A Molecular Basis for Bifidobacterial Enrichment in the Infant Gastrointestinal Tract[1–3]

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ABSTRACT

Bifidobacteria are commonly used as probiotics in dairy foods. Select bifidobacterial species are also early colonizers of the breast-fed infant colon; however, the mechanism for this enrichment is unclear. We previously showed that *Bifidobacterium longum* subsp. *infantis* is a prototypical bifidobacterial species that can readily utilize human milk oligosaccharides as the sole carbon source. MS-based glycoprofiling has revealed that numerous *B. infantis* strains preferentially consume small mass oligosaccharides, abundant in human milks. Genome sequencing revealed that *B. infantis* possesses a bias toward genes required to use mammalian-derived carbohydrates. Many of these genomic features encode enzymes that are active on milk oligosaccharides including a novel 40-kb region dedicated to oligosaccharide utilization. Biochemical and molecular characterization of the encoded glycosidases and transport proteins has further resolved the mechanism by which *B. infantis* selectively imports and catabolizes milk oligosaccharides. Expression studies indicate that many of these key functions are only induced during growth on milk oligosaccharides and not expressed during growth on other prebiotics. Analysis of numerous *B. infantis* isolates has confirmed that these genomic features are common among the *B. infantis* subspecies and likely constitute a competitive colonization strategy used by these unique bifidobacteria. By detailed characterization of the molecular mechanisms responsible, these studies provide a conceptual framework for bifidobacterial persistence and host interaction in the infant gastrointestinal tract mediated in part through consumption of human milk oligosaccharides. *Adv. Nutr. 3: 415S–421S, 2012.*

Introduction

Human milk plays a key role in supporting the survival and development of offspring and is a good example of a nutrient that has been shaped by evolution (1). Strong selective pressures have influenced the composition of this fluid in which factors such as the nutritional and protective needs of the newborn are balanced against the energy cost of milk production by the mother (2).

The WHO defines breastfeeding as the "normal way of providing young infants with the nutrients they need for healthy growth and development" (3,4). Human milk is considered a gold standard for nutrition (5) and is characterized by high amounts of essential nutrients (6), such as lactose, fatty acids, and proteins, as well as macronutrients, such as vitamins and minerals. Human milk is compositionally different from mother to mother, from time to time during lactation, and also during different breastfeeding periods (7,8).

Human milk also contains a wide variety of nonessential nutrients that are not consumed by the infant but display complex and potent bioactive functions (9–11). For example, human milk oligosaccharides (HMO)[7] are an array of complex carbohydrates that do not provide direct nutritional

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[7] Abbreviations used: Gal, galactose; HMO, human milk oligosaccharides; GlcNAc, *N*-acetylglucosamine; LNB, lacto-*N*-biose; LNT, lacto-*N*-tetraose; SBP, solute-binding protein.

value for the infant. Among other putative roles, these molecules act as prebiotics, serving as growth substrates for specific colonic bacteria in breast-fed infants, mainly belonging to the *Bifidobacterium* genus. In this review, we address recent advances in our understanding of the bifidogenic effect of HMO and how specific intestinal bifidobacteria have developed strategies for gaining access to the energy content of HMO.

Human milk oligosaccharides

Oligosaccharides are present in human milk at 5–15 g/L, being the third largest component after lactose and lipids (12). HMO are free soluble carbohydrates that contain 3–15 monosaccharides, linked through a variety of glycosidic bonds. Their concentration is severalfold compared with other mammalian milks (13). HMO composition and abundance progressively change during lactation, with significant variations among individual mothers (14). The question of why these indigestible molecules are present at high concentrations in milk has challenged researchers for decades. HMO are synthesized solely in the mammary gland during lactation, and the complex pathways that result in all the structures observed have not yet been completely elucidated. HMO are minimally affected by transit through the stomach and small intestine, reaching a high concentration in the infant colon (14–16).

Breastfeeding has been associated with a lower incidence of diarrhea and gastrointestinal infections caused by bacterial and viral pathogens (17–20). Many milk oligosaccharides contain structural elements that are homologous to glycoconjugates present in the intestinal mucosa used by pathogens for adherence and invasion. For these reasons, HMO are proposed to act as soluble receptor analogs and inhibit the adhesion of pathogens (19,21).

Neutral HMO are composed of galactose (Gal), *N*-acetylglucosamine (GlcNAc), fucose, and a lactose core, whereas acidic HMO can contain these monosaccharides in addition to one or more molecules of *N*-acetylneuraminic acid or sialic acid (22). A variety of glycosyltransferases are active in HMO synthesis, creating 13 potential glycosidic bonds, which results in more than a thousand possible combinations (12,23). However, only a few hundred have been identified. The Gal unit of the lactose core is linked to subunits of lacto-*N*-biose I (LNB) (Galβ1–3GlcNAc, type 1 chain) and/or *N*-acetyllactosamine (type 2 chain, Galβ1–4GlcNAc) originating specific HMO isomer classes. Fucose and sialic acid residues are generally found attached to these backbones in terminal positions. A high-throughput strategy based on high-accuracy MS combined with nanoliquid chromatography (24) was recently used to annotate the molecular and structural complexity of HMO.

Establishment of the infant microbiota: impact of breast milk on bifidobacteria

Immediately after birth, the newborn is exposed to a nonsterile environment, and microorganisms colonize mucosal surfaces including the gastrointestinal tract, initiating what will be a long-term microbe-host relationship. The initial microbial colonization patterns present in the distal colon have an impact on several aspects of the infant health, and, moreover, they are thought to have a long-term impact on human health (25,26). However, the mechanisms by which some microorganisms are responsible for these health effects are not well understood.

The process of colonization is dependent on the mode of birth. Vaginal delivery allows direct contact of the neonate with several rich bacterial niches including the vaginal and the fecal maternal microbiota (27–30). Others have shown that some infant gut bacterial clades are also present in human milk, suggesting that this could represent another reservoir for microorganisms colonizing the infant gut (31–34). Conversely, the main sources of bacteria for cesarean-born infants are found in the hospital environment (35) and the skin microbiome (29), and a delay in colonization by prominent members of the intestinal microbiota such as *Bifidobacterium*, *Bacteroides*, and *Escherichia coli* have been observed (36,37). Moreover bifidobacterial counts have been shown to be lower in cesarean-born infants (38,39).

The type of infant feeding, either breast or formula milk, is a main factor contributing to the development of the infant gut microbiota. Infant formulas are usually based on cow's milk, supplemented with vegetal fatty acids, minerals, and vitamins. Despite recent advances and new ingredients (40), it is difficult to exactly replicate the wide array of nonessential nutrients present in breast milk, such as oligosaccharides, antibodies, and bioactive proteins.

Breast milk guides the development of the infant gut microbiota by preventing pathogen colonization and also by selecting bacteria that can use HMO as a growth substrate, such as *Bifidobacterium* species. This genus is the most abundant in the infant gut microbiome in both breast-fed and formula-fed infants (33,38,41,42). However, the bifidobacterial species recovered from infants subject to both diets differ. In general, a small number of *Bifidobacterium* species are routinely recovered from the feces of breast-fed infants including *Bifidobacterium longum* subsp. *longum*, *B. longum* subsp. *infantis*, *Bifidobacterium bifidum*, and *Bifidobacterium breve* (43–45). The bifidobacterial species found in feces from bottle-fed infants is more diverse and include the aforementioned species and also *Bifidobacterium adolescentis* and *Bifidobacterium pseudocatenulatum*, which are also commonly found in adults (46).

Molecular adaptations of bifidobacteria to utilize HMO

The inherent complexity of HMO renders them inaccessible to digestive enzymes; therefore, they can reach high concentrations in the distal colon. To grow on HMO as a substrate, a microorganism requires specific transporters and/or enzymatic machinery to process these molecules. Recent advances in our understanding of how bifidobacteria utilize HMO correlates also with a general notion of bifidobacteria providing health benefits to the host, given the probiotic or antipathogenic activities shown for certain strains.

Table 1. Genes in *Bifidobacterium infantis* upregulated during bacterial growth on HMO, associated with its consumption[1]

HMO transport	
Blon_2344-Blon_2347	Import of type 2 HMO, such as lacto-*N*-neotetraose or LacNAc containing oligosaccharides; also bind glycans found in colonic mucins
Blon_2350-Blon_2351	Import of galacto-*N*-biose
Blon_2177	Import of lacto-*N*-tetraose and other type 1 HMO; constitutive expression
Blon_0883	Import of lacto-*N*-biose, galacto-*N*-biose,and certain fucosylated blood sugar oligosaccharides.
Glycosyl hydrolases	
Blon_2348	Exo α-sialidase, active on α2–3/6 linkages
Blon_2355	β-hexosaminidase; active on GlcNAcβ1–3Gal linkages
Blon_0732-Blon_0459	β-hexosaminidase; active on GlcNAcβ1–3/6Gal linkages
Blon_2016	β-galactosidase specific for type 1 HMO (Galβ1–3GlcNAc); constitutive expression
Blon_2334	β-galactosidase specific for type 2 HMO (Galβ1–4GlcNAc); constitutive expression
Blon_2335	α-fucosidase, with preference for Fucα1–2 linkages found in HMO but also active on Fucα1–3/4
Blon_2336	α-fucosidase, specific for Fucα1–3/4 linkages found in HMO

[1] Gal, galactose; GlcNAc, HMO, human milk oligosaccharides; *N*-acetylglucosamine; LacNAc.

From the early studies of Moro in 1905 (47) and Gyorgy in 1954 (48), a relationship between human milk and the selective growth of *Bifidobacterium* was determined. Growth factors in human milk, or bifidus factor, were thought to be GlcNAc containing oligosaccharides and glycoproteins that stimulated the growth of bifidobacteria specifically in breast-fed infants. *Bifidobacterium* species are gram positive and strictly anaerobic bacteria, with a fermentative metabolism that produces acetate and lactate as end products (49). Bifidobacteria are often dominant microorganisms in the breast-fed infant gut microbiota and also are significant members of the adult gut microbiome.

Ward et al. (50), first showed that bifidobacteria can consume HMO as the sole carbon source. The extent of growth observed by *B. infantis* was higher compared with other species in the genus. Later work demonstrated that *B. infantis* preferentially consumed short-chain HMO (degree of polymerization <7), but longer chains were used when the total concentration of HMO was reduced (51).

The genome sequence of *B. infantis* provided an explanation for this phenotype (52). Similar to other bifidobacterial genomes, *B. infantis* possessed a number of genes involved in consumption of complex carbohydrates (53,54). However, gene clusters dedicated to the metabolism of plant polysaccharides in the closely related *B. longum* were replaced in *B. infantis* with gene functions related to HMO consumption. Of particular interest was a 43-kb gene cluster (termed HMO cluster I), which so far has been only found in *B. infantis* strains. It contained several genes predicted to be involved in the import and metabolism of HMO, such as glycosyl hydrolases and oligosaccharide transport proteins, all within a single locus (52). The HMO cluster I, as well as other potentially HMO-associated clusters, were shown to be conserved among different strains of *B. infantis* (55,56). Importantly, a *B. infantis* isolate in which the HMO cluster I was partially deleted could only weakly utilize HMO, suggesting a correlation of the presence of the HMO cluster I with vigorous growth on HMO (57). These observations also confirmed the genetic and functional divergence from *B. longum* strains, which lacked several of these HMO-related clusters.

Several observations suggest that *B. infantis* imports and degrades HMO intracellularly. For example, glycolytic enzymes potentially active on HMOs encoded within this microorganism lack signal peptide sequences, indicating a cytoplasmic localization. In addition, the *B. infantis* genome contains several family 1 solute-binding proteins (SBPs; pfam01547) with predicted affinity for oligosaccharides, suggesting a link to HMO transport. Recently Garrido et al. (58) determined that 10 of 20 SBPs encoded by *B. infantis* exhibit a binding preference for prominent mammalian glycans. The affinities of these SBPs covered great part of the spectrum of HMO linkages, including type 1 and 2 HMO (**Table 1**), also matching the substrates that *B. infantis* is able to consume in vitro (51). Genes encoding SBPs that bind type 1 and 2 chains were specifically expressed during growth on HMO (Table 1), but not on fructooligosaccharides and galactooligosaccharides (58). The consumption of specific HMO such as lacto-*N*-tetraose (LNT), lacto-*N*-neotetraose, and certain fucosylated HMO by *B. infantis* was recently observed, and it was suggested that ABC importers are associated with their import (59).

The enzymatic deconstruction of HMO within *B. infantis* appears to occur sequentially via an array of glycosyl hydrolases (60), and some of them have been associated to HMO consumption given their gene expression patterns (Table 1). The ability to consume sialylated HMO such as sialyl-LNT is likely mediated by Blon_2348, one of two α-sialidase genes in *B. infantis*. Only Blon_2348 is upregulated during growth on HMO, and the encoded enzyme more effectively cleaves both α2–3 and α2–6 linkages found in acidic HMO compared with Blon_0646 (61). Fucosylated HMO are highly abundant in breast milk, and glycoprofiling of HMO consumption revealed that *B. infantis* readily utilizes lacto-*N*-fucopentaoses and lacto-*N*-difucohexaoses, however, only after LNT is consumed first. Two α-fucosidases encoded in the HMO cluster I, Blon_2335 and Blon_2336, are expressed during growth on HMO, and both release fucose from 2′- and 3′-fucosyllactose, Lewis a, Lewis x, and fucosylated HMO such as lacto-*N*-fucopentaose I and III (62). Other fucosidases in the *B. infantis* genome, albeit showing high kinetic rates and activity on certain HMO species, did not show induction during bacterial growth on HMO.

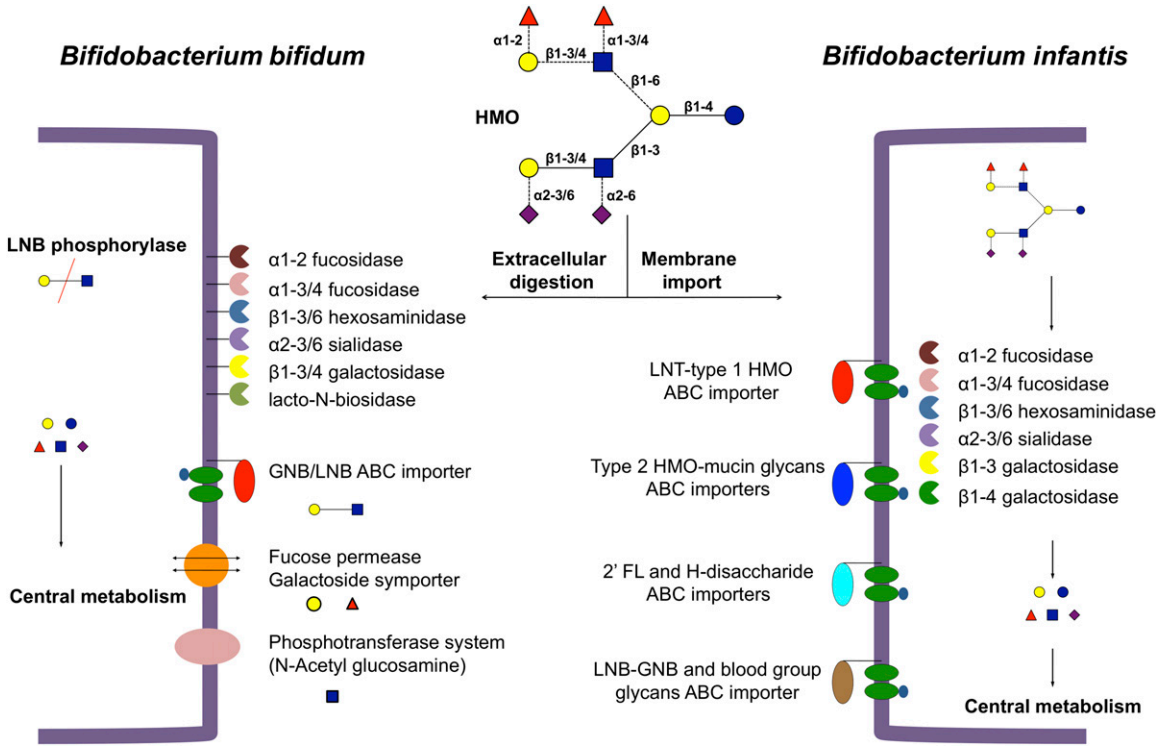

Figure 1 Possible strategies for human milk oligosaccharides (HMO) consumption in *Bifidobacterium bifidum* and *Bifidobacterium infantis*. Yellow circles indicate galactose; red triangles, fucose; blue squares, GlcNAc; purple diamonds, blue circles, glucose. Dashed lines in the HMO figure represent potential linkages. GNB, galacto-*N*-biose; LNB, lacto-*N*-biose.

In addition, two β-galactosidases, Blon_2016 and Blon_2334, were recently shown to be active on type 1 and 2 HMO linkages, respectively (63). The genes encoding these enzymes showed similar gene expression levels between glucose and HMO (63). Finally, two β-hexosaminidases in *B. infantis* are constitutively expressed during growth on HMO and lactose. Blon_2355 seems to be specific for linear GlcNAcβ1–3Gal linkages, whereas Blon_0732 and Blon_0459 can additionally release GlcNAc from branched HMO characterized by GlcNAcβ1-6Gal (64). Together, these observations indicate that glycosyl hydrolases in *B. infantis* are expressed during grown on HMO and can cleave all the different linkages found in these molecules. A summary of the molecular determinants within *B. infantis* associated to HMO import and deconstruction is presented in Table 1.

The utilization of HMO by *B. bifidum* has also been described in detail (59). This infant-borne bacterium possesses a wide array of extracellular glycosyl hydrolases that can cleave linkages found in HMO and mucin oligosaccharides (54,65,66). A single F1SBP is thought to participate in the import of LNB and galacto-*N*-biose, as well as other importers that can import monosaccharides (67). The induction of several of these enzymes has been also determined during growth in vitro on HMO or pig mucin (54). HMO consumption by *B. bifidum* is different from that of *B. infantis* in that it deploys many extracellular glycosyl hydrolases to digest HMO into components, some of which are consumed, whereas others, such as fucose, are left behind (50). It remains to be determined whether these two different HMO consumption strategies undertaken by these two species enable colonization of different niches within the infant colon. A comparison of HMO consumption between these two species is presented in **Figure 1**.

B. longum, as recently described, is routinely found in both infant and adult microbiota (53,55,68,69). However, unlike *B. bifidum* and *B. infantis*, the number of enzymes and transporters involved in the metabolism of HMO in *B. longum* appears to be limited. A membrane-associated endo-*N*-acetylgalactosaminidase has been described in certain *B. longum* strains, suggesting possible mucin oligosaccharide release (70,71). *B. longum*, as well as several infant-associated bifidobacteria, possesses a gene cluster dedicated to the metabolism of LNB and galacto-*N*-biose, linking type 1 HMO and mucin oligosaccharide consumption (55,67).

If the enrichment of bifidobacteria in the infant colon is the result of coevolution of specific bifidobacteria and milk components, one might predict that the host-microbe interface is similarly influenced by milk components. Numerous researchers have demonstrated a beneficial impact of bifidobacterial probiotics on the host in both animal models (72) and human studies (73–75). Recently Fukuda et al. (76) demonstrated that production of acetate, a main end product of bifidobacterial metabolism, is a protective factor modulating intestinal permeability in a mouse model. In that work, production of acetate by certain bifidobacterial

strains was linked to specific sugar transporters, suggesting that select sugar consumption is a driving factor for protective colonization of the host.

If milk glycans evolved as a selective substrate for specific bifidobacterial strains commonly found in infants, it is tempting to speculate that the resultant acetate production by those infant-borne bacteria is 1 mechanism by which milk-driven enrichment of a bifidobacteria protects the infant. However, other HMO-induced protective interfaces are at play as well. Recently, Chichlowski et al. (77) demonstrated that growth on HMO increases intestinal cell binding and enhances protective modulation of tight junction proteins and cytokines. In aggregate, these results advance a concept of a unique relationship between milk glycans, enrichment of specific bifidobacteria, and protection of the infant host.

Although a full mechanistic understanding of how specific human milk components promote infant growth, development, and protection remains elusive, the application of new approaches in analytical chemistry, glycobiology, and genomics have advanced our understanding tremendously. It is now clear that specific structural elements of milk oligosaccharides are crucial for their ability to selectively enrich beneficial bifidobacteria while inhibiting or acting as poor growth substrates for undesirable and pathogenic bacteria. Moreover, the genetic and enzymatic determinants that enable specific bifidobacteria to deconstruct and grow on these unique substrates are increasingly being identified and characterized, providing an emerging mechanistic picture of this enrichment. The consequences of this enrichment for the infant, however, are still relatively unclear. It can be predicted that systems biology tools such as metabolomics and next-generation sequencing of intestinal metagenomes or transcriptomes will help to identify, at a more global level, how breast milk is selective for beneficial microbes, how the target microbes respond to this stimulus, and how they interface with the host. Moreover, it is likely that these approaches will help in the design of more specific nutritional formulations, primed to drive enrichment in the infant gut of specific bacterial strains with a proven and understood health benefit.

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