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Protein-Linked Glycan Degradation in Infants Fed Human Milk

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Abstract

Many human milk proteins are glycosylated. Glycosylation is important in protecting bioactive proteins and peptide fragments from digestion. Protein-linked glycans have a variety of functions; however, there is a paucity of information on protein-linked glycan degradation in either the infant or the adult digestive system. Human digestive enzymes can break down dietary disaccharides and starches, but most of the digestive enzymes required for complex protein-linked glycan degradation are absent from both human digestive secretions and the external brush border membrane of the intestinal lining. Indeed, complex carbohydrates remain intact throughout their transit through the stomach and small intestine, and are undegraded by *in vitro* incubation with either adult pancreatic secretions or intact intestinal brush border membranes. Human gastrointestinal bacteria, however, produce a wide variety of glycosidases with regio- and anomeric specificities matching those of protein-linked glycan structures. These bacteria degrade a wide array of complex carbohydrates including various protein-linked glycans. That bacteria possess glycan degradation capabilities, whereas the human digestive system, perse, does not, suggests that most dietary protein-linked glycan breakdown will be of bacterial origin. In addition to providing a food source for specific bacteria in the colon, protein-linked glycans from human milk may act as decoys for pathogenic bacteria to prevent invasion and infection of the host. The composition of the intestinal microbiome may be particularly important in the most vulnerable humans-the elderly, the immunocompromised, and infants (particularly premature infants).

Keywords: Glycan; Glycoprotein; Protein-linked glycan; Human milk; Infant; Bacteria; Microbiota; Digestion; Glycosidase; Prebiotic

Abbreviations: Fuc: Fucose; Gal: Galactose; GlcNAc: *N*-acetylglucosamine; GalNAc: *N*-acetylgalactosamine; NeuAc: *N*-Acetylneuraminic Acid; Glc: Glucose; Lf: Lactoferrin; HMO: Human Milk Oligosaccharides; PLG: Protein-Linked Glycans; GIT: Gastrointestinal Tract; Man: Mannose

Introduction

Many proteins in human milk are glycosylated, including lactoferrin, mucin 4, α-lactalbumin, lactadherin, κ-casein, butyrophilin, lactoperoxidase, xanthine oxidase, bile salt-stimulated lipase, α-1-antichymotrypsin, α-1-antitrypsin, a variety of immuglobulins, and at least 26 other proteins [1-3]. Protein-linked glycans (PLG) are post-translational modifications comprised of linear or branched chains of monosaccharides. For human milk PLG, these monosaccharides include hexoses (glucose, galactose and mannose), hexosamines (N-acetylglucosamine, N-acetylgalactosamine), fucoses and the sialic acid, *N*-acetylneuraminic acid.

Addition of glycan structures to milk proteins is energetically expensive to the mother [4]. Darwinian evolutionary pressure allows only components that benefit the infant to remain in milk. Therefore, these PLG likely have important, but as-of-yet undescribed, bioactive functions or nutritive value. PLG protect bioactive protein and peptide fragments from degradation and allow their continued functionality. Degradation of the glycan portion will increase protein susceptibility to proteolysis. The observation that bovine lactoferrin is more resistant to trypsin digestion when glycosylated than when unglycosylated supports this hypothesis [5].

Glycoproteins in human milk have many biological effects in the neonate, including host protection against pathogens [6-8], brain development [9], nutrient uptake [10], and immune responsiveness [11-13].

Studies of dietary PLG digestion in the human gastrointestinal tract (GIT) have not been performed in either infants or adults. The various monosaccharides comprising PLG are linked in a variety of positions and with a variety of linkage types, each of which requires a specific glycosidase (also known as glycoside hydrolases) for cleavage. Both adults and infants produce enzymes capable of hydrolyzing disaccharides and starch; however, no studies have demonstrated secreted or external brush border glycosidases specific for the bond types present in human milk PLG. Adult pancreatic juice proteomes include several glycosidases, but none with a specificity matching the individual bond structures of human milk PLG [14]. If PLG-degrading secretory or external brush border glycosidases are produced by the human digestive system, they have minimal activity, as *in vitro* studies show that neither pancreatic juices nor intact intestinal brush border membranes degrade complex carbohydrate (except for starch) [15]. Complex glycans survive intact through the human digestive system to the colon, suggesting that little, if any, degradation by human-produced digestive glycosidases occurs. This finding also suggests that bacterial PLG degradation in the upper GIT is minimal.

In contrast to the lack of PLG-degrading glycosidases produced by the human digestive system, a large number of human GIT bacterial species secrete PLG-degrading glycosidases [16-18]. Genes encoding

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PLG-degrading glycosidases are present in a variety of bacterial species [19-21]. *In vitro* studies show that intestinal bacteria degrade a wide variety of complex carbohydrates including PLG [22-26]. Indeed, complex carbohydrates serve as a major nutrient source for colonic bacteria [27].

Not all human gut bacteria degrade complex carbohydrates, and not all complex carbohydrate-degrading bacteria break down all types of complex carbohydrate bonds. Therefore, specific sugar-sugar linkage types provide a food source for some bacterial species, but not for others. Dietary glycans, therefore, potentially feed specific microbes to the exclusion of others via specific structure. This principle was demonstrated for a group of complex carbohydrates in human milk: human milk oligosaccharides (HMO). HMO=free complex carbohydrates consisting of the same monosaccharide building blocks as PLG=support the growth of particular species of gut microbiota, including *Bifidobacterium longum* subsp. *infantis* ATCC 15697, *B. longum* subsp. *longum* DJO10A, and *B. breve* ATCC 15700 [28], but do not support growth of bacteria such as *Enterococcus faecalis, Streptococcus thermophilus, Veillonella parvula, Eubacterium rectale, Clostridium difficile,* and *Escherichia coli* [22]. This degree of specific promotion of bacteria by HMO suggests a similar role for milk PLG. As both infants and adults seem to lack the ability to produce most of the specific glycosidases required for dietary PLG degradation, PLG do not likely serve a direct nutritional role to the host, rather PLG likely serve a direct nutritional role for some microbial species.

Human Milk Protein-Linked Glycan Bond Structures

A variety of glycosidic linkage types are present in human milk PLG. Glycosidic bonds occur in two stereo isomeric forms-α and β-and glycosidases are typically specific to only a single bond type [29]. Most glycosidases are specific to glycosidic bonds with particular regiochemistry (e.g. 1-3 vs. 1-4 linkage) [29]. For example, a fucose (Fuc) linked to a galactose (Gal) by an α-1,2-linkage can only be cleaved by an enzyme specific to α-1,2-linked Fuc- an α-1,2-fucosidase. Neither an α-1,4-fucosidase nor a β-1,2-fucosidase can cleave this bond. Table 1 summarizes the bond types identified to date in human milk PLG, the corresponding enzymes required for complete degradation, and the source of these enzymes if known. The structures of a relatively low number of human milk PLG are characterized to date, therefore, Table 1 is likely not an exhaustive list.

Glycosidases Produced by Infant and Adult Git

Secretory or external brush border glycosidases vs. intracellular glycosidases

This review makes a distinction between enzymes that are either exported to the gut lumen as digestive secretions or exist on the outer brush border membrane, and those that are internal to gastrointestinal tract cells. Secreted and external brush border membrane glycosidases can interact with luminal PLG, whereas intracellular glycosidases cannot interact unless PLG or PLG fragments are imported into the cell. The intracellular glycosidases of most cells likely have the capacity for complete PLG degradation, as PLG degradation is essential for remodeling glycans and degrading proteins in all eukaryotic cells [4,29]. For example, fucosidases, which are required for complete fucosylated PLG degradation, are present intracellularly in a wide range of human tissues [30], but are not found in human-produced digestive secretions [14]. Large, complex carbohydrates and glycopeptides are unlikely to undergo passive transport because of their size; therefore, their entry into cells would require specific receptor-mediated uptake. However, oligosaccharide import systems on GIT enterocytes have not been

demonstrated, therefore, intracellular glycosidases likely have minimal impact on PLG-degradation.

Many studies show that homogenized human tissues such as the pancreas, stomach, and intestine have glycosidase activity. Homogenization disrupts cell membranes, releasing intracellular glycosidases and making it difficult to distinguish between extra- and intracellular glycosidases.

Digestive glycosidases

Disaccharide-hydrolyzing glycosidases, which cleave disaccharides to monosaccharides, are present in the human digestive system [23]. For example, lactase cleaves lactose to glucose (Glc) and galactose (Gal) [23]. Human digestive secretions degrade structurally and compositionally simple polysaccharides such as starch (an all-Glc polymer) to produce monosaccharides [15]. The large amounts of intact complex carbohydrates in the distal GIT support the concept that the human digestive system does not produce complex carbohydrate-degrading enzymes. Undigested materials in the distal GIT of humans include polysaccharides (including cellulose, xylan, and pectin) from plant cell walls, undigested starch [23], HMO [31], and host-derived mucin-linked glycans and glycosphingolipids [23]. As these complex carbohydrates and human milk PLG have many of the same glycosidic bond structures, human PLG likely survive intact to the large intestine, though this has not been demonstrated.

This section reviews digestive glycosidases produced in the oral, gastric, and intestinal segments of the digestive tract and explains which glycosidase could degrade components of human milk PLG.

Oral glycosidase: At least three glycosidases are present in human saliva. These glycosidases include salivary α-amylase, α-galactosidase, and β-galactosidase. Of these, only β-galactosidase could take part in PLG degradation, but its regiospecificity is currently unknown. Blood group-degrading exoglycosidases that cleave A, H, and Lewis A (Le[a]) antigenic glycosides from non-reducing ends of glycans are present in small amounts in saliva [32].

Salivary α-amylase: Salivary α-amylase (1,4-α-D-glucan glucanohydrolase, E.C.3.2.1.1), which cleaves Glc-α-1,4-Glc bonds of starch (a pure Glc polymer), is present in both term infant and adult saliva [33]. Compared with adult levels, those in term neonate saliva are low, but reach 2/3 of adult levels by 3 months postpartum [33]. Glc-α-1,4-Glc bonds are not present in human milk PLG, so this enzyme probably does not degrade human milk PLG.

α-Galactosidase: α-Galactosidase (EC 3.2.1.22) cleaves α-linked Gal from glycans. This salivary α-galactosidase converts blood group B antigen to H antigen by removing Gal [34]. Saliva from healthy adults has activity levels of 100–300 μunits/mL [35]. Whether this α-galactosidase activity is human produced or produced by oral bacteria (both *Streptococcus mutans* and *Actinomyces* spp. have α-galactosidase activity) remains unknown [36,37]. Studies of human milk PLG structure have not identified α-linked Gal residues, however, so this enzyme may not be important in milk PLG degradation.

β-Galactosidase: β-Galactosidases (EC 3.2.1.23) cleave β-linked terminal Gal from glycans. β-galactosidase activity is present human saliva; however, the regiospecificity was not determined [38]. β-Gal is present in human milk PLG in the 1,3 and 1,4 regiomeric forms [39-43]. Therefore, this enzyme could begin human milk PLG-degradation in the oral cavity if it is regiospecific for 1,3 or 1,4 bonds.

Gastric glycosidases

Glycosidases present in homogenized gastric mucosa in-

Connectivity type	In human milk PLG?	Enzyme required	Host: secretory or external membrane?	Enzyme produced by GIT microbiota?
NeuAc-α-2,3-Gal	✓[42, 56, 84]	α-2,3-neuraminidase	✗	✓ [18, 88, 89]
NeuAc-α-2,6-Gal	✓[40, 41, 43, 58]	α-2,6-neuraminidase	✗	✓ [89]
NeuAc-α-2,6-GlcNAc	✗	α-2,6-sialidase	✗	✓ [89]
Fuc-α-1,2-Gal	✓[56, 57, 85]	α-1,2-fucosidase	✗	✓ [18, 88]
Fuc-α-1,3-Glc	✗	α-1,3-fucosidase	✗	✓ [83, 88]
Fuc-α-1,3-GlcNAc	✓[40, 41, 43, 56, 57, 84, 85]	α-1,3-fucosidase	✗	✓ [83, 88]
Fuc-α-1,4-GlcNAc	✓[56, 57, 60, 85]	α-1,4-fucosidase	✗	✓[83, 88]
Gal-β-1,3-Gal	✓[43]	β-1,3-galactosidase	✗	✓ [18]
Gal-β-1,3-GalNAc	✓[42, 56, 60, 84]	β-1,3-galactosidase	✗	✓ [18]
Gal-β-1,3-GlcNAc	✓[56, 57, 60, 85]	β-1,3-galactosidase	✗	✓ [18]
GlcNAc-β-1,3-Gal	✓[41, 56, 60]	β-1,3-N-acetylglucaminidase	✗	✓ [18]
Gal-β-1,4-Glc	✗	β-1,4-galactosidase	✗	✓ [18]
Gal-β-1,4-GlcNAc	✓[40-43, 56-60, 84, 85]	β-1,4-galactosidase	✗	✓ [18]
Fuc-α-1,6-Gal	✓[43]	α-1,6-fucosidase	✗	✗
Fuc-α-1,6-GlcNAc	✓[40, 43, 58, 59]	α-1,6-fucosidase	✗	✗
GlcNAc-β-1,2-Man	✓[40, 43, 58, 59]	β-1,2-N-acetylglucaminidase	✗	✗
GlcNAc-β-1,4-GlcNAc	✓[40, 43, 58, 59]	β-1,4-N-acetylglucaminidase	✗	✗
GlcNAc-β-1,4-Man	✓[59]	β-1,4-N-acetylglucaminidase	✗	✗
GlcNAc-β-1,6-Gal	✓[56, 60]	β-1,6-N-acetylglucaminidase	✗	✗
GlcNAc-β-1,6-GalNAc	✓[42, 56] [60, 84]	β-1,6-N-acetylglucaminidase	✗	✗
Man-α-1,3-Man	✓[40, 43, 58, 59]	α-1,3-mannosidase	✗	✗
Man-α-1,6-Man	✓[40, 43, 58, 59]	α-1,6-mannosidase	✗	✗
Man-β-1,4-GlcNAc	✓[40, 43, 58, 59]	β-1,4-mannosidase	✗	✗

Table 1: Glycan bonds in human milk protein-linked glycans, enzymes that cleave these bonds, and whether those enzymes are secreted by bacteria within the human gastrointestinal tract. X in Connectivity type means the monosaccharide linkage was unspecified in literature.

clude N-acetyl-β-glucosaminidase, N-acetyl-β-galactosaminidase, α-fucosidase, β-galactosidase, α-mannosidase, and α-glucosidase [44]. Experiments with homogenized tissue, however, cannot determine whether enzyme are on the external membrane or intracellular. These enzymes have not been identified in gastric secretions or in the external membrane of the stomach; however, neither adult nor infant gastric secretory proteomes have been determined. As large amounts of complex carbohydrates remain intact to the distal intestine, the stomach likely has little involvement in complex carbohydrate degradation.

Small intestinal luminal glycosidases

Adult pancreatic juice degrades maltodextrose (a simple polysaccharide control) but not the more complex HMO [15]. Proteomic studies demonstrate α-amylase, glucoamylase and mannosidase α-1,2 member IA, but no other glycosidases in the pancreatic juice of adults [14,45]. Pancreatic α-amylase (EC 3.2.1.1) hydrolyzes dietary starch at α-1,4-linked terminal Glc [46]. Pancreatic α-amylase does not appear in the duodenal fluid of term or preterm infants until after the first month postpartum [47]. Glucoamylase (also called maltase-glucoamylase or glucan 1,4-α-glucosidase; EC 3.2.1.3) cleaves terminal Glc-α-1,4-Glc bonds, and to some extent α-1,6 linked Glc at nonreducing ends of amylose and amylopectin, to yield Glc [48]. Therefore, this enzyme is an α-1,4 (6)-glucosidase. Mannosidase α-1,2 member IA (also known as mannosyl-oligosaccharide 1,2-α-mannosidase, EC 3.2.1.113) cleaves α-1,2-linked mannose (Man) from glycans. Milk PLG with either α-Glc, Glc-α-1,4-Glc, or α-1,2-linked Man bonds remain unidentified, so these enzymes likely have little importance to human milk PLG degradation.

Small intestinal brush-border glycosidases

Neuraminidase, cellobiase, lactase, invertase, sucrase, maltase, isomaltase, α, α-trehalase, and glucoamylase are present in homogenized brush border tissue. Whether these enzymes are present on the external brush border membrane remains unknown. The observation that adult intact intestinal brush border membranes do not degrade HMO, but degrade maltodextrose (a simple polysaccharide control) [15], suggests that basic glucosidases, but not complex carbohydrate-degrading glycosidases, are present on the external brush border. Studies with intact brush borders are needed to determine whether glycosidases are present on the external side of the membrane.

In summary, the reviewed data suggest that neither adults nor infants secrete or possess external brush border glycosidases that are required for breakdown of human milk PLG.

Glycosidases and Anti-Glycosidases in Human Milk

When discussing human milk digestion, it is important to consider the effects of enzymes delivered within the milk to the digestive system. Four glycosidases are present in intact human milk. Amylase does not degrade any of the human milk PLG structures identified to date [49]. The regiospecificities of the other three glycosidases in human milk-α-L-fucosidase [50], N-acetyl-β-D-hexosaminidase [51,52], and sialidase [53]-remain undetermined, so whether they can act on human milk PLGs is unknown. Human milk glycosidases may be soluble, located on the outer membrane of milk fat globules or milk cells, or internal to milk fat globules or cells.

α-L-Fucosidase

α-L-Fucosidases (EC 3.2.1.51) cleave α-Fuc from terminal ends of glycans [54]. Ninety percent of human milk α-L-fucosidase activity is not within cellular components such as leukocytes [50], which suggests that it is a soluble protein. Though the optimal pH for this milk α-L-fucosidase is ~pH 5.0, it maintains 73% activity at normal human milk pH (pH 7.0) [50]. α-L-Fucosidase activity in milk is highest at onset of lactation, then declines until week 2, then after week four continues to increase until day 370 of lactation [55]. However, even in milk samples incubated at body temperature for 16 h, only 170 μg/mL of free Fuc is liberated, which is approximately 5% of the available bound Fuc [51].

These findings show that this enzyme has a minimal overall effect on glycan structures in milk. Human milk PLG contain α-1,2-, 1,3-, 1,4-, and 1,6-linked Fuc [39,40,56,57]. Therefore, more than one type of fucosidase is required to cleave all human milk PLG (see Table 1). The regioselectivity of human milk α-fucosidases remains undetermined.

N-acetyl-β-D-hexosaminidase

N-acetyl-β-D-hexosaminidase (EC 3.2.1.52), which releases β-linked N-acetylglucosamine (GlcNAc) and N-acetylgalactosamine (GalNAc) from glycans, is present and active in the soluble fraction of human milk [51,52]; however, its regioselectivity remains undetermined. Activity levels of *N*-acetyl-β-D-hexosaminidase varied from 800–2,000 nmol/mL^{-h} [51]. GlcNAc-β-1,3-Gal, GlcNAc-β-1,2-Man, GlcNAc-β-1,4-GlcNAc, GlcNAc-β-1,4-Man, GlcNAc-β-1,6-Gal, and GlcNAc-β-1,6-GalNAc exist in human milk PLG [41,58-60] and could be substrates for this N-acetyl-β-D-hexosaminidase, depending on its regioselectivity.

Neuraminidase

Neuraminidases (also known as sialidases, EC 3.2.1.18) release sialic acids such as NeuAc from glycans [61]. Neuraminidase activity is present in human milk [51,53] (at ~10^{-8} units of neuraminidase per mL of milk [53]), but its regioselectivity and stereoisomeric specificity remain undetermined. Human milk PLG contain both NeuAc-α-2,3-Gal and NeuAc-α-2,6-Gal [39,56]. Therefore, this neuraminidase could be important in human milk PLG degradation, depending on its stereo isomeric and regiochemical specificity.

α-Amylase

α-Amylase (EC 3.2.1.1), which cleaves terminal α-1,4 linked Glc from starch, is present in milk of term mothers as early as the first week postpartum [49]. Milk α-amylase activity in English and Gambian mothers between 0.5 and 27 months of lactation range from .08 to 3.53 IU/mL [49]. However, there are no reports of α-1,4 linked Glc on human milk PLG. As such, this glycosidase is not likely to have major human milk PLG-degrading activity.

Anti-glycosidases

Though human milk contains antiproteases, anti-glycosidases are not known to be present in human milk.

α-1,3/4-Fucosyltransferase

α-1,3/4-fucosyltransferase attaches Fuc to glycans in an α-1,3/4 bond type. α-1,3/4-Fucosyltransferase (EC 2.4.1.65) is present and active in human milk [62-64]. The effect of this enzyme on PLG in human milk is not known.

Bacterial Degradation of Glycans in Infants

The lower GIT is poor in mono- and disaccharides, as these sugars are efficiently absorbed in the proximal small intestine via active transport. Without available mono- or disaccharides, carbohydrate-fermenting colonic bacteria must break down complex carbohydrates for nourishment [18,65-67].

Bacterial production of glycosidases

Gut bacteria must degrade complex carbohydrates to monosaccharides in order to use them as nourishment. Degradation of complex carbohydrates requires production of a variety of glycosidic linkage-specific glycosidases [20,21]. The ensemble of human gut microbes produces a wide array of glycosidases, including fucosidases [16], sialidases [16,17], N-acetyl galactosaminidases [18], and galactosidases [18] (see Table 1). Many of these glycosidases have linkage specificities matching PLG structures in human milk. Genomic studies show that carbohydrate-fermenting intestinal bacteria typically have a portion of their genomes for encoding proteins such as glycosidases involved in obtaining fuel from complex carbohydrates [20,21]. For example, in *Bifidobacterium longum* NCC2705, more than 8.5% of the total predicted proteins from the genome analysis are involved in oligo and polysaccharide degradation processes [20].

Species that can degrade all or part of PLG

Some bacteria survive on complex carbohydrates, whereas others do not. For example, *Bifidobacterium longum* subsp. *infantis*, *Bacteroides fragilis,* and *Bacteroides vulgatus* grow well with HMO as the sole carbon source, whereas *Enterococcus faecalis, Streptococcus thermophilus, Veillonella parvula, Eubacterium rectale, Clostridium difficile,* and *Escherichia coli* grow little or not at all on HMO [22].

Independent vs. cooperative complex carbohydrate degradation

Some bacterial species completely degrade a particular class of complex carbohydrates. For example, some *Bacteroides, Ruminococcus,* and *Bifidobacterium* species completely degrade mucin-linked glycans independently [25]. However, not all glycosidase-producing bacteria can produce the complete set of glycosidases necessary for degradation of a particular complex carbohydrate. Therefore, complex carbohydrate degradation sometimes requires the contributions of multiple bacterial species working together.

Bacterial degradation of complex sugars

Some *Bacteroides*, one of the most abundant among the genera in the human distal intestine, degrade a variety of polysaccharides, including xylan [68], *psyllium hydrocolloid* [69], and other plant polysaccharides [70]. *Bifidobacterium longum* subsp. *infantis* ATCC 15697, *Bifidobacterium longum* subsp. *longum* DJO10A, and *Bifidobacterium breve* ATCC 15700 all degrade and grow well with HMO as the only carbon source [28]. *Bifidobacterium* strains including *Bifidobacteria adolescentis* ATCC 15703, *Bifidobacterium breve* ATCC 15700, *Bifidobacterium longum* subsp. *infantis* ATCC 15697, and *Bifidobacterium longum* subsp. *longum* DJO10A degrade and grow on purified galacto-oligosaccharides [71].

Bacterial degradation of protein-linked glycans

Whether PLG of dietary origin are degraded is unknown; however, PLG, such as glycans attached to sloughed intestinal cell mucin, originating from the host are degraded. Host-derived glycans have the same monosaccharide components and many of the same glycosidic linkages as dietary glycans; therefore, digestion of dietary PLG by human gut microbes is likely. Human fecal cultures of anaerobic bacteria do degrade mucin PLG [24-26], and bacteria in human feces degrade both the carbohydrate and protein portions of porcine mucin [24]. Members of the *Bacteroides* genus in human feces degrade host-derived PLG such as chondroitin sulfate [72,73], mucin-linked glycans [70], hyaluronate [74], and heparin [75]. Specifically, *Bacteroides fragilis* NCDO 2217 grows well on porcine mucin [76]. *Bacteroides thetaiotamicron* and *Bacteroides ovatus* degrade ovomucoid-linked glycans from bovine submaxillary mucin [70].

External vs. internal complex sugar degradation

Bacteria can break down complex carbohydrates with either secreted glycosidases, external membrane glycosidases, or after

import of intact or glycan fragments intracellular glycosidases [28,77]. Intracellular glycosidases only act on complex carbohydrates after these carbohydrates are imported into the interior of the bacterial cell. Growth on HMO induces expression in *Bifidobacteria longum* subsp. *infantis* of specific binding proteins that are predicted to help import HMO into the intracellular space [78].

α-1,3-N-acetylgalactosaminidase: α-1,3-*N*-acetylgalactosaminidase cleaves α-1,3-linked GalNAc from glycans and, therefore, converts blood group A to blood group H by removing blood group A's GalNAc-α-1,3-Gal [18,79]. Bacterially-produced secretory α-1,3-N-acetylgalactosaminidase is present in term infant meconium [18]. Bacterial species, including *Ruminococcus torques* strains VIII-239 and IX-70 and *Ruminococcus AB* strain VI-268, secrete this enzyme [18]. This enzyme may not be important in human milk PLG degradation, however, as there is no evidence that α-1,3-linked GalNAc is present on human milk protein glycans.

β-1,3-N-acetylglucosaminidase: β-1,3-*N*-acetylglucosaminidase cleaves β-1,3-linked GlcNAc from glycans. Bacterially-produced secretory β-1,3-*N*-acetylglucosaminidase is present in term infant meconium [18], and *Ruminococcus torques*, *Bifidobacterium bifidum* and *Bifidobacterium infantis* all secrete this enzyme [18]. *B. bifidum* have membrane-bound external β-1,3-*N*-acetylglucosaminidases that were shown to cleave the terminal β-1,3-GlcNac from lacto-N-triose II leaving lactose [80]. Fecal bacteria growing on porcine mucin produced extracellular β-*N*-acetylglucosaminidase activity [26]; however, the regiospecificity of the enzyme is not known. *Bacteroides fragilis* NCDO 2217 produces β-*N*-acetyl glucosaminidase, but secretes only trace amounts, with the rest being cell-bound. Whether the cell-bound activity is exterior or interior is not known, nor is the regiospecificity of the enzyme known [76]. GlcNAc-β-1,3-Gal is present in human milk PLG [41,56,60], so β-1,3-*N*-acetylglucosaminidase will be important in human milk PLG degradation.

α-1,3-Galactosidase: α-1,3-Galactosidase cleaves α-1,3-linked Gal from glycans and, therefore, can convert blood group B glycan epitopes to blood group H by removing the α-1,3-linked D-Gal from the Gal [18,79,81,82]. In term infant meconium, *Ruminococcus AB* strain VI-268 and *Ruminococcus gnavus* secrete α-1,3-galactosidase [18]. However, neither blood group B nor α-1,3 linked Gal is known to be in human milk PLG, so this enzyme may not be important for human milk PLG degradation.

β-1,3-Galactosidase: β-1,3-Galactosidase cleaves β-1,3-linked Gal from glycans. β-1,3-galactosidase is secreted by *R. torques* and *B. bifidum* in term infant meconium [18]. However, a later study of *B. bifidum* suggests β-1,3-Galactosidase is not present in secreted or on the external membrane [83]. β-1,3-linked Gal exists on human milk PLGs attached to Gal [43], GalNAc [42,56,60,84], and GlcNAc [56,57,60,85]. Therefore, this enzyme may have a large impact on human milk PLG degradation.

β-1,4-Galactosidase: β-1,4-Galactosidase cleaves β-1,4-linked Gal from glycans. Two *R. torques* strains in term infant meconium secrete β-1,4-galactosidase [18]. *B. bifidum* has external membrane β-1,4-Galactosidase which can cleave the terminal β-1,4-Gal from lacto-N-neotetraose, releasing lacto-N-triose II [80]. *B. longum* subsp. *infantis* ATCC 15697 has genes for β-galactosidases, but it is not known whether this enzyme is secreted or external, and its regiospecificity is unknown [86]. Fecal bacteria growing on porcine mucin produce secretory β-D-galactosidase [26], but−the regiospecificity is unknown. *Bacteroides fragilis* NCDO 2217 produces trace levels of secretory β-galactosidase and has cell-bound activity, but it is not known whether this activity

is internal or external nor is its regiospecificity known [76]. Gal-β-1,4-GlcNAc is present in human milk [40-43,56-60,84,85]; therefore, bacterially-produced β-1,4-galactosidase will likely impact human milk PLG degradation.

α-1,2-Fucosidase: α-1,2-Fucosidase cleaves α-1,2-linked Fuc from glycans (EC 3.2.1.63). In term infant meconium, *Bifidobacterium bifidum*, *Bifidobacterium infantis*, *Ruminococcus torques* strains VIII-239 and IX-70, and *Ruminococcus AB* strain VI-268 secrete active α-1,2-fucosidase [18]. Lewis blood group B (Le^b) has an α-1,2-linked Fuc attached to a Gal. α-1,2-fucosidase could, therefore, degrade Le^b blood group glycans, converting them to Le^a. α-1,2-fucosidase degrades degrade Blood group H [18] and should be able to degrade A and B blood group epitopes, as each has an α-1,2 Fuc linked to a Gal. Fuc-α-1,2-Gal is present on human milk PLG [56,57,85], so bacterially-produced α-1,2-fucosidase will likely impact human milk PLG degradation.

α-1,4-Fucosidase: α-1,4-Fucosidase cleaves α-1,4-linked Fuc from glycans. Several strains of *Bifidobacterium* and *Ruminococcus* secrete α-1,4-fucosidase in term infant meconium [18]. *B. bifidum* has genes encoding α-1,3/4-L-fucosidase but whether this enzyme is secretory or external is unknown [83]. *B. longum* subsp. *infantis* ATCC15697 also produces α-1,3/4-fucosidases [87]. Le^a and Le^b glycan epitopes have α-1,3/4-linked Fuc to GlcNAc [79] (see Figure 1). Therefore, α-1,4-fucosidase can degrade α-1,4 linked Le^a and Le^b blood groups [79]. Fuc-α-1,4-GlcNAc is found in human milk PLGs [56,57,60,85], so bacterially-produced α-1,4-fucosidase may impact milk PLG degradation.

Neuraminidase: Neuraminidases (EC 3.2.1.18) cleave terminal NeuAc from glycans. *Ruminococcus torques* IX-70 and VIII-239, *Ruminococcus gnavus* VI-268, *Bifidobacterium bifidum* VIII-210, and *Bifidobacterium infantis* VIII-240 isolated from adult feces secrete sialidase when grown on hog gastric mucin, but the anomeric and regiochemical specificity remain unknown [17,72,73]. *B. bifidum* JCM 1254 has genes encoding sialidases; however, neither linkage specificity nor whether the enzyme is secreted or external is known [83]. Human milk PLG contain terminal NeuAc as both α-2,3-NeuAc [42] and α-2,6-NeuAc [59], so this enzyme may impact human milk PLG degradation, depending on its bond specificity.

α-2,3-Neuraminidase: α-2,3-Neuraminidase cleaves α-2,3-NeuAc from glycans. Two *Bifidobacterium* and three *Ruminococcus* strains in term infant meconium secrete active α-2,3-neuraminidase [18]. *Bifidobacterium bifidum* JCM1254 has a α-2,3-neuraminidase gene that has a C-terminal transmembrane region, which suggests this is an extracellular membrane-anchored protein [88]. *B. longum* subsp. *infantis*, commonly found in human breastfed infants, has genes for α-2,3-sialidases; however, this enzyme is not likely secreted nor on the external membrane because it lacks an export signal, transmembrane domain or cell wall anchor motif [89]. α-2,3-sialidase can likely degrade Sialyl Le^x (SLe^x) and Sialyl Le^a (SLe^a) blood groups' α-2,3-linked sialic acids, converting them to Le^x and Le^a, respectively. NeuAc-α-2,3-Gal is found in human milk PLG [42,56,84], therefore this enzyme may be important in human milk PLG degradation.

α-2,6-Neuraminidase: α-2,6-Neuraminidase (also known as α-2-6-sialidase) cleaves α-2,6-linked NeuAc from glycans. *B. longum* subsp. *infantis* ATCC15697 has genes for α-2-6-sialidases [89], but this enzyme is likely intracellular as its gene sequence lacks an identifiable export signal, transmembrane domain, or cell wall anchor motif [89]. Whether any GIT bacterial species secretes or produces external membrane α-2,6-neuraminidase is unknown. α-2,6-NeuAc linked to

Gal is found in human milk PLG [39-41,59]; therefore, α-2,6-sialidase is requisite for complete human milk PLG degradation.

Endo-α-N-acetylgalactosaminidase: Endo-α-*N*-acetylgalactosaminidase (also called endo-α-GalNAcase and glycopeptide α-*N*-acetylgalactosaminidase; EC 3.2.1.97) cleaves *O*-linked Gal-β-1,3-GalNAc (*O*-linked glycan core 1) between Ser/Thr the α-GalNAc [90]. Bacteria, including *Alcaligenes* sp., *Bacillus* sp. [91], *Clostridium perfringens* [92], *Streptococcus pneumoniae* [93-97], and various bifidobacterial strains such as *B. longum*, *B. bifidum*, and *B. breve* [90] secrete endo-α-*N*-acetylgalactosaminidase. Core 1 type *O*-linked glycans have not yet been found in human milk, so it is not known whether this enzyme will impact human milk PLG degradation.

Endo-β-N-acetylglucosaminidase: Endo-β-*N*-acetylglucosaminidase (Endo-β-GlcNAcase) cleaves di-*N*-acetylchitobiose linkages in oligosaccharides and glycoproteins [98]. Bacteria in infant meconium secrete active endo-β-*N*-acetylglucosaminidase [18]. *Bifidobacteria longum* NCC2705 has genes for endo-β-*N*-acetylglucosaminidase, but whether it is secreted or external is unknown [83].

Lacto-N-biosidase: Lacto-*N*-biosidase cleaves lacto-*N*-tetraose (LNT, Gal-β-1,3-GlcNac- β-1,3-Gal- β-1,4-Glc, a major HMO) at the GlcNAc- β-1,3-Gal bond to release the disaccharide Gal-β-1,3-GlcNAc (lacto-*N*-biose; LNB) and lactose [83]. This enzyme cleaves neither fucosylated LNT (lacto-*N*-fucopentaose I and II) nor lacto-*N*-neotetraose (including type II chain) [83]. Some *Bifidobacterium* species possess genes for a lacto-*N*-biosidase with a membrane anchor sequence, suggesting that it is externally active [83,99]. Gal-β-1,3-GlcNAc is present in human milk PLG [56,60,85,90]; however, there is a lack of data showing lacto-*N*-tetraose-like structures in human milk PLG; therefore, this enzyme probably has little importance for PLG breakdown.

Enzymes essential for PLG breakdown that GIT bacteria lack

Enzymes needed for complete PLG degradation, but not yet known to be produced by human gastrointestinal tract bacterial species in external or secreted forms, include α-2,6-neuraminidase, α-1,6-fucosidase, β-1,2-*N*-acetylglucaminidase, β-1,4-*N*-acetylglucaminidase, β-1,6-*N*-acetylglucaminidase, α-1,3-mannosidase, α-1,6-mannosidase, and β-1,4-mannosidase (Table 1). The secreted glycosidases of human intestinal micro flora are incompletely studied, however, so whether some or all of these enzymes are secreted is unknown.

Bifidobacterium longum subsp. *infantis*

Bifidobacterium longum subsp. *infantis* produces α-2,6 and α-2,3 sialidases [89], α-1,2 and α-1,3/4 fucosidases [28,87], and β-galactosidase [19]. This bacterium survives well on HMO and consumes small mass oligosaccharides [28]. The *B. longum* subsp. *infantis* ATCC 15697 genome has five gene clusters with capacity for binding, cleaving, and importing human milk oligosaccharides [19]. Studies suggest, but have not proven, that *B. infantis* can import oligosaccharides inside the bacterial cell.

Development of microbial composition in the neonate

Neonatal gastrointestinal systems are thought to be sterile at birth and colonized during or shortly after birth by maternal and environmental microbes. For infants delivered vaginally, the heavily colonized vaginal canal (typically dominated by *Lactobacillus* and contains *Prevotella* at the time of delivery) provides the source of inoculation of the newborn's gastrointestinal tract and skin surfaces [100,101]. Subsequent to birth, full-term vaginally-delivered neonates are colonized by facultative anaerobes (e.g. *Escherichia* and

Streptococcus spp). Shortly after birth, bifidobacteria are often observed in prominence in the breast-fed infant gut [102]. Data suggest that bifidobacteria are enriched by the uncommon ability to utilize the relatively abundant HMO in mother's milk [19]. These HMO are thought to be partially responsible for the difference between the infant and adult microbial community structure [103]. With the initiation of weaning, the diversity of the obligate anaerobes (e.g. *Bacteroides* and *Clostridium* species) increase [104,105]. Introduction of solid foods to the diet and termination of breastfeeding induces major remodeling of the infant microbial community [102]. Only after weaning does the infant develop the differentiated microbial communities present in adults [106]. By the first year of life, the infant gut microbiota establishes an adult-like composition (i.e. domination by *Firmicutes* and *Bacteroides*), although phylotypic representation appears to vary across individual infants [107].

Whereas the full-term neonate is rapidly colonized by a diverse array of microorganisms, preterm infants exhibit a dissimilar profile, including delayed colonization, less diverse phylogenetic representation, and greater susceptibility to challenges by pathogenic bacteria [108].

The gut microbiota is intimately involved in biological processes that benefit the host over its lifespan. This is particularly evident with the persistent impact on host health throughout infancy. For example, gut microbiota erect a barrier to shield their infant hosts from pathogens, and they activate immunological components that promote the development and regulation of the nascent immune system [109].

Extent of Bacterial Degradation of Complex Carbohydrates

Though little HMO digestion occurs in the upper intestine, extensive hydrolysis likely occurs in the lower intestine/colon, as only about 8% of ingested HMO are found in the infant feces [110]. HMO are abundant in human milk, this lack of intact HMO in stool samples suggests that bacterial digestion of HMO is high. The extent of bacterial digestion of milk PLG breakdown remains unknown.

Bioactive Potential of Intact Glycans in Human Milk

Incomplete digestion of human milk PLG in the small intestine may have beneficial consequences as they may act in the infant gut as prebiotics for specific bacteria, decoys for specific pathogens or by other unknown biological mechanisms.

Specific prebiotic

Human milk PLG may be prebiotic by enhancing the growth of particular complex carbohydrate-metabolizing bacteria. Specific glycan structures may shape the proportions and types of microbes in the infant gut. Shaping the microbiome can have biological advantage; for example, the presence of *Bifidobacteria* spp. in the gut is associated with reduced incidence of diarrheal illnesses, improved lactose digestion, and enhanced immunomodulatory functions [111]. Some strains of gut bacteria produce vitamins K and B$_{12}$, and short-chain fatty acids [112]. Growth of commensal bacteria can also competitively inhibit colonization by pathogenic bacteria [113].

Structural decoy

Human milk PLG may serve a role in host protection against pathogens via immune exclusion [6-8,114-116]. Often, the binding of pathogens to enterocytes is facilitated when receptor proteins on the

pathogen interact with specific glycan motifs on the external membrane of the enterocyte. Once bound, pathogens can invade enterocytes, a process required for bacteria to produce an infection [86,117]. HMO bind pathogens and prevent their adherence to and invasion of cells lining the gastrointestinal, urogenital, and respiratory tracts [115,118-121]. Human milk α-1,2-fucosyloligosaccharides inhibit *Escherichia coli in vitro*, and the secretory diarrhea induced by its toxins both *in vivo* and *in vitro* [122]. Provision of α-1,2 fucosylated HMO to infants reduced occurrences of *Campylobacter*-induced diarrhea [123,124]. Sialylated milk glycoproteins neutralize infectious particles such as rotavirus [125]. By preventing binding to the enterocytes, decoy glycan-pathogen interaction facilitates expulsion of microbes into the feces [126].

Other bioactivities

Human milk glycoproteins have biological functions in the neonate related to brain development [9], nutrient uptake [10], and immune responsiveness [11-13]. The part of this bioactivity due to the glycan component of these glycoproteins is unknown. PLG can bind to glycan binding proteins and mediate cell-cell interactions, extracellular molecule recognition, and cellular recognition [127]. For example, selectins, a type of adhesion molecule, bind glycans and mediate interactions between blood cells and vascular cells [127]. Glycosylation of a protein can also act as a switch to turn a protein's activity on or off, and as a modulator of protein activity [127]. For example, deglycosylated beta-human chorionic gonadotropin hormone binds to its receptor with affinity similar to that of the glycosylated form, but it fails to stimulate adenylate cyclase activity [127].

Conclusion

Human milk proteins are widely glycosylated [1-3]. Increasing resistance to proteolytic degradation may be an important role of glycosylation as it could allow bioactive proteins and peptides to remain intact for later exertion of bioactive effect. PLG may exert biological functions through interaction with receptors such as lectins, by nourishing specific bacterial species or by competitively inhibiting pathogen binding to epithelial glycans.

Both adults and infants can produce an array of enzymes to degrade disaccharides and starches, but neither produces glycosidases in digestive secretions nor in the external brush border membrane capable of breaking down the specific bonds in PLG. Indeed, *in vitro* and *in vivo* studies suggest that little to no degradation of complex carbohydrates, including PLG, occurs in the stomach or small intestine. The lack of complex carbohydrate-specific enzymes produced in the human digestive system and the abundance of these enzymes produced by colonic bacteria suggest that most degradation of complex carbohydrates in the digestive system results from bacterial fermentation. Differences in bacterial populations between term and preterm infants may alter degradation of human milk PLG and change bioactivity of the glycopeptide or glycoprotein.

The particular make-up of the intestinal microbiome has major health effects. Particular intestinal microbial compositions are linked to necrotizing enterocolitis [128], a common devastating disease of premature infants. Attempts to alter the composition of the intestinal microbiota with probiotic supplements and/or human milk feeding are promising in these very high risk infants [129,130].

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