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Linking human milk oligosaccharide metabolism and early life gut microbiota: bifidobacteria and beyond

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SUMMARY Human milk oligosaccharides (HMOs) are complex, multi-functional glycans present in human breast milk. They represent an intricate mix of heterogeneous structures which reach the infant intestine in an intact form as they resist gastrointestinal digestion. Therefore, they confer a multitude of benefits, directly and/or indirectly, to the developing neonate. Certain bifidobacterial species, being among the earliest gut colonizers of breast-fed infants, have an adapted functional capacity to metabolize various HMO structures. This ability is typically observed in infant-associated bifidobacteria, as opposed to bifidobacteria associated with a mature microbiota. In recent years, information has been gleaned regarding how these infant-associated bifidobacteria as well as certain other taxa are able to assimilate HMOs, including the mechanistic strategies enabling their acquisition and consumption. Additionally, complex metabolic interactions occur between microbes facilitated by HMOs, including the utilization of breakdown products released from HMO degradation. Interest in HMO-mediated changes in microbial composition and function has been the focal point of numerous studies, in recent times fueled by the availability of individual biosynthetic HMOs, some of which are now commonly included in infant formula. In this review, we outline the main HMO assimilatory and catabolic strategies employed by infant-associated bifidobacteria, discuss other taxa that exhibit breast milk glycan degradation capacity, and cover HMO-supported cross-feeding interactions and related metabolites that have been described thus far.

KEYWORDS infant microbiome, human milk oligosaccharides, gut microbiota, infant, bifidobacteria

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INTRODUCTION

Human breast milk is primarily composed of water, carbohydrates, lipids, and milk proteins to form an early life source of nutrients, while it furthermore contains various minor, yet functional ingredients, such as antibodies (e.g., IgG and IgA), antimicrobial peptides, lactoferrin, and mucins (1–5). A proportion of the human milk-associated carbohydrates, i.e., those larger than lactose and collectively known as human milk oligosaccharides (HMOs), are non-digestible, structurally diverse, multi-functional glycans. Oligosaccharide concentration and diversity in human milk are significantly higher than that of other mammals (1). The availability of HMOs is primarily through feeding of breast milk, which is the universally accepted gold standard for infant nutrition (6). They serve as substrates for select gut microorganisms as they escape host digestion, thereby playing a significant role in shaping the infant microbiota, including the establishment and persistence of a set of health-promoting gut microbes (7). Initial colonization of the infant intestine shortly after birth establishes a life-long mutualistic relationship between the intestinal microbial community and its respective host. This is crucial for the development of pathogen resistance, utilization of dietary components, and interactions with the immune system (8, 9). The formation of the infant gut microbiota is impacted by numerous factors including, but not limited to, mode of delivery, antibiotic administration, geographical and environmental location, and available nutrients (10, 11).

Prevalent taxa in the early infant gut are highly variable and may include *Escherichia*, *Enterococcus*, *Bifidobacterium*, *Bacteroides*, *Streptococcus*, and *Veillonella* (11, 12). *Bifidobacterium* members are among the earliest colonizers of the infant gut microbiota, in individual infants reaching up to 90% relative abundance (13–15), and certain members within this genus are HMO degraders (11, 13, 16). Specific infant-typical bifidobacterial species, or strains thereof, are equipped with an arsenal of enzymes and transporters to facilitate HMO uptake and metabolism. The first 6–12 months following birth represent a critical stage for the formation and establishment of the human gut microbiota, and infant-associated *Bifidobacterium* species and HMOs are contributors to the configuration of this microbial community. The microbiota evolves in parallel with the host, which is significant to overall infant health (9) due in part to the metabolites produced from substrate fermentation. As a collection of microbes, complex community dynamics are at play when individual or multiple HMOs are present.

This review will describe HMO structure and composition as well as factors affecting early life microbiota, with particular focus on the intricate interactions between select *Bifidobacterium* species, and indeed other taxa, and HMOs. Additionally, we provide an overview of the variety and function of metabolites produced from HMO degradation. We also consider HMOs and their similarities to traditional prebiotics applied in infant formula such as galacto-oligosaccharides (GOS) and fructo-oligosaccharides (FOS) as they relate to the infant gut and how they are consumed. Finally, we identify some future considerations and knowledge gaps while illuminating the broad potential of various HMOs in modulating the infant gut microbial composition and the consequent metabolic functions that ensue.

FACTORS AFFECTING EARLY LIFE MICROBIOTA

Numerous factors influence the infant gut microbiota establishment and development including maternal diet, antibiotic administration, mode of delivery (i.e., vaginal delivery or delivery by cesarean section), feeding regimen (i.e., breast milk versus formula milk), birth term (full term versus preterm), geographical location, human interaction, mother-infant transfer, and the surrounding environment (Fig. 1). The first year of life is critical for the formation of the infant gut microbiota (6), with bifidobacteria being among the first colonizers (11, 17–19). Bifidobacterial absence in the early life microbial community may expose the infant to undesirable bacteria such as *Enterococcus*, *Enterobacter*, *Clostridium*, and *Klebsiella* species (12), possibly through a lack of competitive exclusion and antimicrobial metabolite production (16, 20). Antibiotic administration

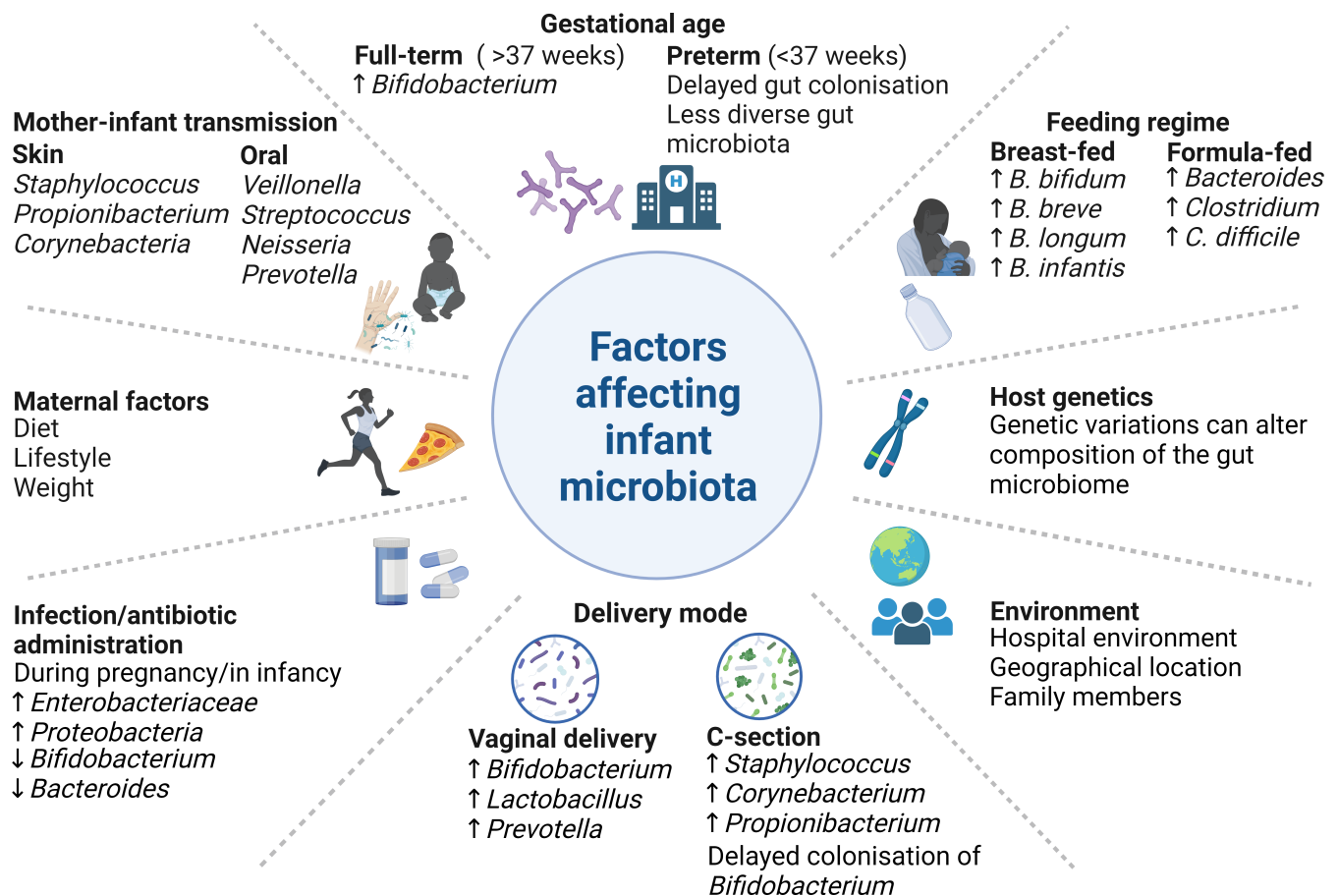


FIG 1 Factors affecting the early infant gut microbiota. Some factors influencing the taxonomic structure, and functionality, of the infant microbiota are displayed here. For further details, see text. Made using BioRender.

during pregnancy can lead to delayed colonization by bifidobacteria in infants (21) and has been associated with a higher abundance of pathogenic bacteria such as members from Enterobacteriaceae and Proteobacteria (22). Antibiotic usage during prenatal, neonatal, and postnatal periods is thought to retard the appropriate development of the gut microbiota and cause a disruption that may not recover for some 6–12 months after birth (23). Environmental factors such as place of birth, i.e., hospital or home, and geographical location may have an indirect effect on early life microbiota, in part due to cultural habits such as bathing practices, e.g., Japan (24), lifestyle, eating habits and food availability, diversity of human interaction, and climate (23). The various factors influencing the neonatal gut microbial composition have previously been subject to in-depth scrutiny (12, 25, 26) and are summarized in Fig. 1.

The composition of the gut microbiota is highly variable during the early stages of life and is often characterized by low microbial diversity (11). Prevalent taxa in the gut of the neonate may include *Escherichia*, *Enterococcus*, *Bifidobacterium*, *Bacteroides*, *Streptococcus*, and *Veillonella* (9, 11, 12). Bifidobacteria populate the gut, at least in part due to vertical, mother-to-baby transmission and membrane rupture during vaginal delivery (19). C-section can interrupt this natural route of vertical transmission, thus resulting in disruption, delay, and/or reduction of bifidobacterial colonization (19, 27). Immediately following birth, *Lactobacillus*, *Prevotella*, or *Sneathia* spp. dominate the gut in vaginally born infants, whereas *Clostridium* dominates in infants born via C-section (28). The alpha diversity of the gut microbiome has been shown to be impacted by birth mode, but also age (18, 28, 29). Feeding mode has an impact with breastfeeding increasing certain *Bifidobacterium* species, such as *B. breve* and *B. bifidum*, as well as

Ruminococcus in the infant gut, with a lower prevalence of *Escherichia coli*, *Clostridioides difficile*, and *Bacteroides fragilis* (21, 30). In contrast, the gut microbiota of formula-fed infants mainly comprised members from Enterobacteriaceae, and genera such as *Streptococcus*, *Bacteroides*, *Bifidobacterium*, *Clostridium*, and *Atopobium* (21, 31, 32). Often, the gut microbiota of a breast-fed infant has a lower microbial diversity, richness, and complexity than that of formula-fed infants (31, 33) but includes a high prevalence of certain bifidobacterial species (13), which will be discussed below. The predominance of bifidobacteria and their intrinsic ability to degrade HMOs is a primary factor in the formation of a less diverse microbial community (29, 34, 35). In contrast to adults, a low microbial diversity, typically dominated by bifidobacteria in breast-fed infants, is favorable due to the health benefits to the infant, e.g., immune system maturation (36), reduction in allergy risk (37), and decreased colonization of potentially undesirable microbes (38). As the gut microbiota develops, bifidobacteria remain present, although to a lesser relative abundance, and compositional shifts are evident at species level (23, 29). The infant microbial composition can be dominated by particular taxa and is much more amenable to change than that of adults, which appears to be crucial, although the true extent remains elusive, in this early stage of microbiota establishment (39). It has been proposed that these taxonomic patterns can be assigned to different infant community state types based on species-level composition (39, 40) and may influence how different substrates, including HMOs, are assimilated and contribute to the corresponding functional benefits.

HUMAN MILK OLIGOSACCHARIDES: STRUCTURE, COMPOSITION, AND VARIATION

One of the most notable aspects of HMOs is their structural diversity. At present, there have been over 200 distinct HMO structures identified in breast milk, with a combined concentration of between 5 and 22 g/L in mature milk (1, 41, 42). Generally, the highest concentration of HMOs is present immediately after birth in colostrum (up to 25 g/L) with a subsequent decrease over the lactation period. HMO concentration and diversity are more abundant in human breast milk when compared to that of other mammals (1). Complex interpersonal variation in HMO composition and structure has been observed due to factors including genetics, lactation stage, and environmental and geographical location. HMOs escape digestion in the small intestine, thus progressing to the colon where they can serve as substrates for select bacteria (1), including certain bifidobacterial populations, thereby facilitating their enhanced abundance.

The structural components of HMOs consist of five monosaccharidic building blocks, namely *D*-glucose (Glc), *D*-galactose (Gal), *N*-acetylglucosamine (GlcNAc), *L*-fucose (Fuc), and *N*-acetylneuraminic acid (Neu5Ac or sialic acid). Through glycosidic bonds they form complex structures. Generally, all HMOs contain a lactose core (Gal β 1-4Glc) at their reducing end, which can be elongated by a β 1-3 or β 1-6 linkage with one or more units of lacto-*N*-biose (LNB; Gal β 1-3GlcNAc) or its isomer *N*-acetyllactosamine (LacNAc; Gal β 1-4GlcNAc) (43). This provides the basis for the designation of type I and type II structures, respectively (Fig. 2A). This core HMO structure can carry fucose or sialic acid substitutions resulting in three categories: neutral (unsubstituted), fucosylated, or sialylated (Fig. 2A). The most abundant HMO present in milk from secretor mothers (see below in Fig. 2B) is 2'-fucosyllactose (2'-FL), a trisaccharide composed of *L*-fucose, *D*-glucose, and *D*-galactose. Other well-characterized structures include 3-fucosyllactose (3-FL), lacto-*N*-tetraose (LNT), lacto-*N*-neotetraose (LNNt), 3'-sialyllactose (3'-SL), and 6'-sialyllactose (6'-SL). The biosynthesis of oligosaccharides occurs following the formation of the lactose core when Gal and Glc are coupled by β -galactotransferase in the presence of α -lactalbumin, which is expressed during lactation (44). Lactose can be fucosylated at the terminal Gal by an α 1-2 linkage to form 2'-FL or at the reducing end Glc by means of an α 1-3 linkage resulting in the formation of 3-FL (45). Lactose can also be sialylated at the terminal Gal in α 2-3 or α 2-6 linkage to form 3'-SL and 6'-SL. The structural diversity and varying composition of HMOs present in human breast milk are comprehensively reviewed

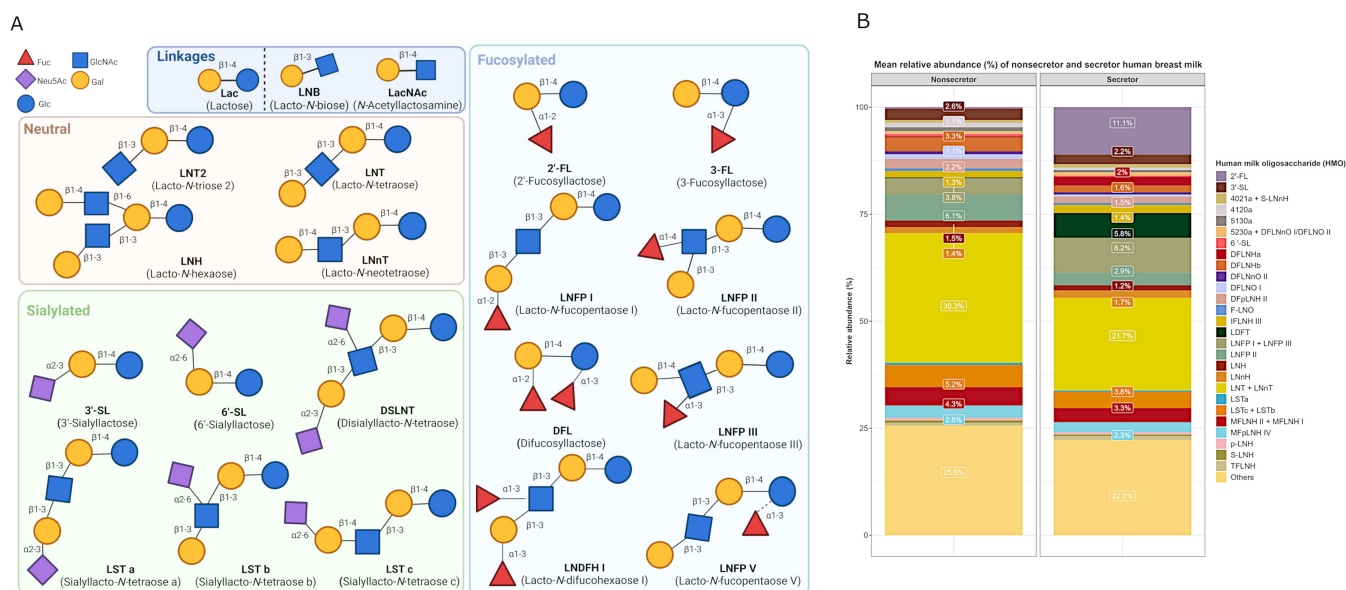


FIG 2 (A) Schematic representation of select HMO structures. Five basic units form HMOs: Fuc, Neu5Ac, GlcNAc, Gal, and Glc. All HMO structures are composed of a lactose core which can be elongated by LNB (type-1 chain) or *N*-acetylglucosamine (type-2 chain) via either β 1–3 or β 1–6 linkages. These structures can be further decorated resulting in three categories: fucosylated, sialylated, and neutral. (B) Mean relative abundance (%) of HMOs in non-secretor and secretor milk profiles. Data adapted from Vinjamuri et al. (47). Percentage labels included for HMOs present >1% relative abundance. Made using BioRender and R (ggplot2 package).

elsewhere, and we therefore refer the interested reader to some excellent recent reviews on this matter (1, 2, 45, 46).

Both inter- and intrapersonal variation influence the type and concentration of HMOs synthesized (45). Specifically, genetic and environmental factors very substantially contribute to the diversity and abundance of (particular) HMOs produced by different women. Such genetic factors, in particular those responsible for the secretor (Se) status and Lewis (Le) blood group, also have a major impact on the composition and concentration of HMOs in breast milk. Approximately 80% of mothers globally, although variations exist among different populations, can express α -1-2-fucosyltransferase (FUT2), enabling them to produce milk containing oligosaccharides such as 2'-FL and lacto-*N*-fucopentaose (LNFP) I (48). Mothers with the Se gene express FUT2, which is responsible for the synthesis of α -1-2 fucosylated HMOs, whereas those with the Le gene express α -1-3/4-fucosyltransferase, thereby synthesizing α -1-3 or α -1-4 fucosylated HMOs (45). The variations in maternal secretor status are likely to have arisen to confer a host advantage. It has been suggested that the non-secretor phenotype, i.e., those lacking a functional FUT2 enzyme, evolved to better protect the host against diseases such as specific norovirus genotypes (49–51) and *Helicobacter pylori* (52, 53). Differences in evolutionary pressures, e.g., the risk of particular pathogens or other geographical factors, in countries or areas may have contributed to Se gene polymorphisms. Four milk phenotypes are thus observed; secretor positive and Lewis positive (Se+, Le+), secretor negative and Lewis positive (Se–, Le+), secretor positive and Lewis negative (Se+, Le–), and secretor negative and Lewis negative (Se–, Le–) (45). Se+, Le+ women produce milk containing a higher concentration and a greater variety of HMOs compared to the other three milk phenotypes, with the main distinction being fucosylation (45). Typically, 50%–80% of HMOs are fucosylated (1); however, in Se–, Le– women, a very small proportion of the HMOs (often <10%, although this can vary) (54, 55) carry fucose substitutions such as 3-FL, which could have a significant impact on the functionality resulting from bacterial HMO degradation. Alterations in the HMO concentration occur across the lactation period, with the highest levels found in colostrum with HMO concentration decreasing throughout lactation as milk matures (45). The HMO composition of human

milk also changes during lactation, e.g., sialylated HMOs are abundant at the beginning of lactation and subsequently decrease over time (45), whereas 3-FL concentration is reported to increase over the lactation course (41). HMO variation is also evident among lactating mothers of preterm versus mothers of full-term infants, with higher oligosaccharide concentrations being present in milk from the latter (45).

Geographical location, ethnicity, and seasonal changes can be a determining factor in HMO composition and concentration (56, 57). Generally, a relatively high proportion of mothers in the United States [up to 95% (42)] and Europe, e.g., Sweden (79%) (42) and Finland (87.2%) (58), are secretors, whereas many non-secretors inhabit parts of Africa, Central Asia, and Pacific regions (42) (Fig. 3). Secretor status type also varies within Africa, e.g., rural Gambia and South Africa have a greater abundance of non-secretors in contrast to Namibia and Malawi (47). Derrien and colleagues recently observed that 71% of their 105 Kenyan cohort were secretors (40) similar to that of Ireland (74.68% secretors) which has recently been reported (19). In Brazil and Peru, countries with a high proportion of secretors (approximately 98% of the population), 2'-FL is more abundant in mother's milk compared to that in other countries such as Ghana and rural Gambia, with 68% and 65% being secretors, respectively (42, 59). Additionally, total concentration of HMOs and 2'-FL was shown to be the lowest in Africa, although LNT concentration was shown to be higher compared to other locations such as Germany, China, and Malaysia (60, 61). Interestingly, milk from non-secretor Bangladeshi women contained higher 2'-FL concentrations than that from non-secretors from other locations (57). Variation of HMOs and secretor status across geographical locations has previously been reported in considerable detail (41, 47, 60). Intra-regional variations are also observed, e.g., a recent study by Lahdenperä and colleagues (58), using a Finnish cohort of breastfeeding

Percentage of secretor mothers globally



FIG 3 Percentage of breast milk donors categorized as secretors worldwide. Secretor breast milk contains large amounts of α 1-2 fucosylated oligosaccharides in contrast to that of non-secretors. Updated from McGuire et al. (42) with additional data from Cheng and Yeung (67), Vinjamuri et al. (47), Asher et al. (68), Feehily et al. (19), Azad et al. (56), Durham et al. (69), Erney et al. (70), Ferreira et al. (59), Lahdenperä et al. (58), Lewis et al. (71), Liu et al. (72), Liu et al. (73), Menzel et al. (74), Musumeci et al. (75), Moya-Alvarez et al. (60), McJarrow et al. (76), Pell et al. (57), Sudarma et al. (77), and Van Leeuwen et al. (78). Fan et al. (79). (Created with Biorender.com.)

women, found that, e.g., exposure to residential green areas was positively associated with HMO diversity and concentration. Variation in HMO composition is also evident between those inhabiting rural and urban areas, which has been observed in countries such as Ethiopia and Gambia (42), where, e.g., milk from mothers in urban Gambia had higher levels of LNnT compared to milk from mothers inhabiting rural parts (42). Additionally in Gambia, lower HMO concentrations were found in mothers' milk during the wet season compared to milk from mothers nursing during the dry season (62). Seasonal differences were also evident in a Canadian study (56), where HMO concentrations varied from winter to spring, e.g., higher LNnT in winter, and higher disialyllacto-*N*-tetraose (DSLNT) and 6'-SL were observed in spring. Diet has a profound impact on host health, with certain dietary components being associated with variations in HMO composition. A varied diet, including whole grains and vitamins, has been positively correlated to 2'-FL abundance (56, 63). Conversely, a high fat diet has been associated with a decrease in sialylated HMOs in breast milk (63–65). In central Africa, decreased food intake was shown to be associated with lower HMO concentrations (60, 66).

While secretor status remains the predominant factor, other parameters, some of which were discussed above, such as the living environment, geographical location, lactation stage, maternal prepregnancy BMI, age, diet, parity, mode of delivery, infant gestational age, and sex, contribute to inter- and intrapersonal HMO variation (58). Difficulty persists when evaluating independent variables impacting HMO composition and concentration as there are often confounding factors influencing the HMO content simultaneously.

BIFIDOBACTERIA AND HMO UTILIZATION

Bifidobacteria, first discovered by Tissier in 1899 from a breast-fed infant fecal sample, are characterized as gram-positive, anaerobic bacteria, often possessing a high G + C genome content. Specific members of *Bifidobacterium*, a genus which from a taxonomic perspective belongs to the Actinomycetota phylum, can be commonly found in the human gut. The overrepresentation of bifidobacteria in the gut of healthy, breast-fed infants is suggestive of their crucial role in supporting early life health and development of their infant host (11, 80). The beneficial effects of bifidobacteria are at least in part believed to be mediated through the production of carbohydrate-derived metabolites, either directly or indirectly through cross-feeding interactions, examples of which are discussed further below. Short-chain fatty acids (SCFAs) are metabolic end products of (bifido) bacterial fermentation of indigestible carbohydrates, including HMOs. These multi-functional compounds can decrease intestinal pH, impact gut barrier integrity, and prevent pathogen establishment (81–83). Other host interactive molecules produced by bifidobacteria include aromatic lactic acids, mentioned later in this review, exopolysaccharides, conjugated linoleic acid, and pili (35, 84, 85). Beneficial impacts exerted by infant-type bifidobacteria to the host are not solely due to carbohydrates but also nitrogen (86). Urea utilization by *B. longum* subsp. *infantis* strains indicates participation in nitrogen homeostasis in early life (87). The higher incidence of pathologies, such as atopy, obesity, and asthma, in infants with reduced bifidobacterial colonization has been reported in several studies (11, 88–90).

Human gut-derived bifidobacteria are generally categorized into two groups: “infant type” and “adult type.” Typical infant gut-associated bifidobacteria include species such as *B. breve*, *B. longum* subsp. *longum* (*B. longum*), *B. bifidum*, *B. pseudocatenulatum*, *B. longum* subsp. *infantis* (*B. infantis*), and *B. catenulatum* subsp. *kashiwanohense* (*B. kashiwanohense*) (13, 91, 92). In contrast, *B. adolescentis*, *B. pseudocatenulatum*, *B. catenulatum* subsp. *catenulatum*, *B. longum*, and *B. dentium* are common in the adult gut (93–95). The ubiquitous nature of *B. longum* has facilitated its identification in the gut microbiota across all age groups (96). This categorization of infant- and adult type is more complex than the presence or absence of one or more species; rather, it is a shift within the bifidobacterial species abundance as the infant develops toward adulthood. One of the defining features between the two categories is the ability of

infant-associated bifidobacteria to consume one or more HMOs and/or HMO degradants, which is not typically observed in the adult-type bifidobacterial species. This proven ability to degrade HMOs and derivatives thereof is referred throughout the text in relation to infant-type bifidobacteria. Infant-associated bifidobacteria encode various enzymes and transporters, which is discussed further, to metabolize HMOs, thus offering a competitive advantage over other (bifido)bacteria in the infant gut. Certainly, this ability of infant-associated bifidobacteria to colonize and persist is no doubt due to a variety of factors but includes their ability to metabolize both diet-derived and host glycans such as mucins and HMOs (11).

The underlying mechanisms involved in bifidobacterial, and other microbial, metabolism of HMOs have been the subject of extensive research with the added complexity that not all (infant-associated) *Bifidobacterium* members can (directly) utilize HMOs. The variation in HMO degradation ability, occurring at both species and strain levels, combined with the substantial number of HMO structures means much remains unanswered. This includes the precise mechanisms involved, the origins of this degradation ability, and the host benefits associated with this metabolic adaptation.

Bifidobacterial HMO utilization *in vitro*

One of the most prominent, and thus comprehensively studied, HMO degraders in the neonate gastrointestinal system is *B. infantis*. It has been demonstrated that strains from this species, including *B. infantis* M-63 and ATCC 15697, can use a broad spectrum of HMO structures, e.g., 2'-FL, 3'-SL, LNnT, and LNT (97–99). There are also strain-specific differences within this species, such as the aforementioned strain M-63 achieving a significantly ($P = 0.0004$) higher maximum optical density (OD) when 2'-FL was made available when compared to *B. infantis* Rosell-33 (R0033) (98). Comparable to *B. infantis*, *B. bifidum* are in general vigorous consumers of HMOs (100, 101), although there does appear to be some strain-level variation. Gotoh et al. (100) demonstrated that *B. bifidum* strains JCM 1254, TMC3108, and MC3115 achieved a higher OD (OD >1 at 20 hours) than strain JCM 7004 (OD <1 at 20 hours) in HMO-supplemented media. In a study using 13 *B. bifidum* strains, most demonstrated a capacity to grow using pooled HMOs from breast milk as well as synthetic HMOs, i.e., LNT, LNnT, 2'-FL, 3-FL, and 6'-SL (102). Four strains, JCM 1255, JCM 1209, JCM 7004, and S28a, struggled to grow using the pooled HMOs (102). Additionally, one of these strains, JCM 1255, was unable to grow using the synthetic HMOs tested, with the exception of LNT (102). Generally, HMO degraders are more common among strains of *B. infantis* and *B. bifidum*, in contrast to *B. breve* and *B. longum* members where HMO degradation is more sporadic. The ability to consume different HMOs is strain dependent among the latter two species, although LNT metabolism is a common property among strains of either of these species (103). Generally, *B. breve* can achieve a high level of *in vitro* growth when either LNT or LNnT is the sole carbohydrate (104, 105). In addition, some *B. breve* strains (e.g., strains SC95 and SC568) have the capacity to assimilate fucosylated HMOs, e.g., 2'-FL and 3-FL, although this is not widespread across the species (104). Often described as a “scavenger” consumer, *B. breve* can capitalize on the HMO degradants, such as fucose and sialic acid, released by other avid HMO degraders. This *B. breve* persistence in the presence of HMOs has been demonstrated *in vitro* using co-culture growth experiments (98, 106). Pertaining to this, the order in which microbes colonize an environment may be deterministic in the formation of the subsequent community structure, and thus, the functions that occur. Ojima et al. (106) examined the facilitative and inhibitory relationships *in vitro* between infant-associated bifidobacteria members in response to a pool of nine HMO structures. The authors observed that even with its comparatively low HMO degradation abilities relative to other bifidobacterial species employed in the study, as reflected in both its genotype and phenotype, *B. breve* was shown to nonetheless achieve a high level of growth in co-culture. Depending on the order in which it was inoculated into the media, *B. breve* UCC2003 was shown to outcompete *B. infantis* ATCC

15697, *B. bifidum* JCM 1254, and *B. longum* MCC10007 by metabolizing carbohydrates such as fucose, which other species provided from assimilating HMOs (106).

The aforementioned cross-feeding phenomenon has also been observed on a broader scale using *in vitro* community-based experiments where the initial microbial composition was an important factor when the fermentation of structurally distinct HMOs occurred (Table 1) (107–109). Xu et al. (107) used fecal microbiota groups dominated by either *B. longum*, *B. breve*, or *Bacteroides* to investigate the impact of structurally different HMOs. Taxonomic composition, SCFA production, and gas profiles differed, not solely owing to the various HMOs but more so due to the starting microbial composition. Similarly, a recent *in vitro* fecal fermentation study (109) using *B. longum*-dominated microbiotas revealed higher levels of community-produced lactate when neutral HMOs, rather than sialylated structures, were introduced. Taken together, bifidobacterial subpopulations may respond varyingly to distinct HMOs. Concomitantly, HMOs can influence the development of the infant gut microbiota by acting as selective agents for the growth of certain infant-associated (bifido)bacteria.

Knowledge regarding HMO assimilation in other infant-associated bifidobacterial members remains limited, although strains from species such as *B. kashiwanohense*, *B. pseudocatenulatum*, and *Bifidobacterium longum* subsp. *suis* (*B. suis*) have demonstrated some degradation abilities. Unlike *B. bifidum* and *B. infantis*, where HMO utilization is generalized to the species, HMO utilization in other bifidobacteria tends to be more sporadic. Studies have shown *B. kashiwanohense* strains DSM 21854 and PV20-2 as well as *B. suis* BSM11-5 preferred fucosyllactoses over sialylated structures, although this was strain dependent (126, 127). Additionally, it has been suggested that lactodifucotetraose (LDFT) and LNFP I may be acquired and metabolized by *B. kashiwanohense* JCM 15439T (128), with LNFP I and II degradation also carried out by strains such as *B. longum* SC596 (129), *B. breve* SC95 (104), and *B. pseudocatenulatum* strains SC595 and MP80 (130). Fucose accumulation in the growth medium as a result of fucosylated HMO fermentation has also been reported for *B. kashiwanohense* DSM 21854 (126), with free fucose unable to support growth (131). However, James et al. (127) deduced that even though growth, as measured using OD, was not substantial with free L-fucose, the metabolite profile altered, indicating some metabolic activity in the presence of this sugar. Additionally, L-fucose accumulation as a by-product from 2'-FL and 3-FL fermentation by *B. kashiwanohense* APCKJ1 was not observed (127). This, at least partial capacity to consume fucose, is highly intriguing and reinforces the importance of HMO and degradant(s) breakdown on a metabolic level. A recent study has demonstrated that fucose contributes to maintaining the reduction-oxidation balance, and in a medium with low levels of amino acids, fucose will promote bifidobacterial growth (132). Pooled HMOs as the primary energy source have supported select *B. pseudocatenulatum* strains (130, 133). For example, out of eight infant-derived isolates, four isolates, namely SC585, MP80, MP86, and DSM 20438, were able to achieve a high level of growth ($0.94 \leq \text{maximum OD} \leq 1.17$), comparable to *B. infantis* ATCC 15697 (maximum OD = 1.13), with this ability also evident using purified 2'-FL and 3-FL (130). It was determined that this consumption divergence between infant isolates was driven by the specific ability to metabolize fucosylated HMOs. Furthermore, LNB supplementation has aided growth in a subset of *B. pseudocatenulatum* tested (33 strains out of 61) (134). For certain strains from both *B. kashiwanohense*, e.g., APCKJ1 and *B. pseudocatenulatum*, e.g., MP80 and DSM 20438, putative mechanistic strategies for fucosylated sugar assimilation and catabolism have been described (127, 130). This fucosyllactose degradation specialization may significantly contribute to the formation of the gut metabolome.

Infant-based studies

Culture- and sequencing-based investigations have been instrumental in unraveling the ability of bifidobacteria and other taxa to utilize HMOs, although this has not been investigated extensively *in vivo*. Many *in vivo* studies have focused on the ability of breast milk, and/or specific HMOs (either individual structures or mixes), to reduce

TABLE 1 Overview of some recent *in vivo*- and *in vitro*-based fermentation investigations of microbial communities as a result of HMO supplementation^a

Type of HMO	Study design	Microbial analysis	Effects on gut microbiota	Metabolite production	Reference
<i>In vivo</i>					
2'-FL and <i>L. reuteri</i> DSM 17938	Double-blind RCT in infants	16S rRNA, qPCR	↑ <i>Bifidobacterium</i> , ↓ <i>C. difficile</i> and <i>K. pneumoniae</i>	↑ Lactate	Alliet et al. (110)
HMO blend (2'-FL, DFL, LNT, 3'-SL, and 6'-SL)	RCT in infants	Shotgun metagenomics	↑ <i>B. infantis</i> , ↓ <i>C. difficile</i>	↑ Acetate and lactate	Bosheva et al. (111)
Amino acid infant formula with HMOs (2'-FL and LNnT)	Single-arm, multicenter, interventional clinical trial in infants with CMPA. No control group	Shotgun metagenomics	↑ <i>B. breve</i> , <i>B. bifidum</i> , <i>B. infantis</i> , and <i>B. longum</i> , ↓ <i>Escherichia</i> spp., and <i>Klebsiella</i> spp.,	↑ Acetate	Gold et al. (112)
Test infant formula with 2'-FL and LNnT (2:1)	RCT in infants	16S rRNA	↑ <i>Bifidobacterium</i> , ↓ <i>Carnobacteriaceae</i> and <i>Escherichia</i>	↑ Acetate, ↓ succinate, butyrate, propionate, and 5-aminovaleate	Dogra et al. (113)
Commercial formula (control) and same formula with 2'-FL (test)	Double-blind RCT in infants	Shotgun metagenomics	2'-FL = ↑ <i>Bifidobacterium</i>	N/A	Wallingford et al. (114)
HMOs (extracted from breast milk) and 2'-FL	Dextran sodium sulphate-induced mouse model 6 weeks old (for prevention of colitis)	16S rRNA	HMOs = ↑ <i>Muribaculaceae</i> , <i>Bacteroides</i> , and <i>R. torques</i> 2'-FL = ↑ <i>Lactobacillus</i> , <i>Muribaculaceae</i> , and <i>R. torques</i> HMOs and 2'-FL = ↓ <i>Streptococcus</i> , <i>Escherichia-Shigella</i> , and <i>Lactococcus</i>	HMOs = ↑ acetate, propionate, and butyrate 2'-FL = ↑ acetate and propionate	Li et al. (115)
2'-FL	Human microbiota-associated mouse model (infant fecal transplant)	16S rRNA	↑ <i>Bifidobacteria</i> , <i>Olsenella</i> , and <i>Blautia</i> ↓ <i>Enterorhabdus</i> and <i>Lachnospiraceae</i> , UCG-006	↑ Acetate and propionate ↓ butyrate	Qingxue Chen et al. (116)
2'-FL	Healthy sucking rats	16S rRNA	↑ <i>Lactobacillus</i> ↓ <i>Enterococcaceae</i> and <i>Streptococcaceae</i>	↑ Butyric acid	Azagra-Boronat et al. (117)
<i>In vitro fermentations</i>					
HMO6 mix (2'-FL, LNnT, diFL, 3'-SL, and 6'-SL), 2'-FL, lactose, and 2'-FL/LNnT	baby M-SHIME	16s rRNA, qPCR	2'-FL = ↑ <i>Bifidobacteriaceae</i> and <i>Coriobacteriaceae</i> 2'-FL/LNnT = ↑ <i>Brucellaceae</i> HMO6 mix = ↑ <i>B. adolescentis</i> and <i>F. prausnitzii</i> , ↓ Firmicutes Lactose = ↑ <i>Rikenellaceae</i> and <i>Veillonellaceae</i>	2'-FL = ↑ propionate HMO6 mix = ↑ acetate and butyrate	Natividad et al. (118)
GOS, 2'-FL, or GOS and 2'-FL (1:1 and 3:1)	microMatrix and baby M-SHIME	16S rRNA	GOS = ↑ <i>Lactobacillus</i> 2'-FL = ↑ <i>Veillonella</i> and <i>Akkermansia</i> GOS and 2'-FL = ↑ <i>Veillonella</i> , <i>Bifidobacterium</i> , <i>Lactobacillus</i> , and <i>Streptococcus</i> GOS and 2'-FL = ↓ <i>Salmonella</i> and <i>Citrobacter</i> GOS or 2'-FL = ↓ <i>Escherichia/Shigella</i>	GOS and/or 2'-FL = ↑ acetate	Lindner et al. (119)

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TABLE 1 Overview of some recent *in vivo*- and *in vitro*-based fermentation investigations of microbial communities as a result of HMO supplementation^a (Continued)

Type of HMO	Study design	Microbial analysis	Effects on gut microbiota	Metabolite production	Reference
2'-FL, GOS, and 2'-FL/GOS (1:4 ratio)	<i>In vitro</i> fermentation	16S rRNA	2'-FL and 2'-FL/GOS = ↑ <i>Bifidobacterium</i> , <i>Escherichia-Shigella</i> , and <i>Enterococcus</i> GOS = ↑ <i>Bifidobacterium</i>	↑ Acetate, lactate, succinate, and propionate	Akkerman et al. (120)
2'-FL, 3-FL, 3'-SL, 6'-SL, LNT, LNnT, FOS, and GOS	<i>In vitro</i> fermentation	16S rRNA	<i>B. longum</i> -dominated inocula: HMOs and GOS = ↑ <i>B. longum</i> <i>B. breve</i> -dominated inocula: HMOs, GOS, and FOS = ↑ <i>B. breve</i> <i>Bacteroides</i> -dominated inocula: 2'-FL and 3-FL = ↑ <i>B. breve</i> , <i>B. fragilis</i> , and <i>B. vulgatus</i> LNT and LNnT = ↓ <i>B. fragilis</i> and <i>B. vulgatus</i> 3'-SL and 6'-SL = ↓ <i>Ba. vulgatus</i> HMOs and GOS = ↑ <i>B. longum</i> and <i>E. coli</i> FOS = ↑ <i>Klebsiella pneumoniae</i>	GOS and HMOs (2'-FL, 3-FL, LNT, and LNnT) = ↑ acetate and lactate	Xu et al. (107)
2'-FL, 3-FL, 3'-SL, 6'-SL, LNT, and LNnT, GOS, and FOS	<i>In vitro</i> fermentation	16S rRNA	LNT2 = ↑ <i>Bifidobacterium</i> (<i>B. longum</i> and <i>B. bifidum</i>) and <i>Collinsella</i> 3-FL = ↑ <i>B. longum</i> , <i>Bacteroides</i> (<i>B. dorei</i>), and <i>Enterococcus</i> genus GOS/inulin = ↑ <i>B. longum</i> LNT2, 2'-FL and GOS/inulin = ↓ <i>Escherichia-Shigella</i>	<i>B. longum</i> -dominant inocula HMOs (2'-FL, 3-FL, LNT, and LNnT) and GOS = ↑ acetate and lactate GOS/inulin = ↑ acetic acid 3-FL = minor amounts of acetic acid (1.1 μmol mg ⁻¹) and lactic acid (0.3 μmol mg ⁻¹) LNT2 = ↑ acetic acid, butyric acid, succinic acid, and lactic acid	Li et al. (109) Kong et al. (121)
3-FL, LNT2, and GOS/inulin (9:1)	<i>In vitro</i> fermentation (in anaerobic glass fermentation bottles)	16S rRNA	Baby 1: 3-FL, 3'-SL, and 6'-SL = ↑ <i>B. breve</i> , <i>P. distasonis</i> Baby 2: 3-FL, 3'-SL = ↑ <i>B. bifidum</i> , <i>B. adolescentis</i> ↑ <i>Bifidobacteriaceae</i> ↓ <i>Veillonellaceae</i> family	3-FL = ↑ propionic acid FOS = ↑ butyric acid	Wiese et al. (122)
2'-FL	M-SHIME	16S rRNA, qPCR	FF-fast degrader: 2'-FL, <i>B. bifidum</i> + 2'-FL, <i>B. infantis</i> + 2'-FL = ↑ <i>Lactobacillaceae</i> family FF-slow degrader: 2'-FL + <i>L. helveticus</i> Rosell, probiotic combination + 2'-FL = ↓ <i>Streptococcaceae</i> BF-slow and BF-fast degraders: 2'-FL + <i>L. helveticus</i> Rosell, probiotic combination and 2'-FL = ↑ <i>Lactobacillaceae</i> family, ↓ <i>Clostridiaceae</i>	↑ Acetate BF-slow degrader: 2'-FL + <i>L. helveticus</i> Rosell, probiotic combination = ↑ lactic acid BF-fast degrader: 2'-FL, <i>B. bifidum</i> + 2'-FL, and <i>B. infantis</i> + 2'-FL = ↑ acetic acid 2'-FL + <i>L. helveticus</i> Rosell, probiotic combination + 2'-FL = ↑ lactic acid FF- fast degrader:	Van den Abbeele et al. (123) Nogacka et al. (108)

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TABLE 1 Overview of some recent *in vivo*- and *in vitro*-based fermentation investigations of microbial communities as a result of HMO supplementation^a (Continued)

Type of HMO	Study design	Microbial analysis	Effects on gut microbiota	Metabolite production	Reference
2'-FL	M-SHIME	16S rRNA, qPCR	Donors 1, 2, and 3 = ↑ <i>Bifidobacterium</i> spp. (<i>B. adolescentis</i>)	2'-FL + <i>L. helveticus</i> Rosell, probiotic combination + 2'-FL, 2'-FL, <i>B. bifidum</i> + 2'-FL, and <i>B. infantis</i> + 2'-FL = ↑ acetic acid, succinic acid, and lactic acid	Van den Abbeele et al. (124)
			Donor 1 = ↑ <i>Lachnospiraceae</i>	FF-slow degrader:	
			Donor 3 = ↑ <i>Acidaminococcaceae</i>	2'-FL + <i>L. helveticus</i> Rosell, probiotic combination + 2'-FL = ↑ lactic acid and succinic acid	
2'-FL	<i>In vitro</i> fecal batch fermentations	16S rRNA	FF-fast degrader = ↑ <i>Lactobacillaceae</i> (<i>Lactobacillus</i>) and <i>Enterococcaceae</i>	Donors 1, 2, and 3 = ↑ acetate, butyrate, and propionate	Nogacka et al. (125)
			FF-slow degrader = ↑ <i>Enterobacteriaceae</i> and <i>Streptococcaceae</i>	Donor 3 = ↑ lactate	
			BF-fast degrader = ↑ <i>Bifidobacteriaceae</i> (<i>Bifidobacterium</i>) and <i>Enterobacteriaceae</i>	FF-fast and slow degraders = ↑ succinic acid	
			BF-slow degrader = ↑ <i>Enterobacteriaceae</i>	FF- and BF-fast degraders = ↑ acetic acid, ↓ isovaleric acid	

^aDFL, 2,3'-di-fucosyl-lactose; CMPA, cow's milk protein allergy; RCT, randomized controlled trial; FF, formula fed; BF, breast fed; SHIME, Simulator of the Human Intestinal Microbial Ecosystem; ↑, increase; ↓, decrease.

the development of infant-associated disorders such as necrotizing enterocolitis and evaluate the safety and tolerance of fortified formula milk (135, 136). Moreover, many *in vivo* investigations focus on 2'-FL and LNnT as these are most frequently added to infant formula. For example, Berger et al. (137) completed a randomized, double-blind study evaluating the gut microbiota of infants (0–14 days old)-fed formula (control) versus the same infant formula with the addition of both 2'-FL and LNnT over a period of 6 months. Following on from this, all infants received the same follow-up formula without HMOs until 12 months of age. Breast-fed infants served as a comparison group with samples taken at 3 and 12 months. At 3 months, the addition of HMOs to infant formula was found to have modulated the microbial composition, bringing it closer to that of the breast-fed infant group, as determined using 16S rRNA sequencing (137). HMO-containing infant formula supported bifidobacterial abundance (median relative abundance at 3 months: 82.81%) compared to the control formula (74.47%) ($P < 0.01$), although it was still less than breast-fed infants (90.87%) ($P < 0.05$, compared to control; non-significant compared to the test group) (137). Lower relative abundances of *Escherichia* and *Peptostreptococcaceae* were observed in the group fed infant formula containing HMOs compared to the control formula group (137). The supplementation of infant formulas with both 2'-FL and LNnT has been shown to support bifidobacterial abundance in clinical studies elsewhere (112, 113, 137). More specifically, Gold et al. (112), using 16S rRNA sequencing, reported that after 1 month, the microbiota became dominated by HMO-utilizing bifidobacterial species, e.g., *B. breve*, *B. infantis*, and *B. longum*, after supplementation with 2'-FL- and LNnT-containing formula. It must be noted that 16S rRNA sequencing alone for bifidobacteria subspecies identification may be problematic as the 16S gene sequence identity is relatively high (up to 99%) for *Bifidobacterium* species (138). A reduction in potential pathogens, such as *Carnobacteriaceae*, *Escherichia* spp., and *Klebsiella* spp., was observed in parallel (112, 113). This shift in microbial composition toward that of a breast-fed infant has also been reported for formula fortified with 2'-FL alone (114).

Investigating the associations between the infant microbiota and HMOs consumed can aid in revealing the apparent complex HMO-microbial dynamics. Borewicz and colleagues (139, 140) evaluated breast milk HMO concentrations and infant fecal microbial composition of 24 mother-infant pairs across three time points (2, 6, and 12 weeks old) (139) and of 121 mother-infant pairs at one sampling point (4 weeks old) (140). Redundancy analysis of the operational taxonomy units (OTUs) and estimated consumption of different HMO structures illuminated the potential association between *Bifidobacterium* OTU 1263 and a high level of HMO consumption. Different HMO structures were shown to be linked with the infant microbiota composition at various time points, such as LNFP III at 2 weeks, 3'-SL at 6 weeks, LNFP I and 2'-FL at 4 weeks ($P < 0.05$), and LNH at 12 weeks (139, 140). It should be noted that, in some instances, sample size was relatively small, further complicated by additional confounding factors, such as infant age and sex, mode of delivery, etc. Further OTU analysis highlighted numerous associations between bifidobacterial members and various HMO structures. Specific examples include *Bifidobacterium* OTU 614 associated with 2'-FL, 2,3'-di-fucosyllactose (DFL), LNDFH I, LNFP II, LNFP III, LNH, and LN(n)T, and the presence of *Bifidobacterium* OTU 418 being significantly correlated ($P < 0.05$) with high consumption levels of 2'-FL, LN(n)T, LNFP III, LNFP II, LN(n)H, LNDFHI, DFL, LNFP I, LSTa, LSTb, and LSTc (139, 140). In a recent larger-scale study, LNH, a non-fucosylated HMO, was positively associated with higher relative abundances of *Bifidobacterium* amplicon sequence variants (141). Elsewhere, in a Chinese cohort of 69 mother-infant pairs, it was reported that *Bacteroides* positively correlated ($R_1 > 0.2$, $P < 0.05$) with 3'-SL present in breast milk, while associations were also observed for certain fecal metabolites, e.g., tryptophan, N-acetyltryptophan, and indolelactic acid (142).

Recent *in vivo* studies focusing on the infant microbiota and HMOs, using either a mix of HMOs (111) or a mix of HMOs in combination with a probiotic (110), have reported the increased abundance of *Bifidobacterium* such as *B. infantis*, although this species-level

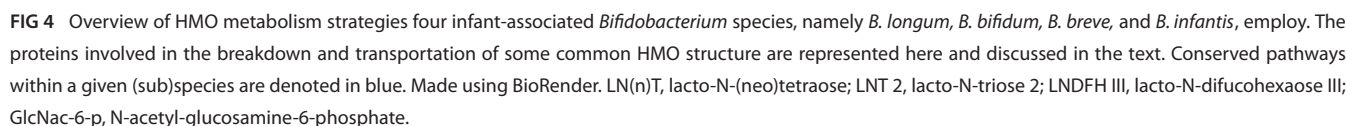
distribution varies between these studies. Both studies demonstrated that the gut microbiota composition of infants-fed formula with added HMO(s) shifted toward that of a breast-fed neonate. Although individual HMOs were not comparatively assessed with the mix of five HMOs (2'-FL, DFL, LNT, 3'-SL, and 6'-SL) in Bosheva et al. (111), the authors postulated that there may be a more pronounced increase of *B. infantis* relative abundance when compared to a formula containing two HMOs (2'-FL and LNnT) (137), which presents an opportunity for further comparison. Additionally, HMO-fortified formula modulated the gut microbial composition of infants delivered via C-section to become closer to that of vaginally born infants (111, 137). Future *in vivo* studies evaluating the microbiota response to pooled HMOs, either alone or combined with other prebiotics such as GOS, would be informative, as HMO structures in breast milk are complex and heterogeneous.

In addition to HMOs, clinical trials have also been carried out with other established prebiotics such as GOS and FOS/inulin. In studies where long chain (Ic) FOS has been referred to, we will denote this as inulin due to the longer chain structure, whereas the short chain structure will simply be referred to as FOS. The percentage of infants colonized by *B. breve* and *B. adolescentis* from enrollment to 4 months of age increased in infants fed GOS more so than the control (143). Furthermore, GOS, when combined with inulin, resulted in a higher abundance of *B. longum*, *B. animalis*, *B. breve*, and *B. pseudocatenulatum*, with the most notable increase evident for the *B. infantis* species (144). Administration of a non-hydrolyzed cow's milk-based formula containing a synbiotic mix of GOS/inulin and *B. breve* M-16V was shown to shift the infant gut microbiota closer to that of breast-fed infants and compensated for delayed bifidobacterial colonization in infants delivered by C-section compared to the same formula without the synbiotic (145). Moreover, lower *Clostridium* viable plate counts and higher abundance of *Bifidobacterium*, as determined using qPCR, were observed in colic infants when supplemented with a GOS-containing formula (146), and similarly, as a result of a GOS-based synbiotic formulation, relative abundance reductions in *Ruminococcus gnavus* and *Blautia* were observed (147). Clinical evidence thus far suggests that prebiotics promote restoration in cases of a disrupted gut microbiota and improve diversity while decreasing potentially pathogenic bacteria, for instance following antibiotic treatment or delivery by C-section (110, 111, 137, 145, 148). Despite these insights, at present, there are very few publications related to *in vivo* studies that compare the effects of HMOs alongside, or in combination with, other prebiotics on the infant gut microbiota.

VARIOUS HMO DEGRADATION STRATEGIES EMPLOYED BY INFANT-ASSOCIATED BIFIDOBACTERIA

Species, subspecies, and, indeed, strain-specific molecular strategies involved in HMO utilization have been the focus of various studies (99, 104, 105, 149). These strain/(sub)species-level differences explain the co-existence of multiple bifidobacterial species and strains in a single niche, as overlapping and distinct metabolic capacities exist within the HMO-degrading bifidobacterial population in the neonatal intestine. Multiple HMO structures can be used by a variety of strains, highlighting their repertoire of human milk carbohydrate utilization mechanisms, many of which are illustrated in Fig. 4, thereby enabling infant-associated bifidobacterial prevalence and abundance. Although HMO metabolism is generally favored by infant-type bifidobacteria, this is not an exclusive ability as bifidobacteria isolated from adults, though probably to a lesser extent, have been shown to metabolize HMOs (150, 151).

The various strategies which infant-related bifidobacteria employ to degrade HMOs can be broadly characterized as intracellular and extracellular approaches (102). The former relies on HMO-specific oligosaccharide transporters, while the latter is based on extracellular HMO-targeting glycosyl hydrolases (GHs). Uptake of intact or partially degraded HMOs is a signature of infant-associated bifidobacteria, although the complete set of molecular mechanisms employed by various bifidobacterial species have yet to be elucidated. *B. infantis* and *B. breve* internalize certain HMOs, e.g., LNnT, LNT, and



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to FL transporter-2 (Blon_2202–2204), which can internalize LDFT and LNFP I, in addition to 2'-FL and 3-FL (128, 155, 158).

Like *B. longum*, *B. breve* can assimilate a rather modest number of HMOs and HMO-derived structures. It was previously thought that fucosylated HMO utilization was uncharacteristic of this species; however, in recent years, strategies for HMO assimilation have been identified. In a study conducted by Ruiz-Moyano et al. (104), a GH95 α -fucosidase was identified in all 24 strains examined, whereas the presence of GH29 α -fucosidase was more variable and only detected in five strains. The ability of these strains to metabolize fucosylated HMOs was more prominent in those possessing a GH29 α -fucosidase, e.g., strains SC95, SC568, and SC154, although some possessing this enzyme were not able to degrade 2'-FL *in vitro* (104), indicating, perhaps, that other activities, such as enzymes or transporters, are necessary. The capacity to utilize acidic HMOs by *B. breve* was also demonstrated in this study, alongside the identification of an α -sialidase (GH33) (104). Glycoprofiling of a subset of *B. breve* strains revealed utilization patterns in the presence of total pooled HMOs, including the preferential consumption of specific sialylated HMOs, such as LSTb and monosialyllacto-*N*-hexaose (S-LNH), over smaller HMOs (104). Interestingly, when individual sialylated HMOs, 3'-SL and 6'-SL, were tested *in vitro*, growth was not apparent using these smaller molecules (104). Neutral HMOs, e.g., LNT and LNnT, have also been shown to support *B. breve* growth, both individual structures and when present in pooled HMOs (98, 104). James et al. (105) characterized various metabolic strategies that *B. breve* UCC2003 employs for LNT (type 1 HMO) and LNnT (type 2 HMO) digestion, determining that overlapping yet distinct metabolic pathways exist for these two HMOs. Reflecting the tactics of *B. infantis*, *B. breve* members also internalize LN(n)T using ABC transporters, making the structure(s) available for degradation by intracellular β -galactosidases. One of these enzymes, encoded by *IntA*, acts on the Gal β -1,3GlcNAc of LNT and Gal β -1,4GlcNAc linkages of LNnT. While the *LntA* β -galactosidase can act on both LNT and LNnT, the first galactose of LNnT can also be cleaved by other β -galactosidases, *LacZ2* and *LacZ6* (105). Both lacto-*N*-triose, which is then hydrolyzed by *NahA*, and galactose are liberated from the hydrolysis of LN(n)T. The breakdown products from either of these HMOs feed directly or indirectly into the bifid shunt metabolic pathway (105). Once thought of as a "scavenger species," intensive efforts have revealed that the ability of *B. breve* strains to acquire and digest HMOs is more extensive than previously recognized.

In contrast to the (predominantly) intracellular approaches described for *B. infantis*, *B. bifidum* deploys extracellular hydrolases, e.g., fucosidases, to metabolize HMOs extracellularly and subsequently import the desired degradant carbohydrates (100). Some of these degradants can remain outside the cell for other (bifido)bacteria to acquire (100). Due to the presence of extracellular enzymes, large HMO structures (degree of polymerization >7) can be degraded as there does not seem to be a size limitation in contrast to bifidobacterial species solely depend on ABC transporters to access HMOs (159). *B. bifidum* provides metabolites, such as fucose and sialic acid, available extracellularly for others to consume as these two HMO-derived carbohydrates are not assimilated by *B. bifidum* itself (160, 161). To achieve this, a collection of secreted enzymes, including α -L-fucosidases (*AfcA* and *AfcB*), lacto-*N*-biosidase (*LnbB*), β -1,4-galactosidase III (*BbgIII*), β -1,3-*N*-acetylglucosaminidase I (*Bbhl*), and sialidase II, is involved in extracellular HMO breakdown (100, 162). Sialidases from other bifidobacterial strains target α -2,3 and α -2,6 linkages, whereas *SiaBb1* and *SiaBb2* in *B. bifidum* target the α -2,3 linkage over the α -2,6 linkage (158, 163). *B. bifidum* also harbors a greater number of carbohydrate-binding modules in comparison to other species, which may enhance its catabolic ability through the increased GH affinity toward carbohydrates (164). Furthermore, an example of LNB utilization occurs in *B. bifidum* JCM1254, whereby a galacto-*N*-biose (GNB)/LNB transporter and GNB/LNB phosphorylase (*GLNBP*) act on LNB, with the ensuing products (galactose-1-phosphate and GlcNAc) then entering the Leloir and amino-sugar metabolizing pathways, respectively, before being routed to the

bifid shunt (100, 101). The extracellularly released LNB can also be utilized by other bifidobacteria that possess a GNB/LNB pathway (158).

In contrast to species such as *B. infantis*, where HMO catabolism is highly conserved, the ability of *B. longum* members to assimilate HMOs is variable, and both intra- and extracellular approaches exist (165). This variability was observed by Garrido et al. (129), where *B. longum* SC596 was shown to vigorously use fucosylated HMOs from pooled breast milk samples, compared to other infant-derived *B. longum* strains tested due to the presence of a particular gene cluster. A subset of *B. longum* strains, e.g., JCM1217, possess the *lnbX* gene (LnbX positive), encoding an extracellular lacto-*N*-biosidase, characterized as part of the GH136 family, which is involved in the degradation of LNT to LNB and lactose (158, 166). Similarly, *B. bifidum* encodes an extracellular GH20 LnbB (encoded by *lnbB*) which also hydrolyzes LNT, thereby liberating LNB and lactose (101), although the exact mechanisms differ between the two species. Both LnbB and LnbX are highly specific for activity on LNT, a core structural unit of many HMOs. The *B. longum* JCM1217-encoded lacto-*N*-biosidase is more flexible in its structural specificity for LNB than its *lnbB*-encoded counterpart, as it can still release LNB units even if its substrate LNT is decorated with other monosaccharides, e.g., fucose (167). This could provide a route for the persistence of *B. longum* in the infant gut in that, even though only a limited number of HMOs can be utilized, there could be flexibility within this restricted range. The liberated LNB from LNT can then be transported across the *B. longum* cell membrane via the GNB/LNB transporter (158, 166). Strain-to-strain variability appears to be more prevalent in HMO consumption by *B. longum* subspecies members when compared to members of the *B. infantis* subspecies (103, 153, 168).

OTHER TAXA AND HMO UTILIZATION

HMO utilization in the infant gut is not a phenomenon exclusive to bifidobacteria. Intense research efforts have led to the identification of a broader scope of HMO-degrading microbes residing in the gut, including members belonging to taxa such as *Akkermansia*, *Lactobacillus*, *Bacteroides*, *Ruminococcus*, and *Roseburia*, which have developed strategies to consume specific HMO structures. *Akkermansia muciniphila* has the advantageous ability to consume host mucosal glycans (169). This species, a member of the Verrucomicrobia phylum that colonizes the mucus layer, can in addition to mucin-associated glycans also utilize certain HMOs. Structural similarities exist between mucin-derived glycans and HMOs; therefore, the presence and survival of *A. muciniphila* in the infant gut may be abetted by its HMO-foraging abilities. Both *in silico* and *in vitro* studies have elaborated on the functional capacity of *A. muciniphila* when HMOs are made available, which followed on from initial studies where positive correlations were observed between the presence of this bacterial species and HMO availability (170). Glycan-degrading enzyme expression has been observed when *A. muciniphila* is grown using HMOs as a sole carbon/energy substrate (171). Kostopoulos et al. (171) identified key enzymes involved in HMO degradation, including α -L-fucosidases, β -galactosidases, exo- α -sialidases, and β -acetylhexosaminidases and the GH families responsible (e.g., GH2, GH20, GH29, GH33, GH35, and GH95 families), and proposed the mechanistic strategies employed by *A. muciniphila*. The capacity to consume different HMO structures varied, e.g., a large proportion of 2'-FL and 3'-SL in the growth medium was degraded in contrast to that of 3-FL (171). This led to the proposal of putative degradation pathways for 2'-FL, 3'-SL, LNT, lacto-*N*-triose II, and lactose by *A. muciniphila* (171). *A. muciniphila* is often categorized into four phylogroups (Aml-AmlV), with the type strain, *A. muciniphila* MucT, assigned to the Aml phylogroup. Luna et al. (172) comparatively assessed the HMO-associated GHs present in 85 *A. muciniphila* genomes, with representatives from all 4 phylogroups included. GH20 (β -hexosaminidase; lacto-*N*-biosidase; β -*N*-acetylglucosaminidase) was consistently the most abundant HMO-associated GH across all phylogroups, with the highest number found in AmlI. The lowest number of potential α -fucosidases (e.g., GH29, GH95, and GH141) and *N*-acetyl β -hexosaminidases (e.g., GH18, GH20, GH84, and GH109) was observed in isolates belonging to Aml (172). The authors

then tested the HMO-utilizing ability of four *A. muciniphila* strains, one representative strain per phylogroup, *in vitro*. Similar to what has been observed for bifidobacterial members, strain-level variation was observed in terms of the final OD reached when cultivated in the presence of 2'-FL, 6'-SL, and LN(n)T, with strain CSUN-19 (AmIV) consistently achieving the highest OD using the four individual HMOs tested (172). A recent study focusing on the structural characterization of *A. muciniphila* fucosidases, specifically four assigned to GH29 (AmGH29A-D) and two assigned to GH95 (AmGH95A and AmGH95B), revealed that both AmGH29C and AmGH29D are able to hydrolyze 3-FL yet that only AmGH29C is able to target this glycosidic linkage within the structurally larger LNFP V (173). Broader enzymatic specificity of AmGH95B compared to AmGH95A was also observed as both were active on 2'-FL, while only AmGH95B exhibited activity on 3-FL (173). Relative abundance increase of *Akkermansia* effected by the inclusion of 2'-FL using *in vitro* infant fecal fermentations has also been observed by Lindner et al. (119).

Although regularly found at a lower prevalence than bifidobacteria, lactobacilli are frequently identified as members of the infant gut microbiota (28). In contrast to bifidobacteria, the ability to use HMOs is not a widely conserved phenomenon among lactobacilli. Correlation analysis, however, has indicated that degradation of 2'-FL, DFL, LNDFH I, LNT and its isomer LNTnT, and LNFP II was significantly associated with a higher relative abundance of lactobacilli (140). In this study, the species level was undetermined, and it is important to consider the impact of other confounding factors. A limited number of strains have the functional capacity to degrade complex HMO structures. One such example is *Lactobacillus plantarum* ATCC BAA-793, for which pooled HMOs moderately supported growth ($OD_{600nm} 0.34 \pm 0.1$) (174). Many lactobacilli can capitalize on the availability of HMO constituents such as glucose, lactose, and GlcNAc to support growth, thereby acting as "secondary degraders" (175). *Lacticaseibacillus rhamnosus* strains GG and HN001 have been demonstrated to grow on fucose as the primary energy source (97, 153), although this was not consistent across all *L. rhamnosus* strains tested (153). Certain HMOs, e.g., LNTnT, can be degraded to support growth, albeit moderately in some cases, of particular strains such as *Lactobacillus acidophilus* strains La-5 and NCFM, *Lactiplantibacillus plantarum* LP-66, and *Limosilactobacillus reuteri* DSM 17938 (97). *L. plantarum* is often identified in a range of environments such as fermented foods, plants, and the human gut (176). This expansive niche colonization is suggestive of the pervasive ability to utilize many substrates, including, to a limited degree, HMOs. Genomic assessment of 54 *L. plantarum* strains revealed that they all encode GH2 (β -galactosidase), whereas the presence of genes encoding GH20 (β -hexosaminidase/lacto-N-biosidase) was lineage dependent, both of which can be involved in HMO breakdown (177). The authors postulated that the presence of these enzymes is related to lactose degradative abilities and the highly diverse environments where *L. plantarum* can colonize (177). Some mechanistic strategies have been described, e.g., a novel phosphoenol phosphotransferase system was identified in *Lacticaseibacillus casei*, which facilitated the importation of LNB (178), and similarly, a system for LacNAc internalization has been described (179); both LNB and LacNAc represent HMO constituents. Additionally, α -L-fucosidase activity as part of the GH29 family (AlfA, AlfB, and AlfC) has been characterized in lactobacilli such as *L. casei*, *Lacticaseibacillus paracasei*, and *L. rhamnosus*, with different strains predicted to carry one, two, or three of such intracellular enzymes (180, 181). Furthermore, an extracellular β -galactosidase described in *L. acidophilus* NCFM can remove galactose from LNTnT resulting in the liberation of LNT2 (97). In a recent study (182), the growth of several *Lactobacillus* strains (*L. acidophilus* ATCC 4796, *Lactobacillus johnsonii* ATCC 33200, *L. paracasei* ATCC 25302, *L. plantarum* ATCC 14917, *Limosilactobacillus fermentum* ATCC 14931, *Lactobacillus gasseri* ATCC 3323, *Lactobacillus delbrueckii* subsp. *bulgaricus* ATCC 11842, and *Levilactobacillus brevis* ATCC 27303) was examined using preterm human milk, preterm formula, or term formula with added 2'-FL. In comparison to the medium control, all strains were shown to grow using preterm milk and preterm formula, with six strains able to grow using

the HMO-supplemented formula (182). Variation occurred between strains when pH was measured after 20 hours growth (182). The authors next scrutinized 452 lactobacilli genomes across 23 species to identify genes encoding putative HMO-degrading GHs (182). Fewer GHs were identified compared to bifidobacterial genomes; nonetheless, most lactobacilli species were predicted to encode members of GH2 and GH42 families, while genes specifying putative GH20 and GH29 family members were less commonly identified (182). Although many lactobacilli do not appear to possess the ability to consume a wide variety of different HMOs, their abundance and functional activity can be stimulated indirectly by extracellularly released carbohydrates and metabolites from HMO degradation by other taxa (183).

Bacteroides species are present in the infant gut population to varying extents (28) and commonly possess extensive saccharolytic activities achieved through the presence of polysaccharide utilization loci. Constituent members such as *Bacteroides thetaiotaomicron*, *Bacteroides fragilis*, and *Bacteroides vulgatus* (now *Phocaeicola vulgatus*) have been shown to exhibit growth in the presence of various HMO structures when used as the primary energy source (153, 184, 185). These include 2'-FL, 3-FL, and 6'-SL, with species differences observed when 3-FL and LDFT were the sole carbohydrate substrates (184). Like *A. muciniphila*, some *Bacteroides* members can consume host-derived, mucus-associated glycans, and it has been determined that *Ba. thetaiotaomicron* and *Ba. fragilis* induce the same genes for HMO utilization as for such host mucus glycan consumption, although species variation was evident (186, 187). The link between mucin and HMO degradation in the infant gut remains unclear. The overlapping enzyme activities employed in HMO and host mucus glycan degradation include GH33 sialidases, GH2 β -galactosidases, and GH95 and GH29 fucosidases (171, 185). The dual ability to metabolize both mucin-associated glycans and HMOs has been observed in only a limited number of bacteria from genera such as *Bacteroides*, *Akkermansia*, and *Ruminococcus* (171, 186, 188). Furthermore, the characterization of mucin utilization enzymes and pathways for *B. bifidum* PRL2010 and JCM1254 has been reported (162, 189). *B. bifidum*, as described above, mainly employs extracellular glycosidases for HMO utilization which could be a significant factor compared to other bifidobacteria which mostly use internal enzymes for HMO hydrolysis, thereby limiting their mucin degradation capacity. As mentioned previously, *A. muciniphila* is a prominent mucin degrader which uses similar GHs for HMO utilization, e.g., GH2, GH20, GH29, GH33, GH35, and GH95 (171). The ability of these species to degrade both HMOs and mucin may have developed to facilitate their persistence in the gut as the infant develops to adulthood. This opportunistic consumption of HMOs may play a role in the persistence of *Bacteroides* members in the infant gut. Interestingly, there appeared to be similar growth patterns depending on the nature of the HMO structure for one *Bacteroides dorei* (now *Phocaeicola dorei*) isolate, i.e., sialylated (3'-SL and 6'-SL), neutral (LNT and LNTT), and fucosylated structures (2'-FL and DFL) (190). This, however, was not the case for a second *P. dorei* isolate as variation in the ability to consume either 2'-FL or DFL was observed. The transcriptional response for one *P. dorei* isolate revealed a broad GH family repertoire facilitating the breakdown of HMOs, including the upregulation of GH2, GH43, and GH97 families (190). However, it appears to be a more generalized response, i.e., no clear association between HMO structure and GH family response when compared to that of bifidobacteria, which apply a more structure-specific approach to HMO degradation (190). For example, and rather unexpectedly, the expression of GH29 and GH95 fucosidases was shown to be upregulated in response to non-fucosylated HMOs, while GH33 sialidases were upregulated in the presence of non-sialylated HMOs (190). Moya-González et al. (191) mined gut metagenomic data from a breast-fed infant to evaluate the presence of GH29 family members. GH29 α -L-fucosidases were annotated in *Ba. thetaiotaomicron*, *Bacteroides caccae*, *Ruminococcus gnavus*, *P. vulgatus*, *P. dorei*, and *Streptococcus parasanguinis* genomes (191). Further characterization of 10 putative α -L-fucosidase-encoding genes revealed substrate specificity. For example, Fuc18 (*Ba. thetaiotaomicron*), Fuc19A (*Ba. caccae*), Fuc35B (*Ba. caccae*), Fuc39 (*Ba. thetaiotaomicron*),

and Fuc1584 (*P. vulgatus*) were shown to elicit hydrolytic activity on α 1,3/4-linked fucose moieties present in 3-FL, while Fuc2358 (*S. parasanguinis*) was demonstrated to exhibit a broad substrate specificity, releasing fucosyl residues from all tested glycans which included 2'-FL and 3-FL (191). In contrast to many infant-associated bifidobacteria, *Bacteroides* members have been shown to elicit growth when cultivated on fucose as the sole carbon/energy source (153), indicating that they may profit from HMO degradants released by other taxa.

Disentangling the metabolic pathways employed by certain *Roseburia* species, e.g., *Roseburia inulinivorans* and *Roseburia hominis*, to metabolize particular HMOs was the focus of a recent investigation (192). A combination of genomic analyses and *in vitro* experiments has highlighted the presence of GH136 homologs involved in HMO degradation as well as an intracellular GH112, an LNB phosphorylase (192), with *R. inulinivorans* DSM 16841 additionally harboring two fucosidases, GH29 and GH95. The putative SBPs of *R. hominis* and *R. inulinivorans* ABC transporters were also described, as upregulation was evident in response to HMOs. Interestingly, *R. inulinivorans* DSM 16841 and *R. hominis* DSM 16839 were shown to exhibit limited growth on the most abundant fucosylated and sialylated HMOs, specifically 2'-FL, 3-FL, 3'-SL, and 6'-SL (192). Failure to grow on sialyllactose was consistent with the lack of sialidases present in the corresponding *Roseburia* genomes. However, growth for both butyrate-producing *Roseburia* strains was observed when purified HMOs, or the HMO constituent LNB, are available. *R. hominis* was also able to grow on LNT in contrast to *R. inulinivorans* (192). In the same study, *Eubacterium ramulus* DSM 15684 was tested, and growth was observed using LNT (192). Another prominent butyrate producer, *Faecalibacterium prausnitzii* A2-165 was the focus of a cross-feeding study with *B. infantis* ATCC 15697, which is discussed in more detail below, where it was proposed that *F. prausnitzii* promotes the *B. infantis* sialidase activity when in co-culture with 6'-SL (193). Moderate growth was observed in *F. prausnitzii* monoculture with 6'-SL (OD_{600nm} 0.5) higher than 2'-FL, 3-FL, and LNT2 (OD_{600nm} 0.2–0.3), although this was less than when in the presence of glucose (final OD_{600nm} of ~1.8) (193). In a clinical trial involving adults with chronic gastrointestinal issues (151), significant increase ($P < 0.05$) in stool counts of *F. prausnitzii* was found between the baseline (0 weeks) and the endpoint (6 weeks) when supplemented with 2'-FL-containing nutritional formula. Although this is a preliminary result and the 12 participants were adults, not infants, the ability of *F. prausnitzii* to consume individual and/or pooled HMOs and the contribution to the microbial metabolic networks that may occur remain largely unexplored and thus require further investigation. Although taxa described here may represent low abundance microbes in the neonatal gut, their presence may have a significant impact on associated microbial functionalities and development of a diverse microbial environment. This could be supported by prominent and efficient HMO users such as bifidobacteria.

Due in part to their structural diversity and specificity, many HMOs remain inaccessible to a range of potentially pathogenic microbes. Salli et al. (153), using *in vitro* growth experiments, demonstrated very limited utilization of 3-FL by a selection of undesirable microbes, such as certain *E. coli* strains, *Clostridium perfringens* E-98861 T, and *Cronobacter sakazakii* DSM4485. However, 2'-FL and DFL did not support growth of these potential pathogens (153). It should also be noted that single-strain utilization *in vitro* generally does not necessarily translate to *in vivo* studies. *R. gnavus*, a mucin degrader and colonizer of the infant and adult gut (194), possesses various GHs and other enzymes for host glycan degradation. The exact role of *R. gnavus* in the intestine has not been fully elucidated as it is often referred to as a commensal (195), while a clade purportedly linked to inflammatory bowel disease in adults was also identified in infants (196). Crost et al. (188) reported the growth of both *R. gnavus* E1 and ATCC 29149 strains when supplemented with 2'-FL and 3-FL but a lack of growth on LN(n)T, and variability between strains on 3'-SL and 6'-SL. However, in *Il10*^{-/-} mice, supplementation with 2'-FL reduced colitis, and an expansion of *R. gnavus* abundance was noted (195). Mechanistic strategies have also been proposed for HMO digestion such as the presence of multiple

α -L-fucosidases (GH95, and GH29A and 29B subfamilies), which can act on fucosyllactoses (197). Strain-level variation has also been observed. For example, *R. gnavus* E1 lacks a GH33 sialidase, unlike the GH33 identified in *R. gnavus* ATCC 29149 and ATCC 35913, which represents an intramolecular *trans*-sialidase (198, 199). The latter two strains also harbor a *nan* gene cluster for sialic acid metabolism (188, 199).

Typically, HMOs do not support vigorous growth of potential (opportunistic) pathogens and are more selective for taxa such as infant-associated bifidobacteria, therefore enabling their dominance, both compositionally and functionally, *in situ*. Concurrently, HMO-mediated reduction of pathogens *in vitro* is widely recognized, including in cases of enteropathogenic *E. coli* (200), group B *Streptococcus* (201–203), *Acinetobacter baumannii* (204, 205), and certain viral infections [reviewed by Moore et al. (206)]. HMOs have anti-pathogenic capabilities by acting as soluble decoy receptors, thus inhibiting pathogen binding and colonization, potentially resulting in reduced infections (207). Although beyond the scope of this review, the HMO-based host immunological impacts have been reviewed in depth elsewhere (46, 208, 209).

METABOLITES LINKED TO OR DIRECTLY RESULTING FROM HMO METABOLISM

The metabolites produced as a result of HMO metabolism are key variables in microbe-mediated interactions with the host. The beneficial impacts exerted can be direct to the respective host and/or indirect through the enrichment, in both abundance and activity, of other microbes in the gastrointestinal environment (210). One of the most prominent microbial metabolite groups studied resulting from saccharolytic fermentation is represented by the SCFAs, in particular acetate, butyrate, and propionate. Generally, SCFAs are recognized as energy sources for colonocytes, interacting with the immune system, e.g., promoting anti-inflammatory cytokine production and preventing growth of pathogens (211). Some of the metabolites described here, including SCFAs, contribute to the acidification of the gut, thus creating an inhospitable environment for pathogens to thrive, thereby preventing the establishment of enteric disorders. Although the literature predominantly demonstrates the benefits of SCFAs (212), these positive impacts have been more recently questioned (213). Instances where an excessive amount of SCFAs are present and factors such as the host health status (213, 214) may lead to less favorable SCFA-mediated host effects. For instance, higher SCFA concentrations in adults have been associated with obesity and hypertension (214), while propionate has been linked to an increased risk of type 2 diabetes (215). Therefore, their status as beneficial metabolites may be considered controversial and more complex than once previously recognized (216). Many investigations have reported the production of SCFAs and other organic acids, such as lactate and formate, as metabolic end products of microbial fermentation of HMOs, both pooled and individual structures (98, 123, 217). Acetate and lactate are two major end products following HMO metabolism by infant-associated bifidobacteria (98, 217, 218). As bifidobacteria are prominent acetate and lactate producers, they contribute to the beneficial effects exerted by SCFAs, although they appear to lack the metabolic machinery to produce butyrate and propionate (16). However, bifidobacterial metabolites, such as lactate, can participate in cross-feeding interactions, thereby supporting formation of other SCFAs such as butyrate by other gut microbes (219). Lactate itself has beneficial host impacts such as supporting epithelial cell barrier activity (220). It must be considered that lactate may also have negative consequences on host physiology (221), such as acting as a growth substrate for certain pathogens, e.g., *Salmonella enterica* serovar Typhimurium (222), and possibly promoting biofilm formation and anti-fungal drug resistance, e.g., *Candida albicans* (223).

HMO structure is a determining factor with respect to microbial utilization, and thus, the metabolic by-products released. For example, variations between fucosylated and sialylated HMOs relating to SCFA production have been noted, with fucosylated HMOs linked to higher amounts of acetate (109). This is particularly true for 2'-FL (123, 124), although it is worth recognizing that it is one of the more well-studied HMOs; therefore, more data are available. Additionally, compared to lactose controls, 2'-FL fermentation

by *B. infantis* Bi-26 resulted in a more diverse metabolite profile, with formate, 1,2-propanediol (1,2-PD), and fucose produced alongside acetic acid and lactate (218). Formate and 1,2-PD can be generated during fucose metabolism as pyruvate is directed away from lactate production (152, 224). This results in an increase in acetyl-CoA, thereby favoring ATP production through a section of the bifid shunt (152, 224). It has been suggested that this shift in metabolic activity, typically resulting in enhanced levels of acetate and formate, enables the restoration of the reduction-oxidation balance and facilitates energy production (98, 218). Fermentation of complex HMOs can liberate core components, e.g., fucose, which subsequently can be utilized and thus result in metabolite production such as 1,2-PD. Therefore, degradation of HMOs, or derivatives thereof, can contribute to propionate production as 1,2-PD can act as a precursor in the formation of this SCFA. Propionate can be involved in the mediation of microbiota and host homeostasis, appetite regulation, and participate in gluconeogenesis in the liver (225, 226). Schwab and colleagues (131) demonstrated that *B. infantis* can metabolize 2'-FL and 3-FL and, when in co-culture with *Eubacterium hallii* (now *Anaerobutyrium hallii/soehngeni*), *B. infantis* produces acetate, lactate, and 1,2-PD, which can be consumed by *A. hallii*, resulting in the formation of butyrate, propionate, and formate. This metabolic scheme has also been identified with *Limosilactobacillus reuteri* ATCC PTA 6475 and *B. breve* UCC2003, whereby the growth of *L. reuteri* was enhanced by the 1,2-PD produced from *B. breve* fucose metabolism (183). This fucose, in turn, was provided by *B. bifidum* PRL2010 following mucin degradation demonstrating the broader microbiota implications of bifidobacteria in supporting other members of the gut (183). Following a similar pattern, *B. infantis* co-cultured with *F. prausnitzii* amassed a significantly ($P < 0.0001$) higher amount of acetate when compared to a *B. infantis* monoculture when 6'-SL was the primary energy source, although this was not the case for 2'-FL, 3-FL, and LNT2 (193). HMO fermentation, therefore, may contribute to beneficial metabolite formation either directly or indirectly through sophisticated cross-feeding relationships.

In vitro fermentations using fecal inocula offer a community-level insight into the metabolites produced when HMOs are obtainable by the microbes present (107, 109, 123) (Table 1). In one such study, *B. longum*-dominant microbial communities were supplemented with individual HMOs (2'-FL, 3-FL, 3'-SL, 6'-SL, LNT, or LNnT), GOS, or FOS (109). The HMOs, varying in structure, and GOS metabolite profiles were comparable, which has also been observed elsewhere (107), with more acetate and lactate produced compared to FOS supplementation. Neutral HMOs, i.e., 2'-FL, 3-FL, LNT, and LNnT, and GOS produced more acetate and lactate compared to sialylated structures. Lactate and succinate, which are intermediate metabolites in SCFA formation, are often positively correlated with microbial HMO degradation, including in the *in vitro* fecal fermentation situation (107). Both lactate and succinate are microbial metabolic end products, yet they may also act as precursors in the formation of propionate by certain gut microbes (211, 227). Elsewhere, using pooled fecal samples from 12-week-old infants, the fermentation of LNT2 was shown to result in higher total SCFAs and organic acids, such as acetic acid, butyric acid, and lactic acid, than 3-FL or GOS/inulin (9:1) (121). The presence of LNT2 also led to the production of succinic acid, which was absent during 3-FL fermentation (121).

The initial microbial assembly is a predominant contributor to the metabolite profile generated from non-digestible carbohydrate fermentation. A *Bacteroides*-dominated inoculum, when supplemented with different HMOs, GOS, and FOS, was shown to result in higher succinate yields than two other *Bifidobacterium*-dominant groups tested, although donor-specific effects were also observed (107). This influence of the starting microbiota on metabolite production has been observed elsewhere, with the designation of "fast degraders" and "slow degraders" by Nogacka et al. (108, 125) contingent on the ability of 2'-FL to be utilized during batch fecal fermentations. Interestingly, both degrader types were evident irrespective of feeding mode, although the low sample size in these two studies means validation is required. The highest levels of acetate and lactate were shown to correspond to fast degraders, which consisted of increased relative abundance of bifidobacteria when compared to that of slow degraders (108,

125). Similarly, Van den Abbeele and colleagues (123, 124), using adapted Simulator of the Human Intestinal Microbial Ecosystem *in vitro* colon models, demonstrated the rapid fermentation of 2'-FL and enriched bifidobacterial relative abundance, which concomitantly resulted in increased acetate production.

A standout study conducted by Laursen et al. (85) evaluated the breast milk-derived bifidobacterial conversion of aromatic amino acids, e.g., tryptophan, phenylalanine, and tyrosine, to aromatic lactic acids, e.g., indole-3-lactic acid (ILA), phenyllactic acid, and 4-hydroxyphenyllactic acid (85). This is achieved via an aromatic lactate dehydrogenase (ALDH) (85). ILA was shown to activate aryl hydrocarbon receptor (AhR) and hydroxycarboxylic acid receptor 3-dependent pathways, which are involved in the mediation of homeostasis and immune system interaction, e.g., through stimulation of IL-22 (85, 228). These results point to a mechanism by which bifidobacteria-derived aromatic lactic acids can provide protective effects by modulating the infant immune system. Generally, the ability of the *Bifidobacterium* tested to produce aromatic lactic acids concurred with a capacity to utilize HMOs, suggesting HMOs provide integral support to bifidobacterial growth and production of these beneficial metabolites (85). An important factor to note is that the bifidobacteria in this study are commonly identified in the microbiota of breast-fed infants and that the aromatic lactic acid-producing ability, including the associated gene encoding the ALDH responsible for this conversion, does not appear to be present in bifidobacteria typically associated with a more mature microbiome. This infant-specific phenomenon has been observed elsewhere in relation to ILA production whereby infant-type bifidobacteria strains produced more ILA (1.84 ± 1.07 $\mu\text{g/mL}$) than those typical of an adult gut (0.4 ± 0.1 $\mu\text{g/mL}$) (229). Furthermore, *B. infantis* ATCC 15697-produced ILA has demonstrated anti-inflammatory potential via IL-8 reduction, which was mediated through AhR interaction, resulting in the prevention of IL-8 transcription (230). In-line with these studies was the observation that *B. infantis* EVC001-produced ILA upregulated immunoregulatory galectin-1 in Th2 and Th17 cells during polarization *in vitro* (36). Taken together, this suggests that specific metabolic activity, resulting in immunological interactions, is linked to breastfeeding, select infant-associated bifidobacteria, and the ability to consume HMOs. The full implications of aromatic lactic acids and why exactly this conversion occurs by infant-associated bifidobacteria remain to be fully elucidated. This underscores a knowledge gap that exists relating to the adapted functional metabolism of infant-associated microbes and the role of HMOs. Metabolites produced from HMO breakdown can also be directed into other microbial metabolic pathways through intricate cross-feeding interactions, as will be discussed below.

HMO-BASED CROSS-FEEDING INTERACTIONS

Bifidobacteria provide a beneficial effect not only through the degradation of HMOs to the infant directly but also indirectly through close associations between species within the genus and, indeed, with those from other genera inhabiting the infant gut. *B. bifidum* provides HMO breakdown products, e.g., fucose, for other species, e.g., *B. breve*, to assimilate (231), thus acting as a driver of community structure through the provision of substrates for others to consume (Fig. 5) (100, 160). The liberation of monosaccharides by *B. bifidum* from HMOs can play a significant role in the prevalence of other bifidobacterial species, particularly *B. breve*, in the infant gut (100). The transient release of 3-FL, when part of pooled HMOs is metabolized by *B. bifidum* R0071, has recently been demonstrated (98). Although *B. bifidum* can subsequently utilize 3-FL itself, or when co-cultured with *B. infantis* R0033, an increase in *B. infantis* R0033 was observed, indicating that (parts of) this HMO is made available for others to consume (98). *B. breve* can vigorously consume breakdown products from HMO assimilation, thereby facilitating its enrichment in the gut (232). Members of this species can utilize fucose, often made available through the breakdown of HMOs, which is unlike many other infant bifidobacterial species (106). In an *in vitro* investigation, *B. breve* M-16V has been shown to exhibit poor growth in mono-culture using 3-FL, 2'-FL, 3'-SL, 6'-SL, and LNnT, although this strain was shown to

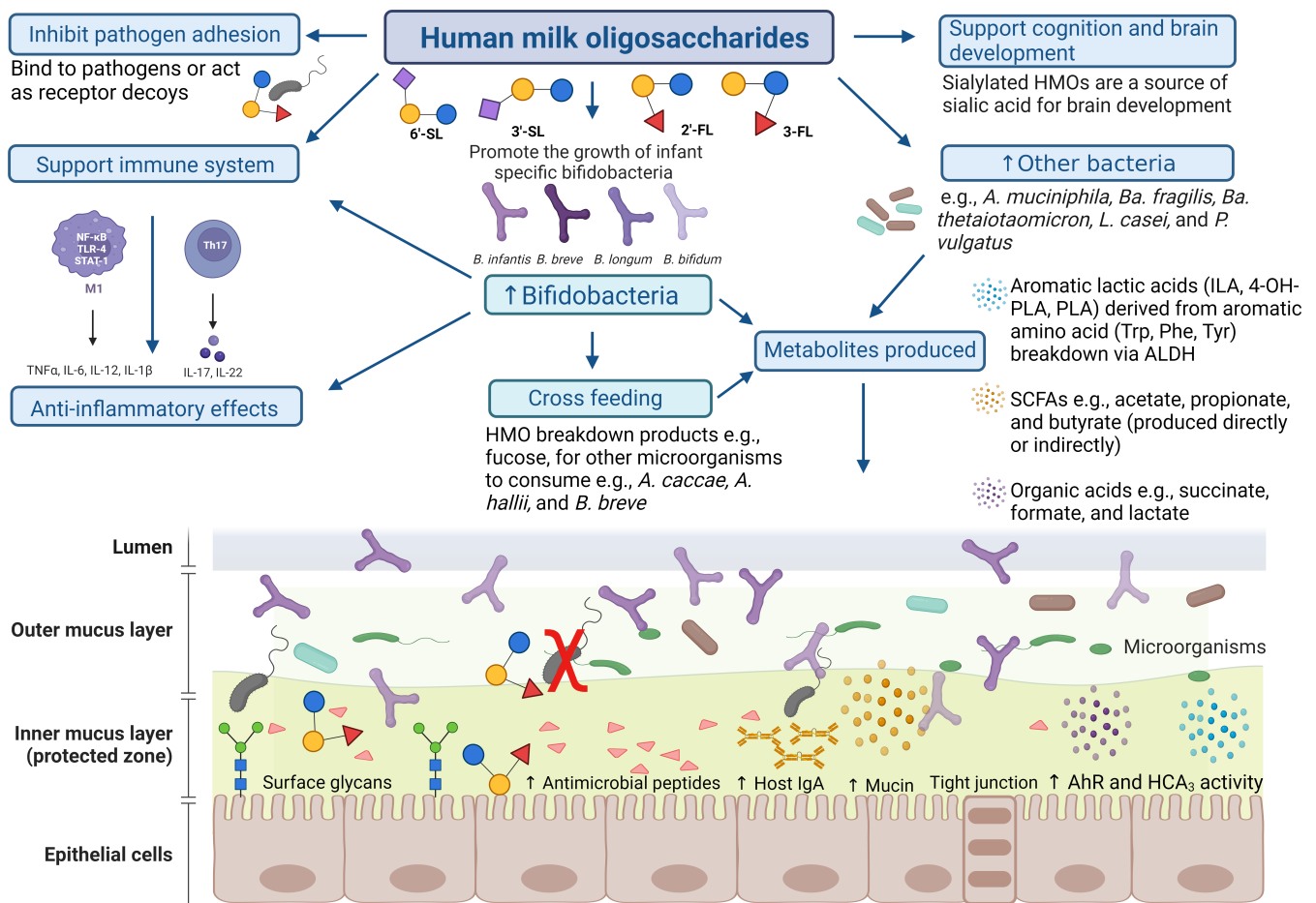


FIG 5 Some of the beneficial impacts that occur from human milk oligosaccharide availability to the infant. Select infant-associated bifidobacteria are highly adapted to digest HMOs. Certain members of other taxa also possess metabolic strategies to degrade specific HMO structures. Made using BioRender.

be able to use the type 1 HMO, LNT (98). However, when in co-culture with *B. bifidum* R0071 and 3'-SL, growth of *B. breve* M-16V prevailed (98), highlighting the capacity of *B. breve* strains to forage on HMO breakdown products for its own prevalence. Cross-feeding between *B. breve* and *B. bifidum* involving sialylated HMOs has also been observed elsewhere (161, 233). The extracellular sialidase-mediated release of sialic acid from 6'-SL by *B. bifidum* ATCC 15696 (161) and 3'-SL by *B. bifidum* PRL2010 (233) can benefit the growth of *B. breve* JCM 7019 and UCC2003, respectively.

An intricate carbohydrate sharing network among infant-associated bifidobacteria may contribute to their dominance in the neonatal gut. This applies to non-HMO utilizing bifidobacteria, which may be supported by the altruistic behavior of HMO utilizers, thus maximizing nutrient usage. Lawson et al. (217) demonstrated that spent or "conditioned" media from HMO degraders consuming 2'-FL (*B. pseudocatenulatum* strains LH9, LH11, LH13, and LH14) supported the growth of *B. longum* LH12, a non-HMO consumer (217). Combinations of strains examined in this study were isolated within the same infant microbial community. However, this mutually beneficial interaction was inconsistent, e.g., *B. infantis* LH23 2'-FL-derived media did not aid the growth of two *B. breve* strains, LH21 and LH24 (217). Conditioned nutritional media from HMO utilizer *B. longum* LH206 grown in the presence of 2'-FL, and to a lesser extent LNnT, were shown to provide modest growth support to *B. infantis* and *B. pseudocatenulatum* strains which have been isolated from the same infant (217). Although the exact mechanisms remain elusive, breakdown products were identified, indicating that fucose, galactose, acetate, and GlcNAc are involved in facilitating HMO-based cross-feeding relationships. This phenomenon may

contribute to the maturation of the gut microbiota as HMO breakdown products may be exploited by typical adult-associated bifidobacteria such as *B. adolescentis*, thus assisting metabolic activity and consequent growth of other bifidobacteria (124). There may be particular HMO-degrading strains, rather than species, that are specifically equipped to participate in nutrient sharing with non-HMO-degrading bifidobacteria.

Interactions between bifidobacteria and other SCFA-producing gut microbes, e.g., *Anaerostipes caccae* (154) and *A. hallii/soehngenii* (ex-*E. hallii*) (131), when HMOs are the primary carbohydrate source have been demonstrated *in vitro*. Trophic interactions can be facilitated by the breakdown of HMOs into simpler carbohydrates, e.g., fucose, and/or providing metabolites, e.g., acetate and 1,2-PD, that encourage the growth of others. As mentioned above, in a study conducted by Cheng et al. (193), the prominent butyrate producer *F. prausnitzii* was shown to support *B. infantis* growth when 6'-SL was present as the primary carbohydrate source in co-culture. However, it was observed that *F. prausnitzii* is not able to substantially consume 6'-SL itself. Additionally, in co-culture, more 6'-SL was consumed, and a significant ($P < 0.0001$) quantity of acetate produced, compared to *B. infantis* monoculture with 6'-SL, with the authors postulating that *F. prausnitzii* promotes *B. infantis* sialidase activity, although the exact mechanism by which this happens remains unknown (193). In the same study, when in the presence of 2'-FL, the co-culture and *B. infantis* monoculture reached similar cell density levels, although the growth rate was faster in co-culture conditions, i.e., stationary phase was reached after 32 hours of cultivation compared to 56 hours (193). Mono- and co-culture-based investigations with the butyrate producer *A. caccae* demonstrated that, in the presence of HMOs, *B. infantis* (154) and *Ba. thetaiotaomicron* (185) generate breakdown products e.g., glucose and galactose, available for *A. caccae* to consume. This, in turn, facilitates the production of butyrate, which, among other beneficial impacts, has been associated with a reduced risk of food allergy development in the infant (234). A recent synbiotic-based study, albeit in adults, showed that a combination of *B. infantis* and an HMO concentrate from human breast milk during and after antibiotic administration promoted the abundance of *B. infantis* (235). At the metabolite level, enhanced amounts of lactate and ILA and decreased *p*-cresol sulfate were measured (235). Additionally, increased levels of *Veillonella* spp., which are lactate consumers, and the stimulation of *Veillonella*-produced propionate, both *in vitro* and *in vivo*, were observed, indicating a cross-feeding occurrence (235). As discussed above, interest relating to *A. muciniphila*, a strict anaerobe which has been identified in 1-month-old infants (236), and its ability to degrade HMOs has been accruing in the scientific literature (171, 172). Not only does *A. muciniphila* have the capacity to assimilate certain complex structures (171, 172), it can also consume constituents such as fucose (171). Although little is known about the dynamics between infant bifidobacterial species and *Akkermansia* with respect to HMO degradation, there is an opportunity to investigate possible metabolic interactions and whether the degradation mechanisms are comparable and/or complementary.

INFANT-ASSOCIATED BIFIDOBACTERIA AND PREBIOTIC CARBOHYDRATE METABOLISM

Well-studied prebiotics as additions to infant formula include GOS, FOS/inulin and, more recently, select HMOs such as 2'-FL and LNnT (237–239). Clinical trials in infants have demonstrated that 2'-FL, LNnT, and HMO mixtures when incorporated in standard formula are safe and support growth in infants (240–242). At present, there are seven commercially available HMOs, namely 2'-FL, LNnT, 6'-SL, 3'-SL, 3-FL, LNT, and DFL (243). As noted throughout this review, the heterogeneous mix of HMO glycans is often selective for breast-fed infant-type bifidobacteria (158); conversely, GOS and FOS/inulin are less selective, i.e., bifidobacteria of both infant and adult origin are supported, and more generally, growth and/or activity of a broader range of taxa can be facilitated (153, 243). There are a variety of overlapping, yet distinct, metabolic strategies to utilize these carbohydrates when provided to the microbiota (Table 2).

TABLE 2 Various mechanisms that members from four infant-associated bifidobacterial species employ for HMO, GOS, and FOS/inulin degradation^a

	HMOs	GOS	FOS/inulin	References
<i>B. infantis</i>	ABC transporters, various GHs present in 43 kb “HMO island,” intracellular Bga42A hydrolyze LNT and Bga2A for LNnT. GlcNAc-6-p deacetylase upregulated in the presence of LNT and LNnT to breakdown HMO-bound GlcNAc. GH29 1,3/1,4- α -L-fucosidase hydrolyzes the 3-fucosyl and 4-fucosyl structures of fucosylated HMOs. Predicted intracellular GH33 2,3-2,6- α -sialidases present.	ABC transporter, intracellular GH2 and GH42 β -galactosidases (Bga42A, Bga42B, and Bga42C) for GOS hydrolysis. Bga42A is encoded for 3'-GL hydrolysis.	ABC transporter, and GH13 (Blon_0128) and GH32 enzymes (Blon_2056) are expressed for FOS and inulin. Blon_0787 is encoded for FOS hydrolysis. An exoinulinase with β -fructofuranosidase and invertase for inulin.	(149, 224, 244–249)
<i>B. bifidum</i>	Extracellular LnbB (GH20) liberates LNB from HMOs. Intracellular GLNBP for LNB degradation. Extracellular GH95 1,2- α -L-fucosidase for 2'-FL, GH29 1,3-1,4- α -L-fucosidase for 3-FL, and GH33 exo- α -sialidase for 6'-SL degradation.	GH42 for hydrolysis of GOS GH2 β -1,4-galactosidase and BIF3 for extracellular hydrolysis of GOS.	Highly variable. GH13 potentially involved.	(7, 159, 189, 244, 247, 250–255)
<i>B. breve</i>	ABC transporters, intracellular GH95 and GH29 α -fucosidases, GH33 α -sialidase encoded for fucosylated and sialylated HMO degradation. β -Galactosidases involved in LN(n)T metabolism.	GL-BG transports 6'-GL into the cells for degradation by GH42 β -galactosidase (e.g., BbrY_0422). Endo- β -galactanase (GH53) encoded for 4'-GL degradation. ABC transporter for internalization.	MFS transporter (<i>lacy</i>), ABC transporter, intracellular β -fructofuranosidase encoded by GH32 family for FOS and inulin hydrolysis. Variable growth observed.	(103, 105, 245, 256, 257)
<i>B. longum</i>	GH136 lacto-N-biosidase LnbX-positive, extracellular GH95 and GH29 α -fucosidases. LnbX-negative intracellular degradation of HMOs. GH112 family, GLNBP. GH33 2,3-2,6- α -sialidase.	Extracellular β -galactosidase I (BL0259), endo- β -galactanase (GH53).	Intracellular GH32 β -fructofuranosidase, ABC transporters (BL1164-BL1165), and putative GHs (BL0529 and BL0544) for inulin.	(129, 244, 247, 249, 258–260)

^a3'-GL, 3' galactosylactose; LN(n)T, lacto-N-(neo)tetraose; LnbX, Lacto-N-biosidase; GH, glycoside hydrolase; GlcNAc-6-p, N-acetyl-glucosamine-6-phosphate; SA-C2, sialic acid utilization cluster 2; GLNBP, GNB/LNB phosphorylase.

One of the most intriguing and distinguishing characteristics of HMOs is their structural complexity, diversity, and specificity. It has been observed in fecal fermentations that 2'-FL results in a greater increase in the relative abundance of bifidobacteria when compared to GOS, and when combined, faster fermentation occurred than when GOS was added alone (120). However, the HMO structure should be taken into consideration when comparing against an established prebiotic such as GOS. For example, when 3-FL or GOS/inulin was tested in a fecal fermentation experiment, the relative abundance of *B. longum* increased but not that of *B. bifidum*, whereas LNT2 increased both *B. bifidum* and *B. longum* relative abundance (121). GOS supports growth of various *Bifidobacterium* members, such as *B. longum* and *B. breve* (107, 109, 120), and infant formula supplemented with GOS has been shown to lead to increased bifidobacterial abundance in infants (143). Structural specificity is also a factor with GOS and FOS, though to a significantly lesser extent than HMOs. Using *B. breve* UCC2003 as a model strain, it was determined that metabolism of large GOS fractions is associated with the ability to produce an extracellular endogalactanase (261). ABC uptake systems have substrate size limitations meaning larger HMO structures (e.g., greater than a degree of polymerization of 8) cannot be internalized unless first degraded extracellularly (232, 262). Some of the enzymes necessary for GOS metabolism are also involved in LN(n)T catabolism, showing functional overlap between GOS and HMO metabolism in *B. breve* (105, 261).

As described above, carbohydrate metabolism by *B. infantis*, LnbX-negative *B. longum*, and *B. breve* relies on transport systems to internalize carbohydrates (e.g., ABC-type

transporters and phosphoenolpyruvate-dependent phosphotransferase system) and intracellular GHs. Conversely, *B. bifidum* and LnbX-positive *B. longum* use extracellular GHs to degrade oligosaccharides, subsequently importing the degradants of choice (100). While the transport systems involved in the internalization of HMOs and GOS are similar, distinct, yet overlapping, GHs encoded for hydrolysis exist. Infant-associated bifidobacteria encode multiple β -galactosidases belonging to GH42 and GH2 families, which participate in the degradation of both HMOs and GOS, in a substrate-specific manner (263). In this study, four bifidobacterial species *B. breve*, *B. bifidum*, *B. longum*, and *B. infantis*, were shown to possess conserved β -galactosidase activities (Bbr_0529, B216_08266, B8809_0415, and Blon_2016), some of which hydrolyze both HMOs, e.g., LNT, and GOS (263, 264). Enzymes such as GH20 β -hexosaminidases, GH33 sialidase, and GH95 α -fucosidase participate in host-derived oligosaccharide (HMOs/mucin) degradation (244). The glycosidic similarities between HMOs and mucin-derived glycans explain this functional overlap (264, 265).

B. breve members can encode GH95 α -fucosidases for HMO degradation, while GH42 β -galactosidases, e.g., encoded by Bbr_1552, are involved in GOS and particular HMO catabolism (105, 263). Studies focusing on *B. breve* UCC2003 have established that the β -galactosidase corresponding to locus tag Bbr_0420 exhibits a more narrower spectrum as its expression is upregulated in the presence of GOS but not by certain HMOs, e.g., LNT and LNTT (105, 261). For GOS utilization, two gene clusters encoding β -galactosidase and two SBPs of ABC transporters are present in *B. breve* involved in 3'-galactosyllactose and 6'-galactosyllactose internalization (256). Galactosyllactoses are components of GOS, but these small oligosaccharides have also been identified in human breast milk (266), whereas FOS/inulin, based on fructose, is not a natural constituent of human milk. Previously, *B. breve* UCC2003 transcription of the *fos* operon, encoding a β -fructofuranosidase, has been demonstrated in the presence of FOS (245). Although fructose is generally not provided in early life, frequent colonizers such as *B. breve* members still have the capacity to utilize fructose (245, 267), demonstrating their broad repertoire for carbohydrate metabolism which contributes to their dominance in the gut microbiota as the infant develops.

B. infantis encodes three GH42 members (Bga42A, Bga42B, and Bga42C) that can act upon GOS and HMOs (246, 258). Although Bga42A in *B. infantis* ATCC 15697 can prioritize the breakdown of 3-galactosylglucose, present in GOS, over LNT (type I HMO), it still can participate in HMO utilization (246). Interestingly, Bga42A is not part of the HMO cluster in *B. infantis* ATCC 15697, indicating a high level of adaptation for the assimilation of complex carbohydrates (246, 259). As mentioned above, GLNBP is involved in the degradation of LNB and is structurally similar to GH42 β -galactosidases which can degrade both HMOs and GOS (159, 247). Furthermore, *B. infantis* has a β -galactosidase encoded by Blon_2016 capable of degrading LNT and LNTT along with GOS (263). Phenotypical differences in GOS degradation exist depending on the structure. Garrido et al. (268) observed that *B. infantis* isolates ($n = 22$) grow vigorously on GOS with a DP of up to 3, but only a subset of isolates tested consumed larger structures. *B. infantis*-encoded β -fructofuranosidases involved in FOS degradation have been characterized, with families such as GH13 (Blon_0128) and GH32 (Blon_2056 and Blon_0787) participating (258, 269). GH29 1,3/1,4- α -L-fucosidase (AfcB) activity is employed for intracellular degradation of fucosylated HMOs such as 3-FL and 2'-FL (270).

B. bifidum DSM 20456 possesses a *lacZ*-encoded extracellular β -galactosidase potentially involved in the degradation of larger GOS structures, i.e., with a degree of polymerization of 5 and 6 (271). Furthermore, *B. bifidum* members can encode GH2 and GH42 β -galactosidases for intracellular hydrolysis of GOS including those encoded by BIF1 and BIF2 (258), and extracellular hydrolysis of GOS via BIF3 (250). Liu and colleagues (244) ascertained that 52 *B. bifidum* strains, both infant- and adult derived, lacked the necessary enzymes for FOS degradation, which translated phenotypically as an absence of growth in the presence of FOS. However, *B. bifidum* growth in the presence of FOS has been reported, although strain variability was noted (267).

Both intra- and extracellular mechanisms to degrade oligosaccharides are reported in the *B. longum* species. LnbX-positive *B. longum* uses extracellular enzymes for HMO degradation, whereas LnbX-negative *B. longum* uses intracellular enzymes (158). Typically, *B. longum* members are associated with plant carbohydrate metabolism, which is reflected in the GH families they encode such as GH43, GH10, and GH13 (257, 272). González et al. (260) identified similarities between *B. longum* LMG 13197 upregulated carbohydrate metabolism genes when grown in infant formula (with added GOS and inulin) and in breast milk *in vitro*. However, differences were observed for those related to the GNB/LNB operon (specifically BL1638 and BL1639), which were upregulated when the *B. longum* strain was grown in breast milk.

Although already an established practice (111), there is incomplete understanding with respect to supplementation of formula milk with HMO mixtures. Additionally, HMOs in combination with other prebiotics may elicit more pronounced effects compared to individual HMO supplementation, highlighting an important knowledge gap. Therefore, more clinical studies are needed to determine if a unique oligosaccharide blend can positively modulate the gut microbiota, and the benefits, if any, of using a prebiotic mixture versus individual prebiotics in infant formula.

CONCLUSIONS AND FUTURE CONSIDERATIONS

HMOs can play a major role in shaping the microbial composition, and thus functions that follow, in the neonate intestine. This is a pivotal point in life where the microbiota is established and is highly vulnerable to modulation. Research pertaining to HMOs and microbial utilization, including the broader health implications, has shifted from fundamental to translational in the last several years. Nevertheless, gaps in the scientific understanding remain, particularly at the microbial community and functional levels which, when addressed, would meaningfully expand our knowledge regarding the complex HMO-mediated effects on the gut microbiota.

First, dynamic and multifaceted sharing interactions, both supportive and inhibitory, between microbes in the infant gut can be facilitated by HMOs. It is perceived that extensive nutrient swapping occurs in the extracellular space, and although steps have been taken to establish some of those connections, the exact subtleties remain unknown, including the role of other breast milk bioactive components. Acquisition of nutrients via cross-feeding is a survival strategy for those within bifidobacteria and, indeed, other gut taxa. The proximity of microbes to one another in the gut to facilitate these cross-feeding interactions remains unclear. This is experimentally challenging to quantify, given the complexity of the gut, i.e., the various environments and micro-compartments that exist. Additional models such as organoids or community level metabolic modeling may help determine the importance of microbial spatial distribution for cross-feeding interactions. Although more intricate than the sharing of one substrate from one microbe to another, the true extent of HMO-supported cross-feeding interactions remains unknown. The expansion of HMO consumers beyond bifidobacteria has enabled a broader view of HMO metabolizers and related networks in the infant gut. Further community-based investigations to untangle the details of these networks are warranted, which could be achieved by combining taxonomic analysis with functional-based approaches, such as metabolomics, metatranscriptomics, and metaproteomics, to determine what HMO structure(s) and/or degradant(s) are being used, the subsequent metabolites produced, and how this is being achieved.

HMO structural diversity, composition, and complexity are key factors in the scientific interest in breast milk glycans accrued thus far. Presently, there have been over 200 structures identified, although the known biological relevance in terms of gut modulation remains limited to just several HMO structures. The impact of other less well-characterized HMOs, e.g., 6'-SL, LNH, and DSLNT (273), and indeed those undetected HMOs, remains less studied. Other HMOs present in breast milk, albeit at lower concentrations, may exert important biological impacts. Irrespective of abundance in breast milk, the wide variety of structures means we have only begun to tip the surface of HMO research.

Linking to this is the high degree of selective structural utilization by specific bacteria. Investigating HMO metabolizing gene expression in response to structurally varying HMOs, and combinations, would be promising in establishing the strain-level preferential assimilation occurring.

Third, recent efforts have begun to explore how the initial microbial composition impacts the degradation of HMOs. Formation and persistence of the early life microbiota are influenced by numerous factors, including delivery, feeding mode, and surrounding environment. HMO structure depletion, and the subsequent metabolites produced, can vary depending on the taxa present in the gut. Few studies have examined this so far, therefore, presenting an exciting opportunity for further investigation. No study to our knowledge has directly compared what different structures and metabolites can be consumed and produced by microbiota dominated by varying levels of overall bifidobacterial abundance. Few studies have evaluated the impact of the initial microbial assembly when an HMO combination, or in conjunction with established prebiotics, is made available to the microbiota.

Finally, further emphasis on establishing the adapted functional capacity of early life *Bifidobacterium*, e.g., the production of aromatic lactic acids which have potential to impact early life immune function (85), would be incredibly informative. Certain infant-associated bifidobacteria encode a suite of genes involved in host glycan degradation compared to more plant-derived degradation abilities observed in the adult-type bifidobacteria. This is significant for immune system development and overall infant health. The provision of HMOs via breast milk or fortified infant formula can support the predominance of bifidobacteria in early life and their respective functionalities.

Taken together, it is apparent that HMOs embody a significant element, although they are part of a larger consortium of factors, in the microbiota development of healthy infants. The prevalence of infant-type bifidobacteria, which have genetically evolved to efficiently consume various HMOs, can likely aid in the establishment of the immune system, including a reduction in GI infection risk, through the production of metabolites and interactions with other taxa. Further research to underpin the true extent of HMO degradation patterns among infant bifidobacteria, and indeed other microbes, and the broader health impacts is crucial when moving toward the development of infant formulae to optimally support infant health.

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