



Molecular Insights Into O-Linked Glycan Utilization by Gut Microbes

Kevin J. González-Morelo, Marco Vega-Sagardía and Daniel Garrido*

Department of Chemical and Bioprocess Engineering, School of Engineering, Pontificia Universidad Católica de Chile, Santiago, Chile

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*Correspondence:

Daniel Garrido
dgarridoc@ing.puc.cl

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O-linked glycosylation is a post-translational modification found mainly in eukaryotic cells, which covalently attaches oligosaccharides to secreted proteins in certain threonine or serine residues. Most of O-glycans have *N*-acetylgalactosamine (GalNAc) as a common core. Several glycoproteins, such as mucins (MUCs), immunoglobulins, and caseins are examples of O-glycosylated structures. These glycans are further elongated with other monosaccharides and sulfate groups. Some of them could be found in dairy foods, while others are produced endogenously, in both cases interacting with the gut microbiota. Interestingly, certain gut microbes can access, release, and consume O-linked glycans as a carbon source. Among these, *Akkermansia muciniphila*, *Bifidobacterium bifidum*, and *Bacteroides thetaiotaomicron* are prominent O-linked glycan utilizers. Their consumption strategies include specialized α -fucosidases and α -sialidases, in addition to endo- α -*N*-acetylgalactosaminidases that release galacto-*N*-biose (GNB) from peptides backbones. O-linked glycan utilization by certain gut microbes represents an important niche that allows them to predominate and modulate host responses such as inflammation. Here, we focus on the distinct molecular mechanisms of consumption of O-linked GalNAc glycans by prominent gut microbes, especially from mucin and casein glycomacropptide (GMP), highlighting the potential of these structures as emerging prebiotics.

Keywords: glycans, prebiotics, GMP, mucin, microbiota, glycoprotein

INTRODUCTION

There is a great interest regarding the impact and modulation of the gut microbiota through our diet. Among several dietary interventions, consumption of fibers and prebiotics has mainly been considered as positive for our health (Sawicki et al., 2017). A recent definition of prebiotic corresponds to a substrate that is selectively utilized by host microorganisms conferring a health benefit (Gibson et al., 2017). These benefits include a reduced microbial load of pathogens (Gibson et al., 2005), stimulation of the immune system (Shokryazdan et al., 2017), and lower allergy rates (Brosseau et al., 2019). These effects are attributed in part to the ability of prebiotics to be fermented by healthy microorganisms and stimulate the production of certain short-chain fatty acids (SCFA), especially butyrate (Rivière et al., 2016).

Traditionally, carbohydrates such as inulin and fructo-oligosaccharides (FOS) have been studied as prebiotics (Vandeputte et al., 2017; Wilson and Whelan, 2017; BeMiller, 2019).

In general, these plant-derived prebiotics have a simple structure containing one monosaccharide and repeats of one linkage. In contrast, host-derived glycans such as human milk oligosaccharides (HMO) and those found in glycoproteins, are structurally more complex and thought to be more suitable and selective toward beneficial members of the gut microbiota. HMO are complex glycans that promote the growth and activity of beneficial gut microbes such as infant-gut associated bifidobacteria, among other effects (Thompson et al., 2018). Several advances have been made to synthesize HMO at the industrial level, and they are currently being added as functional ingredients to infant formula (Puccio et al., 2017).

Besides, some human and bovine milk proteins possess similar glycans to HMO in their structures. Glycosylation is a post-translational modification where oligosaccharides are covalently bound to asparagine (*N*-glycans), or serine or threonine (Ser/Thr; *O*-glycans; Vliegenthart, 2017). These glycans serve as signaling molecules for secretion and other cellular processes, providing increased resistance to proteolysis (Baum and Cobb, 2016). These glycans are being proposed as emerging prebiotics due to their similarity to host-derived glycans compared to plant-derived prebiotics and their enrichment of dominant and health-promoting gut microbes, such as *Bacteroides* spp. (*Ba.*), *Bifidobacterium* spp. (*Bi.*), and *Akkermansia muciniphila* (Bergstrom and Xia, 2013; Kirmiz et al., 2018). Several molecular adaptations for accessing and consuming *N*- and *O*-glycans have been described in gut microorganisms, indicating that the gut microbiota is quite adapted for metabolizing these oligosaccharides. Importantly, some of these bioactive glycans could be also be found in dairy streams, warranting a wide availability for potential prebiotic use for the food industry.

N-linked glycans are complex oligosaccharides that possess *N*-Acetylglucosamine (GlcNAc) as a common core, conjugated with an additional GlcNAc and three mannose (Man) residues, forming a Man₃GlcNAc₂ motif found in all *N*-glycans. *N*-glycans could be further modified by extensive mannosylation (high-mannose *N*-glycans) or by lactosamine chains (Galβ1-4GlcNAc; complex *N*-glycans). Terminal fucose (Fuc) and *N*-acetylneuraminic acid (NeuAc) modifications are commonly added to complex *N*-glycans (Stanley et al., 2017). *N*-glycans are characteristic of immunoglobulins and lactoferrin in milk (Le Parc et al., 2015; Davis et al., 2016). *N*-glycans are an example of host-derived oligosaccharides that can be used by beneficial microbes. *Bifidobacterium longum* subsp. *infantis* ATCC 15697 is a dominant beneficial infant gut microbe that has been shown to access *N*-glycans *in vitro* by a specialized endo-β-*N*-acetylglucosaminidase (Garrido et al., 2012b). It also shows vigorous growth *in vitro* using *N*-glycans (Karav et al., 2016). The released *N*-glycans from milk glycoproteins such as lactoferrin and immunoglobulins have been well characterized (Le Parc et al., 2015). These results, while promising, are yet to be tested *in vivo* for claiming any prebiotic effect.

Less attention has been paid to *O*-linked glycans. These are characterized by an *N*-acetylgalactosamine (GalNAc) residue linked to Ser/Thr as a common core. GalNAc is usually bound to Galβ1-3, forming galacto-*N*-biose (GNB) as a building block in Core 1 and Core 2, which could be further extended forming

larger chains similar to *N*-glycans. *O*-linked glycans are a significant component of mucins (MUCs; **Figure 1**), also found in immunoglobulins and κ-caseins (Magnelli et al., 2011). *O*-glycosylated proteins have a higher resistance to proteolysis (Boutrou et al., 2008; Kesimer and Sheehan, 2012), probably reaching lower sections of the gut and interacting the gut microbiota. Therefore, it is not surprising that gut microbes have evolved strategies to utilize *O*-linked glycans as a carbon source.

Prominent gut microbes such as *A. muciniphila*, *Bifidobacterium bifidum*, and *Bacteroides thetaiotaomicron* are representative species capable of *O*-linked glycan consumption. However, the mechanisms involved and the consequences of this process for the host are not fully known. This review aims to summarize and discuss current research regarding the structures of *O*-linked glycans, their potential prebiotic activity, and consumption by gut microorganisms.

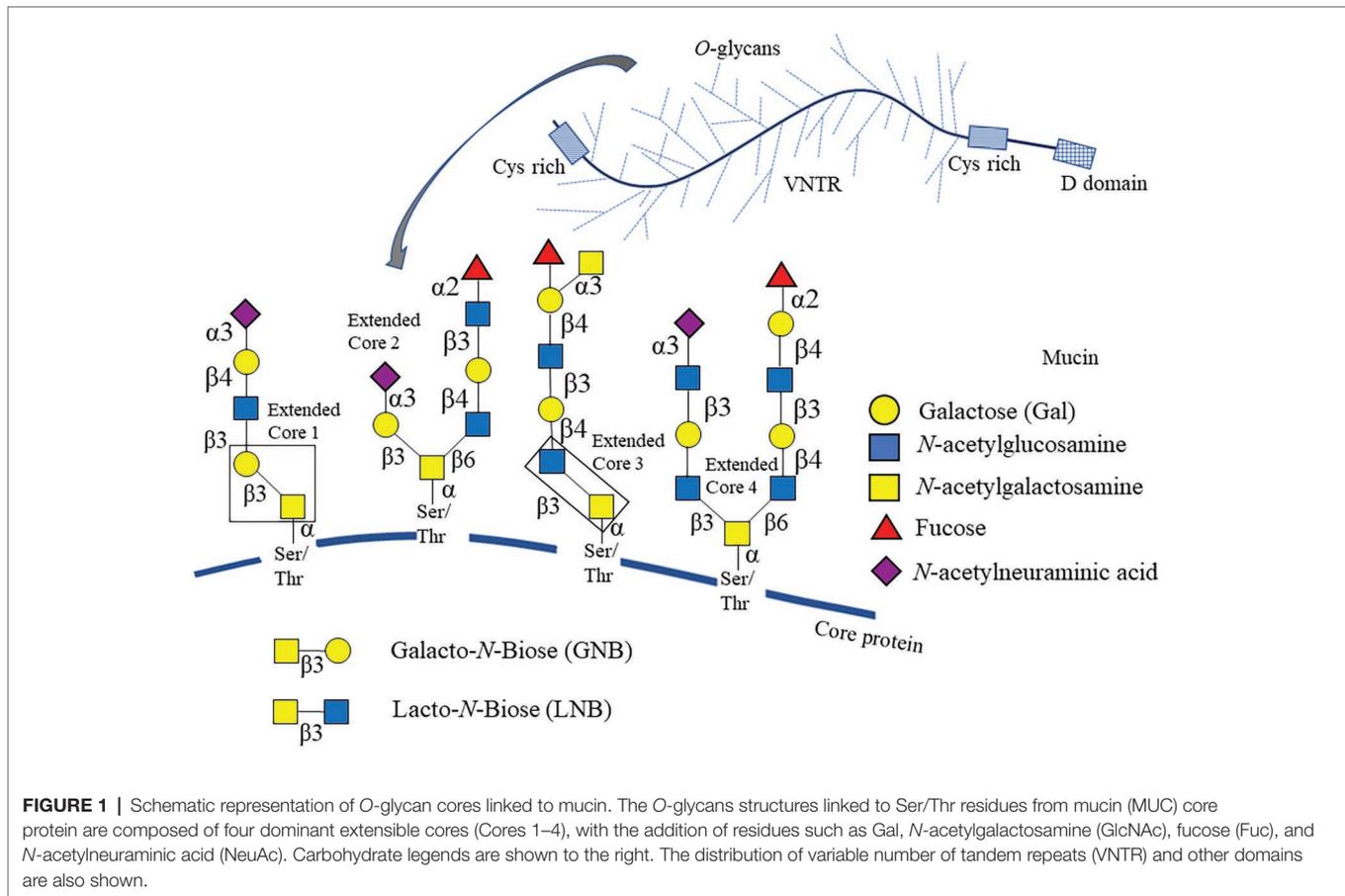
STRUCTURES OF O-LINKED GLYCANS

The elongation of *O*-linked glycans and their attachment to secreted proteins begins in the Golgi apparatus (Brockhausen and Stanley, 2017). A polypeptide-*N*-acetylgalactosaminyltransferase (ppGalNAcT) catalyzes the addition of a GalNAcα1 to a Ser/Thr available as a glycosylation site (Varki and Lowe, 2009). Several glycosyltransferases act in conjunction to attach sugar residues in single *O*-glycans. The Core 1 *O*-glycan (Galβ1-3GalNAcα1-Ser/Thr) is the first synthesized, and then the Core 2 unit [Galβ1-3(GlcNAcβ1,6)GalNAcα1-Ser/Thr; Itano, 2019].

Here, we will focus on two model glycoproteins containing *O*-glycans: MUC and glycomacropptide (GMP). The wide availability of mucins from animal sources makes them reference glycoproteins for the study of their contribution to host processes and their interaction with the gut microbiota. Similarly, GMP is a glycopeptide derived from cheesemaking, available in large quantities. It contains neutral and acidic *O*-glycans, which are shorter and simpler compared to mucin *O*-glycans. Both types of glycans have been shown to interact with the gut microbiota and could be considered promising prebiotics considering their stimulation of beneficial gut microbes.

O-Linked Glycans From Mucin

Mucins are highly glycosylated (up to 90%), high-molecular-weight (200 kDa–200 MDa), and large (Rg 10–300 nm) extracellular glycoproteins that serve as a dense barrier between the intestinal lumen and epithelium (Kesimer and Sheehan, 2012). They are generally found in epithelial tissues in the gastrointestinal tract and certain secretions. They provide a crucial role in forming a physicochemical barrier against the luminal compartment through their gel-forming properties (Bansil and Turner, 2018). Interestingly, mucin serves as a scaffold for the attachment and colonization by certain microorganisms (Ringot-Destrez et al., 2017). Two different mucus layers have been identified: an outer mucus layer (containing 10⁶ microbial cells/g) and a tight inner mucus layer (10⁵ microbial cells/g; Atuma et al., 2001). Mucins can be found in different parts of the human body: kidneys



and bladder, respiratory and urinary tracts, among others (Atuma et al., 2001). They are produced by goblet cells, specialized secretory cells found in the epithelial layer (Lamacchia et al., 2018).

There are at least 20 different MUC genes, whose products are classified into two families: secreted and transmembrane mucins (Corfield, 2015; Dhanisha et al., 2018). Secreted mucins can be small and soluble, or large gel-forming mucins. The latter are able to cross-link, forming complex networks and contributing to the viscosity of mucins. MUC5AC is among the most studied mucin being secreted by epithelial cells in the stomach, while MUC6 is secreted in the deep gastric glands in the stomach and ileum (Magalhães et al., 2016). MUC2 is the most abundant secreted mucin in the small intestine and the colon (Corfield, 2000; Arike et al., 2017). Transmembrane mucins participate primarily in cellular adhesion, while secreted mucins are responsible for viscoelasticity for both the inner and outer mucus layers (Lillehoj et al., 2013; Demouveau et al., 2018). All these differences between secreted and membrane-bound mucins contribute to the dynamic properties of the mucus layer across the GI tract.

Mucins could also be found in secretions and milk. MUC1 is a transmembrane mucin expressed on the apical surface of most epithelial cells (Ross et al., 2015). This type of mucin can be found in breast milk, which is transferred to the milk fat globule membrane upon its secretion from the cells.

MUC15 is also a mucin-type isolated from bovine milk fat globule membranes (Bernard et al., 2018). The function of milk mucins has not been extensively studied, but it is suggested to be mostly structural (Donovan, 2019).

Eight cores of O-glycan structures have been identified in mucins, four of them being the most predominant (Core 1–4; **Figure 1**). These O-glycans are found in the region known as the variable number of tandem repeats (VNTR; Arike and Hansson, 2016). The VNTR section is rich in Ser/Thr, which can make up to 80% of the weight of the mucin. In MUC2, VNTRs are accompanied by two cysteine-rich regions at their ends and a D domain involved in mucin polymerization (Arike and Hansson, 2016). Depending on the tissue, MUC5AC and MUC6 could be glycosylated with predominant Core 1–2 structures (Jin et al., 2017). The O-linked glycans of colonic MUC2 are predominantly Core 1–3 structures (Bergstrom et al., 2017).

Mucin O-glycans can be either branched or linear in structure, depending on their Core (e.g., Core 1 glycans are linear, and Core 2 glycans are branched; Podolsky, 1985; Li and Chai, 2019). O-glycans have been found to contain up to 20 residues and may include blood group determinants of ABO, Lewis groups, and glycan epitopes such as the linear antigen *i* (Gal β 1-4GlcNAc β 1-3Gal; Vliegthart, 2017). Fuc and NeuAc are monosaccharides decorating O-glycans in terminal α -linkages, and usually mucins contain sulfate groups. The latter two confer

a negative charge on the mucin structure, crucial for selective mucin permeability and its rheological properties (De Weirdt and Van de Wiele, 2015). An acidic gradient, based on increasing amounts of NeuAc in mucins, has been shown in the gastrointestinal tract (Robbe et al., 2003).

Several studies have shown how alterations in mucin O-glycosylation patterns participate in disease. In cystic fibrosis patients, the respiratory epithelium shows higher degradation rates for MUC5B and MUC5AC, in addition to reduced sulfation, higher sialylation, and lower fucosylation (Schulz et al., 2007). This disease is characterized by chronic pulmonary infection and severe inflammation, and the changes mentioned above could be used as biomarkers. In the stomach, a broad diversity of O-glycans has been observed, but specific epitopes, such as Lewis b and α 1-4GlcNAc, were found to promote adhesion of *Helicobacter pylori* to the mucus layer (Rossez et al., 2012; Jin et al., 2017). This pathogen is a direct cause of gastrointestinal ulcers and gastric cancer.

Similarly to mucins, glycoproteins in cancer cells show alterations in their O-glycosylation profiles. Truncated O-glycans such as a GalNAc residue with no further glycosylation (GalNAc α -Ser/Thr; Tn antigen) is considered a tumor marker given its presence is abnormal in glycoproteins (Itzkowitz et al., 1989). Sialylated Tn is also a common feature of cancer cells. An increase in core fucosylation is also observed in these cells. Consequently, these changes interfere with cell-cell adhesion processes, promoting tumor cell invasion (Pinho and Reis, 2015).

Importantly, O-linked glycans from human gastric mucin are structurally similar to those from the porcine stomach mucin. The oligosaccharides in both glycoproteins are mainly extended with Core 1 and Core 2 structures. The availability of porcine stomach mucin has facilitated the study of mucin function and its interactions with gut microorganisms. However, oligosaccharides in pig mucins have unique characteristics, like that they could contain extended Core 3 and Core 4 glycans (Padra et al., 2018). Pig gastric mucins are highly sulfated, usually terminating with galactose residues and have lower sialylation (Quintana-Hayashi et al., 2018).

Mucin glycosylation is in part mediated by gut microbiota. Using germ-free (GF) mice as control, it has been shown that the presence of certain members of gut microbiota is critical for the expression of glycosyltransferases participating in mucin O-glycosylation for example, ppGalNAcT, Core 1 β 1,3-Galactosyltransferase (C1GALT1), and Fucosyltransferase (FUT2; Johansson et al., 2015). MUC2 O-linked glycans from colonic tissues of conventionally raised animals were more sialylated, fucosylated, and longer than GF mice (Arike et al., 2017). MUC2 from GF mice showed a reduced abundance of β 1,6-N-acetyl glucosaminyltransferases, enzymes responsible for the formation of Core 2 and Core 4 O-glycans. These GF animals tended to produce shorter O-glycans (Arike et al., 2017). This result suggests that the gut microbiota modulates the O-glycosylation patterns of mucins, which in turn influence the composition the gut microbiota.

Mucin utilization by the gut microbiota is important for host health. It is known that animals that are fed mucin show higher fecal butyrate levels. This compound is an anti-inflammatory

SCFA considered positive for health and significantly decreased in inflammatory diseases such as Ulcerative Colitis (UC; Chen et al., 2018; Yamada et al., 2019). In UC subjects, mucin O-glycan abundance correlates negatively with butyrate production in feces, showing a reduced utilization of O-glycans by the gut microbiota (Yamada et al., 2019). This study presented important *in vivo* evidence linking the foraging of host-derived glycans, the action of the gut microbiota, and inflammatory bowel diseases (IBDs). In a different context, mucin O-glycan consumption by gut microbes could be considered unfavorable for the host. Animals deprived of fiber in their diets show the promotion of gut inflammation and colitis. This was explained partly by their gut microbiota turning into endogenous mucin resources as the last carbon source available. Accessing the mucin layer by mucin degraders permits the colonization by *Citrobacter rodentium*, a mucosal pathogen of mice sharing several pathogenic features with human gastrointestinal pathogens such as enteropathogenic *Escherichia coli* (Collins et al., 2014; Desai et al., 2016).

Mucins and their O-glycans are essential in the gut barrier function. Modifications of their expression, organization, or glycosylation are likely to prevent this effect, as observed in IBD. The altered balance between pro-inflammatory cytokines (TNF, IL-1b, IL-8, and IL-17), anti-inflammatory cytokines (IL-4 and IL-13), and immuno-regulatory cytokines described in IBD is likely to modify mucin expression and glycosylation (Guan and Zhang, 2017). Furthermore, altered glycosylation and sulfation of colonic mucins in IBD subjects could alter the protective role of the colonic mucus barrier (Groux-Degroote et al., 2020).

O-Linked Glycans From GMP

Glycomacropptide is a constituent of whey (20–25% of the protein moiety), derived from κ -casein after chymosin treatment during cheesemaking (Manso and López-Fandiño, 2004; Sunds et al., 2019). GMP is a hydrophilic, negatively charged, 64 amino acid glycopeptide. The molecular weight of GMP ranges from 6.7 to 8 kDa (Córdova-Dávalos et al., 2019). GMP is available in large quantities in dairy streams (Rojas and Torres, 2013).

The glycan portion is simpler compared to mucin oligosaccharides, containing only NeuAc, GalNAc, and Gal. GMP has five distinct O-linked glycans bound to Ser/Thr (Figure 2), corresponding to monosaccharides (0.8%), disaccharides (6.3%), trisaccharides (36.5%), and tetrasaccharides (56%; Saito and Itoh, 1992). The disaccharide GNB (Figure 2) is a building block found in GMP (Fiat and Jollès, 1989). Different O-glycosylation sites have been proposed in GMP, as determined by 2D gel electrophoresis and tandem mass spectrometry (Figure 2; Neelima et al., 2013). Thr in positions 121, 131, 133, 136, and 142 appear to be used as glycosylation sites, and positions 165, 135, and 141 have been proposed as potential sites (Thomä-Worringer et al., 2006). As expected, the functional and adhesion properties of GMP will depend on the position where O-glycans are attached, especially the sialylated. NeuAc is an acid sugar easily recognizable by mucin-degrading bacteria (Neelima et al., 2013).

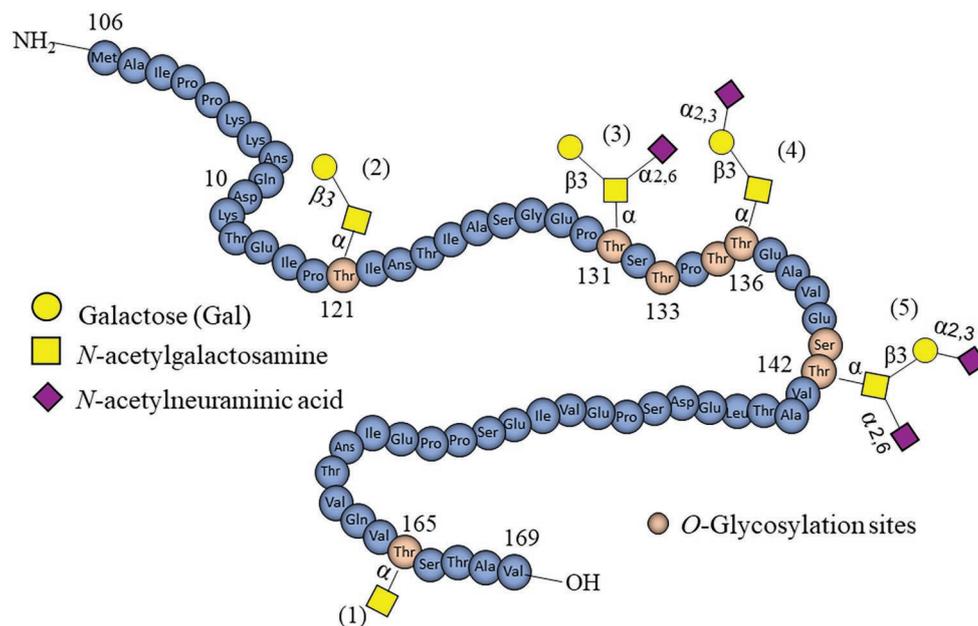


FIGURE 2 | Schematic representation of O-linked glycans found in casein glycomacropeptide (GMP). Within the GMP structure, there are five identified O-glycans containing Gal, GalNAc, and NeuAc (named 1–5). Threonine residues serving as accepting glycosylation sites are also marked in brown.

UTILIZATION OF MUCIN O-GLYCANS BY GUT MICROBES

While certain gut bacteria use mucin glycan as attachment sites, a few others go beyond and additionally deploy enzymatic activities to use mucin O-glycans as a nutrient (Tailford et al., 2015). Interestingly, this enzymatic capability is distributed in members across the four most dominant phyla in the gut microbiota (*Bacteroidetes*, *Actinobacteria*, *Firmicutes*, and *Verrucomicrobia*), suggesting it is an important trait for the gut microbiota.

Mucin O-glycan degradation by intestinal microorganisms requires an extensive array of glycosyl hydrolases (GHs), as expected from the complex structures, compositions, and diverse linkages found (Tailford et al., 2015). Most mucin-degrading bacteria base their strategies on exo-acting enzymes, including sialidases (GH33), fucosidases (GH29 and GH95), exo- and endo- β -*N*-acetylglucosaminidases (GH84 and GH85), β -galactosidases (GH2, GH20, and GH42), among others. Their activities imply sequential degradation. Other enzymes required for mucin utilization are α -*N*-acetylglucosaminidases (GH89) and α -*N*-acetylgalactosaminidases (GH101, GH129), which cleave a monosaccharide-peptide linkage after glycosidase activity. One potential exception to this exo-acting mechanism is a GH16 endoactive O-glycanase with mucin breakdown activities (Crouch et al., 2020). These activities release mono or disaccharides, which could be imported inside the bacterial cell or serve as cross-feeding metabolites (Turrone et al., 2018). Several enzymes related to mucin deglycosylation have been described, but the full mechanisms by which gut microbes utilize these glycans are unknown.

Utilization of O-Linked Glycans by *Bacteroides* spp.

The *Bacteroides* genus is predominant in the adult gut microbiota, being generally considered as commensals. Genomic and functional studies in animals show that these species show a preference for utilizing complex carbohydrates (e.g., HMO), rather than simple carbohydrates (Wexler and Goodman, 2017). *Bacteroides* devote a large part of their genomes to polysaccharide utilization loci (PULs), which correlates with their broad diverse polysaccharide utilization. These gene clusters encode multiple extracellular GHs enzymes, oligosaccharide transporters, and binding proteins.

Early works studied the molecular system of complex glycan degradation in starch utilization in these species (Anderson and Salyers, 1989). *Bacteroides thetaiotaomicron* VPI-5482 is a model bacterium for mucin O-glycan utilization. The microorganism shows a remarkable growth in porcine mucin glycans (Martens et al., 2008). These oligosaccharides can induce the expression of at least 16 PULs in *Ba. thetaiotaomicron*, aiding in identifying loci involved in O-glycan utilization. These genes encode for putative glycolytic enzymes, including an α -L-fucosidase, endo- β -GlcNAc-ase, endo- β -galactosidase, α -GalNAc-ase, in addition to proteases, neuraminidase, and a sulfatase. The enzymes are suggested to play an orchestrated degradation of O-linked glycans (Sicard et al., 2017; Luis et al., 2018). The relevance of these genetic determinants has been confirmed *in vivo* experiments in GF mice, where mutants for these loci show reduced gut colonization.

These enzymes are part of PULs also known as Starch utilization system (Sus), an operon of eight genes (SusRABCDEFGF). Their products are predicted to locate in

the periplasm or the outer membrane (**Figure 3**). Carbohydrates are metabolized in response to the SusR signal, which triggers the expression of hydrolyzing proteins. The processed oligosaccharides are transported by a TonB protein and are metabolized inside the cells (Cockburn and Koropatkin, 2016). SusD homologs possess a binding site for O-glycans (Tailford et al., 2015) and have been implicated in the utilization of O-linked mucin glycans. Interestingly, the transcriptional response mounted to utilize O-glycans is similar to that required for HMO consumption (Marcobal et al., 2011). Four PULs in *Ba. thetaiotaomicron* were found to be induced in HMO but not in the presence of mucin glycans, indicating that this microorganism can respond to building blocks in HMO not found in mucin. Regardless, the deletion of these PULs did not affect the ability of *Ba. thetaiotaomicron* to consume HMO. Apparently, for this strain O-glycans are not a premium substrate since several mono or simple carbohydrates repressed these genes (Pudlo et al., 2015). This was not observed for other *Bacteroides* species, indicating the complex regulation of glycan utilization and diversity on preference for carbon sources even at the species level (Pudlo et al., 2015).

Monocolonization of GF mice with *Ba. thetaiotaomicron* modulates cellular responses in mucin-producing goblet cells, increasing their differentiation and synthesis of mucins, changing their glycosylation patterns with a higher NeuAc content (Wrzosek et al., 2013). In the presence of *Faecalibacterium prausnitzii*, a keystone microbe and butyrate producer, these cellular responses were attenuated, probably maintaining epithelial homeostasis between cell lines.

Bacteroides fragilis is a commensal species, but certain strains could be enterotoxigenic causing serious diseases (Purcell et al., 2017; Chung et al., 2018). This species also has a PUL involved in the consumption of host O-glycans. This PUL is known as

the commensal colonization factor (CCF; Lee et al., 2013). The genes within the CCF are homologous to *Ba. thetaiotaomicron* PULs, which are up-regulated possibly producing extracellular enzymes to sense and mediate O-glycan processing. These enzymes allow the commensal colonization of mucus, specifically in the crypts of the colon. Some of these have been characterized biochemically (Praharaj et al., 2018; Yamamoto et al., 2018).

Utilization of O-Linked Glycans in Firmicutes

This phylum contains a broad diversity of genera, including commensal *Clostridium*, *Eubacterium*, and *Lactobacillus* species. In general, most *Firmicutes* in the gut microbiota prefer the assimilation of smaller rather than complex carbohydrates (Cockburn and Koropatkin, 2016; Ravcheev and Thiele, 2017). Several *Lactobacillus* species are endowed with mucin binding proteins (van Tassel and Miller, 2011), but they apparently lack any mucin utilization mechanism. However, within the *Firmicutes* a few members have acquired the ability to gain access to O-linked glycans, deploying several extracellular GHs.

A few pathogens in this group have evolved mechanisms for O-glycan utilization. *Clostridium perfringens*, an opportunistic pathogenic bacterium, can consume N-glycans and O-glycans from intestinal mucin by releasing extracellular glycosidases such as sialidases (Koutsoulis et al., 2008; Pluvinage et al., 2019). *Enterococcus faecalis* strains possess genes encoding endo- β -N-acetylglucosaminidase and endo- α -N-acetylgalactosaminidase (endo- α -GalNAc-ase) that release N-glycans. However, this pathobiont appears not to be able to utilize mucin O-linked glycans (Roberts et al., 2000; Morio et al., 2019). These microorganisms are not usually

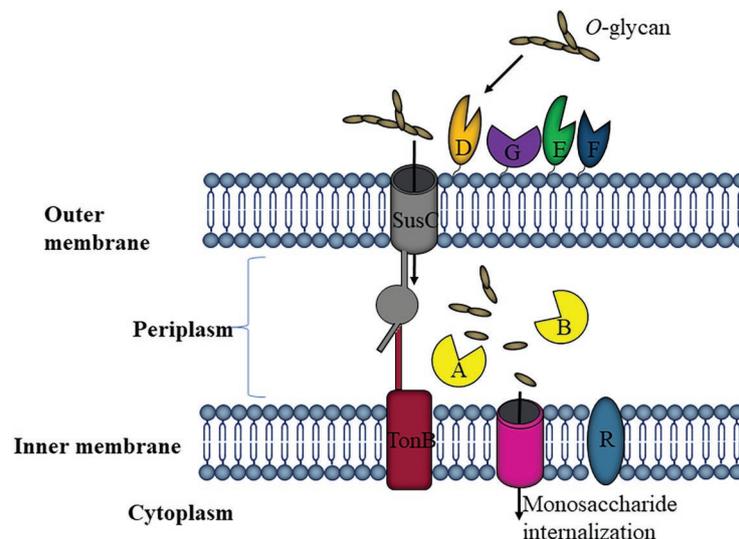


FIGURE 3 | Representation of O-linked glycan utilization by *Bacteroides thetaiotaomicron*. The starch utilization system (Sus) is the machinery best described in *Ba. thetaiotaomicron* for the utilization of mucin-type O-glycans. SusDEF are recognition and binding sites for O-glycans, SusC is a TonB-dependent transporter for sugar importation, SusR is a predicted membrane-spanning regulator, and SusAB are glycosyl hydrolases (GHs; Zhang et al., 2018).

dominant in the gut microbiota, probably due to the barrier effect mounted by this community.

Commensal Firmicutes such as *Ruminococcus torques* and *Ruminococcus gnavus*, belonging to the *Lachnospiraceae* family, can also access the O-linked glycans from mucin, especially MUC2. *Ruminococcus torques* is endowed with an α -sialidase (GH33), α -fucosidase (GH29), β -galactosidase, β -N-acetylgalactosaminidase, β -N-acetylglucosaminidase, sialate O-acetyltransferase, and glycosulfatase activities. Mucin deglycosylation is carried out from a mixture of exo and endo activities (Croft et al., 2016). *Ruminococcus gnavus* contains a similar enzymatic arsenal, but its strategy involves the chemical modification of NeuAc by a trans-sialidase, releasing 2,7-anhydro-Neu5Ac (Bell et al., 2019). Interestingly, this modification allows this microorganism to use this carbohydrate, making it inaccessible for others gut microbes (Bell et al., 2019). This strategy has proven essential for proper colonization of *R. gnavus* in mice.

Ruminococcus gnavus is part of the healthy gut microbiota but appears to be increased in IBDs (Hall et al., 2017). Similar to *Ba. thetaiotaomicron*, *R. gnavus* has the ability to modulate host cellular responses, especially genes encoding MUC2 and certain glycosyltransferases (Graziani et al., 2016). However, both *R. gnavus* and *R. torques* are increased several-fold in the intestinal mucosa of UC and Crohn's disease subjects, suggesting that their mucolytic activities contribute to disease progression (Png et al., 2010).

Sheridan et al. (2016) used *in vitro* growth assays and comparative genomics to identify genes involved in general carbohydrate metabolism in 11 *Roseburia* spp. and *Eubacterium rectale* strains. These bacteria have been suggested next-generation probiotics (Lordan et al., 2019), considered desirable for the host due to the production of butyrate. Two *Roseburia inulinivorans* strains were found to contain a Gram-positive PUL (gpPUL) with putative mucin glycan degradation genes such as a desulfatase, four glycosidases, and an ATP-binding cassettes (ABC) transporter. While this evidence suggests certain butyrate-producers could use mucin O-glycans as a carbon source, none of the strains of this study was able to grow using type 2 or type 3 porcine-derived mucin. Further experiments are required to clarify the ability of these microorganisms to target mucin as carbon source and the role of the gpPUL.

Utilization of O-Linked Glycans by *Bifidobacterium*

A few members of the phylum Actinobacteria can utilize glycans found in mucin, especially *Bifidobacterium* (Turroni et al., 2010; Katoh et al., 2017). This genus contains mostly commensal or beneficial microorganisms, dominant in the infant's gut.

Milani et al. (2016) showed that *Bi. bifidum* PRL2010 has a set of chromosomal loci that allows both N-glycans and O-glycan utilization. Seventy-seven genes, including GHs, glycosyltransferases, and glycosyl esterases were identified. *Bifidobacterium bifidum* contains a complete set of extracellular GHs, including exo- α -sialidases, 1,2- α -L-fucosidase, 1,3/4- α -L-fucosidase, N-acetyl- β -hexosaminidases, and four

β -galactosidases (Figure 4A). Several of these enzymes have been biochemically characterized, showing that they are membrane-bound and essential for mucin O-glycan assimilation (Shimada et al., 2015).

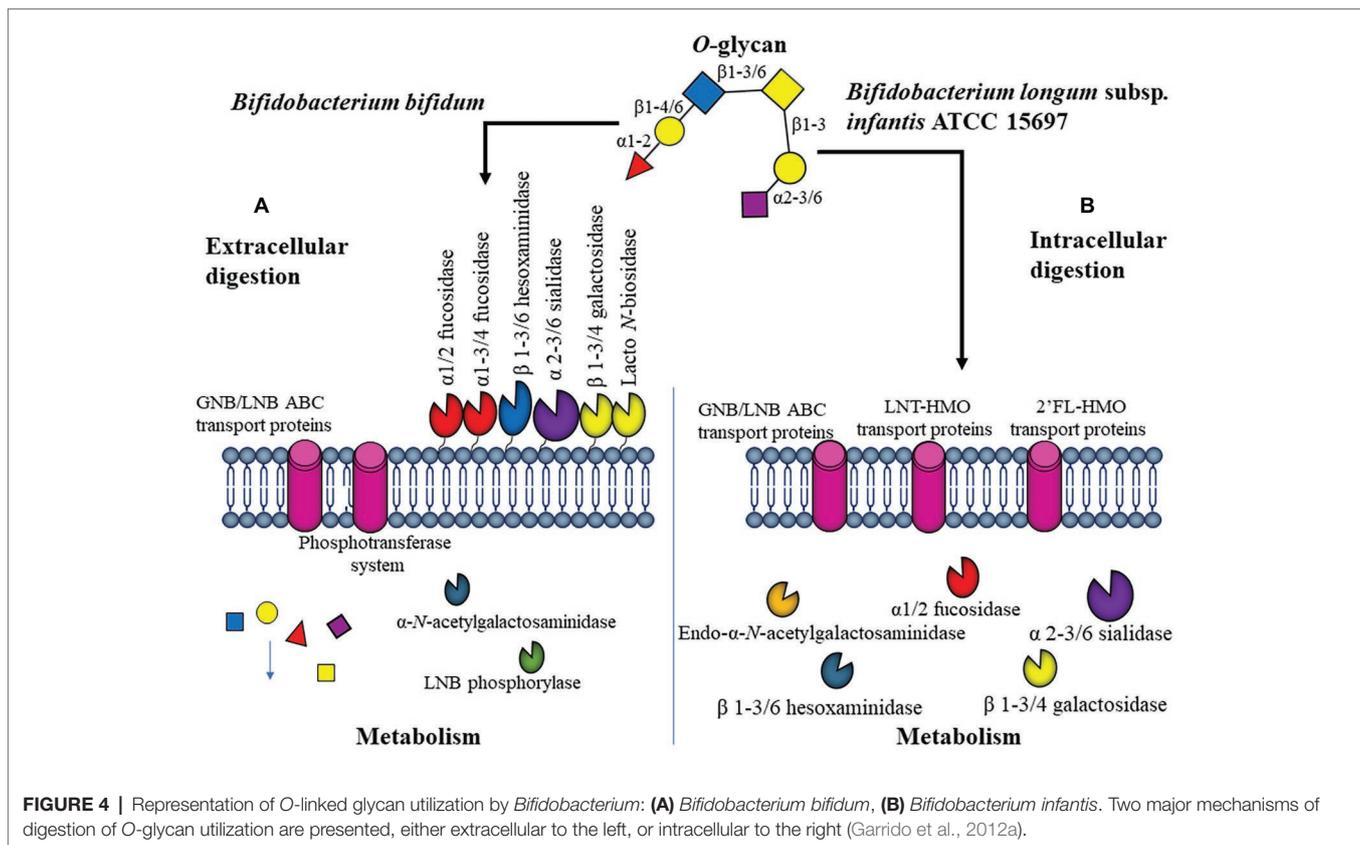
The consumption strategy of *Bi. bifidum* is mostly extracellular, where all these enzymes contain transmembrane domains and participate in mucin deglycosylation rendering GNB as a result. Apparently, *Bi. bifidum* does not utilize Fuc or NeuAc from complex glycans (Garrido et al., 2015). However, sialidase activity in this bacterium appears to promote bacterial adhesion to the epithelium (Nishiyama et al., 2017). This consumption mechanism allows the resulting mono and disaccharides to participate in cross-feeding with other microorganisms, for example, with *Bifidobacterium breve* and *Eubacterium hallii* (Egan et al., 2014; Bunesova et al., 2018).

The Lacto-N-Biose (LNB)/GNB cluster present in most bifidobacteria allows these microorganisms to consume Lacto-N-Tetraose from HMO and GNB resulting from O-glycan degradation. This cluster is present in several infant and adult-associated bifidobacteria. It contains an ABC transporter specific for these two disaccharides and intracellular enzymes for LNB/GNB utilization in the bifid shunt (Thompson et al., 2018).

A few enzymes in this genus have been characterized by their ability to target mucin glycans. Earlier works described an endo- α -GalNAc-ase in *Bi. longum* subsp. *longum* JCM 1217 (EngBF), which is a conserved extracellular GH101 enzyme in this subspecies, being highly specific for Core 1 O-glycans (Fujita et al., 2005). An α -acetylgalactosaminidase (NagBb) from *Bi. bifidum* JCM 1254 is a GH101 enzyme that hydrolyzes O-glycans from Core 1 and Core 3 from mucin MUC2 (Kiyohara et al., 2012). The enzyme shows a high affinity for the Tn antigen (GalNAc α -Ser/Thr). Finally, a GH89 from the same microorganism shows affinity for GlcNAc α 1-4Gal, an epitope commonly found in terminal positions in mucin glycans (Shimada et al., 2015). All these enzymes have great potential as a tool in glycobiology studies, and the obtention of prebiotics from complex mucin sources. Finally, evidence regarding the utilization of mucin O-glycans by *Bifidobacterium* has primarily been studied *in vitro*, and further studies are required to understand the implications of these strategies for the host. *Bi. longum*, as well as inulin, show protective effects against mucus deterioration caused by a mucin-eroding gut microbiota (Schroeder et al., 2018).

Utilization of O-Linked Glycans by *Verrucomicrobia*

In recent years, *A. muciniphila* has received considerable attention for its associations with health. Its abundance inversely correlates with the onset of IBDs and metabolic disorders (Dao et al., 2016; Lopez-Siles et al., 2018; Yassour et al., 2018), and it has been suggested to play an anti-inflammatory role (Cani and de Vos, 2017). It has also been proposed as a candidate next-generation probiotic, which in addition to the modulation of host responses possesses several technological properties compatible with scale-up processes (Cani and de Vos, 2017). Recently, the administration of pasteurized doses of



A. muciniphila to overweight individuals improved certain inflammation and metabolic markers, being well-tolerated and safe (Depommier et al., 2019).

Akkermansia muciniphila is a Gram-negative bacterium that specializes in using mucins as nitrogen and carbon sources. This contrasts with other generalist gut microbes such as *Bacteroides* spp., which can target multiple sources for growth. Its specificity for mucin also presents a remarkable example of microbial adaptations to host conditions. *Akkermansia muciniphila* is the only member of the phylum *Verrucomicrobia* cultured and found in the gut microbiota, representing the 3% of intestinal bacteria detected in adult feces (Everard et al., 2014). Despite being considered a strict anaerobic microbe, recent studies have determined that it colonizes the mucus layer near epithelial cells, characterized by microaerophilic levels of oxygen (Ouwkerk et al., 2016). *Akkermansia muciniphila* cannot synthesize Thr; however, it obtains this amino acid from mucin, which is one of the most abundant amino acids in its structure (van der Ark et al., 2018).

This microorganism is underrepresented in IBD, especially UC patients. Yamada et al. (2019) reported that fermentation of mucin O-glycans is associated with the production of butyrate in healthy subjects. However, subjects with UC show altered mucin O-glycosylation patterns, and the abundance of *A. muciniphila* was inversely proportional to the markers of inflammation (Earley et al., 2019; Yamada et al., 2019). This suggests that the O-glycosylation patterns present in

mucins influence the anti-inflammatory activity of certain commensal microorganisms.

Studies show that the mucolytic activity and metabolite production by *A. muciniphila* promotes beneficial microbial networks (Belzer et al., 2017). Mucin degradation by gut microorganisms might be considered a pathogenic trait because it reduces the protective layer of the complex mucus layer. Intriguingly, in IL10-genetically deficient mice *A. muciniphila* contributes to colitis (Seregin et al., 2017). However, these degrading microorganisms may stimulate mucin accumulation, renewal, and thickening of the mucosal layer. In addition, the complexity of the mucin O-glycans makes these structures protective and inaccessible to most bacteria, except those that have developed consumption mechanisms such as *A. muciniphila* and *Ba. thetaiotaomicron*.

The genome analysis of *A. muciniphila* ATCC BAA-835, isolated from the human gastrointestinal tract, predicted the presence of a large number of mucinases (van Passel et al., 2011). This is a general term referring to GHs or lyases with combined activity against mucin. It was also reported that 26% of its proteome contains a signal peptide site, indicating a preference for extracellular degradation of macromolecules. Consequently, the putative strategy employed by this microorganism is to secrete an extensive array of extracellular proteins that hydrolyze N-glycans and O-glycans in simple sugars, some of which are later internalized (Shin et al., 2019). Regarding its enzymatic machinery, *A. muciniphila* is predicted

to contain α/β -D-galactosidase, α -L-fucosidase, α/β -N-acetylgalactosaminidase, two α -N-Acetyl-glucosaminidases, neuraminidase, and a sulfatase. Few studies have confirmed the activities of some of these enzymes (Wang et al., 2018; Shin et al., 2019; Meng et al., 2020), including sulfatases and proteases targeting MUC2. Major end-metabolites produced by this bacterium are propionate, acetate, and sulfate (Ottman et al., 2017).

UTILIZATION OF GMP-DERIVED GLYCANS BY GUT MICROBES

Glycomacropeptide is a highly sialylated glycopeptide resulting from the cheesemaking process. It is an abundant source of O-glycans. However, not much attention has been paid to its potential applications for promoting a healthy gut microbiota. Compared to mucin, most studies have focused on the utilization of GMP by probiotic microorganisms *in vitro*, sometimes with contradictory results (Córdova-Dávalos et al., 2019). Early work from Idota et al. (1994) showed that certain *Bifidobacterium*, including *Bi. bifidum*, *Bi. breve*, *Bi. longum*, and *Bifidobacterium adolescentis* could grow on GMP with OD_{600nm} values ranging from 0.7 to 2.70. Although purified GMP contains small amounts of lactose, which could contribute to the above results, the latter two microorganisms do not have the sialidases required to hydrolyze the GMP O-glycans (O'Callaghan and van Sinderen, 2016).

Bifidobacterium longum subsp. *infantis* ATCC 15697 (*Bifidobacterium infantis*) is a dominant bacterium in the gut microbiota of newborns. It has been well characterized by its ability to fully utilize several HMO as a carbon source, with protective effects on the infant (Thompson et al., 2018; Henrick et al., 2019). The utilization strategy for this microorganism relies on ABC for internalization of intact glycans, subsequently degraded by intracellular GHs (Figure 4B; Zúñiga et al., 2018). *Bifidobacterium infantis* shows a preference for host glycans containing within its genome 16 GH, including several α -fucosidases, β -galactosidases, β -hexosaminidases, and α -sialidases (Thompson et al., 2018). This microorganism has also evolved determinants and mechanisms for the vigorous utilization of milk-derived N-glycans (Garrido et al., 2012b; Karav et al., 2016).

Considering the structural similarity between HMO, N-glycans, and O-glycans, it could be expected that this microorganism also targets mucins as a growth substrate. Interestingly, *B. infantis* is not able to access the O-glycans of these glycoproteins (Kim et al., 2013; Turrone et al., 2018). Most glycosidases in *B. infantis* are intracellular, and it lacks homologs to α -N-Acetylgalactosaminidases found in *B. bifidum* and *B. longum*.

Strikingly, *B. infantis* has been shown to grow vigorously using GMP (O'Riordan et al., 2018). A $20.6 \pm 3.6\%$ increase in OD_{600nm} was observed in the mid-exponential phase of *B. longum* ssp. *infantis* cultures supplemented with GMP, indicating a growth-promoting effect. *In vitro*, it has been

shown that *B. infantis* growth is proportional to GMP concentration in the culture media. GMP periodate treatment (GMP-P) inactivates the glycan portion of the peptide chain, and growth was compared with native GMP. GMP-P resulted in a substantially lower (5.5%) increase in growth compared with full GMP in the mid-exponential phase. Under these conditions, *B. infantis* was not able to grow using GMP-P, indicating that the GMP O-glycan portion is essential in *B. infantis* growth.

Transcriptomic analysis of the GMP utilization revealed the induction of two intracellular GH25 enzymes, a family related to bacterial lysozymes, and a solute binding protein from an ABC transporter (O'Riordan et al., 2018). These results provide new insights regarding the adaptations of this probiotic microorganism for a milk-derived substrate. However, how this infant-gut associated bacterium can use GMP O-glycans as the sole carbon source is not clear, especially considering it cannot access mucin O-glycans. It is possible that GMP, a smaller and structurally and physicochemical simpler molecule, is easier to access. Another possibility is the participation of proteases, which could facilitate the import of GMP-derived O-glycans into the bacterial cell for intracellular processing.

CONCLUSION

O-linked glycans are complex oligosaccharides decorating host glycoproteins. They are produced endogenously in mucins or could be found in secretions such as milk. These glycans are accessed, released, and consumed by individual members of the gut microbiota, especially beneficial gut microbes such as *Bifidobacterium*, *Bacteroides*, *Akkermansia*, and stimulate the growth of next-generation probiotics such as *Roseburia* and other butyrate-producing bacteria.

O-glycans serve as signaling molecules for cell secretion and provide greater resistance to proteolysis (Boutrou et al., 2008). Under conditions of dietary fiber depletion, endogenous mucin degradation occurs associated with microbial activity, promoting inflammation, and intestinal disease.

O-glycans derived from milk glycopeptides such as in GMP are an attractive opportunity to use them in foods as emerging prebiotics, promoting a healthy gut microbiota. Unfortunately, there are still several limitations to this goal, and no human studies have evaluated its prebiotic effect. While, we have advanced in understanding some of the molecular mechanisms involved in O-linked glycan utilization in single microorganisms, the impact of this utilization in metabolic interactions and networks has been evaluated only in a small number of studies.

These gaps have been hindered in part because we lack enzymes or other tools to recover full O-glycans from dairy streams or mucin sources. In contrast, certain enzymes have been described for the recovery of N-glycans from dairy byproducts. This offers an opportunity for identifying novel enzymes from gut microbes or improving the activity of current endo- α -N-acetylgalactosaminidases.

Moreover, the consequences for the host of the microbial utilization of O-glycans, or glycoproteins containing O-glycans, have not been evaluated and remains a critical question. Whether gut microbes accessing the mucus layer and releasing O-glycans is beneficial for the host is still unclear. Furthermore, the study of O-glycans utilization by the gut microbiota, and how these microbes shape mucin glycosylation patterns, should be further studied in the context of intestinal inflammation and IBDs, as well as cancer progression.

AUTHOR CONTRIBUTIONS

KG-M, MV-S, and DG conceived and wrote the manuscript. KG-M prepared the Figures. DG critically reviewed

the manuscript. All authors approved the final version of the manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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