DOI 10.1002/mnfr.200700121 Mol. Nutr. Food Res. 2008, *52*, 146–163

Review

Bifidobacterium carbohydrases-their role in breakdown and synthesis of (potential) prebiotics

Lambertus A. M. van den Broek, Sandra W. A. Hinz, Gerrit Beldman, Jean-Paul Vincken and Alphons G. J. Voragen

Laboratory of Food Chemistry, Wageningen University, Wageningen, The Netherlands

There is an increasing interest to positively influence the human intestinal microbiota through the diet by the use of prebiotics and/or probiotics. It is anticipated that this will balance the microbial composition in the gastrointestinal tract in favor of health promoting genera such as *Bifidobacterium* and *Lactobacillus*. Carbohydrates like non-digestible oligosaccharides are potential prebiotics. To understand how these bacteria can grow on these carbon sources, knowledge of the carbohydrate-modifying enzymes is needed. Little is known about the carbohydrate-modifying enzymes of bifidobacteria. The genome sequence of *Bifidobacterium adolescentis* and *Bifidobacterium longum* biotype *longum* has been completed and it was observed that for *B. longum* biotype *longum* more than 8% of the annotated genes were involved in carbohydrate metabolism. In addition more sequence data of individual carbohydrases from other *Bifidobacterium* spp. became available. Besides the degradation of (potential) prebiotics by bifidobacterial glycoside hydrolases, we will focus in this review on the possibilities to produce new classes of non-digestible oligosaccharides by showing the presence and (transglycosylation) activity of the most important carbohydrate modifying enzymes in bifidobacteria. Approaches to use and improve carbohydrate-modifying enzymes in prebiotic design will be discussed.

Keywords: Bifidobacteria / Glycoside hydrolases / Prebiotic / Retaining enzymes / Transglycosylation

Received: March 25, 2007; revised: June 20, 2007; accepted: June 25, 2007

1 Introduction

Bifidobacteria are gram-positive, anaerobic, non-spore-forming, and non-motile bacteria and they are often Y- or V-shaped rods [1]. Tissier [2] reported in 1900 the isolation of presumably the first *Bifidobacterium* from the intestine of a child, and named it *Bacillus bifidus communis*. The genus *Bifidobacterium* was already recognized by Orla Jensen as a separate taxon in 1924, but it took 50 years before the genus *Bifidobacterium* was for the first time classified in the Bergey's Manual of Determinative Bacteriology [3]. Presently, 34 species (some different biotypes and subspecies) have been described [4–9]. The major habitat is considered to be the intestine of humans and animals [4] and twelve species have been associated with humans as host. Bifidobacteria rapidly colonize the digestive tract of newly born infants but the number of known *Bifidobacterium* spp. gradually decreases with age while the relative composition of certain bifidobacterial species also changes during ageing (*e.g. B. bifidum*, *B. catenulatum*, *B. pseudocatenulatum* and *B. longum* biotype *infantis* are not found in elderly people) [10–12]. The bifidobacterial content represents 4.4 ± 4.3% of the total fecal microbiota of adults in Northern Europe [13].

Carbohydrates play an important role in the gastrointestinal tract (GIT) of humans and besides their direct physiological effect they also affect the gut ecosystem, which significantly contributes to the well-being of humans [14]. Bifidobacteria are one of the major groups of bacteria in the GIT and it is claimed that they have several health-promoting effects [15–17] such as the prevention of diarrhea [18], reduction of cholesterol level [19], immunostimulation [20], anticarcinogenicity [21–23], improved mineral

Correspondence: Professor Alphons G. J. Voragen, Laboratory of Food Chemistry, Wageningen University, P.O. Box 8129, 6700 EV Wageningen, The Netherlands
E-mail: Fons.Voragen@wur.nl
Fax: +31-317-484-893

Abbreviations: **AXH,** arabinoxylan arabinofuranohydrolases; **CBM,** carbohydrate binding module; **FOS,** fructo-oligosaccharides; **GIT**, gastrointestinal tract; **GH family**, glycoside hydrolase family; **NDOs,** non-digestible oligosaccharides ;**PI,** prebiotic index; **SignP**, signal peptide; **TmD**, transmembrane domain; **TOS**, transgalacto-oligosaccharides absorption [24], and production of vitamins [25]. In order to increase the amount of bifidobacteria in the GIT the concepts of probiotics and prebiotics have been developed. To positively influence the microbiota in the GIT probiotics and/or prebiotics can be applied in the diet as a functional food. The definition of a probiotic is ‚a preparation of or a product containing viable, defined microorganisms in sufficient numbers, which alter the microflora (by implantation or colonization) in a compartment of the host, and by that, exert beneficial health effects in this host' [26]. Bacteria used as probiotic are mainly from the genera *Bifidobacterium* or *Lactobacillus* [27, 28]. A prebiotic can be defined as ‚a non-digestible 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 that have the potential to improve host health' [16]. The combination of probiotics and prebiotics is termed synbiotic. The definition is ‚a mixture of probiotics and prebiotics that beneficially affects the host by improving the survival and implantation of live microbial dietary supplements in the gastrointestinal tract' [16].

This review will focus on the carbohydrate breakdown capacity of bifidobacteria and the utilization and synthesis of prebiotics. Glycosidases play an important role in the breakdown of carbohydrate-based prebiotics to fermentable sugars. In addition, some glycosidases can be very useful to synthesize non-digestible oligosaccharides (NDOs) by their transglycosylation activity *in vitro* [29].

2 Induction of enzymes by carbohydrates

Bifidobacteria play an important role in carbohydrate fermentation in the colon. Most of the oligo- and polysaccharides will ultimately be degraded to monosaccharides and these will be converted to intermediates of the hexose fermentation pathway, also called fructose-6-phosphate shunt or bifid shunt. Subsequently, they will be converted to short chain fatty acids and other organic compounds [30, 31]. In general, gut bacteria degrade polysaccharides to low molecular weight oligosaccharides, which can subsequently be degraded to monosaccharides by the use of a wide range of depolymerizing enzymes. These glycosidases are found extracellularly, associated with the bacterial envelope, or intracellularly.

Prebiotics can cause a change in microbial enzyme activity. For example: the activity of β-galactosidase and α-arabinopyranosidase of *B. longum* biotype *longum* is increased, when growth takes place on larch wood arabinogalactan [32], which could be a potential prebiotic. In addition, Salyers *et al.* [33] and Crociani *et al.* [34] found that in *Bifidobacterium* spp. mainly the *B. longum* biotype *longum* strains were able to grow on arabinogalactan. Growth of *B. adolescentis* on xylose and arabinoxylan-derived oligosaccharides (potential prebiotics) induced the production of two arabinofuranohydrolases [35]. Also *B. longum* biotype *longum* produced arabinofuranosidases during growth on arabinoxylan [36]. So far, *B. longum* biotype *longum* and *B. adolescentis* seem to be the only *Bifidobacterium* spp. with the ability to grow on arabinoxylan [36]. Another example of an enzyme induced by disaccharides and/or oligosaccharides is the sucrose phosphorylase from *B. animalis* subsp. *lactis* and *B. longum* biotype *longum*. It was found that sucrase activity of *B. animalis* subsp *lactis* was induced in the presence of sucrose, raffinose, and in small amounts by oligofructose [37]. Gene expression of sucrose phosphorylase from *B. longum* biotype *longum* was observed in the presence of sucrose and raffinose [38].

The induction of enzymes, which are involved in the degradation of carbohydrates, can be repressed by the presence of glucose [37 – 39]. This repression of enzyme synthesis is a way of bacteria to control the oligo- and polysaccharide metabolism. When a preferred carbon source is present, there will be no unnecessary production of large amounts of enzyme [32].

3 Classification of carbohydrases

3.1 Retaining and inverting glycosidases.

Enzymatic hydrolysis of glycosidic bonds in oligo- and polysaccharides is carried out with one of two stereo chemical outcomes: net retention or net inversion of the anomeric configuration. Therefore, glycosidases are classified as either retaining or inverting as first proposed by Koshland [40]. Retaining glycoside hydrolases have a double displacement (S_N1) mechanism [41] involving a glycosyl-enzyme intermediate. The retaining enzyme has two carboxylic acids in the catalytic center and one will act first as an acid catalyst and protonates the glycosidic oxygen, while the other carboxylic acid acts as nucleophile and assists departure of the leaving group (Fig. 1(A)). Subsequently, the first carboxylic acid will behave as a base catalyst and activate the incoming nucleophile (water), resulting in the hydrolysis of the glycosyl-enzyme intermediate (deglycosylation step). The product formed has the same stereochemistry as the substrate. When instead of water the incoming nucleophile is a sugar molecule this can lead to the formation of oligosaccharides with a higher degree of polymerization or containing a new linkage type. Such reactions are called transglycosylation [42 – 44].

Inverting glycosyl hydrolases have a single displacement (S_N2) mechanism [41] and have different carboxyl acids acting as acid and base (Fig. 1(B)). In this case the protonation of the glycosidic oxygen and departure of the leaving group are accompanied by a concomitant attack of a water molecule, which is activated by the carboxylic base catalyst. The product has the opposite stereochemistry as the substrate. Inverting enzymes do not have the capability to synthesize oligosaccharides [42 – 44].

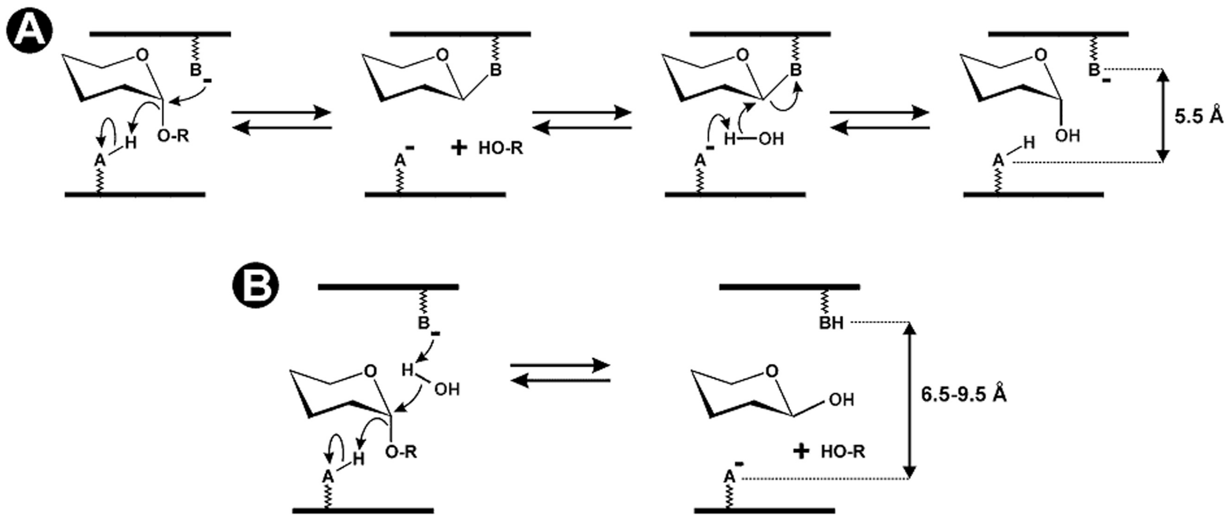

Figure 1. Mechanism of retaining (A) and inverting (B) glycoside hydrolases. Details are explained in section Retaining and inverting glycosidases.

3.2 Glycoside hydrolase (GH) family classification

In 1991 Henrissat introduced a classification of glycoside hydrolases based on their amino acid sequence similarities. Glycoside hydrolases with a high degree of sequence homology were assigned to the same glycoside hydrolase family (GH family) [45]. This classification has predictive value with respect to (i) the structural features (fold) of the enzymes, (ii) the evolutionary relationship between the enzymes, and (iii) catalytic mechanism [45, 46]. This classification system is complementary to the International Union of Biochemistry enzyme nomenclature (EC numbers), which is based on substrate specificity of the enzyme [47]. The database is regularly updated [46, 48] and available on the internet (http://www.cazy.org). Currently, more than 100 GH families are known.

Enzymes in one GH family can have different substrate specificity but also a different mode of action (endo or exo). If an enzyme acts randomly on a polymeric substrate it has an endo mode of action. Exo-enzymes split off terminally linked carbohydrate units. However, it appears that within a GH family the catalytic mechanism, retaining or inverting, is conserved [49]. The catalytic residues are also conserved within a GH family [48] as well as the protein fold [42, 50]. Although only limited information is available about the 3D structures of enzymes from bifidobacteria [51–53], the 3D structure of more than 50 GH families is known, and these might be used for predicting structural features of carbohydrases from bifidobacteria.

4 *Bifidobacterium* carbohydrases

Japanese researchers have performed most of the early work (1980–1987) on isolation and characterization of bifidobacterial enzymes [54–58]. An overview of isolated and characterized carbohydrate modifying enzymes is shown in Table 1. All the enzymes were isolated from *Bifidobacterium* species present in humans except for *B. pseudolongum*.

Initially most of the investigations were directed towards the hydrolytic activity of the enzymes. However, studies with glycosidases showed that some enzymes have transglycosylation activity (for explanation see Section 3.1) besides hydrolytic activity. Dumortier *et al.* [59] showed that one of the β-D-galactosidases from *B. bifidum* was able to synthesize transgalacto-oligosaccharides (TOS), whereas other purified β-D-galactosidases were not able to produce TOS under the same conditions.

Most of the data about heterologously produced enzymes from *Bifidobacterium* spp. has been published in the last decade; these data are also summarized in Table 1. All enzymes are from bifidobacteria species present in humans, with the exception of those from *B. animalis* subsp. *lactis*, which is commonly found in fermented milk. Most of the carbohydrases are α-galactosidases (GH family 36), β-galactosidases (GH family 2 or 42), and enzymes active against gluco-oligosaccharides like α-glucosidases and sucrose phosphorylases (GH family 13).

Since the genome of *B. longum* biotype *longum* NCC2705 was published [60], more information about carbohydrate modifying enzymes of this organism became available. The percentage of annotated genes of *B. longum* biotype *longum* coding for carbohydrate modifying enzymes (like glycoside hydrolases and glycoside esterases) and carbohydrate binding molecules (CBMs) is 4.4%. This percentage is 4.8% for *B. adolescentis* ATCC15703, which genome sequence was recently released at the NCBI-database (http://www.ncbi.nlm.nih.gov/). In contrast bacteria in the GIT like *L. lactis plantarum* WCFS1 contains

Table 1. Glycoside hydrolases from bifidobacteria

Species/ Strain	Enzyme	Mol. Mass (kDa) SDS/ native	pH_{opt}	T_{opt} (°C)	p*I*	Accesion Number	Gene	GH Family	Referen- ces
B. adolescentis									
	D-xylo-isomerase	53/168	7	60	4.3				[149]
DSM 20083	AXHd3	–/100	6	30	–				[35, 82]
		~60/–	6.0	–	–	AF233379	*axhD3*	43	[81]
	AXHm23	–/160	6	37	–				[35]
	α-glucosidase A	~71/68	6.6	37	–	AF358444	*aglA*	13	[130]
	α-glucosidase B	73/149	6.8	47		AF411186	*aglB*	13	[130]
	α-galactosidase	83/330	6	45	–	AF124596	*aga*	*36*	[122]
		79/344	5.5	55	–				[124]
		83/332	6.5	45	–				[123]
	β-galactosidase	89/350	6	35	–				[106]
		81/235	6.0	50	-	AY359872	*bgal II*	42	[109]
	sucrose phosphorylase	58/129	6.0–6.5	48	–	AF543301	*sucP*	13	[132]
G1	β-fructofuranosidase	74/75	6.1	–	4.5				[93, 94]
E194a	α-glucosidase I	97/490	6.0	50	–				[54, 55]
	α-glucosidase IIa and b	60/120	6.0	50	–				[54, 55]
Int-57	α-amylase	66/–	5.5	50	5.2				[129]
	α-amylase	–	–	–	–	AY240946	*amyB*	13	[141]
	β-galactosidase	–	–	–	–	AF213175	*gal*		–
	β-glucosidase	–	–	–	–				[150]
B. animalis* subsp. *lactis									
	sucrose phosphorylase	–	–	–	–	AF441242	*scrP*	13	[37]
DSM 10140	β-fructofuranosidase	60/60	6.5	40	–	AJ437479	*bfrA*	32	[96]
		60/–	–	–	–	AY509036		32	[90]
B. bifidum									
A3	β-galactosidase	–	7.0	50	–				[104]
DSM 20082	β-galactosidase	163–190/362	6.5	37–39	5.25				[59]
	β-1,3-galactosyl-*N*-acetyl-hexosamine phosphory-lase	–/140	6–6.5	40–45	–				[135]
DSM 20125	β-galactosidase	–/620	–	–	–	AJ272131	*BIF1*		[84]
		130/236	–	–	–	AJ224434	*BIF2*	2	[84]
		180–360/182	–	–	–	AJ224435	*BIF3*	2	[151]
						AX319625			[84]
JCM1254	1,2-α-L-fucosidase	–	–	–	–	AY303700	*afcA*	95	[136]
B. breve									
203	α-galactosidase	39/330	5.5	–	3.7				[57]
		80/160	5.5–6.5	–	–	AF406640	*aga*	36	[120]
	β-glucosidase I	48/96	6.0	45	–				[56]
	β-glucosidase II	–/450	5.5	40	–				[56]
203 clb	β-glucosidase	48/47	5.5	45	4.3				[152, 153]
		50/50	5.5	45	4.3	D88311		1	[153, 154]
K-110	α-L-arabinofuranosidase	60/60	4.5	45	–				[114]
	α-L-arabinopyranosidase	77/310	5.5–6.0	40	–				[114]
	β-D-xylosidase	49/49	5.0	37	–				[115]
UCC2003	β-fructofuranosidase	60/–	6.0	37		AY549965	*fosC*		[89]
YIT4010	β-galactosidase	–	–	–	–	E05040			[155]
B. longum* biotype *infantis									
ATCC15697	β-fructofuranosidase	68/72	6.0	37	4.3				[95]
	α-galactosidase	–	6.0	40	–				[102]
	β-galactosidase	–	7.0	40	–				[102]
DSM 20088	β-galactosidase	77/140	–	–	–	AJ224436	*INF1*	42	[84]
HL96	β-galactosidase	–	–	–	–	AF192265	b*galI*	2	[99, 101]
HL96	β-galactosidase	–	–	–	–	AF192266	b*galIII*	42	[99, 101]
JCM 7007	β-fructofuranosidase	75/232	6.0–6.2	55	–				[156]

Table 1. Continued

Species/ Strain	Enzyme	Mol. Mass (kDa) SDS/ native	pH_{opt}	T_{opt} (°C)	p*I*	Accesion Number	Gene	GH Family	References
B. longum* biotype *longum									
401	β-galactosidase	–/330	6.0	40	–				[58]
	lactase	–/700	6.5	45–50	–				[58]
B667	α-L-arabinofuranosidase	–	–	–	–	AY259087	*abfB*	51	[113]
CCRC 15708	β-galactosidase	–/357	7.0	50					[98]
CRL 849	α-galactosidase	–	5.8	40–45					[121]
MB219	β-galactosidase	–	–	–	–	AJ242596	*lacZ*	2	[157]
NCC490	endo-galactanase	94/329	5.0	37		NC_004307	*YvfO*	53	[83]
JCM 1217	endo-α-*N*-acetylgalactos-aminidase	200/200	5.0	60		AY836679	*engBF*	101	[137]
SJ32	sucrose phosphorylase	56/–	–	–	–	AY236071	*scrP*	13	[158]
VMKB44	α-galactosidase	–	–	–	–	AF160969	*aglL*	36	–
B. pseudolongum									
NCFB 2244	α-glucosidase	126/126	–	–	4.2				[39]

only 2.9% of the annotated genes coding for carbohydrate modifying enzymes [61], whereas the value for *Clostridium perfringens* is 3.1%, *E. coli* K12 1.4%, *Bacillus subtilis* 1.7%, and *Mycobacterium tuberculosis* 1.1% [62]. Only *Bacteroides thetaiotaomicron* contains a higher number (7.8%) of annotated carbohydrate modifying enzymes as compared to bifidobacteria [63]. As shown in Table 2 there are differences in the presence of glycoside hydrolases and CBMs for the two bifidobacterial strains. As an example *B. longum* biotype *longum* contains more glycoside hydrolases belonging to GH 51 that are classified as arabinofuranosidases, whereas *B. adolescentis* contains more glycoside hydrolases belonging to GH 13 that are involved in the degradation of α-glucosidic bonds. This results in a different preference for carbon sources between *Bifidobacterium* spp.

From the genome sequence of *B. longum* biotype *longum* NCC2705 [60], only a few genes that code for carbohydrate modifying enzymes contained a signal peptide (SignP), and it is therefore suggested that most carbohydrases are intracellularly located. Only four enzymes were annotated to have an "endo" working mechanism. This shows that *B. longum* biotype *longum* has probably a preference for oligosaccharides, which are transported into the cell for further utilization. This is in agreement with the presence of a high number of sugar transport systems identified in the genome of *B. longum* biotype *longum* NCC2705 [64]. This indicates that bifidobacteria are very well adapted for the utilization of especially oligosaccharides in the colon. In addition, results from fermentation studies showed a preference for (potential) prebiotic oligosaccharides amongst bifidobacteria [65–68].

5 Possible strategies of bifidobacteria for carbohydrate uptake

Bacteria can employ different strategies for utilizing carbohydrates as a carbon source. These strategies have in common that they comprise a battery of intra- and extracellular enzymes with activity towards oligo- and polysaccharides, linked to an extensive set of sugar transporters nested in membranes. Examples of carbohydrate imported by *Bifidobacterium*, described in some detail, include that of fructose, glucose and arabinose in *B. breve* [69, 70], lactose, glucose, and galactose in *B. bifidum* [71, 72], lactose and glucose in *B. longum* biotype *longum* [73, 74], and of galacto-oligosaccharides [75] and sucrose in *B. animalis* subsp. *lactis* [37]. It is also hypothesized that some *Bifidobacterium* species are able to import xylo-oligosaccharides [36, 76]. *B. adolescentis* showed a preference for di- and oligosaccharides compared to their monosaccharide moieties [77].

The first publication on the genome of *B. longum* biotype *longum* included eight high-affinity MalEFG-type oligosaccharide transporters and one PTS-type sugar transporter [60]. A comprehensive proteomic study revealed that in *B. longum* biotype *longum* glucose and fructose were catabolized via the same degradation pathway and that the uptake of fructose may be conducted by a specific transport system [78]. A more detailed study of the *B. longum* biotype *longum* genome, analyzed by *in silico* analysis combined with RNA assays, revealed 19 permeases for carbohydrates uptake [64]. However, a validated system for arabinose and arabinogalactan uptake was not found or not recognized, most likely because the specificity of these transporters is not yet known.

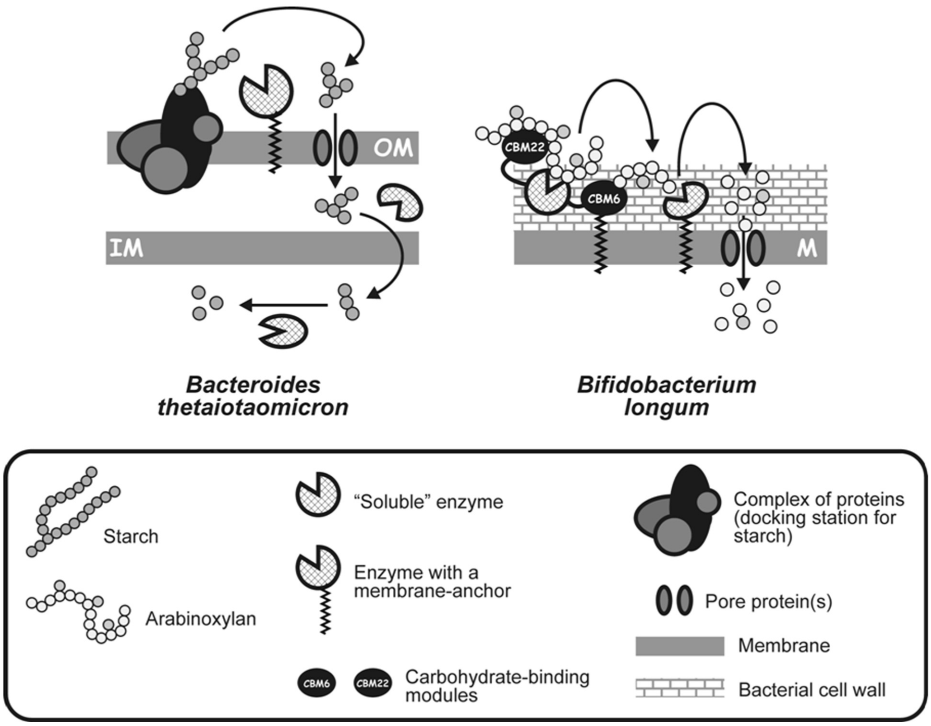

Figure 2. Schematic illustration of different (putative) strategies adopted by various microbes to secure carbohydrate nutrients for their own benefit. IM = inner membrane; OM = outer membrane; M = membrane; *B. longum* = *B. longum* biotype *longum*.

As an example, we will discuss the mechanism of starch utilization of *Bact. thetaiotaomicron* (Fig. 2). We hypothesize that *B. longum* biotype *longum* may use a strategy for utilization of (arabino) xylan oligosaccharides (potential prebiotic), which is reminiscent of that of *Bact. thetaiotaomicron*.

Bact. thetaiotaomicron makes use of a kind of docking station to capture starch molecules and bind these to the bacterial cell surface without loosing them to nearby competitors [79]. The docking station is a complex of proteins, which is present in the outer membrane of the bacterium (Fig. 2). Proximal to this complex can be an α-amylase, which is anchored to the cell surface by a transmembrane domain (TmD). The enzyme degrades the starch molecule to linear maltodextrins, sufficiently small to pass through a porin which gives access to the periplasmic space. Subsequently, the oligosaccharides are further degraded and internalized by an unknown mechanism.

The genome sequence of *B. longum* biotype *longum* indicates that this organism is equipped with a number of modular glycanases. A few examples of such enzymes will be discussed, together with their putative role in (prebiotic) carbohydrate utilization. The most striking multi-domain glycanase seems to be the putative endo-xylanase (BL1543), which is also present in *B. longum* biotype *suis* [80], SignP–CBM22–GH43–CBM6–TmD (Fig. 2). The SignP indicates that this enzyme is secreted, whereas the TmD indicates that the enzyme is anchored in the cell membrane. The catalytic domain belongs to GH family 43, and shows homology to other endo-xylanases. The exact substrate specificity of the GH43 domain is unknown, *i.e.* it is not known whether the enzyme is hindered by the presence of arabinosyl substituents as in arabinoxylans. For this reason it is not possible to state with certainty that arabinoxylan can be utilized by *B. longum* biotype *longum*. CBM6 and CBM22, flank the GH43 domain. Enzymes with similar CBMs have been shown to interact with xylan. Therefore, we postulate that the two CBMs may function as a kind of docking station, which is an intrinsic part of the endo-xylanase, contrary to the machinery of *Bact. thetaiotaomicron* in which the docking station and the hydrolase are separate proteins. Interestingly, *B. longum* biotype *longum* also seems to contain an extracellular exo-xylanase (BL1544), which is also equipped with a TmD. In contrast the genome sequence of *B. adolescentis* does not encode for any extracellular exo-xylanase. It is possible that the exo-xylanase from *B. longum* biotype *longum* degrades the products of the endo-xylanase further to monosaccharides, which are subsequently transported across the bacterial membrane by a transporter protein that remains to be identified. The presence of the bacterial cell wall may prohibit diffusion of these nutrients away from the transporter. The fate of arabinosylated xylo-oligosaccharides, which are also expected to be formed by the endo-xylanase, is unknown. They may be lost as a nutritional source; alternatively, they may be internalized by di- or trisaccharide transporters, after which they are further degraded by arabinofuranosidases inside the cell. The genome sequence of *B. longum* biotype *longum* has not revealed extracellular arabinofuranosidases to degrade arabinoxylan-oligosacchar-

Table 2. The presence of annotated genes coding for carbohydrate active enzymes in the whole genome of *Bifidobacterium adolescentis* ATCC15703 and *Bifidobacterium longum* biotype *longum* NCC2705 available at the CAZy database (http://www.cazy.org) [159].

GH[a)]	*B. adolescentis*	*B. longum biotype longum*
1	2	–
2	3	2
3	6	3
5	2	3
8	1	–
13	13	8
20	–	1
25	1	–
26	1	–
27	1	1
30	1	–
31	1	3
32	2	1
35	1	–
36	2	1
38	1	3
42	4	2
43	7	9
51	2	5
53	–	1
77	2	2
85	–	1
101	–	1
GT[b)]	*B. adolescentis*	*B. longum biotype longum*
2	9	12
4	5	3
28	1	1
35	1	1
51	2	2
CBM[c)]	*B. adolescentis*	*B. longum biotype longum*
6	–	1
13	–	1
22	–	2
23	1	–
32	–	2
41	2	–
48	3	3

a) GH family
b) Glycosyl transferase family
c) CBM family

ides further [60]. However, it is also possible that the exo-xylanase serves another purpose. Crittenden and coworkers [36] observed that *B. longum* strains grow well on xylo-oligosaccharides (dp 2–5) and much less on xylose. In addition it can be mentioned that the amino acid sequence shows a high similarity with an arabinoxylan arabinofuranohydrolase (AXH) from *B. adolescentis* (AXHd3). This enzyme releases only C3-linked arabinosyl residues from double-substituted xylose residues [35, 81, 82] suggesting that the "exo-xylanase" is acting as an arabinofuranosidase and removes arabinosyl residues from arabinoxylan.

Besides, a putative system for (arabino) xylan utilization, it is likely that *B. longum* biotype *longum* can also thrive on (arabino) galactan a (potential) prebiotic. From fermentation studies it is known that *B. longum* biotype *longum* can utilize arabinogalactan [32–34]. The genome sequence reveals the presence of a membrane-bound, extracellular endogalactanase, which is not present in the genome sequence of *B. adolescentis,* suggesting different preferences between bifidobacteria species. The enzyme was cloned and characterized and the enzyme was able to liberate galacto-trisaccharides from type I galactan. The enzyme acts with a processive mechanism, *i. e.* after an initial mid-chain (or endo) cleavage the enzyme remains attached to the galactan and liberates galacto-trisaccharides in an exo-fashion [83]. Typically, *B. longum* biotype *longum* does not appear to have an extracellular β-galactosidase. This may indicate that this microorganism has a mechanism to internalize galacto-oligosaccharides, which has also been suggested for *B. animalis* subsp. *lactis* based on growth studies with these oligosaccharides [75]. It is also unclear whether galacto-oligosaccharides with arabinosyl side chains (formed upon arabinogalactan degradation) can be taken up by *B. longum* biotype *longum*. A number of enzymes, which may have a role in removing arabinosyl substituents, have been annotated [60]. The enzyme annotated as an *endo*-α-L-arabin*osidase* (BL183), which is equipped with a signal peptide sequence, may be involved in this process. The genome sequence has not revealed any other extracellular arabinofuranosidases or endo-arabinanases. The exact mechanism of action remains to be established, since the prefix "*endo*" suggests that the enzyme acts randomly on arabinans, whereas the ending "*osidase*" suggests an exo mode of action. The fact that the enzyme is classified in GH family 43 is not conclusive on its mode of action, because both endo- and exo-enzymes have been assigned to this GH family.

Import of larger galacto-oligosaccharides is not necessarily a common feature of bifidobacteria. For instance, Møller *et al.* [84] described a β-galactosidase (BIF3) from *B. bifidum* having a SignP, which suggests that this protein is secreted by the bacterium. Besides, the enzyme contained a C-terminal CBM with high homology to cell surface-attached galacto-binding domains, sometimes referred to as lectins. We speculate that this enzyme will be attached to carbohydrates of the *Bifidobacterium* cell wall through the galactose-recognizing CBM, because galactose is an important constituent glycosyl residue in the backbone of *Bifidobacterium* cell wall polysaccharides, and the predominant residue in their (single unit) side chains [85, 86]. If this β-galactosidase is located extracellularly and attached to the cell wall, oligosaccharides will be degraded by the β-galactosidase to monomers. In that case no special oligosaccharides-transporter will be needed, but galactose may be internalized through the more common hexose transporters.

6 *Bifidobacterium* carbohydrases for oligosaccharide utilization and production

A whole range of carbohydrates has been tested for use as prebiotic like fructo-oligosaccharides (FOS), β-galacto-oligosaccharides, α-galacto-oligosaccharides, α-gluco-oligosaccharides, and lactulose. The prebiotic ‚effect' was studied in most cases by (i) fermentation of the carbohydrates by bifidobacteria [33, 34] and/or (ii) the enumeration of bifidobacteria was investigated in human and animal studies after intake of the carbohydrates [17]. However, to confirm the prebiotic effect, well double-blind placebo-controlled human studies have to be used. In this review we want to pay attention to the degradation of (potential) prebiotics by bifidobacterial glycoside hydrolases, as well as the potential to produce new classes of prebiotics by showing the presence and (transglycosylation) activity of the most important carbohydrate modifying enzymes in bifidobacteria.

6.1 FOS

The application of FOS as a prebiotic ingredient in dairy products and other foods such as breakfast cereal and soft drinks is increasing [87; http://www.ingredientsdirectory.com/reports/report2.pdf]. FOS is the best studied prebiotic to date. It is present in nature in various plant sources (*i. e.* onion and chicory) and can have the general structure (β-D-Fru*f*-(2→1))$_n$-D-Fru*f* or can be prepared from sucrose through the transfructosylation action of enzymes, namely β-fructofuranosidase and β-D-fructosyltransferase, which results in the general structures (β-D-Fru*f*-(2→1))$_n$-β-D-Fru*f*-(2↔1)-α-D-Glc*p*. Inulin is the polymeric form of the former. Bifidobacteria are known to ferment FOS rapidly [88], making use of the enzyme β-fructofuranosidase. Sequence analysis of all known β-fructofuranosidases from bifidobacteria revealed that no secretion or membrane-anchoring sequences were identified suggesting that all these enzymes are intracellularly located [89]. Therefore, the efficacy of inulin as prebiotic needs probably other gut bacteria to digest the polymer to smaller oligosaccharides.

In a FOS-mixture the shorter oligosaccharides are first utilized by bifidobacteria [90, 91]. Well-described *Bifidobacterium* β-fructofuranosidases are those from *B. adolescentis* [92–94], *B. breve* [89], *B. longum* biotype *infantis* [95], and *B. animalis* subsp. *lactis* [90, 96]. Muramatsu *et al.* [94] differentiate β-fructofuranosidases into those active towards sucrose, inulin, and FOS. Their β-fructofuranosidase from *B. adolescentis* G1 was purified and appeared rather specific for FOS. Although hydrolysis is 63-times more effective than transglycosylation, some transglycosylation products with β-D-Fru*f*-(2→1)-β-D-Fru*f*-(2↔1)-α-D-Glc*p* (GF_2) as substrate were formed. Products obtained were GF_3, neokestose (β-D-Fru*f*-(2↔6)-α-D-Glc*p*-(1↔2)-D-Fru*f*), and β-D-Fru*f*-(2→1)-β-D-Fru*f*-(2↔6)-α-D-Glc*p*-(1↔2)-D-Fru*f*.

A β-fructofuranosidase with almost the same physico-chemical properties was isolated from *B. longum* biotype *infantis* ATCC15697 [95], and the enzyme showed also preference for the hydrolysis of oligosaccharides (over that of sucrose and inulin). This β-fructofuranosidase was inactive towards raffinose, which shows that it is different from a heterologously expressed β-fructofuranosidase from *B. animalis* subsp. *lactis* DSM10140 that was able to release fructose at a low rate from this substrate [96]. The highest activity was found with sucrose, while FOS were hydrolyzed at a slower rate. However, another group cloned the same gene and they reported highest activity toward FOS instead of sucrose [90]. Recently Ryan *et al.* [89] cloned and expressed a β-fructofuranosidase from *B. breve* UCC2003 that specifically catalyzed the hydrolysis of the β-(2→1) glycosidic bond between glucose and its neighboring fructose moiety in sucrose and in short FOS. No detectable activity was observed towards the β-(2→1) glycosidic bonds between fructose moieties. Comparison of the amino acid sequence of the β-fructofuranosidase from *B. breve* and from other bifidobacteria showed 97–94, 83, and 71% identity with *B. longum* biotype *longum* (ABN04092, AAN23970, ZP_00121244), *B. adolescentis* (BAF39931), and *B. animalis* subsp. *lactis* (AAS87041), respectively.

It is also reported that bifidobacteria can degrade levan oligosaccharides β-(2→6)-linked fructose oligosaccharides [97].

6.2 β-Galacto-oligosaccharides

β-Galactosidases are essential enzymes for bifidobacteria to be able to grow on milk or milk-based substrates such as lactose and lactose-derived TOS that contain β-galactosidic-linkages. A diversity of *Bifidobacterium* strains has been studied with respect to their β-galactosidase activity, in more or less detail. These strains include *B. longum* biotype *longum* [58, 98], *B. longum* biotype *infantis* [84, 99–102], *B. bifidum* [59, 84, 103–105], *B. adolescentis* [106], and *B. animalis* subsp. *lactis* [75]. Initially these studies on β-galactosidases focused on the hydrolytic degradation of lactose but gradually more and more attention was paid to their transferase activity towards lactose, for the synthesis of TOS. In this respect also *B. angulatum* and *B. pseudolongum* can be mentioned as sources for β-galactosyl-transferring β-galactosidases [107]. Obviously, the strains with the transglycosylating β-galactosidases also showed the ability to hydrolyze the galacto-oligosaccharides again.

The genomic sequence of *B. longum* biotype *longum* NCC2705 revealed the existence of multiple forms of β-galactosidases in this species [60]; these enzymes belong to

GH family 2 or 42 (Table 1). In a crude extract of *B. longum* biotype *infantis* ATTC 27920 and HL96, three different β-galactosidase bands were observed upon native electrophoresis [99, 102]. Two β-galactosidase genes from *B. longum* biotype *infantis* HL96 (β-GalI and β-GalIII) [99–101] and one from *B. longum* biotype *infantis* DSM20088 (INF1) [84] have been cloned. Although the amino acid sequence of β-GalIII displays high (74%) identity to that of INF1, their transglycosylation activity is rather different. β-GalI is six times more effective in transferring galactosyl groups to lactose than β-GalIII.

Also, several β-galactosidases from *B. bifidum* were found to display transferase activity [59, 84, 103, 105, 108]. Dumortier *et al*. [59] purified a transgalactosidase and from a diversity of monomeric sugars only glucose and xylose could act as acceptors for transgalactosidase, using lactose as donor. Galactose was only an acceptor when present in a glycosidic linkage, *i. e.* α-methyl–D-galactose and lactose. Three other genes from *B. bifidum* DSM20215, cloned and expressed in *E. coli* resulted in β-galactosidases named BIF1, BIF2, and BIF3. Only BIF3 was likely to be extracellular, since it contained a signal peptide. Besides hydrolysis, all showed transferase activity with lactose as substrate. The efficiency of transferase activity of BIF3 could be increased tremendously by truncation of this β-galactosidase at the C-terminal end by 580 amino acids [105]. This C-terminal end contains a galactose binding domain. In *Bact. thetaiotaomicron* also a β-galactosidase was found, which contained a similar galactose binding domain [63]. Although with many β-galactosidases transfer reactions are only observed with a high lactose concentration, the truncated form of BIF3 resulted in 90% transfer and only 10% hydrolysis over a wide range of lactose concentrations (10–40% lactose). The molecular mechanism behind this increase in transferring power of the enzyme is yet unknown. Jørgensen *et al.* [105] hypothesized that the truncated β-galactosidase may have a more open structure which facilitates transglycosylation.

To compare the β-galactosidases genes a dendrogram was constructed (Fig. 3). The β-galactosidase genes from the *B. longum* biotype *longum* genome, annotated by Schell *et al.* [60], were included in this dendrogram as well. Two groups of β-galactosidases were found, namely the β-galactosidases from GH family 2 and the β-galactosidases from GH family 42. The enzymes from GH family 2 have different properties than the ones from GH family 42. The members of GH family 2 have a higher lactase activity and higher transferase activity than the β-galactosidases from GH family 42 [84, 101]. Taking just the catalytic domain of BIF3 for the purpose of constructing the phylogenetic tree, it would be clustered in GH family 2. The available genome sequences of *Lactobacillus* strains revealed also enzymes in GH family 2 and GH family 42, whereas for *Bacteroides* and *Clostridium* strains different glycoside hydrolases were found in GH 2 but depending on the strain no or only one enzyme was classified in GH family 42 (http://www.cazy.org).

In *B. adolescentis* DSM20083 the presence of at least two β-galactosidases was demonstrated by native gel electrophoresis (β-Gal I and β-Gal II), using 4-methylumbelliferyl-β-galactoside as substrate [106]. β-Gal I was a typical lactose hydrolyzing enzyme, while β-Gal II appeared unable to do so. Growth of *B. adolescentis* on TOS appeared to be a 'two-phase' process. In the first phase lactose was utilized (by β-Gal I) until the cell density reached a plateau level, followed by a second phase in which larger oligosaccharides were fermented and the formation of β-gal II was observed. Experiments with a heterologously produced β-Gal II showed that this enzyme belongs to GH family 42 and showed preference for β-(1-4)-galactosides, such as in arabinogalactan-oligosaccharides derived from potato galactan instead of lactose [109].

The concept of using strain-specific β-galactosidases for the production of oligosaccharides (see also Section 8), with a (potential) prebiotic function for that specific strain, was nicely demonstrated for a series of *Bifidobacterium* strains [107]. β-galactosidase extracts were used to produce transgalacto-oligosaccharides with different linkage compositions from lactose. As a general rule the specific strain showed the highest growth rate on the oligosaccharide mixture produced by its own β-galactosidase. Only the *B. adolescentis* ANB-7 strain was an exception to this rule. For the production of β-galacto-oligosaccharides also whole cell extract of bifidobacteria can be used directly in the desired product as demonstrated for *B. bifidum* NCIMB 41171 [110]. In addition, for the development of a synbiotic, Lamoureux *et al.* [111] used mixed cultures of bifidobacteria in the preparation of yoghurts, which were also used to produce oligosaccharides in these yoghurts.

6.3 Arabinan, arabinogalactan, and arabinoxylan

Arabinofuranosyl-containing oligosaccharides derived from plant cell wall polysaccharides arabinan, arabinogalactan, and arabinoxylan can be fermented by bifidobacteria [112] and may be prebiotics. In these oligosaccharides arabinose is mainly present as single unit side chains. The first well-characterized enzymes from *Bifidobacterium* spp. able to degrade arabinoxylan and arabinoxylo-oligosaccharides have been described by Van Laere and coworkers [35, 82]. Two different arabinofuranosidases were purified from a cell-extract of *B. adolescentis* DSM20083. Both enzymes were very specific for arabinoxylan (or oligosaccharides thereof, see further). Therefore, these enzymes were named AXH. These enzymes were found to have a different preference for the glycosidic linkage type. AXHd3, which was recently cloned [81], hydrolyzed only C3 linked arabinofuranosyl residues of doubly substituted xylopyranosyl residues of arabinoxylan or oligosaccharides thereof. AXHm23 released only arabinosyl units that were

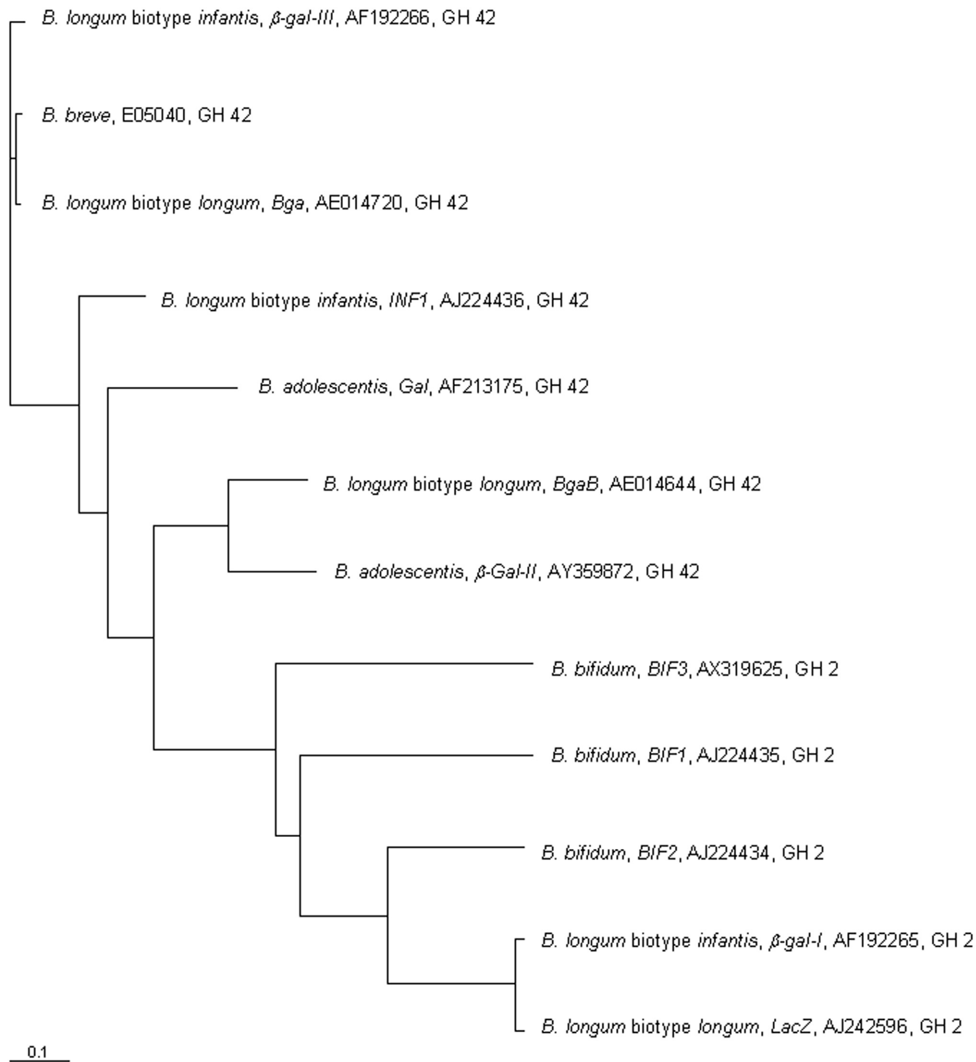

Figure 3. Phylogenetic dendrogram of β-galactosidases from *Bifidobacterium* spp. was constructed using Clustel W and neighbor-joining analysis of the alignment. Results from the bootstrap analysis (n=100) revealed that all junctions were 99 or 100. GH 42 or 2 is glycoside hydrolase family 42 or 2, respectively.

linked to the C2 or C3 position of single substituted xylose residues in arabinoxylo-oligosaccharides. Both enzymes were induced specifically by arabinoxylo-oligosaccharides containing doubly substituted xylopyranosyl residues. Besides AXHd3 and AXHm23 also a β-xylosidase active towards linear xylo-oligosaccharides is produced by *B. adolescentis* DSM20083, enabling complete degradation of the branched oligosaccharides to monosaccharides.

The relatively large number of genes coding for putative arabinoxylan degrading enzymes in *B. longum* biotype *longum* NCC2705 [60] demonstrates also the importance of these enzymes for survival of this microorganism in its environment. The deduced amino acid sequence of an α-L-arabinofuranosidase gene (*abfB*) from *B. longum* biotype *longum* B667 [113] showed more than 99% identity to the deduced amino acid sequence of one of the *B. longum* biotype *longum* NCC275 arabinosidase genes (NC004307; BL1166). The enzyme showed only exo-activity and broad substrate specificity when compared to AXHd3 and AXHm23 from *B. adolescentis*. Shin *et al.* [114] investigated an α-L-arabinopyranosidase and an α-L-arabinofuranosidase from *B. breve* K110*,* but were not able to detect activity towards arabinogalactan from larch wood. These enzymes were shown to be important for bioconversion of two types of glycosylated ginsenosides named Rb2 and Rc. In a parallel study by the same investigators, a β-D-xylosidase was purified from *B. breve* K110 [115]. Besides *p*-nitrophenyl-β-D-xylose hydrolyzing activity, this enzyme also released xylose from xylan and from the ginsenosides Ra1 and Ra2, which are the β-D-xylosylated forms of Rb2 and Rc, respectively. In contrast, preliminary results of the genome sequence of *B. breve* UCC2003 showed the absence of arabinoxylan degrading enzymes (personal communication Van Sinderen). It is concluded that arabinoxylan-oligosaccharides have some selectivity within the genus *Bifidobacterium*, and is supported by *in vitro* fermentation studies of arabinoxylan by Crittenden and coworkers [36].

Fermentation studies of bifidobacteria with arabinoxylan and arabinogalactan revealed that the rate of degradation of these polymers is rather low as compared to the oligosaccharides derived thereof. It was assumed that, despite the high number of glycosidases, other microorganisms are needed for the hydrolysis of polymeric arabinoxylan and arabinogalactan into oligosaccharides [32, 116]. Feed-crossing by *e.g. Bact. thetaiotaomicron* is possible, because most of their glycosyl hydrolases are predicted to be extracellular [63]. In contrast, the *B. longum* biotype *longum* [60] and *B. adolescentis* genomes contained mainly glycoside hydrolases that did not have a signal peptide for extracellular secretion, although *B. longum* biotype *longum* has an extracellular endo-galactanase. In addition the genome sequences of *Lactobacillus* and *Clostridium* strains do not reveal any glycoside hydrolase in GH family 43 en GH family 51, which contain enzymes encoding for arabinofuranosidases and xylosidases. The genome sequences of *Bacteroides* strains revealed many enzymes in GH family 43 but only a few in GH family 51.

6.4 α-Galacto-oligosaccharides and galactomannan

Bifidobacteria are known to grow very well on α-galactosyl oligosaccharides from soymilk such as raffinose and stachyose [117–119]. The α-galactosidase responsible for the degradation of these types of substrates has been studied for *B. breve* [57, 120], *B. longum* biotype *infantis* [102], *B. longum* biotype *longum* [121], and *B. adolescentis* [122–124]. Besides raffinose and stachyose, also melibiose is easily degraded by these α-galactosidases. Sakai *et al.* [57] mentioned the release of galactose from galactomannan using α-galactosidase from *B. breve*.

The α-galactosidase from *B. adolescentis* has been well characterized [122–124] and was found to be active toward melibiose, α-D-Gal*p*-(1→3)-D-Gal*p*, raffinose, α-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-D-Glc*p*, stachyose, and verbascose. Besides hydrolytic activity, the *B. adolescentis* α-galactosidase showed strong transgalactosylation activity. Starting from melibiose, raffinose, or stachyose elongated oligosaccharides could be formed. The structure of the synthesized products from melibiose were determined and appeared to be the trisaccharide α-D-Gal*p*-(1→6)-α-D-Gal*p*-(1→6)-D-Glc*p* and the tetrasaccharide α-D-Gal*p*-(1→6)-α-D-Gal*p*-(1→6)-α-D-Gal*p*-(1→6)-D-Glc*p*. It was concluded that selective transgalactosylation at the C6-hydroxyl group took place [124]. Similar results were found for the heterologously produced enzyme [123, 125, 126]. In contrast to the preferred formation of 1→6 linkages during the transfer reaction, Leder *et al.* [122] showed that 1→3 linkages are hydrolyzed at a higher rate than 1→6 linkages. Based on the amino acid sequence [123], the α-galactosidase was classified in GH family 36, which is consistent with the observed retention of configuration of the galactose residue during hydrolysis (a→α).

6.5 Starch and α-gluco-oligosaccharides

Although bifidobacteria are known to utilize starch [34, 127], only the purification of one α-amylase from *B. adolescentis* Int-57 is described [128, 129]. More research is published about α-glucosidases that are able to hydrolyze oligosaccharides derived from starch and other α-glucosides. From *B. adolescentis* type a, E194a three different α-glucosidases were purified (type I and types IIa and IIb) using maltitol and maltose as substrates, but none of them was able to degrade starch [54, 55]. Type I was relatively specific for nigerose, kojibiose, and maltitol, while sucrose was not hydrolyzed; on the other hand types IIa and IIb showed broader substrate specificity, including degradation of sucrose. Also, Van den Broek and coworkers [130] demonstrated the existence of at least two different α-glucosidases in *B. adolescentis* DSM20083. The cloned genes were named *aglA* and *aglB*, both belonging to GH family 13. The enzymes showed high activity towards isomaltose (α-D-Glc*p*-(1→6)-α-D-Glc*p*) but not towards starch. With respect to substrate specificity AglA was different from type I, IIa, and IIb α-glucosidase from *B. adolescentis* type a, E194a, while AglB resembles types IIa and IIb. AglA could play a role in the utilization of isomalto-oligosaccharides that can be used as prebiotic, because the enzyme showed high activity toward isomaltotriose [130]. Two cell wall associated α-glucosidases were identified and partially purified from *B. pseudolongum*, but none of them were able to hydrolyze starch. Also the genomic sequence of *B. longum* biotype *longum* does not indicate the presence of an α-amylase in that strain [60] and only one α-glucosidase was reported. In contrast to these findings, Wang *et al.* [131] showed multiple forms of α-amylase in extracts from *B. bifidum* and *B. pseudolongum*, using SDS-PAGE and reactivation of the denatured enzymes and also Ryan *et al.* [127] have described a number of starch-degrading bifidobacteria .

In parallel to the other glycosidases described above, also the α-glucosidases from *B. adolescentis* DSM20083 showed transferase activity [130]. AglA produced oligosaccharides from trehalose and sucrose, while AglB was able to synthesize oligosaccharides from maltose, sucrose, and melizitose. The structures of the newly formed products were not determined, but it can be assumed that they were obtained by α-glucosyl transfer.

Sucrose phosphorylase is another enzyme in *B. adolescentis* that is able to catalyze α-glucosyl transfer [132]. This enzyme (SucP) belongs to the same GH-family as the α-glucosidases (GH family 13). SucP can also perform transglucosylation with glucose 1-phosphate as donor and a large number of monomeric sugars (or their alditols) as acceptors. Oligosaccharides could not serve as acceptor substrate. The transglucosylation product with D-arabinose as acceptor was determined to be a novel non-reducing disaccharide with the structure α-Glc*p*(1↔1)β-Ara*f*. The crystal structure of SucP was determined [52] and the structural rearrangement of sucrose phosphorylase from *B. adolescentis* during sucrose conversion was also determined [53].

Sucrose phosphorylase produces glucose 1-phosphate from sucrose and free phosphate in the cell. This reaction is energetically advantageous, because it bypasses the ATP-requiring step of the hexokinase reaction to phosphorylate glucose in preparation for glycolysis. In this way, sucrose phosphorylase can play an important role in the fermentation of sucrose obtained after hydrolysis of raffinose, stachyose, and FOS. The sucrose phosphorylase gene was also cloned from *B. longum* biotype *longum* [73] and *B. animalis* subsp. *lactis* [37] and for the last enzyme it was shown that the enzyme was repressed by glucose.

7 Mucin

The epithelial cell of the human intestine express and/or secrete mucin glycoprotein [133] but these glycoproteins are not classified as prebiotic. The mucin-type oligosaccharides are involved in several important biological events including cell-to-cell communication in higher eukaryotes and bacterial adhesion to host cells [134]. Derensy-Dron and co-workers [135] characterized a β-1,3-galactosyl-*N*-acetylhexosamine phosphorylase from *B. bifidum* DSM20082, which was active towards mucin. Recently, two other enzymes have been identified that were able to act on mucin-type glycoproteins. A specific 1,2-α-L-fucosidase was cloned from *B. bifidum* [136] and an endo-α-*N*-acetylgalactosaminidase was cloned from *B. longum* biotype *longum* [137]. From these data it appears that some bifidobacteria can have the capacity to degrade/modify mucins.

8 Future directions

Over the years, various approaches have been used to improve the interaction between bifidobacteria and their potential substrates. Striking examples of this are the efforts of *Bifidobacterium* strain improvement by chemical mutagenesis, which was aimed at creating probiotic strains with improved lactose utilization [138]. Another approach could be the introduction of foreign DNA into *Bifidobacterium* strains, although this still requires the development of efficient transformation protocols [80, 139]. However, recently some progress has been made with respect to the heterologous expression of enzymes like phytase from *E. coli* [140], α-amylase from *B. adolescentis* [141], and glutamate decarboxylase from rice [142] in *B. longum* biotype *longum*. Such approaches may hold potential in the long run, at least when they are accompanied by extensive safety studies while also securing consumer acceptance. These two approaches will not be discussed further here, but rather we focus on (i) strategies for efficient prebiotic oligosaccharide production using *Bifidobacterium*'s own carbohydrases, and (ii) exploiting *Bifidobacterium*'s genome sequence for rationalizing prebiotic development.

8.1 Strategies for efficient prebiotic oligosaccharide production

It has been suggested above that the carbohydrases of bifidobacteria (or, more in general, beneficial intestinal microbes) can be used for the production of prebiotic oligosaccharides, at least when these enzymes have transglycosylation activity. The rationale behind this is that oligosaccharides can be obtained, that can easily be utilized by bifidobacteria, because all the tools for degradation and uptake are ready to use. The transglycosylation reactions proceed most favorably at high substrate concentration, making it desirable that the substrate is highly water-soluble, or that the reaction can take place at elevated temperatures. Usually, rather cheap oligosaccharides or sugars, such as sucrose and lactose, serve as a starting point for chain elongation. These synthesized oligosaccharides might be incorporated in food products to influence the microbial composition in the more distal parts of the colon [29], because most of the gut disorders are taking place in that region of the colon [17]. As mentioned before, *Bifidobacterium* strains showed the highest growth rate on the oligosaccharide mixture produced by their own enzymes, *e.g.* β-galactosidase [107]. Another advantage could be that a different degree of polymerization and/or degree of branching might lead to less flatulent prebiotics [143] and also a lower osmolarity can be obtained. A useful tool to investigate if (novel) synthesized oligosaccharides have the potential to be used as prebiotic is to determine their prebiotic index (PI). The PI has been defined as a relationship between changes in the "beneficial" and "undesirable" group of microorganisms in the microflora, all of them related to their starting levels. The bacterial groups incorporated into the PI equation were bifidobacteria, lactobacilli, clostridia and bacteroides [144–146]. However, these kind of assays should be interpreted carefully, because only a limited group of intestinal bacteria are included (http://www.afssa.fr/ftp/afssa/28500-28501.pdf).

The main disadvantage of transglycosylation with retaining enzymes is that they possess also hydrolytic activity (besides transglycosylation activity). In most cases, the former is more predominant than the latter. For *Bifidobacterium* α-galactosidase we have found that, under the right conditions 69% of the cleaved galactose units are used in the transglycosylation reaction with melibiose as substrate [126]. Usually, the balance between hydrolysis and transglycosylation is much less favorable. Different methods can be used to improve or optimize enzyme activities. Most of these methods are based on molecular genetic tools. Although not all of these methods have been applied for the improvement of *Bifidobacterium* carbohydrases, they can have potential to generate more efficient enzymes for industrial application.

Site-directed mutagenesis can be used to modify the enzyme activity. For bifidobacteria only one attempt is reported. In this case the transglycosylation properties of α-galactosidase from *B. adolescentis* were changed by site-directed mutagenesis [125]. The highest increase in transglycosylation activity obtained was 16% for one single mutant, whereas most of the other single mutants showed an increase of only 2–5%. Combining of successful single mutations in double mutants resulted in a maximal increase in transglycosylation activity of 10–16% compared with the wild type enzyme.

8.2 Rational prebiotic design

Fermentation studies of poly- and oligosaccharides and ‚beneficial' bacteria were in most cases the basis for detection of prebiotic preparations. The genome sequence of *B. longum* biotype *longum* and *B. adolescentis* offers opportunities to rationalize prebiotic design, because a much clearer picture of the carbohydrate-degrading potential is now available. In addition the presence of glycoside hydrolases may differ for *Bifidobacterium* spp. (Table 2). For instance, it appears that *B. longum* has an array of enzymes involved in the degradation of arabinoxylans, suggesting that these polysaccharides and/or oligosaccharides may be a suitable prebiotic. It should be realized that, although the completion of *Bifidobacterium* genome sequences is a leap forward, there are still many aspects that should be approached with caution. (i) Polysaccharides (or oligosaccharides) can have many subtle structural details, which greatly influence their degradability by carbohydrases. For instance, cereal arabinoxylans can contain a large number of xylosyl residues that are doubly substituted by arabinose. Utilization of oligosaccharides containing such structural signatures strongly depends on having the appropriate enzymes. *B. adolescentis* is known to contain such an enzyme [35, 81, 82], but it is certainly not self-evident that all arabinofuranosidases can act on these side chains. Thus, available sequence information needs a thorough biochemical back-up at some stage. (ii) Feed-crossing by other microorganism may also play an important role for the availability of oligosaccharides in the GIT. (iii) It will be necessary to verify whether the extracellularly formed oligosaccharides can actually be internalized by *Bifidobacterium*. It is therefore important to establish a link between oligosaccharide structure and selectivity of the various transporter proteins. (iv) Last but not least, there is always the question whether a rationally designed *Bifidobacterium* prebiotic is truly a selective substrate. An effective prebiotic use is dependent not only on the ability of bifidobacteria to utilize the compounds. In the human intestine it is proposed that more than 1000 bacterial species are present [147, 148], which can potentially all compete with bifidobacteria for these substrates.

The authors have declared no conflict of interest.

9 References

[1] Scardovi, V., in: Sneath, P. H. A., Mair, N. S., Sharpe, M. E., Holt, J. G. (Eds.), *Bergey's manual of systematic bacteriology* vol. 2., Williams & Wilkins, Baltimore MD 1986, pp. 1418–1434.

[2] Tissier, M. H., PhD Thesis, University of Paris, 1900.

[3] Rogosa, M., in: Buchanan, R. E., Gibbons, N. E. (Eds.), *Bergey's manual of determinative bacteriology* vol 8., Williams & Wilkins, Baltimore MD 1974, pp. 669–676.

[4] Biavati, B., Vescovo, M., Torriani, S., Bottazzi, V., Bifidobacteria: history, ecology, physiology and applications, *Ann. Microbiol.* 2000, *50*, 117–131.

[5] Hoyles, L., Inganäs, E., Falsen, E., Drancourt, M., *et al.*, *Bifidobacterium scardovii* sp. nov., from human sources, *Int. J. Syst. Evol. Microbiol.* 2002, *52*, 995–999.

[6] Masco, L., Ventura, M., Zink, R., Huys, G., Swings, J., Polyphasic taxonomic analysis of *Bifidobacterium animalis* and *Bifidobacterium lactis* reveals relatedness at the subspecies level: Reclassification of *Bifidobacterium animalis* as *Bifidobacterium animalis* subsp. *animalis* subsp. nov. and *Bifidobacterium lactis* as *Bifidobacterium animalis* subsp. *lactis* subsp. nov., *Int. J. Syst. Evol. Microbiol.* 2004, *54*, 1137–1143.

[7] Sakata, S., Kithara, M., Sakamoto, M., Hayashi, H. *et al.*, Unification of *Bifidobacterium infantis* and *Bifidobacterium suis* as *Bifidobacterium longum*, *Int. J. Syst. Evol. Microbiol.* 2002, *52*, 1945–1951.

[8] Simpson, P. J., Ross, R. P., Fitzgerald, G. F., Stanton, C., *Bifidobacterium psychraerophilum* sp. nov. and *Aeriscardovia aeriphila* gen. nov., sp. nov. isolated from a porcine caecum, *Int. J. Syst. Evol. Microbiol.* 2004, *54*, 401–406.

[9] Zhu, L., Li, W., Dong, X., Species identification of genus *Bifidobacterium* based on partial HSP60 gene sequences and proposal of *Bifidobacterium thermacidophilum* subsp. *porcinum* subsp. nov., *Int. J. Syst. Evol. Microbiol.* 2003, *53*, 1619–1623.

[10] Hopkins, M. J., Macfarlane, G. T., Changes in predominant bacterial populations in human faeces with age and with *Clostridium difficile* infection, *J. Med. Microbiol.* 2002, *51*, 448–454.

[11] Hopkins, M. J., Sharp, R., Macfarlane, G. T., Variation in human intestinal microbiota with age, *Digest. Liver Dis.* 2002, *34*, S12–S18.

[12] Mitsuoka, T., Bifidobacteria and their role in human health, *J. Ind. Microbiol.* 1990, *6*, 263–268.

[13] Lay, C., Rigottier-Gois, L., Holmstrom, K., Rajilic, M., *et al*., Colonic microbiota signatures across five northern European countries, *Appl. Environ. Microbiol*. 2005, *71*, 4153–4155.

[14] Falk, P. G. L., Hooper, T., Midvedt, T., Gordon, J. I., Creating and maintaining the gastrointestinal ecosystem: what we know and need to know from gnotobiology, *Microbiol. Mol. Biol. Rev.* 1998, *62*, 1157–1170.

[15] Gibson, G. R., Dietary modulation of the human gut microflora using prebiotics, *Br. J. Nutr.* 1998, *80*, S209–S212.

[16] Gibson, G., Roberfroid, M., Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics, *J. Nutr.* 1995, *125*, 1401–1412.

[17] Gibson, G. R., Ottaway, P. B., Rastall, R. A., *Prebiotics: New developments in functional foods*, Chadwick House Group Ltd., London 2000.

[18] Saavedra, J. M., Bauman, N. A., Oung, I., Perman, J. A., Yolken, R. H., Feeding of *Bifidobacterium bifidum* and *Streptococcus thermophilus* to infants in hospital for prevention of diarrhea and shedding of rotavirus, *Lancet* 1994, *344*, 1046–1049.

[19] Beena, A., Prasad, V., Effect of yoghurt and bifidus yoghurt fortified with skim milk powder, condensed whey and lactose-hydrolyzed condensed whey on serum cholesterol and triacylglycerol concentrations in rat, *J. Dairy Res*. 1997, *64*, 453–457.

[20] Roller, M., Rechkemmer, G., Watzl, B., Prebiotic inulin enriched with oligofructose in combination with the probiotics *Lactobacillus rhamnosus* and *Bifidobacterium lactis* modulates intestinal immune functions in rats, *J. Nutr.* 2004, *134*, 153–156.

[21] Burns, A. J., Rowland, I. R., Anti-carcinogenicity of probiotics and prebiotics, *Curr. Issues Intestinal Microbiol*. 2000, *1*, 13–24.

[22] Reddy, B. S., Prevention of colon cancer by pre- and probiotics: Evidence from laboratory studies, *Brit. J. Nutr.* 1998, *80*, S219–S223.

[23] Wollowski, I., Rechkemmer, G., Pool-Zobel, B. L., Protective role of probiotics and prebiotics in colon cancer, *Am. J. Clin. Nutr.* 2001, *73*, 451S–455S.

[24] Scholz-Aherns, K. E., Schaafsma, G., van den Heuvel, E. G. H. M., Schrezenmeir, J., Effects of prebiotics on mineral metabolism, *Am. J. Clin. Nutr.* 2001, *73*, 459S–464S.

[25] Deguchi, Y., Morishita, T., Mutai, M., Comparative studies on synthesis of water-soluble vitamins among human species of Bifidobacteria, *Agric. Biol. Chem*. 1985, *49*, 13–19.

[26] Schrezenmeir, J., De Vrese, M., Probiotics, prebiotics, and synbiotics–approaching a definition, *Am. J. Clin. Nutr.* 2001, *73*, 361S–364S.

[27] O'Sullivan, D. J., Screening of intestinal microflora for effective probiotic bacteria, *J. Agric. Food Chem*. 2001, *49*, 1751–1760.

[28] Tomasik, P. J., Tomasik, P., Probiotics and prebiotics, *Cereal Chem*. 2003, *80*, 113–117.

[29] Voragen, A. G. J., Technological aspects of functional food-related carbohydrates, *Trends Food Sci. Technol*. 1998, *9*, 328–335.

[30] De Vries, W., Ph.D. Thesis, Vrije Universiteit Amsterdam, 1969.

[31] Scardovi, V., Trovatelli, L. D., The fructose-6-phosphate shunt as peculiar pattern of hexose degradation in the genus *Bifidobacterium*, *Ann. Microbiol. Enzimol.* 1965, *15*, 19–29.

[32] Degnan, B. A., Macfarlane, G. T., Arabinogalactan utilization in continuous cultures of *Bifidobacterium longum*, Effect of co-culture with *Bacteroides thetaiotaomicron*, *Anaerobe* 1995, *1*, 103–112.

[33] Salyers, A. A., West, S. E. H., Vercellotti, J. R., Wilkins, T. D., Fermentation of plant polysaccharides by anaerobic bacteria from the human colon, *Appl. Environ. Microbiol*. 1977, *34*, 529–533.

[34] Crociani, F., Alessandrini, A., Mucci, M. M., Biavati, B., Degradation of complex carbohydrates by *Bifidobacterium* spp., *Int. J. Food Microbiol*. 1994, *24*, 199–210.

[35] Van Laere, K. M. J., Voragen, C. H. L., Kroef, T., Van den Broek, L. A. M., *et al*., Purification and mode of action of two different arabinoxylan arabinofuranohydrolases from *Bifidobacterium adolescentis* DSM 20083, *Appl. Microbiol. Biotechnol*. 1999, *51*, 606–613.

[36] Crittenden, R., Karppinen, S., Ojanen, S., Tenkanen, M., *et al*., *In vitro* fermentation of cereal dietary fibre carbohydrates by probiotic and intestinal bacteria, *J. Sci. Food Agric*. 2002, *82*, 781–789.

[37] Trindade, M. I., Abratt, V. R., Reid, S. J., Induction of sucrose utilization genes from *Bifidobacterium lactis* by sucrose and raffinose, *Appl. Environ. Microbiol*. 2003, *69*, 24–32.

[38] Kullin, B., Abratt, V. R., Reid, S. J., A functional analysis of the *Bifidobacterium longum cscA* and *scrP* genes in sucrose utilization, *Appl. Microbiol. Biotechnol*. 2006, *72*, 975–981.

[39] Degnan, B. A., Macfarlane, G. T., Synthesis and activity of α-glucosidase produced by *Bifidobacterium pseudolongum*, *Curr. Microbiol*. 1994, *29*, 43–47.

[40] Koshland, D. E., Stereochemistry and the mechanism of enzymatic reactions, *Biol. Rev. Camb. Philos. Soc*. 1953, *28*, 416–436.

[41] Sinnott, M. L., Catalytic mechanisms of enzymatic glycosyl transfer, *Chem. Rev.* 1990, *90*, 1171–1202.

[42] Davies, G., Henrissat, B., Structures and mechanisms of glycosyl hydrolases, *Structure* 1995, *3*, 853–859.

[43] McCarter, J. D., Withers, S. G., Mechanisms of enzymatic glycoside hydrolysis, *Curr. Opin. Struct. Biol*. 1994, *4*, 885–892.

[44] Withers, S. G., Mechanisms of glycosyl transferases and hydrolases, *Carbohyd. Polym*. 2001, *44*, 325–337.

[45] Henrissat, B., A classification of glycosyl hydrolases based on amino acid sequence similarities, *Biochem. J.* 1991, *280*, 309–316.

[46] Henrissat, B., Bairoch, A., New families in the classification of glycosyl hydrolases based on amino acid sequence similarities, *Biochem. J.* 1993, *293*, 781–788.

[47] IUB, Enzyme Nomenclature: Recommendations of the Nomenclature Committee of the international Union of Biochemistry on the Nomenclature and Classification of Enzyme Catalyzed Reactions. Academic Press, London and New York 1984.

[48] Henrissat, B., Bairoch, A., Updating the sequence-based classification of glycosyl hydrolases, *Biochem. J.* 1996, *316*, 695–696.

[49] Gebler, J., Gilkes, N. R., Claeyssens, M., Wilson, D. B., *et al*., Stereoselective hydrolysis catalyzed by related β-1,4-glucanases and β-1,4-xylanases, *J. Biol. Chem*. 1992, *267*, 12559–12561.

[50] Henrissat, B., Davies, G., Structural and sequence-based classification of glycoside hydrolases, *Curr. Opin. Struct. Biol*. 1997, *7*, 637–644.

[51] Iwata, S., Ohta, T., Molecular basis of allosteric activation of bacterial L-lactate dehydrogenase, *J. Mol. Biol.* 1993, *230*, 21 – 27.

[52] Sprogøe, D., Van den Broek, L. A. M., Mirza, O., Kastrup, J. S., *et al.*, Crystal structure of sucrose phosphorylase from *Bifidobacterium adolescentis*, *Biochemistry* 2004, *43*, 1156 – 1162.

[53] Mirza, O., Skov, L. K., Sprogøe, D., Van den Broek, L. A. M., *et al.*, Structural rearrangements of sucrose phosphorylase from *Bifidobacterium adolescentis* during sucrose conversion, *J. Biol. Chem.* 2006, *281*, 35576 – 35584.

[54] Igaue, I., Watabe, H., Oda, T., Oyamada, K., Isolation and purification of maltitol-hydrolyzing enzyme (α-glucosidase) from a strain of *Bifidobacterium adolescentis*, *Nippon Nôgeikagaku Kaishi* 1983, *57*, 985 – 994.

[55] Igaue, I., Watabe, H., Oda, T., Oyamada, K., Some characteristics of maltitol-hydrolyzing enzyme (α-glucosidase) from a strain of *Bifidobacterium adolescentis*, *Nippon Nôgeikagaku Kaishi* 1983, *57*, 995 – 999.

[56] Sakai, K., Tachiki, T., Kumagai, H., Tochikura, T., Isolation and characterization of two β-D-glucosidases from *Bifidobacterium breve* 203, *Agric. Biol. Chem.* 1986, *50*, 2287 – 2293.

[57] Sakai, K., Tachiki, T., Kumagai, H., Tochikura, T., Hydrolysis of α-D-Galactosyl oligosaccharides in soymilk by α-galactosidase of *Bifidobacterium breve* 203, *Agric. Biol. Chem.* 1987, *51*, 315 – 322.

[58] Tochikura, T., Sakai, K., Fujiyoshi, T., Tachiki, T., Kumagai, H., *p*-Nitrophenyl glycoside-hydrolyzing activities in bifidobacteria and characterization of β-D-galactosidase of *Bifidobacterium longum* 401, *Agric. Biol. Chem.* 1986, *50*, 2279 – 2286.

[59] Dumortier, V., Brassart, C., Bouquelet, S., Purification and properties of a beta-D-galactosidase from *Bifidobacterium bifidum* exhibiting a transgalactosyl reaction, *Biotechnol. Appl. Biochem.* 1994, *19*, 341 – 354.

[60] Schell, M. A., Karmirantzou, M., Snel, B., Vilanova, D., *et al.*, The genome sequence of *Bifidobacterium longum* reflects its adaptation to the human gastrointestinal tract, *Proc. Natl. Acad. Sci. USA* 2002, *99*, 14422 – 14427.

[61] Kleerebezem, M., Boekhorst, J., van Kranenburg, R., Molenaar, D., *et al.*, Complete genome sequence of *Lactobacillus plantarum* WCFS1, *Proc. Natl. Acad. Sci. USA* 2003, *100*, 1990 – 1995.

[62] Henrissat, B., Couthino, P. M., in: Teeri, T. T., Svensson, B., Gilbert, H. J., Feizi, T. (Eds.), *Carbohydrate bioengineering: Interdisciplinary approaches*, The Royal Society of Chemistry, Cambridge 2002, pp. 171 – 177.

[63] Xu, J., Bjursell, M. K., Himrod, J., Deng, S., *et al.*, A genomic view of the human-*Bacteroides thetaiotaomicron* symbiosis, *Science* 2003, *299*, 2074 – 2076.

[64] Parche, S., Amon, J., Jankovic, I., Rezzonico, E., *et al.*, Sugar transport systems of *Bifidobacterium longum* NCC2705, *J. Mol. Microbiol. Biotechnol.* 2007, *12*, 9 – 19.

[65] Gibson, G. R., Wang, X., Bifidogenic properties of different types of fructo-oligosaccharides, *Food Microbiol.* 1994, *11*, 491 – 498.

[66] Hopkins, M. J., Cummings, J. H., Macfarlane, G. T., Interspecies differences in maximum specific growth rates and cell yields of bifidobacteria cultured on oligosaccharides and other simple carbohydrate sources, *J. Appl. Microbiol.* 1998, *85*, 381 – 386.

[67] Anderson, J. P., Steele, F. M., Schaalje, B. G., An inter-species growth comparison of bifidobacteria using sources of commercially available inulin, *Int. Sugar J.* 2001, *103*, 505 – 511.

[68] Vernazza, C. L., Gibson, G. R., Rastall, R. A., Carbohydrate preference, acid tolerance and bile tolerance in five strains of *Bifidobacterium*, *J. Appl. Microbiol.* 2006, *100*, 846 – 853.

[69] Mazé, A., O'Connell-Motherway, M., Fitzgerald, G. F., Deutscher, J., van Sinderen, D., Identification and characterization of a fructose phosphotransferase system in *Bifidobacterium breve* UCC2003, *Appl. Envriron. Microbiol.* 2007, *73*, 545 – 553.

[70] Degnan, B. A., Macfarlane, G. T., Transport and metabolism of glucose and arabinose in *Bifidobacterium breve*, *Arch. Microbiol.* 1993, *160*, 144 – 151.

[71] Krzewinski, F., Brassart, C., Gavini, F., Bouquelet, S., Characterization of the lactose transport system in the strain *Bifidobacterium bifidum* DSM 20083, *Curr. Microbiol.* 1996, *32*, 301 – 307.

[72] Krzewinski, F., Brassart, C., Gavini, F., Bouquelet, S., Glucose and galactose transport in *Bifidobacterium bifidum* DSM 20082, *Curr. Microbiol.* 1997, *35*, 175 – 179.

[73] Kim, T. B., Song, S. H., Kang, S. C., Oh, D. K., Quantitative comparison of lactose and glucose utilization in *Bifidobacterium longum* cultures, *Biotechnol. Prog.* 2003, *19*, 672 – 675.

[74] Parche, S., Beleut, M., Rezzonico, E., Jacobs, D., *et al.*, Lactose-over-glucose preference in *Bifidobacterium longum* NCC2705: *glc*P, encoding a glucose transporter, is subject to lactose repression, *J. Bacteriol.* 2006, *188*, 1260 – 1265.

[75] Gopal, P. K., Sullivan, P. A., Smart, J. B., Utilization of galacto-oligosaccharides as selective substrates for growth by lactic acid bacteria including *Bifidobacterium lactis* DR10 and *Lactobacillus rhamnosus* DR20, *Int. Dairy J.* 2001, *11*, 19 – 25.

[76] Palframan, R. J., Gibson, G. R., Rastall, R. A., Carbohydrate preferences of *Bifidobacterium* species isolated from the human gut, *Curr. Issues Intest. Microbiol.* 2003, *4*, 71 – 75.

[77] Amaretti, A., Tamburini, E., Bernadi, T., Pompei, A., *et al.*, Substrate preference of *Bifidobacterium adolescentis* MB 239: Compared growth on single and mixed carbohydrates, *Appl. Micriobiol. Biotechnol.* 2006, *73*, 654 – 662.

[78] Yuan, J., Zhu, L., Liu, X., Li, T., *et al.*, A proteome reference map and proteomic analysis of *Bifidobacerium longum* NCC2705, *Mol. Cell. Proteomics* 2006, *5*, 1105 – 1118.

[79] Hooper, L. V., Midtvedt, T., Gordon, J. I., How host-microbial interactions shape the nutrient environment of the mammalian intestine, *Annu. Rev. Nutr.* 2002, *22*, 283 – 307.

[80] Klijn, A., Mercenier, A., Arigoni, F., Lessons from the genome of bifidobacteria, *FEMS Microbiol. Rev.* 2005, *29*, 491 – 509.

[81] Van den Broek, L. A. M., Lloyd, R., Beldman, G., Verdoes, J. C., *et al.*, Cloning and characterization of arabinoxylan arabinofuranohydrolase-D3 (AXHd3) from *Bifidobacterium adolescentis* DSM20083, *Appl. Microbiol. Biotechnol.* 2005, *67*, 641 – 747.

[82] Van Laere, K. M. J., Beldman, G., Voragen, A. G. J., A new arabinofuranohydrolase from *Bifidobacterium adolescentis* able to remove arabinofuranosyl residues from double-substituted xylose units in arabinoxylan, *Appl. Microbiol. Biotechnol.* 1997, *47*, 231 – 235.

[83] Hinz, S. W. A., Pastink, M. I., Van den Broek, L. A. M., Vincken, J. P., Voragen, A. G. J., An endo-galactanase from *Bifidobacterium longum* liberates galactotriose from type I galactans, *Appl. Environ. Microbiol.* 2005, *71*, 5501 – 5510.

[84] Møller, P. L., Jørgensen, F., Hansen, O. C., Madsen, S. M., Stougaard, P., Intra- and extracellular β-galactosidases from *Bifidobacterium bifidum* and *B. infantis*, Molecular cloning, heterologous expression, and comparative characterization, *Appl. Environ. Microbiol.* 2001, *67*, 2276–2283.

[85] Nagaoka, M., Hashimoto, S., Shibata, H., Kimura, *et al.*, Structure of a galactan from cell walls of *Bifidobacterium catenulatum* YIT4016, *Carbohydr. Res.* 1996, *281*, 285–291.

[86] Nagaoka, M., Shibata, H., Kimura, I., Hashimoto, *et al.*, Structural studies on a cell wall polysaccharide from *Bifidobacterium longum* YIT4028, *Carbohyd. Res.* 1995, *274*, 245–249.

[87] Francks, A. M. E., in: Gibson, G., Angus, F. (Eds.), *LFRA Ingredients handbook. Prebiotics and Probiotics*. Leatherhead Publishing, Leatherhead 2000, pp. 1–18.

[88] Durieux, A., Fougnies, C., Jacobs, H., Simon, J. P., Metabolism of chicory frutooligosaccharides by bifidobacteria, *Biotechnol. Lett.* 2001, *23*, 1523–1527.

[89] Ryan, S. M., Fitzgerald, G. F., van Sinderen, D., Transcriptional regulation and characterization of a novel β-fructofuranosidase-encoding gene from *Bifidobacterium breve* UCC2003, *Appl. Environ. Microbiol.* 2005, *71*, 3475–3482.

[90] Janer, C., Rohr, L. M., Peláez, C., Laloi, M., *et al.*, Hydrolysis of oligofructose by the recombinant β-fructofuranosidase from *Bifidobacterium lactis*, *Syst. Appl. Microbiol.* 2004, *27*, 279–283.

[91] Perrin, S., Warchol, M., Grill, J. P., Schneider, F., Fermentations of fructo-oligosaccharides and their components by *Bifidobacterium infantis* ATCC 15697 on batch culture in semi-synthetic medium, *J. Appl. Microbiol.* 2001, *90*, 859–865.

[92] Muramatsu, K., Onodera, S., Kikuchi, M., Shiomi, N., The production of beta-fructofuranosidase from *Bifidobacterium* spp., *Biosci. Biotechnol. Biochem.* 1992, *56*, 1451–1454.

[93] Muramatsu, K., Onodera, S., Kikuchi, M., Shiomi, N., Purification and some properties of β-fructofuranosidase from *Bifidobacterium adolescentis* G1, *Biosci. Biotechnol. Biochem.* 1993, *57*, 1681–1685.

[94] Muramatsu, K., Onodera, S., Kikuchi, M., Shiomi, N., Substrate specificity and subsite affinities of beta-fructofuranosidase from *Bifidobacterium adolescentis* G1, *Biosci. Biotechnol. Biochem.* 1994, *58*, 1642–1645.

[95] Warchol, M., Perrin, S., Grill, J. P., Schneider, F., Characterization of a purified β-fructofuranosidase from *Bifidobacterium infantis* ATCC 15697, *Lett. Appl. Microbiol.* 2002, *35*, 462–467.

[96] Ehrmann, M. A., Korakli, M., Vogel, R. F., Identification of the gene for β-fructofuranosidase of *Bifidobacterium lactis* DSM10140^T and characterization of the enzyme expressed in *Escherichia coli*, *Curr. Microbiol.* 2003, *46*, 391–397.

[97] Marx, S. P., Winkler, S., Hartmeier, W., Metabolization of β-(2,6)-linked fructose-oligosaccharides by different bifidobacteria, *FEMS Microbiol. Lett.* 2000, *182*, 163–169.

[98] Hsu, C. A., Yu, R. C., Chou, C. C., Purification and characterization of a sodium-stimulated β-galactosidase from *Bifidobacterium longum* CCRC 1578, *World J. Microbiol. Biotechnol.* 2005, *22*, 355–361.

[99] Hung, M. N., Lee, B. H., Cloning and expression of β-galactosidase gene from *Bifidobacterium infantis* into *Escherichia coli*, *Biotechnol. Lett.* 1998, *20*, 659–662.

[100] Hung, M. N., Lee, B. H., Purification and characterization of a recombinant beta-galactosidase with transgalactosylation activity from *Bifidobacterium infantis* HL96, *Appl. Microbiol. Biotechnol.* 2002, *58*, 439–445.

[101] Hung, M. N., Xia, Z., Hu, N. T., Lee, B. H., Molecular and biochemical analysis of two β-galactosidases from *Bifidobacterium infantis* HL96, *Appl. Environ. Microbiol.* 2001, *67*, 4256–4263.

[102] Roy, D., Blanchette, L., Savoie, L., Ward, P., Chevalier, P., Alpha-galactosidase and beta-galactosidase properties of *Bifidobacterium infantis*, *Milchwissenschaft* 1992, *47*, 8–21.

[103] Garman, J., Coolbear, T., Smart, J., The effect of cations on the hydrolysis of lactose and the transferase reactions catalysed by beta-galactosidase from six strains of lactic acid bacteria, *Appl. Microbiol. Biotechnol.* 1996, *46*, 22–27.

[104] Iwasaki, T., Yoshioka, Y., Kanauchi, T., Study on the metabolism of *Lactobacillus bifidus* Part III. Purification and some properties of β-galactosidase of a strain of *L. bifidus*, *Nippon Nôgeikagaku Kaishi* 1971, *45*, 207–215.

[105] Jørgensen, F., Hansen, O. C., Stougaard, P., High-efficiency synthesis of oligosaccharides with a truncated β-galactosidase from *Bifidobacterium bifidum*, *Appl. Microbiol. Biotechnol.* 2001, *57*, 647–652.

[106] Van Laere, K. M. J., Abee, T., Schols, H. A., Beldman, G., Voragen, A. G. J., Characterization of a novel β-galactosidase from *Bifidobacterium adolescentis* DSM 20083 active towards transgalactooligosaccharides, *Appl. Environ. Microbiol.* 2000, *66*, 1379–1384.

[107] Rabiu, B. A., Jay, A. J., Gibson, G. R., Rastall, R. A., Synthesis and fermentation properties of novel galacto-oligosaccharides by β-galactosidases from *Bifidobacterium* species, *Appl. Environ. Microbiol.* 2001, *67*, 2526–2530.

[108] Dumortier, V., Montreuil, J., Bouquelet, S., Primary structure of 10 galactosides formed by transglycosylation during lactose hydrolysis by *Bifidobacterium-bifidum*, *Carbohyd. Res.* 1990, *201*, 115–123.

[109] Hinz, S. W. A., Van den Broek, L. A. M., Beldman, G., Vincken, J. P., Voragen, A. G. J., β-Galactosidase from *Bifidobacterium adolescentis* DSM20083 prefers β(1,4)-galactosides over lactose, *Appl. Microbiol. Biotechnol.* 2004, *66*, 276–284.

[110] Tzortzis, G., Goulas, A. K., Gibson, G. R., Synthesis of prebiotic galactooligosaccharides using whole cells of a novel strain, *Bifidobacterium bifidum* NCIMB 41171, *Appl. Microbiol. Biotechnol.* 2005, *68*, 412–416.

[111] Lamoureux, L., Roy, D., Gauthier, S. F., Production of oligosaccharides in yoghurt containing Bifidobacteria and yoghurt cultures, *J. Dairy Sci.* 2002, *85*, 1058–1069.

[112] Van Laere, K. M. J., Hartemink, R., Bosveld, M., Schols, H. A., Voragen, A. G. J., Fermentation of plant cell wall derived polysaccharides and their corresponding oligosaccharides by intestinal bacteria, *J. Agric. Food Chem.* 2000, *48*, 1644–1652.

[113] Margolles, A., De Los Reyes-Gavilán, C. G., Purification and functional characterization of a novel alpha-L-arabinofuranosidase from *Bifidobacterium longum* B667, *Appl. Environ. Microbiol.* 2003, *69*, 5096–5103.

[114] Shin, H. Y., Park, S. Y., Sung, J. H., Kim, D. H., Purification and characterization of α-L-arabinopyranosidase and α-L-arabinofuranosidase from *Bifidobacterium breve* K-110, a human intestinal anaerobic bacterium metabolizing ginsenoside Rb2 and Rc, *Appl. Environ. Microbiol.* 2003, *69*, 7116–7123.

[115] Shin, H. Y., Lee, J. H., Lee, J. Y., Han, Y. O., *et al*., Purification and characterization of ginsenoside Ra-hydrolyzing β-D-xylosidase from *Bifidobacterium breve* K-110, a human intestinal anaerobic bacterium, *Biol. Pharm. Bull.* 2003, *26*, 1170–1173.

[116] Hopkins, M. J., Englyst, H. N., Macfarlane, S., Furrie, E., *et al*., Degradation of cross-linked and non-cross-linked arabinoxylans by the intestinal microbiota in children, *Appl. Environ. Microbiol.* 2003, *69*, 6354–6360.

[117] Garro, M. S., de Valdez, G. F., Oliver, G., de Giori, G. S., Hydrolysis of soya milk oligosaccharides by *Bifidobacterium longum* CRL 849, *Z. Lebensm. Unters. Forsch. A* 1999, *208*, 57–59.

[118] Minami, Y., Yazawa, K., Tamura, Z., Tanaka, T., Yamamoto, T., Selectivity of utilization of galacto-oligosaccharides by bifidobacteria, *Chem. Pharm. Bull.* 1983, *31*, 1688–1691.

[119] Yazawa, K., Imai, K., Tamura, Z., Oligosaccharides and polysaccharides specifically utilizable by Bifidobacteria, *Chem. Pharm. Bull.* 1978, *26*, 3306–3311.

[120] Xiao, M., Tanaka, K., Qian, X. M., Yamamot, K., Kumagai, H., High-yield production and characterization of α-galactosidase from *Bifidobacterium breve* grown on raffinose, *Biotechnol. Lett.* 2000, *22*, 747–751.

[121] Garro, M. S., de Giori, G. S., de Valdez, G. F., Oliver, G., α-D-Galactosidase (EC 3.2.1.22) from *Bifidobacterium longum*, *Lett. Appl. Microbiol.* 1994, *19*, 16–19.

[122] Leder, S., Hartmeier, W., Marx, S. P., α-Galactosidase of *Bifidobacterium adolescentis* DSM 20083, *Curr. Microbiol.* 1999, *38*, 101–106.

[123] Van den Broek, L. A. M., Ton, J., Verdoes, J. C., Van Laere, K. M. J., *et al*., Synthesis of α-galacto-oligosaccharides by a cloned α-galactosidase from *Bifidobacterium adolescentis*, *Biotechnol. Lett.* 1999, *21*, 441–445.

[124] Van Laere, K. M. J., Hartemink, R., Beldman, G., Pitson, S., *et al*., Hydrolase and transgalactosylation activity of *Bifidobacterium adolescentis* α-galactosidase, *Appl. Microbiol. Biotechnol.* 1999, *52*, 681–688.

[125] Hinz, S. W., Doeswijk, C. H. L., Schipperus, R., Van den Broek, L. A., *et al*., Increasing the transglycosylation activity of α-galactosidase from *Bifidobacterium adolescentis* DSM20083 by site-directed mutagenesis, *Biotechnol. Bioeng.* 2005, *93*, 122–131.

[126] Van den Broek, L. A. M., Hinz, S. W. A., Beldman, G., Doeswijk-Voragen, C. H. L., *et al*., Glycosyl hydrolases from *Bifidobacterium adolescentis* DSM20083, *Lait* 2005, *85*, 125–133.

[127] Ryan, S. M., Fitzgerald, G. F., van Sinderen, D., Screening for and identification of starch-, amylopectin-, and pullulan-degrading activities in bifidobacterial strains, *Appl. Environ. Microbiol.* 2006, *72*, 5289–5296.

[128] Ji, G. E., Han, H. K., Yun, S. W., Rhim, S. L., Isolation of amylolytic *Bifidobacterium* sp. Int-57 and characterization of amylase, *J. Microbiol. Biotechnol.* 1992, *2*, 85–91.

[129] Lee, S. K., Kim, Y. B., Ji, G. E., Note: Purification of amylase secreted from *Bifidobacterium adolescentis*, *J. Appl. Microbiol.* 1997, *83*, 267–272.

[130] Van den Broek, L. A. M., Struijs, K., Verdoes, J. C., Beldman, G., Voragen, A. G. J., Cloning and characterization of two α-glucosidases from *Bifidobacterium adolescentis* DSM20083, *Appl. Microbiol. Biotechnol.* 2003, *61*, 55–60.

[131] Wang, X., Conway, P. L., Brown, I. L., Evans, A. J., *In vitro* utilization of amylopectin and high-amylose maize (amylomaize) starch granules by human colonic bacteria, *Appl. Environ. Microbiol.* 1999, *65*, 4848–4854.

[132] Van den Broek, L. A. M., van Boxtel, E. L., Kievit, R. P., Verhoef, R., *et al*., Physico-chemical and transglucosylation properties of recombinant sucrose phosphorylase from *Bifidobacterium adolescentis* DSM20083, *Appl. Microbiol. Biotechnol.* 2004, *65*, 219–227.

[133] Gum, J. R., Byrd, J. C., Hicks, J. W., Toribara, N. W., *et al*., Molecular cloning of human intestinal mucin cDNAs. Sequence analysis and evidence for genetic polymorphism, *J. Biol. Chem.* 1989, *264*, 6480–6487.

[134] Varki, A., Cummings, R., Esko, J., Freeze, H., *et al*., *Essentials of glycobiology*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 1999.

[135] Derensy-Dron, D., Krzewinski, F., Brassart, C., Bouquelet, S., β-1,3-Galactosyl-*N*-acetylhexosamine phosphorylase from *Bifidobacterium bifidum* DSM 20082: Characterization, partial purification and relation to mucin degradation, *Biotechnol. Appl. Biochem.* 1997, *29*, 3–10.

[136] Katayama, T., Sakuma, A., Kimura, T., Makimura, Y., *et al*., Molecular cloning and characterization of *Bifidobacterium bifidum* 1,2-α-L-fucosidase (AfcA), a novel inverting glycosidase (glycoside hydrolase family 95), *J. Bacteriol.* 2004, *186*, 4885–4893.

[137] Fujita, K., Oura, F., Nagamine, N., Katayama, T., *et al*., Identification and molecular cloning of a novel glycoside hydrolase family of core 1 type *O*-glycan-specific endo-α-*N*-acetylgalactosaminidase from *Bifidobacterium longum*, *J. Biol. Chem.* 2005, *280*, 37415–37422.

[138] Ibrahim, S. A., O'Sullivan, D. J., Use of chemical mutagenesis for the isolation of food grade beta-galactosidase overproducing mutants of bifidobacteria, lactobacillii and *Streptococcus thermophilus*, *J. Dairy Sci.* 2000, *83*, 923–930.

[139] Van der Werf, M. J., Venema, K., Bifidobacteria: Genetic modification and the study of their role in the colon, *J. Agric. Food Chem.* 2001, *49*, 378–383.

[140] Park, M. S., Seo, J. M., Kim, J. Y., Ji, G. E., Heterologous gene expression and secretion in *Bifidobacterium longum*, *Lait* 2005, *85*, 1–8.

[141] Rhim, S. L., Park, M. S., Ji, G. E., Expression and secretion of *Bifidobacterium adolescentis* amylase by *Bifidobacterium longum*, *Biotechnol. Lett.* 2006, *28*, 163–168.

[142] Park, K. B., Ji, G. E., Park, M. S., Oh, S. H., Expression of rice glutamate decarboxylase in *Bifidobacterium longum* enhances γ-aminobutyric acid production, *Biotechnol. Lett.* 2005, *27*, 1681–1684.

[143] Cummings, J. H., Macfarlane, G. T., Englyst, H. N., Prebiotic digestion and fermentation, *Am. J. Clin. Nutr.* 2001, *73*, 415S–420S.

[144] Sanz, L. M., Gibson, G. R., Rastall, R. A., Influence of disaccharide structure on prebiotic selectivity *in vitro*, *J. Agric. Food Chem.* 2005, *53*, 5192–5199.

[145] Olano-Martin, E., Gibson, G. R., Rastall, R. A., Comparison of the *in vitro* bifidogenic properties of pectins and pectic-oligosaccharides, *J. Appl. Microbiol.* 2002, *93*, 505–511.

[146] Palframan, R. J., Gibson, G. R., Rastall, R. A., A development of a quantitative tool for the comparison of the prebiotic effect of dietary oligosaccharides, *Lett. Appl. Microbiol.* 2003, *37*, 281–284.

[147] Heselmans, M., Reid, G., Akkermans, L. M. A., Savelkoul, H., *et al*., Gut flora in health and disease: potential role of probiotics, *Curr. Issues Intest. Microbiol*. 2004, *6*, 1 – 8.

[148] Whitfield, J., Features-Science and health; microbial soup of life is sieved for treasure. Financial Times, 2004.

[149] Kawai, Y., Konishi, H., Horitsu, H., Sakurai, H., *et al*., Purification and characterization of D-xylose isomerase from *Bifidobacterium adolescentis*, *Biosci*. *Biotechnol*. *Biochem*. 1994, *58*, 691 – 694.

[150] Choi, Y. J., Kim, C. J., Ji, G. E., A partially purified β-glucosidase from *Bifidobacterium adolescentis* converts cycasin to a mutagenic compound, *Lett*. *Appl*. *Microbiol*. 1996, *22*, 145 – 148.

[151] Jørgensen, F., Hansen, O. C., Stougaard, P., Enzyme isolated from a *Bifidobacterium*, Patent US 6555348-A 2, 2003.

[152] Nunoura, N., Ohdan, K., Yano, T., Yamamoto, K., Kumugai, H., Purification and characterization of β-D-glucosidase (β-D-fucosidase) from *Bifidobacterium breve* clb acclimated to cellobiose, *Biosci*. *Biotechnol*. *Biochem*. 1996, *60*, 188 – 193.

[153] Nunoura, N., Ohdan, K., Tanaka, K., Tamaki, H., *et al*., Cloning and nucleotide sequence of the beta-D-glucosidase gene from *Bifidobacterium breve* clb, and expression of a beta-D-glucosidase activity in *Escherichia coli*, *Biosci*. *Biotechnol*. *Biochem*. 1996, *60*, 2011 – 2018.

[154] Nunoura, N., Ohdan, K., Yamamoto, K., Kumagai, H., Expression of the β-D-glucosidase I gene in *Bifidobacterium breve* 203 during acclimation to cellobiose, *J. Ferment*. *Bioeng*. 1997, *83*, 309 – 314.

[155] Iono, T., Shimakawa, Y., Morishita, T., DNA fragment containing beta-galactosidase gene and plasmid containing the same DNA fragment thereinto, JP 1993146296-A 1, 1993.

[156] Imamura, L., Hisamitsu, K., Kobashi, K., Purification and characterization of β-fructofuranosidase from *Bifidobacterium infantis*, *Biol*. *Pharm*. *Bull*. 1994, *17*, 596 – 602.

[157] Rossi, M., Altomare, L., Gonzàlez Vara y Rodriguez, A., Brigidi, P., Matteuzzi, D., Nucleotide sequence, expression and transcriptional analysis of the *Bifidobacterium longum* MB219 *lacZ* gene, *Arch*. *Microbiol*. 2000, *174*, 74 – 80.

[158] Kim, M., Kwon, T., Lee, H. J., Kim, K. H., *et al*., Cloning and expression of sucrose phosphorylase gene *from Bifidobacterium longum* in *E. coli* and characterization of the recombinant enzyme, *Biotechnol*. *Lett*. 2003, *25*, 1211 – 1217.

[159] Coutinho, P. M., Henrissat, B., in: Gilbert, H. J., Davies, G., Henrissat, B, Svensson, B. (Eds.), *Recent Advances in Carbohydrate Bioengineering*, The Royal Society of Chemistry, Cambridge 1999, pp. 3 – 12.