrow1)$ fructans. HPAEC-PAD analysis of (A) scInulin Frutafit® CLR, (B) Oligofructose Frutalose OFP, and (C) GF3 nystose and compounds derived in culture supernatants of *L. paracasei* W20 at mid-exponential growth phases (Fig. 1A *L. paracasei*, $t = 12$ h).

Identification and activity of a putative extracellular family GH32 β -fructosidase. Following growth on several $\beta(2\rightarrow1)$ fructans, we detected β -fructosidase activity in *L. paracasei* W20 culture supernatants. This activity degraded scInulin into sucrose, fructose and glucose (Fig. S1). Supernatants obtained from growth of *L. paracasei* W20 on glucose or fructose clearly had much lower activities. When searching the Carbohydrate Active enZyme (www.cazy.org) database (dbCAN) (21), the genome of *L. paracasei* W20 was found to encode one putative β -fructosidase protein of 1031 amino acids (family GH32), including a cell wall anchor motif for Gram-positive bacteria (LPQAG) (GenBank accession no. MH047828). Prediction of the subcellular localization of this protein is >99% extracellular. The protein sequence showed 97% identity to β -fructosidases/sucrose-6-phosphorylases present in various strains of *L. paracasei*/*L. casei* and around 30% identity to exo-inulinases from *Bacteroides* species and *Bc. subtilis* (22, 23). In order to characterize the activity of this putative GH32 enzyme, we cloned the predicted catalytic domain (residues 188–739) into the pET15bLIC vector which added an N-terminal 6-His-tag (from hereon called *LpGH32*). Overexpression of *LpGH32* in *E. coli* BL21 yielded a dominant band on SDS-PAGE at the expected height of 61 kD; this protein could be purified using His-trap column affinity chromatography. Incubation of 0.9 $\mu\text{g/ml}$ (excess amounts) of the purified *LpGH32* with $\beta(2\rightarrow1)$ fructans revealed activity on inulin and oligofructose (only at pH 5.0) which was observed before with culture supernatants. Final products of enzymatic conversion after 40 h were identified as monosaccharides (Fig. 4A, inulin and oligofructose), subsequently separated and identified by HPAEC-PAD as glucose and fructose. This activity was similar to what was observed before within the culture supernatants of *L. paracasei* W20 (Fig. S1). We further tested activity of the enzyme with sucrose and the $\beta(2\rightarrow6)$ fructan levan (Fig. 4A, levan and sucrose). While all sucrose was converted into fructose and glucose after 40 h, levan was not a substrate at pH 4.0 and only partly converted into fructose at pH 5.0 (indicated by a remaining spot at the bottom of the TLC plate) (Fig. 4A levan, lanes 3 and 4). Product formation at 40 h was also analyzed by adding a minimal amount (0.15 $\mu\text{g/ml}$) of *LpGH32* activity to incubations with inulin. Besides monosaccharides glucose and fructose, GF sucrose and GF2 1-kestose accumulated from inulin conversion (Fig. 4B). With oligofructose we observed an increase of DP 2 F2 plus DP 2 sucrose and DP 3 1-kestose (derived from GF impurities present in the oligofructose) (Fig. 4C). These results show that larger $\beta(2\rightarrow1)$ fructan chains are preferably converted

by the *LpGH32* enzyme leading to intermediate accumulation of shorter DP 2–DP 3 $\beta(2\rightarrow1)$ fructan chains.

Fig. 4 Substrate specificity of *LpGH32* and release of breakdown products during conversion of different $\beta(2\rightarrow1)$ fructans. **A** Products formed by purified *LpGH32* enzyme (0.9 $\mu\text{g/ml}$) incubated with fructan ($\beta(2\rightarrow1)$ and $\beta(2\rightarrow6)$) substrates: native inulin (from chicory, $\beta(2\rightarrow1)$), oligofructose ($\beta(2\rightarrow1)$), levan ($\beta(2\rightarrow6)$, bacterial origin) and sucrose ($\alpha(1\rightarrow2)$). St Standard containing fructose (Fru), sucrose (G), 1-kestose (GF₂) and nystose (GF₃). Incubation was at pH 4.0 (lane 3) and pH 5.0 (lane 4) for 40 h; lane 2 substrate control. **B**, **C** Accumulation of intermediate breakdown products liberated by *LpGH32* (excess, 0.9 $\mu\text{g/ml}$, and minimal, 0.15 $\mu\text{g/ml}$, amounts) during incubations with GF-type fructan native inulin and FF-type fructan oligofructose (also contains GF-type fructans) and stopping reactions after 40 h. Main product released was fructose, accumulation of intermediate breakdown products highlighted with arrows.

Genome analysis explains different fructan utilization patterns in probiotic bacterial strains. Our results show that *L. paracasei* W20 employs an extracellular *exo*-inulinase (EC 3.2.1.80, family GH32) to degrade prebiotic $\beta(2\rightarrow1)$ fructans (*LpGH32*). Among the bacteria tested in this study, this enzyme accounts for the ability of *L. paracasei* W20 to degrade $\beta(2\rightarrow1)$ fructans independent of their structural parameters (GF- and FF-types; DP 2–60). Due to the preference of *LpGH32* to degrade fructan chains larger than DP 3 first, seen both with the purified enzyme and in supernatants of *L. paracasei* W20, the intermediate breakdown products GF and GF2 for GF-type fructans and GF, GF2 and F2 for FF-type fructans accumulated (Fig. 3 and 4).

We searched bacterial genomes for genes involved in known pathways of fructose utilization, sucrose utilization and fructo-oligosaccharide /raffinose utilization (22, 24–31) in order to explain the strain differences in utilization of GF-type and FF-type components. Specifically we identified key glycoside hydrolases and transporters involved in fructose metabolism. We found that these strains differed highly when comparing genes from all three pathways mentioned above (Fig. 5 and Table S1). None of these strains combined all three pathways, rather one or two pathways were predicted by the genomic annotation. The genomes of *L. paracasei* W20 and *L. salivarius* W57 both encode a fructose utilization pathway with a PTS transport system, which may account for uptake of the free fructose released by *LpGH32*. The *L. salivarius* W57 genome contains a predicted sucrose utilization system with a sucrose PTS transport system, which was not found in the *L. paracasei* W20 genome. The *Bifidobacterium* strains studied lacked a fructose utilization pathway (also observed experimentally, bifidobacterial strains tested could not grow on pure fructose, Fig. 1B), but sucrose utilization was predicted in the bifidobacterial representative strain *B. longum* NCC 2705 with a sucrose permease and in addition a FOS/raffinose utilization pathway with an ABC transporter and an intracellular β -fructosidase (Fig. 5 and (31)). Based on the results from the genome analyses from these probiotic strains, we hypothesize that the diverse distribution of these pathways may allow inter-species interactions resulting in cross-feeding during the degradation of inulin. If inter-species interactions occur, these may generate interesting biotechnological opportunities in the application of combinations of probiotic strains with the prebiotic inulin, as novel synbiotic mixtures based on inter-species cross-feeding properties.

Fig. 5 Metabolism of prebiotic $\beta(2\rightarrow1)$ fructans by *L. paracasei* W20, *L. salivarius* W57, and *B. longum* NCC 2705. Fructose uptake via a Fructose-PTS transport system (FT-PTS); Sucrose uptake via a Sucrose-PTS system (PTS_ScrA), or a Sucrose permease (SucP); FOS/raffinose utilization pathway with a FOS-ABC transport system (Msm_ABC) followed by intracellular β -fructosidases ScrP/GtfA and ScrB/SacA.

Inter-species interactions between *L. salivarius* W57 and *L. paracasei* W20 involving LpGH32 degradation products. In order to test the inter-species cross-feeding hypothesis, and to identify possible involvement of particular enzymes and transporters encoded by probiotic bacteria in degradation of prebiotic fructan components, we studied inter-species interactions of the two probiotic strains *L. paracasei* W20 and *L. salivarius* W57. We chose to use these strains based on their genome analysis and their different carbohydrate-utilization profiles: while *L. salivarius* incubated with scInulin showed very specific utilization of fructan compounds with DP <4 (Fig. 2A), *L. paracasei* was able to utilize all components of $\beta(2\rightarrow1)$ fructans employing an extracellular exo-inulinase. Both strains differ at the transporter level, with a single sucrose transporter present in *L. salivarius* W57 (PTS_ScrA, Fig. 5), but lacking in *L. paracasei* W20.

To identify any preference of *L. salivarius* W57 for GF-type fructans with distinct size, the strain was grown on pure 1-kestose (GF2, DP 3 FOS) and nystose (GF3, DP 4 FOS). The results show that *L. salivarius* only grew on 1-

kestose, while *L. paracasei* grew on both 1-kestose and nystose compounds (Fig. 6A). Nystose thus is the smallest DP fructo-oligosaccharide that demonstrates selectivity between the two *Lactobacillus* strains and therefore requires the activity of *LpGH32* in order to be metabolized. We then looked for the possible beneficial cross-feeding effects of *LpGH32* by adding the purified enzyme to *L. salivarius* W57 cultures incubated with nystose. *L. salivarius* W57 is unable to grow on nystose (see Fig. 6A), while the addition of *LpGH32* enabled the strain to completely metabolize this DP4 fructan (Fig. 6B). To identify products of *LpGH32* produced by *L. paracasei* W20 that are potentially available for cross-feeding by *L. salivarius* W57, we grew *L. paracasei* W20 and *L. salivarius* W57 on several *LpGH32* inulin products. While *L. salivarius* W57 grew equally well on fructose, sucrose and 1-kestose, reaching stationary phase after 5 h, *L. paracasei* W20 used fructose rapidly (stationary after 9 h), but grew quite poorly on 1-kestose (after a long lag-phase of 12 h) and on sucrose (Fig. 6C). These results suggest that *L. paracasei* W20 overall has high affinity towards fructose, while GF-type compounds sucrose and GF2 1-kestose are used with less preference. Therefore sucrose and 1-kestose liberated by *LpGH32* activity may be available as cross-feeding products from scInulin for metabolism by other bacteria that are in close proximity of *L. paracasei*.

To test for *in vivo* cross-feeding, we co-cultured *L. salivarius* W57 and *L. paracasei* W20 on two types of $\beta(2\rightarrow1)$ fructans, scInulin containing mainly GF-type fructan chains and oligofructose (Frutalose OFP) containing mostly pure fructose composed $\beta(2\rightarrow1)$ fructan chains. In the case of scInulin, co-culturing resulted in a clear increase in colony forming units (CFU/ml) for *L. salivarius* W57 in the co-cultures compared to the single cultures (Fig. 7A). Cross-feeding thus occurred between the two strains which stimulated the growth of *L. salivarius* W57 benefiting from the presence of *L. paracasei* W20 employing the extracellular GH32 enzyme for degradation of the GF-type fructan scInulin (resulting in accumulation of short-chain FOS DP 2–3 compounds). Degradation of oligofructose yields free fructose, which does not result in cross-feeding of *L. salivarius* W57 (Fig. 7B).

Fig. 6 Beneficial effects of *LpGH32* on growth of *L. salivarius* W57 and abilities of *L. salivarius* W57 and *L. paracasei* W20 to grow on *LpGH32* breakdown products liberated from prebiotic inulin conversion **A** Anaerobic growth of *L. paracasei* W20 and *L. salivarius* W57 individually on 5 mg/ml pure 1-kestose (GF2) and nystose (GF3). **B** Growth of *L. salivarius* W57 on 5 mg/ml glucose (positive control) or nystose (DP4 FOS, GF3) with (0.5 µg/ml) or without added *LpGH32* enzyme activity. mMRS modified MRS-medium, 100 mM NaCH₃COO enzyme buffer. **C** Growth of *L. salivarius* W57 and *L. paracasei* W20 on 5 mg/ml of individual *LpGH32* (intermediate) inulin degradation products: Fructose (Fru), sucrose (Suc), 1-kestose (GF2). All values shown are means of n = 3 biological replicates.

Fig. 7 Growth (colony forming units/ml) of *L. paracasei* W20 plus *L. salivarius* W57 in single- and co-cultures on 5 mg/ml scInulin (Frutafit® CLR) (**A**) and oligofructose (Frutalose OFP) (**B**). Co-cultures and single cultures of *L. paracasei* W20 were grown for 18 h, single cultures of *L. salivarius* W57 8 h. Values shown are means of n = 3 biological replicates. **p < 0.01 and ***p < 0.001 (Student's t test).

Discussion

Selective stimulation of growth of beneficial bacteria in the human gut may yield *in vivo* health effects. This study reports a structural characterization of commercial prebiotic $\beta(2\rightarrow1)$ fructans and their utilization for growth by selected probiotic bacteria.

The carbohydrate utilization properties of probiotic strains from the genera *Lactobacillus* and *Bifidobacterium* have been investigated previously. The ability of *Lactobacillus* strains to utilize prebiotic carbohydrates appeared a lot more limited as compared to *Bifidobacterium* strains (32). Transporters and GH enzymes encoded by the genomes of these probiotic bacteria varied even amongst strains from the same species (13). For instance, Hutkins et al. investigated *L. paracasei* 1195 for its ability to degrade FOS, attributing its FOS utilization to an intracellular β -fructosidase enzyme (30). Later, Hutkins et al identified through whole genome microarray analysis the *fos* utilization operon in the genome of *L. paracasei* 1195 encoding an extracellular β -fructosidase enzyme and a fructose/mannose transporter (22). An extracellularly located β -fructosidase was also found in *L. paracasei* DSM 23505, used to effectively convert FOS/inulin into lactic acid (33). Both studies characterized the extracellular enzyme activity on FOS/inulin of *L. paracasei* 1195 and *L. paracasei* DSM 23505 as *exo*-inulinase, EC 3.2.1.80. To characterize the extracellular β -fructosidase of *L. paracasei* W20, we purified the *LpGH32* enzyme after overexpression in *E.coli* and identified it as an *exo*-inulinase (EC 3.2.1.80). Hutkins et al observed for *L. paracasei* 1195 that degradation of FOS DP 4 and DP 5 occurred first, resulting in a temporary increase of glucose, sucrose and FOS DP 3. Temporary increase of short-chain fructo-oligosaccharides was also mentioned in the study using *L. paracasei* 8700:2, a human isolate releasing fructose, GF2 and F2 upon oligofructose degradation or fructose, glucose and sucrose upon inulin degradation (20). Cultures of *L. paracasei* W20 showed preferential utilization of FOS components $>DP\ 3$ and thus temporal accumulation of sucrose and DP 3 FOS. This degradation pattern also was observed using purified *LpGH32*. These data show that preferential degradation of FOS/inulin components with $DP > 3$ is due to activity of *LpGH32* synthesized by *L. paracasei* W20 and not to selective uptake systems in the host (Fig. 3 and 4).

Unlike *L. paracasei* W20, *L. salivarius* W57 displayed a highly specific fructan utilization profile which enabled the strain to exclusively utilize GF-type

$\beta(2\rightarrow1)$ fructan with DP 2–3. Preferential utilization of FOS components with a low DP has not been described for *L. salivarius* strains before, however Saulnier et al. reported that *Lactobacillus plantarum* WCFS1 consumes GF2 and GF3 compounds. Using whole genome microarrays, three genes were identified participating in scFOS metabolism of *L. plantarum*, a sucrose PTS uptake system, a β -fructofuranosidase and a fructokinase (34). Our annotation of the *L. salivarius* W57 genome revealed the presence of a PTS_ScrA gene, explaining preference of *L. salivarius* W57 for scFOS compounds, that in comparison to *L. plantarum* WCFS1, shows higher specificity for GF-type FOS components of only DP 2–3.

To specifically stimulate growth of human gut microorganisms, possibly even at the species/strains level, requires generation of a given metabolic activity which may then lead to a given health effect. Where bacterial cells are limited in their ability to utilize prebiotic FOS/inulin compounds, the presence of extracellular β -fructosidase enzymes may stimulate degradation and thus result in improved growth of a probiotic bacterium on a given prebiotic (17, 35). For instance, bifidobacterial growth is very well-known to be stimulated by prebiotic FOS/inulin utilization. The enrichment of this genus through FOS/inulin utilization is so dominant that this was labelled as bifidogenic effect. *Bifidobacterium* strains in pure culture however only are able to use FOS/inulin components $<DP\ 20$ (12). When growing bifidobacteria in co-cultures with (commensal) gut species expressing extracellular enzymes from family GH32, cross-feeding occurs and results in an increased bifidobacterial growth (35). As shown in the present study, *L. paracasei* W20 exhibits the ability to degrade FOS/inulin extracellularly using a family GH32 enzyme, an exo-inulinase (*LpGH32*) that yields intermediate degradation products of GF and FF-type $\beta(2\rightarrow1)$ fructans, respectively (Fig. 4). GF-type fructan use results in accumulation of the short-chain FOS DP 2–3 compounds, whereas FF-type fructan use only yields fructose as product from *LpGH32* activity. This accumulation of intermediate FOS DP 2–3 products leads to stimulation of growth of another *Lactobacillus*, *L. salivarius* W57, that specifically takes up GF (sucrose) and GF2 (1-kestose) using a sucrose transporter. In contrast, any released fructose (e.g. from FF-type fructans) is more likely to be directly taken up by *L. paracasei* W20 using a fructose/mannose transporter (Fig. 5). Based on the results of our study, we can conclude that the ability of a *Lactobacillus* strain to benefit of cross-feeding during growth on prebiotic $\beta(2\rightarrow1)$ fructans is highly dependent on the use of GF-type (cross-feeding stimulating) or FF-type

(relatively low cross-feeding) fructans. In comparison, cross-feeding mediated by bifidobacteria towards butyrate producing members of the gut microbiota was mediated by short fractions of oligofructose (FF-type fructan) and thus not the GF-type fructan as observed in our study (15, 36). Moreover, we conclude that the extent of cross-feeding amongst lactobacilli also depends on fructan chain-length: $\beta(2\rightarrow1)$ fructans with a relatively low DP contain at the molar level a relatively high content of GF-type molecules, thus resulting in higher concentrations of the breakdown products GF-type DP 2–3. Our results therefore show that the extent of bacterial cross-feeding amongst probiotic lactobacilli is clearly controlled by the structure of the prebiotic fructan substrate used.

These results provide insights in the mechanism and role of cross-feeding by $\beta(2\rightarrow1)$ fructan degradation products, stimulating growth of bacteria living in the same environment. To stimulate growth of probiotic bacteria at the strain level, the prebiotic $\beta(2\rightarrow1)$ fructan should be carefully chosen, taking into account its structural composition (Table 1). In synbiotic mixtures using *L. paracasei* W20 employing an extracellular GH32 enzyme, FF-type FOS is preferred when only *L. paracasei* W20 is supposed to benefit from prebiotic fructan supplementation (Table 2). When aiming to stimulate growth of another strain, preferably GF-type fructan of relatively low chain-length should be considered. Our results further highlight the advantageous properties of *L. paracasei* and its extracellular GH32 enzyme mediated through release of intermediate degradation products from long-chain inulin demonstrating its potential ability to act as keystone species amongst probiotic lactobacilli.

Table 2 Prebiotic $\beta(2\rightarrow1)$ fructans used in this study, ordered according to their potential to stimulate cross-feeding between *L. paracasei* W20 and *L. salivarius* W57; Top (starting with 1-kestose; high cross-feeding) to bottom (ending with oligofructose; low cross-feeding). This is based on the activity of the extracellular *L. paracasei* W20 *LpGH32* enzyme releasing more of the GF (sucrose) and 1-kestose (GF2) molecules from the shorter $\beta(2\rightarrow1)$ fructans and from fructans comprising more GF-type molecules (and not from oligofructans).

Commercial name	Description
1-Kestose	GF2
Nystose	GF3
scFOS P6	scFOS
Frutafit CLR	scInulin
Inulin P2	native inulin
Frutafit HD	native inulin
Frutafit TEX	lcInulin
FOS P1	oligofructose
Frutalose OFP	oligofructose

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Supplemental

Fig. S1 TLC analysis of enzyme activities in culture supernatants of *L. paracasei* W20 cells grown on scInulin using a Urea-staining solution specific for fructose-type of compounds. St Standard containing sucrose, 1-kestose (GF₂) and nystose (GF₃), lane 2 fructose control, lane 3 scInulin control, lanes 4–9 scInulin incubated with *L. paracasei* W20 culture supernatants grown with glucose (4), fructose (5), native Inulin Frutafit® HD (6), IcInulin Frutafit® TEX! (7), scInulin Frutafit® CLR (8), oligofructose Frutalose OFP (9).

Table S1 IDs of genes of *Lactobacillus* strains involved in known pathways of fructose, sucrose, FOS/raffinose degradation. ‘-’ indicates that genes for a certain pathway are not present in the organism.

Fructose metabolism

	Reg	FruK	FT PTS
<i>L. paracasei</i> W20	1597.45.peg.1690	1597.45.peg.2629	1597.45.peg.1692
<i>L. salivarius</i> W57	1624.75.peg.901	1624.75.peg.902	1624.75.peg.903

Sucrose metabolism

	Reg	PTS_scrA
<i>L. paracasei</i> W20	-	-
<i>L. salivarius</i> W57	1624.75.peg.526	1624.75.peg.528

Fructan metabolism

	FosA	FosB	FosC
<i>L. paracasei</i> W20	1597.45.peg.2457	1597.45.peg.2458	1597.45.peg.2459
<i>L. salivarius</i> W57	-	-	-

	FosD	FosX	FosE
<i>L. paracasei</i> W20	1597.45.peg.2460	1597.45.peg.2461	<i>LpGH32</i>
<i>L. salivarius</i> W57	-	-	-

Chapter 3

Structural and functional characterization of a family GH53 β -1,4-galactanase from *Bacteroides thetaiotaomicron* that facilitates degradation of prebiotic galactooligosaccharides

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Abstract

Galactooligosaccharides (GOS) are prebiotic compounds synthesized from lactose using bacterial enzymes and are known to stimulate growth of beneficial bifidobacteria in the human colon. *Bacteroides thetaiotaomicron* is a prominent human colon commensal bacterial species that hydrolyzes GOS using an extracellular Glycosyl Hydrolase (GH) family GH53 endo-galactanase enzyme (BTGH53), releasing galactose-based products for growth. Here we solved the 3-dimensional structure of this *Bt. thetaiotaomicron* GH53 endo-galactanase. BTGH53 has a relatively open active site cleft which was not observed with the bacterial enzyme from *Bacillus licheniformis* (BLGAL). BTGH53 acted on GOS with degree of polymerization ≤ 3 and therefore more closely resembles activity of fungal GH53 enzymes (e.g. *Aspergillus aculeatus* AAGAL and *Meripileus giganteus* MGGAL). Probiotic lactobacilli that lack galactan utilization systems constitute a group of bacteria with relevance for a healthy (infant) gut. The strains tested were unable to use GOS $\geq$ DP 3. However, they completely consumed GOS in the presence of BTGH53, resulting in clear stimulation of their extent of growth. The extracellular BTGH53 enzyme thus may play an important role in carbohydrate metabolism in complex microbial environments such as the human colon. It also may find application for the development of synergistic synbiotics.

Introduction

The human gut is colonized by trillions of bacteria which are collectively called the human gut microbiota. Gut bacteria have tremendous impact on our health and enable humans to degrade most of the food we ingest. In particular bacteria that colonize the human colon are highly specialized in harvesting energy from complex carbohydrates, such as those found in plant material, in complex human glycans and in fungal and bacterial carbohydrates (1). To acquire energy from carbohydrates in such extremely competitive environments, bacteria express specific enzymes and transporters to target and degrade these carbohydrate substrates. The presence of particular carbohydrates therefore may largely dictate which bacteria will grow and persist in the colon. This may allow us to nurture our colonic bacteria by eating a diet full of complex carbohydrates or by ingesting functional carbohydrates as food supplements, such as prebiotics (2).

A prebiotic is generally defined as a “substrate that is selectively utilized by host microorganisms conferring a health benefit” (3). People may ingest prebiotics as a way to promote the growth of healthy gut bacteria in times of intestinal distress, e.g. during antibiotic treatment, infant colic, inflammatory bowel disease or colorectal cancer (4). Galactooligosaccharides (GOS) represent one class of prebiotics synthesized from lactose comprising often glucose at the reducing end and a galactose-chain with a degree of polymerization varying from 2–6, constituting a mixture of non-digestible carbohydrates based on galactose. GOS are most commonly used in infant formula. Their application in infant formula dates back to the discovery of the stimulatory effect of human milk on colonic bacteria in the last century. Initially, the growth of a prominent infant gut bacterium, *Lactobacillus bifidus* (now known as *Bifidobacterium bifidum*), was linked to milk from breastfeeding mothers (5–10). Later it was found to be based on consumption of human milk oligosaccharides, or HMOs (11, 12). HMOs thus became known as the “bifidus factor” and since then, much research has gone into determining the structures of HMOs (of which there are over 200) and into finding compounds and molecules that mimic the bifidus effect (13, 14). In the late 20th century it was shown that enzymatically synthesized GOS are bifidogenic and, similar to HMOs, stimulate the growth of bifidobacteria (15, 16). HMOs are complex structures and expensive and laborious to synthesize, while GOS are relatively easy to produce at industrial scale and have similar effects on growth of infant colonic microbiota (17, 18). From the early 2000’s onwards the infant nutrition industry started to use GOS

to produce an infant formula that mimics the more natural effects of mother's milk (19).

With the increasing awareness of how strongly the human colonic microbiota is linked to our overall well-being, researchers and food nutritionists alike aim to build up and maintain a healthy and balanced colonic microbiota (20). GOS at least partly fulfill similar roles as HMOs, but further information is required about the specific mechanisms of GOS utilization. Previously we reported that a subset of branched GOS mimic HMOs in the activation of bacterial mucin utilization pathways, which may allow bacterial species to adhere to the mucosal layer and colonize the GI tract (21). A second mechanism involves direct use of GOS as a nutritional source by colonic bacteria; GOS has been shown to activate galactan/galactose utilization systems in *Bifidobacterium* and *Bacteroides* (21). One particular enzyme from the galactan utilization system identified as important for GOS utilization by gut bacteria is an extracellular endo-galactanase from the GH53 family as described in the CAZy database (www.cazy.org) (22). Coincidentally, several probiotic bifidobacteria and prominent colonic commensal bacterial species that benefit from HMOs for growth also harbor galactan utilization systems and GH53 enzymes (21, 23). Recently, (Böger et al., Chapter 4) reported that lactobacilli, another group of probiotic bacteria with relevance for a healthy (infant) gut, lack galactan utilization systems and thus poorly grow on prebiotic GOS. They are able to grow on lactose, however, involving a dedicated uptake system and an intracellular β -galactosidase. As allochthonous members of the human colon microbiota, they may instead benefit from GOS breakdown products generated by other autochthonous members (24).

As a proven prebiotic, beneficial effects of GOS may also occur through application in synbiotics. In such synergistic synbiotics the prebiotic is supplied as a specific growth substrate for the cognate probiotic bacterium (25). Some studies already have reported the use of GOS in synbiotics, i.e. in combination with a given probiotic strain, but often the synergistic effect of the combination remains to be proven (4, 26).

In earlier work we have identified an extracellular GH53 enzyme from *Bt. thetaiotaomicron* that is implicated in both pectic galactan and prebiotic GOS utilization and releases galactobiose and galactose as final products of pectic galactan degradation (21). In the present study, we further characterized this endo-galactanase enzyme in terms of three-dimensional structure and enzymatic activity, in order to further understand its role in GOS degradation. We observed

that this enzyme degrades β -1,4-galactan (potato galactan) but not β -1,3-galactan (as found in larch wood arabinogalactan). Its three-dimensional crystal structure revealed that BTGH53 is similar to fungal GH53s rather than to the bacterial GH53 enzyme characterized from *Bacillus licheniformis* (27, 28). Specifically, BTGH53 shows a different organization of $\beta\alpha$ -loops 6, 7 and 8 lacking additional substrate binding sites which is not observed for the *Bc. licheniformis* GH53 enzyme, along with an additional D331 residue in BTGH53 which stabilizes the axial C4 hydroxyl group of galactose in the -1 subsite position. As a result BTGH53 is able to degrade shorter GOS and *p*NP-galactose. Finally, we demonstrate that in the presence of BTGH53 probiotic *Lactobacillus* strains become fully competent to degrade and grow on larger DP GOS. These results show in more detail how GOS are metabolized by GH53 enzymes, and further our understanding of how extracellular CAZymes facilitate growth of beneficial probiotic *Lactobacillus* strains in complex microbial environments.

Materials and Methods

Materials. Azo-galactan and galactobiose were purchased from Megazyme (Bray, Ireland). Purified GOS (pGOS derived from Vivinal® GOS with glucose, galactose and lactose largely removed from the commercial product) was provided by FrieslandCampina Domo (Amersfoort, The Netherlands), and P5 GOS by Winclove Probiotics (Amsterdam, the Netherlands). Composition of pGOS has been analyzed and described previously (21). P5 GOS composition was analyzed as described below. All other compounds were purchased from Sigma (Zwijndrecht, NL). *Bacteroides thetaiotaomicron* VPI-5482 was purchased from DSMZ (DSM 2079, ATCC 29148) (Braunschweig, Germany). *Lactobacillus paracasei* W20, *Lactobacillus casei* W56 and *Lactobacillus salivarius* W57 were a kind gift from Winclove Probiotics (Amsterdam, NL). Crystallization materials were purchased from Molecular Dimensions (Newmarket, UK).

***Bt. thetaiotaomicron* growth experiments.** *Bt. thetaiotaomicron* growth was carried out as described previously (21). Briefly, overnight cultures of *Bt. thetaiotaomicron* were grown at 37°C in rich medium (Brain Heart infusion broth + 5 mM L-cysteine) under anaerobic conditions in an anaerobic jar (Oxoid) with a GasPak (BD). The following day, 1 ml of a 50-fold dilution of a *Bt. thetaiotaomicron* overnight culture was used to inoculate carbon-limited minimally defined medium with 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 mM hematin, 200 mM L-histidine, 100 nM MgCl_2 , 1.4 nM $\text{FeSO}_4 \cdot 7 \text{H}_2\text{O}$, 50 mM CaCl_2 , 1 mg/ml vitamin K3, 5 ng/ml vitamin B12 and individual carbon sources (0.5%, w/v). Culture tubes containing 2 ml total volume were prepared in Hungate tubes under anaerobic conditions using Hungate techniques under 100% CO_2 gas (29). Growth curves were obtained by incubating tubes at 37°C and taking optical density readings at 600 nm (OD600) at ~1–2 h time intervals over a 4 day period.

Protein production, crystallization, data collection and structure determination. Production and purification of BTGH53 protein was carried out as described previously (21). Further purification steps were used to obtain fully purified BTGH53 protein for crystallization experiments. Size exclusion chromatography was performed using Sephadex S75 in buffer containing 20 mM Tris pH 7.5, 150 mM NaCl. Fractions containing the purified protein were pooled and concentrated using an Amicon centricon filter (Millipore) with a 10 KDa MWCO membrane.

Crystallization trials were performed at room temperature using the sitting-drop vapor diffusion method using commercially available crystallization screens. MRC2 Plates were setup by pipetting 100 nL; 10 mg/ml protein and 100 nL screen condition together using a Mosquito pipetting robot (TTP Labtech Ltd). Crystals were found after a few days up to several weeks at various conditions. Crystals from condition 20% PEG6000, 200 mM NH_4Cl , MES pH 6.0 (Wizard Classic 3 & 4 screen, Rigaku) were recognized as most promising single crystals based on size (approximately 185 x 80 x 80 μm) and sharp edges. For determination of the apo protein structure the crystal was transferred in a new drop with a higher PEG6000 concentration of 22% to reach cryo conditions, and directly flash frozen in a stream of nitrogen gas at 110K. Diffraction data was collected at 110K using a Microstar rotating anode (Cu) X-ray source (Bruker AXS GmbH) in combination with Helios optics (Incoatec GmbH) and a MAR345dtb detector (Marresearch GmbH). Diffraction images for 130° were recorded with 1° oscillation.

For determination of the galactose bound protein structure, the crystal was transferred to a new drop for 30 sec containing a reservoir solution with 50 mM pGOS and flash frozen in liquid nitrogen. The data collection was performed under cryo conditions at the P13 EMBL Beamline (Hamburg, Germany). Diffraction images for 135° were recorded with 0.05° oscillations.

Diffraction images were indexed, integrated, scaled and merged with programs XDS and Aimless (30). The structure of the apo BTGH53 protein was

determined by the molecular replacement method using Phaser (31) where four homology search models were used. These search models were obtained from the FFAS server blast and the top four score structural homologues (PDB codes: 1FHL; 1HJQ; 4BF7; 1HJS) with 28–31% sequence identity were used to build homology models using the SCWRL modelling method in the mixed atom mode by replacing template residues with serine for unconserved residues between the target and the template except for glycines and alanines (32, 33). After Phaser, building the final model was performed with the ARP/wARP program and refinement was done with Refmac5, both from the CCP4 software suite (34, 35). For the crystals used in the substrate soak experiment the search coordinates from the apo structure were used.

Crystals belonging to space group $P2_1$ contained 2 protein molecules in the asymmetric unit having a solvent content of 39.1%. A summary of the data collection statistics is given in Table 1 and of the refinement statistics in Table 2.

Table 1 Data collection & statistics. Between brackets: Statistics highest resolution shell
*: Department of Biophysical Chemistry.

	Apo BTGH53 (PDB ID 6GP5)	BTGH53 + Galactose (PDB ID 6GPA)
X-ray Facility	University Groningen*	EMBL Hamburg
Wavelength (Å)	1.54	0.98
Space group	$P2_1$	$P2_1$
mol/A.U	2	2
Solvent content (%)	39.11%	41.24
Unit-cell parameters		
<i>a</i> , <i>b</i> , <i>c</i> (Å)	47.2, 44.7, 137.6	47.5, 45.6, 138.8
α , β , γ (°)	90.0, 99.4, 90.0	90.0, 99.8, 90.0
Resolution range high (Å)	1.93 (1.98–1.93)	1.79 (1.83–1.79)
Resolution range low (Å)	46.58	46.85
R_{merge}	0.073 (0.324)	0.110 (0.403)
$R_{\text{p.i.m}}$	0.053 (0.247)	0.085 (0.328)
Total No. of observations	110321 (5637)	138347 (6516)
Total No. of unique reflections	40545 (2339)	53053 (2687)
Mean $I/\sigma(I)$	14.06 (3.28)	8.6 (2.8)
Completeness (%)	94.8 (81.1)	96.1 (80.5)
Multiplicity	2.7 (2.4)	2.6 (2.4)

Table 2 Refinement statistics

	Apo BTGH53 (PDB ID 6GP5)	BTGH53+galactose (PDB ID 6GPA)
Resolution (Å)	1.93	1.79
R	0.1735	0.170
R _{free}	0.2247	0.220
RMSD from target geometry		
Bond lengths (Å)	0.010	0.0115
Bond angles (°)	1.356	1.4558
Tot. number of atoms	5369	5678
Number of amino acids	625	624
Number of water molecules	430	724
Ramachandran (%)		
Favoured (%)	96	97
Allowed (%)	4	3
Outliers (%)	0	0

Determination of BTGH53 specific activities. The activity of BTGH53 on the synthetic substrate *p*NP-galactose was determined by measuring the release of 4-nitrophenol at 405 nm. Assays were run using different buffers over a range of pH values: 50 mM sodium acetate pH 5.5, 6.0; 50 mM sodium phosphate pH 6.0, 6.5, 7.0; 20 mM HEPES pH 7.5; 20 mM Tris pH 7.5, 8.0. The molar extinction coefficients used to calculate *p*NP release were 2.31 mM⁻¹ cm⁻¹ (pH 5.5), 3.66 mM⁻¹ cm⁻¹ (pH 6.0), 7.38 mM⁻¹ cm⁻¹ (pH 6.5), 11.39 mM⁻¹ cm⁻¹ (pH 7.0), 14.22 mM⁻¹ cm⁻¹ (pH 7.45), 17.26 mM⁻¹ cm⁻¹ (pH 8.0). Reactions were initiated by the addition of BTGH53 to a final concentration of 5 μM. Reaction rates were plotted against pH producing a bell shaped curve. Optimum pH conditions were found to be 50 mM phosphate buffer pH 7.0 which was used for all subsequent studies.

Enzyme activity was measured with 0.1–15 mM *p*NP-galactose in 50 mM phosphate buffer pH 7.0 using the conditions described above. Reaction rates were monitored continuously at 405 nm for 600 sec, the rates were taken as the slope between 300 and 360 sec. The data were fitted to the Michaelis-Menten equation in GRAFIT which was used to calculate the K_M and V_{max} values. The V_{max} was adjusted for the enzyme concentration to give the k_{cat} value.

BTGH53 endo-galactanase specific activity was determined using the dyed substrate Azo-galactan according to manufacturer's instructions. A reaction mixture containing 500 μl 2% Azo-galactan in 50 mM phosphate buffer pH 7.0

plus 500 μ l of 5 μ M BTGH53 in the same buffer was incubated for 10 min at 37°C. After incubation, the reaction was stopped and the residual high molecular weight polymer was precipitated by the addition of 2.5 ml of cold 100% ethanol. The reaction mixture was centrifuged for 10 min at 2800 x g and the produced low-molecular weight dyed fragments in the supernatant assayed by measuring absorbance at 590 nm. Units of enzyme activity were calculated from absorbance values by using a standard curve supplied by the manufacturer that standardized values of absorbance at 590 nm to amount of reducing sugar ends formed obtained according to the method Nelson/Somogyi (36). One unit of activity (GalU/mg) was defined as the amount of enzyme required to release 1 μ mol of galactose reducing sugar equivalents from galactan per min.

***Lactobacillus* growth curves.** *Lactobacillus* growth curves were measured in microtiter plates as described previously (37). In brief, *Lactobacillus* strains were grown in modified MRS medium containing 5 mg ml⁻¹ GOS. BTGH53 treated GOS was prepared by incubating 10 mg/ml GOS with 200 μ M BTGH53 in 50 mM sodium phosphate buffer pH 7.0 at 37°C for 24 h. After digestion, BTGH53 was inactivated by heat treatment (10 min at 80°C), any precipitant removed by centrifugation (5 min, 16,000 x g) and samples were filter sterilized using 0.2 μ m cellulose acetate filters. After *Lactobacillus* growth, culture supernatants were analyzed for any remaining GOS molecules using high pressure anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) as previously described (21).

Analysis of GOS composition. P5 GOS was analyzed using comparative HPAEC-PAD with known structural components (covering >99% of the substrate composition) of Vivinal® GOS as reference (38). In brief, P5 GOS was dissolved to 1 mg/ml in 80% (vol·vol⁻¹) dimethyl sulfoxide and its composition analyzed on a Dionex DX500 work station equipped with an ED40 pulsed amperometric detection system (HPAEC-PAD). Components were separated on a CarboPac PA-1 column (250 by 5 mm; Dionex) using a system of buffer A = 0.1 M NaOH, buffer B = 0.6 M NaOAc in 0.1 M NaOH, buffer C = deionized water and buffer D = 50 mM NaOAc. Separation was carried out 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 with 10% A, 85% B and 5% D for 7 min. Furthermore, P5 GOS was subjected to NMR spectroscopy and known structural-reporter group signals used to evaluate the ¹H NMR spectrum of P5 GOS as described by (39). Briefly, P5 GOS was lyophilized and exchanged

twice with 99.9% atom D₂O (Cambridge Isotope Laboratories Ltd, Andover, MA). Then, the sample was dissolved in 650 µl 99.9% atom D₂O, containing 25 ppm acetone as internal standard. The 1D ¹H NMR spectra were recorded at a probe temperature of 298K in a Varian Inova 600 spectrometer (NMR department, University of Groningen, The Netherlands).

Protein Data Bank accession codes. Structures of BTGH53 were deposited at PDB with the codes PDB ID 6GP5 (Apo BTGH53) and 6GPA (BTGH53 + Galactose).

Results and Discussion

BTGH53 is specific for β-1,4-galactans. We previously reported that BTGH53 is important for degradation of GOS by *Bt. thetaiotaomicron* and that the enzyme is specific for β-1,4-linked galactans (pectic galactan) and not for the β-1,3-linkage type as found in larch wood arabinogalactans (21). In the present study BTGH53 was further characterized, both structurally and enzymatically. First, the specific activity of BTGH53 on high molecular weight galactan was determined using the Azo-galactan colorimetric substrate (derived from potato galactan). BTGH53 specific activity with Azo-galactan was as 105.8 ± 0.9 GalU/mg, which is a 10-fold difference lower to the other characterized GH53 enzymes (40). Previously, we observed that also significant levels of galactose were formed after BTGH53 activity on galactan, similar to what was observed for fungal GH53 enzymes (21). Therefore exo-activity of BTGH53 was tested with the chromogenic substrate 4-nitrophenyl-β-D-galactopyranoside (*p*NPG). This indeed resulted in yellow color formation due to the release of 4-nitrophenyl, with a pH optimum at pH 7.0 (not shown). BTGH53 displayed Michaelis-Menten kinetics, with a K_M value of 69.7 ± 5.7 µM and a k_{cat} value of 29.6 ± 0.9 sec⁻¹ (k_{cat}/K_M value is 425 mM⁻¹ s⁻¹). The moderate turnover rate of BTGH53 on the *p*NPG substrate is similar to that observed for the fungal GH53 enzymes AAGAL and MGGAL (41). Hydrolysis decreased above concentrations of 15 mM *p*NPG which may be due to transglycosylation activity of BTGH53, as was also suggested for the fungal GH53 enzymes. BTGH53 was inactive on lactose and galactobiose (mixture of β-D-Galp-(1→3)-D-Gal plus β-D-Galp-(1→4)-D-Gal) but able to cleave DP 3 GOS (21). The ability of BTGH53 to hydrolyze galactose from *p*NPG and to cleave DP 3 GOS, makes this enzyme more similar to the fungal GH53 endo-galactanases than to the bacterial BLGAL enzyme from *Bc. licheniformis*.

Fig. 1 3-Dimensional structure of the BTGH53 enzyme in complex with a galactose molecule (orange) showing the organization of a $(\beta\alpha)_8$ -barrel. Residues highlighted in pink are the catalytic residues D132 and E180, and stabilizing residue W130.

Structure of BTGH53. In order to gain further insights into the catalytic mechanism of BTGH53, we determined the three-dimensional structure of BTGH53. Native crystals were soaked in 50 mM pGOS in order to get a high resolution structure of BTGH53 in complex with products. We obtained a high resolution data set (1.79 Å) and solved the structure by molecular replacement as described (see Methods). The final model of BTGH53 consisted of amino acid residues 37–350 with two molecules in the asymmetric unit. Each BTGH53 molecule contained one galactose residue in the active site. The high resolution model was refined to give an overall R/Rfree of 0.17/0.22.

Fig. 2 Structural characteristics original to BTGH53. **(A+B)** $2Fo-Fc$ omit electron density maps for the original unrefined density, contoured to 1σ , showing the bound galactose. D331 is proposed to play an important role in forming the -2 subsite and we speculate its flexibility, as indicated by the different positions within the individual protein molecules in the asymmetric unit, provides BTGH53 the ability to cleave shorter galactooligosaccharide substrates. **(A)** protein 1 active site showing hydrogen bonds between D331 and C4 and C6 OH groups, **(B)** protein 2 active site where D331 is rotated 180 degrees about C_β with respect to protein A. Amino acids are indicated in grey, galactose molecules are indicated in green, D331 highlighted in blue oval. **(C)** Amino acid sequence alignment of BTGH53 and BLGAL (PDB code 1R8L) showing the missing loop 8 region in BTGH53; β α -loops numbered according to the structure of BLGAL (40). Loop 8 is highlighted in light blue in BLGAL (residues 328–378; missing in BTGH53); alignment prepared with ClustalOmega and ESPript (42, 43). **(D)** 3-dimensional organization of the active sites of BLGAL (bound with galactobiose in orange) and **(E)** BTGH53 (bound with galactose in orange). Catalytic residues of

BLGAL (D117 and E165) and corresponding residues D132 and E180 in BTGH53 are shown. Loop 8 region lacking in BTGH53 is highlighted by the (empty) blue circle.

The overall fold and architecture of BTGH53 is similar to the other structurally characterized bacterial GH53 enzyme, BLGAL from *Bc. licheniformis* (PDB code 1UR4) (core RMSD 1.48 Å, 32.5% sequence identity) and the closely related fungal AAGAL enzyme of *Aspergillus aculeatus* (PDB code 1FHL, core RMSD 1.49 Å, 29.5% sequence identity) (Fig. 1). The closest structure for the bacterial homologue *Bc. licheniformis* 1UR4 (based on sequence identity) did not work for finding the phases for the BTGH53 structure. An alternative approach to solving the BTGH53 structure was then attempted using the FFAS blast algorithm (<http://ffas.sanfordburnham.org/ffas-cgi/cgi/ffas.pl>) to obtain the closest structural homologues based on fold and function (32). The top 4 from the FFSA search (PDB codes 4BF7, 1FHL, 1HJS and 1HJQ) were used for finding the phases.

BTGH53 exhibits a $(\beta\alpha)_8$ TIM barrel fold with the catalytic machinery positioned within the center of the barrel (Fig. 1). The candidate catalytic residues are D132 as acid/base and E180 as nucleophile, based on the position of these catalytic residues within all GH53 enzymes which belong to clan GH-A (CAZy database GH53 https://www.cazypedia.org/index.php/Glycoside_Hydrolase_Family_53; (22)). These residues align with the catalytic residues D117 and E165 of the GH53 enzyme from *Bc. licheniformis* (PDB code 1UR4).

In one of the protein molecules in the asymmetric unit, the galactose molecule occupying the -1 subsite was stabilized by direct hydrogen bonding with D331 to its C4 and C6 hydroxyl groups, while W130 facilitated stacking interactions with the galactose molecule (Fig. 2A). In the other protein molecule in the asymmetric unit, we observed that D331 is rotated 180 degrees about C_b and does not form hydrogen bonds with galactose occupying the -1 subsite (Fig. 2B). Therefore we speculate that D331 plays an important role in forming the -2 subsite, where in the presence of galactobiose, the -2 galactose is stabilized by D331, while in its absence, D331 forms hydrogen bonds with galactose in -1. This explains why BTGH53 is active on *p*NP-galactose.

BTGH53 lacks a large loop region within the substrate binding site which is present in BLGAL (Fig. 2C, D, E). In BLGAL an extended loop 8 from the beta strand at position 8 forms a substrate binding site region with the -3 and -4 substrate binding subsites. The loop 8 region in BLGAL restricts this enzyme from cleaving GOS smaller than DP 4 (40). With the loop 8 region missing in BTGH53, the enzyme is able to bind to and act upon smaller substrates, such as galactotriose, producing galactobiose and galactose (21). This activity makes BTGH53 much more similar to the fungal GH53 enzyme AAGAL (28).

Due to a truncated loop 8, BTGH53 loop 7 is not linked to loop 8, which is important for active site stability in all other structurally characterized GH53 enzymes (40). In both AAGAL and BLGAL, loops 7 and 8 are linked via coordination of a calcium ion (BLGAL) or via a disulfide bond (AAGAL). In all fungal GH53 enzymes, the cysteine residues involved in disulfide bond formation are conserved (27). To correct for this, BTGH53 also has a truncated loop 7 region creating a large gap which is filled by an extended loop 6 region. This loop 6 region forms part of the BTGH53 active site. This organization has not been observed in any other GH53 structures studied to date. These structural changes in loops 6, 7 and 8 in BTGH53 may have clear effects on its activity and specificity. BTGH53 is active on β -1,4-linked galactan but not on larch arabinogalactan which is primarily β -1,3-galactan (Megazyme Larch Wood arabinogalactan, product code P-ARGAL). The structural changes observed in the substrate binding site of BTGH53 thus may select for only the β -1,4-linkage type and accommodate molecules of shorter DP length as observed previously (21).

The acid/base D132 residue forms a hydrogen bond with the galactose C2 hydroxyl group, where the E180 carboxyl group can perform nucleophilic attack on C1. Because of the loop 8 truncation in BTGH53, an important W363 residue (Fig. 2D) which forms stacking interactions with the galactose in the BLGAL -2 subsite is missing in the BTGH53 structure which may explain why we did not observe a second galactose molecule in the BTGH53 active site (Fig. 2D+E).

A total of five GH53 structures (http://www.cazy.org/GH53_structure.html) have been solved to date, four from eukaryotic soil fungi and one from a soil bacterium. BTGH53 is the second bacterial GH53 enzyme whose structure has been solved, the first from a bacterium that resides in the human gastrointestinal tract. The particular niche environment where a microbe resides may influence how its enzymes evolve also with respect to substrate specificity. *Bt. thetaiotaomicron* is resident in the distal gut, and the extracellular BTGH53 may

target non-digestible GOS that reach the distal gut. The observed changes to the BTGH53 active site thus may allow *Bt. thetaiotaomicron* to efficiently degrade galactan compounds of the β -1,4-linkage type down to its smallest constituents. The *Bt. thetaiotaomicron* galactan utilization system is part of a higher order system that allows degradation of all pectin components with a backbone of β -1,4-galactan, such as rhamnogalacturonans (44). Only exposure to pectic galactan, and not arabinogalactan of the β -1,3-linkage type (such as larch wood), upregulates the expression of this system and its BTGH53 enzyme. The structural similarity of higher DP GOS to pectic galactan also activates the expression of BTGH53. Therefore the production of the GH53 enzyme is a feature associated with efficient GOS utilization in *Bt. thetaiotaomicron*.

Effects of BTGH53 activity on growth of probiotic lactobacilli.

Lactobacilli constitute probiotic bacterial strains that very specifically utilize GOS with a degree of polymerization ≤ 2 (45). Pure cultures of *Lactobacillus* strains tested hardly consumed prebiotic GOS and thus were unable to grow with GOS as carbon sources. Therefore we subsequently tested whether treatment of GOS with BTGH53 activity actually stimulated growth of probiotic lactobacilli on GOS mixtures.

Two commercial GOS preparations were used, pGOS and P5 GOS. Their composition was analyzed recently using HPAEC-PAD and NMR spectroscopy to elucidate structural identity of nearly all their components (40 and 26, respectively) (38, 46); Fig. S1, S2). In brief, in pGOS we identified a total of 40 compounds with different degrees of polymerization (DP) (mostly DP 3–6) and/or glycosidic linkages (Fig. 3A). Its major component by peak height in the HPAEC-PAD chromatogram was the trisaccharide 4'-galactosyllactose (peak 11, Fig. 3A). Also DP 4 compounds, linear and branched, constituted a major fraction of pGOS (28% of total GOS mixture). Comparative HPAEC-PAD analysis of pGOS and P5 GOS, followed by NMR analysis of P5 GOS, also allowed elucidation of the P5 GOS structures (Fig. S1, S2). All 26 GOS structures found in P5 GOS were also found in pGOS, but in clearly different ratios (Fig. 3B). The two major components were DP 3 4'-galactosyllactose (as in pGOS) and the corresponding elongated product DP 4 β -D-Galp-(1 $\rightarrow$ 4)- β -D-Galp-(1 $\rightarrow$ 4)- β -D-Galp-(1 $\rightarrow$ 4)-D-Glc (Fig. 3B). Together these two linear DP3 and DP 4 components constituted up to 44% of total GOS composition in P5 GOS (24% in pGOS). P5 GOS and pGOS differed at the level of branched DP 3 and DP 4 components. Compounds eluting as peak 10 and 16 were nearly absent in P5 GOS (7% and 5% in pGOS) (Fig. 3B, Fig. S1). Also compounds with DP5

and larger were reduced in P5 GOS (5%) in comparison to pGOS (10%). Interestingly, two compounds from the DP 2 pool clearly found in pGOS were absent in P5 GOS, allolactose β -D-Galp-(1 $\rightarrow$ 6)-D-Glc and β -D-Galp-(1 $\rightarrow$ 6)-D-Gal (Fig. 4). Finally, lactose was still present in P5 GOS (13%) which was removed in pGOS.

Fig. 3 HPAEC-PAD analysis of 1 mg/ml pGOS (**A**) and P5 GOS (**B**) untreated (grey) and after incubation with BTGH53 (black). Adjacent tables show degree of polymerization of GOS components of corresponding peaks. Peak numbers with increased peak height due to BTGH53 activity are highlighted in bold, underlined peak numbers refer to compounds recalcitrant to BTGH53.

Fig. 4 Structural identity of GOS compounds comprising the DP 2 fraction. **3** β -D-Galp-(1 $\rightarrow$ 6)-D-Gal, **4** β -D-Galp-(1 $\rightarrow$ 6)-D-Glc (allolactose), **5** β -D-Galp-(1 $\rightarrow$ 4)-D-Glc (lactose), **7** β -D-Galp-(1 $\rightarrow$ 4)-D-Gal (galactobiose), **8a** β -D-Galp-(1 $\rightarrow$ 2)-D-Glc, **8b** β -D-Galp-(1 $\rightarrow$ 3)-D-Glc. Numbers indicate HPAEC-PAD chromatogram peaks shown in Fig. 3.

Growth of probiotic lactobacilli with pGOS and P5 GOS yielded OD 600 nm values at stationary phase between 19–63% of positive controls (with glucose) (Table 3). Final OD values for *L. paracasei* W20 and *L. casei* W56 were clearly lower for pGOS than for P5 GOS (Fig. 5). In contrast, *L. salivarius* W57 reached similar OD values with both GOS preparations (0.53 with P5 GOS and 0.49 with pGOS). The results reflect specific GOS utilization preferences of these lactobacilli. HPAEC-PAD analysis of the bacterial culture supernatants at stationary phase showed that these lactobacilli differ most clearly in utilization of the various DP 2 to 3 GOS. *L. salivarius* W57 consumed any DP 2 GOS (peaks 3–8, Fig. 6) present in both GOS and additionally the DP 3 GOS component β -D-Galp-(1 $\rightarrow$ 2)-[β -D-Galp-(1 $\rightarrow$ 4)-]D-Glc (peak 9, Fig. 6). *L. casei* W56 specifically utilized the DP 2 components and *L. paracasei* W20 any DP2 component, but not allolactose (Fig. 6). Similar to *L. paracasei* W20, Thongaram et al.(45) recently identified *L. paracasei* LCV-1 as strong utilizer of lactose and a GOS substrate comprising 59% DP2 GOS.

Table 3 Growth of *Lactobacillus* strains on 5 mg/ml GOS and GOS pre-incubated with BTGH53. No exponential growth phase was detected for *L. paracasei* W20 on pGOS. OD600 nm values obtained at stationary growth phase are expressed relative to corresponding OD600 nm values of glucose controls set as 1. All values shown are means of n=3 biological replicates. n.a., not applicable.

	Relative OD600 at stationary phase (16 h)			
	P5 GOS	P5 GOS + BTGH53	pGOS	pGOS + BTGH53
<i>L. salivarius</i> W57	0.46 ± 0.01	0.97 ± 0.01	0.41 ± 0.02	0.99 ± 0.03
<i>L. paracasei</i> W20	0.47 ± 0.01	0.99 ± 0.02	0.19 ± 0.02	0.86 ± 0
<i>L. casei</i> W56	0.63 ± 0	0.93 ± 0	0.47 ± 0	0.88 ± 0

	Growth rates (h ⁻¹)			
	P5 GOS	P5 GOS + BTGH53	pGOS	pGOS + BTGH53
<i>L. salivarius</i> W57	0.27 ± 0.01	0.37 ± 0	0.21 ± 0	0.38 ± 0.02
<i>L. paracasei</i> W20	0.07 ± 0	0.18 ± 0	n.a.	0.16 ± 0
<i>L. casei</i> W56	0.10 ± 0	0.20 ± 0	0.07 ± 0	0.2 ± 0

Fig. 5 Anaerobic growth of *L. paracasei* W20 (A), *L. salivarius* W57 (B), *L. casei* W56 (C) with 5 mg/ml pGOS and P5 GOS, pure and (*) BTGH53 digested. Glucose (5 mg/ml) served as positive control. In some cases error bars are smaller than symbols and therefore not apparent.

When incubating the two commercial GOS with BTGH53, composition shifted from DP 3 to DP 4 size dominating in untreated GOS to DP 2 components in BTGH53 treated GOS. Galactobiose (peak 7, Fig. 3) became the dominating component released to similar levels by BTGH53 treatment. In BTGH53 treated pGOS also the DP 2 components β -D-Galp-(1 $\rightarrow$ 2)-D-Glc, β -D-Galp-(1 $\rightarrow$ 3)-D-Glc (peaks 8a and 8b, see Fig. 3 and Fig. 4) increased, but they

remained nearly the same in BTGH53 treated P5 GOS. Besides galactobiose, lactose was the second major component in BTGH53 treated P5 GOS, whereas in BTGH53 treated pGOS the peak heights of lactose and other DP 2 components were more equally distributed. Analysis of pGOS products after BTGH53 treatment showed that it was inactive on DP 3 branched GOS, the linear DP 3 component 6'-galactosyllactose and the DP 2 units β -D-Galp-(1 $\rightarrow$ 2)-D-Glc, β -D-Galp-(1 $\rightarrow$ 3)-D-Glc, β -D-Galp-(1 $\rightarrow$ 6)-D-Gal, β -D-Galp-(1 $\rightarrow$ 6)-D-Glc. Similar observations were made with P5 GOS. The β -D-Galp-(1 $\rightarrow$ 6)-D-Gal, β -D-Galp-(1 $\rightarrow$ 6)-D-Glc compounds were absent in P5 GOS. They also were not found in the chromatogram of BTGH53 treated P5 GOS, thus also not liberated by enzymatic activity with GOS of higher DP. With both GOS samples BTGH53 treatment caused a significant increase in the DP 2 compounds β -D-Galp-(1 $\rightarrow$ 2)-D-Glc, β -D-Galp-(1 $\rightarrow$ 3)-D-Glc, β -D-Galp-(1 $\rightarrow$ 4)-D-Gal and β -D-Galp-(1 $\rightarrow$ 4)-D-Glc (Fig. 3 and 4). This DP 2 fraction may specifically stimulate growth of probiotic lactobacilli.

All three lactobacilli clearly benefited from the BTGH53 treatment of the GOS substrates and reached OD values at stationary phase close to positive controls (glucose) (Fig. 5, Table 3). BTGH53 hydrolysis products of P5 GOS stimulated the extent of growth of *L. paracasei* W20 better than the ones obtained from pGOS. BTGH53 treated GOS also resulted in reduced lag phases of growth for *L. paracasei* W20 and *L. casei* W56 (Fig. 5).

HPAEC-PAD analysis demonstrated that lactobacilli consumed a broader range of prebiotic GOS molecules in BTGH53 treated samples than in untreated ones. *L. salivarius* W57 consumed the broadest range of GOS by utilizing any component besides the branched trisaccharides β -D-Galp-(1 $\rightarrow$ 2)-[β -D-Galp-(1 $\rightarrow$ 6)-]D-Glc and β -D-Galp-(1 $\rightarrow$ 3)-[β -D-Galp-(1 $\rightarrow$ 4)-]D-Glc (peak 10, Fig. 6). These two components were also not used by *L. casei* W56 and *L. paracasei* W20 and in addition these two strains didn't consume β -D-Galp-(1 $\rightarrow$ 2)-[β -D-Galp-(1 $\rightarrow$ 4)-]D-Glc (peak 9, Fig. 6) which was also observed with untreated samples. These remaining compounds weren't cleaved by BTGH53 in enzymatic digestions of GOS mixtures (Fig. 3). Overall, pre-treating GOS mixtures with BTGH53 enabled the strains to benefit from a much broader range of GOS molecules with higher DP (Fig. 6).

Fig. 6 Utilization of GOS components by probiotic *Lactobacillus* strains analyzed by HPAEC-PAD. Profiles of pGOS and P5 GOS shown in grey (control). Composition of the two GOS preparations in bacterial culture supernatants harvested at stationary growth phase (s. Fig. 5) are shown in black (untreated GOS) and blue (BTGH53 treated GOS). Any GOS components not utilized by probiotic strains in untreated GOS are numbered according to Fig. 3. Peaks arising from medium (mMRS) components are indicated with *.

Conclusions

In this paper we have characterized the *Bt. thetaiotaomicron* BTGH53 β -1,4-galactanase in structural and functional detail. The data shows that BTGH53 activity on prebiotic GOS mixtures results in an increase of DP 2 GOS with

clear stimulatory effects on growth of lactobacilli. *Bt. thetaiotaomicron* is a dominant inhabitant of the human colon and probiotic lactobacilli are known to be present in the same compartment (47, 48), with potential cross-feeding effects *in vivo*. Similar stimulatory effects may be exerted by extracellular GH53 enzymes from other colon bacteria, but this also depends on their GOS product spectra. The *Bifidobacterium breve* *galA* gene for instance encodes an extracellular GH53 enzyme (23). GalA is an endo-acting galactanase that releases galactotriose as major product instead of galactobiose. Our study shows that lactobacilli are very specific for DP 2 GOS utilization and thus will not benefit from products of GalA activity.

Second, our results show that addition of BTGH53 to preparations of probiotic lactobacilli and prebiotic GOS mixtures provides a synbiotic combination: GOS DP 2 products released by BTGH53 from longer GOS DP specifically can be utilized for growth by lactobacilli. Such synbiotic combinations may be prepared either by adding (purified) carbohydrate-active enzymes or by pairing probiotic bacteria that (mutually) benefit from each other by producing complementary enzymes on a particular prebiotic substrate.

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Supplemental data

Fig S1 Comparative HPAEC-PAD analysis of Vivinal GOS derived pGOS and P5 GOS. In total 26 out of the 40 GOS structures found in pGOS were found in P5 GOS with different intensity in HPAEC peak height.

Fig S2 1D ^1H NMR spectra of P5 GOS and Vivinal GOS. Signals have been assigned their structure using structural-reporter-group signals as shown by van Leeuwen et al. (1, 2).

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Chapter 4

Structural identity of galactooligosaccharide molecules selectively utilized by single cultures of probiotic bacterial strains

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Abstract

Various β -galactosidase enzymes catalyze a trans-glycosylation reaction with lactose. The resulting galactooligosaccharide (GOS) mixtures are widely used in infant nutrition to stimulate growth of beneficial gut bacteria. GOS consist mainly of compounds with a degree of polymerization (DP) varying from 2–8, and with diverse glycosidic linkages. In recent years we have elucidated in detail the composition of several commercial GOS mixtures in terms of DP and the structural identity of the individual compounds. In this work thirteen (single) probiotic strains of gut bacteria, belonging to 11 different species, were grown to stationary phase with a Vivinal® GOS derived sample purified to remove lactose and monosaccharides (pGOS). Growth among probiotic strains varied strongly between 30–100% of OD600nm relative to positive controls with glucose. By identifying the components of the pGOS mixture that remain after growth, we showed that strains varied in their consumption of specific GOS compounds. All strains commonly used most of the GOS DP 2 pool. *Lactobacillus salivarius* W57 also utilized the DP 3 branched compound β -D-Galp-(1→4)-[β -D-Galp-(1→2)]-D-Glc. Bifidobacterial strains tended to use GOS with higher DP and branching than lactobacilli; *Bifidobacterium breve* DSM 20091, *Lactobacillus acidophilus* W37 and *Bifidobacterium infantis* DSM 20088 were exceptional in using 38, 36 and 35 compounds, respectively, out of the 40 different structures identified in pGOS. We correlated these bacterial GOS consumption profiles with their genomic information and were able to relate metabolic activity with presence of genome-encoded transporters and carbohydrate-active enzymes. These detailed insights may support the design of synbiotic combinations pairing probiotic bacterial strains with GOS compounds that specifically stimulate their growth. Such synbiotic combinations may be of interest in food/feed and/or pharmacy/medicine applications.

Introduction

Probiotic bacteria influence human health in various ways, i.e. by modulating the immune system, assisting in fermentation of dietary non-digestible elements into short-chain fatty acids (SCFA) and by inhibiting growth of pathogens. Imbalanced microbiota composition (dysbiosis) has been related to many diseases, including coronary heart disease, fatty liver diseases, rheumatoid arthritis, irritable bowel syndrome and inflammatory bowel disease (1–4). Changes in microbiota composition may occur naturally during a lifetime, but can also be caused by diseases and through the use of antibiotics. These changes require readjustment towards a healthy bacterial composition in the recovery phase. Stimulating growth and activity of beneficial bacteria in the human gut has become a valuable approach to sustain and restore human health (5). A growing number of prebiotic molecules, mostly carbohydrates, have been shown to affect the presence and to increase the numbers of distinct bacterial groups in the human colon that may ultimately drive the health effect (6).

At the beginning of life the human colon is a nearly sterile environment that is rapidly colonized primarily by bifidobacteria forming the microbiota of the healthy infant gut (7). Human milk oligosaccharides were found to be the main drivers in infant gut microbiota development (8). Infant nutritional supplements, as replacement for human milk, aim to stimulate the development of an infant gut microbiota with a composition as close to the natural situation as possible. For example, current infant formulas often contain mixtures of GOS and fructooligosaccharides (FOS) which have been shown to support the formation of a gut microbial environment that closely resembles that of breastfed infants (9, 10). GOS are primarily produced by incubating β -galactosidase enzymes at high lactose concentrations. These enzymes use lactose as donor substrate for transfer of galactose residues onto lactose or any prior formed GOS. These GOS molecules exhibit often a terminal reducing glucose residue elongated with galactose residues towards the non-reducing end. GOS yield and product composition are strongly influenced by the microbial origin of the (mutant) enzyme, reaction conditions and substrate concentrations used, resulting in a growing number of industrially produced GOS mixtures of different compositions (11, 12). These GOS mixtures vary mostly in their DP ranging from DP 2–8 and the presence of different ratios of glycosidic linkages of β -(1 $\rightarrow$ 2), β -(1 $\rightarrow$ 3), β -(1 $\rightarrow$ 4), and/or β -(1 $\rightarrow$ 6). It is unknown whether and how these individual GOS structures differ functionally.

To study the selective stimulatory effects of GOS on strains of bacteria that are associated with the human gut, we followed bacterial growth *in vitro* using a GOS mixture as sole carbon source. Previously we have analysed the composition of 7 commercial GOS mixtures (13). Vivinal GOS was most diverse in GOS composition (with over 40 different molecules), and hence it was selected for this study. This allowed us to identify the specific GOS compounds, differing in DP and glycosidic linkages present, that these bacteria use for growth. Furthermore, we correlated bacterial growth to the distinct set of genome encoded enzymes and transporters that the bacteria may produce in the presence of GOS substrates (14). In previous work, Gopal et al. studied two strains, *Bifidobacterium lactis* DR10 and *Lactobacillus rhamnosus* DR20 that utilized different GOS components within GOS mixtures: *B. lactis* DR10 consumed GOS with higher degree of polymerization (DP) while *L. rhamnosus* preferred use of galactose and GOS disaccharides (15). Another study tested growth of 68 human derived strains from the genera *Lactobacillus* and *Bifidobacterium*, on a broad range of prebiotics including GOS. While many strains grew well on GOS compared to other substrates, no clear selectivity of the strains towards certain components of GOS was found (16). Recently, a study revealed three subsets of strains of lactobacilli and bifidobacteria regarding GOS utilization: one group utilizing only GOS DP 2, a second utilizing GOS $\leq$ DP 3 and a third utilizing all GOS oligomers (17). Another study has observed that different strains of bifidobacteria have a different preference for GOS molecules, but was unable to identify the specific GOS structures (18). Kittibunchakul et al. (2018) tested fermentability of 3 GOS mixtures (including Vivinal GOS) using 8 *Lactobacillus* spp. strains and 3 *Bifidobacterium* spp. strains. Highest growth scores were obtained with a novel GOS mixture that contained mostly oligosaccharides with β -(1 $\rightarrow$ 3) and β -(1 $\rightarrow$ 6) glycosidic linkages. The precise identity of the GOS molecules degraded was not studied however (19). It thus is clear that there is a strain-based preference for specific GOS DPs, but detailed information about the structural identity of GOS molecules consumed in terms of glycosidic linkages is still missing.

We recently introduced methodologies for rapid identification of the complete structural composition of GOS mixtures (13, 20). Here we applied this comparative HPAEC-PAD analysis to identify GOS structures that specifically were utilized by probiotic bifidobacteria and lactic acid bacteria. The results show that individual strains differ strongly in utilization of pGOS in terms of polymerization degree and type of glycosidic linkages. These results provide a

more detailed understanding of how GOS structures found in (commercial) prebiotic samples stimulate growth of (commercial) probiotic bacteria at the level of individual strains and may be of interest in designing novel synbiotic mixtures.

Materials and Methods

Materials. The commercial probiotic strains *Lactobacillus paracasei* subsp. *paracasei* W20, *Lactobacillus acidophilus* W37, *Lactobacillus salivarius* W57, *Lactobacillus casei* W56, *Enterococcus faecium* W54 and *Pediococcus acidilactici* W143, *Bifidobacterium animalis* subsp. *lactis* W51, *Bifidobacterium animalis* subsp. *lactis*, *Bifidobacterium animalis* subsp. *lactis* W53 were supplied by Winclove Probiotics (Amsterdam, The Netherlands). *Bifidobacterium longum* subsp. *infantis* DSM 20088, *Bifidobacterium breve* DSM 20091, *Bifidobacterium adolescentis* DSM 20083, and *Bifidobacterium bifidum* DSM 20456 were purchased from DMSZ (Braunschweig, Germany). Purified GOS (pGOS), derived from Vivinal® GOS with galactose, glucose and lactose largely removed, was kindly provided by FrieslandCampina Domo (Amersfoort, The Netherlands). The modified de Man-Rogosa-Sharpe-medium (mMRS-medium) was prepared as described previously (21). In brief, 1 liter of mMRS-medium contained: peptone 10 g; granulated yeast extract 2.5 g; tryptose 3 g; Tween80 1 g; K₂HPO₄ 3 g; KH₂PO₄ 3 g; ammonium citrate 2 g; pyruvic acid sodium salt 0.2 g; MgSO₄·7H₂O 0.575 g; MnSO₄·H₂O 0.12 g; FeSO₄·7H₂O 0.034 g. All components were dissolved in aqua bidest. H₂O and the medium heated to 60°C to dissolve all components. After adjusting pH to 6.8, the medium was sterilized by autoclaving (15 min, 121°C) and supplemented with filter-sterilized 0.5 g/l Cys-HCl (final concentration). The carbon-source free *Bifidobacterium* medium (cfBM) contained (g/l) (22): trypticase peptone 10 g; yeast extract 2.5 g; tryptose 3 g; K₂HPO₄ 3 g; KH₂PO₄ 3 g; triammonium citrate 2 g; pyruvic acid 0.3 g; Tween 80 1 g; MgSO₄·7H₂O 0.574 g; MnSO₄·H₂O 0.12 g; NaCl 5 g. All components were dissolved in aqua bidest. H₂O and, after boiling the medium, the pH was adjusted to 6.8. The medium was sterilized by autoclaving as stated for the mMRS-medium and afterwards supplemented with sterile 0.5 g/l Cys-HCl (final concentration).

Growth of probiotic bacterial strains. Lactic acid bacteria (LAB) were cultured in MRS-medium (Oxoid, Basingstoke, UK) in anaerobic culture tubes flushed with Nitrogen using the Hungate technique (23). Under these

conditions, strains were pre-cultured twice overnight at 37°C before the growth experiments with GOS; purity of cultures was frequently checked under the microscope. For growth experiments with pGOS, cultures of LAB strains were harvested by centrifugation (2,300 x g, 2 min) and bacterial pellets diluted 25-fold in 2x sterilized mMRS. Diluted cultures were mixed 1:1 with sterile pGOS (dissolved in MilliQ water at 10 mg/ml) in microtiter plates (96 well, flat-bottom) (Greiner Bio-One, Frickenhausen, Germany) yielding final volumes of 160–200 µl per well (24). Glucose (5 mg/ml) and medium without any carbon-source added served as positive and negative control, respectively. Inoculation of microtiter plates was carried out inside a glove box (Bohlender, Grünsfeld, Germany) constantly flushed with N₂ in order to ensure anaerobic conditions. Afterwards, plates were sealed airtight (Simport, Beloeil, Canada) and transferred into a microtiter plate reader (BioTek, Winooski, VT) incubating plates at 37°C. Plates were shaken continuously at medium speed and OD_{600nm} measured every 5 min. *Bifidobacteria* were cultured in Medium 58 following the recipe of the supplier (DSMZ, Braunschweig, Germany), using anaerobic culture tubes flushed with CO₂. Every strain was pre-cultured twice overnight at 37°C prior to growth experiments with pGOS. Bacterial cultures were harvested as described above for LAB strains and bacterial pellets diluted 25-fold in 2x carbon-source free *Bifidobacterium* medium. For the actual growth experiments, the diluted bacterial cultures were mixed 1:1 with 10 mg/ml of sterilized pGOS in anaerobic glass tubes and cultures flushed with 100% CO₂. During the growth experiments cultures were maintained at 37°C and the cell density meter WPA CO 8000 (Biochrom, Cambridge, UK) was used for direct measurement of OD_{600 nm} within anaerobic glass tubes. All cultures were inoculated as independent n = 3 triplicates.

pH measurements of cultures were carried out at the time of inoculation (0 h) and stationary growth phase (18 h for LAB strains, 25–32 h *Bifidobacterium* strains) using a pH electrode VWR pH100 (VWR International, Leuven, Belgium).

Carbohydrate structural analysis. GOS composition in commercial mixtures and bacterial cultures was profiled using high performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) as described (13). Bacterial cultures were harvested at stationary phase (see Figure 1 for time points) by centrifugation (2 min, 16,000 x g). Supernatants were transferred immediately into HPAEC vials and diluted 5-fold in 80% DMSO. GOS molecules were separated on a CarboPac PA-1 column (250 by 2

mm; Dionex, Amsterdam, The Netherlands) with a complex gradient of buffer A = 0.1 M NaOH, buffer B = 0.6 M NaOAc in 0.1 M NaOH, buffer C = deionized water and buffer D = 50 mM NaOAc as described (13). The remaining pGOS composition after growth was analyzed per strain in $n = 3$ independent samples, each derived from a different biological replicate. Samples containing pure pGOS and medium without carbon source added served as positive and negative controls, respectively.

Genome analysis. Genbank files of bacterial genomes including plasmids for the DSMZ strains were downloaded (<https://www.ncbi.nlm.nih.gov>) at 07-11-2018 or supplied by Winclive. A comparative analysis for different GOS catabolic pathways identified in reference strains was performed. Genomes were submitted to RAST (Rapid Annotation using Subsystem Technology; <http://rast.nmpdr.org/rast.cgi>) using RASTtk as default method (25). Annotated bacterial genomes were compared for lactose and galactose utilization to identify candidate genes encoding the LacS/LacZ, LacEF/LacG and LacY pathways. Whole CAZomes were established using dbCAN2 (automated CAZyme annotation; <http://cys.bios.niu.edu/dbCAN2/blast.php>) to identify candidate genes encoding galactose-specific glycoside hydrolases (GH) of families 1, 2, 35, 42 and 53 (26). Searches for signal sequences for secretion in GH proteins were performed with dbCAN2 and PSORTb version 3.0.2 (bacterial localization prediction tool; <http://www.psorth.org/psorth/>) (27). As the RAST subsystem for lactose and galactose utilization does not contain genes of the GosDEC and GalCDE pathways, the presence of these genes in bacterial genomes was checked by cross BLAST searches using the GosDEC gene from *B. lactis* 04 (Balac_0485, Acc. nr. WP_004268783.1; Balac_0486, Acc. nr. WP_004269047.1) and GalCDE gene from *B. breve* UCC2003 (Bbr_0417_galc, Acc. nr. WP_015438440.1; Bbr_0418_galD, Acc. nr. WP_025262769.1; Bbr_0419_galE, Acc. nr. WP_003828491.1) as reference. Total number of candidate genes for each enzyme in these pathways was retrieved and on this basis a heat map was created using the program GraphPrism (version 7.0).

Statistical analysis. All growth experiments are biological triplicates; the OD600 values are expressed as averages. Medium without bacterial inoculation was used to obtain blank values during OD600 measurements.

Nucleotide accession numbers. The newly identified genes and gene clusters putatively involved during GOS utilization of probiotic bacterial strains were assigned the following nucleotide accession numbers: *B. lactis* W51 lactose operon, MN102123; *B. lactis* W51 glycoside hydrolase family 2 beta-

galactosidase, MN102124; *B. lactis* W51 glycoside hydrolase family 42 beta-galactosidase, MN102125; *B. lactis* W51 Gos utilization gene cluster, MN102126; *B. lactis* W52 lactose operon, MN102119; *B. lactis* W52 glycoside hydrolase family 2 beta-galactosidase, MN102120; *B. lactis* W52 glycoside hydrolase family 42 beta-galactosidase, MN102121; *B. lactis* W52 Gos utilization gene cluster, MN102122; *B. lactis* W53 lactose operon, MN102115; *B. lactis* W53 glycoside hydrolase family 2 beta-galactosidase, MN102116; *B. lactis* W53 glycoside hydrolase family 42 beta-galactosidase, MN102117; *B. lactis* W53 Gos utilization gene cluster, MN102118; *L. salivarius* W57 lactose operon, MN191511; *L. acidophilus* W37 Lactose and galactose utilization gene cluster, MN191512; *L. acidophilus* W37 LacZ glycoside hydrolase family 2 beta-galactosidase, MN191514; *L. acidophilus* W37 glycoside hydrolase family 42 beta-galactosidase, MN191513.

Results and Discussion

Growth of probiotic bacterial strains on pGOS. Utilization of GOS compounds was studied by growing strains under appropriate anaerobic conditions with 5 mg/ml pGOS, or appropriate controls. Most bifidobacterial strains grew well on pGOS, with lag phases and growth rates close to positive controls (glucose 5 mg/ml) (Figure 1A). *B. breve* DSM 20091 and *B. lactis* W51–52 preferred pGOS as growth substrates over glucose clearly indicating saccharolytic capabilities for more complex carbon sources, as observed for bifidobacteria (28, 29). Strains of lactic acid bacteria often grew to a limited extent on pGOS compared to their positive controls, with OD600nm values ranging between 32%–49% of Glc controls (Figure 1B). *L. acidophilus* W37 was an exception and reached an OD600nm over 70% of its positive control. Growth results on pGOS were supported by final culture pH, close to (for bifidobacteria) or above (for most lactobacilli) their positive controls. These results show that LAB strains grew on pGOS only to a limited extent and that these strains thus differ from most *Bifidobacterium* strains in their metabolic capabilities to use pGOS.

Fig. 1 **A** Bifidobacterial strains grown with 5 mg/ml pGOS; glucose (5 mg/ml) and modified MRS-medium served as positive and negative controls (Neg. control), respectively. Growth was followed by measuring ΔOD manually in anaerobic glass tubes (s. Material and Methods). **B** Strains from lactic acid bacteria (LAB) grown with 5 mg/ml pGOS; glucose (5 mg/ml) and modified MRS-medium served as positive and negative controls, respectively. OD600nm values were acquired by growing strains in microtiter plates and following ΔOD using a microtiter plate reader. All values shown are means from 3 biological replicates. Most standard deviations are smaller than the size of the symbols and therefore not apparent.

Structural identity of pGOS compounds consumed by probiotic strains. The pGOS mixture contained 40 different GOS compounds (Fig. 2, 3) as identified by peak height differences above the baseline. Analysis of GOS compounds remaining at stationary growth phase (harvesting time points for *Bifidobacterium* strains, 25 or 32 h, depending on time required to reach stationary phase; for LAB strains 18 h, see Fig. 1) revealed strain dependent GOS consumption profiles. The three *B. lactis* strains W51–W53 completely utilized the DP 2 compounds β -D-Galp-(1→2)-D-Glc, β -D-Galp-(1→3)-D-Glc (peak 8) and partially utilized the linear DP4 β -D-Galp-(1→4)- β -D-Galp-(1→4)- β -D-Galp-(1→4)-D-Glc (peak 17) (Fig. 2A). In addition, HPAEC-PAD chromatograms of strains W51 and W52 showed an increase in galactose (peak 1, Fig. 2A) while strain W53 had consumed virtually all galactose at the time cultures were harvested, as previously observed for various lactobacilli (21). As pH in these cultures remained above controls, this effect is most likely linked to a lactose-galactose anti-porter involved in pGOS metabolism present in the *B. lactis* strains (further described below) (30, 31). The *B. breve* strain DSM 20091 showed the broadest utilization of GOS compounds amongst all strains tested. It consumed 38 out of the 40 compounds in pGOS; the remaining compounds were the DP 4 branched compounds β -D-Galp-(1→4)- β -D-Galp-(1→4)-[β -D-Galp-(1→6)]-D-Glc and β -D-Galp-(1→4)-[β -D-Galp-(1→4)- β -D-Galp-(1→6)]-D-Glc (peak 14a and b). Partially consumed compounds (detected with reduced peak height) represented the two linear elongated DP 4 and DP 6 structures (peaks 17 and 24). In the chromatogram of this strain, the galactose peak was found to be reduced compared to the pGOS standard. Previously, Watson et al. (21) studied growth and GOS consumption by various other *B. breve* strains, revealing that strain dependent differences may occur. Similar to *B. breve* DSM 20091, the *B. adolescentis* strain DSM 20083 did not use the two DP 4 branched compounds eluting under peak 14 nor the DP3 β -D-Galp-(1→3)- β -D-Galp-(1→4)-D-Glc (peak 12). Interestingly, the latter structure has been linked to cross-reactive allergies in Japan (32, 33). Partial utilization was observed with the β -(1→4) linear elongated products of 4'Galactosyllactose (peak 11); i.e. DP 4 (peak 17), DP 5 (peak 22) and DP 6 (peak 24). The latter two compounds were preferentially utilized by the *B. bifidum* strain DSM 20456. Furthermore, this strain selectively left the DP 3 branched compounds β -D-Galp-(1→6)-[β -D-Galp-(1→2)]-D-Glc and β -D-Galp-(1→6)-[β -D-Galp-(1→2)]-D-Glc (peaks 10 a and b) while the linear β -D-Galp-(1→3)- β -D-Galp-(1→4)-D-Glc compound (peak 12) was consumed. *B. bifidum* DSM 20456 also did not utilize the DP 3

compounds β -D-Galp-(1 $\rightarrow$ 4)- β -D-Galp-(1 $\rightarrow$ 2)-D-Glc, β -D-Galp-(1 $\rightarrow$ 4)- β -D-Galp-(1 $\rightarrow$ 3)-D-Glc, β -D-Galp-(1 $\rightarrow$ 4)- β -D-Galp-(1 $\rightarrow$ 4)-D-Glc (peaks 13 and 11, Fig. 2A). The DP 4 branched compounds eluting as peaks 14, 15 and 16 were not utilized; neither was the β -(1 $\rightarrow$ 4) linear elongation of 4'-galactosyllactose (peak 17). Similarly to *B. breve*, the *B. longum infantis* strain DSM 20088 consumed the majority of GOS molecules. Only DP 5+ and 3'-galactosyllactose (peak 12) were left by this strain. Overall these results show that most *Bifidobacterium* strains grew well on pGOS and consumed GOS molecules with distinct degree of polymerization and different glycosidic linkage composition. Most of the branched structures (peaks 10, 13, 15, 16) are only consumed by a limited number of strains, particularly by *B. infantis*, *B. breve*, *B. adolescentis* and *L. acidophilus*. Peak 14 is only consumed by *B. infantis* DSM 20088 and *L. acidophilus* W37. Branched structure 6a is fermented by all strains tested (Fig. 2 and 3).

Rather opposite effects were seen for the lactic acid bacteria (LAB) strains that we tested. These strains mostly utilized a narrow and specific range of GOS molecules. *P. acidilactici* W143 hardly used GOS molecules, only the disaccharides β -D-Galp-(1 $\rightarrow$ 6)-D-Gal (peak 3) and β -D-Galp-(1 $\rightarrow$ 4)-D-Gal (peak 7; Figure 2B, Figure 3). *E. faecium* W54, *L. salivarius* W57, *L. paracasei* W20 and *L. casei* W56 utilized in addition to these structures also the disaccharides β -D-Galp-(1 $\rightarrow$ 2)-D-Glc and β -D-Galp-(1 $\rightarrow$ 3)-D-Glc (peak 8). Strains *B. lactis* W51, *B. lactis* W53, *L. salivarius* W57 and *L. casei* W56 also consumed allolactose (peak 4). *L. salivarius* W57 also consumed branched trisaccharide peak 9. *L. acidophilus* W37 was exceptional amongst the LAB strains in utilizing a broad range of GOS molecules, similar to what was observed for *B. infantis* strain DSM 20088. However, residual levels of the linear components β -D-Galp-(1 $\rightarrow$ 4)- β -D-Galp-(1 $\rightarrow$ 4)-D-Glc and β -D-Galp-(1 $\rightarrow$ 4)- β -D-Galp-(1 $\rightarrow$ 4)- β -D-Galp-(1 $\rightarrow$ 4)-D-Glc were still found, while *B. infantis* completely utilized those compounds (Fig. 2A and 2B). The results show that LAB strains often utilized only the DP 2–3 GOS molecules. This is in line with the results reported in a recent study (17). In our work, we were further able to identify the precise molecules consumed from this DP 2–3 fraction by the individual LAB strains. These results show that lactic acid bacteria grow to a limited extent on pGOS and often only utilize the DP 2–3 GOS molecules. Strain dependent differences may occur however: Watson et al. (21) studied *L. casei* DN-144-001 and reported use of virtually all Vivinal® GOS derived components.

Fig. 2 HPAEC-PAD chromatograms of pGOS (control, first line) and pGOS received from growth of probiotic strains (**A**) bifidobacteria and (**B**) lactic acid bacteria. For each strain, pGOS composition was analyzed in $n = 3$ biological replicates and shown in one chromatogram above. Numbers indicate single pGOS compounds that were not utilized by the strains at stationary growth phase (Fig. 1). *Bifidobacterium* strains were grown in carbon-source free *Bifidobacterium* medium with 5 mg/ml pGOS added for 25–32 h. LAB strains were grown in modified MRS-medium with 5 mg/ml pGOS added for 18 h. Peaks marked * are non-GOS peaks stemming from the growth medium.

Fig. 3 Differential utilization of pGOS components for growth as observed for *Bifidobacterium* and LAB strains highlighting their diverse capabilities to consume GOS of a specific DP level and different glycosidic linkages present.

Genome analysis. Bacterial strains employ specific catabolic pathways to utilize GOS and several possible routes were identified in bifidobacterial and lactic acid bacterial strains (Fig. 4 and 5). The most common route observed for lactobacilli involves a lactose permease transporter (LacS, identified in *L. acidophilus* NCFM) in combination with one or more (intracellular) β -galactosidases of family GH2 (LacZ/LacLM) (Fig. 4) (34). This pathway is often found in genomes of (probiotic) lactobacilli (35). A second route was identified in *L. casei* using a lactose phosphotransferase system (Lac_PTS, LacEF) and an intracellular family GH1 phospho- β -galactosidase (LacG) (36). We searched the available genomes of the probiotic LAB strains studied here, to identify the catabolic pathways that may be involved in degradation of pGOS compounds. We found that most of the LAB strains encoded genes for the LacS/LacZ pathway, however they differ in total number of candidate genes for LacZ (GH2 β -galactosidase) (Fig. 4). As an exception, *L. paracasei* W20 encoded genes for the lactose phosphotransferase system (Lac_PTS, LacEF). In growth experiments, *L. paracasei* W20 reached an OD600nm clearly below values of other strains (Fig. 1B) and structural analysis of pGOS compounds utilized by this strain revealed specific utilization of only 7 out of 40 pGOS components, mostly from the DP 2 fraction. It is known, that this lactose PTS is specific for lactose uptake and therefore may explain the low number of pGOS compounds used by *L. paracasei* W20 (15). While all other LAB strains employ genes for the LacS/LacZ pathway, the common presence of this pathway in these strains doesn't explain the individual differences observed in growth and pGOS utilization (Fig. 1B and 3). For example, *L. salivarius* W57 utilized DP 2 GOS, but also metabolized branched DP 3 component β -D-Galp-(1 $\rightarrow$ 4)-[β -D-Galp-(1 $\rightarrow$ 2)]-D-Glc (Fig. 3). It was previously shown that the GH2 β -galactosidases associated with the LacZ/LacLM system cleaved GOS with a wide variety of glycosidic linkages including β (1 $\rightarrow$ 2,3,4,6) and DP (2–6) (37). Therefore, we suggest that the GH2 β -galactosidase or the LacS transporter is the limiting factor in utilization of GOS. The substrate specificity and (3-dimensional) structural organization of this transporter needs to be further characterized within probiotic strains, in order to identify differences that may explain differential utilization of pGOS components among probiotic LAB strains.

In comparison to lactobacilli, bifidobacteria employ diverse catabolic systems to degrade GOS. In *B. breve* UCC2003 (and in *Bacteroides thetaiotaomicron*), it was demonstrated that efficient GOS utilization, particularly of higher DP compounds, correlated with expression of a membrane

associated GH53 endo-galactanase (GalA) (38). The gene encoding this enzyme was part of a galactan utilization operon encoding also an ABC transporter (GalCDE) and an intracellular GH42 β -galactosidase (GalG). Another GOS utilization system was identified in *B. lactis* BI-04 which employs the LacS/LacZ pathway and in addition the GosDEC (ABC transporter) with GosG (intracellular GH42 β -galactosidase) (39). Other strains with GH53 GalA homologues were found in *B. longum*, but lacking among *B. longum* subsp. *infantis* strains (40). The strains in the present study showed variation in the set of genes potentially involved in the above mentioned pathways. The *B. lactis* strains W51–W53 studied here encode an enzyme and a transporter of the GosG/GosDEC pathway, in addition to the LacS pathway (39). The genome of the *B. infantis* strain DSM 20088 contained multiple candidate genes for the GosDEC and GalDEC transporter, but no extracellular GH53 enzyme. The lack of a GH53 enzyme, as reported for *B. infantis* strains (40), may explain why the strain DSM 20088 used in this study was unable to use larger GOS (Fig. 3). The *B. breve* strain DSM 20091 encoded the highest number of genes possibly expressed as GosDEC and GalCDE transporters among all strains tested (8 and 9, respectively, Fig. 4 and 5) as well as an extracellular GH53 enzyme. Our structural analysis of pGOS component utilized by *B. breve* showed that strain DSM 20091 used 38 out of the 40 identified components and only left the DP 4 branched molecules β -D-Galp-(1 $\rightarrow$ 4)- β -D-Galp-(1 $\rightarrow$ 4)-[β -D-Galp-(1 $\rightarrow$ 6)]-D-Glc and β -D-Galp-(1 $\rightarrow$ 4)-[β -D-Galp-(1 $\rightarrow$ 4)- β -D-Galp-(1 $\rightarrow$ 6)]-D-Glc (Fig. 3). As shown in *B. breve* UCC2003, the GH53 enzyme and not the GalCDE transporter was essential for GOS utilization (41); this most likely shows that the *B. breve* GH53 galactanase is inactive on these two components. Interestingly, the genome of *B. adolescentis* DSM 20083 encoded almost no genes for ABC transporters although the strain used a broad range of pGOS molecules. We do not know whether the presence of LacS and GalDEC genes is responsible for GOS uptake in *B. adolescentis* DSM 20083 or that another (unidentified) system is active in this strain. *B. adolescentis* was the only *Bifidobacterium* strain in this study that encoded one family GH35 gene. Another extracellular enzyme (*lacZ* β -galactosidase, Acc. Nr. WP_021648433) was found in *B. bifidum* DSM 20456. This extracellular galactosidase may allow the strain to degrade larger GOS molecules of DP 5 and DP 6 (specifically utilized by this strain, Fig. 3). At the same time, *B. bifidum* DSM 20456 did not utilize the DP 4 and DP 3 branched GOS (compounds nr. 10, 14–16). Together with the β -1,4 linearly elongated structures β -D-Galp-(1 $\rightarrow$ 4)- β -D-Galp-(1 $\rightarrow$ 2)-D-Glc, β -D-Galp-(1 $\rightarrow$ 4)- β -D-

Galp-(1→3)-D-Glc, β-D-Galp-(1→4)-β-D-Galp-(1→4)-D-Glc (nr. 11 and 13, Fig. 3) these branched molecules may be unsuitable substrates for the extracellular GH2 enzyme of *B. bifidum*. The genetic organization of this strain shows similarity to that of the *B. lactis* strains W51–W53 (Fig. 5). An additional extracellular GH2 enzyme may enable the strain to access larger DP GOS compounds that comprise β-1,4 linear elongated galactose residues at the non-reducing end while these molecules are not accessible for the *B. lactis* strains.

Fig. 4 Candidate genes involved in galactooligosaccharide catabolism in probiotic bacterial strains. **(A)** Candidate genes in known pathways for LacEF/LacG, LacS/LacZ, GosDEC/GosG and GalA/GalCDE/GalG retrieved from BLAST searches of reference genes against bacterial genomes. Family and number of glycoside hydrolases annotated with dbCAN2. **(B)** Signal sequences for extracellular secretion searched by dbCAN2 and PSORTb 3.0 (26, 27). Supernatant +: signal sequence predicted to be present.

Overall, the diversity of utilization of GOS compounds with different DP and glycosidic linkages is reflected in the variable catabolic systems encoded in bacterial genomes. The presence or absence of a particular system together with different numbers of candidate genes reflects the differences that are observed between strains in terms of consumption of GOS molecules. We need further data to show why strains that encode the same system differ in utilization of

particular GOS molecules. Further characterization (biochemically and/or 3-dimensional structure) may identify what differences at the protein level result in the use of (or inability to use) certain components.

Moreover, the role of strains encoding extracellular enzymes during potential cross-feeding on GOS should be further characterized. These strains may together with their (purified) extracellular enzymes find application in synergistic synbiotics comprising a mixture of probiotic strains and GOS.

Fig. 5 Catabolic routes identified in *Bifidobacterium* and lactic acid bacteria to degrade β-galactooligosaccharide compounds from pGOS. *Lactobacillus* strains employ genes of (i) the LacEF/LacG pathway to utilize mostly pGOS compounds with similar structure of lactose (labelled as fraction ‘1’) or (ii) the LacS/LacZ pathway to utilize mostly DP 2 compounds of pGOS and certain DP 3 compounds (labelled as fraction 1 and 2, respectively). *Bifidobacterium* strains employ (i) also the LacS/LacZ pathway to utilize DP 2 compounds from pGOS and in addition (ii) the GosDEC/GosG and/or the GalA/GalCDE/GalG pathway(s) to utilize GOS compounds with higher DP.

Conclusions

This study has characterized the GOS consumption profiles of 13 probiotic bifidobacteria and LAB strains, using a purified Vivinal® GOS derived sample (pGOS), with 40 different GOS molecules, as carbon source for anaerobic growth. For this purpose, we identified GOS compounds remaining in the medium at the stationary growth phase of these bacteria when incubated with pGOS, not only identifying the degree of polymerization (DP) of the individual GOS compounds left but also their glycosidic linkage composition.

The results revealed bacterial species dependent profiles of GOS compound utilization: The different bacterial strains examined selectively consumed a variable number of GOS molecules. The DP 4 branched compounds β -D-Galp-(1 $\rightarrow$ 4)- β -D-Galp-(1 $\rightarrow$ 4)-[β -D-Galp-(1 $\rightarrow$ 6)]-D-Glc and β -D-Galp-(1 $\rightarrow$ 4)-[β -D-Galp-(1 $\rightarrow$ 4)- β -D-Galp-(1 $\rightarrow$ 6)]-D-Glc were hardly utilized by these strains and apparently are poorly accessible for the GOS catabolic systems encoded in their bacterial genomes. GOS mixtures comprise mostly compounds that at the molecular level mimic structures close to lactose and β -galactan, thus, GOS utilization correlated well with the different lactose uptake/degradation and/or β -galactan degradation systems genomically encoded by these strains. Dietary supplementation with this GOS mixture is likely to result in enrichment for bacterial strains encoding such catabolic systems.

At the non-reducing end, pGOS majorly comprises compounds with β (1 $\rightarrow$ 4) linked galactose. Only three strains studied were able to use the GOS DP 3+ fraction, providing interesting potential selectivity. In future work we aim to characterize whether GOS mixtures enriched in β (1 $\rightarrow$ 3) or β (1 $\rightarrow$ 6) elongations at the non-reducing end are degraded similarly, involving the catabolic systems described here or whether other pathways are involved in selective metabolism of these prebiotic GOS compounds.

The results also show that pGOS, applied as functional ingredient in infant nutrition, stimulates growth of a broad range of probiotic bacteria. This may be advantageous in generation of a diversified gut microbiota close or beyond the composition of breast-fed infants. These data also show that synbiotic mixtures of this type of GOS with *B. breve*, *B. infantis* and/or *L. acidophilus* are most likely to be successful. Although beyond the scope of this paper, such data may also be used to prepare GOS mixtures with a tailored composition to restore dysfunctional microbiota composition in patients with gut related diseases.

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Chapter 5

***Lactobacillus reuteri* strains convert starch and maltodextrins into homo-exopolysaccharides using a cell-associated 4,6- α -glucanotransferase enzyme**

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Abstract

Exopolysaccharides (EPS) of lactic acid bacteria (LAB) are of interest for food applications. LAB are well-known to produce α -glucan from sucrose by extracellular glucansucrases. Various *Lactobacillus reuteri* strains also possess 4,6- α -glucanotransferase (4,6- α -GTase) enzymes. Purified 4,6- α -GTases (e.g., GtfB) were shown to act on starches (hydrolysates), cleaving $\alpha(1\rightarrow4)$ linkages and synthesizing $\alpha(1\rightarrow6)$ linkages, yielding isomalto-/maltopolysaccharides (IMMP). Here we report that also *L. reuteri* cells with these extracellular, cell-associated 4,6- α -GTases synthesize EPS (α -glucan) from starches (hydrolysates). NMR, SEC, and enzymatic hydrolysis of EPS synthesized by *L. reuteri* 121 cells showed that these have similar linkage specificities but generally are much bigger in size than IMMP produced by the GtfB enzyme. Various IMMP-like EPS are efficiently used as growth substrates by probiotic *Bifidobacterium* strains that possess amylopullulanase activity. IMMP-like EPS thus have potential prebiotic activity and may contribute to the application of probiotic *L. reuteri* strains grown on maltodextrins or starches as synbiotics.

Introduction

Lactic acid bacteria (LAB) are a group of Gram-positive, nonsporulating bacteria, of which *Lactobacillus* strains are regarded as probiotics. Some LAB are “Generally Recognized As Safe” (GRAS) strains and have been used in fermented foods such as cheese and yoghurt since ancient time. LAB produce lactic acid, diacetyl/acetoin, and carbon dioxide that contribute to the texture, flavor, and shelf life of fermented foods (1–3). Some LAB also produce exopolysaccharides (EPS), which find application in the improvement of food texture. They also may have interesting health effects for humans, e.g., antitumor activity and immune stimulation (4, 5).

Depending on their composition and mechanism of biosynthesis, EPS produced by LAB can be classified into two groups: hetero-EPS and homo-EPS (6). Hetero-EPS consist of more than one carbohydrate moiety, and generally are synthesized intracellularly from activated sugars (7). LAB homo-EPS consist of a single type of monosaccharide, generally D-glucose or D-fructose, and can be divided into α -glucans (e.g., dextran, mutan, alternan, and reuteran) and β -fructans (e.g., inulin and levan) (8–10). Most LAB homo-EPS known are produced from sucrose by extracellular glucansucrase and fructansucrase enzymes (6, 11, 12). *Lactobacillus reuteri* 121 uses the GtfA glucansucrase to convert sucrose into an α -glucan (reuteran) with both $\alpha(1\rightarrow4)$ and $\alpha(1\rightarrow6)$ linkages, mostly alternating (9, 13–15). Glucansucrases are glycoside hydrolase enzymes belonging to family GH70 as classified by Carbohydrate Active Enzyme Database (www.CAZy.org).

Compared to sucrose, starch is a cheaper and more abundant carbon source for LAB industrial cultivation and product formation (16). In the past 30 years, a number of amylolytic LAB strains have been isolated and their starch-acting enzymes investigated (17, 18). Most of these are starch-hydrolyzing enzymes while only few enzymes with starch transglycosylation activity have been characterized (19–24). Recently, various 4,6- α -glucanotransferase (4,6- α -GTase) enzymes have been characterized from *L. reuteri* strains (25, 26). These 4,6- α -GTase transglycosylating enzymes constitute a GH70 subfamily that produces isomalto-/maltopolysaccharides (IMMP), which is a new type of soluble dietary fiber, using starch or starch hydrolysates as substrate rather than sucrose (27, 28). The *L. reuteri* 121 strain GtfB enzyme has been purified, and its IMMP products have been characterized in detail (27, 28). The *gtfA* and *gtfB* genes in *L.*

reuteri 121 are located next to each other in the genome (25, 29). The *in vivo* functional role of 4,6- α -GTases remains to be studied.

In this study, 4 *Lactobacillus reuteri* strains possessing 4,6- α -GTase genes are shown to produce homo-EPS from maltodextrins *in vivo*. The model strain *L. reuteri* 121 with the *gtfB* gene was found to produce homo-EPS from starch or starch hydrolysates. The EPS products of *L. reuteri* 121 from different types of starch were further investigated with respect to structures and sizes, and compared to the IMMP produced by the GtfB enzyme *in vitro*. Finally, the *L. reuteri* 121 EPS derived from different maltodextrins as well as from sucrose (reuteran) were tested as carbon source for growth of various probiotic *Bifidobacterium* strains in order to evaluate their potential prebiotic activity.

Materials and Methods

Growth conditions. *Lactobacillus reuteri* strains, including *L. reuteri* 121 (LMG 18388), *L. reuteri* DSM 20016, *L. reuteri* TMW1.106 (kindly provided by Prof. Rudi F. Vogel in Technische Universität München) (30), *L. reuteri* ML1 (LMG 20347), *L. reuteri* 180 (LMG 18389), and *L. reuteri* ATCC5573, were subcultured (from stocks stored at -80°C) in 10 mL of sugar-free MRS medium (liquid cultures or agar plates) supplemented with 1% glucose. The fresh cultures were inoculated anaerobically in modified sugar-free MRS medium with different carbon sources with or without 1% glucose (w/v). One liter of medium contained 10 g of bactopectone, 4 g of yeast extract, 5 g of sodium acetate, 2 g of triammonium citrate, 0.2 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05 g of $\text{MnSO}_4 \cdot \text{H}_2\text{O}$; and 1 mL of Tween 80, supplemented with 5 g of starch or 50 g of maltodextrins. For agar medium, 15 g/L agar was added. The native starches, maltodextrins, and amylose V were provided by AVEBE (Veendam, The Netherlands) (Table 1).

The *Bifidobacterium* strains *B. breve* DSM 20091, *B. adolescentis* DSM 20083, and *B. dentium* DSM 20436 were subcultured (from stocks stored at -80°C) in 5 mL of *Bifidobacterium* medium (BM) described by Ryan et al. supplemented with 1% glucose (31). After autoclaving BM was supplemented with 0.05% (w/v) filter-sterilized cysteine-HCl, and strains were grown at 37°C under anaerobic conditions maintained by GasPak EZ anaerobe container system (BD, New Jersey, US). Aliquots (1%) from overnight cultures were inoculated into 5 mL of BM supplemented with different EPS (0.5%, w/v). BM without an

added carbon source and BM with 0.5% (w/v) glucose were used as negative and positive controls, respectively.

Extraction of Exopolysaccharides (EPS). EPS were produced by *L. reuteri* strains in the sugar-free MRS medium supplemented with various maltodextrins (5.0%, w/v) or starch (0.5%, w/v). Reuteran was produced and purified from *L. reuteri* 35-5, a fructansucrase negative mutant derived from *L. reuteri* 121, using the sugar-free MRS medium supplemented with sucrose (5.0%, w/v) (32). Strains were grown anaerobically at 37 °C for 72 h. For cultures grown with maltodextrins, EPS were extracted from supernatants obtained by centrifugation of liquid MRS cultures (33). Two volumes of cold ethanol (−20 °C) was added to supernatants and held at 4 °C overnight. Precipitates were harvested by centrifugation (12000 x g, 30 min, 4 °C) and redissolved in 1 volume of dd H₂O. EPS were reprecipitated with 2 volumes of ethanol at 4 °C overnight. After centrifugation, the harvested precipitates were dissolved in water, and then dialyzed (10 kDa MWCO, Thermo Scientific) against water for 48 h with changes of dd H₂O every 12 h (34). For cultures grown with starch, the EPS were collected according to a modified method based on the protocol of Lee et al. (35). Total EPS, including soluble and insoluble EPS, and cells were precipitated with 2 volumes of cold ethanol (−20 °C) at 4 °C overnight. Samples collected from tubes were centrifuged at 10000 x g for 10 min. The polysaccharides were solubilized by addition of 10 mL of 2 M NaOH. The cells were separated by centrifugation at 10000 x g for 15 min. EPS present in the supernatant was neutralized by the addition of an appropriate amount of 2 M HCl (36). The ethanol precipitation procedure described above was used to further purify EPS.

Expression of truncated GtfB-ΔN, full-length GtfA, and full-length GtfB. Truncated GtfB-ΔN and full-length GtfA and GtfB were produced as described before (37–39).

Extraction of *L. reuteri* cell proteins and activity staining of EPS synthesizing enzymes. The cells from *L. reuteri* 121 cultures (10 mL) grown with maltodextrins (AVEBE MD20) were collected by centrifugation (10000 x g) and then suspended in 200 μL of B-PER protein extraction buffer (Thermo, Rockford, IL, US). The suspensions were frozen in liquid nitrogen and thawed in a 37 °C water bath three times. Then 2 μL of lysozyme (150,000 U) was added to the suspension. After 1 h incubation at room temperature, 10 μL of each suspension was boiled with 5 μL of SDS loading buffer for 10 min. After centrifugation (12000 x g, 5 min), the supernatants were analyzed by SDS-

PAGE. The gels were washed 3 times with dd H₂O (30 min each), allowing protein renaturation, and incubated overnight at 37 °C in sodium acetate buffer (25 mM, pH 5.0, 1 mM CaCl₂) with 5% (w/v) maltodextrins (AVEBE MD20). The activity of EPS synthesizing enzymes in the gel was detected by staining for the polysaccharides by a periodic acid-Schiff (PAS) procedure (29). The periodic acid oxidizes the vicinal diols in sugars, creating a pair of aldehydes, which react with the Schiff reagent to give a purple-magenta color. The full-length GtfB expressed in and purified from *E. coli* was loaded as a reference.

Amylose V conversion by washed *L. reuteri* 121 cells. The *L. reuteri* 121 cells were collected by centrifugation (5000 x g, 30 min, 4 °C). Cell pellets were washed twice using sodium acetate buffer (25 mM, pH 5.0, 1 mM CaCl₂, 0.02% (w/v) NaN₃) in order to remove any unbound proteins. The cells were resuspended in the same sodium acetate buffer and were incubated with amylose V (0.25%, w/v) on a shaker at 40 °C to measure enzyme activity and to synthesize the enzyme products (37, 39). For amylose V conversion, cells (0.03 U of total GtfB activity per mL reaction volume) with an activity equivalent to that of 60 nM GtfB-ΔN enzyme were incubated with amylose V (0.25%, w/v) on a shaker at 40 °C and pH 5.0 for 24 h.

Preparation of Isomalto-/Maltopolysaccharides (IMMP) with GtfB-ΔN enzyme. IMMP were produced according to Leemhuis et al. (27). Different types of starches and maltodextrins (0.5%, w/v) were incubated with GtfB-ΔN enzyme (1.4 U per g substrate) in 10 mM sodium acetate buffer pH 5.0, supplemented with 1 mM CaCl₂ and NaN₃ (0.02% w/v) at 37 °C. Native starches were pregelatinized by autoclaving (15 min, 121 °C).

NMR analysis. EPS samples produced by the GtfB enzyme in vitro and by *L. reuteri* 121 cells *in vivo* were analyzed by NMR according to Leemhuis et al. (27). Resolution-enhanced 1D 500 MHz ¹H NMR spectra were recorded in D₂O (99.9 at % D, Cambridge Isotope Laboratories, Inc.) on a Varian Inova spectrometer (NMR Center, University of Groningen) at probe temperatures of 300 K. Spectra were recorded with 16k complex points, using a 5000 Hz spectral width. Data are expressed relative to internal acetone (δ¹H 2.225). Prior to analysis samples were exchanged twice with D₂O with intermediate lyophilization and then dissolved in 0.6 mL of D₂O. Spectra were processed using MestReNova 5.3 software (Mestrelabs Research SL, Santiago de Compostella, Spain), using Whittaker Smoother baseline correction and zero filling to 32k complex points.

Enzymatic debranching of polysaccharides and enzymatic hydrolysis of produced EPS. Starches and the IMMP and EPS products derived were suspended in citrate buffer (50 mM, pH 4.0, 0.02% NaN₃) to a final concentration of 10 mg/mL and slowly heated to 99 °C with a thermomixer (Thermo Fisher Scientific Inc., Waltham, MA, US). The suspension was left at 99 °C for 1 h while shaking. Afterward, the polymers were debranched at 40 °C for 16 h with 20 U of isoamylase from *Pseudomonas* sp. (Megazyme, Wicklow, Ireland). The debranched samples were dialyzed (1000 Da MWCO tube, Sigma-Aldrich, US) against Milli-Q and freeze-dried. Samples of 1 mg of soluble EPS were dissolved in 100 µL of citrate buffer (50 mM, pH 3.5–5.0 based on the instructions for these commercial enzymes), and excess amounts of the following enzymes were added: pullulanase M1 from *Klebsiella planticola* (Megazyme, Ireland), dextranase from *Chaetomium erraticum* (Sigma-Aldrich, US), α -amylase from *Aspergillus oryzae* (Megazyme, Ireland), isoamylase from *Pseudomonas* sp. (Megazyme, Ireland), and isopullulanase from *Aspergillus niger* (Megazyme, Ireland), as well as α -amylase plus isoamylase. Samples were incubated for 72 h at 40 °C. The samples were boiled for 10 min to terminate the enzymatic hydrolysis and used for further analysis. The products after α -amylase and isoamylase treatment were fractionated by size-exclusion chromatography on a Bio-Gel P-2 column (100 × 0.9 cm) (Bio-Rad) using ammonium bicarbonate (10 mM) as eluent at a rate of ~10 mL/h and then subjected to NMR analysis.

Size Exclusion Chromatography (SEC). DMSO-LiBr (0.05M) was prepared by stirring for 3 h at room temperature followed by degassing for 15 min using an ultrasonic cleaner (Branson 1510, Branson, Danbury, CT). Samples were dissolved at a concentration of 4 mg/mL in DMSO-LiBr by overnight rotation at room temperature, followed by 30 min heating in an oven at 80 °C, obtaining clear sample solutions. The samples were cooled to room temperature and filtered through a 0.45 µm Millex PTFE membrane (Millipore Corporation, Billerica, MA). The SEC system setup (Agilent Technologies 1260 Infinity) from PSS (Mainz, Germany) consisted of an isocratic pump, an auto sampler, an online degasser, an inline 0.2 µm filter, a refractive index detector (G1362A 1260 RID Agilent Technologies), a viscometer (ETA-2010 PSS, Mainz), and MALLS (SLD 7000 PSS, Mainz). WinGPC Unity software (PSS, Mainz) was used for data processing. Samples (100 µL) were injected into a PFG guard column using an autosampler at a flow rate of 0.5 mL/min and DMSO-LiBr as eluent. The separation was done by three PFG-SEC columns

with porosities of 100, 300, and 4000 Å. The columns were held at 80 °C, the refractive index detector at 45 °C, and the viscometer was thermostated at 60 °C. A standard pullulan kit (PSS, Mainz, Germany) with molecular masses from 342 to 805000 Da was used. The specific RI increment value dn/dc was measured by PSS and is 0.072 (obtained from PSS company).

High-pH Anion-Exchange Chromatography (HPAEC). The products of GtfB were injected onto a 4 × 250 mm CarboPac PA-1 column connected to an ICS3000 workstation (Dionex). Samples were run with a gradient of 30–600 mM NaAc in 100 mM NaOH (1 mL/min), and detected by an ICS3000 pulsed amperometric detector. A mixture of glucose, isomaltose, isomaltotriose, maltose, panose, maltotriose, maltotetraose, maltopentaose, maltohexaose, and maltoheptaose was used as references for qualitative determination of position of each component.

Total carbohydrate measurements. Total carbohydrates were measured using the total carbohydrate colorimetric assay kit (Biovision, Milpitas, USA). Of each liquid sample, 5–15 µl was added into a 96-well plate. The total volume was adjusted to 30 µl per well by adding ddH₂O. Then, 150 µl of concentrated H₂SO₄ (98%, v/v) was added per well. The plate was mixed for 1 min on a shaker at room temperature and incubated in an oven at 90°C for 15 min. Afterwards, 30 µl of developer reagent was added and mixed with sample for 5 min on a shaker at room temperature. The optical density was measured at 490 nm. A calibration curve was made using 0, 4, 8, 12, 16 and 20 µg glucose per well.

Results and Discussion

***Lactobacillus reuteri* strains produce homoexopolysaccharides (EPS) from maltodextrins.** Protein blast searches in the NCBI database (<http://www-ncbi-nlm-nih-gov.proxy-ub.rug.nl/BLAST/>) using GtfB of *L. reuteri* 121 as a query sequence revealed that approximately 36 *Lactobacillus* strains carry a 4,6- α -GTase encoding gene. Nine out of these 36 are *L. reuteri* strains. Previously, we have expressed and purified some of these 4,6- α -GTases in *E. coli*, e.g., GtfB from *L. reuteri* 121, GtfW from *L. reuteri* DSM20016, and GtfML4 from *L. reuteri* ML1, and shown that they produce IMMP from maltodextrins (Fig. 1A) (25–27). The question remained whether also *in vivo* these *L. reuteri* strains and their genes/enzymes support formation of IMMP-like EPS.

Using maltodextrins 13–17 (dextrose equivalent 13–17, Sigma-Aldrich) as carbon sources for growth, *L. reuteri* 121 (GtfB), *L. reuteri* DSM 20016 (GtfW), *L. reuteri* TMW1.106 (Gtf106b), and *L. reuteri* ML1 (GtfML4), which all possess a 4,6- α -GTase gene, clearly produced ethanol-precipitable EPS, while *L. reuteri* strains lacking a 4,6- α -GTase gene, such as *L. reuteri* 180 and *L. reuteri* ATCC 55730, did not. The yields of EPS produced by the *L. reuteri* DSM 20016 (GtfW) and *L. reuteri* ML 1 (GtfML4) strains were relatively low (data not shown), probably because the relatively high hydrolytic activity (39) of their enzymes impaired the formation of polysaccharides that can be precipitated by cold ethanol. These EPS are all composed of glucosyl units (monosaccharide analysis, data not shown) linked by $\alpha(1\rightarrow4)$ and $\alpha(1\rightarrow6)$ linkages (Fig. 1B). The peaks at δ 3.50 in the spectra also support the occurrence of the $-(1\rightarrow6)-\alpha$ -D-Glcp- $(1\rightarrow6)-$ element, which is not present in maltodextrin substrate (40, 41). The variation of area of peaks at δ 3.50 suggested that the percentage of $-(1\rightarrow6)-\alpha$ -D-Glcp- $(1\rightarrow6)-$ element in EPS produced by *L. reuteri* 121 is the highest while the percentage in EPS synthesized by *L. reuteri* TMW 1.106 is the lowest. Thus, these *L. reuteri* strains are able to produce α -glucan EPS from maltodextrins, which may be highly relevant for their survival and activities in natural and industrial environments.

Fig. 1 (A) Schematic representation of the IMMP product of 4,6- α -GTase (e.g., GtfB) with maltodextrins (28). (B) NMR spectra of the EPS isolated from *L. reuteri* 121, *L. reuteri* DSM 20016, *L. reuteri* ML1, and *L. reuteri* TMW1.106 cultures grown on maltodextrins AVEBE MD20 (5%, w/v)-MRS medium.

4,6- α -GTase enzymes are essential for EPS formation by *L. reuteri* *in vivo*. Using the previously established 4,6- α -GTase amylose-iodine assay with amylose V as substrate,(39), no activity was detectable in cell-free culture supernatants. However, cell-associated activities were detected in the washed and resuspended cell pellets of all 4 *L. reuteri* strains with a (putative) 4,6- α -GTase enzyme (Fig. 2A). Amylose V is too large a molecule to pass the *L.*

reuteri cell membrane; therefore intracellular enzymes are not involved here. The combined data indicate that the amylose V modifying enzymes are extracellular and cell-associated and not released into the culture fluid during growth. This is in accordance with the primary structures of 4,6- α -GTases, showing the presence of signal peptides for secretion (42). The 4,6- α -GTases, like glucansucrases, also have variable N-terminal regions. Although the functional role of this N-terminal region has not been established, glucansucrases with this region are always cell wall bound in lactic acid bacteria (32). The same appears to be true for 4,6- α -GTases.

To further analyze the functional in vivo role of 4,6- α -GTase, the *L. reuteri* 121 system was studied in detail as model strain. Periodic acid-Schiff staining (PAS) is a well-established method that has been used to quickly detect polysaccharides produced by glucansucrase enzymes. Under mild conditions, denatured glucansucrases can be refolded and regain activity in SDS-PAGE gels (29). Previously, we already reported that also the denatured GtfB enzyme can be easily renatured in double distilled water (37). PAS staining (Fig. 2B, lane 2) showed that *E. coli* expressed and purified GtfB protein (~179 kDa) in SDS-PAGE gels produced polysaccharides from maltodextrins. Following SDS-PAGE of *L. reuteri* 121 total cellular proteins and protein renaturation, incubation with maltodextrins and PAS staining revealed a band, representing an enzyme with a similar molecular mass, (Fig. 2B, lane 1). Previously we reported that *L. reuteri* 121 GS GtfA (~199 kDa) activity also can be detected by PAS staining of SDS-PAGE gels when using sucrose as substrate (resulting in reuteran formation) (29). GtfA is the only carbohydrate-acting protein in the genome of *L. reuteri* 121 that has a similar molecular mass as GtfB (Gangoiti et al., in preparation). Unlike the PAS results with sucrose as substrate, no SDS-PAGE band was observed following incubation of *E. coli* expressed and purified GtfA protein with maltodextrins (Fig. 2B, lane 3). In addition, the resuspended *L. reuteri* 121 cell pellet was incubated with amylose V (0.25%, w/v) until all substrate was consumed as no color formed when using the iodine-staining assay developed previously (39). NMR analysis revealed that an α -glucan product was formed with 54% of $\alpha(1\rightarrow4)$ linkages and 46% of $\alpha(1\rightarrow6)$ linkages (Fig. 2C). The *L. reuteri* 121 cell associated 4,6- α -GTase enzyme and the purified GtfB enzyme thus have the same product specificity, cleaving $\alpha(1\rightarrow4)$ linkages and forming $\alpha(1\rightarrow6)$ linkages. The combined data shows that the GtfB enzyme is responsible for EPS formation by *L. reuteri* 121 cells.

Fig. 2 (A) 4,6- α -GTase activity associated with cells of *L. reuteri* strains using amylose V (0.25%, w/v) as substrate (39). “+” and “-” indicate the presence and absence of activity. (B) Periodic acid-Schiff (PAS) stained SDS-PAGE gels of whole-cell-extracted proteins of *L. reuteri* 121 (lane 1) and *E. coli* expressed and purified GtfB (lane 2) and GtfA (lane 3) after incubating with maltodextrins (AVEBE MD20, 5.0%, w/v) overnight at pH 5.0 and 40 °C. (C) 1D ^1H NMR analysis of the products (with 54% of $\alpha(1\rightarrow4)$ linkages and 46% of $\alpha(1\rightarrow6)$ linkages) derived from amylose V (0.25%, w/v) incubated with *L. reuteri* 121 cells. R1 α / β represent the reducing $-(1\rightarrow4)\text{-D-Glcp}$ units and D-Glcp units.

Table 1 Percentages of $\alpha 1 \rightarrow 6$ linkages in EPS formed during growth of *L. reuteri* 121 in MRS-medium with or without 1% of glucose in the presence of different types of starch and maltodextrins, determined by ^1H NMR spectroscopy. (n/a, not applicable). The final pH values of the *L. reuteri* 121 cultures with 1% glucose all were in the range of pH 4.5-4.6.

Substrate	Amylose content (%) (apparent)	Average DP ^a	Degree of branching ($\alpha 1 \rightarrow 4,6$) (%)	% ($\alpha 1 \rightarrow 6$) in IMMP produced by GtfB enzyme ^d	% ($\alpha 1 \rightarrow 6$) in EPS from <i>L. reuteri</i> 121 grown with 1% glucose	% ($\alpha 1 \rightarrow 6$) in EPS from <i>L. reuteri</i> 121 grown without 1% glucose
AVEBE Maltodextrins						
Paselli SA2	n/a	50		32		35
Paselli	n/a	20		34		19
AVEBE	n/a	6		25		26
AVEBE	n/a	4		24		31
AVEBE Starches						
Etenia 457	n/a	n/a	- ^c	33	22	
Arrow root	20.8	n/a	-	24	14	
Barley	25.5	n/a	-	21	15	
Corn	29.4	n/a	3.6	21	22	
Corn, waxy	0	n/a	4.8	7	11	
Mung bean	37.9	n/a	-	34	21	
Pea, yellow	30.9	n/a	-	35	26	
Pea,	52.8	n/a	-	71	52	
Potato	36.0	n/a	3.1	28	17	
Rice, round	25.0	n/a	4.1	13	12	
Rice, waxy	0	n/a	4.9	7	11	
Sorghum	23.7	n/a	-	22	18	
Tapioca	23.5	n/a	3.9	20	12	
Wheat	28.8	n/a	3.7	22	23	

^aDegree of polymerization; ^bn/a, not applicable;

^cnot available in literature; ^ddata adapted from Leemhuis et al. (27).

NMR analysis of EPS produced by *L. reuteri* 121 cells from maltodextrins or starches. Subsequently, we investigated whether *L. reuteri* 121 cells in liquid cultures also are able to convert starch. Various starches were supplied at a concentration of 5 g/L in order to prevent retrogradation (27).

Cultures were supplemented with glucose as carbon and energy source because *L. reuteri* 121 does not grow on starch alone; no extra glucose (Table 1) was added in the case of maltodextrins. The isolated EPS were subjected to NMR analysis. All starches tested were modified during the growth of *L. reuteri* 121 as is apparent from the increased percentages of $\alpha(1\rightarrow6)$ linkages obtained in all cases (Table 1). Growth of *L. reuteri* 121 in the presence of starch with a high amylose content and a lower degree of branching resulted in formation of EPS with higher percentages of $\alpha(1\rightarrow6)$ linkages. Highest conversion of $\alpha(1\rightarrow4)$ into $\alpha(1\rightarrow6)$ linkages was obtained with wrinkled pea starch, which contains the highest amylose (52.8%, w/w) content. This is in accordance with the *in vitro* data for starch conversion by GtfB, showing that the amylose content is positively, but the degree of branching is negatively, correlated with the percentage of $\alpha(1\rightarrow6)$ linkages in the produced IMMP (27). Compared to these IMMP (27), the *L. reuteri* 121 EPS derived from the same starch or maltodextrin substrates always have around 0–20% lower percentages of $\alpha(1\rightarrow6)$ linkages (Table 1). The *L. reuteri* 121 whole cell system provides more complicated reaction conditions, e.g., with pH variations, and the presence of other enzymes and components. Also, in whole cell systems the GtfB enzyme is cell wall bound. These factors are likely to influence product formation.

Size Exclusion Chromatography Analysis of EPS and IMMP Derived from Maltodextrins. The sizes of the IMMP products of the GtfB enzyme have not been reported before. Therefore, the IMMP-like EPS (Table 1) produced by *L. reuteri* 121 cells and the IMMP produced by the GtfB enzyme from different maltodextrins were analyzed by SEC in comparison with their original substrate. EPS derived from AVEBE SPG30 (<1.0 kDa) and AVEBE MD20 (approximately 1.0 kDa) (Table 1) both contain two peaks of much bigger polymers (Fig. 3A,B) of relatively low (region II, 132 kDa for EPS SPG30, 706 kDa for EPS MD20) and relatively high (region I, 1.7 MDa for EPS SPG30, 3.3 MDa for EPS MD20) apparent average molar mass (MM) (Fig. 3). The high MM products in all cases represented only a small percentage (2.5%). In general, these EPS thus are much larger than the IMMP produced by the GtfB enzyme from the same maltodextrins (Fig. 3). A possible explanation is that the reaction conditions for the *L. reuteri* 121 cell-associated GtfB enzyme and the free purified GtfB enzyme differ. Any glucose and maltose produced by the *L. reuteri* 121 cell-associated GtfB enzyme can be consumed by the cells as carbon source and thus cannot be used as acceptor substrate in further reactions. On the contrary, the glucose and maltose produced in the *in vitro* GtfB reaction can be

used as acceptor substrates (25), resulting in relatively high yields of oligosaccharides, which have no clear increase in chain length compared to the maltodextrin substrate.

Fig. 3 SEC chromatograms of the different maltodextrin substrates (**A**, SPG30; **B**, MD20), the EPS produced by *L. reuteri* 121 cells, and IMMP produced by the free GtfB- Δ N enzyme. Elution volumes of the pullulan standards corresponding to 366, 200, 113, 48.8, 21.7, 10, 6.2, 1.32, and 0.342 kDa are shown as gray dots.

SEC Analysis of EPS and IMMP Derived of Etenia 457 and Wheat Starch. NMR analysis showed that *L. reuteri* 121 cells convert various starches into EPS with increased percentages of $\alpha(1\rightarrow6)$ linkages in linear chains (Table 1). To compare starch conversion by the *L. reuteri* 121 cell-associated GtfB enzyme and by the free GtfB enzyme, Etenia 457 and wheat starch (28.8% amylose) were used as substrates (Table 1). The sizes of these substrates and the EPS and IMMP produced were analyzed using SEC.

Etenia 457 is produced from potato starch by amylomaltase (4- α -glucanotransferase) treatment in which the amylose part gradually is transferred to the nonreducing ends of the side chains of amylopectin, resulting in elongated external chains (43). As shown in Fig. 4A, the *L. reuteri* 121 EPS derived from Etenia 457 (approximately 350–400 kDa) has almost the same SEC profile as Etenia 457 itself (thus no clear change in MM) although the NMR results (Table 1) showed that this EPS has more $\alpha(1\rightarrow6)$ linkages. By contrast, the SEC profile of the IMMP produced by GtfB from Etenia 457 clearly shows that Etenia 457 was converted into two main products with smaller MM (approximately 200 kDa in region II and approximately 10 kDa in III). The material present in region II most likely represents Etenia 457 derived products with shortened nonreducing end side chains, used as donor substrate or hydrolyzed by GtfB. Region III represents Etenia 457 derived smaller MM products.

Fig. 4 SEC chromatograms of Etenia 457, the EPS produced from Etenia 457 by *L. reuteri* 121, and IMMP produced from Etenia 457 by the free GtfB- Δ N enzyme, before (A) and after (B) isoamylase treatment. Elution times of the pullulan standards corresponding to 366, 200, 113, 48.8, 21.7, 10, 6.2, 1.32, and 0.342 kDa are shown as gray dots.

As any size difference between Etenia 457 and the IMMP and EPS product derived might be covered by their relatively broad size distribution in the SEC analysis, these three polymers were debranched by isoamylase and subsequently analyzed again by SEC (Fig. 4B) (44). After debranching, the side chains of Etenia 457/isoamylase all eluted in region II'. The debranched products from IMMP and EPS were both smaller than those from the Etenia 457 substrate, indicating that the original amylopectin side chains were modified. However, the EPS and IMMP differed as isoamylase treated EPS formed by *L. reuteri* 121 cells contained some larger MM material, eluting in region I'.

Starches with high amylose content have been described as preferred substrates for GtfB (27). In *L. reuteri* 121 cultures, starches with a higher amylose content also yielded higher percentages of $\alpha(1\rightarrow6)$ linkages (Table 1). For instance, the EPS and IMMP derived from wheat starch both have approximately 22% of $\alpha(1\rightarrow6)$ linkages, which is much higher than the original 3.7% (Table 1). As shown in Fig. 5A, the graph representing wheat starch shows a peak in region I representing amylopectin content and a peak in region II representing amylose which are overlapping (44). After fermentation with *L. reuteri* 121, the amylopectin content decreased with an increase of products in region II. The SEC profile of the IMMP produced by GtfB from wheat starch is largely different from that of the wheat starch substrate. Wheat starch clearly was converted into two main products with smaller MM (approximately 300 kDa and approximately 15 kDa). The data indicates that GtfB also is capable of modifying the amylopectin part in native wheat starch.

Fig. 5 SEC chromatograms of wheat starch and the EPS produced from wheat starch by *L. reuteri* 121, and IMMP produced from wheat starch by the free GtfB- Δ N enzyme, before (**A**) and after (**B**) isoamylase treatment. Elution times of the pullulan standards corresponding to 366, 200, 113, 48.8, 21.7, 10, 6.2, 1.32, and 0.342 kDa are shown as gray dots.

After debranching, the SEC curve of wheat starch was divided into two parts: one is the amylose part, which cannot be processed by isoamylase (region I'); the other is the bimodal peak representing the amylopectin branches including side and inner chains (Fig. 5B) (44). For EPS derived from wheat starch, the SEC curve shows significant differences. The peak in region III' representing the long branches of amylopectin in wheat starch decreased a lot, suggesting that *L. reuteri* 121 also modifies the side chains of amylopectin in wheat starches. The peak representing amylose in region I' (>400 kDa) shifted dramatically and formed a new peak in region II' (approximately 200 kDa), indicating that the amylose content of wheat starch was converted by *L. reuteri* 121 and new, smaller products were generated. By contrast, the amylose part in region I' was completely converted *in vitro* by the GtfB enzyme as the peak representing amylose disappeared. The amount of the short chains after the debranching process increased.

To conclude, *L. reuteri* 121 cells and the GtfB enzyme are capable of modifying the amylose molecule as well as the side chains of amylopectin molecules in starches, introducing higher percentages of $\alpha(1\rightarrow6)$ linkages. The EPS produced have similar size distribution as the starch substrates although the percentages of $\alpha(1\rightarrow6)$ linkages increased significantly compared to the starch substrates. The produced IMMP are clearly different from the produced EPS and starch substrates with regard to their size distribution. This may result from the minor endoacting activity (Bai et al., in preparation) as detected when elucidating the reaction mechanism of the GtfB enzyme based on its three-dimensional structure. However, during the *L. reuteri* 121 fermentations, the interactions of the starch substrate with the cell-associated GtfB enzyme clearly are different from those with the purified GtfB enzyme. Conceivably, the endoacting activity of the cell-associated GtfB enzyme is impaired compared to the free GtfB enzyme. In addition, any glucose released by the *L. reuteri* 121 cells may be consumed whereas the free GtfB enzyme is able to use glucose as acceptor substrate (25), resulting in smaller size products. This might influence product synthesis, resulting in the size differences between IMMP and EPS.

Structural analysis of EPS derived from maltodextrins by enzymatic digestion. IMMP produced by the GtfB enzyme from maltodextrin substrates were characterized as soluble fibers (27). They are structurally different from the known starch and maltodextrin molecules as GtfB builds linear consecutive $(1\rightarrow6)$ - α -glucan chains onto the nonreducing end of $(1\rightarrow4)$ - α -glucan chains. Thus, IMMP derived from maltodextrins are mainly composed of

sequences of α -D-Glcp-(1 $\rightarrow$ 6)- α -D-Glcp-(1 $\rightarrow$ 4)-, α -D-Glcp-(1 $\rightarrow$ 6)- α -D-Glcp-(1 $\rightarrow$ 6)-, and α -D-Glcp-(1 $\rightarrow$ 4)- α -D-Glcp-(1 $\rightarrow$ 4)-, as well as some branching α -D-Glcp-(1 $\rightarrow$ 4,6)- α -D-Glcp-(1 $\rightarrow$ 4)- originating from the substrates (Fig. 6B) (27).

To analyze whether the IMMP-like EPS produced by *L. reuteri* 121 cells and cultures are structurally similar to IMMP produced by the GtfB enzyme, the AVEBE MD6 derived EPS were subjected to hydrolysis by several enzymes that are typical for structural analysis of polysaccharides. α -Amylase (Fig. 6B) is an endoacting enzyme cleaving α (1 $\rightarrow$ 4) glycosidic linkages inside starch molecules mainly producing maltose. Dextranase of *Chaetomium erraticum* is a (1 $\rightarrow$ 6)- α -glucan 6-glucanohydrolase that endohydrolyzes (α 1 $\rightarrow$ 6)- α -glucosidic linkages (Fig. 6B) in dextran mainly producing glucose and isomaltose. As shown in Fig. 6A, glucose and maltose were released by α -amylase hydrolysis of EPS, suggesting that this EPS contains α -D-Glcp-(1 $\rightarrow$ 4)- α -D-Glcp-(1 $\rightarrow$ 4)-sequences. Likewise, the presence of α -D-Glcp-(1 $\rightarrow$ 6)- α -D-Glcp-(1 $\rightarrow$ 6)-sequences was also confirmed because dextranase hydrolysis generated glucose and isomaltose.

Pullulanase type I specifically attacks α (1 $\rightarrow$ 6) linkages in both α -D-Glcp-(1 $\rightarrow$ 6)- α -D-Glcp-(1 $\rightarrow$ 4)- sequences (Fig. 6B) in pullulan and α -D-Glcp-(1 $\rightarrow$ 4,6)- α -D-Glcp-(1 $\rightarrow$ 4)- branch points (Fig. 6B) in starch molecules (45). Isoamylase exclusively cleaves the branching α (1 $\rightarrow$ 4,6) linkages (46). AVEBE MD6 derived EPS were hydrolyzed by both pullulanase and isoamylase, forming similar products (Fig. 6A and Fig. S1). These EPS thus possess α -D-Glcp-(1 $\rightarrow$ 4,6)- α -D-Glcp-(1 $\rightarrow$ 4)- branch points which also exist in IMMP (27). Isopullulanase from *Aspergillus niger* exclusively cleaves the α (1 $\rightarrow$ 4) linkages in α -D-Glcp-(1 $\rightarrow$ 6)- α -D-Glcp-(1 $\rightarrow$ 4)- sequences (Fig. 6B) in pullulan but has no activity on starches (47). After treatment of EPS with isopullulanase, oligomers appeared (Fig. 6A), confirming the presence of linear α -D-Glcp-(1 $\rightarrow$ 6)- α -D-Glcp-(1 $\rightarrow$ 4)- sequences. These enzymatic hydrolysis results thus showed that the EPS produced by *L. reuteri* 121 and IMMP produced by GtfB are structurally similar since both contain the same fragments.

Fig. 6 (A) HPAEC analysis of the hydrolysis products of EPS (from AVEBE MD6) following incubation with an excess of different enzymes, including α -amylase, dextranase, pullulanase, isoamylase, and isopullulanase. A combination of glucose (G1), isomaltose (isoG2), isomaltotriose (isoG3), maltose (G2), panose (P), maltotriose (G3), maltotetraose (G4), maltopentaose (G5), maltohexaose (G6), maltoheptaose (G7), and maltooctaose (G8) were used as standard. EPS without enzymatic treatment was applied as negative control. **(B)** Schematic representation of the IMMP products of 4,6- α -GTase (e.g., GtfB) with starch and maltodextrins. GtfB synthesizes (1 $\rightarrow$ 6)- α -glucan chains (with occasionally an internal (1 $\rightarrow$ 4) linkage) onto the nonreducing end of (1 $\rightarrow$ 4)- α -

glucan chains (28). Schematic diagram of the α -amylase, dextranase, pullulanase, isoamylase, and isopullulanase enzymatic cleavage points in IMMP. Amylopullulanase enzymes provide a combination of both α -amylase and pullulanase activities (not shown).

To further investigate the presence of consecutive $\alpha(1\rightarrow6)$ linkages that are typical in IMMP, the EPS derived from AVEBE MD6 was treated with a combination of pullulanase and α -amylase to remove the consecutive $\alpha(1\rightarrow4)$ linkages, and the $\alpha(1\rightarrow6)$ linkages in connecting $-\alpha\text{-D-Glcp-}(1\rightarrow6)\text{-}\alpha\text{-D-Glcp-}(1\rightarrow4)\text{-}$ sequences and $-\alpha\text{-D-Glcp-}(1\rightarrow4,6)\text{-}\alpha\text{-D-Glcp-}(1\rightarrow4)\text{-}$ branches. The hydrolyzed products were fractionated on Bio-Gel P2, yielding nine fractions, denoted F1–F9. F8 and F9 were mainly composed of maltose and glucose (TLC, data not shown) that resulted from consecutive $\alpha 1\rightarrow4$ linkages hydrolyzed by α -amylase. With the increase of DP, the percentage of $\alpha(1\rightarrow6)$ linkages increased (Table S1). In fractions 1 and 2, the polymers were mainly composed of α -glucan with 94% $\alpha(1\rightarrow6)$ linkages. This shows that linear consecutive $\alpha(1\rightarrow6)$ linked fragments inside EPS are present with a broad DP distribution.

Fermentation of *L. reuteri* EPS by probiotic *Bifidobacterium* strains. The EPS produced by *L. reuteri* 121 has a special structure which is similar to IMMP but different from known α -glucans such as starch, dextran, and pullulan. Previously, IMMP were shown to be soluble dietary fibers (27). EPS thus may partially escape digestion in the upper part of the gastrointestinal tract and small intestine, to serve as typical carbon sources for bacterial fermentation in the large intestine. Alternatively, intake of probiotic *L. reuteri* 121 most likely results in formation of EPS rich in $\alpha(1\rightarrow6)$ linkages in the upper gastrointestinal tract by 4,6- α -GTase enzymatic modification of the available (partially hydrolyzed) starch. The *in vivo* synthesized EPS may selectively stimulate the growth of (other) probiotic bacteria in the large intestine.

Bifidobacterium is one of the major genera of bacteria that make up the colon microbiota. Some of them are used as probiotics in the food industry as they may exert a range of beneficial health effects (48, 49). To date, many type II pullulanases (amylopullulanases) with both α -amylase and pullulanase activity have been identified in probiotic *Bifidobacterium* species from the human gut (31, 50, 51). It was also reported that amylopullulanase is the only extracellular polysaccharide hydrolyzing enzyme in lactobacilli (17). Based on the above data (Fig. 6 and Fig. S1), *L. reuteri* 121 EPS derived from maltodextrins can be partially hydrolyzed by both α -amylase and pullulanase. Previously, we reported

that reuteran derived from sucrose can be hydrolyzed by pullulanase (9). Thus, the *Bifidobacterium* strains producing extracellular amylopullulanase have strong potential to degrade both *L. reuteri* 121 EPS derived from maltodextrins, and reuteran, and to use the degradation products as carbon and energy source. Growth of *Bifidobacterium breve* DSM 20091 and *Bifidobacterium dentium* DSM 20436, both containing amylopullulanase activity (50), was studied using EPS of *L. reuteri* 121 as carbon source. Both *B. breve* DSM20091 (Fig. 7B) and *B. dentium* DSM20436 (Fig. 7C) grew well with different EPS. After 12 h of growth, OD₆₀₀ values of *B. breve* DSM20091 all reached 80% with EPS derived from MD20, MD6, or SPG30 (Table 1), and 67.5% with EPS derived from MD4–7, compared to a 100% control grown on glucose. Growth curves of *B. dentium* with EPS followed closely the glucose control reaching all a final OD₆₀₀ value of 2.00 after 8.5 h. The exponential growth phases with the EPS growth substrate did not show any differences from the glucose control, indicating that these EPS are highly suitable as growth substrates. In addition, total carbohydrate measurements and determination of OD₆₀₀ values and pH values (Table 2), as well as TLC detection (Fig. S2), also showed that EPS was partially or completely used by strains.

Fig. 7 Growth of *B. adolescentis* DSM20083 (A), *B. breve* DSM20091 (B), and *B. dentium* DSM20436 (C) on different EPS (5 mg/mL) derived from different maltodextrins. Glucose (5 mg/mL) served as positive control.

Table 2 OD₆₀₀, pH values and total carbohydrate consumption values were determined after growth of *B. breve* DSM 20091 and *B. dentium* DSM 20436 for 24 h with *L. reuteri* 121 EPS derived from different maltodextrins, and reuteran derived from sucrose. Experiments were done in duplicate and the average data are shown.

	<i>Bifidobacterium breve</i> DSM 20091			<i>Bifidobacterium dentium</i> DSM 20436		
	OD ₆₀₀	pH	Total carbohydrate consumption (%)	OD ₆₀₀	pH	Total carbohydrate consumption (%)
Control	0.03	6.8	-	0.03	6.8	-
Glucose	1.68	4.9	100	1.85	5.0	100
EPS Avebe SPG30	1.54	5.2	74	1.68	5.2	84
EPS Avebe MD20	1.46	5.3	81	1.56	5.3	85
EPS Avebe MD6	1.63	5.2	91	1.68	5.1	88
EPS MD 4-7	1.62	5.2	82	1.70	5.1	86
Reuteran	1.56	5.0	84	1.80	5.0	95

As reported, the EPS produced by *L. reuteri* 121 from maltodextrins is hardly degraded by human gut symbiont *Bt. thetaiotaomicron* VPI-5482, which does not express amylopullulanase (<http://www-ncbi-nlm-nih-gov.proxy-ub.rug.nl/genome/>) (52). In this study, *B. adolescentis* DSM 20083 that cannot degrade starch (data not shown) was also investigated, showing that it cannot grow with any of the EPS derived from maltodextrins (Fig. 7A). Therefore, EPS of *L. reuteri* 121 derived from both maltodextrins and sucrose are potential prebiotics which typically stimulate growth of *Bifidobacterium* strains which have amylopullulanase activity.

To conclude, in this study, novel homo-EPS produced by the probiotic bacterium *L. reuteri* strains from starch or maltodextrins as substrates were characterized as IMMP-like EPS. The data show that the cell-associated 4,6- α -glucanotransferase enzyme plays an important role in the formation of such IMMP-like EPS in *L. reuteri* strains. The *L. reuteri* 121 EPS are structurally similar to the IMMP produced by the free GtfB enzyme, with percentages of $\alpha(1\rightarrow6)$ linkages ranging between 11% and 52% (Table 1). However, the *L. reuteri* 121 EPS derived from either maltodextrins or starches are generally much larger than the IMMP produced by the *in vitro* GtfB enzyme. The SEC results also demonstrated that *L. reuteri* 121 is capable of modifying starches by converting both the amylose part and the side chains of amylopectin molecules. Finally, the EPS derived from different maltodextrins were shown to be

excellent growth substrates for some probiotic *Bifidobacterium* strains that have amylopullulanase activity. These EPS thus have potential prebiotic activity and may contribute to the application of probiotic *L. reuteri* strains grown on maltodextrins or starches as synbiotics.

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Supplemental data

Fig. S1 TLC analysis of the enzymatic hydrolysis of EPS (from AVEBE MD6) by different enzymes, α -amylase, L1; dextranase, L2; pullulanase, L3; isoamylase, L4; and isopullulanase, L5; control (EPS), C.

Fig. S2. TLC analysis of the saccharides in the cell-free supernatant before (1–5) and after (1'–5') growth of *Bifidobacterium breve* DSM 20091 and *Bifidobacterium dentium* DSM 20436 incubated with *L. reuteri* 121 EPS derived from different maltodextrins (1, SPG30, 2, MD20, 3, MD6, 4, MD4-7), and reuteran (5) derived from sucrose. BM medium without carbon source and with glucose was spotted as negative control (C) and positive control before (6) and after (6') fermentation.

Table S1 The amylase-pullulanase treated EPS produced from Paselli MD6 was fractionated by Biogel P2 column into 9 fractions (fraction 1 represents the saccharides with highest DP and fraction 9 represents the saccharides with lowest DP). The linkage specificity of each fraction was recorded by ^1H NMR spectroscopy.

Fractions	α-1,4 (%)	α-1,6 (%)
1	6	94
2	6	94
3	11	88
4	20	80
5	51	49
6	69	31
7	94	6
8	98	2
9	100	0

Chapter 6

Summary and Conclusion

Diet is increasingly considered as an important functional aspect for sustaining the human body with the right food ingredients at various life stages. Moreover, the human society is facing a growing number of resistances against antibiotics that served as effective treatments against bacterial infections for the last century. This has resulted in a growing interest in specific oligo- and polysaccharides that beneficially influence human health and decrease the risk of bacterial infections by maintaining a stable gut microbiota composition. These carbohydrates are referred to by the term prebiotics, mostly comprised by fructooligosaccharides (FOS), galactooligosaccharides (GOS) and inulin. Certain bacteria also exert beneficial effects on human health. These bacteria are classified under the term probiotic, 'live microorganisms that, when administered in adequate amounts, confer a health benefit on the host'.

Prebiotics are usually non-digestible oligo- and polysaccharides, i.e. they escape human digestive enzymes and reach distal sites in the human gastrointestinal tract where they become available for the various members of the gut microbiota. Gut bacteria ferment those carbohydrates using various enzymes and transporters to process them and use them as carbon- and energy source in their metabolism. Their bacterial fermentation leads to synthesis and secretion of Short Chain Fatty Acids (SCFAs) that play a crucial role in establishing various health effects through interaction with gut epithelial cells and regulatory functions at distal sites in the human gut and body. Prominent prebiotics are FOS and GOS. FOS comprise fructose monomers that are connected by $\beta(2\rightarrow1)$ glycosidic linkages, rarely also by $\beta(2\rightarrow6)$ linkages. Often they contain a sucrose residue at one terminal site. GOS are formed enzymatically from lactose and comprise galactose units linked through $\beta(1\rightarrow2,3,4,6)$ linkages, often with a terminal glucose residue. Other (candidate) prebiotics are arabinoxylan oligosaccharides (AXOS), isomalto-/malto polysaccharide (IMMP) (originating from starch) or vitamins.

Probiotic bacteria are found among lactic acid bacteria, commonly in the genus *Lactobacillus*, and actinobacteria (genus *Bifidobacterium*), and potential others such as *Faecalibacterium prausnitzii*. These bacteria exert their probiotic function through exclusion of pathogenic bacteria, strengthening of the epithelial barrier, production of anti-pathogenic compounds; production of vitamins, antioxidants, polyphenols and conjugated linoleic acids, modulation of host immune responses and decrease of luminal pH by the production of acids. Bifidobacteria are among the first microorganisms that colonize the infant gut

and thus play a crucial role during maturation of the immune system at early life stage.

Prebiotics may directly stimulate probiotic bacteria in growth and metabolism. This effect is the basis for synbiotics, effective combinations of pre- and probiotics. A number of ‘classical’ microbiological culturing studies investigated the direct effect of prebiotic carbohydrates on growth of probiotics. McLaughlin et al. found that among 32 human-derived isolates from *Bifidobacterium* and *Lactobacillus*, bifidobacteria showed more diverse catabolic capabilities to degrade prebiotic carbohydrate substrates than lactobacilli (1). Lactobacilli and bifidobacteria thus ferment prebiotic oligosaccharides with a wide range in structural composition differentially. This provides a promising basis for the design of synbiotic combinations. In practice, however, *in vivo* tests with synbiotics often didn’t yield the expected synergistic effects. In this PhD thesis, we therefore studied the degradation of (prebiotic) carbohydrates by probiotic bacteria in more detail. We investigated the complete structural composition of various (commercial) prebiotic mixtures using (comparative) HPAEC-PAD, NMR, HPSEC techniques, before and after growth. This was followed by genomic annotation (with prior assembly of bacterial genomes where needed) to identify the likely pathways and Carbohydrate Acting (CAZy) enzymes that probiotic bacteria employ to degrade prebiotic oligosaccharides. This provided a detailed overview of how individual prebiotic molecules may be degraded by probiotic bacteria, essential for design of effective synbiotics.

In **Chapter 1** current knowledge on the ability of probiotic bacteria to ferment prebiotic oligo- and polysaccharides has been reviewed. Among these carbohydrates, FOS and GOS are some of the most studied prebiotics. In *Lactobacillus acidophilus* NCFM a FOS utilization pathway was solved involving an ABC transporter MsmEFGK and two family GH32 enzymes BfrA and GtfA. Other pathways for FOS-utilization were identified in *Lactobacillus plantarum* (involving sucrose phosphoenolpyruvate-dependent phosphotransferase uptake system (PTS) and an intracellular β -fructofuranosidase) and in *Lactobacillus paracasei* 1195 (involving an extracellular β -fructofuranosidase FosE and a fructose PTS transporter FosABCDX). IMMP degradation remained to be studied.

In **Chapter 2** a detailed structural characterization of 9 commercial FOS/inulin products was conducted. These prebiotic products differed in their degree of polymerization between 2 and 60 and the presence of terminal glucose

or fructose residues. Growth of (commercial) probiotic bacteria was studied with short-chain Inulin, comprising still minor amounts of glucose, fructose and sucrose, and with FOS/inulin substrates of both the GF- and FF-type with DP 2–40. Growth varied strongly among the probiotic bacteria tested, yielding often OD_{600nm} values around 40% of positive glucose controls. Analysis of known genes for bacterial FOS/inulin utilization revealed the presence of a sucrose PTS transporter (e.g. *Lactobacillus salivarius*) or a sucrose permease (e.g. *Bifidobacterium longum*) and an extracellular β -fructofuranosidase of family GH32 (*LpGH32*) in combination with a fructose PTS transporter in *L. paracasei* W20. The latter was also observed in *L. paracasei* 1195 and DSM 23505. The extracellular β -fructofuranosidase *LpGH32* acted as *exo*-inulinase cleaving off terminal fructose residues of FOS/inulin chains yielding fructose as major product. Characterization of fructan degradation over time with the recombinantly expressed and purified enzyme *LpGH32* showed that also various intermediate degradation products were formed. For GF-type of fructans, these were sucrose and 1-kestose, while for FF-type of fructans this was F2. Co-cultivation of *L. paracasei* W20 and *L. salivarius* (the latter using a sucrose PTS transporter for FOS uptake) stimulated growth of *L. salivarius* by cross-feeding on GF-type of fructans, but not on FF-type. These experiments demonstrated the differential capabilities of probiotic commercial lactobacilli to utilize individual FOS/inulin compounds present in prebiotic mixtures. *L. paracasei* possesses an extracellular family GH32 enzyme (*exo*-inulinase) that may allow cross-feeding in microbial communities on intermediate fructan degradation products. Occurrence of extracellular glycoside hydrolases is a rare feature among gut related firmicutes (2). The *LpGH32* enzyme enables *L. paracasei* to grow on a broad range of FOS/inulin substrates including those with a high DP (DP20 and higher) that are inaccessible for many other probiotic gut bacteria. *L. paracasei* W20 uses fructose (and glucose) released by *LpGH32*, but cannot use intermediate degradation such as sucrose and 1-kestose, lacking selective transporters. Therefore these products become temporarily available for other bacteria colonizing in proximity to *L. paracasei* W20. The role of *L. paracasei* W20 shows features of a potential keystone species that may play a key role during the entire degradation of prebiotic FOS/inulin chains. This however needs further study, e.g. by using fecal inocula. An example of a proven keystone function is that of *Ruminococcus bromii* acting as such in resistant starch degradation (3). Among lactobacilli this role was assigned only to the strain *L. reuteri* DSM 17938 (4) so far.

The results obtained are of high interest and provide a better basis for designing novel synbiotics, i.e. combinations of pre- and probiotics with increased selectivity of one for the other (5).

In **Chapter 3** we have characterized the family GH53 β -1,4-galactanase of *Bacteroides thetaiotaomicron* in structural and functional detail, and report its involvement in bacterial metabolism of galactooligosaccharides (GOS). These prebiotic compounds are known to stimulate growth of beneficial bifidobacteria (see chapter 4). Lactobacilli also constitute a group of bacteria with relevance for a healthy (infant) gut, but they lack such galactan utilization systems. Previously, the activity of a GH53 enzyme GalA of *Bifidobacterium breve* was studied with various galactan substrates showing that it acts as endo-galactanase releasing galactotriose as major product. We observed involvement of a GH53 enzyme in the metabolism of prebiotic GOS by the human gut symbiont *Bacteroides thetaiotaomicron*. Of its many Polysaccharide Utilization Loci (PUL), growth of *Bt. thetaiotaomicron* on prebiotic GOS clearly correlated with up-regulation of galactan/mucin degradation pathways (6). This PUL encoded a family GH53 galactanase that we previously studied biochemically (6). BTGH53 acted on galactan substrates releasing galactotriose and galactobiose as main products. We studied BTGH53 activity, testing for endo-/exo-activity, and solved its three dimensional structure. BTGH53 acted on the chromogenic substrate *p*NP-galactose releasing 4-nitrophenyl following Michaelis-Menten kinetics. Its three dimensional protein structure showed the organization of a $(\beta\alpha)_8$ TIM barrel. The candidate catalytic residues conserved among all other known GH53 galactanases were D132 (acid/base) and E180 (nucleophile). In comparison with the other known bacterial GH53 galactanase from *Bacillus licheniformis* (BLGAL), BTGH53 lacked loop region 8 (residues 328–378 in BLGAL). In BLGAL, this region forms substrate binding sites –3 and –4 which are therefore lacking in BTGH53. The loop 8 region in BLGAL restricts this enzyme from cleaving GOS smaller than DP 4 (7). With the loop 8 region missing in BTGH53, this enzyme is able to bind to and act upon smaller substrates, such as galactotriose, producing galactobiose and galactose (6). This activity makes BTGH53 much more similar to the fungal GH53 enzyme AAGAL (8). Various lactobacilli were tested for growth with GOS mixtures. In general they were unable to use GOS $\geq$ DP 3. However, when incubated with BTGH53 digested GOS, they grew close to positive controls (glucose). The extracellular BTGH53 enzyme thus facilitates degradation of prebiotic GOS mixtures by lactobacilli. *Bt. thetaiotaomicron* is a dominant inhabitant of the

human colon and probiotic lactobacilli are known to be present in the same compartment, with potential cross-feeding effects *in vivo*. The BTGH53 enzyme may play an important role in carbohydrate metabolism in complex microbial environments such as the human colon. It also may find application in the development of synergistic synbiotics. Our results show that addition of BTGH53 to preparations of probiotic lactobacilli and prebiotic GOS mixtures provides a symbiotic combination: GOS DP 2 products released by BTGH53 from longer GOS DP specifically can be utilized for growth by lactobacilli. Such synbiotic combinations may be prepared either by adding (purified) carbohydrate-active enzymes or by pairing probiotic bacteria that (mutually) benefit from each other by producing complementary enzymes on a particular prebiotic substrate (9).

In **Chapter 4**, selective utilization of individual pGOS components was studied with individual *Bifidobacterium* and lactic acid bacterial (LAB) strains. GOS mixtures are widely used in infant nutrition to stimulate growth of such beneficial gut bacteria. In recent years we have elucidated in detail the composition of several commercial GOS mixtures and reported the structural identity of the individual compounds (10, 11). GOS consist mainly of compounds with a degree of polymerization (DP) varying from 2–8, and with diverse glycosidic linkages. This allowed us to study utilization of GOS components by probiotic bacteria at the level of individual molecules. In this work thirteen (single) probiotic strains of gut bacteria, belonging to 11 different species, were grown to stationary phase with a Vivinal® GOS derived sample purified to remove lactose and monosaccharides (pGOS). Growth among probiotic strains varied strongly between 30–100% of OD600nm relative to positive controls with glucose. By identifying the components of the pGOS mixture that remained after growth, we showed that strains varied in their consumption of specific GOS compounds. This revealed that lactobacilli usually utilized a narrow range of GOS molecules, of DP 2 and DP 3. Bifidobacterial strains tended to use GOS with higher DP and branching than lactobacilli. However, the number of GOS molecules utilized was species/strain dependent. The three *Bifidobacterium lactis* W51–53 strains mostly used DP 2 GOS molecules as observed for lactobacilli. *Bifidobacterium breve* DSM 20091, *Lactobacillus acidophilus* W37 and *Bifidobacterium infantis* DSM 20088 were exceptional in using 38, 36 and 35 compounds, respectively, out of the 40 different structures identified in pGOS. Utilization profiles of GOS molecules correlated with the presence of known genes/gene clusters previously identified

during bacterial GOS utilization. LAB strains mostly encoded genes of the lactose based LacZ and LacLM pathways. These involve lactose permeases and intracellular β -galactosidases (often encoded by lactose operons). Bifidobacteria encoded *lac* operons (LacZ/LacS) as well, and in addition GOS utilization gene clusters involving ABC-type transporters and enzymes of glycoside hydrolase families 42 and 53. An exception was *L. acidophilus* W37 that as only *Lactobacillus* strain also encoded family 42 β -galactosidases. These results provide a more detailed understanding of how GOS structures found in (commercial) prebiotic samples stimulate growth of (commercial) probiotic bacteria at the level of individual strains. The detailed insights may support the design of synbiotic combinations pairing probiotic bacterial strains with GOS compounds that specifically stimulate their growth. Such synbiotic combinations may be of interest in food/feed and/or pharmacy/medicine applications (12).

In **Chapter 5** we studied the IsoMalto-/MaltoPolysaccharides (IMMP) synthesized by *Lactobacillus reuteri* strains from starch and maltodextrins using an extracellular and cell-associated 4,6- α -glucanotransferase (4,6- α -GTase). Lactic acid bacteria synthesize IMMPs as exopolysaccharides (EPS) which are of interest for food applications. Various *L. reuteri* strains possess extracellular 4,6- α -GTase enzymes, e.g. GtfB. Purified GtfB was shown to act on starches (hydrolysates), cleaving $\alpha(1\rightarrow4)$ linkages and synthesizing $\alpha(1\rightarrow6)$ linkages, yielding IMMP. Here we report that also *L. reuteri* cells with these extracellular, cell-associated 4,6- α -GTases synthesize EPS (α -glucan) from starches (hydrolysates). NMR, SEC, and enzymatic hydrolysis of the α -glucans synthesized by *L. reuteri* 121 cells showed that these have similar linkage specificities but generally are much bigger in size than IMMP produced by the purified GtfB enzyme. Various IMMP-like EPS were efficiently used as growth substrates by probiotic *Bifidobacterium* strains that possess extracellular type II amylopullulanase activity. IMMP-like EPS thus have potential prebiotic activity and may contribute to the application of probiotic *L. reuteri* strains grown on maltodextrins or starches as synbiotics (13). They may also serve as prebiotic food source for bacteria in the gut.

In conclusion, this PhD study investigated the structure-function relationships of certain (prebiotic) carbohydrates (GOS, FOS and IMMP) and probiotic bacterial strains. The results obtained provide a more in depth knowledge about the structural composition of prebiotic carbohydrates. At the level of probiotic bacterial strains, multiple (putative) pathways were identified that allow involvement of these strains in prebiotic carbohydrate degradation. In

some cases, these pathways were described in more detail biochemically and cross-feeding was tested. Bacterial FOS/inulin degradation usually involves family GH32 enzymes and, for lactobacilli, transporters of the sucrose and fructose utilization pathways. Bifidobacteria encode ABC-type transporters to utilize FOS/inulin compounds. Family GH1, 2, 42 and 53 enzymes are found encoded in genomes of probiotic bacteria that utilize GOS. These enzymes allow lactose, galactose and galactan utilization. The results thus show how individual (probiotic) members of the human microbiota are involved in degradation of complex prebiotic carbohydrates. At the industrial level, these results may stimulate development of synbiotic combinations by adding prebiotics with greater selectivity to probiotic mixtures.

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Samenvatting en Vooruitzichten

De samenstelling van ons dieet met de juiste voedingsstoffen is van groot belang voor het onderhoud van ons lichaam; tevens dient het dieet afgestemd te worden op de verschillende levensfasen. Over de jaren is het inzicht verkregen dat de koolhydraatsamenstelling van ons dieet hierbij een belangrijke rol speelt. Onze samenleving is in de afgelopen jaren ook geconfronteerd met een groeiend aantal microbiële antibioticaresistenties waardoor specifieke bacteriële infecties minder effectief bestreden kunnen worden. Deze twee ontwikkelingen hebben geresulteerd in een groeiende belangstelling voor specifieke oligo- en polysachariden die positieve effecten hebben op de menselijke gezondheid. Zij verlagen het risico op bacteriële infecties doordat ze bijdragen aan het handhaven van een goede en stabiele samenstelling van de populatie aan micro-organismen in ons darmkanaal (het darmmicrobioom). Deze zogenaamde prebiotica bestaan vooral uit fructo-oligosachariden (FOS), galacto-oligosachariden (GOS) en inuline. Specifieke bacteriën hebben eveneens positieve effecten op de menselijke gezondheid. Deze bacteriën worden ook wel probiotica genoemd, 'levende micro-organismen die, wanneer toegediend in de juiste hoeveelheden, een positief gezondheidseffect hebben op de gastheer'.

Prebiotica zijn gewoonlijk niet-verteerbare oligo- en polysachariden: zij worden niet afgebroken door menselijke enzymen en bereiken de verderop gelegen delen van het darmkanaal (dikke darm, endeldarm) waar ze worden afgebroken door darmbacteriën. Met behulp van verschillende enzymen en transportsystemen fermenteren de bacteriën deze koolhydraten en gebruiken ze aldus als koolstof- en energiebron voor de groei. Deze bacteriële afbraak resulteert in de synthese en uitscheiding van kortketenige vetzuren die een cruciale rol spelen in verschillende gezondheidseffecten door interacties met epitheelcellen in de menselijke darm. FOS bestaan uit fructose-monomeren die met elkaar verbonden zijn door $\beta(2\rightarrow1)$ -glycosidische bindingen en in mindere mate door $\beta(2\rightarrow6)$ -bindingen. Ze bevatten vaak een sucrose-molecuul aan hun uiteinde. GOS worden enzymatisch gesynthetiseerd uit lactose en bestaan uit galactose-eenheden die met elkaar verbonden zijn door $\beta(1\rightarrow2,3,4,6)$ -bindingen, vaak met een glucose-molecuul aan hun uiteinde. Diverse andere (potentiële) prebiotica worden momenteel bestudeerd, zoals arabinoxylaan-oligosachariden (AXOS), isomalto-/malto-polysachariden (IMMP) (uit zetmeel) en vitamines.

Bekende probiotische bacteriestammen zijn vooral melkzuurbacteriën van het geslacht *Lactobacillus* en actinobacteriën van het geslacht *Bifidobacterium*, maar potentieel ook verschillende andere bacteriën zoals *Faecalibacterium prausnitzii*. Deze bacteriën hebben een probiotisch effect door het buitensluiten

van pathogene bacteriën of het versterken van de epitheel-barrière; door de productie van anti-pathogene verbindingen; door de productie van vitamines, antioxidanten, polyfenolen of geconjugeerde linolzuren; door de modulering van de immuunreactie van de gastheer; en/of door de daling van de pH in het lumen door de productie van zuren. Bifidobacteriën zijn dominant aanwezig onder de eerste micro-organismen die het darmkanaal van pasgeboren baby's koloniseren en spelen een cruciale rol in de ontwikkeling van het immuunsysteem in de vroege levensfase.

Prebiotica kunnen probiotische bacteriën stimuleren in hun groei en stofwisseling. Dit stimulerende effect vormt de basis voor zogenaamde synbiotica: effectieve combinaties van pre- en probiotica die van belang zijn voor voeding en farmacie. De directe effecten van prebiotische koolhydraten op de groei van probiotica kunnen met klassieke microbiologische kweektechnieken bestudeerd worden. McLaughlin et al. onderzocht 32 uit de mens geïsoleerde *Bifidobacterium*- en *Lactobacillus*-stammen. Deze Bifidobacteriën en Lactobacilli bleken duidelijk te verschillen in hun vermogen om prebiotische oligosachariden te fermenteren. De geteste Bifidobacteriën beschikten over een grotere afbraakcapaciteit voor prebiotische koolhydraten, ook met verschillende structuren, dan de Lactobacilli (1). Deze verschillen maken het mogelijk om een grotere variatie aan synbiotische combinaties te ontwikkelen.

In de praktijk lieten *in-vivo*-tests met synbiotica echter vaak niet de verwachte synergistische effecten zien. In dit proefschrift is daarom de afbraak van (prebiotische) koolhydraten door probiotische bacteriën in meer detail bestudeerd. Hierbij werden de detailstructuren van de verschillende (commerciële) prebiotische mengsels, voor en na de groei van deze bacteriën, geanalyseerd met HPAEC-PAD-, NMR-, HPSEC-technieken. Vervolgens werden de beschikbare genoomsequenties onderzocht op mogelijke routes en koolhydraat-actieve (CAZy) enzymen die deze probiotische bacteriën gebruiken om prebiotische oligosachariden af te breken. Deze benaderingen gaven een gedetailleerd inzicht in de wijze waarop de individuele prebiotische moleculen wel of niet afgebroken kunnen worden door de verschillende probiotische bacteriën. Dit is essentiële informatie voor de ontwikkeling van effectieve synbiotica.

Hoofdstuk 1 geeft een overzicht van de huidige kennis over probiotische bacteriën en hun vermogen om prebiotische oligo- en polysachariden te fermenteren. FOS en GOS behoren tot de meest bestudeerde prebiotische

koolhydraten. De FOS-afbraakroute in *Lactobacillus acidophilus* NCFM bestaat uit een ABC-transporter MsmEFGK en twee familie GH32-enzymen BfrA en GtfA. Andere FOS-afbraakroutes zijn opgehelderd in *Lactobacillus plantarum* (met een sucrose-fosfoenolpyruvaat-afhankelijk fosfotransferase-opnamesysteem (PTS) en een intracellulair β -fructofuranosidase enzym) en in *Lactobacillus paracasei* 1195 (met een extracellulair β -fructofuranosidase FosE-enzym en een fructose-PTS-transporter FosABCDX). IMMP-afbraakroutes waren nog onbekend.

Hoofdstuk 2 rapporteert onze gedetailleerde karakterisering van de structuren van 9 commerciële FOS/inuline-producten. Deze prebiotica verschillen in hun ketenlengte (tussen de 2 en 60 fructose-eenheden, DP 2-60) en de identiteit van de aanwezige terminale residuen (glucose of fructose). Vervolgens werd de groei van (commerciële) probiotische bacteriën met kort-ketenig inuline bestudeerd. Ook werd de groei bestudeerd met FOS/inuline-substraten (zowel GF- als FF-type) met een ketenlengte tussen de 2 en 40 fructose-eenheden. De geteste probiotische bacteriën varieerden sterk in hun groei, vaak ook met OD600nm-eindwaarden die ongeveer 40% waren van die van de positieve controles met glucose. De genomen van deze bacteriën zijn vervolgens geanalyseerd op genen met een bekende functie bij bacterieel FOS/inuline-gebruik. Dit leidde tot de identificatie van een sucrose-PTS-transporter (bijvoorbeeld in *Lactobacillus salivarius*) of een sucrose-permease (bijvoorbeeld in *Bifidobacterium longum*) en een extracellulair β -fructofuranosidase-enzym van familie GH32 (*LpGH32*) in combinatie met een fructose-PTS-transporter in *L. paracasei* W20. Dit laatste werd ook waargenomen in *L. paracasei* 1195 en DSM 23505. De extracellulaire β -fructofuranosidase *LpGH32* had *exo*-inulinase-activiteit en knipte terminale fructose-eenheden van de FOS/inuline-ketens af waardoor fructose als belangrijkste product vrijkwam. Gebruikmakend van recombinant-DNA-technieken is dit *LpGH32*-enzym tot expressie gebracht en opgezuiverd. Incubatie van dit *LpGH32*-enzym met fructaan-substraten liet zien dat ook diverse intermediaire producten gevormd werden. Dit waren sucrose en 1-kestose uit GF-type fructanen, en F2 uit FF-type-fructanen. In mengculturen met zowel *L. paracasei* W20 als *L. salivarius* (laatstgenoemde gebruikt een sucrose-PTS-transporter voor FOS-opname) werd de groei van *L. salivarius* met GF-type-fructanen gestimuleerd door ‘cross-feeding’; dit werd niet waargenomen met FF-type-fructanen. Deze experimenten demonstreerden dat probiotische commerciële Lactobacilli onderling verschillen in hun vermogen om individuele

FOS/inuline-verbindingen die aanwezig zijn in prebiotische mengsels te gebruiken voor hun groei. *L. paracasei* beschikt over een extracellulair familie GH32-enzym (*exo*-inulinase) dat verantwoordelijk is voor ‘cross-feeding’ in microbiële gemeenschappen bij groei op intermediaire fructaan-afbraakproducten. Extracellulaire glycoside hydrolase-enzymen komen weinig voor in darmbacteriën behorende tot de Firmicutes (2). Het *LpGH32*-enzym maakt het mogelijk voor *L. paracasei* om te groeien op een brede keuze aan FOS/inuline-substraten, waaronder die met langere ketens (20 fructose-eenheden en langer) die niet kunnen worden afgebroken door andere probiotische darmbacteriën. *L. paracasei* W20 groeit op de fructose (en glucose) die afgeknipt wordt door *LpGH32*, maar kan niet groeien op intermediaire producten zoals sucrose en 1-kestose vanwege het ontbreken van specifieke transporters. Daardoor komen deze intermediaire producten tijdelijk beschikbaar voor andere bacteriën die aanwezig zijn in gemeenschappen met *L. paracasei* W20. *In vivo* zou *L. paracasei* W20 dus wel eens een sleutelrol kunnen spelen bij de totale afbraak van prebiotische FOS/inuline-ketens. Dit moet echter nog nader bestudeerd worden, bijvoorbeeld door gebruik te maken van mengsels van fecale bacteriën. *Ruminococcus bromii* heeft een vergelijkbare sleutelrol bij de afbraak van zogenaamd ‘resistant starch’ (3). Onder Lactobacilli is een dergelijke sleutelrol alleen toegekend aan stam *L. reuteri* DSM 17938 (4).

Deze resultaten zijn niet alleen wetenschappelijk interessant, maar vormen ook een goede basis voor het ontwikkelen van nieuwe synbiotica: met deze nieuwe kennis wordt het beter mogelijk om op rationele wijze combinaties te maken van pre- en probiotica met hoge selectiviteit voor elkaar (5).

Hoofdstuk 3 beschrijft de functionele karakterisering van de familie GH53- β -1,4-galactanase van *Bacteroides thetaiotaomicron*, de 3D-structuur van dit eiwit, en de rol van dit enzym in de afbraak van galacto-oligosachariden (GOS). Deze prebiotische verbindingen stimuleren de groei van probiotische Bifidobacteria (zie ook hoofdstuk 4). Lactobacilli hebben ook een positieve rol bij de ontwikkeling en handhaving van een gezonde darmomgeving (bij baby’s), maar zij beschikken niet over dit galactaan-(polygalactose)-hydrolyserende enzym. De activiteit van het GH53-enzym GalA van *Bifidobacterium breve* met verschillende galactaan substraten is al eerder bestudeerd. Hierbij bleek dat dit enzym werkt als een endo-galactanase met galactotriose als belangrijkste product. Een andere darmbacterie, *Bacteroides thetaiotaomicron*, beschikt eveneens over een GH53 enzym en dit blijkt een belangrijke rol te spelen bij de afbraak van prebiotische GOS. Het genoom van *Bt. thetaiotaomicron* bevat vele

zogenaamde ‘Polysaccharide Utilization Loci’ (PUL) en groei van deze bacterie op prebiotische GOS resulteerde in duidelijk verhoogde expressie van specifiek de galactaan/mucine-afbraakroutes (6). De betrokken PUL codeert voor een familie GH53 galactanase (BTGH53) die we eerder al biochemisch gekarakteriseerd hadden (6). BTGH53 was actief met galactaan-substraten en maakte galactotriose en galactobiose als belangrijkste producten. Tevens hebben we de BTGH53 activiteit gemeten, getest of er sprake was van endo- of exo-activiteit, en de 3D-structuur van het eiwit opgehelderd. BTGH53 was actief met het chromogene substrate *p*NP-galactose, splitste hier 4-nitrophenyl vanaf en volgde hierbij Michaelis-Menten-kinetiek. Uit de 3D-structuuranalyse bleek dat het eiwit georganiseerd is als een zogenaamde $(\beta/\alpha)_8$ TIM-barrel-structuur. De mogelijke katalytische residuen, geconserveerd in alle andere bekende GH53 galactanasen, werden geïdentificeerd als D132 (zuur/base) en E180 (nucleofiel). Vergeleken met de andere bekende bacteriële GH53-galactanase, van *Bacillus licheniformis* (BLGAL), ontbrak in BTGH53 de zogenaamde ‘loop region’ 8 (aminozuurresiduen 328-378 in BLGAL). In BLGAL vormt deze ‘loop region’ de substraat-bindingsplaatsen -3 en -4, die dus ook ontbreken in BTGH53. In BLGAL beperkt de aanwezigheid van deze ‘loop region’ het enzym in zijn mogelijkheden om GOS kleiner dan DP 4 te hydrolyseren (7). BTGH53 daarentegen kan kleinere substraten binden en hydrolyseren, bijvoorbeeld galactotriose dat wordt omgezet in galactobiose en galactose (6). Deze activiteit maakt dat BTGH53 veel meer lijkt op het schimmel-GH53-enzym AAGAL (8). We hebben verschillende Lactobacilli getest op hun vermogen om te groeien op GOS-mengsels. In het algemeen waren ze niet in staat om GOS met een ketenlengte van 3 galactose-eenheden of langer te gebruiken. Na hydrolyse van GOS mengsels door BTGH53 groeiden Lactobacilli echter tot een vergelijkbare celdichtheid als de positieve controle met glucose. De activiteit van dit extracellulaire BTGH53-enzym faciliteert dus de afbraak van prebiotische GOS-mengsels door Lactobacilli. *Bt. thetaiotaomicron* is een dominante bewoner van de menselijke endeldarm, samen met probiotische Lactobacilli, zodat ‘cross-feeding’ potentieel zou kunnen plaatsvinden. Het betreffende BTGH53-enzym zou dus een belangrijke rol kunnen spelen in koolhydraatafbraak in complexe microbiële milieus zoals de menselijke endeldarm. Dit enzym zou ook gebruikt kunnen worden voor de ontwikkeling van synbiotica. Onze resultaten laten zien dat BTGH53 met probiotische Lactobacilli en prebiotische GOS-mengsels in feite een effectieve synbiotische combinatie vormt: DP 2 GOS-producten die vrijkomen door hydrolyse van langere GOS-moleculen door BTGH53 kunnen

door *Lactobacilli* gebruikt worden voor de groei. Dergelijke synbiotische combinaties kunnen bereid worden door de toevoeging van (gezuiverde) koolhydraat-actieve enzymen aan een bacteriekweek, of door mengsels van probiotische bacteriën te gebruiken die kunnen profiteren van elkaars enzymactiviteiten op specifieke prebiotische substraten (9).

In **hoofdstuk 4** hebben we het selectieve gebruik van specifieke GOS-moleculen door reïnculturen van *Bifidobacteria* en melkzuurbacteriën bestudeerd. GOS-mengsels worden veel gebruikt in baby- en kleutervoeding om de groei van deze probiotische bacteriën te stimuleren. In de afgelopen jaren hebben we de samenstelling van verschillende commerciële GOS-mengsels opgehelderd en de gedetailleerde structuren van de individuele GOS-moleculen beschreven (10, 11). GOS-mengsels bevatten moleculen met een polymerisatiegraad van 2-8 met verschillende glycosidische bindingen. Gewapend met deze kennis konden we het gebruik van individuele GOS-moleculen door probiotische bacteriën analyseren. Hiertoe werden 13 reïnculturen van probiotische darmbacteriën, behorende tot 11 verschillende soorten, gekweekt tot in de stationaire fase op een Vivinal® GOS-mengsel, gezuiverd van lactose, galactose en glucose (pGOS). De groei van deze probiotische stammen op pGOS varieerde sterk, met eindwaarden tussen de 30-100% van de OD600nm-niveaus die bereikt werden op de positieve controles met glucose. Door de identificatie van de GOS-moleculen die overbleven na groei op het mengsel konden we aantonen dat deze bacteriestammen sterk verschilden in hun vermogen om specifieke GOS-moleculen te consumeren. Vrijwel alle *Lactobacilli* gebruikten slechts een beperkte serie GOS-moleculen, van DP 2 en DP 3. *Bifidobacteria* gebruikten over het algemeen GOS-moleculen met een grotere ketenlengte en ook wel vertakte GOS. Het aantal GOS-moleculen dat gebruikt werd varieerde echter met de geteste bacteriesoort/-stam. De 3 *Bifidobacterium lactis* W51-53-stammen gebruikten vooral DP 2 GOS-moleculen, zoals ook waargenomen werd voor *Lactobacilli*. *Bifidobacterium breve* DSM 20091, *Lactobacillus acidophilus* W37 en *Bifidobacterium infantis* DSM 20088 bleken een uitzonderlijk sterk vermogen te hebben tot GOS-gebruik: respectievelijk maar liefst 38, 36 en 35 verschillende moleculen werden gebruikt van de 40 structuren die door ons in pGOS geïdentificeerd zijn. Deze profielen van GOS-consumptie correleerden goed met de aanwezigheid van bekende genen/genclusters die eerder geïdentificeerd zijn als betrokken bij bacterieel GOS-gebruik. De genomen van de meeste melkzuurbacteriën bevatten genen van de LacZ- en LacLM-routes voor lactose

gebruik. Deze routes gebruiken lactose-permeases en intracellulaire β -galactosidases (vaak gecodeerd in lactose-operonen). *Bifidobacteria*-genomen bevatten ook *lac*-operonen (*LacZ/LacS*), maar daarnaast ook genclusters voor GOS-afbraak met ABC-type-transporters en enzymen van glycoside hydrolase families 42 en 53. *L. acidophilus* W37 was uitzonderlijk als de enige *Lactobacillus*-stam die ook een familie 42- β -galactosidase bevatte. Deze resultaten laten in detail zien welke GOS-structuren in (commerciële) prebiotische mengsels de groei stimuleren van reinculturen van (commerciële) probiotische bacteriën. Op basis van deze informatie kunnen synbiotische combinaties van probiotische bacteriestammen met specifieke GOS-mengsels gemaakt worden die de groei stimuleren. Dergelijke synbiotica zijn potentieel interessant in voedingsmiddelen en voor toepassingen in de farmaceutische industrie (12).

In **hoofdstuk 5** hebben we de IsoMalto-/MaltoPolysacharides (IMMP) gekarakteriseerd die uit zetmeel en maltodextrines gesynthetiseerd worden door *Lactobacillus reuteri* stammen die beschikken over een extracellulair, aan de celwand gehecht 4,6- α -glucanotransferase-enzym (4,6- α -GTase). *Lactobacilli* synthetiseren IMMPs, zoals exopolysachariden (EPS) die interessant zijn voor toepassingen in voedingsmiddelen. Vooral *L. reuteri* stammen hebben een dergelijk extracellulair 4,6- α -GTase-enzym, gedefinieerd als GtfB. Gezuiverd GtfB is actief met zetmeel(-hydrolysaten), knipt $\alpha(1\rightarrow4)$ -bindingen en synthetiseert $\alpha(1\rightarrow6)$ -bindingen, resulterend in IMMP. In dit hoofdstuk laten we zien dat ook *L. reuteri*-cellen met deze extracellulaire, aan de celwand gehechte 4,6- α -GTasen in staat zijn om EPS (α -glucan) te synthetiseren uit zetmeel(-hydrolysaten). NMR, SEC en enzymatische hydrolyse van de α -glucanen gesynthetiseerd door *L. reuteri* 121-cellen toonde aan dat deze dezelfde bindingstypes hebben maar over het algemeen veel grotere afmetingen dan de IMMP geproduceerd met een gezuiverd GtfB-enzym. Verschillende soorten IMMP-achtige EPS werden efficiënt gebruikt als groeisubstraat door probiotische *Bifidobacterium* stammen die beschikken over extracellulaire type II-amylopullulanase-enzymen. IMMP-achtige EPS zijn dus potentiële prebiotica en na groei van probiotische *L. reuteri* stammen op maltodextrines of zetmelen zou dit mengsel dus als synbiotica toegepast kunnen worden (13).

In dit proefschrift zijn de structuur-functie-relaties van specifieke (prebiotische) koolhydraten (GOS, FOS en IMMP), evenals de groei en het metabolisme van probiotische bacteriën bestudeerd. De resultaten geven in de eerste plaats meer inzicht in de precieze structurele samenstelling van deze

prebiotische koolhydraten. Tevens werden meerdere (potentiële) metabole routes voor de afbraak van deze prebiotica opgehelderd in de individuele probiotische bacteriestammen. In een paar voorbeelden konden deze routes in meer biochemisch detail worden beschreven en kon ‘cross-feeding’ worden waargenomen. Familie GH32 enzymen zijn gewoonlijk betrokken bij de bacteriële FOS/inuline-afbraak en transporters van de sucrose- en fructose-afbraakroutes in *Lactobacilli*. Bifidobacteriën maken gebruik van ABC-type-transporters bij de afbraak van FOS/inuline-verbindingen. Genomen van probiotische bacteriën die GOS metaboliseren coderen voor familie GH1-, 2-, 42- en 53-enzymen waarvan bekend is dat ze lactose, galactose en galactaan kunnen afbreken. Samenvattend laten deze resultaten zien hoe individuele (probiotische) menselijke darmbacteriën betrokken zijn bij de afbraak van complexe prebiotische koolhydraten. Op basis van deze kennis en inzichten kunnen nieuwe, of betere en meer selectieve, synbiotica ontwikkeld worden.

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Appendix

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Markus Böger
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About the author

Markus Böger was born in the south of Germany, close to Nürnberg, the latter known as the second biggest city in Bavaria and famous for its medieval architecture as well as for some important inventions such as Germany's first operating railway track opened 1835. During his high school period at the Gymnasium Roth, he started to focus on research topics and carried out his first work dealing with the influence of Sodium Chloride, brought every year during winter periods in huge amounts onto roads, on survival and fitness of various wildflower seeds. Participating in the competition 'jugend forscht', this work was awarded a first place at its regional competition in 2006. After high school, Markus moved to Würzburg for his undergraduate studies in Biology at Würzburg's University. Completing this period in 2009, he continued his studies focusing on biochemistry, cell biology and organic chemistry at the University of Würzburg. In 2009, he also joined the newly established research group of Prof. Jürgen Seibel to start working on carbohydrate synthesis during an internship. In this work a bacterial enzyme was used to produce new sucrose-analogues with interesting properties such as a low glycemic index. Inspired by their broad structural diversity, Markus decided to continue his work in the carbohydrate field and therefore went abroad in 2010 to join the lab of Lubbert Dijkhuizen at the University of Groningen, NL. In this work, he focused on an enzyme from a probiotic *Lactobacillus* strain synthesizing prebiotic fructooligosaccharides. Collecting a robust data set, he graduated with this work at the University of Würzburg achieving his 'Diplom' (Master of Science) in 2013. The same year he joined the lab of Lubbert Dijkhuizen to start working on his PhD project. Using previous knowledge about carbohydrate structure, he focused on degradation of (prebiotic) carbohydrates by selected probiotic members of the human gut microbiota. Early 2020, he will be defending his work at the University of Groningen.

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