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Milk glycan metabolism by intestinal bifidobacteria: insights from comparative genomics

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Abstract

Bifidobacteria are early colonizers of the human neonatal gut and provide multiple health benefits to the infant, including inhibiting the growth of enteropathogens and modulating the immune system. Certain *Bifidobacterium* species prevail in the gut of breastfed infants due to the ability of these microorganisms to selectively forage glycans present in human milk, specifically human milk oligosaccharides (HMOs) and *N*-linked glycans. Therefore, these carbohydrates serve as promising prebiotic dietary supplements to stimulate the growth of bifidobacteria in the guts of children suffering from impaired gut microbiota development. However, the rational formulation of milk glycan-based prebiotics requires a detailed understanding of how bifidobacteria metabolize these carbohydrates. Accumulating biochemical and genomic data suggest that HMO and *N*-glycan assimilation abilities vary remarkably within the *Bifidobacterium* genus, both at the species and strain levels. This review focuses on the delineation and genome-based comparative analysis of differences in respective biochemical pathways, transport systems, and associated transcriptional regulatory networks, providing a foundation for genomics-based projection of milk glycan utilization capabilities across a rapidly growing number of sequenced bifidobacterial genomes and metagenomic datasets. This analysis also highlights remaining knowledge gaps and suggests directions for future studies to optimize the formulation of milk-glycan-based prebiotics that target bifidobacteria.

Keywords

Bifidobacteria; HMO; *N*-glycans; carbohydrate metabolism; comparative genomics; transcriptional regulation; probiotics; prebiotics

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Disclosure statement

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Introduction

The human gastrointestinal tract harbors a complex community of microorganisms, referred to as the gut microbiota (GM, the list of all abbreviations is given in Table 1). The GM of an adult person comprises ~10^{13}–10^{14} cells (Sender et al. 2016), spanning hundreds of species and strains (McDonald et al. 2018; Pasolli et al. 2019). The enormous complexity of the adult GM is not primordial; it gradually develops through a succession of various microbial taxa during the first years postpartum (Vatanen et al. 2018; Raman et al. 2019; Tannock 2021). One of the key factors shaping the development of GM in infants is breastfeeding (Stewart et al. 2018). Breast milk is a complex mixture of nutrients and biologically active compounds that confer health benefits to the infant and modulate the composition of the neonatal GM (Ballard and Morrow 2013; Masi and Stewart 2022). Specifically, glycans found in human milk promote the colonization and growth of particular *Bifidobacterium* species, resulting in GMs predominated by bifidobacteria (Tannock et al. 2013; De Leoz et al. 2015; Jones et al. 2020).

The most prevalent *Bifidobacterium* species that inhabit the gut of breastfed children include *Bifidobacterium longum* subsp. *infantis* (*B. infantis*), *Bifidobacterium longum* subsp. *longum* (*B. longum*), *Bifidobacterium breve*, and *Bifidobacterium bifidum* (Bäckhed et al. 2015; Vatanen et al. 2016, 2019, 2022; Olm et al. 2022; Taft et al. 2022). The predominance of these microorganisms is generally considered beneficial for the infant due to their favorable properties, such as protection from enteropathogens (Fukuda et al. 2011; Casaburi and Frese 2018; Casaburi et al. 2019) and modulation of the host immune system (Henrick et al. 2019, 2021; Huda et al. 2019; Laursen et al. 2021). In contrast, a decreased abundance of bifidobacteria has been associated with persisting GM immaturity observed in preterm infants (Alcon-Giner et al. 2020; Beck et al. 2022) and children with severe acute malnutrition (Subramanian et al. 2014; Barratt et al. 2022). In the latter case, disrupted GM development has been shown to contribute to the pathogenesis of malnutrition (Blanton et al. 2016; Gehrig et al. 2019).

Therapeutic approaches aimed at repairing GM immaturity include the administration of exogenous *Bifidobacterium* species (e.g. *B. infantis*) as probiotics or/and food formulas supplemented with glycan-based prebiotics that would selectively promote the growth of autochthonous or administered bifidobacteria (Underwood et al. 2015; Frese et al. 2017; Berger et al. 2020; Bajorek et al. 2021; Nguyen et al. 2021; Barratt et al. 2022; O'Brien et al. 2022). In that regard, one of the key challenges is establishing a rational approach toward the personalized selection of respective strains and carbohydrates. Because of the direct link between breastfeeding and *Bifidobacterium*-rich GMs, glycans found in human milk, namely, human milk oligosaccharides (HMOs) and *N*-linked glycans decorating milk proteins, are widely considered "natural" prebiotics (Thomson et al. 2018; Duman et al. 2021). A collateral notion is that strains with the largest repertoire of HMO utilization genes might serve as better probiotic candidates (Duar et al. 2020). In addition to these considerations, a deeper understanding of how bifidobacteria metabolize milk glycans is required to enable the optimization of probiotics, prebiotics, and mixtures thereof (synbiotics) prior to clinical trials.

The molecular mechanisms of milk glycan utilization by bifidobacteria have received considerable attention in the last two decades. In addition to biochemical methods, recent advances in genetic manipulation enabled functional delineation of HMO utilization machinery in various *Bifidobacterium* species (Matsuki et al. 2016; Nishiyama et al. 2017; Sakanaka, Hansen, et al. 2019; Ojima, Asao, et al. 2022). However, many aspects of respective catabolic pathways (especially related to *N*-glycan catabolism) remain underexplored. Bioinformatic approaches combined with transcriptomics allow us to fill some of these knowledge gaps (Arzamasov et al. 2022; Barratt et al. 2022). Additionally, comparative genomics enables tentative identification of candidate genomic loci *via* linking their occurrence profiles with differences in HMO and *N*-glycan utilization capabilities among the members of the *Bifidobacterium* genus (Asakuma et al. 2011; Garrido, Nwosu, et al. 2012; Ruiz-Moyano et al. 2013; Matsuki et al. 2016; Gotoh et al. 2018; Duar et al. 2020; Kujawska et al. 2020; Lawson et al. 2020).

Recent studies have demonstrated that *O*-glycans decorating milk glycans can also be metabolized by bifidobacteria, such as *B. bifidum* (Fukudome et al. 2021), likely *via* pathways used for the foraging of mucin *O*-glycans (Fujita et al. 2005; Kitaoka et al. 2005; Turroni et al. 2010; Kiyohara et al. 2012; Shimada et al. 2015; Katoh et al. 2017; Ashida et al. 2018). The structure of these pathways was recently summarized in the excellent review by Katoh et al. (2020). Therefore, we have limited the scope of this review to a genome-based comparative analysis of HMO and *N*-glycan utilization pathways in human-residential bifidobacteria. To this end, we integrated the experimental knowledge and bioinformatic predictions of genes/gene clusters encoding transporters, catabolic enzymes, and transcriptional regulators constituting the respective pathways.

Major carbohydrates in human milk: HMOs and *N*-glycans

Human milk is a complex mixture of macronutrients (proteins, lipids, and carbohydrates), micronutrients (e.g. B-vitamins), and a myriad of bioactive components, such as growth factors, cytokines and chemokines, hormones, immunoglobulins, and antimicrobials (Allen 2012; Ballard and Morrow 2013). Among the carbohydrates found in human milk, lactose (Lac) is the most abundant (67–78 g/L) and serves as the primary nutritional source for the infant (Ballard and Morrow 2013). In contrast, HMOs, the second most abundant type of milk glycans (5–20 g/L), are minimally assimilated in the small intestine and do not provide nutritional value to the infant (Bode 2012).

HMOs are linear or branched oligosaccharides (DP 3–20) composed of five monosaccharide building blocks: *D*-glucose (Glc), *D*-galactose (Gal), *N*-acetyl-*D*-glucosamine (GlcNAc), *L*-fucose (Fuc), and *N*-acetylneuraminic acid (Neu5Ac) (Figure 1) (Kunz et al. 2000). Most HMOs contain a Lac core (Galβ1–4Glc) at the reducing end, reflecting shared steps in the biogenesis of these glycans (Kellman et al. 2022). The Lac core can be elongated at the C-3 position of the Gal residue with a lacto-*N*-biose (LNB, Galβ1–3GlcNAc) or *N*-acetyllactosamine (LacNAc, Galβ1–4GlcNAc) unit(s). The resulting HMO structures are denoted as type I and type II chains, respectively, with lacto-*N*-tetraose (LNT) and lacto-*N*-neotetraose (LNnT) as archetypes (Figure 1). LacNAc can also modify the C-6 position of the Gal moiety in the Lac core, forming branched type I and II oligosaccharide structures

(Figure 1). Type I/II chains and the Lac core are often decorated by Fuc and Neu5Ac residues *via* various α-glycosidic bonds. Fuc can be attached to Gal (α−1,2), Glc (α−1,3), or GlcNAc units (α−1,3 or α−1,4), whereas Neu5Ac can decorate Gal (α−2,3 or α−2,6) or GlcNAc (α−2,6) units (Figure 1). Among the most abundant fucosylated and sialylated HMOs are 2′/3-fucosyllactose (2′FL and 3FL) and 3′/6′-sialyllactose (3′SL and 6′SL), respectively (Wu et al. 2010; Chen 2015).

A plurality of possible modifications of the Lac core yields a tremendous structural diversity of HMOs. Indeed, around 170 structurally distinct HMO species have been identified so far (Urashima et al. 2018, 2021). However, only 19 species account for >90% of total HMO content (Bode 2012). 2′FL and type I HMOs, such as LNT and lacto-*N*-fucopentaoses (LNFP) I/II are generally predominant in human milk (Urashima et al. 2012). The predominance of type I HMO structures in human milk is speculated to have evolved to promote the establishment of *Bifidobacterium*-rich GMs (Urashima et al. 2022). The worldwide variability of HMO composition is quite prominent (McGuire et al. 2017), reflecting various environmental and genetic factors. For example, mutations in the galactoside α−1/2-L-fucosyltransferase 2 (*FUT2*) and α−3/4-L-fucosyltransferase (*FUT3*) genes may markedly decrease the abundance of α1–2-fucosylated and α1–4-fucosylated HMOs, respectively (Kunz et al. 2017; McGuire et al. 2017).

Human milk proteins (10–20 g/L) provide another important nutritional source for infants (Ballard and Morrow 2013). By some estimates, ~70% of the most abundant milk proteins, such as lactoferrin, tenascin, bile salt-stimulated lipase, secretory IgA, and casein, are glycosylated (Froehlich et al. 2010). Thus, while not as abundant as HMOs, *N*-linked glycans constitute a sizeable fraction of total milk glycans. All *N*-glycans share a common core sugar sequence, Manα1–3(Manα1–6)Manβ1–4GlcNAcβ1–4GlcNAcβ, which is covalently attached by an *N*-glycosidic bond to the asparagine residue of the Asn-X-Ser/Thr sequon. *N*-glycans are classified into three structural types: (i) oligomannose, in which only *D*-mannose (Man) residues extend the core; (ii) complex, in which antennae containing GlcNAc residues extend the core; and (iii) hybrid, which combine structural features of both types (Figure 1) (Takimori et al. 2011; Nwosu et al. 2012; Stanley et al. 2015). Compared to *N*-glycans from bovine milk, a distinct feature of human milk *N*-glycans is a high (~75%) degree of fucosylation (Nwosu et al. 2012; Bai et al. 2018). So, one of the core GlcNAc residues is often fucosylated at the C-6 position (“core fucosylation”) by fucosyltransferase 8 (Fut8) (Yamaguchi et al. 2000).

HMO utilization by bifidobacteria

For the last two decades, the ability to utilize HMOs was considered a hallmark of several *Bifidobacterium* species and rarely reported in other gut bacteria (Ward et al. 2006; Marcobal et al. 2010; Yu et al. 2013). While recent studies have challenged this paradigm by demonstrating HMO assimilation by members of the *Bacteroides, Lactobacillus, Roseburia,* and *Akkermansia* genera (Marcobal et al. 2011; Bidart et al. 2014, 2016; Pichler et al. 2020; Kijner et al. 2022; Luna et al. 2022), collectively, bifidobacteria possess the largest and most diverse arsenal of transporters, glycoside hydrolases (GHs), catabolic enzymes, and transcriptional regulators involved in HMO utilization. As a result, these bacteria can

metabolize a wide spectrum of undecorated, fucosylated, and sialylated HMOs (Thomson et al. 2018; Sakanaka, Gotoh, et al. 2019).

Overall, bifidobacteria implement two distinct strategies of HMO utilization. The first strategy includes the transport of HMO molecules inside the cell followed by step-by-step degradation to monosaccharides by intracellular GHs. This strategy is exemplified by *B. infantis*, which possesses multiple gene clusters encoding HMO transporters and intracellular hydrolytic enzymes (Figures 2(A,B)) (Sela et al. 2008; LoCascio et al. 2010), enabling this bacterium to metabolize dozens of various HMOs species (Ward et al. 2007; Ruiz-Moyano et al. 2013). Certain *B. breve, Bifidobacterium catenulatum* subsp. *kashiwanohense (B. kashiwanohense), Bifidobacterium pseudocatenulatum,* and *B. longum* strains also implement this strategy (Bunesova et al. 2016; Garrido et al. 2016; James et al. 2016; Matsuki et al. 2016; James et al. 2019; Lawson et al. 2020; Ojima, Asao, et al. 2022; Shani et al. 2022). However, their sets of HMO utilization genes are more narrow than those of *B. infantis* (Table S1), limiting the spectrum of HMOs that these species can metabolize (LoCascio et al. 2007; Ruiz-Moyano et al. 2013).

The second HMO utilization strategy is primarily used by *B. bifidum* and involves partial extracellular degradation by membrane-attached GHs, followed by import and catabolism of specific HMO constituents, namely LNB and Lac (Figure 3) (Asakuma et al. 2011). Unlike other species, *B. longum* shows a remarkable variation in HMO utilization strategies. For example, *B. longum* SC596 metabolizes LNT and fucosylated HMOs (2′FL, 3FL) intracellularly (Garrido et al. 2016), whereas *B. longum* JCM 1217 uses an extracellular GH to degrade LNT (Table S1) (Yamada et al. 2017; Katoh et al. 2020). The following subsections provide an overview of the uptake machinery and hydrolytic enzymes driving the two HMO utilization strategies.

HMO uptake—Bifidobacteria primarily employ Carbohydrate Uptake Transporter-1 (CUT1) family ATP-binding cassette-type (ABC) transport systems for HMO uptake. CUT1 transporters typically comprise an extracellular substrate-binding protein (SBP) that dictates specificity to particular glycans, two transmembrane permease components, and two energizing ATPase subunits (Rees et al. 2009; Saier et al. 2021). The latter subunits can be shared by multiple CUT1 transport systems within the cell (Marion et al. 2011). Historically, determining the substrate-specificity of bifidobacterial ABC transporters has been a daunting task and generally relied on accessing the binding of recombinant SBPs to various glycan arrays *in vitro*. However, a limitation of this biochemical approach is that it may not always capture all substrates of a particular transport system (Sakanaka, Hansen, et al. 2019). Therefore, an approach using a *B. longum* strain engineered for evaluating HMO transporter specificities has been implemented (Sakanaka, Hansen, et al. 2019).

The genome of *B. infantis* ATCC 15697 encodes two dozen SBPs of CUT1 transport systems (Garrido et al. 2011). Among them, GltA (Blon_2177) binds LNB, galacto-*N*-biose (GNB), and LNT *in vitro* (Garrido et al. 2011), suggesting that the cognate transporter, GltABC, imports these glycans into the cell (Figure 2(B)). Notably, *B. infantis* strains lacking the *gltABC* genes within the *lnp* locus (Figure 2(A)) can still efficiently grow in the medium supplemented with LNT, indicating the presence of yet unidentified

alternative LNT-specific transport system in this bacterium (Duar et al. 2020; Barratt et al. 2022). GltABC orthologs can be identified in *B. longum, B. breve, B. bifidum,* and *B. pseudocatenulatum* strains (Table S1). However, the substrate specificity of this transport system might vary at the species and strain levels. For example, while GltA in *B. longum* JCM1217 preferentially binds LNB/GNB but not LNT (Suzuki et al. 2008), its ortholog in *B. longum* SC596 displays comparable affinities both to LNB/GNB and various type I HMO moieties *in vitro* (Garrido et al. 2016). These observations imply that the ability to uptake LNB/GNB is conserved within *B. longum*, whereas the ability to uptake LNT varies at the strain level. The exact substrate specificities of GltA orthologs in other LNT consumers, such as *B. breve* and *B. pseudocatenulatum* (Ruiz-Moyano et al. 2013; James et al. 2016; Ojima, Asao, et al. 2022) remain to be determined.

The HMO cluster I (H1), a unique genomic feature of all *B. infantis* and particular human *B. longum* subsp. suis (*B. suis*) strains (Barratt et al. 2022, Vatanen et al. 2022), encodes two paralogous ABC transporters (HmoABC and HmoA2B2C2) and four paralogous SBPs (HmoA3-A6) implicated in HMO uptake (Figure 2(A)) (Sela et al. 2008). The gene composition of H1 varies at the strain level, as certain *B. infantis* strains lack *hmoA5* and *hmoA2B2C2* genes (Barratt et al. 2022). *In vitro* glycan binding assays demonstrated that HmoA (Blon_2347) binds LNnT and various type II HMO moieties (Garrido et al. 2011), implying the role of HmoABC in LNnT uptake (Figure 2(B)). HmoA2 (Blon_2344) also binds type II HMO moieties, albeit with very low affinity. HmoA3-A6 (Blon_2350/2351/2352/2354) do not bind LNnT or any other HMO species despite all sharing over 70% sequence identity with HmoA (Garrido et al. 2011). The exact substrate specificities of these SBPs are unknown. Transcriptomic data, namely the upregulation of *hmo* genes during the growth of *B. infantis* ATCC 15697 on various HMOs (Garrido et al. 2015; Arzamasov et al. 2022), suggest that these SBPs may potentially be involved in the transport of certain HMO species by sharing permease components of HmoABC or HmoA2B2C2.

B. breve UCC2003 imports LNnT *via* a different non-orthologous ABC transporter; its SBP is termed NahS. Knockout of the *nahS* gene abolishes the growth of this bacterium in the medium supplemented with LNnT (James et al. 2016). Almost all *B. breve* strains possess *nahS* orthologs (Sakanaka, Gotoh, et al. 2019). In addition, approximately 50% of *B.* infantis genomes possess a truncated version of a *nahS* ortholog (Figure 2(A)) (Sakanaka, Gotoh, et al. 2019), but its role in the uptake of LNnT remains to be elucidated.

A distinct set of ABC transporters endows bifidobacteria with the ability to import fucosylated HMOs. A pioneering study demonstrated that two paralogous SBPs in *B. infantis* ATCC 15697, FL1-BP (Blon_0343) and FL2-BP (Blon_2202) (Figure 2(A)), bind 2′FL *in vitro* (Garrido et al. 2011). Similar results were obtained for an FL2-BP ortholog in *B. longum* SC596 (Garrido et al. 2016). The functionality and substrate specificities of corresponding ABC transport systems (termed FL1 and FL2) from various *Bifidobacterium* species were later delineated using targeted gene knockouts and a cell-based platform for evaluating HMO transporter specificities (Matsuki et al. 2016; Sakanaka, Hansen, et al. 2019; Ojima, Asao, et al. 2022).

Based on amino acid sequences of the SBP, bifidobacterial ABC transporters for fucosylated HMOs can be classified into four phylogenetic groups: 1-IV, 2-I, 2-II, and 2-III (Sakanaka, Hansen, et al. 2019; Ojima, Asao, et al. 2022). Group I-IV representative, FL1 in *B. infantis* ATCC 15697, transports 2′FL and 3FL (Figure 2(B)) (Sakanaka, Hansen, et al. 2019). Group 2-I and 2-II representatives (FL2 orthologs in *B. pseudocatenulatum* JCM 1200 and *B. infantis* ATCC 15697, respectively) can also import lactodifucotetraose (LDFT) and LNFP I in addition to 2′FL and 3FL (Table S1) (Sakanaka, Hansen, et al. 2019; Ojima, Asao, et al. 2022). Finally, an FL2 ortholog in *B. kashiwanohense* JCM 15439 representing group 2-III has the broadest substrate specificity and transports 2′FL, 3FL, LDFT, LNFP I, LNFP II, and lacto-*N*-difucohexaoses (LNDFH) I/II (Table S1) (Ojima, Asao, et al. 2022).

While most *B. infantis* genomes encode FL1 or FL2 (or both), only a small subset of *B. longum* (3%), *B. breve* (4–8%), and *B. pseudocatenulatum* (13%) strains harbor the loci encoding these transport systems (Sakanaka, Gotoh, et al. 2019). Despite the rarity, these gene clusters may confer the respective strains a selective advantage in the neonatal gut during breastfeeding (Matsuki et al. 2016; Ma et al. 2022). Several gaps in knowledge regarding the transport of fucosylated HMOs in bifidobacteria still exist. For example, the mechanisms by which *B. infantis* imports LNFP II/III and LNDFH I/II remain to be elucidated (Sakanaka, Gotoh, et al. 2019).

Glycoprofiling data indicate that multiple *B. infantis* and *B. breve* strains can consume sialyllacto-*N*-tetraose (LST) b (Sela et al. 2011; Ruiz-Moyano et al. 2013), suggesting the presence of a transport system(s) for sialylated HMOs. The uptake of LST b can potentially be mediated by GltABC orthologs in these two species since GltA in *B. infantis* ATCC 15697 was shown to bind this oligosaccharide *in vitro*, albeit with low affinity (Garrido et al. 2011). Currently, *B. infantis* is the only *Bifidobacterium* species described as capable of consuming and metabolizing 3′SL and 6′SL inside the cell (Ruiz-Moyano et al. 2013; Garrido et al. 2015; Barratt et al. 2022). Nevertheless, no candidate 3′SL/6′SL-specific transporters have been predicted or described, pointing to an important knowledge gap to be addressed in future studies.

Intracellular HMO degradation—Bifidobacteria use a repertoire of intracellular exoglycosidases to step-by-step release terminal monosaccharide residues from the non-reducing end of various HMOs. Degradation of LNT and LNnT is initiated by β-galactosidases, which can be split into two groups based on their specificity. Particular GH42 family enzymes, such as Bga42A in B. *infantis*, preferentially release β−1,3 linked Gal residues from LNT and thus are involved in LNT degradation (Figure 2(B)) (Yoshida et al. 2012; Viborg et al. 2014). A Bga42A ortholog in *B. breve* UCC2003 (termed LntA) possesses the same substrate-specificity and is essential for LNT but not LNnT utilization (James et al. 2016). Certain GH2 family enzymes, such as Bga2A in *B. infantis*, participate in LNnT and Lac degradation by cleaving β−1,4 linked Gal residues (Figure 2(B)) (Yoshida et al. 2012; Viborg et al. 2014). Bga2A is not essential for LNnT utilization in *B. breve* UCC2003, consistent with the presence of multiple GH2 family enzymes with overlapping specificities in this bacterium (James et al. 2016; Ambrogi et al. 2019). Orthologs of Bga42A and Bga2A are conserved among various *Bifidobacterium* species (Table S1) (Sakanaka, Gotoh, et al. 2019).

After the removal of the terminal Gal moiety, the next step of LNT and LNnT degradation involves the breakdown of the GlcNAcβ1–3Gal bond in lacto-*N*-triose II by GH20 family β-*N*-acetylglucosaminidases (Figure 2(B)). Three such homologous GHs, Hex1 (Blon_0732), NahA (Blon_0459), and Hex2 (Blon_2355), have been characterized in *B. infantis* ATCC 15697 (Garrido, Ruiz-Moyano, et al. 2012). All three GH release terminal β−1,3-linked GlcNAc residues from lacto-*N*-triose II, whereas Hex1 and NahA can additionally cleave β−1,6 glycosidic bonds found in branched neutral HMOs. Orthologs of Hex1 and NahA from *B. longum* JCM1217 and *B. breve* UCC2003, respectively, were shown to possess the same activity toward lacto-*N*-triose II (Honda et al. 2013; James et al. 2016) and are widespread among bifidobacteria (Table S1) (Sakanaka, Gotoh, et al. 2019). Furthermore, NahA is essential for both LNT and LNnT utilization in *B. breve* UCC2003 (James et al. 2016).

Intracellular α-L-fucosidases cleave terminal Fuc residues found in fucosylated HMOs. The genome of *B. infantis* ATCC 15697 encodes five α-L-fucosidases. However, only two of them, BiAfcA and BiAfcB, encoded within the H1 locus (Figure 2(A)), preferentially act on fucosylated HMOs (Sela et al. 2012). BiAfcA (GH95) releases Fuc residues by preferentially cleaving α−1,2 glycosidic bonds in 2′FL and LNFP I (Figure 2(B)). This GH also has moderate activity toward α−1,3 linkages. In contrast, BiAfcB (GH29_B) cleaves α−1,3/4 glycosidic bonds in 3FL and LNFP III (Figure 2(B)). Orthologs of BiAfcA and BiAfcB in *B. longum* SC596, *B. kashiwanohense* APCKJ1, and *B. pseudocatenulatum* MP80 demonstrate highly similar substrate preferences, acting on α−1,2 and α−1,3/4 linkages, respectively (Garrido et al. 2016; James et al. 2019; Shani et al. 2022). In contrast to *B. infantis*, in other species, orthologs of *biAfcA* and *biAfcB* genes co-localize with genes encoding FL transporters (Table S1) (Ojima, Asao, et al. 2022).

Intracellular α-sialidases cleave terminal Neu5Ac residues from sialylated HMOs. *B. infantis* ATCC 15697 possesses two α-sialidases: NanH1 encoded within the *nan* cluster, and NanH2 encoded within the H1 cluster (Figure 2(A)). Both GHs exhibit similar activities toward synthetic sialylated substrates *in vitro*, cleaving α−2,3 and α−2,6 linkages (Sela et al. 2011). However, only NanH2 shows activity toward sialylated HMOs, particularly mono- and di-sialylated LNT (Sela et al. 2011). This observation, together with the absence of *nanH2* orthologs in other *Bifidobacterium* species (Table S1), suggests that NanH2 might be a unique determinant of the SL utilization ability of *B. infantis* (Figure 2(B)).

Extracellular HMO degradation—The extracellular HMO degradation strategy is exemplified by *B. bifidum*. This species possesses multiple extracellular exo- and endo-acting GHs that also contribute to *O*-glycan degradation (Katoh et al. 2020). For LNT utilization, *B. bifidum* employs endo-acting lacto-*N*-biosidase LnbB (GH20), which cleaves the GlcNAcβ1–3Gal bond, releasing LNB and Lac (Wada et al. 2008) (Figure 3). Certain *B. longum* strains possess non-orthologous lacto-*N*-biosidase LnbX (GH136), capable of hydrolyzing the same glycosidic bond (Sakurama et al. 2013). However, these two GHs have different substrate preferences. While LnbB hydrolyzes only unmodified LNT, LnbX also acts on LNFP I and LST a (Wada et al. 2008; Sakurama et al. 2013). LnbX requires a chaperone protein (LnbY) for the correct folding (Sakurama et al. 2013).

B. bifidum degrades LNnT by a coordinated action of two membrane-anchored GHs, BbgIII and BbhI (Figure 3). BbgIII is a GH2 family β-galactosidase that cleaves β−1,4-linked terminal Gal residues from LNnT and LacNAc and is not active toward LNT, LNB, or fucosylated HMOs (Miwa et al. 2010). BbhI is a GH20 family β-*N*-acetylglucosaminidase that subsequently cleaves the GlcNAcβ1–3Gal bond in lacto-*N*-triose II, releasing GlcNAc and Lac (Miwa et al. 2010).

As mentioned above, fucosylated and sialylated HMOs are not readily degraded by LnbB and BbgIII, indicating that Fuc and Neu5Ac decorations must be removed first by other GHs. Terminal Fuc residues are released by extracellular α-L-fucosidases (Figure 3): BbAfcA (GH95) that preferentially acts on α−1,2 glycosidic bonds in 2′FL, LDFT, LNFP I, and LNDFH I, and BbAfcB (GH29_B) that cleaves α−1,3/4 linkages in 3FL, LNFP II/III (Katayama et al. 2004; Ashida et al. 2009). Terminal Neu5Ac residues are released by membrane-anchored α-sialidases SiaBb1 and SiaBb2 (GH33) (Figure 3). Both GHs preferentially cleave α−2,3 bonds and are also active toward α−2,6 linkages, releasing Neu5Ac from 3′SL, 6′SL, and DSLNT (Kiyohara et al. 2011; Ashida et al. 2018; Nishiyama et al. 2018). While both sialidases share similar substrate-specificities, SiaBb2 is presumed to be more important for the utilization of sialylated HMOs since knockout of the *siaBb2* gene significantly decreases the growth of *B. bifidum* on 6′SL (Nishiyama et al. 2017, 2018).

A noteworthy consequence of the extracellular hydrolysis of HMO by *B. bifidum* is that certain HMO degradation products can be salvaged by other bifidobacteria. *B. bifidum* primarily consumes LNB and Lac produced during the hydrolysis of LNT and type I HMOs (Figure 3) (Asakuma et al. 2011). However, the release rate of LNB/Lac by extracellular GHs surpasses their consumption rate (Asakuma et al. 2011), suggesting that a portion of these disaccharides may become available to other bifidobacteria. In addition, *B. bifidum* releases Gal and GlcNAc residues by degrading type II HMOs (Figure 3). While *in vitro* growth data indicates that certain *B. bifidum* strains can grow in the medium supplemented with Gal or GlcNAc (Turroni et al. 2010, 2012), the direct consumption of these monosaccharides during HMO degradation by this bacterium has not been evident (Asakuma et al. 2011; Ojima, Jiang, et al. 2022), suggesting other bacteria can benefit from foraging these monosaccharides. Finally, *B. bifidum* strains do not utilize Fuc and Neu5Ac cleaved by extracellular GHs (Asakuma et al. 2011) due to a lack of transporters and downstream catabolic pathways for these monosaccharides (Figure 3 and Table S1). Thus, Fuc and Neu5Ac released by *B. bifidum* can be foraged by *B. infantis* and *B. breve* (Figure 3) (Egan, O'Connell Motherway, Ventura, et al. 2014; Egan, O'Connell Motherway, Kilcoyne, et al. 2014; O'Connell Motherway et al. 2018; Gotoh et al. 2018; Nishiyama et al. 2018; Centanni et al. 2019; Walsh et al. 2022; Ojima, Jiang, et al. 2022; Yokoi et al. 2022) and potentially other gut bacteria.

Extracellular HMO degradation contributes to so-called facilitative priority effects (the concept reviewed in Ojima, Yoshida, et al. 2022) exerted by *B. bifidum*. Species like *B. breve* can benefit from facilitative effects by utilizing Fuc and other HMO constituents provided by *B. bifidum* and dominate neonatal microbial communities, despite having limited HMO-utilization ability (Ojima, Jiang, et al. 2022).

N-linked glycan utilization by bifidobacteria

To be absorbed in the small intestine, most dietary proteins undergo digestion by gastric and pancreatic proteases, and the released large peptides are further hydrolyzed to amino acids or di- and tripeptides by peptidases present on the enterocytic brush border (Miner-Williams et al. 2014). However, *N*-glycosylation shields proteins from complete proteolytic degradation (O'Riordan et al. 2014), allowing bulky *N*-glycopeptides to reach the large intestine, where they can be foraged by commensal gut bacteria (Garrido, Nwosu, et al. 2012; Briliūté et al. 2019; Becerra et al. 2020). Pioneering work by Garrido et al. demonstrated that multiple strains of *B. infantis*, *B. longum*, and *B. breve* release *N*-glycan chains from glycoproteins (Garrido, Nwosu, et al. 2012). Subsequent studies have revealed that *B. infantis* can readily consume a multitude of *N*-glycan species found in human and bovine milk (Karav et al. 2016) in a process accompanied by high activity of multiple intracellular GHs (Wang WL et al. 2017; Mao et al. 2022). These findings indicate the presence of a dedicated bifidobacterial *N*-glycan utilization pathway, which can be divided into three stages: (i) release of *N*-glycan chains from glycosylated peptides/proteins, (ii) transport of the released moieties into the cell, (iii) degradation to monosaccharides by a concerted action of intracellular GHs (Figure 4(B)).

Release of N-glycans from glycosylated proteins—Endo-*β*-*N*-acetylglucosaminidases (ENGases) are the key enzymes that release *N*-glycans from glycosylated proteins by cleaving the *N,N′*-diacetylchitobiose core structure (Figure 4(B)) (Garrido, Nwosu, et al. 2012; Karav et al. 2016, 2019). Bifidobacterial ENGases are classified into two GH families: GH18 and GH85 (Drula et al. 2022). The GH18 family members found in *B. infantis* strains, EndoBI1 and EndoBI2, act on individual glycoproteins from human and bovine milk, such as bovine/human lactoferrin, IgA, and IgG, releasing a variety of oligomannose and core-fucosylated bi- or triantennary complex *N*-glycan moieties (Garrido, Nwosu, et al. 2012). In contrast, EndoBB (GH85) from *B. longum* DJO10A acts on RNaseB (containing oligomannose *N*-glycan) but not lactoferrins (Garrido, Nwosu, et al. 2012). Furthermore, an ortholog of EndoBB from *B. longum* NCC2705 was shown to cleave the *N,N′*-diacetylchitobiose core in $Man_5GlcNAc_2$, $Man_9GlcNAc_2$, and $GlcMan_9GlcNAc_2$ structures (Cordeiro et al. 2023). Certain *B. longum* and *B. suis* genomes encode another GH85 family ENGase, EndoBB2 (Figure 4(A)); however, its activity has not been experimentally characterized.

The ENGase genes are sporadically distributed among *Bifidobacterium* genomes (Figure 4(A) and Table S1). *B. infantis* strains typically possess at least one GH18 family enzyme (EndoBI1 or EndoBI2), whereas *B. suis*, *B. breve*, and *B. longum* generally rely on GH85 family enzymes (EndoBB or EndoBB2) (Garrido, Nwosu, et al. 2012; Barratt et al. 2022; Cordeiro et al. 2023). Linking the substrate specificity of ENGases with their distribution among bifidobacterial genomes suggests that *B. infantis* is adapted to liberate multiple *N*-glycan types (oligomannose, hybrid, and complex) from a variety of milk glycoproteins. In contrast, most other *Bifidobacterium* species likely preferentially release oligomannose *N*-glycans.

N-glycan uptake—Localization of GH activity in *B. infantis* combined with the tracing of fluorescently-labeled substrates has provided strong evidence that *N*-glycan chains released by ENGases are not degraded extracellularly but instead transported inside the cell (Wang WL et al. 2017; Mao et al. 2022). Previous studies suggested that an ABC transporter (termed hereof MnaABC) encoded in the same operon as *N*-glycan acting β-mannosidase BlMan5B (GH5_18) is responsible for *N*-glycan internalization in *B. longum* (Cordeiro et al. 2019, 2023; Higgins et al. 2021). An additional piece of evidence supporting this hypothesis is the predicted co-regulation of *mnaABC* and *endoBI1* genes by a LacI-family transcription factor (TF) MnaR in *B. infantis* ATCC 15697 (Figure 4(A)) (Barratt et al. 2022). MnaABC belongs to the CUT1 family of oligosaccharide transporters (Saier et al. 2021). Orthologs of this transport system can be found in multiple *Bifidobacterium* genomes (Figure 4(A) and Table S1), suggesting that the ability to import *N*-glycans is not confined to *B. infantis* and *B. longum.*

Certain *B. infantis* strains (e.g. Bg_2D9) isolated from Bangladeshi and Malawian infants possess the Ngl cluster (Figure 4(A)) that encodes: (i) a BlMan5B paralog termed BiMan5, (ii) β-*N*-acetylglucosaminidase Hex3 (GH20), and (iii) another predicted ABC transport system for *N*-glycans, NglABC (Barratt et al. 2022). Although the SBPs of MnaABC and NglABC are likely paralogous, their sequence identity in *B. infantis* Bg_2D9 is only 40%, indicating potential functional divergence. Furthermore, the co-localization of *nglABC* and *hex3* genes in the same operon suggests that NglABC may preferentially transport GlcNAc-decorated (complex) *N*-glycan chains. Further experiments are needed to test the role of MnaABC and NglABC in *N*-glycan uptake in bifidobacteria and elucidate the specificities of these transport systems toward different *N*-glycan types.

Intracellular degradation of *N*-glycans—Monosaccharide residues constituting *N*-glycans are interconnected by various glycosidic linkages, which would require a coordinated action of multiple intracellular exo-acting GHs to break down (Figure 4(B)). The molecular mechanisms of oligomannose *N*-glycan degradation have been recently elucidated (Cordeiro et al. 2023). The terminal α-linked Man residues are cleaved by α-mannosidases from the GH38 and GH125 families. The three paralogous GH38 enzymes in *B. longum* NCC2705 (BlMan38A, BlMan38B, BlMan38C) share the capacity to cleave α−1,2-, α−1,3-, and α−1,6-mannoside linkages. However, while BlMan38B is a generalist enzyme capable of releasing all α-Man residues from both $Man_9GlcNAc$ and $Man_5GlcNAc$, BlMan38A and BlMan38C completely degrade $Man_9GlcNAc$ only when combined (Cordeiro et al. 2023). The hydrolytic activity of GH38 enzymes is complemented by a specialist GH125 α−1,6-mannosidase BlMan125 (Cordeiro et al. 2023). The GH5_18 family β-mannosidase BlMan5B (also known as BlGH5_18) performs the final step of intracellular *N*-glycan degradation by hydrolyzing the Man-β−1,4-GlcNAc disaccharide, releasing Man and GlcNAc (Cordeiro et al. 2019; Higgins et al. 2021). The *blMan38A, blMan38B, blMan125, mnaABC*, and *blMan5B* genes are conserved among various bifidobacteria (Figure 4(B) and Table S1), supporting the idea that multiple bifidobacterial species (beyond *B. infantis* and *B. longum*) are capable of *N*-glycan utilization.

Additional exo-GHs with α-sialidase, β-galactosidase, and β-*N*-acetylglucosaminidase activities would be required to release monosaccharide residues from the antennae of

complex *N*-glycans. Although *B. infantis* employs a set of such GHs to degrade HMOs (Sela et al. 2011; Garrido, Ruiz-Moyano, et al. 2012; Yoshida et al. 2012; Viborg et al. 2014), the activities of these enzymes toward *N*-glycans have not been tested experimentally. Nevertheless, the structural similarities between HMOs and complex *N*-glycans suggest that bifidobacterial α-sialidases (NanH2), β-galactosidases (Bga2A), and β-*N*-acetylglucosaminidases (Hex1/Hex2/Hex3/NahA) may contribute to both HMO and *N*-glycan utilization (Figure 4(B)). Additional studies are required to elucidate the details of the complex *N*-glycan degradation process inside bifidobacterial cells.

Comparative-genomic analysis suggests that specific bifidobacteria may encode unique yet-uncharacterized elements of intracellular *N*-glycan degradation. For example, *Bifidobacterium scardovii* JCM 12489 contains a gene cluster that shares similarities with the Ngl cluster from *B. infantis* Bg_2D9 (Barratt et al. 2022) and also encodes a putative α-fucosidase (GH29) and β-mannosidase (GH2) (Figure 4(A)). Moreover, another *N*-glycan utilization cluster found in this species encodes a putative anaerobic carbohydrate sulfatase (BsAtsX) and sulfatase maturing enzyme (BsAtsY) (Figure 4(A)). A distant homolog of BsAtsX was described in *B. breve* UCC2003, where it removes the sulfate group from *N*-acetylglucosamine-6-sulfate (Egan et al. 2016). We speculate that the presence of BsAtsX allows *B. scardovii* to metabolize sulfated complex *N*-glycans, which decorate proteins found in various body sites (Wang JR et al. 2017; Huang et al. 2022).

N-glycan core structure utilization—Core fucosylation of milk *N*-glycans has been associated with an increased abundance of *Bifidobacterium* and *Lactobacillus* genera in the gut of breastfed infants (Li et al. 2019), suggesting that these microorganisms could benefit from metabolizing *N*-glycopeptides released during the cleavage of the fucosylated *N,N′*-diacetylchitobiose core by bacterial ENGases (Figure 4(C)). Indeed, a utilization pathway for Fucα1–6GlcNAcβ1-Asn (6′FN-Asn) has been characterized in *Lactobacillus* casei BL23 (Becerra et al. 2020). This bacterium employs MFS superfamily permease AlfH to import 6′FN-Asn inside the cell and α-L-fucosidase AlfC (GH29_A) to cleave the α1,6-linked Fuc residue, which is not metabolized and excreted to the culture medium. The generated GlcNAc-Asn is processed by glycosylasparaginase AsnA2 (EC 3.5.1.26) to aspartate and GlcNAc; the latter is used as a carbon source. The gene cluster (*alf-2*) harboring *alfH*, *alfC*, and *asnA2* genes also encodes a putative peptidase PepV, indicating that L. casei may also utilize longer *N*-glycopeptides.

The *alf-2* cluster is conserved among multiple *Lactobacillus* and several *Bifidobacterium* species (Becerra et al. 2020). Among infant gut-colonizing bifidobacteria, *B. infantis* strains possess two genomic loci homologous to *alf-2* (Becerra et al. 2020) that could have been acquired *via* horizontal gene transfer from *Lactobacillus* by displacing the arabinose utilization operon in the common ancestor of *B. infantis* and *B. longum* (Sela et al. 2008). These loci, previously termed H2 and H3 (LoCascio et al. 2010), have an identical operon structure and encode orthologs of AlfH and AlfC: AfcT and BiAfcC, respectively (Figure 4(A)). BiAfcC cleaves α1,6-linked Fuc residues from 6′FN-Asn-peptide but not from intact core-fucosylated glycoproteins (Ashida et al. 2020), suggesting that the core must be first cleaved by ENGases for further utilization. Therefore, a putative pathway for *N*-glycopeptide utilization in *B. infantis* includes uptake by AfcT and a subsequent release of

the Fuc moiety by BiAfcC (Figure 4(B)). *B. infantis* lacks AsnA2 orthologs needed for the hydrolysis of the amide bond between the Asn and the GlcNAc residue. Thus, this bacterium either uses a different enzyme to cleave this bond or, unlike *L. casei*, catabolizes only the Fuc residue of the core structure, highlighting potential differences and complementarity of *N*-glycan foraging strategies of commensal gut bacteria.

Metabolism of mono- and disaccharide constituents of milk glycans

Most monosaccharides that are released during HMO and *N*-glycan degradation are converted to fructose-6-phosphate (Fru6P) by downstream catabolic pathways (Figure 5). Fru6P enters the central phosphoketolase pathway termed the “bifid shunt”, which is driven by the key enzyme fructose-6-phosphoketolase (EC 4.1.2.2). The bifid shunt enables bifidobacteria to ferment both hexose and pentose sugars into lactate/acetate and produce more energy in the form of ATP than fermentative pathways in other commensal gut bacteria (Palframan et al. 2003; Pokusaeva, Fitzgerald, et al. 2011). Unlike other monosaccharides constituting milk glycans, Fuc is catabolized through an oxidative pathway to 1,2-propanediol (1,2-PD) and formate (Bunesova et al. 2016; James et al. 2019; Dedon et al. 2020; Tsukuda et al. 2021). The fermentation end-products, such as acetate, lactate, and 1,2-PD, not only confer beneficial properties to the host but also can be crossfed to other commensal gut bacteria (Belenguer et al. 2006; Fukuda et al. 2011; Rios-Covian et al. 2015; Rivière et al. 2015; Schwab et al. 2017).

Glucose and galactose—Bifidobacteria catabolize Glc and Gal via a pathway conserved among various *Bifidobacterium* species (Table S1) (Pokusaeva, Fitzgerald, et al. 2011) and other gut bacteria (Bettenbrock and Alpert 1998; Holden et al. 2003). Glc is first phosphorylated to glucose-6-phosphate (Glc6P) by either glucokinase (GlcK; EC 2.7.1.2) or polyphosphate glucokinase (PpgK; EC 2.7.1.2) and then converted to Fru6P by glucose-6-phosphate isomerase (Gpi; EC 5.3.1.9). Gal is converted to Glc6P through the Leloir pathway (De Bruyn et al. 2013). The first step of this pathway involves the phosphorylation of Gal to galactose-1-phosphate (Gal1P) by galactokinase (GalK; EC 2.7.1.6) (Li et al. 2012). Next, galactose-1-phosphate uridylyltransferase (GalT; EC 2.7.7.10) converts Gal1P to UDP-Gal using UDP-Glc as the uridine diphosphate source and simultaneously releases glucose-1-phosphate (Glc1P), whereas UDP-galactose 4-epimerase (GalE; EC 5.1.3.2) recycles UDP-Gal to UDP-Glc (De Bruyn et al. 2013). Finally, phosphoglucomutase (Pgm; EC 5.4.2.2) converts Glc1P to Glc6P.

Mannose—Bifidobacteria metabolize Man to Fru6P *via* a metabolic route that differs from pathways described in *Escherichia coli* and *Bacillus subtilis*, where phosphoenolpyruvatedependent phosphotransferase systems mediate the active transport and concomitant phosphorylation of Man to mannose-6-phosphate, which is then converted to Fru6P by mannose-6-phosphate isomerase (Erni et al. 1987; Sun and Altenbuchner 2010). Instead, bifidobacterial mannose isomerase (ManI; EC 5.3.1.7) converts Man to fructose (Cordeiro et al. 2023). The latter is phosphorylated to Fru6P by bifidobacterial fructokinase (Frk; EC 2.7.1.4) (Caescu et al. 2004).

Aminosugars—The bifidobacterial metabolism of aminosugars revolves around the GlcNAc catabolic pathway, which is encoded by the *nag* genes (Figures 2(A) and 5). GlcNAc liberated during the breakdown of HMOs and complex *N*-glycans is phosphorylated to *N*-acetylglucosamine-6-phosphate (GlcNAc6P) by a predicted ROK family N-acetylglucosamine kinase (NagK; EC 2.7.1.59) (Uehara and Park 2004; Egan et al. 2016). The predicted specificity of NagK is based on genomic context and the confirmed co-regulation of *nagK* with other GlcNAc catabolism genes by the same transcription factor (NagR) (Figure 2(A)) (Arzamasov et al. 2022). GlcNAc6P is then deacetylated by *N*-acetylglucosamine-6-phosphate deacetylase (NagA; EC 3.5.1.25) and subsequently converted to Fru6P by glucosamine-6-phosphate deaminase (NagB; EC 3.5.99.6) (Egan, O'Connell Motherway, Ventura, et al. 2014).

Neu5Ac released during the degradation of internalized milk glycans can be directly routed to the GlcNAc catabolic pathway via enzymes encoded within the *nan* locus (Figures 2(A) and 5) (Egan, O'Connell Motherway, Ventura, et al. 2014). First, *N*-acetylneuraminate lyase (NanA; EC 4.1.3.3) cleaves Neu5Ac to pyruvate and *N*-acetylmannosamine. The latter compound is phosphorylated by *N*-acetylmannosamine kinase (NanK; EC 2.7.1.60) to *N*-acetylmannosamine-6-phosphate, which, in turn, is converted to GlcNAc6P by N-acetylmannosamine-6-phosphate epimerase (NanE; EC 5.1.3.9).

Another important component of aminosugar metabolism in bifidobacteria is the unique lacto-*N*-biose/galacto-*N*-biose pathway encoded within the *lnp* cluster (Figures 2(A) and 5). As the name implies, the primary function of this pathway is the catabolism of LNB and GNB, which are released during extracellular degradation of type I HMOs (Wada et al. 2008; Sakurama et al. 2013; Yamada et al. 2017) and mucin O-glycans (Fujita et al. 2005; Ashida et al. 2008), respectively. The LNB/GNB pathway was first described in *B. longum* (Kitaoka et al. 2005; Nishimoto and Kitaoka 2007) and later identified in *B. infantis, B. bifidum*, and *B. breve* (Table S1) (Xiao et al. 2010). In the first step of the pathway, β−1,3-D-galactosyl-D-hexosamine phosphorylase (LnpA; EC 2.4.1.211) cleaves LNB/GNB to Gal1P and GlcNAc or *N*-acetylgalactosamine (GalNAc), respectively, without consuming ATP (Kitaoka et al. 2005). Then *N*-acetylhexosamine kinase (LnpB; EC 2.7.1.162) phosphorylates the α-anomeric hydroxyl groups of emerging hexosamines, yielding *N*-acetylglucosamine-1-phosphate (GlcNAc1P) or *N*-acetylgalactosamine-1-phosphate (GalNAc1P) (Nishimoto and Kitaoka 2007). GalNAc1P can be converted to GlcNAc1P by a concerted action of UTP-hexose-1-phosphate uridylyltransferase (LnpC; EC 2.7.7.10) and UDP-hexose 4-epimerase (LnpD; EC 5.1.3.2). LnpC and LnpD can also convert Gal1P to Glc1P, albeit with much lower efficiency than GalT/GalE from the Leloir pathway (De Bruyn et al. 2013).

Despite the accumulated data, bifidobacterial aminosugar metabolism still contains several gaps in knowledge. So, certain *B. pseudocatenulatum* strains that lack the LNB/GNB pathway (Table S1) can utilize LNB (Xiao et al. 2010). One possible explanation is that in *B. pseudocatenulatum*, LNB is cleaved to Gal and GlcNAc by β−1,3-galactosidase (Bga42A ortholog) (Sakanaka, Gotoh, et al. 2019). Another unsolved question is the fate of GlcNAc1P formed in the LNB/GNB pathway. Generally, bacteria utilize bifunctional *N*-acetylglucosamine-1-phosphate uridyltransferase/glucosamine-1-phosphate acetyltransferase

(GlmU; EC 2.7.7.23 and 2.3.1.157) to convert GlcNAc1P to UDP-*N*-acetylglucosamine, which is then used in the cell wall biosynthesis (Mengin-Lecreulx and van Heijenoort 1994). In eukaryotes, GlcNAc1P can be isomerized to GlcNAc6P by phospho-*N*-acetylglucosamine mutase (PAGM; EC 5.4.2.3). However, no prokaryotic PAGMs have been described so far (Stiers et al. 2017), indicating that additional studies are required to determine whether GlcNAc1P emerging during LNB/GNB metabolism is indeed routed to the GlcNAc catabolic pathway or is primarily utilized for cell wall biogenesis.

Fucose—Although the enzymes involved in Fuc catabolism in bifidobacteria have not been biochemically characterized, genomic and metabolomic data (Bunesova et al. 2016; James et al. 2019) suggest that these microorganisms metabolize Fuc *via* a route I non-phosphorylating pathway similar to the one described in *Campylobacter jejuni* and *Veillonella ratti* (Figure 5) (Stahl et al. 2011; Watanabe 2020). In *B. infantis* strains, the genes encoding the elements of this pathway are split between two genomic loci (H1 and *fuc*) (Figure 2(A)), whereas in *B. longum*, *B. kashiwanohense*, and *B. pseudocatenulatum*, they form a single gene cluster (Table S1).

The first step of Fuc catabolism includes the isomerization of α-L-Fuc to β-L-Fuc by fucose mutarotase (FumB; EC 5.1.3.29). The *fumB* gene has a notable distribution pattern among *Bifidobacterium* genomes; it is present in genomes encoding both GH29 and GH95 family α-L-fucosidases but absent in genomes encoding only GH95 enzymes (Table S1) (Garrido et al. 2016; James et al. 2019; Ojima, Asao, et al. 2022; Shani et al. 2022). Such a pattern is consistent with distinct mechanisms of action of these GHs. Indeed, while GH95 α-L-fucosidases invert the anomeric configuration of cleaved Fuc residues from α to β (Katayama et al. 2004), GH29 family enzymes have a retaining mechanism, suggesting the requirement of FumB. Emerging β-L-fucose is oxidized to L-fuconate by a concerted action of L-fuco-β-pyranose dehydrogenase (FumC; EC 1.1.1.122) and L-fuconolactone hydrolase (FumD; EC 3.1.1.-). Then L-fuconate dehydratase (FumE; EC 4.2.1.68) converts L-fuconate to an acid intermediate 2-keto-3-deoxy-L-fuconate, which is then cleaved to L-lactaldehyde and pyruvate by 2-keto-3-deoxy-L-fuconate aldolase (FumF; EC 4.1.2.18) (Watanabe 2020). Finally, lactaldehyde reductase (FucO; EC 1.1.1.77) reduces L-lactaldehyde to 1,2-PD.

Transcriptional regulation of milk glycan utilization

One of the essential physiological aspects of bacterial adaptation to the constantly changing environment of the human gut is the ability to rapidly induce the expression of genes encoding carbohydrate utilization pathways in the presence of corresponding dietary glycans. In bifidobacteria, this process is regulated by a multitude of TFs from the LacI, ROK, GntR, and other families. Most of these TFs function as carbohydrate-sensing transcriptional repressors (Khoroshkin et al. 2016; Kelly et al. 2021).

Historically, experimental methods for studying bifidobacterial TFs have been based on (i) transcriptome profiling upon genetic inactivation of a particular TF gene and (ii) biochemical characterization of the TF to identify its cognate transcriptional effector molecule(s) and binding sites (operators) in promoter regions of regulated genes (Ryan et al. 2005; Pokusaeva et al. 2010; Pokusaeva, O'Connell-Motherway, et al. 2011; O'Connell

Motherway et al. 2011; O'Connell et al. 2014; Egan et al. 2015, 2016; James et al. 2018; Lanigan et al. 2019). An orthogonal approach is based on *in silico* reconstruction of gene regulatory networks (regulons) and permits the prediction of cognate operator sequences of a particular TF on a genome-wide scale (Rodionov 2007; Khoroshkin et al. 2016; Arzamasov et al. 2018). This approach has proven instrumental in understanding transcriptional regulation of HMO and *N*-glycan utilization in bifidobacteria (Arzamasov et al. 2022; Barratt et al. 2022).

Regulation of LNT and LNnT utilization—Previous studies have demonstrated that growth in the medium supplemented with LNT or LNnT induces a profound and surprisingly similar transcriptomic response in *B. infantis* ATCC 15697. Specifically, both oligosaccharides induce the expression of (i) GlcNAc catabolism genes (*nagA, nagB, nagK*) within the *nag* cluster, (ii) *gltABC* and the LNB/GNB pathway genes in the *lnp* cluster, (iii) multiple genes within the H1 cluster (*hmoABC*, *hmoA2B2C2*) encoding HMO transporters (Figure 2(A)) (Garrido et al. 2015; Özcan and Sela 2018). Based on these observations, Garrido et al. hypothesized that the H1 cluster acts as an LNT/LNnT-inducible unit and is co-regulated with the GlcNAc catabolic pathway (Garrido et al. 2015). The mechanism behind this co-regulation has been deciphered in a recent study, which combined *in silico* regulon reconstruction with transcriptomics to implicate NagR, a ROK family TF, as a repressor of *nag, lnp*, and H1 cluster genes (Figures 2(A) and 6) and thus a global regulator of LNB/GNB and HMO utilization in *B. infantis* ATCC 15697 (Arzamasov et al. 2022).

Biochemical assessment of NagR binding to its operator sequences has revealed that several intermediate products of GlcNAc metabolism (GlcNAc, GlcNAc6P, and GlcNAc1P) disrupt the NagR-operator complex *in vitro* and thus serve as potential transcriptional effectors of this TF (Arzamasov et al. 2022). The proposed model stipulates that the release of GlcNAc during intracellular degradation of LNT/LNnT and other GlcNAc-containing glycans results in the derepression of genes in the NagR regulon. The size of the NagR-mediated transcriptional network in *B. infantis* provides a remarkable example of an evolutionary adaptation of this bacterium to synchronous foraging of multiple HMOs. The translational implication of this observation is that using a mixture of LNT and LNnT (and potentially other HMOs) rather than individual oligosaccharides as a prebiotic may be a more efficient solution for the selective stimulation of *B. infantis* growth in the neonatal gut.

NagR regulon composition markedly varies among the *Bifidobacterium* species suggesting an evolutionary scenario of gradual regulon expansion (Arzamasov et al. 2022). In *B. breve* UCC2003, NagR represses the *nag* and lnp clusters, thereby regulating LNB/GNB and LNT utilization, while its paralog, NahR, represses the *nahS* gene encoding the SBP of an LNnT transporter (James et al. 2018). NagR and NahR in *B. breve* were reported to respond to different carbohydrate effectors, GlcNAc6P and GlcNAc, respectively (James et al. 2018). In *B. bifidum*, the NagR regulon is predicted to have genes encoding extracellular GHs involved in the degradation of HMOs and *O*-glycans, in contrast to *B. infantis*, where this regulon includes multiple H1 cluster genes that encode HMO transporters (Arzamasov et al. 2022). These observations illustrate how the catabolic machinery associated with two distinct strategies of HMO utilization (intracellular *vs*. extracellular) may have converged toward transcriptional control by the same TF.

Regulation of fucosylated HMO utilization—Multiple studies have demonstrated that 2′FL induces a distinct transcriptomic response compared to LNT/LNnT in various *B. infantis* strains (Garrido et al. 2015; Dedon et al. 2020; Zabel et al. 2020; Tsukuda et al. 2021). Although there are substantial discrepancies in gene expression profiles between the datasets (which might be attributed to differences in used strains and controls), a shared trend is the upregulation of *FL1/FL2* and *fuc* gene clusters encoding ABC transporters for fucosylated HMO and enzymes involved in Fuc catabolism, respectively (Figure 2(A)). Based on *in silico* regulon reconstruction, these loci are predicted to be regulated by two local transcriptional repressors from the LacI-family: FucR, controlling the *fuc* cluster, and FumR, controlling the FL1 and FL2 loci (Figures 2(A) and 6) (Khoroshkin et al. 2016; Barratt et al. 2022). Although the transcriptional effector molecules of FucR and FumR are unknown, Fuc is a good candidate since both Fuc and 2′FL induce the expression of fuc and FL2 clusters in *B. infantis* IN-F29 (Tsukuda et al. 2021).

It is interesting to note that individual 2′FL, in contrast to pooled HMOs, does not induce the expression of H1 cluster genes encoding Fuc catabolic enzymes (FumB, FumC, FumE, FumF) and α-L-fucosidases (BiAfcA, BiAfcB) in *B. infantis* ATCC 15697 (Garrido et al. 2015; Dedon et al. 2020; Zabel et al. 2020). Although this observation is consistent with the absence of predicted FucR or FumR operators in the promoter regions of these genes (Figure 2(A)), it suggests that other yet unidentified mechanisms control the expression of H1 cluster genes involved in the metabolism of fucosylated HMOs. Insufficient upregulation of GH genes (*biAfcA* and *biAfcB*), which products are required for the breakdown of fucosylated HMOs, by individual 2'FL may explain why this oligosaccharide is partially consumed when 2'FL is used as the sole carbon source (Zabel et al. 2020) but completely consumed from a pooled HMO mixture (Asakuma et al. 2011).

In *B. longum, B. kashiwanohense*, and *B. pseudocatenulatum* strains, 2′FL induces the expression of a single genomic locus encoding all elements (transporters, GHs, and Fuc catabolic enzymes) of the fucosylated HMOs utilization pathway (Table S1) (Garrido et al. 2016; James et al. 2019; Shani et al. 2022). This locus also encodes a FumR ortholog, suggesting that transcriptional regulation of 2′FL utilization by this TF is conserved among FL-utilizing members of the *Bifidobacterium* genus. The *fuc* clusters in *B. suis* and *B. breve* are potentially regulated by FucR orthologs (Table S1) (Khoroshkin et al. 2016).

Regulation of N-glycan utilization—The details of transcriptional regulation of *N*-glycan utilization in bifidobacteria remain largely unexplored due to the scarcity of available transcriptomic datasets. One study demonstrated that a cluster of *N*-glycan utilization genes in *B. longum* NCC2705 (*blMan38A, blMan38C, mnaABC, blMan5B, blGlc31,* and *endoBB*) is downregulated during co-colonization with *Bacteroides thetaiotaomicron* in mice (Sonnenburg et al. 2006). These data are compatible with potential regulation by a LacI family TF, MnaR, which is predicted to control the expression of gene clusters encoding elements of the *N*-glycan utilization pathway (ENGases, transporters, intracellular GHs, ManI) in multiple *Bifidobacterium* species (Figures 4(A) and 6) (Barratt et al. 2022). A non-homologous TF from the ROK family, NglR, is predicted to control the expression of the Ngl cluster found in certain *B. infantis* and *B. scardovii* strains (Figures 4(A) and 6) (Barratt et al. 2022). The transcriptional effectors of MnaR and NglR are unknown. We

surmise that Man or Man-containing oligosaccharides may function as effector molecules of MnaR, whereas NglR likely senses a GlcNAc-containing product of intracellular *N*-glycan degradation, given that this TF is a paralog of a group of GlcNAc-sensing TFs (NagR and NahR). Overall, further studies are required to experimentally test the role of MnaR and NglR in regulating *N*-glycan utilization in bifidobacteria.

A previous study demonstrated that growth in the medium supplemented with bovine or human lactoferrin induces the expression of genes not directly involved in *N*-glycan utilization in *B. infantis* ATCC 15697, namely *gltA, gltF, hmoA,* and *hmoA2* encoding SBPs of HMO transporters (Garrido, Nwosu, et al. 2012). Since these genes are repressed by NagR (Arzamasov et al. 2022), their upregulation can be explained by the release of GlcNAc (NagR effector molecule) during intracellular catabolism of *N*-glycan moieties. The derepression of NagR-controlled genes during the metabolism of GlcNAc-containing carbohydrates other than HMOs supports the notion that *B. infantis* is adapted to the utilization of mixtures of glycans present in human milk.

Concluding remarks and future perspectives

Bifidobacteria are often considered “good” bugs of the neonatal GM, conferring multiple beneficial properties to the infant. The ability of various *Bifidobacterium* species to colonize and prevail in the GMs of breastfed children has been associated with the capacity of these microorganisms to metabolize milk glycans, specifically HMOs. This notion prompted the administration of these carbohydrates as prebiotic supplements to infant formulas to restore the bifidobacterial population in children with persistent GM immaturity. However, given the enormous complexity of the glycan composition of human milk, one of the key questions regarding prebiotics is which glycan species should be used. Will a particular individual oligosaccharide suffice? Or should a mixture of two or more glycans be administered? Understanding the physiology and mechanisms of milk glycan metabolism by bifidobacteria would provide guidelines for answering these questions.

Indeed, milk glycan utilization by bifidobacteria has received considerable recognition in the last two decades, particularly relating to pathways driving the metabolism of various HMOs. Hence, multiple HMO transporters and HMO-acting GHs have been characterized using biochemical and cell-based assays. The details of *N*-glycan and downstream monosaccharide catabolic pathways have received less attention, with multiple “pathways gaps” still present. Comparative genomic techniques, such as the analysis of genomic context and reconstruction of TF regulons, are a powerful approach for reconstructing metabolic pathways and predicting novel pathway elements (“missing genes”). However, the exact functions and properties of these putative elements remain to be experimentally characterized. Thus, future studies employing computational, biochemical, structural, and genetic approaches will be required to fully characterize pathways driving the metabolism of HMOs and *N*-glycans by members of the *Bifidobacterium* genus.

Comparative genomic analysis additionally provides a foundation for exploring differences in pathway structures at the species and strain levels, which remains another largely unexplored aspect of milk glycan metabolism by bifidobacteria. Most current studies focus on a limited set of type strains of *Bifidobacterium* species that may only partially represent

the functional metabolic diversity of strains isolated from various populations worldwide. Studying such strains would enable the discovery of novel elements of catabolic machinery involved in milk glycan utilization and permit the personalized design of pro- and prebiotics targeting these populations.

Studying transcriptional regulation provides additional evidence that certain *Bifidobacterium* species have evolved to be adapted for synchronous foraging of multiple milk glycans. This notion suggests that prebiotics based on mixtures of milk glycans may stimulate bifidobacterial growth better than individual glycans. Another important aspect of using HMOs (and potentially *N*-glycans) as prebiotics requires accounting for priority effects and trophic interactions between members of the *Bifidobacterium* genus and other gut commensals. These interactions may include cross-feeding of HMO degradation products mediated by *B. bifidum* or competition with other gut bacteria that metabolize HMOs. Therefore, studies assessing the dynamics of bifidobacteria in co-culture with other gut microorganisms under HMO supplementation *in vitro* and *in vivo* are necessary to take the first steps toward personalization and clinical development of new pre- and synbiotics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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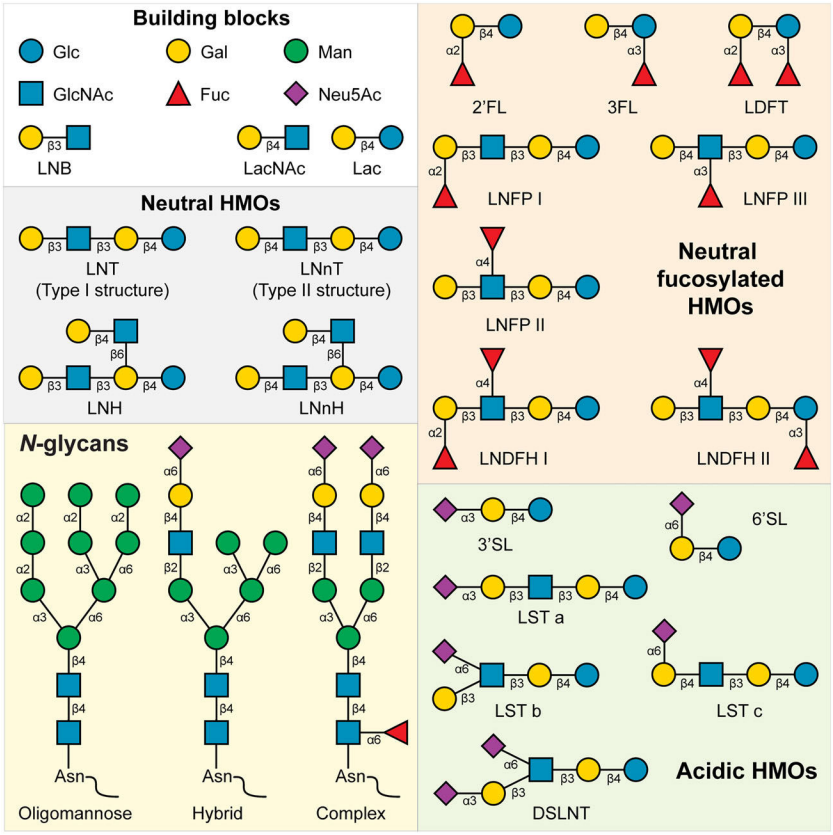

Figure 1.

Structures of representative HMOs and *N*-glycans. Glc: D-glucose; Gal: D-galactose; Man: D-mannose; GlcNAc: *N*-acetyl-D-glucosamine; Fuc: L-fucose; Neu5Ac: *N*-acetylneuraminic acid; LNB: lacto-*N*-biose; LacNAc: *N*-acetyllactosamine; Lac: lactose; LNT: lacto-*N*-tetraose; LNnT: lacto-*N*-neotetraose; LNH: lacto-*N*-hexaose; LNnH: lacto-*N*-neohexaose; 2′FL: 2′-fucosyllactose; 3FL: 3-fucosyllactose; LDFT: lactodifucotetraose; LNFP I/II/III: lacto-*N*-fucopentaose I/II/III; LNDFH I/II: lacto-*N*-difucohexaose I/II; 3′SL: 3′-sialyllactose; 6′SL: 6′-sialyllactose; LST a/b/c: sialyllacto-*N*-tetraose a/b/c; DSLNT: disialyllacto-*N*-tetraose.

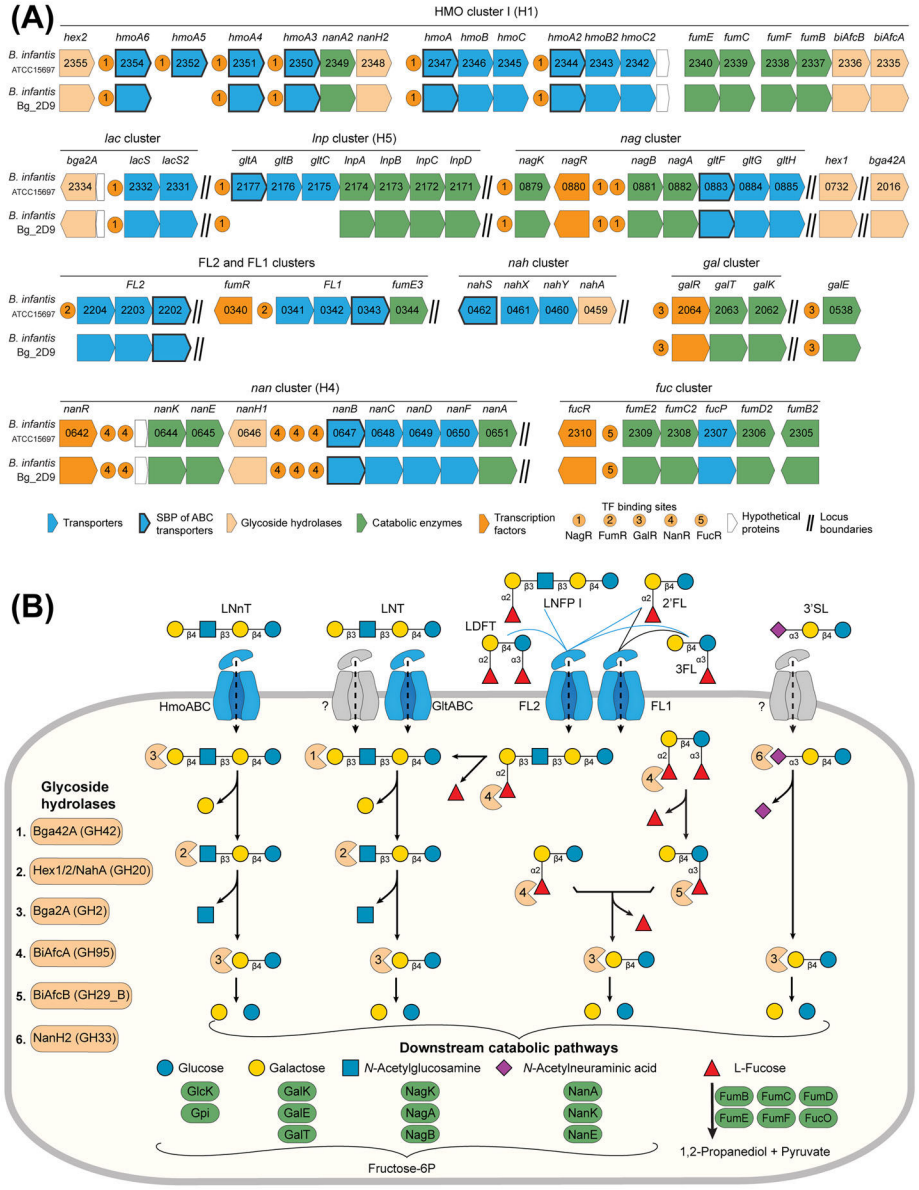

Figure 2.

HMO utilization by *B. infantis*. (A) Gene clusters involved in HMO utilization in *B. infantis*. Genes are colored according to the function of encoded proteins; gene annotations are given in Table S1. Numbers inside gene arrows represent locus tags in the Blon_XXXX format (GenBank accession no. CP001095.1). (B) Schematic representation of HMO utilization pathways in *B. infantis*. *Stage 1:* HMOs are transported into the cell by ABC (CUT1) transporters. Uncharacterized transporters whose existence can be guessed based on growth data are in grey. *Stage 2:* once inside the cell, HMOs are degraded from the non-reducing end by a coordinated action of exo-acting GHs. *Stage 3:* released monosaccharides (except Fuc) are converted to fructose-6-phosphate and enter the bifid shunt. Fuc is catabolized to 1,2-propanediol and pyruvate *via* a separate pathway (through fuconolactone). This schematic is adapted from Barratt et al. (2022).

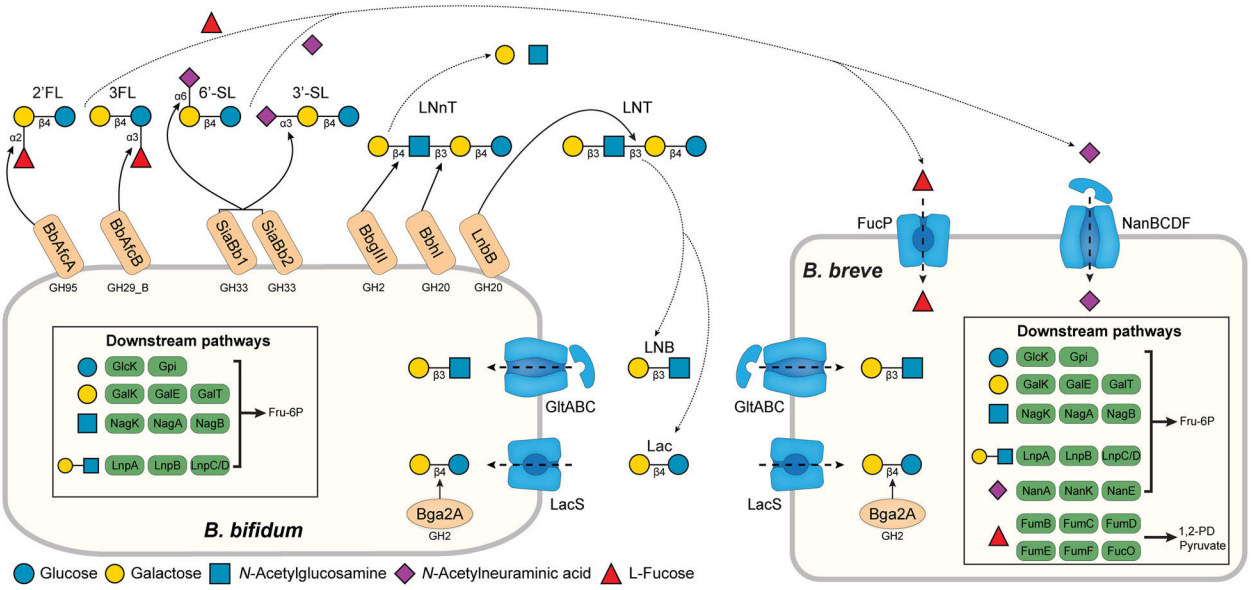

Figure 3.

HMO utilization by *B. bifidum* and cross-feeding with *B. breve*. Transporters are in blue, GHs are in orange, and downstream catabolic enzymes are in green. Annotations are given in Table S1. Solid arrows represent the activity of membrane-attached GHs toward specific glycosidic linkages. Dotted lines represent the release of specific HMO degradation products. LNB: lacto-*N*-biose; Lac: lactose; LNT: lacto-*N*-tetraose; LNnT: lacto-*N*-neotetraose, 2′FL: 2′-fucosyllactose; 3FL: 3-fucosyllactose; 3′SL: 3′-sialyllactose; 6′SL: 6′-sialyllactose; Fru-6P: fructose-6-phosphate; 1,2-PD: 1,2-propanediol.

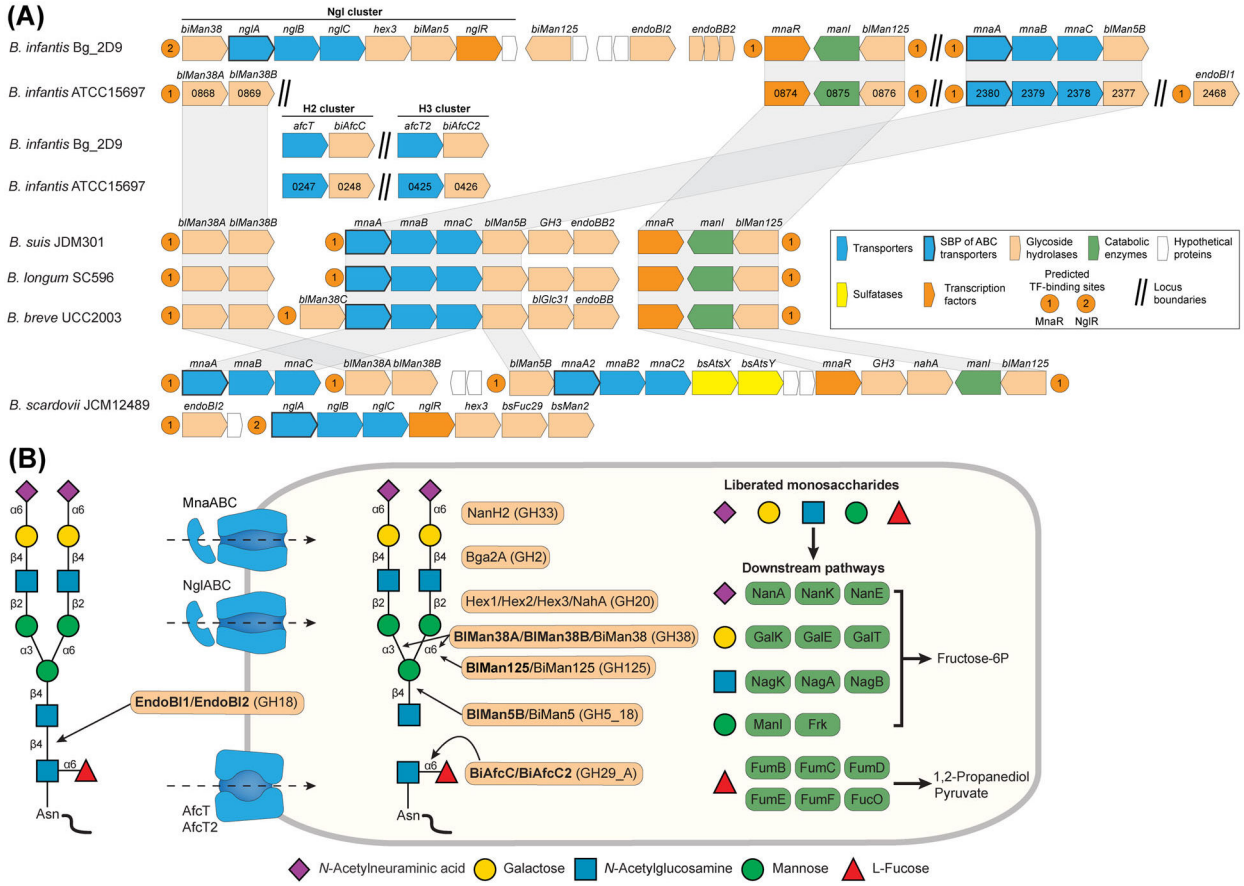

Figure 4.

N-glycan utilization by bifidobacteria. (A) Gene clusters involved in *N*-glycan utilization in *B. infantis* and other *Bifidobacterium* species. Orthologous genes and loci are marked by gray shading. Genes are colored according to the function of encoded proteins; gene annotations are given in Table S1. Numbers inside gene arrows represent locus tags in the Blon_XXXX format (GenBank accession no. CP001095.1). (B) Schematic representation of the proposed utilization pathway for complex *N*-glycans in *B. infantis*. *Stage 1:* *N*-glycans are released from glycosylated proteins by membrane-attached ENGases. *Stage 2:* the *N*-glycan moiety is imported into the cell by ABC (CUT1) transporters. Glycopeptides are imported by MFS transporters. *Stage 3:* once inside the cell, glycans are step-by-step degraded from the non-reducing end by a coordinated action of exo-acting GHs. The breakdown of glycosidic bonds is carried out by specific GHs (as shown in orange boxes). Names of enzymes whose activities toward *N*-glycans have been experimentally characterized are in bold. *Stage 4:* released monosaccharides (except Fuc) are converted to fructose-6-phosphate and enter the bifid shunt. Fuc is catabolized to 1,2-propanediol and pyruvate *via* a separate pathway (through fuconolactone). This schematic is adapted from Barratt et al. (2022).

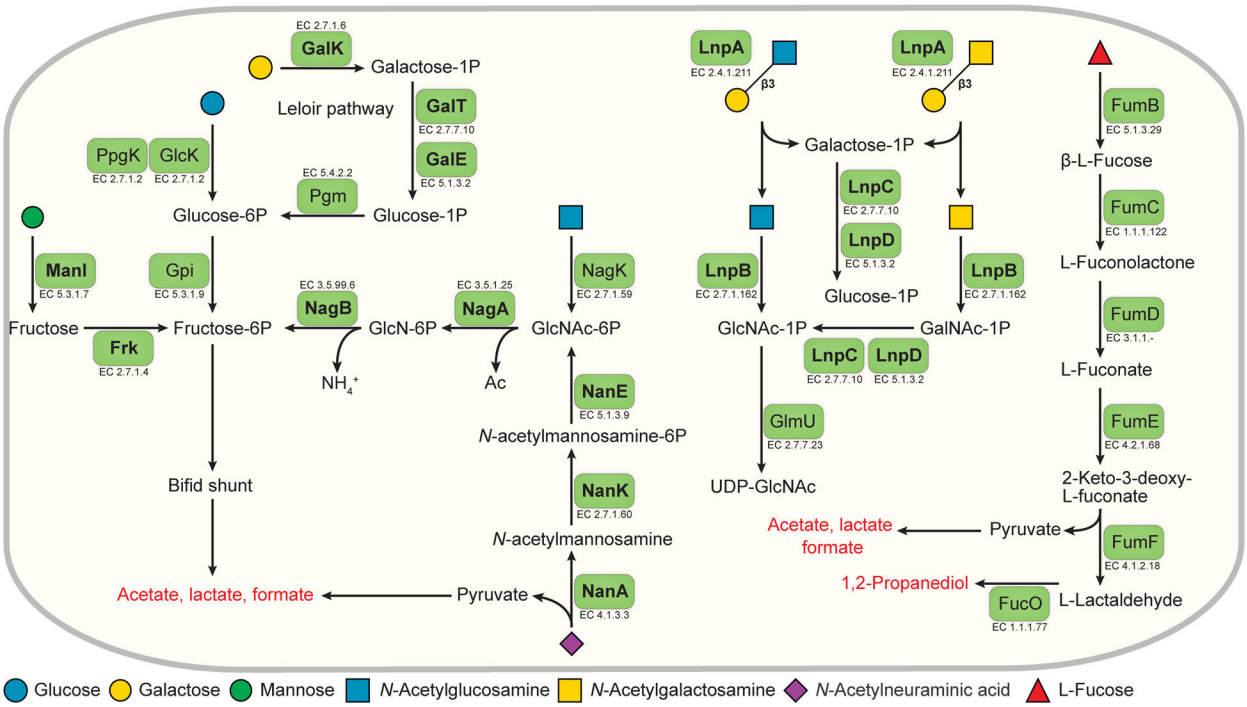

Figure 5.

Downstream catabolic pathways in bifidobacteria. Names of experimentally characterized enzymes are in bold; annotations are given in Table S1. Fermentation end products are in red.

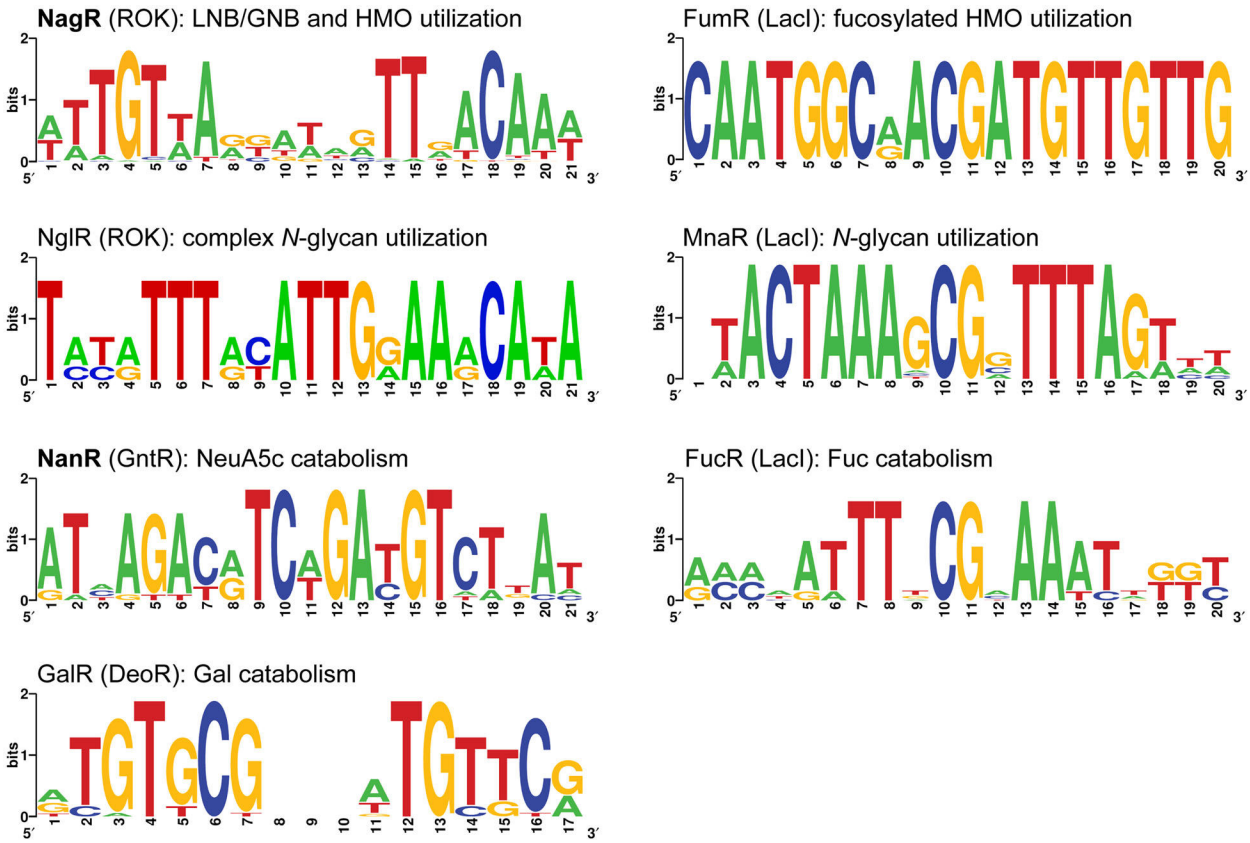

Figure 6.

Binding motifs of transcription factors that control gene clusters involved in the metabolism of HMOs, *N*-glycans, and their constituents. TF family classification is indicated in parentheses. Names of experimentally characterized TFs (Egan et al. 2015; James et al. 2018; Arzamasov et al. 2022) are in bold. Motifs were built based on operator sequences from Khoroshkin et al. (2016), Arzamasov et al. (2022), and Barratt et al. (2022).

Table 1.

List of abbreviations.

GM	Gut microbiota
HMOs	Human milk oligosaccharides
GH	Glycoside hydrolase
ENGase	Endo-β-*N*-acetylglucosaminidase
CUT1	Carbohydrate uptake transporter-1
ABC	ATP-binding cassette-type
SBP	Substrate-binding protein
FL1/FL2	Fucosyllactose ABC transporter 1/2
TF	Transcription factor
Glc	D-glucose
Glc1P	Glucose-1-phosphate
Glc6P	Glucose-6-phosphate
Gal	D-galactose
Gal1P	Galactose-1-phosphate
Man	D-mannose
Fru6P	Fructose-6-phosphate
Fuc	L-fucose
GlcNAc	*N*-acetyl-*D*-glucosamine
GlcNAc1P	*N*-acetylglucosamine-1-phosphate
GlcNAc6P	*N*-acetylglucosamine-6-phosphate
GalNAc	*N*-acetylgalactosamine
GalNAc1P	*N*-acetylgalactosamine-1-phosphate
Neu5Ac	*N*-acetylneuraminic acid
Lac	Lactose
LNB	Lacto-*N*-biose
GNB	Galacto-*N*-biose
LacNAc	*N*-acetyllactosamine
LNT	Lacto-*N*-tetraose
LNnT	Lacto-*N*-neotetraose
2’FL	2’-Fucosyllactose
3FL	3-Fucosyllactose
3’SL	3’-Sialyllactose
6’SL	6’-Sialyllactose
LNFP	Lacto-*N*-fucopentaose
LDFT	Lactodifucotetraose
LNDFH	Lacto-*N*-difucohexaose
LST	Sialyllacto-*N*-tetraose
6’FN-Asn	Fucα1–6GlcNAcβ1-Asn
1,2-PD	1,2-Propanediol