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Review

Bifidobacterium longum subspecies *infantis*: champion colonizer of the infant gut

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Oligosaccharides are abundant in human milk. Production of these highly diverse structures requires significant energy expenditure by the mother and yet these human milk oligosaccharides offer no direct nutritive value to her infant. A primary function of human milk oligosaccharides is to shape the infant's intestinal microbiota with life-long consequences. *Bifidobacterium longum* subspecies *infantis* (*B. infantis*) is unique among gut bacteria in its prodigious capacity to digest and consume any human milk oligosaccharide structure, the result of a large repertoire of bacterial genes encoding an array of glycosidases and oligosaccharide transporters not found in other bacterial species. *In vitro*, *B. infantis* grows better than other bacterial strains in the presence of human milk oligosaccharides, displays anti-inflammatory activity in premature intestinal cells, and decreases intestinal permeability. In premature infants, *B. infantis* given in combination with human milk increases *B. infantis* and decreases Enterobacteriaceae in the feces. Probiotics containing *B. infantis* decrease the risk of necrotizing enterocolitis in premature infants. Colonization with *B. infantis* is also associated with increased vaccine responses. Probiotic organisms have historically been selected based on ease of production and stability. The advantages of *B. infantis*, selected through coevolution with human milk glycans, present an opportunity for focused manipulation of the infant intestinal microbiota.

The colonization of the fetal gut begins *in utero* with swallowing of amniotic fluid. At that point, infants begin a life-long relationship with their gut microbiota. Major shifts in the community of microbes inhabiting the intestinal tract (the gut microbiota) and the genes expressed by these microbes (the gut microbiome) and presumably the health consequences of the phenotype of the gut microbiota occur with rupture of the fetal membranes, birth, initiation of feeding, addition of solid foods, weaning, and interventions such as antibiotics, acid-suppression, and prebiotic or probiotic dietary supplements. The predominance of "bifid" microbes in the stools of healthy infants was described more than 100 y ago, prompting the hypothesis that human milk contained "bifidogenic factors" that stimulated the growth of these bifidobacteria (1).

Prebiotics are dietary supplements that promote health benefits by stimulating the growth and/or activity in the gut lumen of commensal microbes (ideally without stimulating potential pathogens); they do not contain live organisms. Probiotics are dietary supplements that do contain live organisms and are intended to promote health benefits through a variety of mechanisms. This article will focus on the coevolution of a collection of complex prebiotic oligosaccharides found in abundance in human milk and a single bacterial subspecies, *Bifidobacterium longum* subsp. *infantis* (*B. infantis*) unique in its capacity to consume these oligosaccharides. Note that the species *B. longum* has two subspecies: *B. longum* subsp. *infantis* and *B. longum* subsp. *longum*. These subspecies will be abbreviated in this review as *B. infantis* and *B. longum*, respectively.

HUMAN MILK OLIGOSACCHARIDES SHAPE THE INFANT INTESTINAL MICROBIOTA

Humans stand at the end of the long evolution of mammals, with differences in milk conspicuous for the volume, number of structures, and complexity of milk oligosaccharides (**Figure 1**) (2–4). Human milk oligosaccharides (HMOs) are the third largest solid component of human milk (after lactose and fat) even in times of famine (5), and yet these free glycans are not digestible by the infant as the human gut does not produce the glycosidases necessary to cleave the HMO linkages. The obvious evolutionary question is: What benefit is provided to the infant that justifies the mother's tremendous expenditure of energy to produce these varied and complex molecules with no apparent nutritional value? The answer to this question comes from careful analyses of the rare capacity of select gut microbes to deconstruct and consume HMOs (6,7). Among multiple microbial species studied, only two genera, *Bifidobacterium* and *Bacteroides*, are able to comprehensively utilize HMOs as a primary food source (**Table 1**) (8,9). This relative resistance to microbial consumption allows the HMOs to arrive intact in the distal small bowel and the colon where the largest numbers of commensal bacteria thrive.

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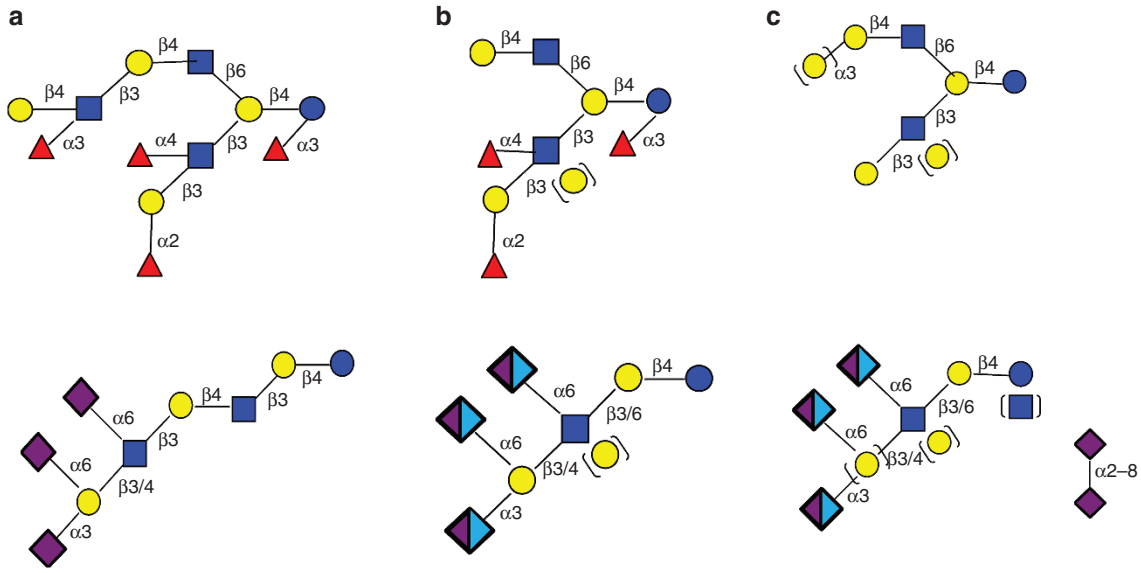

Figure 1. Systematic structural analysis of milk oligosaccharides from multiple mammalian species. Human milk (**a**) has a higher degree of oligosaccharide polymerization with about 70% fucosylated structures (upper structure) and less than 20% sialylated structures (lower structure, exclusively *N*-acetylneuraminic acid). Nonhuman primate milk (**b**) varies with species with 20–65% fucosylated structures (upper structure) and 10–45% sialylated structures (lower structure, both *N*-acetylneuraminic acid and *N*-glycolylneuraminic acid). Other mammals (**c**) show the least degree of polymerization, less than 5% fucosylated structures and up to 70% sialylated structures (lower structure, both *N*-acetylneuraminic acid and *N*-glycolylneuraminic acid) (2,79–83). Red triangle = fucose, yellow circle = galactose, blue square = *N*-acetylglucosamine, blue circle = glucose, purple diamond = *N*-acetylneuraminic acid, and light blue diamond = *N*-glycolylneuraminic acid.

Table 1. Consumption of single human milk oligosaccharide (HMO) structures by different bacterial species

	HMO structure				
Bacterial species (*n*)	2′FL	3-FL	LDFT	3′SL	6′SL
Escherichia coli (1)	–	–	–	–	–
Clostridium (2)	–	–	–	–	–
Lactobacillus (2)	–(1)/+(1)	–(1)/+(1)	–	–	–(1)/++(1)
Enterobacter (2)	–	–	–	–	–
Enterococcus (2)	–(1)/+(1)	–(1)/+(1)	–	–	–
Staphylococcus (2)	–	–	–	–	–
Streptococcus (1)	+	+	–	–	–
Bacteroides (3)	++	++	–(1)/++(2)	–(1)/+(1)/++(1)	+(1)/++(2)
Bifidobacterium (10)	+(1)/++(9)	++	+(1)/++(9)	–(2)/+(1)/++(7)	–(1)/+(1)/++(8)

2′FL, 3-FL, and LDFT are abundant fucosylated HMO structures, and 3′SL and 6′SL are abundant sialylated HMO structures. The numbers in parentheses are the number of strains tested. The symbols represent consumption of <10% (–), consumption between 10 and 40% (+), and consumption of >40% (++) (from ref. 8).

The gut of healthy term infants is initially colonized by bacteria acquired at birth. These "pioneer" bacteria are predominately facultative anaerobes with composition heavily influenced by mode of delivery (10). Within the first days to weeks, two obligate anaerobes, *Bacteroides* and *Bifidobacterium*, generally become the most abundant genera (11–13). Previous dogma was that the pioneer bacteria create a low-oxygen environment in which the obligate anaerobes then become dominant; however, two recent observations suggest that this second wave of colonization is more complex. First, species of both *Bacteroides* and *Bifidobacterium* are found in maternal feces, human milk, and infant feces suggesting direct inoculation through breastfeeding and maternal–infant contact (14). Second, species of both *Bacteroides* and *Bifidobacterium* are aggressive consumers of HMOs (15). Early reports of a relative absence of *Bifidobacterium* species in the stools of healthy infants (16) were likely due to limitations in methods, e.g., imprecise PCR primers and lack of bead-beating in bacterial DNA extraction (17). Healthy term breastfed infants are colonized by a small number of subspecies including *B. infantis*, *B. longum*, and *B. breve* and to a lesser extent *B. bifidum* and *B. pseudocatenulatum*, whereas healthy term formula-fed infants are colonized by a more diverse population, including the above species plus bifidobacterial species seen in adults such as *B. adolescentis* (18–20). In adults, increased diversity in the intestinal microbial population is generally considered beneficial (21); however, this may not be the case in the healthy neonate where a predominance of a

few subspecies of bifidobacteria is associated with improved growth (22).

MECHANISTIC EVIDENCE FOR COLONIZATION BY *B. INFANTIS*

Early studies demonstrated that a strain of *B. infantis* was better able to grow in a culture medium wherein HMOs were the only carbon source, than strains of *B. longum*, *B. breve*, or *B. adolescentis* (23,24). The sequencing of this strain of *B. infantis* demonstrated a large number of genes involved in catabolism of complex carbohydrates (25). Comparison of the closely related subspecies *B. longum* and *B. infantis* demonstrated that the former encodes enzymes for the digestion of plant oligosaccharides, while the latter has evolved the capacity to digest HMOs. Most of the strains of *B. infantis* sequenced to date contain a 43-kb gene cluster (HMO cluster I) that encodes a variety of oligosaccharide transport proteins and glycosyl hydrolases; this gene cluster is not found in other bifidobacterial species (26,27). The one *B. infantis* strain analyzed to date, which showed weak growth in the presence of HMO, has a partial deletion of this gene complex (24,26). Most HMO structures contain either fucose or sialic acid (**Figure 1**); among species of *Bifidobacterium*, only *B. infantis*, *B. breve*, and *B. bifidum* produce fucosidases and sialidases, and only *B. infantis* is able to digest all HMO structures (**Table 2**) (28).

B. infantis strains, when grown in the presence of HMOs, upregulate expression of two groups of bacterial genes. First, transporter proteins that bind to specific HMO linkages, including a number of solute-binding proteins with an affinity for HMOs, are upregulated in *B. infantis* grown on HMO but not in *B. infantis* grown on the simpler prebiotic oligosaccharides fructo-oligosaccharide or galacto-oligosaccharide (29,30). This suggests that *B. infantis* is able to transport intact HMOs into its cytoplasm and that this capacity is "turned on" by the HMOs. Second, glycosidases with specificity for every linkage in HMOs are upregulated in *B. infantis* grown on HMO. The 16 glycosyl hydrolases expressed by *B. infantis* include α-fucosidases, β-galactosidases, β-hexosaminadases, and α-sialidases, facilitating complete digestion of HMOs within the bacterial cytoplasm that is not possible for other bifidobacteria (6,31–34). *B. infantis* also differs markedly from *B. bifidum* and *Bacteroides* in its specificity for HMOs. *B. infantis* is unable to deconstruct the O-glycans in human mucus in spite of structural similarity to HMOs (30), while *B. bifidum* and *Bacteroides* are able to consume both HMOs and mucus glycans (35). Indeed, unlike *B. infantis*, *B. bifidum* and *Bacteroides* species deploy extracellular glycosyl hydrolases that deconstruct complex glycans outside of the cell enabling import and consumption of specific glycan components while other components of the digested glycans (e.g., mono and disaccharides) are left outside the cell (15). In a mouse model, this consumption mode has recently been shown to liberate sugars that promote the growth of pathogens that otherwise would be unable to utilize host glycans (36). These results suggest the hypothesis that *Bacteroides* species and *B. bifidum* may not be ideal as probiotics for correction of dysbiosis in human milk-fed premature infants as the byproducts of HMO consumption may stimulate growth of intraluminal pathogens.

HMOs are not the only human milk components of "interest" to *B. infantis*. Acidic glycolipids (gangliosides) are found arrayed on the surface of fat globules in human milk and may play a role in neurodevelopment (37), pathogen binding within the gut lumen, and shaping the intestinal microbiota (38). Among six species of bifidobacteria tested, *B. infantis* and *B. bifidum* were best able to consume the two major gangliosides GM3 and GD3 (39). These data demonstrate that human milk gangliosides have a selective prebiotic effect in addition to that of HMOs. Evolutionary selective pressure has equipped *B. infantis* with multiple enzymes for deconstructing milk glycans, and as a result this subspecies is able to outcompete even other bifidobacteria as well as other commensals and pathogens in the gut lumen of the healthy breastfed infant. This advantage extends beyond free glycans and glycolipids to glycoproteins. In ongoing studies, *B. infantis* produces an endo-β-*N*-acetylglucosaminidase that is able to cleave the *N*-glycans associated with human glycoproteins like lactoferrin, IgA, and IgG. Human milk incubated with this bacterial enzyme undergoes significant *N*-deglycosylation and *B. infantis* grown in the presence of lactoferrin upregulates expression of this enzyme (40). These data suggest two possibilities: that human milk glycoproteins serve a prebiotic role and that these *B. infantis* endoglycosidases release biologically active peptides from human glycoproteins.

Table 2. *Bifidobacterium* species and the number of glycoside hydrolases encoded in their genomes (from ref. 21)

Species/subspecies	Total glycoside hydrolases	α-Sialidase	α-L-Fucosidase
B. adolescentis	22	0	0
B. angulatum	13	0	0
B. bifidum	17	2	2
B. breve	19	1	1
B. catenulatum	21	0	0
B. dentium	31	0	1
B. longum subsp. *longum*	26	0	0
B. longum subsp. *infantis*	24	2	5
B. minimum	2	0	0
B. pseudocatenulatum	25	0	1
B. pseudolongum	14	0	1
B. subtile	3	0	0
B. thermacidophilum	9	0	0

B. INFANTIS IN PREMATURE INFANTS

Premature infants have a markedly different intestinal microbiota than term infants. While term babies progress from colonization with maternal microbes obtained at birth to microbes influenced mostly by diet, premature infants are generally

colonized with Firmicutes (predominantly staphylococci, streptococci, and enterococci) and Proteobacteria (predominantly Gram-negative Enterobacteriaceae) with a marked absence of bifidobacteria for several weeks or months (41). This dysbiosis (defined as an alteration in the fecal microbiota) in premature infants is likely due to a combination of environmental factors inherent in neonatal intensive care, hygiene, antibiotic use, and endogenous factors including genetics and immaturity of the intestinal immune responses. Dysbiosis appears to be a significant risk factor in susceptibility to necrotizing enterocolitis (NEC), a common and devastating disease that predominantly affects premature infants. Careful studies have demonstrated associations between NEC and early dysbiosis (42), antibiotic administration (43), and acid suppression (44), as well as worsening of dysbiosis just prior to the onset of NEC (45).

Attempts to alter the intestinal microbiota of premature infants with prebiotics alone have yielded mixed results. The incidence of NEC in human milk-fed premature infants is significantly lower than in those receiving formula (46). Milk from mothers delivering preterm does not differ dramatically from milk from mothers delivering at term in numbers of total HMOs; however, the variability of fucosylated HMOs was found to be significantly higher in the former than the latter (47). It is unclear whether the paucity of bifidobacteria in premature infants is due to these small differences in HMO composition, to lack of introduction of bifidobacteria, or to extrinsic factors such as antibiotics and environmental factors. Results to date suggest that any single intervention is insufficient to significantly impact the premature infant gut microbiota. In a small dose escalation trial of added galacto-oligosaccharide or HMOs in formula-fed premature infants, there were not significant differences in the fecal microbiota with either prebiotic intervention (48). A larger study in premature infants that showed significant changes in the fecal microbiota with antibiotics and only minimal changes with administration of a mixture of galacto-oligosaccharides and fructo-oligosaccharides (49), failed to show significant decreases in the incidence of NEC or sepsis (50) or in neurodevelopment (51) between infants that received the prebiotic mixture and those that received the placebo.

Administration of probiotics to premature infants in most of the clinical trials performed to date is associated with a decreased incidence of NEC (52,53). Routine administration of probiotics to all premature infants has been proposed and is common practice in many countries (54,55). The question of which probiotic product to provide to a premature infant is challenging given the lack of direct comparisons between products and the lack of rigorous standardization of live bacteria as a therapeutic intervention. For example, a high number of discrepancies between the stated contents of commercial probiotic products and the measured contents has been reported (56,57). Furthermore, most current commercial probiotics were developed years ago and selection criteria for organisms were based on stability and ease of industrial production rather than specific mechanistic criteria of the organisms selected. It is now possible to establish standards of strain specificity, dosing accuracy, optimum combinations of paired prebiotics and probiotics, and analysis of changes in the gut microbiota composition. Differences among bifidobacteria illustrate this principle. We compared pure formulations of *B. infantis* and *B. animalis* subsp. *lactis* (*B. lactis*, a common *Bifidobacterium* in yogurts and commercial probiotics) in dose escalation and cross-over trials and found that *B. infantis* was better able to colonize the intestine than *B. lactis* in both formula-fed and human milk-fed premature infants. In the infants receiving *B. lactis*, even at high doses, the numbers of total bifidobacteria in the stool were low, and the general bifidobacteria that were present were not the administered species. There was no additive effect of human milk and *B. lactis*—a result that was not surprising given that the *B. lactis* was chosen for this study because, unlike *B. infantis*, it does not grow in culture medium where HMO is the only carbon source. The highest numbers of fecal bifidobacteria were seen in infants receiving a combination of human milk and *B. infantis* at a dose of 10^9 organisms twice daily (58). Of the more than 20 published randomized controlled trials of probiotics in premature infants, six have included administration of *B. infantis* alone or in combination; five of these trials showed a decreased incidence of NEC in the probiotic group (53,59–63). A meta-analysis of four studies of administration of *B. lactis* to premature infants showed no decrease in the incidence of NEC (64).

MECHANISMS OF OBSERVED PROTECTIVE EFFECTS

Recent studies have demonstrated four promising mechanisms by which bifidobacteria decrease the risk of NEC in premature infants. First, as described above, *B. infantis* has a competitive advantage in the presence of human milk components; therefore, increased colonization resulting in decreased diversity of the gut microbiota and fewer luminal pathogens is one likely mechanism of protection. In addition to a selective growth advantage, *in vitro* studies reveal that *B. infantis* cells grown on HMO bind to cultured intestinal cells at a higher rate suggesting that the unique ability to grow on HMOs coincides with an increased ability to bind and colonize the intestinal mucus layer (65,66).

Second, *B. infantis* has been shown to be anti-inflammatory in several *in vitro* and animal studies. An immature and poorly modulated immune response to bacterial translocation is believed to be a key trigger of NEC (67). In an elegant series of experiments, explants of both immature and mature human neonatal intestinal tissue were exposed to the supernatant from *B. infantis*. The *B. infantis* supernatant suppressed the exuberant production of the proinflammatory cytokines IL-6 and IL-8 and toll-like receptors TLR2 and TLR4 triggered by lipopolysaccharide and IL1β in the immature tissue explants. In the mature tissue explants, expression of these cytokines and TLRs was less marked and not significantly different with exposure to the *B. infantis* supernatant. Similar observations were seen with enterocytes from premature infants with NEC and with immature human enterocytes. These experiments suggest that *B. infantis* produce exogenous substances that promote maturation of the immature innate immune response (68). This

supernatant from *B. infantis* attenuates *Cronobacter sakazakii*-induced enteritis in a newborn mouse model (*C. sakazakii* is a contaminant of powdered infant formulas associated with both sepsis and NEC in premature infants) (69). In a rat model of NEC, administration of *B. infantis* decreased expression of IL6, IL8, TNFα, IL23, and iNOS, decreased the expression of antimicrobial peptides, altered expression of intestinal mucus-related proteins, and decreased the incidence of NEC (17). *In vitro*, Caco-2 cell expression of anti-inflammatory IL10 was increased when these cells were exposed to *B. infantis* grown in the presence of HMOs, but not when exposed to *B. infantis* grown in the presence of lactose. This series of studies suggest that HMOs "turn on" the repertoire of genes in *B. infantis* within the infant that are important in controlling inflammation (65).

Third, *B. infantis* decreases intestinal permeability. In mice colonized with human fecal microbes, increased numbers of bifidobacteria are associated with decreased bacterial translocation while increased numbers of *Bacteroides* and *Clostridia* are associated with increased bacterial translocation (70). In a neonatal mouse NEC model, *B. infantis* decreased intestinal permeability, increased stabilization of the tight junction proteins claudin 4 and occludin, and decreased the incidence of NEC (71). *In vitro*, *B. infantis* grown in the presence of HMO increased expression of junctional-associated molecule (JAM-A) in Caco-2 cells and tight junction protein ZO-1 in HT-29 cells compared with *B. infantis* grown in the presence of lactose, once again confirming that growth on HMOs is necessary to "turn on" genes in *B. infantis* associated with host intestinal permeability (65).

Fourth, many commensal bacteria produce short chain fatty acids (SCFA, particularly butyrate, propionate, and acetate) with direct and indirect effects on the host. Healthy breastfed infants have higher levels of fecal acetate than formula-fed infants, likely due to increased bifidobacteria. Measurement of fecal SCFA has been proposed as a measure of carbohydrate fermentation and therefore a marker of dysbiosis. However, evaluation of the effects of SCFA is challenging, as these volatile products are both produced and consumed in the colon by bacteria and absorbed by the enterocyte to enter the portal circulation. In adults, increased acetate production may be associated with obesity and inflammation, while butyrate and propionate appear to be protective (72). It has been hypothesized that excessive production of butyrate increases the risk of NEC and limited data from animal models support this hypothesis (73,74). The influence of HMOs, commercial prebiotics, and probiotics on SCFA production remains unclear. In premature infants, administration of *B. breve* was associated with a decrease in fecal butyrate (75), administration of *B. lactis* was associated with increased fecal acetate (76), while administration of a combination product containing *B. infantis* did not alter fecal SCFA compared with placebo (59).

B. INFANTIS IS ASSOCIATED WITH IMPROVED GROWTH AND VACCINE RESPONSES IN TERM INFANTS

A recent cohort study of infants in Dhaka, Bangladesh found that infants there were heavily colonized with bifidobacteria. The dominant species of bifidobacteria in these infants (96% of whom were breastfed) was *B. infantis*. Correlations among this cohort showed that the infants with the most *B. infantis* in their stools had better weight gain, increased thymic index, and better responses to the oral polio, tuberculosis, and tetanus vaccines (22). The observed correlation does not establish causality. It is possible that the healthiest babies have improved growth, better immune responsiveness, and increased fecal bifidobacteria without the latter causing either of the former; however, these observations support the hypothesis that the composition of the infant microbiota is critical to immune development and surveillance. Probiotic organisms have been demonstrated to boost immune response to polio vaccine in adults (77), but pediatric studies have been equivocal to date, perhaps due to the choice of probiotic strain (78).

CONCLUSION

HMOs are able to transit the stomach and proximal small bowel of infants without being altered or consumed. In the distal gut, HMOs are selectively consumed by *B. infantis* creating a microbiota that is limited in diversity but associated with improved growth and vaccine responsiveness in term infants and decreased NEC in premature infants. HMOs activate a variety of genes in *B. infantis* that allow it to dominate the gut microbiota and benefit the host by accelerating maturation of the immune response, limiting excessive inflammation, improving intestinal permeability, and increasing acetate production. This symbiotic relationship is a compelling example of coevolution of two species to temporarily protect the full term neonate and nourish a healthy gut microbiota prior to weaning. In the premature infant, this colonization is disrupted and the provision of both human milk and probiotic *B. infantis* appears to be both restorative and protective.

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