

Review

Consumption of human milk glycoconjugates by infant-associated bifidobacteria: mechanisms and implications

Daniel Garrido, David C. Dallas and David A. Mills

Correspondence

David A. Mills
damills@ucdavis.edu

Department of Food Science and Technology and Foods for Health Institute, University of California Davis, One Shields Ave, Davis, CA 95616, USA

Human milk is a rich source of nutrients and energy, shaped by mammalian evolution to provide all the nutritive requirements of the newborn. In addition, several molecules in breast milk act as bioactive agents, playing an important role in infant protection and guiding a proper development. While major breast milk nutrients such as lactose, lipids and proteins are readily digested and consumed by the infant, other molecules, such as human milk oligosaccharides and glycosylated proteins and lipids, can escape intestinal digestion and transit through the gastrointestinal tract. In this environment, these molecules guide the composition of the developing infant intestinal microbiota by preventing the colonization of enteric pathogens and providing carbon and nitrogen sources for other colonic commensals. Only a few bacteria, in particular *Bifidobacterium* species, can gain access to the energetic content of milk as it is displayed in the colon, probably contributing to their predominance in the intestinal microbiota in the first year of life. Bifidobacteria deploy exquisite molecular mechanisms to utilize human milk oligosaccharides, and recent evidence indicates that their activities also target other human milk glycoconjugates. Here, we review advances in our understanding of how these microbes have been shaped by breast milk components and the strategies associated with their consumption of milk glycoconjugates.

Introduction

After birth, the profound and intimate connection between a mother and her newborn continues in several ways. Breast milk represents a physical representation of this relationship: an intriguing fluid synthesized at the mother's expense, shaped throughout evolution to nourish the infant and improve its rate of survival. Human milk is perhaps the most personalized food, where the molecular make-up varies from mother to mother and across lactation, providing the infant all the nutrients needed in a concentrated form (Allen *et al.*, 1991; Mitoulas *et al.*, 2002). Breastfeeding is regarded as the 'normal way of providing young infants with the nutrients they need for healthy growth and development' (Fewtrell *et al.*, 2007). Exclusive breastfeeding is recommended for up to 6 months of age (American Academy of Pediatrics Section on Breastfeeding, 2012), and its benefits are multifold and some can last beyond childhood (Hernell, 2011; Le Huërou-Luron *et al.*, 2010).

Human milk is a complex food matrix and its composition reflects all the nutritional and physiological demands of the newborn. Essential nutrients in human milk such as lactose, fatty acids and proteins are absorbed by the small intestine at a rate that is limited by the developing conditions of the gastrointestinal (GI) tract of the infant (Neu, 2007). Other micronutrients such as nucleotides,

vitamins and minerals are also highly bioavailable for the infant (Picciano, 2001).

Numerous studies have shown that breastfeeding is associated with a lower risk of infections and diarrhoea. This has been associated with the activity of milk immunoglobulins (Xanthou *et al.*, 1995), antimicrobial agents such as lactoferrin and lysozyme (Håversen *et al.*, 2002; Jollès & Jollès, 1961; Lönnerdal, 2009), and human milk glycoconjugates (Newburg *et al.*, 2005). Several of these molecules are not readily absorbed by the small intestine and transit throughout the GI tract (Dallas *et al.*, 2012), but their impact and biological activities are poorly understood. These bioactive compounds play additional roles in protection and/or stimulate development regardless of their nutritive value (Hamosh, 2001; Lönnerdal, 2010). Bioactives in human milk represent a significant difference between breast milk and bovine milk-based formulas (Hernell, 2011; Le Huërou-Luron *et al.*, 2010).

A common characteristic of these bioactive agents is that they are glycosylated molecules. Glycans in milk can be found as free human milk oligosaccharides (HMO), or conjugated via glycosidic bonds to proteins or lipids. Among other functions, human milk glycans represent the main driving force for bacterial colonization of the distal large intestine of the breast-fed infant (Scholtens *et al.*, 2012). The high concentrations of HMO and conjugated

oligosaccharides processed after intestinal digestion are thought to be the main contributors to the predominance of *Bifidobacterium* species in the infant gut. The genome sequences of bifidobacteria show that these micro-organisms are highly adapted to the intestinal environment (Schell *et al.*, 2002), and the genomes of infant gut-associated bifidobacteria have been shaped by complex carbohydrates (Sela & Mills, 2010). In this review, we examine recent advances in our understanding of how milk oligosaccharides and other glycoconjugates influence the dominance of beneficial micro-organisms in the gut microbiota, especially *Bifidobacterium*, and of the mechanisms and strategies that these micro-organisms have devised for using milk components as a carbon source.

HMO

Structures of HMO

A great amount of the energy invested in human milk production is dedicated to synthesize complex free oligosaccharides. These molecules represent the third most abundant component in breast milk after lactose and fatty acids (Petherick, 2010). HMO consist of a pool of soluble carbohydrates with a degree of polymerization of 3 to 15 and linked through a variety of glycosidic bonds (Kunz *et al.*, 2000; Urashima *et al.*, 2012). HMO are composed of five monosaccharides: glucose (Glc), galactose (Gal), *N*-acetylglucosamine (GlcNAc), fucose (Fuc) and *N*-acetylneuraminic acid (NeuAc; sialic acid). Fig. 1 presents a representative HMO structure and the diversity of linkages that can be found. All HMO are characterized by a terminal lactose molecule, modified by fucose or sialic acid in the case of the shorter HMO such as 2'-fucosyllactose (FL), 3FL and sialyllactose (SL), or by repeats of building blocks of lacto-*N*-biose type 1 (LNB; Gal β 1-3GlcNAc) or *N*-acetylglucosamine (LacNAc; Gal β 1-4GlcNAc). These repeats can be further decorated by fucose or sialic acid in α -linkages, adding more complexity and diversity to these molecules (Bode & Jantscher-Krenn, 2012). HMO can be classified as acidic or neutral depending on the presence of the negatively charged sialic acid. Neutral HMO can be further categorized by the presence of fucose on their structures. Over 200 oligosaccharide structures have been identified in human milk (Wu *et al.*, 2010, 2011).

Significant differences exist in HMO abundance and composition among different mothers and across lactation stages (Coppa *et al.*, 1999; De Leoz *et al.*, 2012; Niñonuevo *et al.*, 2008). An important association also exists between HMO and the blood group type of the mother represented by the Lewis system and the secretor genes, which generates four different groups of milks (Totten *et al.*, 2012). Another important characteristic of human milk is the overabundance of type 1 HMO, containing type 1 LNB, over type 2 HMO containing LacNAc (Urashima *et al.*, 2012). Type 1 predominance and large amounts of fucosylated HMO are characteristic of human milk but much less so for other mammals (Tao *et al.*, 2011; Taufik *et al.*, 2012).

Great efforts have been made to elucidate the composition and structures of HMO. As recently reviewed (Kunz, 2012), milk carbohydrates research started in the early 1900s. Despite recent technological advances, structural elucidation of oligosaccharides from breast milk still remains a challenge, mainly due to the variety of possible isomeric forms of any given composition. MS has become a method of choice for oligosaccharide analysis, and current methods allow isomer differentiation with high resolution (Ruhaak & Lebrilla, 2012).

Physiological effects of HMO

GI enzymes in the infant are not capable of breaking down the diversity of HMO linkages synthesized by glycosyltransferases in the mammary gland (Dallas *et al.*, 2012), which emphasizes the role of milk bioactive molecules in functions beyond nutrition. HMO are minimally affected by transit through the stomach and small intestine, reaching a high concentration in infant faeces (Chaturvedi *et al.*, 2001; Engfer *et al.*, 2000; Gnath *et al.*, 2000). Excreted faecal HMO in breast-fed infants are a reflection of the mother secretor status, and the course of oligosaccharide excretion is apparently individual-specific and intermediate degradation products can be observed (Albrecht *et al.*, 2011). Furthermore, small amounts of certain HMO can be found in urine (Rudloff & Kunz, 2012), suggesting that these molecules can exert physiological effects not only locally in the GI tract but also systemically.

HMO are well known for their ability to prevent adherence and invasion of several pathogens (Imberty & Varrot, 2008; Morrow *et al.*, 2005). This is probably due to the structural similarity between HMO and glycoconjugates present in the intestinal mucosa. Fucose- and sialic acid-containing HMO are particularly important in pathogen deflection as they are found at terminal positions in these molecules. Therefore, the abundance of HMO and other milk glycoconjugates can explain in great part how breast milk helps to prevent infant diarrhoea and GI infections in breast-fed infants (Coppa *et al.*, 2006; Hakkarainen *et al.*, 2005; Hong *et al.*, 2009; Martín-Sosa *et al.*, 2002; Morrow *et al.*, 2004; Newburg *et al.*, 2004; Ruiz-Palacios *et al.*, 2003).

Establishment of the infant intestinal microbiota

At birth, the newborn is first exposed to the extrauterine environment, entering a microbial-laden world that results in quick colonization of different body sites, typically in a non-pathogenic fashion (Dominguez-Bello *et al.*, 2010). Of particular interest is how the intestinal microbiota is established, given the potential impact it has on subsequent health and disease (Reinhardt *et al.*, 2009; Scholtens *et al.*, 2012). Patterns of early intestinal colonization can have both short-term and long-term health effects (Bager *et al.*, 2008; Cho *et al.*, 2012; Collado *et al.*, 2012; Kalliomäki *et al.*, 2008; Salvini *et al.*, 2011). Bacterial colonization of

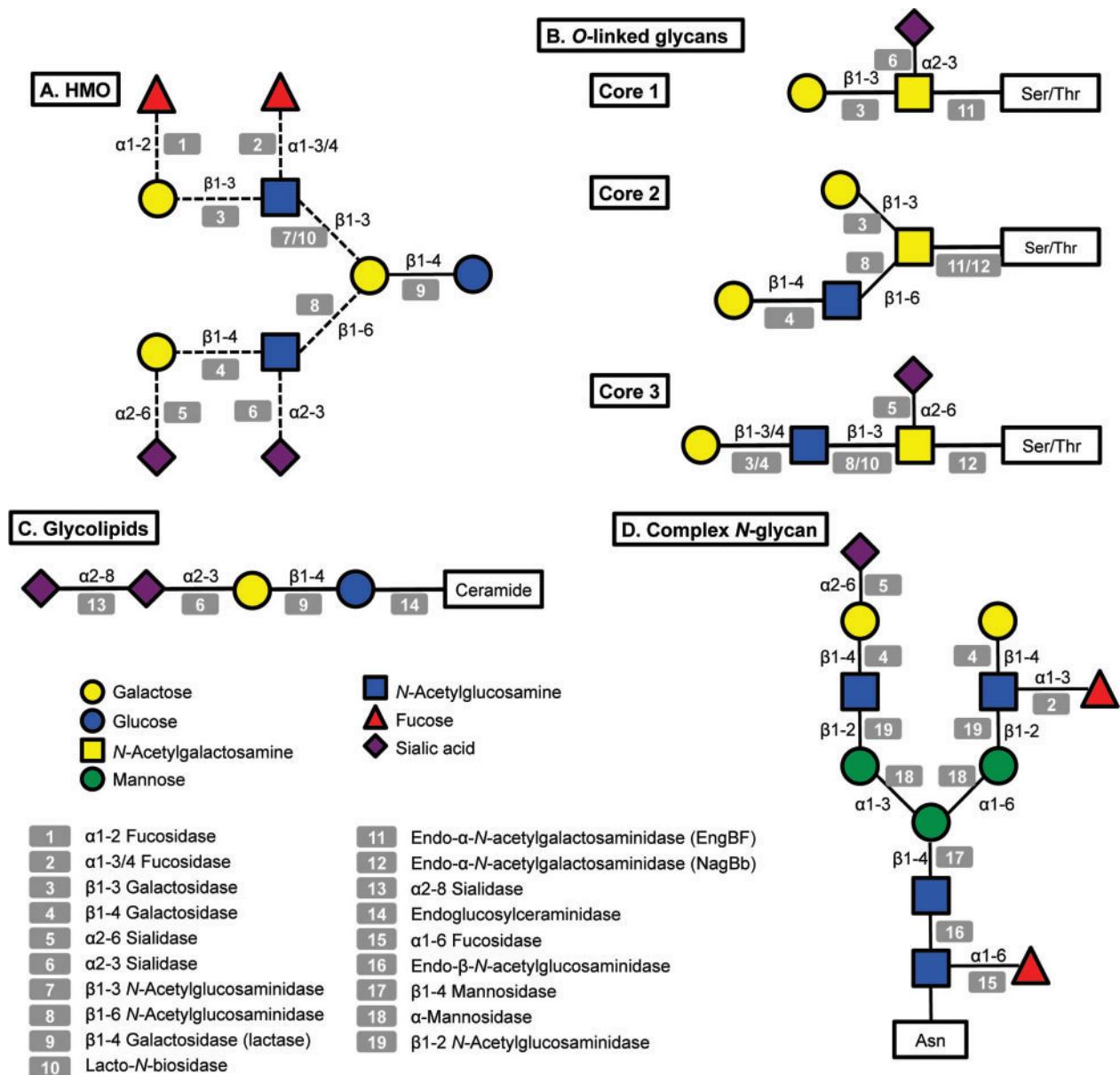


Fig. 1. Structural diversity of glycans in human milk and corresponding glycosyl hydrolases in infant-gut associated bifidobacteria. Legends at the bottom left indicate monosaccharide composition and the corresponding potential glycolytic enzymes in bifidobacteria acting at specific linkages. A: illustrative structure of HMO; B: three different cores found in human O-linked glycans; C: glycolipids, the structure of ganglioside GD3 is shown; D: a complex N-glycan.

the intestine is key in several aspects: bacteria provide essential nutrients for the infant such as vitamins and short-chain fatty acids, they stimulate the development of the immune system, especially adaptive responses, and they provide general protection against pathogen colonization, among several other functions (Hooper *et al.*, 2012; Nicholson *et al.*, 2012). A contribution of the intestinal microbiota has been established in the onset of obesity and type 2 diabetes (Harris *et al.*, 2012; Ley *et al.*, 2005). The establishment of the microbiota in the infant colon has been described as an orchestrated, but chaotic, succession

of bacteria (Koenig *et al.*, 2011), wherein the composition depends on a diverse number of factors such as mode of delivery, type of feeding, and genetic, cultural and geographical determinants (Scholtens *et al.*, 2012). The first colonizers of the intestinal tract are facultative anaerobic bacteria, such as *Escherichia coli*, enterococci and streptococci, which predominate in the first days of life. These bacteria will consume the oxygen in the intestinal lumen, creating an anaerobic environment more favourable for strict anaerobes, such as *Bacteroides*, *Clostridium* and *Bifidobacterium* (Jost *et al.*, 2012).

Mode of delivery is one of the most important factors that dictate the composition of the infant intestinal microbiota in the first months of life. Normal vaginal delivery exposes the infant to the vaginal and faecal microbiota of the mother (Dominguez-Bello *et al.*, 2010; Makino *et al.*, 2011). Human breast milk has been also considered another source of micro-organisms that can potentially contribute to gut colonization (Cabrera-Rubio *et al.*, 2012; Grönlund *et al.*, 2007; Makino *et al.*, 2011); however, this remains controversial, since the microbiota in breast milk can be instead a reflection of the skin or faecal microbiota. In the other hand, the hospital environment (Martirosian *et al.*, 1995) and the skin microbiota (Dominguez-Bello *et al.*, 2010) are considered sources of bacteria for caesarian-born infants. A delay in microbial colonization by prominent members of the intestinal microbiota such as *Bifidobacterium*, *Bacteroides* and *E. coli* has also been observed (Adlerberth *et al.*, 2006; Mitsou *et al.*, 2008), and bifidobacterial counts are also lower in caesarian-born infants (Chen *et al.*, 2007; Penders *et al.*, 2006).

Significant differences are found in the early composition of the infant intestinal microbiota relative to the type of diet. Infant formulas are traditionally based on bovine milk, and great advances have been made to improve their composition by adding supplements such as minerals, vitamins and prebiotics, in order to simulate the essential components in breast milk (Hernell, 2011; Koletzko, 2010). Unfortunately, some of the bioactive molecules in human milk are not found in bovine milk, and therefore replicating their effects is challenging (Dewey *et al.*, 1995; Le Huërou-Luron *et al.*, 2010).

Bifidogenic effect of HMO

Culture-based and current large-scale metagenomic studies show that *Bifidobacterium* is a dominant genus in the intestinal microbiota of breast-fed infants, in some cases representing approximately 75 % of total bacteria (Harmsen *et al.*, 2000; Roger & McCartney, 2010; Sakata *et al.*, 2005; Yatsunenکو *et al.*, 2012). The overrepresentation of bifidobacteria in this environment is less observed in formula-fed infants, who show a more diverse microbiota (Fallani *et al.*, 2010; Penders *et al.*, 2006). Therefore, differences in bacterial colonization between breast-fed and formula-fed infants can be explained in great part by the non-essential components in human milk.

The predominance of bifidobacteria in breast-fed infant faeces was first noticed over 100 years ago (Moro, 1905). Moro and Tissier suggested that breast milk contained certain molecules that stimulated the growth of these bacteria, defined as *bifidus factors* (Moro, 1905). Gynolactose was later described as a mixture of milk oligosaccharides containing GlcNAc that stimulated the growth of certain *Bifidobacterium* strains (Polonowski & Lespagnol, 1931). These studies first suggested a prebiotic role for oligosaccharides in breast milk.

The ability of these micro-organisms to metabolize HMO might therefore represent an example of co-evolution with

their host, and the physiology and mechanism of this consumption has been addressed. Ward and co-workers first showed that *Bifidobacterium longum* subsp. *infantis* (*B. infantis*) can grow vigorously on HMO *in vitro* as the sole carbon source (Ward *et al.*, 2006). *B. infantis* displays a preference for shorter HMO but can use larger oligosaccharides as well (LoCascio *et al.*, 2007). The ability to consume HMO *in vitro* has been demonstrated for additional strains of *B. infantis* and also *Bifidobacterium bifidum*, and to a lesser extent strains of *Bifidobacterium breve* and *Bifidobacterium longum* (Asakuma *et al.*, 2011; LoCascio *et al.*, 2009; Turroni *et al.*, 2010). These four species are usually present in breast-fed infant faeces (Turroni *et al.*, 2012; Yatsunenکو *et al.*, 2012; Avershina *et al.*, 2013; Boesten *et al.*, 2011; Matsuki *et al.*, 2002).

Hence, the enrichment in bifidobacteria in breast-fed infant faeces can be explained in great part by their ability to consume and metabolize HMO. Moreover, the prebiotic character of HMO seems to be selective for infant bifidobacteria and a few *Bacteroides* species, and not for adult bifidobacteria or other prominent members of the intestinal microbiota such as *Clostridium* and enterobacteria (Marcobal *et al.*, 2010). Bottle-fed infants display higher numbers of Firmicutes and *Bacteroides* and less of *Bifidobacterium* in their faeces, and the bifidobacteria characteristic of formula-fed infants include additionally *Bifidobacterium adolescentis* and *Bifidobacterium pseudocatenulatum*, which are more commonly associated with the adult intestinal microbiota (Haarman & Knol, 2005; Mangin *et al.*, 2006).

Bifidobacterial strategies for HMO consumption

Bifidobacteria possess a fermentative metabolism, and they are almost exclusively associated with the GI tract of animals (Lee *et al.*, 2008; Sela *et al.*, 2010). They are considered to be beneficial for the human host, and several strains of bifidobacteria are commercialized as probiotics. In general they devote a significant portion of their genomes to the consumption of complex oligosaccharides (O'Connell Motherway *et al.*, 2011b; Schell *et al.*, 2002; Sela *et al.*, 2008; Turroni *et al.*, 2010), either of plant origin in the case of adult-associated species or host-derived in the case of species better adapted to the nursing period. Analysis of genome sequences of bifidobacteria isolated from breast-fed infants has enabled predictions regarding the HMO consumption phenotype. In particular, *B. infantis* ATCC 15697 and *B. bifidum* PRL2010 are prototypical members of the infant intestinal microbiota that have possibly co-evolved with their host to consume milk or host oligosaccharides (Sela *et al.*, 2008; Turroni *et al.*, 2010).

Physiologically, *B. infantis* can simultaneously consume distinct classes of HMO *in vitro* with high efficiency, reaching higher cell densities compared to other infant-associated bifidobacteria (Asakuma *et al.*, 2011; Ward *et al.*, 2006). A hallmark of the genome of this species is a

conserved cluster of genes, the HMO cluster I (Fig. 2), containing several glycosyl hydrolases and ABC transporters (Sela *et al.*, 2008). Other potentially important clusters for HMO consumption are also conserved among other *B. infantis* strains but are absent in *B. longum* strains (LoCascio *et al.*, 2010). The overall overrepresentation of genes encoding family 1 solute binding proteins (SBPs) and also intracellular glycosyl hydrolases (GHs) with putative affinity for or activity on HMO in the genome of *B. infantis* is suggestive of a consumption strategy based on the import of intact HMO structures and their intracellular enzymic degradation (Zivkovic *et al.*, 2011). Several of these predictions regarding HMO consumption have been addressed and genes encoding functions in HMO import and hydrolysis have been identified (Fig. 2).

For example, a large array of oligosaccharide-binding SBPs in *B. infantis* ATCC 15697 is biased towards mammalian glycans, especially HMO (Garrido *et al.*, 2011). Their substrate affinities are diverse and include neutral HMO containing either LNB or LacNAc (type 1 or type 2 HMO), or fucosylated HMO such as 2'FL and Lewis epitopes. Chemical blockage of ABC transporters inhibits the ability of *B. infantis* to consume lacto-*N*-tetraose (LNT; type 1 HMO) as the sole carbon source *in vitro* (Yoshida *et al.*, 2012). In addition, genes encoding SBPs with affinity for HMO are exclusively induced during exponential growth on HMO (Fig. 2). In addition, some of these proteins are able to bind epithelial surfaces *in vitro*, probably due to the structural similarities between HMO and epithelial glycoconjugates. These results also suggested physiological differences between *B. infantis* cells growing on either simple lactose or HMO, where epithelium-binding SBPs are induced only during growth on HMO. In agreement with these observations, *B. infantis* cells growing on HMO but not lactose display increased binding to intestinal epithelial cells, and under these conditions they enhance the production of anti-inflammatory cytokines and tight junction proteins (Chichlowski *et al.*, 2012).

Another relevant aspect of bacterial HMO utilization is the enzymic processing of these molecules by glycosyl hydrolases. Interestingly, the microbiome of breast-fed infants is enriched in fucosidases and sialidases (Yatsunenkov *et al.*, 2012). The genome of *B. infantis* contains an army of glycosyl hydrolases active on these carbohydrates, including two genes encoding α -sialidases, five α -fucosidases, five β -galactosidases and three β -*N*-acetylglucosaminidases (Garrido *et al.*, 2012a). Recent functional studies on the enzymic properties of these enzymes and their induction by HMO have greatly advanced our understanding of HMO consumption by *B. infantis* (Fig. 2). Acidic HMO such as 3'SL, 6'SL and sialyl-LNT represent nearly 15% of total HMO. These are probably imported inside the cells by systems different from ABC transporters, and membrane permeases of the major facilitator family are likely candidates. NanH2 is an α -sialidase in *B. infantis* (Blon_2348 in HMO cluster I, Fig. 2) that removes sialic acid from α 2-3 and α 2-6 sialyl linkages found in individual

HMO such as mono and disialyl-LNT (Sela *et al.*, 2011). The expression of NanH2, but not a second encoded sialidase NanH1, was significantly increased during bacterial growth on HMO. In addition, two fucosidases in *B. infantis* have significant activity in fucosylated HMO and blood group oligosaccharides. Blon_2335 and Blon_2336 are located in HMO cluster I and belong to GH families 95 and 29, respectively (Fig. 2). Growth on pooled HMO, LNT or LNnT (a type 2 HMO) upregulates their gene expression (Sela *et al.*, 2012). Blon_2335 is a highly efficient α 1-2 fucosidase that has also considerable activity towards α 1-3 and α 1-4 fucosyl linkages, contrary to the AfcA fucosidase in *B. bifidum*, which acts exclusively on Fuc α 1-2 linkages (Ashida *et al.*, 2009). Blon_2335 can release fucose from several HMO such as 2'FL, 3FL and lacto-*N*-fucopentaoses I and III, and also from fucosylated epitopes found in epithelial glycoconjugates such as Lewis^a [Gal β 1-3(Fuc α 1-4)GlcNAc], Lewis^x [Gal β 1-4(Fuc α 1-3)GlcNAc] and the H-disaccharide (Fuc α 1-2Gal). In the other hand Blon_2336 is specific for α 1-3/4 linkages, such as those found on 3FL, lacto-*N*-fucopentaose III and Lewis^x (Sela *et al.*, 2012).

Galactose and *N*-acetylglucosamine constitute the building blocks of HMO, and these monosaccharides are generally fermentable carbon sources for bifidobacteria. Galactose is found in simple carbohydrates such as lactose and complex oligosaccharides of mammalian or plant origin. In *B. infantis*, two β -galactosidases with glycolytic activity on HMO are constitutively expressed (Yoshida *et al.*, 2012). Bga2A, encoded by Blon_2334 in HMO cluster I (Fig. 2), belongs to GH family 2 and has a preference for lactose, also efficiently removing galactose from type 2 HMO such as LacNAc and LNnT. Complementing this activity is Bga42A (encoded by Blon_2016; Fig. 2), a GH42 β -galactosidase highly specific for LNT, one of the most abundant HMO (Yoshida *et al.*, 2012). Interestingly, this enzyme has considerably less activity on LNB, suggesting that each residue in LNT is crucial for its enzymic activity. Finally, β -*N*-acetylglucosaminidases participate in this process (Garrido *et al.*, 2012c). Blon_0459, Blon_0732 and also Blon_2355 in the HMO cluster I are expressed mostly during early exponential growth on HMO, and while the three enzymes can cleave the GlcNAc β 1-3Gal linkage found in linear HMO such as LNT or LNnT, only Blon_0459 and Blon_0732 are active on branched HMO, characterized by GlcNAc β 1-6Gal as found in lacto-*N*-hexaose. These results support the concept of sequential hydrolysis of HMO in *B. infantis*, releasing large amounts of monosaccharides that can be fermented in central metabolic pathways.

Parallel studies have provided important details on the mechanisms of HMO utilization by *B. bifidum*, another member of the infant intestinal microbiota. *B. bifidum* and *B. infantis* are competitive in HMO consumption but using different strategies (Garrido *et al.*, 2012a). While *B. infantis* has specialized in the import and intracellular deglycosylation of several HMO, *B. bifidum* uses an array of

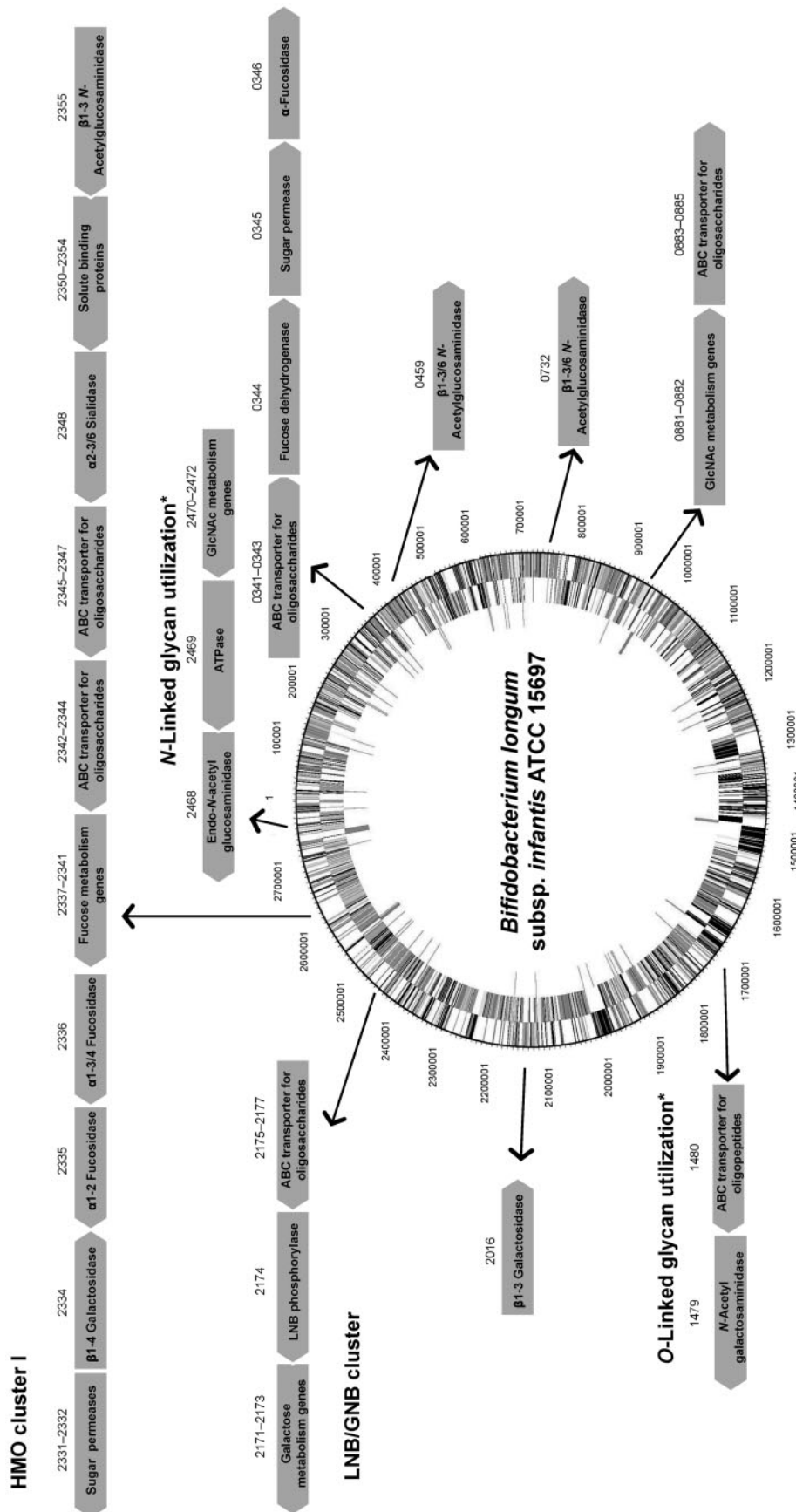


Fig. 2. Clusters of genes in *B. infantis* ATCC 15697 with assigned or putative functions in the utilization of milk glycans. Numbers above the arrows correspond to the respective locus tags (Blon_###). Genes are not drawn to scale, and the genome circle was adapted from Sela *et al.* (2008). SBPs from ABC transporters with affinities for HMO and expressed during growth on these substrates were identified by Garrido *et al.* (2011). An α -sialidase and two α -fucosidases were characterized by Sela *et al.* (2011, 2012). Two β -galactosidases and three β -hexosaminidases active on different linkages in representative HMO are also included (Garrido *et al.*, 2012c; Yoshida *et al.*, 2012). Finally, potential gene clusters for *N*-linked and *O*-linked glycan utilization (*) are depicted (Garrido *et al.*, 2012b; Kiyohara *et al.*, 2012).

membrane-associated glycosyl hydrolases, including extracellular α -sialidases (Kiyohara *et al.*, 2011), α -fucosidases (Ashida *et al.*, 2009), β -galactosidases and β -*N*-acetylglucosaminidases (Miwa *et al.*, 2010), which efficiently remove monosaccharides from complex HMO. Another important difference between these strategies is the presence of membrane lacto-*N*-biosidase activity in *B. bifidum* (Wada *et al.*, 2008). This endoglycosidase generates LNB and lactose from LNT, and some of the mono- and disaccharides released can be internalized and metabolized, especially LNB (Asakuma *et al.*, 2011). The binding protein for this disaccharide is a family 1 SBP, part of a gene cluster found in several bifidobacteria, the LNB/GNB cluster (Kitaoka *et al.*, 2005; Nishimoto & Kitaoka, 2007). Genes encoding ABC permeases, a lacto-*N*-biose phosphorylase that generates galactose 1-phosphate and glucose from LNB, and two other genes in the Leloir pathway for galactose metabolism (Fig. 2), are adjacent to this SBP. The LNB/GNB cluster is actually conserved across all infant gut-associated bifidobacteria, including *B. bifidum*, *B. infantis*, *B. longum* and *B. breve* isolates (LoCascio *et al.*, 2010; Xiao *et al.*, 2010).

Relatively little attention has been paid to *B. breve* and *B. longum* regarding HMO consumption, even considering that they are normally dominant in infant faeces, and *B. breve* seems to be found exclusively in this environment (Avershina *et al.*, 2013). *B. longum* ATCC 15707 and *B. breve* ATCC 15700 show only modest growth on pooled HMO and apparently can metabolize solely LNT, leaving other HMO unmodified (Asakuma *et al.*, 2011; LoCascio *et al.*, 2007). An association between the LNB/GNB cluster and HMO consumption in *B. longum* ATCC 15707 was suggested after induction of these genes during growth on human milk (González *et al.*, 2008). Several species of bifidobacteria, including strains of *B. breve* and *B. longum*, are able to grow *in vitro* using LNB as the sole carbon source (Xiao *et al.*, 2010). This consumption can be explained solely by the presence and activity of the LNB/GNB cluster in these strains that can import and metabolize this disaccharide.

Human milk glycoconjugates

The complexity of human milk is far from understood, and one example of this is the multiplicity of functions played by several bioactive agents. While the high concentrations of oligosaccharides in human milk can explain in great part the enrichment in bifidobacteria in breast-fed infant faeces,

glycans conjugated to other molecules in milk, such as proteins, peptides or lipids, can also have a prebiotic role. Here we address some of the biological properties of these glycoconjugates and what the mechanisms are that infant bifidobacteria have devised to use these glycoconjugates as a carbon source.

Human milk glycolipids

Lipids make up 3–5 % of the total volume of human milk, of which 98 % are triacylglycerols (Jensen, 1999). A fraction of the remaining fats in human milk consists of glycolipids, mostly associated with the milk fat globule membrane (Newburg & Chaturvedi, 1992). Glycolipids are composed of a lipid chain of ceramide, a fatty acid linked to a sphingoid base, covalently attached to one or more monosaccharides. Milk glycolipids can be classified as neutral, including galactosylceramide (Gal β 1-1Cer) and glucosylceramide (Glc β 1-1Cer; Bouhours & Bouhours, 1979), or acidic glycosphingolipids (or gangliosides), containing sialic acid (Laegreid *et al.*, 1986). The most abundant gangliosides in human milk are GD3 (Fig. 1) and GM3 (NeuAc α 2-3Gal β 1-4Glc β 1-1Cer; Lee *et al.*, 2011).

The glycan portion of milk glycolipids plays an important role in pathogen deflection, similar to other milk glycoconjugates. Binding the epithelium is the first line of entry for pathogens or their toxins, and this process is usually mediated by glycolipids. Therefore, milk-borne glycolipids associated with milk fat globule membranes can prevent bacterial, viral or toxin binding to the intestine (Lindberg *et al.*, 1987; Miller-Podraza *et al.*, 2005; Newburg, 2009; Otnaess *et al.*, 1983). In addition, several intestinal commensals are able to bind glycolipids *in vitro* (Mukai *et al.*, 2004; Neeser *et al.*, 2000; Strömberg *et al.*, 1988; Yamamoto *et al.*, 1996).

Milk fat globules are degraded by diverse lipases in the GI tract (Lindquist & Hernell, 2010), releasing lipids that are readily absorbed into the small intestine. However, the fate of milk glycolipids is not yet understood, and it is possible that they transit distal portions of the GI tract. Only a few studies have addressed the degradation of milk or epithelial glycolipids by members of the infant intestinal microbiota, and evidence has indicated that they possess enzymes that can hydrolyse in great part these glycoconjugates (Larson *et al.*, 1988). Right after establishment, the intestinal microbiota is responsible for the degradation of glycolipids observed in breast-fed infant faeces (Gustafsson *et al.*, 1986; Midtvedt *et al.*, 1988). The degree of this hydrolytic activity

is however lower than that in adults, but higher compared to newborns or germ-free mice (Larson *et al.*, 1987; Larson & Midtvedt, 1989).

It has been observed *in vitro* that glycosidases from *Ruminococcus torque*, *B. bifidum* and *B. infantis* degrade several glycosides containing certain blood group determinants, including the H disaccharide, Lewis^a and Lewis^b (Falk *et al.*, 1991; Larson *et al.*, 1988). Lactosylceramide is a common end product of their reactions (Falk *et al.*, 1990). The ability of bifidobacteria to release sialic acid from predominant milk gangliosides such as GD3 and GM3 has been observed (Falk *et al.*, 1990), suggesting that certain bifidobacteria possess α 2-8 and α 2-3 sialidase activity (Fig. 1).

Human milk glycoproteins

Protein glycosylation is a post-translational modification in which a glycan is covalently linked to predetermined amino acids in the protein structure. There are two major types of oligosaccharides attached to eukaryotic proteins: *N*-linked and *O*-linked glycans. These conjugated carbohydrates play several biochemical and physiological roles, for example in protein synthesis, folding, trafficking and secretion, resistance to proteolysis, and prevention of pathogen colonization of epithelial surfaces among several others (Barboza *et al.*, 2012; Gopal & Gill, 2000; Newburg *et al.*, 2005; Peterson *et al.*, 1998; Rudd *et al.*, 1994). In human milk a large number of human milk proteins are glycosylated, including lactoferrin, immunoglobulins and κ -casein among several others (Froehlich *et al.*, 2010).

N-Linked glycans are attached to the protein via specific asparagines in the sequence Asn-xxx-Ser/Thr (Stanley *et al.*, 2009). All *N*-linked glycans have in common a pentasaccharide with the structure Man₃GlcNAc₂, where the last GlcNAc is linked to the asparagine via a β -linkage (Fig. 1). This pentasaccharide can sometimes be modified with core α 1-6 fucosylation or a bisecting GlcNAc. *N*-Glycans can be heterogeneous and tissue-specific, but three main classes of *N*-glycans have been described based on further modifications of the pentasaccharide: (a) high mannose, consisting of branches of α -mannose; (b) complex, characterized by core α 1-6 fucosylation of the basal GlcNAc and by two or more antennae (Gal β 1-4GlcNAc repeats) that can be additionally decorated by fucose or sialic acid in terminal positions (Fig. 1); and (c) the hybrid type, which consists of a mixture of these two types. The human milk *N*-glycome has been recently described in detail, and in general human milk *N*-glycans are largely fucosylated and present in larger concentrations compared to bovine milk (Dallas *et al.*, 2011; Nwosu *et al.*, 2012). In contrast, bovine *N*-glycans are also more sialylated and characterized by the presence of *N*-glycolylneuraminic acid (NeuGc) instead of *N*-acetylneuraminic acid (Nwosu *et al.*, 2012).

O-Linked glycans differ from *N*-linked glycans by attachment to serine or threonine residues, with no obvious surrounding amino acid consensus sequence. Eight different

core structures have been described, each beginning with an α -GalNAc attachment to the amino acid (Brockhausen *et al.*, 2009). Only four of these core structures (cores 1–4) are usually found in humans (Brockhausen *et al.*, 2009). These *O*-linked structures can be further elongated by *N*-acetyllactosamine chains and decorated by fucose, sialic acid or GalNAc at terminal positions (Fig. 1).

Bifidobacterial consumption of human milk glycoproteins

In milk, protein glycosylation increases the resistance of proteins to proteolysis (van Berkel *et al.*, 1995), probably contributing to the excretion of considerable amounts of intact or partially degraded milk proteins in breast-fed infant faeces (Davidson & Lönnerdal, 1987; Prentice *et al.*, 1989). Milk proteins vary in their digestibility (Le *et al.*, 2012; Ye *et al.*, 2011), and non-glycosylated proteins such as β -casein and α -lactoglobulin are more digested in comparison to lactoferrin, IgA and milk mucins (Jakobsson *et al.*, 1982; Lindh, 1975; Prentice *et al.*, 1989).

Therefore, breast milk glycoproteins, in conjunction with mucosal secretions and shed epithelial cells, transit the GI tract of the breast-fed infant and can play a role in shaping the developing intestinal microbiota. Evidence indicates that these microbes largely modify host glycoconjugates (Hoskins *et al.*, 1985; Variyam & Hoskins, 1981). Germ-free mice secrete intact mucins in their faeces, while conventionalized animals are able to degrade and metabolize mucins completely (Corfield *et al.*, 1992; Midtvedt *et al.*, 1987). In addition, bacteria extracted en masse from adult and infant stools produce a variety of extracellular glycosidases that degrade the glycans of hog gastric mucin under anaerobic conditions (Midtvedt *et al.*, 1988; Variyam & Hoskins, 1981). In addition, individual members of the infant and adult intestinal microbiota have been well studied for their ability to deglycosylate mucins in order to gain access to the bound oligosaccharides as a carbon source (Derrien *et al.*, 2010; Wright *et al.*, 2000). Several species of *Bacteroides* deploy exquisite mechanisms for mucin glycan degradation based on membrane-bound glycosyl hydrolases and importers that are crucial for the survival and predominance of these species in the adult microbiota (Bäckhed *et al.*, 2005; Martens *et al.*, 2009). Interestingly, certain *Bacteroides* species can utilize HMO (Marcobal *et al.*, 2010), and the transcriptional responses elicited during growth *in vitro* on mucin are highly similar to those witnessed on HMO for *Bacteroides thetaiotaomicron*, suggesting that these substrates are energetically similar for this species (Marcobal *et al.*, 2011).

Some bifidobacteria are also well-known mucin degraders (Crociani *et al.*, 1994; Hoskins *et al.*, 1985). To date, this phenotype seems to be exclusive to *B. bifidum* and certain isolates of *B. longum* (Ruas-Madiedo *et al.*, 2008). Pivotal to the release of *O*-linked glycans from mucins are endo- α -*N*-acetylgalactosaminidases (EngBF, glycosyl hydrolase family 101). Functional studies have shown that these

extracellular enzymes cleave Core 1 O-linked glycans (Gal β 1-3GalNAc α -Ser/Thr) found in mucins (Fig. 1; Ashida *et al.*, 2008; Fujita *et al.*, 2005). This hydrolysis releases galacto-*N*-biose (Gal β 1-3GalNAc; GNB), a disaccharide structurally similar to LNB from HMO that can be directly used as a carbon source by *B. longum* via an ABC importer and enzymes in the LNB/GNB cluster (Kitaoka *et al.*, 2005; Nishimoto & Kitaoka, 2007). Since EngBFs are highly specific, GNB release and import probably require the previous action of several glycosyl hydrolases on mucin glycans, such as α -fucosidases, α -sialidases and lacto-*N*-biosidase. These enzymes are also active on HMO, and the genes encoding these activities are highly expressed during growth *in vitro* on hog gastric mucin as well as on HMO (Turroni *et al.*, 2010).

An alternative mucin utilization pathway has been recently described in bifidobacteria, which might represent a more accurate representation of intestinal mucin degradation by these micro-organisms *in vivo* (Kiyohara *et al.*, 2012). The glycans on colonic mucins contain mostly Core 3 O-linked glycans based on the structure GlcNAc β 1-3GalNAc α 1-Ser/Thr (Fig. 1), which are inaccessible for EngBF. A novel endo- α -*N*-acetylgalactosaminidase (NagBb, GH129) in *B. bifidum* is specific for the Tn antigen (GalNAc α -Ser/Thr). This structure is potentially produced after extracellular degradation by glycosyl hydrolases. While this mechanism remains to be validated, this novel enzyme was shown to be present in several genomes of infant gut-associated bifidobacteria including *B. infantis* (Fig. 2), *B. breve* and *B. longum* (Kiyohara *et al.*, 2012), suggesting a common route to degradation of Core 3 O-linked glycans (Fig. 1).

The ability of bifidobacteria to access O-linked glycans in human or bovine milk proteins has been less explored; however, we hypothesize that similar mechanisms to those described above are prevalent for human milk mucins. As mentioned above, milk mucins contain a majority of Core 2 O-glycans (Fig. 1). It is possible that after gastric and intestinal digestive processes a higher concentration of mucin-derived glycopeptides is available for infant-associated bifidobacteria. This is not a new concept, as the bovine κ -casein-derived GMP is a highly sialylated glycopeptide that has been suggested to have prebiotic effects (Gomes *et al.*, 1998; Janer *et al.*, 2004; Petschow & Talbott, 1990).

We recently explored the ability of bifidobacteria to gain access to *N*-glycans from host glycoproteins using a representative panel of 76 strains of these micro-organisms isolated from infant faeces (Garrido *et al.*, 2012b). Endo- β -*N*-acetylglucosaminidases (EC 3.2.1.96; endoglycosidases) hydrolyse the *N,N'*-diacetyl-chitobiose core common to all *N*-glycans (Fig. 1). Genes encoding these enzymes were found in several isolates of *B. longum*, *B. infantis* and *B. breve*, and their presence correlated with the ability of these micro-organisms to release the *N*-linked glycan of bovine RNase B. Among these enzymes, those belonging to glycosyl hydrolase family 18 (GH18) were able to remove

the *N*-glycans from bovine and human lactoferrin (Garrido *et al.*, 2012b), containing high mannose and complex *N*-glycans, respectively (Nwosu *et al.*, 2011; Yu *et al.*, 2011). Further characterization by MALDI-Fourier transform ion cyclotron resonance (FTICR) MS of endoglycosidase EndoBI-1 from *B. infantis* ATCC 15697 (Blon_2468, Fig. 2) revealed that this enzyme can deglycosylate common host glycoproteins such as IgA and IgG in their native forms in addition to human and bovine lactoferrin. Surprisingly, EndoBI-1 cleavage specificity was wide, releasing *N*-glycans with a variety of structures including high mannose *N*-linked glycans, or complex glycans with core α 1-6 fucosylation, chain sialylation or fucosylation, and bi- and tri-antennary structures (Fig. 1). Furthermore, incubation of the enzyme with fresh breast milk samples led to a complete removal of milk protein *N*-glycans.

Replicating the bifidogenic effect of breast milk

For some mothers, breastfeeding is not possible, and therefore there is an increased need for human milk substitutes. Commercial production of synthetic mimics of HMO or other milk glycoconjugates is challenging, given the diversity of complex glycans involved. However, commercial production of more simple HMO species such as LNnT, 2'FL and 6'SL is now possible, as is the ability to test these molecules for bifidogenicity in animal (Marcobal *et al.*, 2011) and human trials. Other prebiotics such as fructooligosaccharides (FOS), galactooligosaccharides (GOS) and inulin (Gibson *et al.*, 2004; Torres *et al.*, 2010) are commonly included in infant formula. GOS are synthetic substrates derived enzymically from the transglycosylation of lactose, with a degree of polymerization of 3–15 (Barboza *et al.*, 2009). It has been suggested that GOS resemble galactan chains found in plant oligosaccharides (O'Connell Motherway *et al.*, 2011a). We recently observed that the consumption of GOS with a large degree of polymerization is strain-dependent in *B. infantis* isolates (Garrido *et al.*, 2013), and discrete mechanisms for import and intracellular degradation are active in *B. infantis* strain ATCC 15697. On the other hand, FOS and inulin are naturally found in chicory plants. The wide availability of FOS and GOS has enabled numerous *in vitro*, animal and human studies of their prebiotic effects, and their bifidogenic effect is currently accepted (Bakker-Zierikzee *et al.*, 2005; Brunser *et al.*, 2006; Davis *et al.*, 2011).

Analysis of the milk of other mammals indicates that they do not possess the high concentration of oligosaccharides combined with a high level of fucosylation witnessed in human milks. For example, mature bovine milk is very low in free oligosaccharides, which are mainly sialylated (Sundekilde *et al.*, 2012; Tao *et al.*, 2008). More efficient analytical tools have recently revealed the presence of low concentrations of several neutral fucosylated oligosaccharides that resemble derivatives of lacto-*N*-neohexaose present in early milk HMO (Barile *et al.*, 2010; Sundekilde *et al.*, 2012). At present, new approaches are

being applied to use dairy streams from cheese production to recover bovine milk oligosaccharides (BMO) in larger quantities (Zivkovic & Barile, 2011).

Conclusion and future directions

The complexity of breast milk is intriguing and still far from being understood. The fundamental role of human milk as a nutrient source for the infant has been the focus of study for decades, with the critical goal of understanding and improving nutritional deficiencies during the neonatal period. However, the influence of breastfeeding beyond nutrition is increasingly being revealed, demonstrating that milk provides much more than protection against pathogens. Breastfeeding has been associated with a variety of long-term health impacts including lowered incidence of obesity (Kalliomäki *et al.*, 2008), diabetes (Mayer *et al.*, 1988; Owen *et al.*, 2006; Pettitt *et al.*, 1997) and allergies (Gdalevich *et al.*, 2001; Snijders *et al.*, 2007). A future challenge will be to identify a mechanistic basis for these benefits.

An infant gut microbiota dominated by bifidobacteria has long been associated with health; however, our understanding of this process is still unclear. Recently the protective role of production of short-chain fatty acids by certain species of bifidobacteria against pathogenic *E. coli* challenge (Fukuda *et al.*, 2011) has been demonstrated. This work highlighted the importance of *in situ* metabolism of complex carbohydrates by bifidobacteria in host-microbe interactions. However, the amounts of protective acetate and lactate produced by bifidobacteria can be different depending on the growth substrate, which might have direct consequences for the host (Garrido *et al.*, 2013). Consumption of certain HMO can also be a selective colonization factor; for example, *B. infantis* grows vigorously on lacto-*N*-neotetraose, and this ability enables the bacterium to outcompete *Bacteroides thetaiotamicron* in a mouse model, emphasizing the selectivity and bifidogenic activities of these unique glycans (Marcobal *et al.*, 2011).

A clear benefit of mechanistic research is the rapid nature by which this information can be translated to address a range of intestinal maladies (Gordon *et al.*, 2012). The ability to purify, or synthesize, HMO-like oligosaccharides and/or glycoconjugates at commercial scales is increasingly becoming a reality. This ability, combined with an expanding number of well-characterized bifidobacterial strains that grow on these complex milk glycans, will help to design tailored synbiotic formulations to target specific at-risk populations such as premature and malnourished infants. Given that milk is the product of millions of years of mammalian evolution, it is not surprising that it displays a constellation of health benefits for the infant. With the advances in nanotechnology and systems biology perhaps this 'constellation' will become more comprehensible and inspire new opportunities for protective modulation of the human GI tract.

Acknowledgements

We acknowledge all the researchers in the UC Davis Foods for Health Institute and the Milk Bioactives Program for their enthusiasm, imagination and collective contribution to this subject matter. Work by the Milk Bioactives Program has been supported by University of California Discovery Grant Program, the California Dairy Research Foundation, the Bill and Melinda Gates Foundation and National Institutes of Health awards R01HD059127, R01HD065122, R01HD061923, R21AT006180, R01AT007079. D.G. has been supported in part by a Fulbright-Conicyt Chile scholarship and a National Milk Producers Federation scholarship. D.A.M. acknowledges support as the Peter J. Shields Endowed Chair in Dairy Food Science.

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