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Nursing our microbiota: molecular linkages between bifidobacteria and milk oligosaccharides

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Abstract

As the sole nutrition provided to infants, bioactive molecules dissolved in milk influence the development of our gut microbiota. Accordingly, human milk oligosaccharides (HMOs) are minimally digested by the infant and persist to negatively and positively regulate gut microbiota. Accordingly, infant-type bifidobacteria utilize these soluble carbohydrate oligomers by convergent mechanisms. *Bifidobacterium longum* subsp. *infantis* efficiently consumes several small mass HMOs and possesses a large gene cluster and other loci dedicated to HMO metabolism. In contrast, adult-associated bifidobacteria such as the closely related *B. longum* subsp. *longum*, are deficient for HMO utilization, although they retain the capacity to ferment plant oligosaccharides and constituent pentose sugars. Thus, the ability to subsist on HMO may demark infant-associated ecotypes as these bifidobacteria may have adapted to colonize the nursing infant.

Milk and the neonatal superorganism

Lactation emerged as a nutritional strategy following the divergence of mammals more than 160 million years ago [1]. Most scientific inquiry into milk's biological role has focused on the linear transfer of material and energy from mother, furnished for the sole privilege of her progeny. However, as the sole source of exogenous material, the provision of breast milk to the neonate has considerable implications in the development of the infant intestinal microbiota.

Several molecules incorporated into mature milk and colostrum (see Glossary) supplement innate immunity, impacting the composition of the infant microbiota. Antimicrobial factors, several of which are activated by partial digestion of milk, include milk-borne fatty acids and peptides [2,3]. In addition, milk components such as secretory IgA, lactoferrin, lysozyme, lipoprotein lipase as well as soluble signals modulate local and systemic neonate immunity (reviewed in Ref. [4]). Whereas milk's inhibition of pathogens is well established, milk may also positively select for beneficial microbes of the gastrointestinal tract (GIT). Indeed there is nascent evidence that mother's milk supplies bacterial cells and products to potentially inoculate or tune tolerogenic responses in the infant [5,6].

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Paradoxically evading digestion, human milk oligosaccharides (HMOs) do not directly nourish the infant, but are growth factors that enrich for commensals proficient at utilizing these atypical carbohydrates [7,8]. Accordingly, microbiomes of nursing infants are often enriched for bifidobacteria and thus HMOs are believed to act as bifidogenic oligosaccharides. Numerous nutritional and clinical interventions have sought to identify bifidogenic molecules to promote desirable health outcomes linked to bifidobacteria [9,10]. This review introduces the concepts and evidence for a mammalian nutritive strategy in which milk influences, in part, the composition of the infant intestinal microbiota.

Early bifidobacterial colonization of the infant

Bifidobacteria belong to the phylum *Actinobacteria* which encompass Gram-positive bacteria characterized by chromosomes enriched for guanine and cytosine content [11]. In addition, bifidobacteria are non-motile, non-sporulating, and do not produce gas through fermentative metabolism. Whereas most species are strict anaerobes, a few have been characterized as microaerophillic [12]. Bifidobacteria are isolated from several mammalian-associated ecological niches (i.e. the oral cavity, genitourinary tract and GIT) in addition to certain insect and bird GITs as well as sewage [13]. Bifidobacteria derive their name from a characteristic bifid morphology, although they are pleomorphic *in vitro* depending on culture conditions.

Bifidobacteria are often overrepresented in the breastfed infant microbiome relative to their appearance in adults. This phenomenon has been observed in culture-based studies, and more recently, verified with molecular methodologies including those applied in a large prospective study [14–18]. There are, however, infant microbiomes characterized by a limited bifidobacterial population. In these instances, it is possible that functionally redundant microbes have actively supplanted or opportunistically seized this vacated niche [19,20]. A recent review details the establishment of gut microbiota in Western infants and potential consequences to infant health [21].

Breastfed infants typically harbor *Bifidobacterium longum*, *Bifidobacterium breve*, and/or *Bifidobacterium bifidum* within their distal GIT microbiome [22]. Though the extent, or mechanism, by which a particular bifidobacterial phylotype dominates a geographical region or correlates to the host genotype is not currently well understood. This issue is also confounded by the inability to distinguish closely related bifidobacterial taxa exclusively by 16S rDNA-based approaches. While *B. longum* subsp. *infantis* is primarily isolated from infants, the habitats which *B. longum* subsp. *longum* colonize may be more diverse [23]. Furthermore, the definition of a strict infant-type bacterium, one which is consistent from an evolutionarily perspective, is hindered by contemporary practices including cesarean delivery, the supplementation of nursing with formula and exposure of infants to antibiotics and other microbially-active compounds in the first months of life. Nevertheless, in general, infant-type bifidobacteria subsist on milk oligosaccharides or derivatives, congruent with their frequent predominance in the breastfed infant colon [24].

HMO structure and properties

HMOs are a heterogeneous mix of soluble glycans that can often exceed the protein content of human breast milk [25]. The precise concentration of milk oligosaccharides vary by individual and declines over the course of lactation, but they are generally present at ≥ 4 g/L in milk, with higher levels observed in colostrum [26]. Milk oligosaccharides contain N-acetylglucosamine with a degree of polymerization (DP) ≥ 4 and incorporate D-glucose, D-galactoseL-fucose and N-acetylneuraminic acid (Neu5Ac) residues in an assortment of isomeric configurations. Lactose (Galβ1-4Glc) is found at the reducing end with lacto-*N*-biose I units (LNB; Galβ1-3GlcNAc) or lactosamine (Galβ1-4GlcNAc) in the more rare type II structures. Both LNB and lactosamine are elongated from a β1–3 linkage to the lactosyl

terminus, with an additional β1–6 linkage in branched forms. Major variations in DP arise from serial integration of multiple LNB units. Further complexity is provided by terminal fucosylation via α1-2/3/4 linkages and/or α2-3/6 sialylation. Absent fucosidase and sialidase activities, these residues obstruct HMO core structures from microbial fermentation. Milk oligosaccharides have also been characterized in domesticated animals including cow and goat, although they are generally lower in abundance and vary in prevalence of specific oligosaccharide compositions [27,28]. In addition, animal milk oligosaccharides include N-glycolylneuraminic acid residues in contrast with HMO, as modern humans lost the ability to synthesize this sialic acid[29]. A structural comparison of HMO to other bifidogenic substrates intended to mimic these milk sugars is depicted in Figure 1.

Interestingly, only ~200 distinct compositions have been identified in pooled breast milk despite HMO's immense combinatorial potential [30]. Indeed, the constraints on HMO synthesis is suggestive of an adaptation for structure-specific functions, such as the prevalence of type I (β1–3) linkages in core LNB of human oligosaccharides. Accordingly, HMO structures do vary among maternal genotypes, with the particular oligosaccharide complement present in milk dependent on Lewis blood group and secretor status [31].

In addition to bifidogenic enrichment, HMOs participate in innate immunity to pathogen colonization by saturating the lower GIT with soluble ligands [32]. HMO motifs reflect the same glycan structures found in mucins and epithelial glycoconjugates. Thus the numerically dominant HMOs outcompete preferred host epitopes for ligation by pathogen adhesins. A well-characterized example of this ligand mimicry is provided by α1,2-fucosylated HMOs which mimic the H-2 epitope to minimize *Campylobacter jejuni* binding and infection *in vivo* [33]. Similar barriers to autochthonous colonization have been described in the intestinal mucosa [34], the laryngopharynx [35] and the urinary tract since some HMOs are also absorbed and excreted by the infant [36]. In addition, HMOs affect epithelial glycosyltransferase expression which, in turn, modulates the glycan topology presented to microbes [37,38]. Finally, there is mounting evidence that HMOs initiate signal cascades in immune and other cell types [39, 40]. Further information on HMO structure and function is provided by recent reviews of the topic [41].

The genomics of bifidobacterial oligosaccharide metabolism

The 20 draft and finished *Bifidobacterium* genomes (March 2009) have considerably advanced our understanding of bifidobacterial metabolism [42]. Accordingly, both glycosidases and membrane-spanning transporters are essential to avail oligosaccharides or derivatives to the bifidobacterial fructose-6-phosphate phosphoketolase central metabolic pathway. These oligosaccharide-processing genes are typically clustered within conserved modules consisting of upstream regulatory elements, ABC (ATP-binding cassette) transporters with high-affinity carbohydrate binding proteins and one or more glycosyl hydrolase [43–46]. Several of these oligosaccharide clusters contain recently duplicated genes and/or mobile elements suggesting rapid innovation of polysaccharide metabolism to process available carbohydrate substrates. Accordingly, ~12% of bifidobacterial genes assigned a COG have been designated as carbohydrate transport or metabolism-related, as one would predict in a saccharolytic microorganism (Figure 2). Likewise, a sizable fraction of bifidobacterial genomes (~5%) are predicted to encode carbohydrate transport proteins (Figure 2) [47]. Evidently, bifidobacteria seize environmental carbohydrates through their extensive complement of ATP-dependent transporters, cation symporters, phosphotransferase systems as well as other translocation mechanisms [43,45,48,49]. This contrasts starkly with *Bacteroides* and other members of the GIT consortium that secrete an arsenal of glycosidases to compete for complex carbohydrates [50,51]. *Bifidobacterium animalis* subsp. *lactis* is a notable exception as its genome is relatively

deficient for glycosidases, and ABC transporter genes appear independent of an operon structure [46].

Bifidobacterial metabolism of HMO core structures

Select bifidobacteria, those typically isolated from infants, are proficient at capturing and utilizing HMO as a sole carbon source [24,52–54]. The infant commensal *B. longum* subsp. *infantis* achieves high cell density on this purified substrate and is regarded as the archetypical HMO consumer [45]. HMO consumption is conserved in the *B. longum* subsp. *infantis* lineage, whereas other infant-type bifidobacteria exhibit more strain-specific phenotypic variation [24]. This variation is manifested in *B. bifidum* ATCC 29521 which degrades HMO, although it does not consume portions of the hydrolyzed oligosaccharide. Yet other bifidobacteria which readily utilize monosaccharides liberated from HMO are incapable of cleaving intact HMO as was observed for *B. breve* ATCC 27539 [24,53]. This phenotypic diversity hints at niche partitioning within the infant GIT consortium and potential protocooperation among bifidobacterial phylotypes. While unable to directly access HMO carbon, certain bacteria may release oligosaccharide-bound monosaccharides to the benefit of scavenging heterologous consortium members (e.g. *B. breve*) (Figure 3). Accordingly, a mixed-species transcriptome of the breastfed infant microbiome is generally enriched for bifidobacterial carbohydrate utilization, suggesting that milk sugars are actively metabolized by phylotypes incapable of utilizing intact HMO under *in vitro* isolation [55].

To further resolve the HMO utilization phenotype, mass spectrometry has been employed to glycoprofile the specific HMO masses consumed in axenic fermentations [30]. Most HMO utilizing bifidobacteria metabolize only a single composition corresponding to lacto-*N*-tetraose (LNT; Galβ1-3GlcNAc β1-3 Galβ1-4Glc) [24,54]. The LNT tetrasaccharide is a core structure found invariably in higher molecular weight HMO. While *B. longum* subsp. *infantis* consumes LNT to extinction, *B. longum* subsp. *longum*, *B. breve* (Figure 4) and *B. bifidum* [24]exhibits a more modest degradation of this molecule. Thus unmodified LNT (i.e. non-fucosylated or sialylated) is susceptible to enzymatic degradation by several representative bifidobacteria. Interestingly, adult-type bifidobacteria, *B. adolescentis* and *B. animalis* do not degrade LNT or other HMO species. This may be due in part to the absence of an identifiable lacto-*N*-biose phosphorylase gene (EC 2.4.1.211) in these species.

Moreover, type I glycans such as HMO incorporate repeating LNB, a disaccharide that can be synthesized *in vitro* by enzymes derived from *B. bifidum* and *B. longum* subsp. *longum* [56]. Human milk differs from most other animal milks by the predominance of LNB as the repeating unit instead of N-acetlyllactosamine (Galβ1-4GlcNAc), which has led some to postulate that LNB is the essential bifidogenic factor delivered in human breast milk [57]. LNB promotes bifidobacterial growth in species that are able to utilize it as a sole carbon source [58]. In these instances, LNB is a proxy for intact HMO molecules as it is not abundant in milk as a soluble dissacharide, and lacks the full structural diversity, and thus function, of HMO. Nevertheless, synthetic LNB offers a significant advance in HMO research as large-scale oligosaccharide purification from fluid milk remains a challenge. Indeed infant-type bifidobacteria have been demonstrated to consume purified LNB whereas adult-type phylotypes generally do not [59].

In apparent contrast with *B. longum* subsp. *infantis*, *B. bifidum* acquires HMO-bound LNB with extracellular LNB liberating enzymes as depicted in Figure 3. Accordingly, *B. bifidum* JCM1254 secretes an extracellular 1,2-α-L-fucosidase (AfcA) and 1-3/4-α-L-fucosidase (AfcB) which cleave terminal fucosyl linkages, permitting further degradation of the LNB core structure to proceed [60–62]. Subsequent to defucosylation, a lacto-*N*-biosidase (EC 3.2.1.140) liberates LNB from lacto-*N*-tetraose and other HMO compositions lacking fucosylated or sialylated LNB moieties [63]. Once released, LNB is translocated across the cell membrane

by an ABC transporter associated with an LNB-specific SBP [64,65]. Interestingly, *B. longum* subsp. *longum* possesses an endo-α-*N*-acetylgalactosaminidase (EC 3.2.1.97) which liberates galacto-*N*-biose (GNB) from *O*-linked mucin glycoproteins [66]. Indeed, the presence of both a endo-α-*N*-acetylgalactosaminidase and fucosidase has been linked to the *B. bifidum* mucin degradation phenotype, with expression of both genes induced in the presence of porcine mucin [67]. An intracellular phosphorylase cleaves the LNB-GNB disaccharide derived from HMO or mucin [57,68]. Finally, a modified Leloir pathway feeds galactose into the central fructose-6-phosphate phosphoketolase pathway to generate cellular ATP [69].

B. longum subsp. *infantis* consumes a range of short chain HMO

B. longum subsp. *infantis* has evolved within a host whose sole source of exogenous nutrition is mother's milk [45]. As a potential consequence, *B. longum* subsp. *infantis* exhibits vigorous growth on several neutral small mass oligosaccharides that are secreted coincident with the start of lactation, whereas other bifidobacteria only partially consume LNT, if at all (Figure 4) [52,54]. Although hypothesized, the extent to which bifidobacteria utilize the less abundant sialylated milk oligosaccharides is not currently well understood.

Subsistence on a broad assortment of milk oligosaccharides is now known to be a chromosomally encoded trait [45]. *B. longum* subsp. *infantis* possesses several HMO-active gene clusters encoding sialidases (EC 3.2.1.18) and fucosidases (EC 3.2.1.51), which are expressed to process sialylated and fucosylated glycans, respectively [54]. This hydrolytic capability broadens its oligosaccharide utilization range to include terminally substituted HMO by enabling access to LNT or LNB core structures. These HMO-active gene clusters are scattered throughout the *B. longum* subsp. *infantis* genome and are conspicuously absent from the subspecies *longum* genome. Of particular significance is the identification of a contiguous (43 kbp) genomic tract consisting of HMO-related genes in *B. longum* subsp. *infantis* [42]. As with other bacterial genomes, genetic linkage suggests a greater likelihood of lateral gene transfer preceding fixation within the subspecies *infantis* clade. Likewise, there is an increased probability for transfer of this locus to other members of the GIT consortium. Moreover, the orientation of genes in a concerted transcriptional direction may be indicative of co-regulation. Finally, and most notable with regards to milk utilization, gene co-localization is a hallmark of participation in a common metabolic operation. This HMO cluster, and other milk-active glycolytic clusters, is not unique to the fully sequenced type strain of *B. longum* subsp. *infantis* ATCC 15697, as confirmed by sequencing of additional subspecies *infantis* genomes [45].

The 43 kbp HMO cluster encodes glycosyl hydrolases active on the four milk oligosaccharide glycosidic linkages (i.e. α-fucosidase, α-sialidase, β-galactosidase and β-N-hexosaminidase). These four glycosidases are interspersed amid an array of ABC transporters and their associated solute binding proteins (SBPs) predicted to bind oligosaccharides. Interestingly, these SBPs exhibit a pronounced sequence divergence relative to other family 1 SBPs observed in ATCC 15697 as well as *B. longum* subsp. *longum* NCC2705 and *B. adolescentis* ATCC15703. The importance of oligosaccharide transport is further accentuated by the predicted intracellular localization of subspecies *infantis* glycosidases, which generally lack identifiable transmembrane domains, secretion signals or Gram-positive cell wall anchor motifs. This is consistent with the glycoprofile which clearly demonstrates that ATCC 15697 preferably utilizes HMO compositions *in vitro* that are DP ≤8 with a molecular mass < 1400 Da (Figure 4). This restriction may be due to transporter specificity or steric factors that inhibit translocation of large substrates across the cell envelope. The precise identities of oligosaccharide structural isomers consumed are an area of active research.

Many glycosidases, transporters and catabolic enzymes predicted to be active on HMO and derivatives were detected by proteomics while growing on HMO. This partially verified their participation in HMO catabolism. Furthermore, *in vivo* metabolic flux analysis has demonstrated that metabolites derived from HMO and sialic acid Neu5Ac are metabolized via the fructose-6-phosphate phosphoketolase pathway in *B. longum* subsp. *infantis* ATCC 15697 [45].

Divergence of carbohydrate utilization in *B. longum*

Significant metabolic disparities are expected between bacterial species, particularly those isolated from dissimilar habitats. Somewhat surprisingly, a conspecific genome comparison between *B. longum* subsp. *longum* and subsp. *infantis* has revealed a vastly divergent intraspecies strategy for carbohydrate acquisition [45]. Subspecies *infantis* oligosaccharide catabolic clusters are generally dedicated to mammalian glycan metabolism, many of which are fucosylated and/or sialylated. **Evidently, xylose and arabinose metabolism has been supplanted by sialic acid and fucose catabolism, as pentose metabolic gene clusters present in subspecies *longum* are absent or degraded in subspecies *infantis*.**.

The inability of subspecies *infantis* to ferment arabinogalactan or constituent arabinose residues provides a functional manifestation of this reorientation to mirror its host's primary dietary polysaccharide [23]. In *B. longum* subsp. *longum*, however, an extracellular endogalactanase liberates galactotrisaccharides from arabinogalactan, an arabinose-substituted galactan produced by various plants such as potato and soybean. Interestingly, in the analogous chromosomal locus, *B. longum* subsp. *infantis* has a severely truncated endogalactanase gene (Blon_0440) located adjacent to a similarly degraded β-galactosidase in the absence of the sugar ABC transporter (Figure 5) [43]. Evidently, these genes became expendable subsequent to the divergence of subspecies *infantis* from *longum*, as the endogalactanase appears to have been acquired following the divergence of the progenitors of *B. longum* and *B. adolescentis*.

Furthermore, *B. longum* subsp. *longum* possesses an arabinose isomerase (*araA*) and L-ribulose-5-phosphate 4-epimerase (*araD*), both of which are absent in *B. longum* subsp. *infantis*. In *B. longum* subsp. *longum* these genes enable conversion of L-arabinose to L-xylulose-5-phosphate with the aid of an L-ribulokinase. A recent non-orthologous displacement appears responsible for arabinose gene decay in *B. longum* subsp. *infantis*. Remarkably, a fucosidase and a permease (Blon_0425 and Blon_0426) are situated in place of the three arabinose catabolic genes, with a vestigial kinase fragment to demarcate the ancestral arabinose cluster's location [42]. This strongly suggests selection for fucosylated mammalian glycan metabolism in lieu of arabinose in a bifidobacterial clade that ceased to encounter plant-derived sugars during its evolution. The apparent selection that has culled arabinose catabolism from subspecies *infantis* has likely encouraged a similar purge in other infant-type bifidobacteria. Both *B. bifidum* NCIMB 41171 and *B. breve* DSM 20213 are missing this arabinose cluster while preserving a small fragment of *araD* in a similar genomic context to indicate its ancestral presence, although fucose-related genes have not been inserted in its place [70].

Furthermore, among bifidobacteria that ferment pentoses, xylose is isomerized (XylA; EC 5.3.1.5) and phosphorylated (XylB; EC 2.7.1.17) prior to entry into central metabolism as xylulose-5-phosphate. Consistent with its xylose-negative phenotype, the *B. longum* subsp. *infantis* ATCC 15697 genome is devoid of a recognizable xylose isomerase (*xylA*) in clear contrast to other bifidobacteria, including subsp. *longum*, which localizes *xylA* upstream of a gene cluster dedicated to xylose metabolism including *xylB* [43,45]. As was evident in the loss of arabinose metabolism, *xylA* is absent from the infant-types *B. bifidum* NCIMB 41171 and *B. breve* DSM 20213 [70]. In *B. longum* subsp. *infantis* ATCC 15697, the severely truncated

xylB gene is likely non-functional (Blon_0572) as only a third of the 5′ terminus is retained while absent from the *B. bifidum* NCIMB 41171 genome altogether.

Milk oligosaccharide mimetics

Numerous applications have been developed to mimic the bifidogenic properties of milk oligosaccharides, though they lack the inherent complexity of HMOs in terms of monosaccharide composition, linkage diversity and structural organization (Figure 1). Nevertheless, there has been much attention given to commercial bifidogenic substrates, as alternatives to HMO, since *in vitro* and clinical evidence support their bifidogenic efficacy [71–73].

Milk oligosaccharide mimetics include fructo-oligosaccharides (FOS) commonly extracted as inulin from chicory or other plant sources. FOS is broadly bifidogenic and is utilized by most bifidobacteria, in contrast to HMO's observed specificity [53]. Galacto-oligosaccharides (GOS) are enzymatically synthesized from dairy galactose (reviewed in Ref. [74]). Similar to FOS, their structural potential is markedly less than that of milk oligosaccharides albeit more diverse than linear FOS fructans. The basic structure of GOS incorporates lactose at the reducing end which is typically elongated with up to six galactose residues ([Gal(β1-3/4/6)$_{1–6}$)Gal(β1-4)Glc). There is some confusion concerning the relative contribution of naturally occurring GOS to the human milk glycome [75,76]. While limited short-chain galactosyllactose (Gal(β1-3/4/6)Gal(β1-4)Glc) has been detected at millimolar concentrations in breast milk, this is 3–4 orders of magnitude lower than the total concentration of the HMO pool [77]. There is little evidence for high DP (≥4) GOS in human milk, despite an entrenched, and often repeated, misperception.

It remains unclear, however, if these oligosaccharide analogs retain similar immunological and pathogen deflection functions and whether they selectively increase bifidobacterial populations to the exclusion of undesirable genera. Importantly, nutraceutical oligosaccharides do not reflect the genomic and physiological links between infant-type bifidobacteria and HMO; instead they target the bifidobacterial population non-specifically.

Milk oligosaccharide utilization by other bacteria

Although the *B. longum* subsp. *infantis* HMO consumption phenotype is predicated on intracellular glycosidases and solute transporters, the presence of similar genes in other species may not be indicative of a coevolutionary relationship with milk. To wit, several genera such as soil *Streptomyces*, do not encounter HMO in their ecological niche, but secrete a wide range of glycosidases which may enable milk glycan utilization upon *in vitro* assay. A less definitive case is provided by mucolytic gut commensals such as *Bacteroides*, since *Bacteroides fragilis* and *Bacteroides vulgatus* have been observed to ferment HMO *in vitro* (D. Mills, unpublished data). *Bacteroides* typically dominate the adult distal GIT, and colonize infants more variably and at lower concentrations than bifidobacteria [15]. Whether early colonizing *Bacteroides* utilize HMO *in vivo* or subsist on other infant or milk glycoconjugates is not yet known. One may speculate that bifidobacteria successfully compete with *Bacterioides* in the infant colon by virtue of a superior ability to sequester soluble HMO or degradation products, although a thorough vetting of this hypothesis remains to be performed.

Conclusions and future directions

Milk defines infant nutrition and, by extension, is integral to the natural history of the earliest symbionts to colonize *Homo sapiens*. Indigestible milk oligosaccharides negatively regulate the infant microbiota via ligand mimicry, in addition to positively selecting for bifidobacteria. Accordingly, infant-type bifidobacteria possess the requisite gene suite active on mammalian

glycans to enable hydrolysis of atypical HMO structures. In particular, *B. longum* subsp. *infantis* imports and subsequently hydrolyzes HMO with sialidases and fucosidases to present LNT and other core glycan domains for further processing [45,53]. The presence of a large gene cluster dedicated to HMO metabolism is consistent with this subspecies's efficient utilization of an assortment of small mass milk oligosaccharides. This utilization trait contrasts markedly with *B. longum* subsp. *longum*, which does not metabolize milk oligosaccharides to the same degree, although it retains the capacity to ferment plant oligosaccharides and their constituent pentose sugars [23,24]. The selective pressures that have restricted plant oligosaccharide metabolism in infant-type bifidobacteria are as profound as those that actuated milk glycan utilization in the same lineages. Moreover, convergent strategies to utilize HMO by *B. breve* and *B. bifidum* have likely evolved independently subsequent to associating with mammals. In addition to evaluating mechanisms that contribute to these physiological disparities, it is of great interest to discriminate traits shared through lateral gene transfer from those that were innovated vertically. This would help describe infant-associated ecotypes within the genus *Bifidobacterium*, as those that have likely adapted to subsist on milk molecules.

Reconciling a causal linkage of *in vivo* bifidobacterial colonization to the act of nursing currently transcends available methodologies, although barriers that have thwarted genetic manipulations of bifidobacteria have begun to be addressed [78]. In addition, reliable genetic tools would help further resolve the metabolic definition of the HMO utilization phenotype. Separation or synthesis of gram quantities of pure HMO species is critical in this regard, along with a systems-level understanding of metabolic regulation and function in HMO consuming organisms.

While it is tempting to classify bacterial oligosaccharide metabolism according to binary models such as intracellular versus extracellular utilization, mammalian versus plant glycan preference, *Bifidobacterium* versus *Bacteroides* and so forth, such discrete schema do not satisfactorily describe the emergent biology inherent to the infant microbiota. As with many biological systems, the boundaries delineating microbial actors and their biochemical actions positioned in syntrophic networks are amorphous and frustrate rigid classification. Thus it is somewhat surprising that milk's influence on the infant microbiota is so readily identifiable in a particular symbiont rather than only detectable in the aggregate microbiome. This provocatively suggests a vital role for *B. longum* subsp. *infantis*, particularly if it is determined to be one of several functionally redundant core members of the infant microbiome. That this phylotype, and other infant-associated bifidobacteria, specialize to the degree that they are unable to persist past weaning is a question fundamental to evolution of human development.

Clearly milk is one of several principle factors that dictate the population structure of the infant microbiome. As such, microbial population fluxes observed during weaning are likely due to the alleviation of suppressive antimicrobials in milk, removal of growth factors and the addition of foods that encourage microbial diversity. The evolved codependence between humans and our earliest symbionts may ensure the proper development of the superorganism, and is expected to have profound and persistent implications to host homeostasis throughout life. Assuming that milk's evolutionary vector is generally oriented towards promoting infant fitness, orchestration of the neonate's microbiota via oligosaccharides and other molecules may have been an adaptive consequence.

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Glossary

ATP-binding cassette (ABC) transporter	these hydrolyze ATP to catalyze solute translocation
Bifidogenic oligosaccharide	a human-indigestible carbohydrate oligomer that is used as a fermentation substrate by bifidobacteria and enriches the representation of these commensals in the distal GIT
Clusters of orthologous groups of proteins (COG)	protein family classification based on orthology in three or more phylogenetic lineages
Colostrum	yellowish fluid secreted by mammals during the first few days of lactation prior to the production of true milk. This is particularly abundant in growth factors, immunological molecules and oligosaccharides among other bioactive molecules
Core microbiome	the set of shared microbial genes in a given niche which is invariable across human microbiomes
Degree of polymerization (DP)	the number of monosaccharide residues in a given oligosaccharide composition
Ecotype	a genetically distinct population within a species that has adapted to a specific habitat
Glycoprofile	mass spectrometry employed to monitor the abundances of a range of oligosaccharide masses. Often used with regards to microbial consumption of oligosaccharides
Lewis blood group	human blood typing system based on two primary Lewis antigens. Depending on which one is present, this antigen strongly correlates with secretion of blood type antigens in fluids by those termed secretors, or not, in non-secretor phenotypes
Microbiota	a microbial community that has been established in a given habitat. Typically used to refer to the microbial population associated with a healthy individual or tissue
Microbiome	the aggregate genomes present in a particular microbiota
Neonate	a newborn infant within its first 28 days of life.[GT1]
Niche partitioning	the process by which selection has segregated competing phylotypes into different patterns of resource use
Phylotype	a phylogenetic cluster of microbes which share a common ancestor and transcended a threshold of molecular divergence from closely-related lineages
Protocooperation	an interaction between two phylotypes that benefits both and not necessarily required for growth or survival of either
Syntrophy	a relationship by which a microbial phylotype is dependent on another to provide a nutritional requirement

References

1. Oftedal OT. The mammary gland and its origin during synapsid evolution. J Mammary Gland Biol Neoplasia 2002;7:225–252. [PubMed: 12751889]
2. Phadke SM, et al. Antimicrobial peptides in mucosal secretions: the importance of local secretions in mitigating infection. J Nutr 2005;135:1289–1293. [PubMed: 15867326]
3. Thormar H, Hilmarsson H. The role of microbicidal lipids in host defense against pathogens and their potential as therapeutic agents. Chem Phys Lipids 2007;150:1–11. [PubMed: 17686469]
4. Lawrence RM, Pane CA. Human breast milk: current concepts of immunology and infectious diseases. Curr Probl Pediatr Adolesc Health Care 2007;37:7–36. [PubMed: 17157245]
5. Martin R, et al. Cultivation-independent assessment of the bacterial diversity of breast milk among healthy women. Res Microbiol 2007;158:31–37. [PubMed: 17224259]
6. Perez PF, et al. Bacterial imprinting of the neonatal immune system: lessons from maternal cells? Pediatrics 2007;119:e724–732. [PubMed: 17332189]
7. Gnoth MJ, et al. Human milk oligosaccharides are minimally digested in vitro. J Nutr 2000;130:3014–3020. [PubMed: 11110861]
8. Engfer MB, et al. Human milk oligosaccharides are resistant to enzymatic hydrolysis in the upper gastrointestinal tract. Am J Clin Nutr 2000;71:1589–1596. [PubMed: 10837303]
9. Moro G, et al. A mixture of prebiotic oligosaccharides reduces the incidence of atopic dermatitis during the first six months of age. Arch Dis Child 2006;91:814–819. [PubMed: 16873437]
10. Arslanoglu S, et al. Early dietary intervention with a mixture of prebiotic oligosaccharides reduces the incidence of allergic manifestations and infections during the first two years of life. J Nutr 2008;138:1091–1095. [PubMed: 18492839]
11. Ventura M, et al. Genomics of *Actinobacteria*: tracing the evolutionary history of an ancient phylum. Microbiol Mol Biol Rev 2007;71:495–548. [PubMed: 17804669]
12. Kawasaki S, et al. Response of the microaerophilic *Bifidobacterium* species, *B. boum* and *B. thermophilum*, to oxygen. Appl Environ Microbiol 2006;72:6854–6858. [PubMed: 16950914]
13. Turroni F, et al. Human gut microbiota and bifidobacteria: from composition to functionality. Antonie Van Leeuwenhoek 2008;94:35–50. [PubMed: 18338233]
14. Harmsen HJM, et al. Analysis of intestinal flora development in breast-fed and formula-fed Infants by using molecular identification and detection methods. Journal of Pediatric Gastroenterology and Nutrition 2000;30:61–67. [PubMed: 10630441]
15. Penders J, et al. Factors influencing the composition of the intestinal microbiota in early infancy. Pediatrics 2006;118:511–521. [PubMed: 16882802]
16. Yoshioka H, et al. Development and differences of intestinal flora in the neonatal period in breast-fed and bottle-fed infants. Pediatrics 1983;72:317–321. [PubMed: 6412205]
17. Favier CF, et al. Molecular monitoring of succession of bacterial communities in human neonates. Appl Environ Microbiol 2002;68:219–226. [PubMed: 11772630]
18. Mariat D, et al. The *Firmicutes*/*Bacteroidetes* ratio of the human microbiota changes with age. BMC Microbiol 2009;9:123. [PubMed: 19508720]
19. Palmer C, et al. Development of the human infant intestinal microbiota. PLoS Biol 2007;5:e177. [PubMed: 17594176]
20. Kurokawa K, et al. Comparative metagenomics revealed commonly enriched gene sets in human gut microbiomes. DNA Res 2007;14:169–181. [PubMed: 17916580]
21. Adlerberth I, Wold AE. Establishment of the gut microbiota in Western infants. Acta Paediatr 2009;98:229–238. [PubMed: 19143664]
22. Matsuki T, et al. Genus- and species-specific PCR primers for the detection and identification of bifidobacteria. Current Issues in Intestinal Microbiology 2003;4:61–69. [PubMed: 14503690]
23. Mattarelli P, et al. Proposal to reclassify the three biotypes of *Bifidobacterium longum* as three subspecies: *Bifidobacterium longum* subsp. *longum* subsp. nov., *Bifidobacterium longum* subsp. *infantis* comb. nov. and *Bifidobacterium longum* subsp. *suis* comb. nov. Int J Syst Evol Microbiol 2008;58:767–772. [PubMed: 18398167]

24. LoCascio RG, et al. A versatile and scalable strategy for glycoprofiling bifidobacterial consumption of human milk oligosaccharides. Microbial Biotechnology 2009;2:333–342.
25. Coppa GV, et al. Changes in carbohydrate composition in human milk over 4 months of lactation. Pediatrics 1993;91:637–641. [PubMed: 8441573]
26. Asakuma S, et al. Variation of major neutral oligosaccharides levels in human colostrum. Eur J Clin Nutr 2008;62:488–494. [PubMed: 17375110]
27. Tao N, et al. Bovine milk glycome. J Dairy Sci 2008;91:3768–3778. [PubMed: 18832198]
28. Urashima T, et al. Oligosaccharides of milk and colostrum in non-human mammals. Glycoconj J 2001;18:357–371. [PubMed: 11925504]
29. Chou HH, et al. A mutation in human CMP-sialic acid hydroxylase occurred after the *Homo-Pan* divergence. Proceedings of the National Academy of Sciences of the United States of America 1998;95:11751–11756. [PubMed: 9751737]
30. Ninonuevo MR, et al. A strategy for annotating the human milk glycome. J Agric Food Chem 2006;54:7471–7480. [PubMed: 17002410]
31. Kunz C, et al. Oligosaccharides in human milk: structural, functional, and metabolic aspects. Annu Rev Nutr 2000;20:699–722. [PubMed: 10940350]
32. Newburg DS. Neonatal protection by an innate immune system of human milk consisting of oligosaccharides and glycans. J Anim Sci 2009;87:26–34. [PubMed: 19028867]
33. Ruiz-Palacios GM, et al. *Campylobacter jejuni* binds intestinal H(O) antigen (Fuc alpha 1, 2Gal beta 1, 4GlcNAc), and fucosyloligosaccharides of human milk inhibit its binding and infection. J Biol Chem 2003;278:14112–14120. [PubMed: 12562767]
34. Newburg DS, et al. Innate protection conferred by fucosylated oligosaccharides of human milk against diarrhea in breastfed infants. Glycobiology 2004;14:253–263. [PubMed: 14638628]
35. Andersson B, et al. Inhibition of attachment of *Streptococcus pneumoniae* and *Haemophilus influenzae* by human milk and receptor oligosaccharides. J Infect Dis 1986;153:232–237. [PubMed: 3484772]
36. Obermeier S, et al. Secretion of 13C-labelled oligosaccharides into human milk and infant's urine after an oral [13C]galactose load. Isotopes Environ Health Stud 1999;35:119–125. [PubMed: 10902537]
37. Angeloni S, et al. Glycoprofiling with micro-arrays of glycoconjugates and lectins. Glycobiology 2005;15:31–41. [PubMed: 15342550]
38. Vanmaele RP, et al. Role of lactosyl glycan sequences in inhibiting enteropathogenic *Escherichia coli* attachment. Infect Immun 1999;67:3302–3307. [PubMed: 10377105]
39. Velupillai P, Harn DA. Oligosaccharide-specific induction of interleukin 10 production by B220+ cells from schistosome-infected mice: a mechanism for regulation of CD4+ T-cell subsets. Proc Natl Acad Sci U S A 1994;91:18–22. [PubMed: 7904066]
40. Eiwegger T, et al. Human milk--derived oligosaccharides and plant-derived oligosaccharides stimulate cytokine production of cord blood T-cells in vitro. Pediatr Res 2004;56:536–540. [PubMed: 15295093]
41. Bode L. Recent advances on structure, metabolism, and function of human milk oligosaccharides. J Nutr 2006;136:2127–2130. [PubMed: 16857829]
42. Liolios K, et al. The Genomes On Line Database (GOLD) in 2007: status of genomic and metagenomic projects and their associated metadata. Nucleic Acids Res 2008;36:D475–479. [PubMed: 17981842]
43. Schell MA, et al. The genome sequence of *Bifidobacterium longum* reflects its adaptation to the human gastrointestinal tract. Proc Natl Acad Sci U S A 2002;99:14422–14427. [PubMed: 12381787]
44. Lee JH, et al. Comparative genomic analysis of the gut bacterium *Bifidobacterium longum* reveals loci susceptible to deletion during pure culture growth. BMC Genomics 2008;9:247. [PubMed: 18505588]
45. Sela DA, et al. The genome sequence of *Bifidobacterium longum* subsp. *infantis* reveals adaptations for milk utilization within the infant microbiome. Proc Natl Acad Sci U S A 2008;105:18964–18969. [PubMed: 19033196]
46. Barrangou R, et al. Comparison of the complete genome sequences of *Bifidobacterium animalis* subsp. *lactis* DSM 10140 and Bl-04. J Bacteriol 2009;191:4144–4151. [PubMed: 19376856]

47. Ventura M, et al. Genome-scale analyses of health-promoting bacteria: probiogenomics. Nat Rev Microbiol 2009;7:61–71. [PubMed: 19029955]
48. Maze A, et al. Identification and characterization of a fructose phosphotransferase system in *Bifidobacterium breve* UCC2003. Appl Environ Microbiol 2007;73:545–553. [PubMed: 17098914]
49. Parche S, et al. Sugar transport systems of *Bifidobacterium longum* NCC2705. J Mol Microbiol Biotechnol 2007;12:9–19. [PubMed: 17183207]
50. Xu J, et al. A genomic view of the human-*Bacteroides thetaiotaomicron* symbiosis. Science 2003;299:2074–2076. [PubMed: 12663928]
51. Ramsay AG, et al. Cell-associated alpha-amylases of butyrate-producing Firmicute bacteria from the human colon. Microbiology 2006;152:3281–3290. [PubMed: 17074899]
52. Ward RE, et al. In vitro fermentation of breast milk oligosaccharides by *Bifidobacterium infantis* and Lactobacillus gasseri. Appl Environ Microbiol 2006;72:4497–4499. [PubMed: 16751577]
53. Ward RE, et al. In vitro fermentability of human milk oligosaccharides by several strains of bifidobacteria. Mol Nutr Food Res 2007;51:1398–1405. [PubMed: 17966141]
54. LoCascio RG, et al. Glycoprofiling of bifidobacterial consumption of human milk oligosaccharides demonstrates strain specific, preferential consumption of small chain glycans secreted in early human lactation. J Agric Food Chem 2007;55:8914–8919. [PubMed: 17915960]
55. Klaassens ES, et al. Mixed-species genomic microarray analysis of fecal samples reveals differential transcriptional responses of bifidobacteria in breast- and formula-fed infants. Appl Environ Microbiol 2009;75:2668–2676. [PubMed: 19286790]
56. Nishimoto M, Kitaoka M. Practical preparation of lacto-N-biose I, a candidate for the bifidus factor in human milk. Biosci Biotechnol Biochem 2007;71:2101–2104. [PubMed: 17690443]
57. Kitaoka M, et al. Novel putative galactose operon involving lacto-N-biose phosphorylase in *Bifidobacterium longum*. Appl Environ Microbiol 2005;71:3158–3162. [PubMed: 15933016]
58. Kiyohara M, et al. Prebiotic effect of Lacto-N-biose I on bifidobacterial growth. Biosci Biotechnol Biochem 2009;73:1175–1179. [PubMed: 19420691]
59. Xiao JZ, et al. Distribution of in vitro fermentation ability of Lacto-N-Biose I, a major building block of human milk oligosaccharides, in bifidobacterial strains. Appl Environ Microbiol 2009;76:54–59. [PubMed: 19854932]
60. Katayama T, et al. Molecular cloning and characterization of *Bifidobacterium bifidum* 1,2-alpha-L-fucosidase (AfcA), a novel inverting glycosidase (glycoside hydrolase family 95). J Bacteriol 2004;186:4885–4893. [PubMed: 15262925]
61. Nagae M, et al. Structural basis of the catalytic reaction mechanism of novel 1,2-alpha-L-fucosidase from *Bifidobacterium bifidum*. J Biol Chem 2007;282:18497–18509. [PubMed: 17459873]
62. Ashida H, et al. Two distinct alpha-L-fucosidases from *Bifidobacterium bifidum* are essential for the utilization of fucosylated milk oligosaccharides and glycoconjugates. Glycobiology 2009;19:1010–1017. [PubMed: 19520709]
63. Wada J, et al. *Bifidobacterium bifidum* lacto-N-biosidase, a critical enzyme for the degradation of human milk oligosaccharides with a type 1 structure. Appl Environ Microbiol 2008;74:3996–4004. [PubMed: 18469123]
64. Suzuki R, et al. Structural and thermodynamic analyses of solute-binding protein from *Bifidobacterium longum* specific for core 1 disaccharide and lacto-N-biose I. J Biol Chem 2008;283:13165–13173. [PubMed: 18332142]
65. Wada J, et al. Purification, crystallization and preliminary X-ray analysis of the galacto-N-biose-/lacto-N-biose I-binding protein (GL-BP) of the ABC transporter from *Bifidobacterium longum* JCM1217. Acta Crystallogr Sect F Struct Biol Cryst Commun 2007;63:751–753.
66. Fujita K, et al. Identification and molecular cloning of a novel glycoside hydrolase family of core 1 type O-glycan-specific endo-alpha-N-acetylgalactosaminidase from *Bifidobacterium longum*. J Biol Chem 2005;280:37415–37422. [PubMed: 16141207]
67. Ruas-Madiedo P, et al. Mucin degradation by *Bifidobacterium* strains isolated from the human intestinal microbiota. Appl Environ Microbiol 2008;74:1936–1940. [PubMed: 18223105]
68. Nishimoto M, Kitaoka M. Identification of the putative proton donor residue of lacto-N-biose phosphorylase (EC 2.4.1.211). Biosci Biotechnol Biochem 2007;71:1587–1591. [PubMed: 17587697]

69. Nishimoto M, Kitaoka M. Identification of N-acetylhexosamine 1-kinase in the complete lacto-N-biose I/galacto-N-biose metabolic pathway in *Bifidobacterium longum*. Appl Environ Microbiol 2007;73:6444–6449. [PubMed: 17720833]
70. Markowitz VM, et al. The integrated microbial genomes (IMG) system in 2007: data content and analysis tool extensions. Nucleic Acids Res 2008;36:D528–533. [PubMed: 17933782]
71. Kruse HP, et al. Effects of inulin on faecal bifidobacteria in human subjects. Br J Nutr 1999;82:375–382. [PubMed: 10673910]
72. Roberfroid MB, et al. The bifidogenic nature of chicory inulin and its hydrolysis products. J Nutr 1998;128:11–19. [PubMed: 9430596]
73. Gibson GR, et al. Selective stimulation of bifidobacteria in the human colon by oligofructose and inulin. Gastroenterology 1995;108:975–982. [PubMed: 7698613]
74. Boehm G, Stahl B. Oligosaccharides from milk. J Nutr 2007;137:847S–849S. [PubMed: 17311985]
75. Macfarlane GT, et al. Bacterial metabolism and health-related effects of galacto-oligosaccharides and other prebiotics. J Appl Microbiol 2008;104:305–344. [PubMed: 18215222]
76. Boehm G, et al. Prebiotic carbohydrates in human milk and formulas. Acta Paediatr Suppl 2005;94:18–21. [PubMed: 16214760]
77. Sumiyoshi W, et al. Galactosyllactoses in the milk of Japanese women: changes in concentration during the course of lactation. Journal of Applied Glycoscience 2004;51:341–344.
78. O'Connell Motherway M, et al. Characterization of ApuB, an extracellular type II amylopullulanase from *Bifidobacterium breve* UCC2003. Appl Environ Microbiol 2008;74:6271–6279. [PubMed: 18689518]

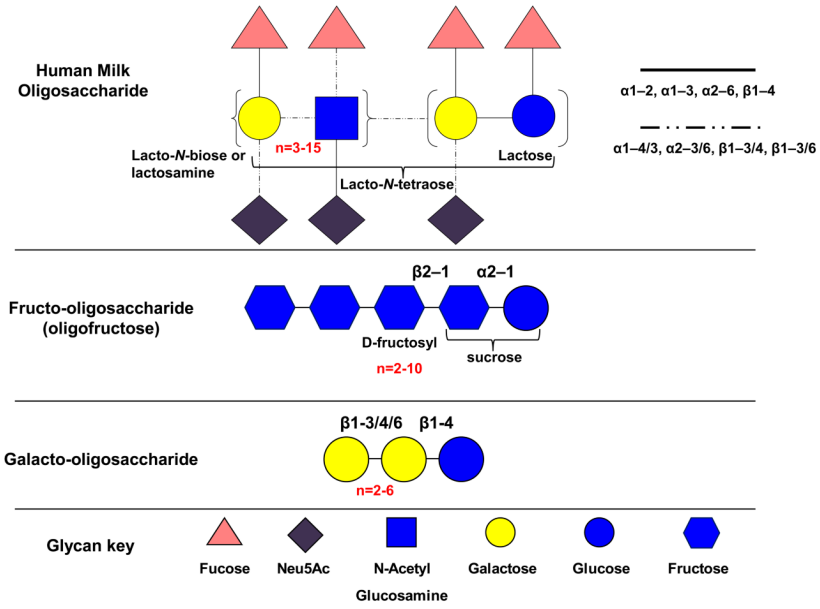

Figure 1.
Basic structures of human milk oligosaccharides and glycan mimetics. HMO possesses much more structural variability in monosaccharide constituents and linkages compared to fructo-oligosaccharides and galacto-oligosaccharides.

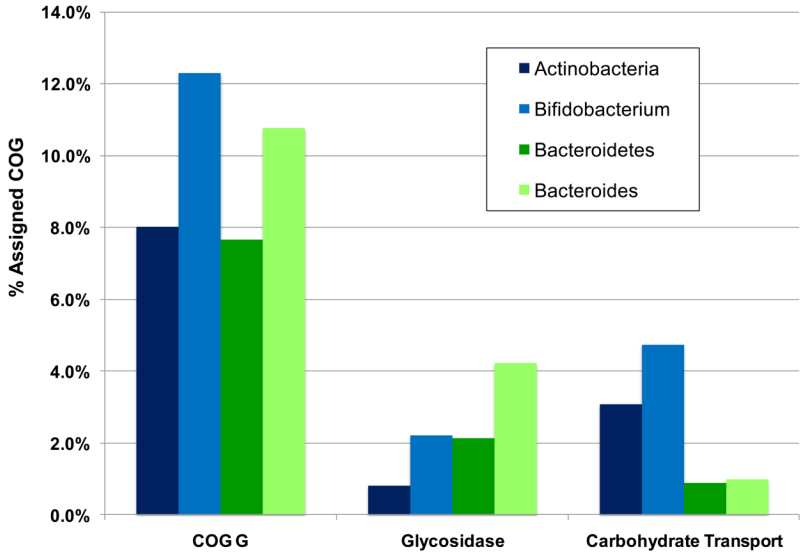

Figure 2.

Percent of COG genes assigned carbohydrate processing function in *Bifidobacterium* and *Bacteroides*. The phyla *Actinobacteria* and *Bacteroidetes* have approximately equivalent percentages of carbohydrate transport and metabolism COG category G proteins, while these are enriched in member genera *Bifidobacterium* and *Bacteroides*. *Bifidobacterium* are enriched for transport-related COGs while *Bacteroides* possesses an abundance of glycosyl hydrolases.

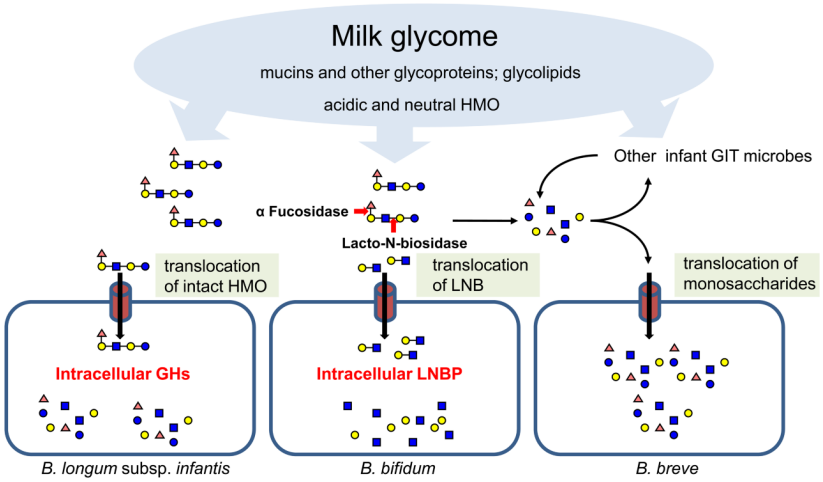

Figure 3.

Putative modes of accessing milk glycans in the infant gut by bifidobacteria. *B. longum* subsp. *infantis* captures intact HMO, while *B. bifidum* secretes extracellular enzymes prior to translocating lacto-*N*-biose degradation products. *B. breve* utilizes HMO monosaccharides cleaved by extracellular enzymes secreted by heterologous members of the consortium.

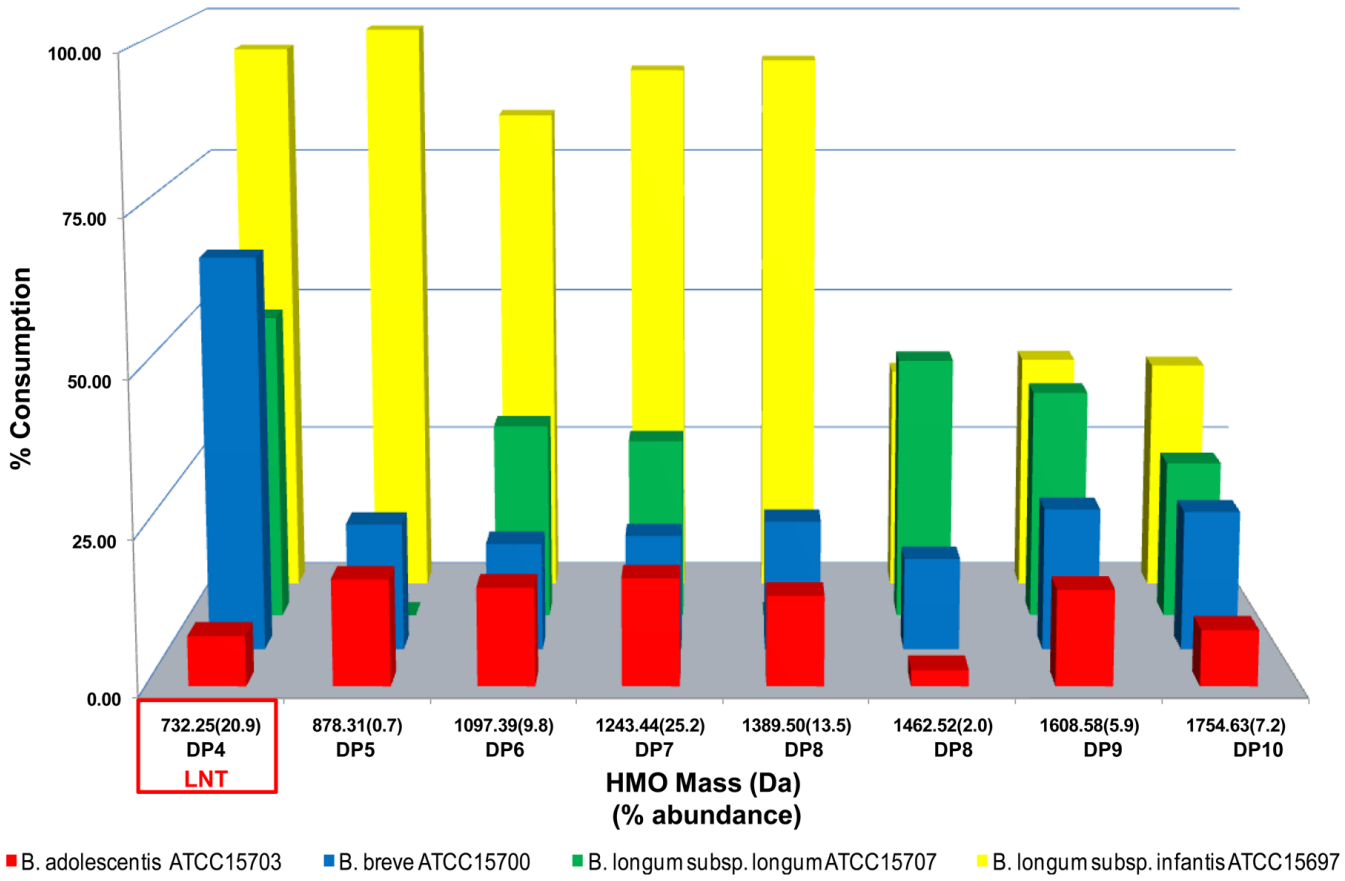

Figure 4.

Phenotypic variation of HMO utilization in bifidobacteria. The consumption of HMO as a sole carbon source data is derived from Ref. [24], and the relative abundances were originally presented in Ref. [54]. Abbreviations: LNT, Lacto-*N*-tetraose-like.

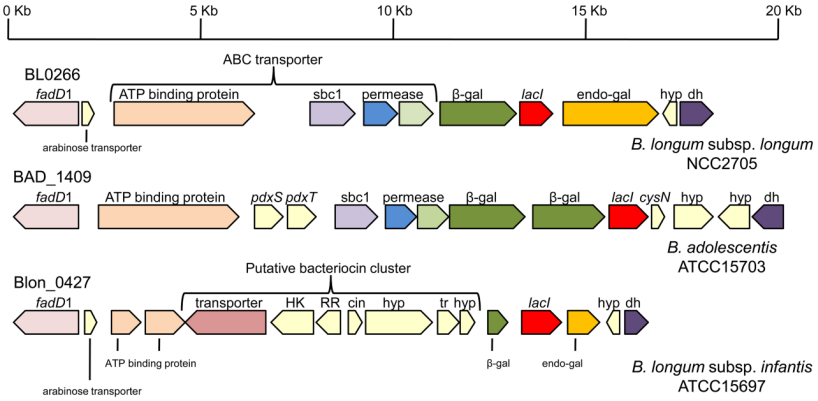

Figure 5.

Endo-galactanase locus in *B. longum* subsp. *longum* NCC2705 and variations in other bifidobacteria. The gene cluster is conserved in *B. adolescentis* ATCC15703 with the exception of the endo-galactanase. The closely related *B. longum* subsp. *infantis* ATCC15697 is missing or possesses degraded homologs of this cluster. Gene fragments are denoted in smaller text below the genes. The locus number for the *fadD1* gene is listed above. Abbreviations: sbp1, solute binding protein family 1; β-gal, β-galactosidase; endo -gal, endo-galactosidase; hyp, hypothetical protein; dh, dehydrogenase; HK, histidine kinase; RR, response regulator; cin, bacteriocin; tr, transport related; fadD1, long-chain acyl-CoA synthetase; pdxS, pyridoxine biosynthesis gene; pdxT, glutamine amidotransferase; lacI, lacI family transcriptional regulator; cysN, sulfate adenylyltransferase subunit 1.