


Relationship Between Oligosaccharides and Glycoconjugates Content in Human Milk and the Development of the Gut Barrier

Susana Figueroa-Lozano  and Paul de Vos

Abstract: The intestinal immune barrier is considered to be the gatekeeper of the human body and rapidly develops directly after birth. Many pre- and postnatal factors influence the development of the gut-barrier, which is composed of the microbiota, the mucus, the epithelial layer and the mucosal immune system. Even minor disturbances during barrier development can have consequences for health far into adulthood. Here we critically discuss the current knowledge on which pre- and postnatal factors influence development, maturation, and maintenance of the gut immune barrier. Human milk has a unique composition and is the gold standard for adequate development of the intestinal immune barrier. Not only the influence of human milk oligosaccharides (HMOs) but also that of glycoproteins (HMGP) is reviewed. We discuss the influence of maternal genetic factors, such as the secretor and Lewis phenotypes on breast milk fucosylation and sialylation of HMOs and HMGP. This diversity in HMOs and HMGP influences microbiota composition and also the development of the immune barrier. Cow milk-derived infant formula is often being used as an alternative for human breast milk. The consequences of this for proper development of the intestinal immune barrier and, in particular, the differences in the type of oligosaccharides and glycosylation patterns (sialic and fucose composition) between cow and human milk are critically discussed. Current and prospective strategies to promote proper gut-immune maturation are proposed. These might include more personalized infant formulas when breast milk is not an option.

Keywords: functional properties, HMOs, human milk glycoproteins, infant formula, intestinal barrier

Introduction

The gastrointestinal immune barrier is considered to be the gatekeeper of the human body. The intestinal immune barrier is responsible for the passage and absorption of nutrients but also functions as a barricade for pathogens to enter the human body. In addition, it regulates the crosstalk between the luminal microbiota and macromolecules and maintains tolerance and immune responses (König et al., 2016; Takiishi, Morales-Fenero, Olsen, & Camara, 2017). Directly after birth this gatekeeper function of the intestinal barrier is not fully developed yet and, therefore, this period after birth is considered to be a vulnerable period for disease development. Any disruption of the neonatal gut barrier function has been found to have long-term effects and may play a role in the development of gastrointestinal infections (Halpern & Denning, 2015) and inflammatory bowel disease (IBD). Also, it can influence the development of other diseases during childhood and adulthood, such as obesity (Argenio & Salvatore, 2015) and allergies (Groschwitz & Hogan, 2011). While the etiology of

these diseases remains to be fully elucidated, multiple factors, such as nutrition (Jacobi & Odle, 2012), feeding practice (O'Sullivan, Farver, & Smilowitz, 2015), microbial colonization (Houghteling & Walker, 2015), delivery mode (Rutayisire, Huang, Liu, & Tao, 2016), maternal genetic factors and immunity (Ganal-Vonarburg, Fuhrer, & Gomez de Agüero, 2017), and host-genetic factors (Ussar, Fujisaka, & Kahn, 2016) are considered to be crucial to shape the gut microbiota and the gastrointestinal immune response.

In the vulnerable period after birth, the mother supports the gastrointestinal barrier of the newborn by sharing its immune system with the baby by supplying antibodies, cytokines, chemokines, hormones, and even leucocytes in breast milk (Cacho & Lawrence, 2017). However, during recent years it has also been shown that proteins, lipids, and carbohydrates in breast milk can contribute to the nonspecific defense against pathogens (Jakaitis & Denning, 2014). Proteins, lipids, and carbohydrates in breast milk modulate immune responses to self- and nonself-antigens, orchestrate microbial colonization, and drive the morphological and immunological maturation of the gut barrier (Turfkruyer & Verhasselt, 2015). Particularly, milk carbohydrates have emerged recently as essential molecules from human milk that protect the newborn from infections and educate the immune barrier system in early life (Smilowitz, Lebrilla, Millis, German, & Freeman, 2014).

In this review we discuss the current insight in how pre- and postnatal factors influence development and maturation of the

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intestinal immune barrier. To this end, we first review the diverse barriers that exist in the intestine and the crosstalk between these barrier components that together form the human intestinal gatekeeper. Next, we review and critically discuss the current knowledge according to pre- and postnatal factors influence development, maturation, and maintenance of the gut immune barrier. We focus not only on the role of human milk oligosaccharides (HMOs), which have been reviewed in many papers (Holscher, Bode, & Tappenden, 2017; Kuntz, Rudloff, & Kunz, 2008), but also on glycoproteins (HMGP) (Lönnerdal, 2003). Cow milk-derived infant formula is often being used as an alternative for human breast milk. The consequences of this for proper development of the intestinal immune barrier, and in particular the differences in the type of oligosaccharides and glycosylation patterns (sialic and fucose composition) between cow and human milk, are critically discussed. Finally, we discuss the influence of maternal genetic factors that affect the fucosylation and sialylation on HMO and HMGP diversities and the associated differences in microbiota composition and development of the immune barrier.

Intestinal Barrier

The intestinal barrier has primarily the function to absorb nutrients while at the same time prevent undesired molecules and organisms to enter the organism. The barrier has evolved into a complex multilayer system to appropriately fulfill this function (Scalaferrri, Pizzoferrato, Gerardi, Lopetuso, & Gasbarrini, 2012). The human intestinal barrier can be seen or categorized as microbial, physical/chemical, and immunological barriers, which are under tight control of the enteric nervous system (Figure 1). The different components interact with each other and have, at the same time, specific functions which contribute to the homeostasis of the infant gut.

Microbial barrier

The microbial barrier develops rapidly after birth. It starts with colonization with the maternal microbiota. This microbiota is an indispensable component of the gut intestinal barrier. The microbiota forms a barrier for pathogens that can adhere to the intestinal wall, but the microbiota can also change the microenvironment making adhesion of undesired intruders impossible (Gritz & Bhandari, 2015). Unlike the adult microbiota, the infant microbiota is highly susceptible during the first 1 to 2 years of life to environmental changes (Weng & Walker, 2013). Disturbances may lead to changes in composition and microbiota diversity (Yang et al., 2016). The adult gut can host up to 1000 bacterial species. This is currently the total amount identified worldwide in diverse individuals (Argenio & Salvatore, 2015). The neonate is during the first weeks of life only populated by 15% of the adult species (Gritz & Bhandari, 2015).

Also, products made by the microbiota are essential for the adequacy of the intestinal immune barrier. Fermentation products from gut bacteria, such as short-chain fatty acids (SCFAs) are immune regulating and serve as nutrients for epithelial cells (Iraporda et al., 2015). Fermentation products, such as SCFA also affect other functions of the gut barrier, such as proliferation and differentiation of immune cells (Blottière, Buecher, Galmiche, & Cherbut, 2003), enhance gut motility that can lead to expedited disposal of pathogens via the fecal route (Karakı & Kuwahara, 2010; Koh, De Vadder, Kovatcheva-Datchary, & Bäckhed, 2016), and strengthening of the tight junctions between gut epithelium (Ohata, Usami, Ph, & Miyoshi, 2005).

Physical and chemical barrier

The intestinal epithelial cells create a physical and biochemical barrier that separates the microbiota from the gut epithelium, and at the same time facilitates the crosstalk between the microbes, immune, and nervous system (Yoo & Mazmanian, 2017).

The physical barrier refers to the columnar epithelial cells that separate the lumen from the lamina propria (Groschwitz & Hogan, 2011). The layer is mostly composed of enterocytes and secretory intestinal epithelial cells which include Paneth, goblet, and enteroendocrine cells (Okumura & Takeda, 2017). The integrity of the gut barrier depends on the ability of enterocytes to maintain the gut permeability and induce oral tolerance (Snoeck, Goddeeris, & Cox, 2005). The permeability of the epithelium relies on a network of desmosomes, adherens junctions, and tight junctions that regulate the passage of fluids, nutrients, and micro/macromolecules from the lumen to the submucosa or vice versa (Arrieta, Bistritz, & Meddings, 2006). The proteins that form the adherens junctions and desmosomes are formed by cadherin-catenin complexes, whereas tight junctions are formed by occludins, claudins, junctional adhesion molecules, and tricellulin (Anderson, 2001). Alterations in the formation of these networks result in epithelial barrier dysfunction (Förster, 2008).

The expression of the proteins that form the tight junctions is known to be modulated by cytokines, microbiota, and immune cells (Groschwitz & Hogan, 2011). Likewise, food components have been found to increase or reduce gut permeability *in vitro* and *in vivo* (Kosińska & Andlauer, 2013; D. Ulluwishewa et al., 2011). Isolated food components, such as nondigestible oligosaccharides (Akbari et al., 2017), bovine lactoferrin (J. Wu et al., 2014), lectines (Ohno, Naganuma, Ogawa, & Muramoto, 2006), curcumin, glutamine (Rapin & Wiernsperger, 2010), and dietary flavonoids (genistein, myricetin, quercetin, and epigallocatechin) (Suzuki & Hara, 2011) have shown to protect the intestinal barrier from inflammation and oxidative stress associated to tight junction opening. In contrast, food components, such as deoxynivalenol, a contaminant of cereals, can cause permeability changes by altering the expression of claudins (Pinton et al., 2009).

Food additives, such as sucrose monoester (Mine & Zhang, 2003), and synthetic surfactants, such as cremophor EL or sodium taurocholate (Hamid, Katsumi, Sakane, & Yamamoto, 2009), disturb the tight junction distribution. The understanding of how food components can modulate the permeability of the gut barrier by altering gene expression is relevant because it has given new insights on the impact of this feature in health and disease (Smyth, 2017). Furthermore, it has open opportunities to use this knowledge for therapeutical interventions (Odenwald & Turner, 2016). For instance, capsaicin may improve the paracellular transport of drugs that are poorly absorbed (M. Kaiser, Chalapala, Gorzelanny, Perali, & Goycoolea, 2016), whereas specific prebiotic carbohydrates have the ability to ameliorate the symptoms of patients with “leaky gut” (Akbari et al., 2015).

The chemical barrier consists of the mucus layer that separates the epithelium from the microbiota and luminal content. The mucus layer is mostly formed by the secretory products from goblet, Paneth, and enteroendocrine cells (L. W. Peterson & Artis, 2014). The most abundant component of the mucus layer are mucins, which are large glycoproteins synthesized at the epithelial level, specifically in the goblet cells (Birchenough, Johansson, Gustafsson, & Hansson, 2015). Goblet cells secrete other proteins that maintain the integrity of the mucus barrier (TFF3, CLCA1),

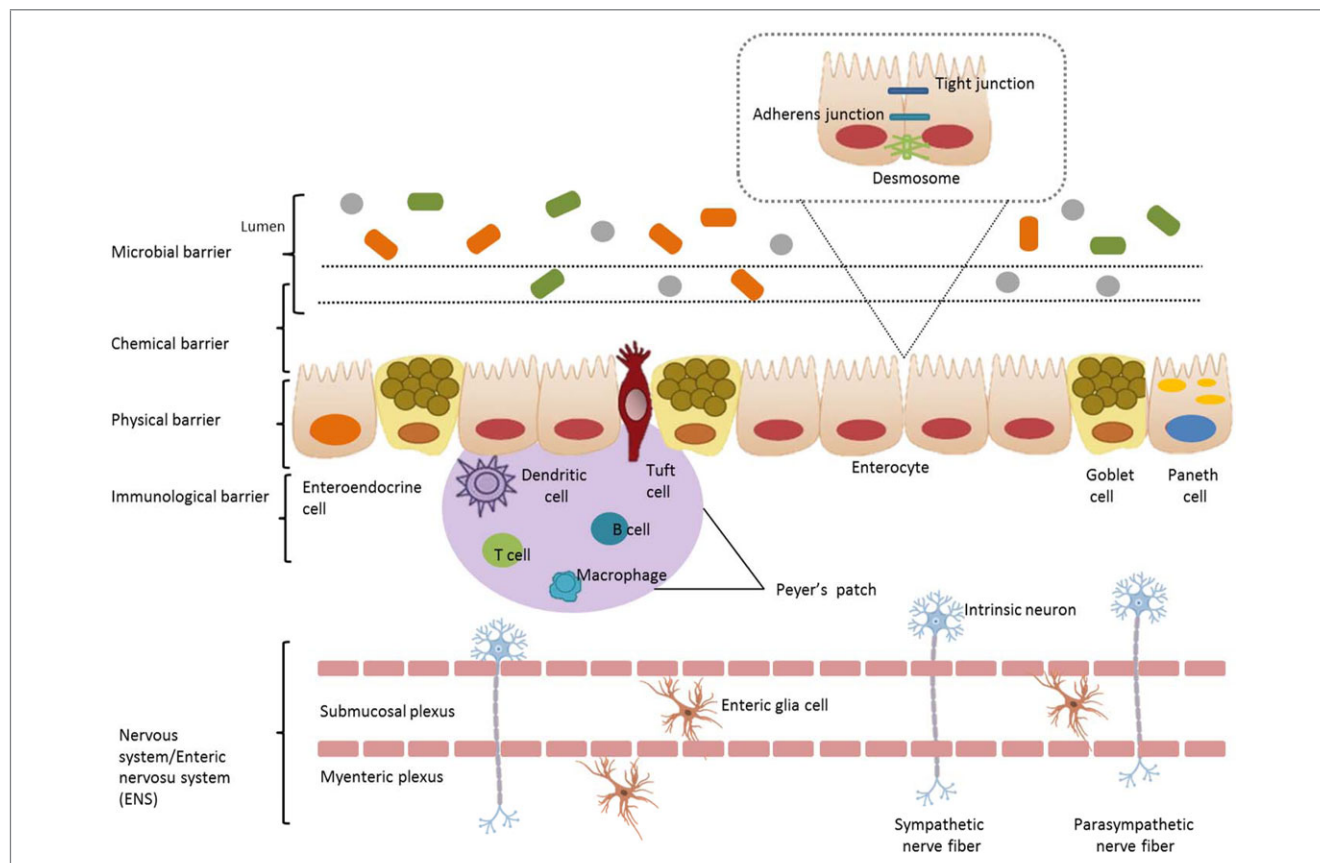


Figure 1—Anatomy of the intestinal barrier. The gastrointestinal tract is composed of different barriers that interact with each other. These components can be categorized as microbial, physical/chemical and immunological barriers, which are under control of the nervous system/ENS. The microbial layer in the proximal colon is composed of two mucus layers. The microbiota and the metabolic products from their digestion interact with all the other components of the gut barrier. The physical barrier is formed by enterocytes, goblet cells, Paneth and enteroendocrine cells (EECs), which secrete a broad array of molecules that facilitate the separation between barrier but at the same time facilitate the cross-talk between components. In the lamina propria many immunological cells reside in the Peyer's patches and lymphoid tissue. These immune cells are in close contact with the glia and neurons in the submucosa and myenteric plexus.

regulate susceptibility to colonic inflammation (RELM), and maintain the separation between the microbial bed and the epithelial layer (ZG16) (Birchenough et al., 2015). The goblet cells not only produce mucus, they also have an active role in the gate-keeping function by delivering luminal content to dendritic cells (Pelaseyed et al., 2014), and by regulating immune responses (Johansson & Hansson, 2014).

The mucus layer separates intestinal compartments but also provides substrate for growth of bacteria. In return, gut microorganisms and their fermentation products alter the composition and gel properties of the mucus (Jakobsson et al., 2014). There is a continuous crosstalk between the microbiota, luminal products, and goblet cells. For example, luminal dietary components (Bhatia et al., 2015), or cytokines derived from the epithelium can alter the secretory function of the goblet cells (Johansson et al., 2014).

Paneth cells are abundant in the small intestine and can be found in the proximal but not distal colon (Simmonds, Furman, Karanika, Phillips, & Bates, 2014). Their function is to secrete antimicrobial components, such as lysozymes, defensin α (HD5-HD6), and β (hBD-1, -2, -3), phospholipase A2, angiogenin 4, and cathelicidins (Gassler, 2017). These molecules modulate the proliferation of microbial communities along the intestine and act by stimulating pattern recognition receptors (PRRs), such as Toll-like receptors (TLR), specifically via TLR4/Myd88 and

nucleotide-binding oligomerization domain-containing protein 2 (NOD2) pathways (H. Wang et al., 2017). Such activations elicit immune pathways that maintain the intestinal epithelial integrity (Halpern & Denning, 2015). Food components have been observed to alter the secretion of Paneth cells. In experiments with mice, the consumption of a high-fat diet, altered the composition of the microbiota and the expression of antimicrobial peptides by the Paneth cells, stimulating intestinal inflammation (Guo et al., 2017).

Finally, the enteroendocrine cells (EECs) contribute to barrier function. EECs comprise only 1% of the epithelial cell layer and its population is generally less diverse in the colon than in the small intestine (Gunawardene, Corfe, & Staton, 2011). There are at least 15 subtypes of EECs that secrete regulatory factors, such as glucagon-like peptide, vasoactive inhibitory peptide, and gastric inhibitory peptides that modulate gut motility, gut transit, and food intake (Hooper, 2015). EECs play a pivotal role in sensing luminal components derived from diet. These intestinal hormones signal for intestinal satiety (Posovszky & Wabitsch, 2015). TLRs reside also on the surface of EECs and because of these, they are considered to operate also as immune sensors (Bogunovic et al., 2007). Many studies suggest that dysfunctional EECs function make the intestine prone to inflammatory disorders (Moran, Leslie, Levison, & McLaughlin, 2008).

Immunological barrier

The immunological barrier consists of cells, such as activated T-cells, mast-, plasma-, dendritic- cells, and macrophages that are in constant communication with the epithelial cells to sense immune status and secrete cytokines that prevent, for instance, invasion of pathogens (Groschwitz & Hogan, 2011). The crosstalk between the luminal side and the immune barrier counter regulates anti-inflammatory responses in order to maintain the balance between immune defense and tolerance. An altered cytokine environment might translate to a pro-inflammatory response when pathogens are sensed. In intestinal inflammatory diseases a Th1-polarized activation has been observed (Strober & Fuss, 2011). In these patients the presence of high levels of TNF α and IFN γ increases gut permeability by inhibiting the myosin light chain kinase (MLCK) phosphorylation (Cunningham & Turner, 2012). This process is key to maintain tight junction integrity and thus barrier function. Therefore, such inhibition increases gut permeability (Wang et al., 2005).

Role of nervous system in barrier integrity

The permeability of the intestinal epithelial lining is regulated by a network of glia and neurons called the enteric nervous system (ENS) which is located in the submucosa and the myenteric plexus. The ENS regulates many gastrointestinal tract functions: secretion (peptides, water, electrolytes), epithelial transport, motility, microvascular circulation, vascular tone, release of hormones, and immunological defense (Rao & Gershon, 2016). The regulation by the ENS is autonomous in certain aspects (Yoo & Mazmanian, 2017). The glia and myofibroblasts secrete neurotransmitters and even cytokines that facilitate the communication with the epithelium, promote epithelial growth, and contribute to the maintenance of gut-barrier function by regulating the expression of tight-junction-associated proteins (Puzan, Hosis, Ghio, & Koppes, 2018).

Alterations in the regulatory mechanism actions of the ENS have been shown to be directly associated to the development of intestinal and neurological disorders (Cani & Knauf, 2016), illustrating its essential role in gut barrier development. This process begins at birth. Together with the microbial, physical, chemical, and immunological barrier, the ENS (re)wires connections and adapts rapidly to the fast changing microenvironment in the gut during the first 2 weeks of the postnatal period (Hao et al., 2013). This adaptation process remains active during later life, by continuously reshaping the nervous responses upon changes in microbiota composition and changes in dietary habits (Heuckeroth & Schäfer, 2016).

Glia and enteric neurons can respond to changes in microbiota by TLRs. Enteric glia and neurons express TLR-4 and TLR-7 that recognize LPS from Gram-negative bacteria and viral RNA, respectively (Barajon et al., 2009). Furthermore, microbial metabolism products can increase nerve density in the submucosa (Kabouridis & Pachnis, 2015). Nutrition is important for adequate development of the ENS and it has been shown that cytokines, growth factors in human milk as well as neurotrophic factors shape the ENS (Fichter et al., 2011). Culturing myenteric plexus from newborn rats with breast milk showed differences in the amounts of neuronal networks and neuronal densities compared to cultures without breast milk (Schäfer, Ginneken, & Copray, 2009). Diet can also influence the production of the hormone serotonin (5-HT), which is responsible for the modulation of mood, sleep, and cognition at the CNS. Although 5-HT is produced in the brain and accumulate in platelets, 80% of the 5-HT secretion occurs in

the gastrointestinal tract where it regulates the motility and the secretory function of the intestine. Studies in patients with IBD and healthy controls showed that the depletion of the natural precursor for the biosynthesis of 5-HT, tryptophan, had an impact in the ENS, affecting for instance visceral perception, pain and, diarrhea episodes (Kilkens, Honig, Van Nieuwenhoven, Riedel, & Brummer, 2004).

Pre- and Postnatal Events Involved in Intestinal Barrier Maturation

In order to achieve an operational response in the gut many temporal and spatial factors interact to induce homeostasis of the gut barrier function. Here we cluster these factors in four groups: nutrition, microbial colonization and maternal (genetic and non-genetics) factors, and host genetic factors (Figure 2).

Nutrition

Nutrition by breast feeding. As the needs of the gastrointestinal immune system change rapidly during the first days of life, the composition, abundance, and diversity of glycans and protein glycosylation in breast milk varies during lactation. Human milk glycans are present as free human milk oligosaccharides (HMOs) or as glycoproteins (HMGP) and glycolipids (HMGLs) (Smilowitz et al., 2014). HMOs are made of linear or branched monosaccharides, such as glucose, galactose, *N*-acetylglucosamine, fucose, and sialic acid (Lars Bode, 2012). HMOs have a lactose core and are enzymatically elongated by β 1-3 and β 1-6 linkages to units of lacto-*N*-biose and *N*-acetylglucosamine, respectively. The HMOs are further elongated by the addition at the terminal positions of units of fucose and sialic acid (Figure 3). The fucose units are linked by α 1-2, α 1-3, or α 1-4 bonds, whereas sialic acid residues are linked by α 2-3 or α 2-6 bonds. In term breast milk 35–50% of HMOs are fucosylated, 12–14% are sialylated, and 42–55% are nonfucosylated neutral HMOs (Totten et al., 2012).

These structures are indigestible for the neonate and can reach the colon without being digested and serve as substrate for the immature microbiota (Davis et al., 2016). However, HMOs modulate the maturation of the intestinal barrier at more levels. Thanks to their diverse and complex glycosylation pattern HMOs promote enterocyte differentiation (Holscher, Davis, & Tappenden, 2014), induce indirectly the production of mucus (Cornick, Tawiah, & Chadee, 2015), and serve as decoy for pathogenic microorganisms (L Bode, 2015). Some Bifidobacteria strains can metabolize HMOs, such as 2-fucosyllactose (2'-FL), whereas other strains of species consume other types of HMOs. The utilization of 2'-FL pathways by Bifidobacteria has shown to alter the profile of metabolites produced and to shape the infant intestinal microbiota (Matsuki et al., 2016; Underwood et al., 2015).

The posttranslational modification that attaches a glycan to a protein backbone is called glycosylation. Glycans can be covalently attached to a protein via threonine or serine (*O*-glycosylation) or asparagine (*N*-glycosylation). While the protein core of glycoproteins is determined genetically, the glycan decoration does not follow a fixed template, which results in a diverse array of possible glycoprotein structures (Froehlich et al., 2011). For instance, a protein with three active glycosylation sites and ten different glycans in each site can display at least 1000 different glycoforms of the protein (Nagae & Yamaguchi, 2012). There is growing evidence that this diversity in milk glycoproteins is functional and essential for immune recognition. The structural diversity in glycosylation determines the function of the glycoprotein and type of immune

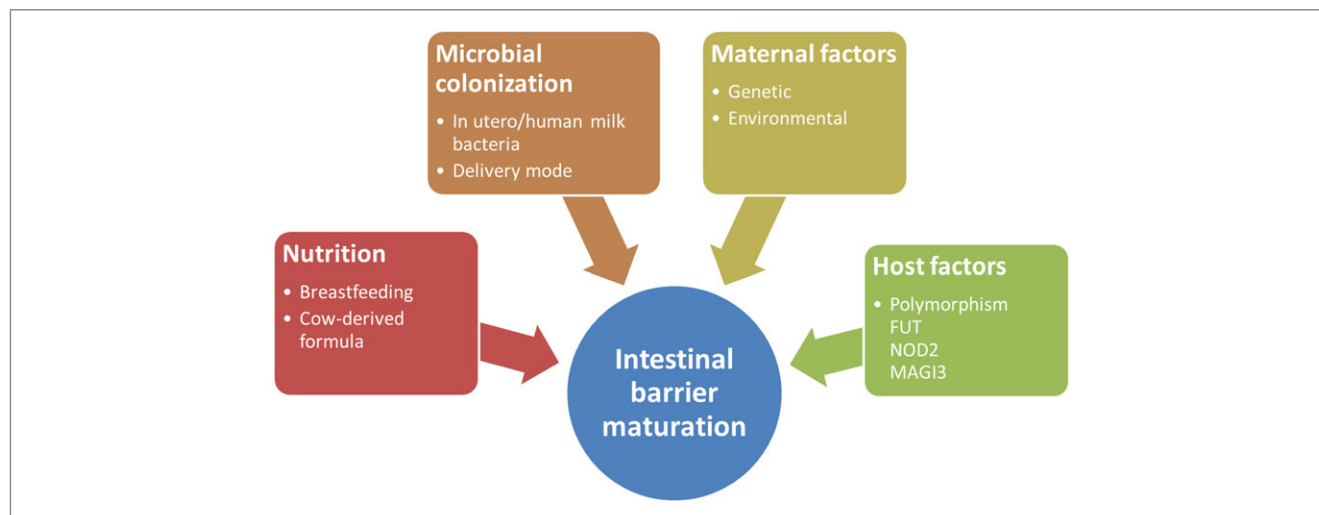


Figure 2—Factors that influence intestinal barrier function maturation. Current insight in the interplay between genetic and nongenetic factors from the mother and the infant in the intestinal barrier development.

response that is induced (Figure 4A and B) (Molinari, Casadio, Hartmann, Arthur, & Hartmann, 2013).

Glycosylation affects many characteristics of the glycoprotein, such as the inter- and intra-cellular signaling (Zhao et al., 2008), proper folding (Kleizen & Braakman, 2004), solubility, protein targeting and trafficking, ligand recognition and binding, immunogenicity, pharmacological and biological activity, pharmacokinetic profile, and structure stability (Yu et al., 2011). Glycosylation can limit the access of proteases, thereby improving the overall resistance of the molecule to proteolysis. The glycosylation pattern is responsible for which glycopeptides are produced (Nishiyama, Kimura, Uchida, Ochi, & Yamaguchi, 2000). In the intestinal barrier these glycopeptides can resemble HMOs and serve as growth substrate for gut bacteria (Liepke et al., 2002). Most commensal bacteria, such as *Bifidobacterium* and *Bacteroides*, are equipped with endoglycosidases and able to further cleave the glycans from the glycoproteins and glycopeptides and use them as substrate for fermentation and growth (Marcobal & Sonnenburg, 2012).

The HMOs and HMGP's composition and glycosylation varies during lactation. Colostrum typically contains in average 23 g/L of HMOs and it decreases to an average of 7 g/L in mature milk (Gabrielli et al., 2011). Some of the major HMGP's and their average concentrations in human milk are: mucins (729 mg/L), secretory immunoglobulin A (200 mg/L), lactoferrin (1–7 g/L), lactoperoxidase (0.77 mg/L), lacto-adherin (93 mg/L), leptin (0.003 mg/L), lysozyme (21 mg/L), and α -lactalbumin (2.4 g/L) (Liu & Newburg, 2013) (Table 2). During lactation the composition of breast milk and quantity of these molecules change (Froehlich et al., 2010). For instance, the lactoferrin content decreases from 5.8 g/L in colostrum to 2 g/L in mature milk (Montagne, Cuilliere, Mole, Bene, & Faure, 2001). Besides, the profile of glycosylation changes from day 1 to day 15 from less fucosylated to highly fucosylated structures (Barboza et al., 2012).

The structures of more than 200 HMOs have been identified, whereas more than 400 HMGP's have been characterized in human milk (Picariello, Ferranti, Mamone, Roepstorff, & Addeo, 2008). The changes in concentration and composition during lactation vary accordingly with the infant developmental needs (Orczyk-Pawilowicz, Berghausen-Mazur, Hirnle, & Kątnik-Prastowska, 2015). At the beginning of lactation the highly

fucosylated and sialylated structures of breast milk protect the gut from bacterial and viral infection by inhibiting pathogen adhesion, while promoting the colonization of beneficial communities of bacteria (Lis-Kuberka, Kątnik-Prastowska, Berghausen-Mazur, & Orczyk-Pawilowicz, 2015). The survival of neonatal gut microbiota relies highly on the glycans present in human milk. HMOs, HMGP's, and HMGLs facilitate the adhesion of beneficial bacteria to the mucus layer and act as decoy for pathogenic bacteria, parasites, or viruses promoting their clearance from the intestine.

The type of HMGLs found in human milk are also known as glycosphingolipids (Figure 4C). These molecules consist of a ceramide moiety attached via an amide-linked acyl group to a glycan portion. Based on the type of glycan they are attached to, they are categorized as cerebrosides and gangliosides. In cerebrosides, the ceramide moiety is attached to an uncharged glycan, whereas in gangliosides the reducing terminal of the ceramide contains sialic acid (Hernell, Timby, Domellöf, & Lönnnerdal, 2016). While the ceramide confers hydrophobicity to the molecule, the glycan portion confers charge to the glycosphingolipid (Merrill, 2011). They can be bound to macromolecules, such as human milk fat globule membrane (MFGM). While the ceramide portion is inserted in the lipidic layer, the glycans are exposed towards the hydrophilic environment (Lopez & Ménard, 2011).

The total amount of gangliosides in mature human milk ranges from 1.6 to 68.6 mg/L (Ma et al., 2015). While 61–86% (0.9–20.3 mg/L) of the glycolipid composition in colostrum corresponds to the Sia α 2–8 Sia α 2–3 Gal β 1–4 Glc β 1–1 ceramide (GD3), the most abundant (4.3–21.4 mg/L) ganglioside in mature milk is the Sia α 2–3 galactose (Gal) β 1–4 glucose (Glc) β 1–1 ceramide (GM3) (Pan & Izumi, 2000; Perea-Sanz, Garcia-Llatas, & Lagarda, 2018). These changes in ganglioside composition during lactation are attributed to changes in the expression of glycosyltransferases in the mammary tissue. They confer protective effects to the intestinal and brain development of the neonate (Palmano, Rowan, Guillermo, Guan, & McJarrow, 2015).

Like HMOs and HMGP's, HMGLs prevent the adhesion of pathogens and bacterial toxins derived from microorganism, such as *E. Coli* and *Giardia muris*. HMGP's have shown antiviral activity against human respiratory syncytial virus and enterovirus 71 (R. Peterson, Cheah, Grinyer, & Packer, 2013; Yamayoshi,

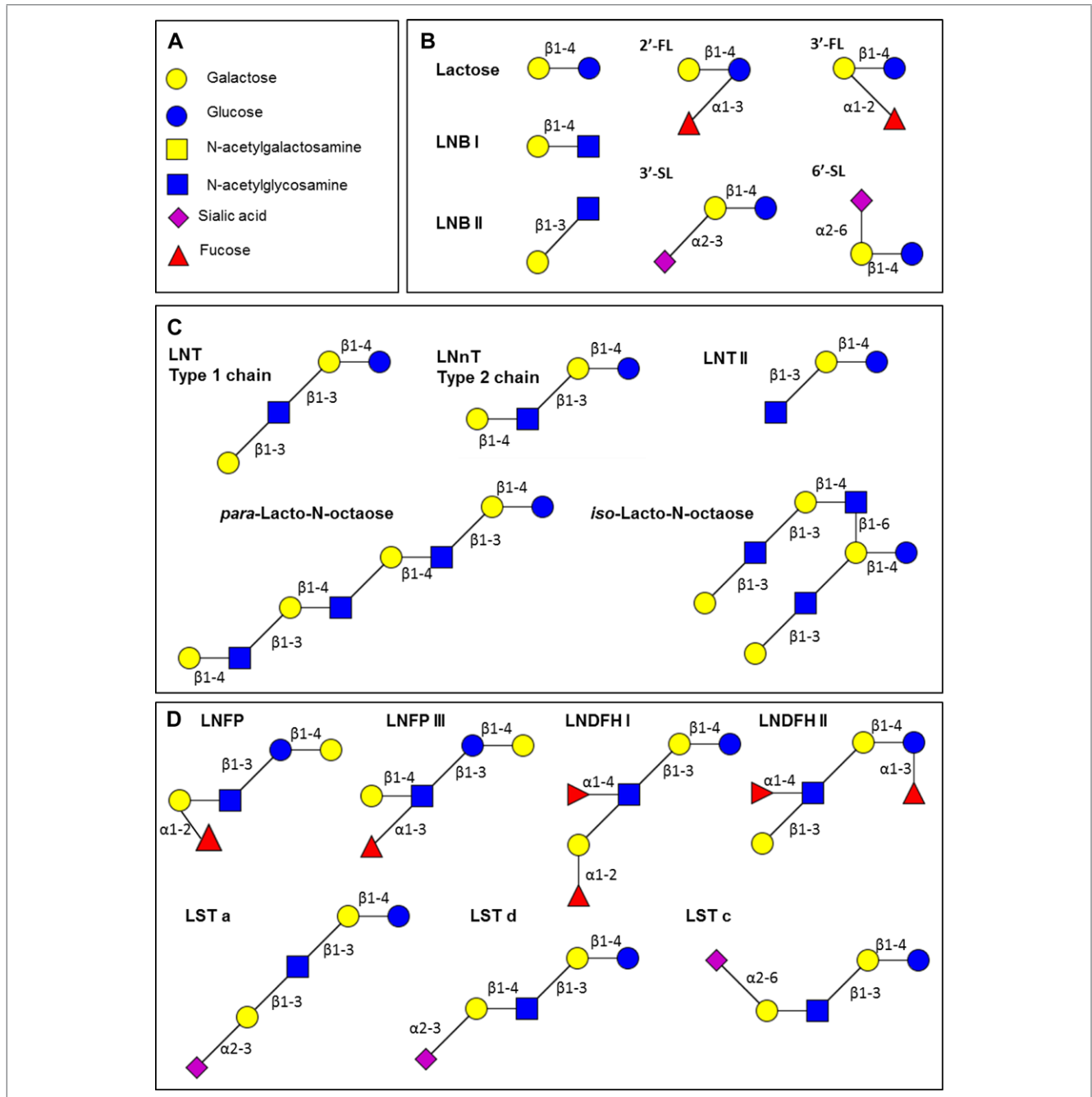


Figure 3—Structure of HMOs. (A) Monosaccharides that compose HMOs. (B) Lactose is an essential building block for larger and more complex oligosaccharides. It is present at the reducing end of all core structures. Lactose can be further elongated with Lacto-*N*-biose (LNB I) and *N*-acetylglucosamine (LNB II) to generate branched structures, such as lacto-*N*-tetraose (LNT), lacto-*N*-neotetraose (LNnT), or isomeric forms, such as Lacto-*N*-tetraose II (LNT II), *para*, and *iso*-HMOs. (C) Additionally, lactose can be further elongated with Lacto-*N*-biose (LNB I) and *N*-acetylglucosamine (LNB II) to generate branched structures, such as lacto-*N*-tetraose (LNT), lacto-*N*-neotetraose (LNnT), or isomeric forms, such as Lacto-*N*-tetraose II (LNT II), *para*, and *iso*-HMOs. (D) Finally, the elongated oligosaccharides can undergo fucosylation or sialylation at different linkage positions, which in turn produce different isomeric forms: Lacto-*N*-fucopentaose I (LNFP), lacto-*N*-fucopentaose III (LNFP III), lacto-*N*-difucohexaose I (LNDFH I), lacto-*N*-difucohexaose II (LNDFH II), LS-tetrasaccharide a (LSTa), LS-tetrasaccharide d (LSTd), LS-tetrasaccharide c (LSTc). Image elaborated with GlycoWorkbench 1.0.

Fujii, & Koike, 2014). It has been reported that glycosphingolipids maintain intestinal tolerance by modulating dendritic cell maturation (Rueda, 2007). Besides, they support the colonization of the neonatal gut. Some Bifidobacteria species can hydrolyze sialic acid from HMGLs and use it as growth substrate, whereas other species consume sialic acid only from HMOs (H. Lee, Garrido, Mills, & Barile, 2014).

Cow-derived infant formula. According to UNICEF and the WHO, exclusive breastfeeding is recommended for the first 6 months of life (WHO, 2006). However, according to their 2017 report not one country complies with this recommendation and considerable variations exist across regions. In Europe it is estimated that only 20 to 40% of infants between 0 to 5 months old are exclusively fed with breast milk. Many medical and social factors are responsible

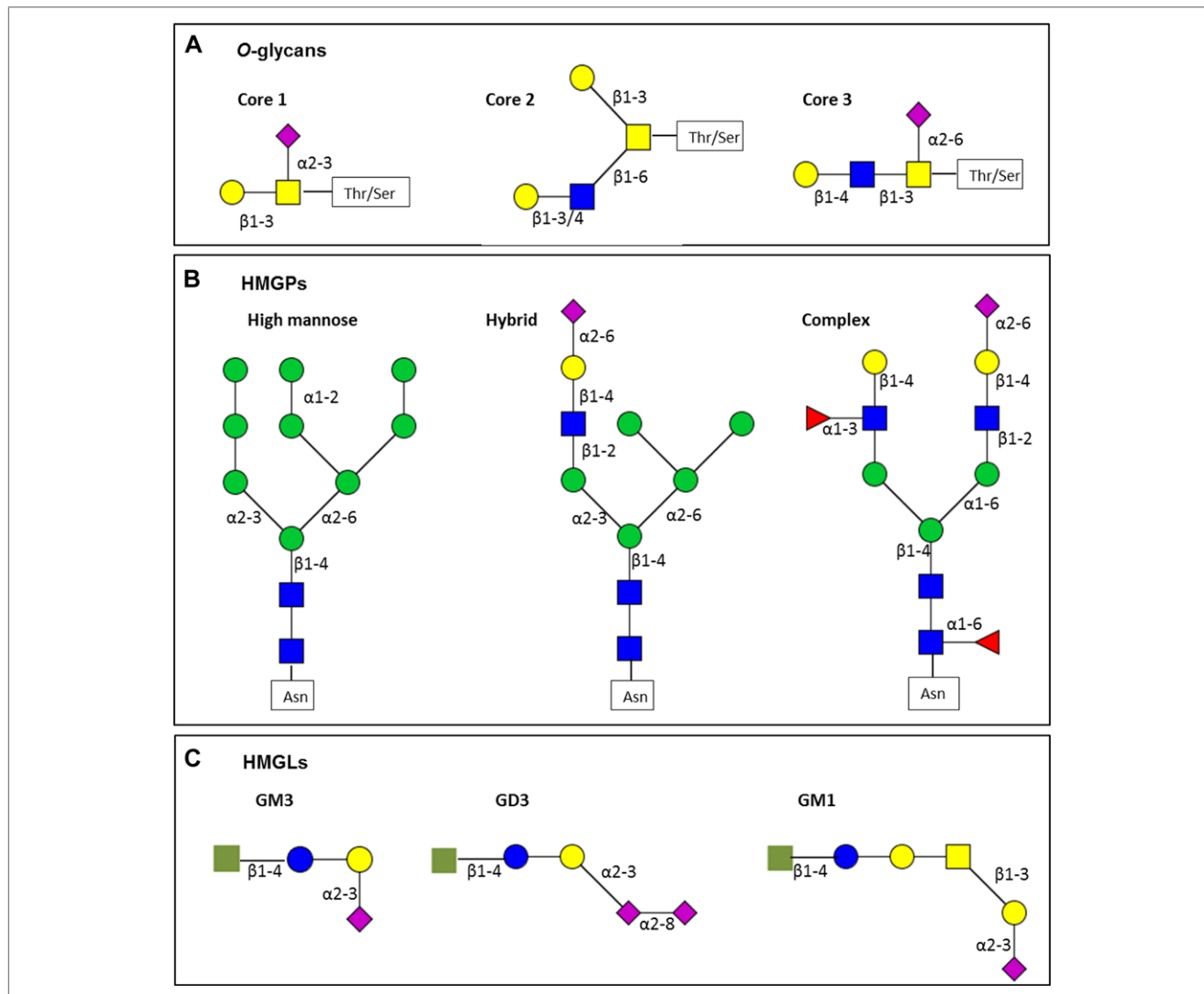


Figure 4—Glyconjugates in human milk. (A) Many proteins, such as mucins in human milk are *O*-glycosylated. Unlike, *N*-glycans, *O*-glycans do not share a common core structure. The three cores structures depicted are present in different tissues. However, human milk contains only core 2 type structures (Wilson et al., 2008). (B) HMGP are very heterogeneous. High mannose glycans contain unsubstituted terminal mannose units whereas on hybrid glycans, some mannose residues are substituted with an *N*-acetylglucosamine linkage. Complex glycans differ from the high mannose and the hybrid type because they do not contain mannose residues apart from those at the core structure. (C) Although HMGLs constitute a small fraction of the total lipid contained in human milk, they have a great impact on intestinal maturation and immunity.

for this (Amaral et al., 2015). In those cases supplementation or substitution of breastfeeding with infant formulas prepared from cow milk is the most common alternative for breast milk (Stevens, Patrick, & Pickler, 2009). These cow milk-based infant formulas should mimic as much as possible, the nutritional and functional profile of human milk (Martin, Ling, & Blackburn, 2016). However, the composition and patterns of oligosaccharides and glycosylation of proteins is different between human and bovine milk (Table 1).

While the concentration of milk oligosaccharides in human colostrum is approximately 7 g/L, bovine colostrum contains only 1g/L of oligosaccharides, and this concentration decays rapidly after 48 hours (Tao, Depeters, German, Grimm, & Lebrilla, 2009). Also, bovine milk GPs, such as lactoferrin, are found in lower concentrations (0.02–0.035 g/L) compared with human milk (Cheng et al., 2008). In fact, lactoferrin is one of the most abundant glycoproteins in human milk (Dallas et al., 2011).

Table 1—Differences in human and cow milk composition.

Component	Human	Bovine
Protein (g/dL) ¹	0.9 to 1.2	3.3
Fat (g/dL) ¹	3.2 to 3.6	3.7
Lactose (g/dL) ¹	6.7 to 7.8	4.5
Oligosaccharides (g/dL) ¹	0.7 to 1.2	0.1
No. of identified oligosaccharides ²	<200	approximately 40
% fucosylated ²	35% to 50%	approximately 1%
% sialylated ²	12% to 14%	Less than 25%

Sources:¹ (Ballard & Morrow, 2013), ² (Totten et al., 2012).

The composition of bovine and human milk glycans varies also in complexity and composition. HMOs can be classified into 13 core groups. These cores are elongated by β 1–3 or β 1–6 linkages to lactosamine units attached to fucose by α 1–2, α 1–3, α 1–4 linkage or to sialic by α 2–3 and α 2–6 linkages (Zivkovic

Table 2—Glycoproteins found in human milk. HMGP's vary greatly in structure and abundance. The concentrations reported correspond to those found in mature human milk.

HMGP's	concentration (g/dL)
Mucin ¹	72.9
Secretory immunoglobulin A ¹	20
Lactoferrin ²	0.1 to 0.7
Lactoperoxidase ¹	0.077
Lactoadherin ¹	0.0093
Lysozyme ²	0.0021
α -lactalbumin ¹	0.00024

Source:¹(Liu & Newburg, 2013),²(G. G. Kaiser et al., 2017).

& Barile, 2011). Bovine milk oligosaccharides (BMOs) contain N-acetylhexosamine, N-acetylneuraminic acid (Neu5Ac), and N-glycolylneuraminic acid (Neu5Gc), or sialic acid. The composition and variation, but also quantity, is lower compared to human milk. Neu5Gc is found in other mammals but not in humans due to the lack of the hydrolase that converts CMO-Neu5Ac to CMP-Neu5Gc in lactating mammary glands (Urashima, Taufik, Fukuda, & Asakuma, 2014). Structures, such as N-acetyl-D-lactosamine (LAcNAc), N,N'-diacetyllactosamine (LacdiNAc) and N-acetyl-D-galactosamine (GalNAc(β 1-4)) Glc have been detected in cow colostrum but not in human colostrum (Van den Nieuwenhof, Schiphorst, Van Die, & Van den Eijnden, 1999).

The reducing terminal sugar of the glycan chains determine its nature. Glycan chains containing fucose are classified as neutral oligosaccharides, whereas glycans with sialic acid as terminal sugar are classified as acidic oligosaccharides. In human milk, neutral oligosaccharides are more abundant than the acidic ones and the ratio of fucose is higher, whereas in cow milk acidic oligosaccharides predominate over neutral oligosaccharides and the amount of fucose is very low (Martín-Sosa, Martín, García-Pardo, & Hueso, 2003). As a result, the content of sialic acid in cow-derived formulas is less than 25% of the amount of sialic acid found in human milk (B. Wang, Brand-Miller, Mcveagh, & Petocz, 2001) and it contains trace amounts of fucose (Choi et al., 2015).

These differences in fucose and sialic acid content have a great impact on neonate health. At birth, neonates have a reduced capacity for de novo synthesis of sialic acid and, for this reason, the intake of this compound from breast milk is essential (Sprenger & Duncan, 2012). Sialic acid is involved in the development of the gastrointestinal tract and of the nervous system, in pathogen adhesion and in cellular recognition (Claumarchirant, Sanchez-Siles, Matencio, Alegría, & Lagarda, 2016). Especially in the gut, sialic acid prevents the adhesion of pathogens and promotes the growth of Bifidobacteria (Egan, Motherway, Ventura, & van Sinderen, 2014). From a nutritional point of view, sialic acid is crucial for immune and cognitive development (Röhrig, Choi, & Baldwin, 2017). However, the presence of some glycans, such as the Gal (α 1-3)Gal epitope present in bovine glycoproteins and not in HMGP's, have been linked with undesirable effects, such as allergic reactions (Rispen, Derksen, Commins, Platts-Mills, & Aalberse, 2013).

Likewise, fucosylated oligosaccharides have both indirect and direct effects on the intestinal barrier. Indirectly, fucose glycans exert defense mechanisms and promote gut maturation by supporting bifidogenesis. The release of SCFAs by *Bifidobacterium* stimulates gastrointestinal motility and development of the enteric nervous system (Bienenstock et al., 2013). The low amount of fucose in infant formula has been associated with the development of the

microbiota which then is significantly different from that of infants fed with breast milk (Zivkovic, German, Lebrilla, & Mills, 2011).

The differences in the type of oligosaccharides and glycosylation patterns among human milk and other species, such as bovines, have been speculated to be related to the system glycosylase/glycosyltransferases or to the enzymatic activity of epithelial cells (Nwosu et al., 2012). The effect of such differences is evidenced by the maturation of the intestinal barrier in infants fed with human milk or infant formula. Infants fed with cow-derived formula have a risk of having a longer period of enhanced gut permeability and a delayed colonization of beneficial bacterial communities (Stuebe, 2009).

Because infant formulas differ substantially from human milk, efforts are continuously made to improve their composition (Bourlieu et al., 2017). As described above the addition of nondigestible carbohydrates (NDCs) to infant formula increases the prebiotic value of the formulation. However, in order to mimic the content of fucose and sialic acid other compounds have to be added. Clinical studies have shown that the enrichment of infant formula with 2'-FL and lacto-N-neotetraose (LNnT) is well tolerated by infants (Puccio 2017) and might compensate for some of the differences between bovine milk and human milk. To compensate for the lack of sufficient amounts of sialic acid in cow infant formula, bovine whey is added to infant formula aiming to achieve sialic acid levels similar to human milk. However, it has been shown that the levels of sialic acid in infants from 0 to 6 months fed with infant formula supplemented with sialic acid were lower than the levels of sialic acid in infants fed with human milk (Claumarchirant et al., 2016).

Short duration or lack of breastfeeding, and the introduction of formula feeding before 6 months of age has been found to increase gut permeability, induce a shift of gut microbial colonization towards an adult pattern, and allows the translocation of pro-inflammatory bacteria (Houghteling & Walker, 2015). Formula-fed infants are colonized with a more diverse flora that includes *Escherichia coli*, *Clostridium difficile*, and *Bacteroides fragilis* compared to breastfed infants (Penders et al., 2005). The abundance of *Clostridium spp.* is associated with the development of atopic disorders (Penders, Stobberingh, van den Brandt, & Thijs, 2007). Furthermore, formula-feeding is regarded to be a risk factor for the development of type 1 diabetes (Patelarou et al., 2012) and metabolic disorders (Stuebe, 2009). However, in many instances breastfeeding is not possible and cow milk formula is the only viable alternative. Since HMOs cannot be manufactured on a large scale, milk formulas are supplemented with NDCs that mimic the biological activities of HMOs (Chen & Michael, 2017; Nauta & Garssen, 2013). NDCs are fermented by gut microbiota reducing the intestinal pH and favor the growth of *Bifidobacterium* and reduce the proliferation of pathogenic bacteria (Bouhnik et al., 2004). Infant formulas are enriched with NDCs produced by enzymatic synthesis, such as of galacto-oligosaccharides (GOS), fructo-oligosaccharides (FOS), or inulin (Akkerman, Faas, & de Vos, 2018; Vandenplas, Greef, & Veereman, 2014). The difference among compounds is reflected in their monosaccharide composition, branching, chain length, type of glycosidic linkage, and purity (Mussatto & Mancilha, 2007).

Cow milk formulas cannot prevent specific infections in infants that are neutralized by HMOs in breast-fed children. Noroviruses is an example of that. This virus is an important cause of diarrhea in infants (Shang et al., 2013), which is neutralized in breast fed infants by HMOs that resemble the glycan histo-blood structure (Hanisch, Hansman, Morozov, Kunz, & Schrotten, 2018). These

complex HMOs are not present in cow milk and, as a consequence, it has been found that infants fed with cow formula have a higher incidence of infectious diarrhea before the age of 4 months (Quigley, Kelly, & Sacker, 2007). Also among HMGPs, Lactoferrin is unique and has a broad spectrum of properties. This glycoprotein interacts directly with the anionic lipid A from Gram-negative bacteria damaging the microorganism membrane (Latorre, Puddu, Valenti, & Gessani, 2010). Using the same mechanism, lactoferrin clears the presence of parasites, such as *Entamoeba histolytica* (León-Sicairos et al., 2006).

Although cow milk is life-saving and the best alternative for breastfeeding it still cannot replace the effects of human milk on infant health (Victora et al., 2016). For instance, preterm infants fed exclusively with human milk have significantly lower intestinal permeability compared with infants that received a combined diet of breast milk and cow milk formula or only cow milk formula (Taylor, Basile, Ebeling, & Wagner, 2009). This effect is translated to reduced incidence of necrotizing enterocolitis, reduced parenteral nutrition duration, and reduced morbidity compared with pre-term infants fed with milk formula (Reisinger et al., 2014). Exclusive breastfeeding for at least the first 4–6 months of life is considered a protective factor to reduce the incidence of atopic diseases in susceptible individuals (Greer, Sicherer, & Burks, 2008), thus illustrating the importance of breast milk ingredients for healthy gut immune development and the need to further improve the alternatives for breast milk.

Microbial colonization

Infant acquisition of microbiota *in utero* and via human milk bacteria. An essential part of the development of immunity and barrier function is the healthy development of an individual's microbiota. The neonatal gut colonization has been suggested to occur by different mechanisms: early colonization *in utero* (Walker, Clemente, Peter, & Loos, 2017), entero-mammary route of transmission (Jost, Lacroix, Braegger, Rochat, & Chassard, 2014), and, maternal skin/infant oral cavity flow back during lactation (Biagi et al., 2017; Ramsay, Kent, Owens, & Hartmann, 2004).

The initial colonization of the surfaces of infant respiratory, urinary and gut mucosa starts, according to recent insights, *in utero* (Collado, Rautava, Aakko, Isolauri, & Salminen, 2016; Perez-Muñoz, Arrieta, Ramer-Tait, & Walter, 2017; Walker et al., 2017). This process occurs mostly in the last stage of pregnancy (Donnet-Hughes et al., 2010; Rodriguez, 2014). Recent studies have characterized the placenta meconium bacterial profile, showing that it includes Firmicutes, Tenericutes, Proteobacteria, Bacteroidetes, and Fusobacteria species (Aagaard et al., 2014; Cao, Stout, Lee, & Mysorekar, 2014). Likewise, the human placenta (Stout et al., 2013), and umbilical cord contain microorganisms, such as *Enterococcus faecium*, *Propionibacterium acnes*, *Staphylococcus epidermidis*, and *Streptococcus sanguinis* (Jiménez et al., 2005). Although the translocation mechanism is not clear, it has been suggested that bacteria from the maternal gut and oral cavity are transferred to the fetus through the placenta (Dong et al., 2015). Other mechanism is the ingestion of amniotic liquid which, not only contain growth factors, hormones, and immunomodulating proteins, but also microorganisms. During the third semester the fetus swallows 700 to 1000 mL of amniotic liquid per day (Magann, Sandlin, & Ounpraseuth, 2011). Therefore, fetal swallowing allows the exposure to these molecules and bacteria into the developing gastrointestinal tract (Chong, Bloomfield, & O'Sullivan, 2018).

As a support mechanism, to protect the infant from pathogens, the mother transfers beneficial bacteria to the neonate via breastfeeding (Prince et al., 2015). The transmission of intestinal bacteria from the maternal gut to the mammary gland has been suggested to occur via dendritic cells and the mesenteric lymph nodes (Murphy et al., 2017). This is known as the entero-mammary pathway. At least 200 bacterial species have been isolated from human milk but the great diversity is linked to the variations in the culture methods or geographical differences (Li et al., 2017). Bacteria from the phylum Proteobacteria, Firmicutes (Pannaraj et al., 2017), and Bifidobacteria have been found in human milk (Cabrera-Rubio, Mira-Pascual, Mira, & Collado, 2016). Although it is unknown how the stage of lactation affects the composition of bacteria in human milk, it has been observed that it affects the abundances of specific genera (Simpson et al., 2017).

Human milk introduces and supports beneficial bacteria that protect breastfed infants from diarrhea, respiratory and, metabolic disorders (Hunt et al., 2011). Furthermore, it stimulates the transitory gut microbiota in the infant and has a great impact on colonization of bacteria (Toscano, Grandi, Grossi, & Drago, 2017). The composition of HMOs is a predictor of the composition of human milk microbiota. The abundances of Bifidobacteria are positively linked to the presence of HMOs, such as, monofucosyllacto-N-hexaose III (MFLNH III), sialyllacto-N-tetraose (LSTb) and lactodifucotetraose (LDFT) (LoCascio et al., 2007; M. Wang et al., 2015). In contrast, in human milk containing 2'-FL and lacto-N-fucopentaose (LNFP 1), the prevalence of *Bacteroides* has been observed to be high given that this genus prefers to consume branched HMOs (Asakuma et al., 2011).

Effects of the delivery mode. The microbial colonization in the intestine with vaginal beneficial bacteria has long-lasting effects on the infant's health as for example, illustrated by the numerous comparative studies on differences in microbiota development in infants delivered vaginally or via C-section (Bjorksten, 2004; Eggesbø, Botten, Stigum, Nafstad, & Magnus, 2003; Negele et al., 2004). The newborn intestine is an aerobic environment which, within a week, ultimately becomes anaerobic (Johnson & Verwsalovic, 2012). In the first few days of life, after vaginal delivery, Bifidobacteria and Bacteroidetes will be predominantly present. *Bifidobacterium spp.* releases bioactive factors that rapidly reduce intestinal permeability (Ewaschuk et al., 2008). The same effect is achieved when *Lactobacillus acidophilus* bacteria colonize the gut. These species can alter and maintain the phosphorylation of the tight junction proteins (Cui et al., 2017). Via these mechanisms the composition of the gut microbiota can shape the immunological barrier, T-cell differentiation and activation (N. Lee & Kim, 2017). Furthermore, bacterial fermentation products can directly influence immunity. For example, butyrate from bacterial metabolism can induce Treg cell differentiation (Zhang et al., 2016).

In contrast, infants delivered by C-section have a lower microbiota diversity during the first 2 years of life (Jakobsson et al., 2014). Given that infants delivered by C-section miss the vaginal-derived bacteria, they depend for supply of bacteria on the breast skin microbiota of the mother which is composed of species from the genera *Staphylococcus*, *Corynebacterium* and *Propionibacterium spp.* and have low numbers of species from *Bacteroides* and *Bifidobacterium* (Dominguez-Bello et al., 2010). C-section delivery is not a risk factor for the development of intestinal bacterial infections in infants (Bager, Simonsen, Ethelberg, & Frisch, 2010), but the differences in microbial transmissions and colonization have been found to influence colonization of the microbial barrier and to

negatively affect the chemical, physical, and immunological components of the intestinal barrier (Chu et al., 2017).

Maternal factors

Genetic determinants. Secretor status and Lewis blood epitopes of the mother determine the composition of HMOs, HMGP, and HMGL and thereby influence the development of the gastrointestinal immune barrier of the neonate. These blood group variables determine the fucosylation of such molecules. The secretor gene encodes for α 1,2 fucosyltransferase (FUT2), whereas the Lewis blood type is determined by the Lewis gene, which encodes for α 1,3/1,4 fucosyltransferase (FUT3) (De Vries, Knechtel, Holmes, & Macher, 2001). Due to variations in the maternal fucosyltransferases the quantity and type of carbohydrates are differently expressed in pyloric or duodenal mucus and intestinal secretions (Mattos, 2016). Although HMOs and HMGP fucosylation has been extensively studied (Doherty, Lodge, Dharmage, Dai, & Lowe, 2018), HMOs and HMGP sialylation is not well understood yet (Grabaric, Csernák, Balogh, & Béni, 2017; Yan et al., 2018). Approximately 70–77% of the HMOs are fucosylated, whereas approximately 28% are sialylated (Ninonuevo et al., 2006).

The presence or absence of any of the secretor (Se) and Lewis (Le) phenotypes have been used to categorize human milk into four groups according to their neutral oligosaccharides: Se^+Le^+ [Le (a-b+)], Se^-Le^+ [Le (a+b-)], Se^+Le^- [Le (a-b-)], and Se^-Le^- [Le (a-b-)] (Thurl et al., 2010). Every lactating woman has a unique pattern of oligosaccharides (Blank, Dotz, Geyer, & Kunz, 2012; Thurl et al., 2010). However, data suggest that ultimately the composition of the HMOs relies more on the maternal Se status than on the Le blood type (Totten et al., 2012).

The content of Sialylated HMOs is high in colostrum (1500 mg/L) and decreases in mature milk (to 300 mg/L) (Ten Bruggencate, Bovee-Oudenhoven, Feitsma, van Hoffen, & Schoterman, 2014). The concentrations of acid oligosaccharides have been found to be not dependent on the Lewis blood group, and sialyltransferases are found broadly distributed in all human cells, including those of the lactating mammary gland (Maksimovic, Sharp, Nicholas, Cocks, & Savin, 2011). Four families of sialyltransferases have been described to be responsible for the synthesis of acidic glycoconjugates: a) ST6Gal1 and ST6Gal2, b) ST3GAL1 to ST3GAL6, c) ST6GALNAC1 to ST6GALNAC6, and d) ST8SIA1 to ST8SIA6 (Weiss & Hennes, 2012). HMOs sialylation is mediated by the ST3GAL and ST6GAL families (Bing, 2009).

These sialic and fucose epitopes help to shape the composition of the microbiota as they serve as adhesion receptors for pathogens and as sources of energy for microorganisms that produce blood group antigen-specific alpha-glycosidases, such as *Bifidobacterium* and *Bacteroides* (Hoskins et al., 1985; Wacklin et al., 2011). Both communities reduce gut permeability and produce lactate and short-chain fatty acids that modulate the immune cells and the mucus composition (Ríos-Covián et al., 2016). Infants that are fed with milk from nonsecretor mothers have a delayed colonization with *Bifidobacterium*, and as a result, they are more susceptible to diarrhea and bacterial translocation (Lewis et al., 2015).

Infants breastfed with secretor milk are protected against diarrhea caused by *Campylobacter*, calciviruses, and *E. Coli* enterotoxins (Morrow, Ruiz-palacios, Jiang, & Newburg, 2005). They are protected as well from protozoan parasites (Jantscher-Krenn et al., 2012). Therefore, the lack of these epitopes on milk glycans has consequences for the function of the intestinal barrier. Given

that nonsecretor individuals do not express FUT2, they are not able to express ABH antigens in their mucus and other secretions (Jaff, 2010). The nonsecretor phenotype has been genetically associated with increased risk for Crohn's disease and necrotizing enterocolitis (Forni et al., 2014). The reason behind this effect is that without certain glycan epitopes bacterial communities of *Bifidobacterium* cannot proliferate and in exchange opportunistic bacteria can adhere or penetrate the epithelial layer (H. Wu et al., 2017).

Sialylated HMOs, such as 6'-sialyllactose (6'-SL) and 3'-sialyllactose (3'-SL) act well as decoy receptors to prevent the adhesion of influenza virus (Stencel-Baerenwald, Reiss, Reiter, Stehle, & Dermody, 2014), *H. pylori* (Lindén, Wickström, Lindell, Gilshenan, & Carlstedt, 2008), and *E. coli* (Angeloni et al., 2005). Although HMOs, in general, are known to stimulate the growth of beneficial bacteria, studies on fucosylated and sialylated HMOs have shown that *Bifidobacteria* have variable strategies for the digestion of individual HMOs (Garrido et al., 2015); some strains survive from the utilization of fucosylated structures but do not metabolize 6'-SL. Among the sialylated structures there are also differences: *Bifidobacterium longum* JCM1210, *B. fragilis* ATCC25285, *Lactobacillus spp.*, and *Clostridium spp.* in the infant gut have shown preference for 6'-SL HMOs but are unable to digest 3'-SL (Yu, Chen, & Newburg, 2013).

In vitro experiments have shown that sialylated HMOs inhibit leukocyte rolling and adhesion to endothelial cells, while neutral HMOs do not exert such effect (Lars Bode et al., 2004). The presence of sialic acid attenuates development of necrotizing enterocolitis (NEC) in infants. In rat models it has been shown that the dimeric structure, disialyllactose, and not the sialylated oligosaccharides containing one, three, or four sialic acid groups, was effective in preventing disease progression, indicating that specific structures are required to obtain specific protective effects (Jantscher-Krenn et al., 2012).

Maternal environmental factors. Factors, such as maternal diet, body weight and antibiotic use have shown to influence milk composition (D. Munblit, Boyle, & Warner, 2015). However, there is not enough information about how these factors affect specifically the composition of oligosaccharides and glycoconjugates in human milk.

The influence of maternal diet on milk composition is not homogeneous over all milk components. For instance, no changes have been observed in proteins, and mineral composition (Innis, 2014). However, diet can affect the content of fatty acids and vitamins, such as vitamin B1 and C composition (Bravi et al., 2016). As a consequence it has been suggested that diet can affect the concentrations of gangliosides, such as GM3 and GD3 in human milk (Giuffrida, Elmelegy, Thakkar, Marmet, & Destailats, 2014). Although it has not been found that lactose composition changes upon dietary intake, Kunz *et al.* showed that after oral administration of galactose to lactating mothers, this monosaccharide was incorporated to lactose and acidic and neutral oligosaccharides (Kunz, Rudloff, Baier, Klein, & Strobel, 2000). Also, the supplementation of the maternal diet with probiotics (*Lactobacillus rhamnosus* and *Bifidobacterium animalis*) was shown to reduce the risk of eczema in breastfed infants (Rautava, Kainonen, Salminen, & Isolauri, 2012). These results suggest that modulating the maternal diet might have therapeutic potential for breast milk to offer secondary prevention towards allergic disorders during childhood (Daniel Munblit et al., 2017). Current guidelines orient practitioners and pregnant women to maintain a healthy intake of micro- and macronutrients in order to protect mother and

developing fetus (Kominiarek & Rajan, 2017). However, in the practice no medical guidelines recommend women to avoid or include specific types of food during pregnancy and lactation in order to improve their milk composition or prevent allergies in their offspring (Best, Gold, Kennedy, Martin, & Makrides, 2016).

Maternal obesity has not been observed to influence milk composition. However, it has been suggested that obese mothers can transfer via breast milk inflammatory cytokines and markers of oxidative stress, which can contribute to weight gain in the infant (Young et al., 2017). Although it has been shown that the profile of some fatty acids in the breast milk from obese women is different compared to lean women, their total fat, protein and carbohydrate content does not differ (Panagos et al., 2016). It remains to be determined which factor in mother milk is responsible for this undesired effect.

Host genetic factors

The etiology of IBD, obesity, and allergies arise from complex interactions between genes (nature) and the environment (nurture) (Ruiz-Núñez, Pruimboom, Dijck-Brouwer, & Muskiet, 2013). These interactions vary among individuals, ethnicities, and gender (Szarc vel Szic, Ndlovu, Haegeman, & Vanden Berghe, 2010). Host genetic defects might for example affect where and how microorganism colonize in the intestine (Knights, Lassen, & Xavier, 2013). Some of these effects can be mitigated by changes in the diet. The gene FUT2 encodes for epitopes that compose glycans that support the proliferation of *Bacteroides* and prevent the adhesion of pathogens. Studies in mice with a nonfunctional FUT2 gene have shown that after the animals had been fed with complex oligosaccharides they developed a fecal microbiome profile similar to that of mice with functional FUT2 genes, illustrating that diet might compensate for genetic differences (Kashyap et al., 2013).

Conversely, in some diseases, gene determinants remain the strongest risk factor to develop an intestinal disease (Turpin, Goethel, Bedrani, & Croitoru, 2018). For example, genes responsible for enhanced intestinal permeability are considered important in the pathogenesis of IBD (McCole, 2014). Two genetic alterations have been found to hinder the maturation of the gut barrier. The first is a deficiency of NOD2, which induces a hyper inflammatory response to gut microbiota. Mutations in this gene disrupt proper sensing of the immune pathways that respond to host microbiota allowing an overgrowth of pathological bacteria, such as *E. coli* (Ramanan, Tang, Bowcutt, Loke, & Cadwell, 2014). As a consequence, *Enterobacteriaceae* is a family of bacteria enriched in patients with IBD (Brantley Hall, Tolonen, & Xavier, 2017). The other genetic alteration interfering with the maturation of the gut immune barrier is polymorphisms in the regions that encode the genes that regulate tight junctions (Membrane Associated Guanylate Kinase-MAGI3). Clinically this genetic defect reduces the expression of tight junctions, increases gut permeability, and induces pro-inflammatory mucosal cytokine secretion (Norén, Almer, & Söderman, 2017).

Conclusion and future challenges. Different nongenetic and genetic factors have been shown to be important for neonatal development of an adequate gut immune barrier and for preventing inflammatory diseases at a later age. Therefore, modulation of the gastrointestinal immune barrier, to improve health, has become a potent strategy to manage or prevent inflammatory diseases (De Santis, Cavalcanti, Mastronardi, Jirillo, & Chieppa, 2015). From all the nongenetic factors that affect the infant intestinal barrier perhaps the only readily applicable one is nutritional or dietary change.

Although no nutraceutical can replace the benefits of human milk, the ultimate way to manage proper development of the gastrointestinal barrier via infant formula is the addition of HMOs and/or HMGP. Not all HMOs and HMGP that have an impact on gut barrier maturation have been identified yet but this might happen soon as analytical techniques to characterize, isolate, and identify HMOs, HMGP, and synthetic oligosaccharides are improving (Cao et al., 2017; Robinson, Colet, Tian, Poulsen, & Barile, 2018). Fucosylation profiles of HMOs and HMGP have been more broadly studied because of their abundance in human milk, whereas sialylated profiling is challenging since it requires the removal of dominant fucosylated structures and lactose (Yan et al., 2018). Nonetheless, differences in molecular structure among species, or among human individuals, are now better documented (Chaturvedi et al., 2001; Sischo, Short, Geissler, Bunyatratchata, & Barile, 2017; W. L. Wang et al., 2017) and might lead to more personalized advice on type and variation of HMOs and HMGP in infant formulas.

However, before HMOs and HMGP can be produced in a more personalized fashion another challenge has to be overcome. This is to isolate and produce sufficient molecules in a cost-effective manner. In the past 20 years enzymatic *in vitro* synthesis has been the most widely employed method to produce oligosaccharides (Ruffing & Chen, 2006). The major limitation continues to be the development of enzymatic systems that can produce more complex HMOs than 2-FL and LNnT at an industrial scale. Major efforts are oriented towards engineered transferases capable to add fucose or sialic acid into desired locations on the glycan backbone. Additionally, for this large-scale production, more efficient methods of purification are needed (Baumgärtner, Seitz, Sprenger, & Albermann, 2013). Since *E. coli* is the most common system used, and there is much and good information about the mutants with no virulent capacity, the downstream production requires controls that guarantee endotoxin-free production (Sprenger, Baumgärtner, & Albermann, 2017).

NDCs should not be abandoned. Many of the carbohydrates used to fortify infant formula have been extensively investigated as food ingredient for their prebiotic properties (Ackerman, Craft, & Townsend, 2017). Unfortunately, it has not been fully confirmed that they can substitute for other relevant functions of HMOs (Kent, 2006). Studies on diverse *in vitro* models have shown that NDCs can have a direct role in the regulation of the secretory function of highly differentiated cells from the epithelial barrier or even stimulate barrier function by supporting tight junction production (Dulantha Ulluwishewa et al., 2011). Therefore, the study of nonprebiotic effects of individual or combinations of NDCs might further improve efficacy of infant formula compositions.

During recent years it has become more accepted that many nutrients are not solely a source of energy or as factors involved in the development of the organism; nutrients are also able to regulate gene expression (Ferguson, 2015). This new field is called nutrigenomics (Pavlidis, Nebel, Katsila, & Patrinos, 2016). As in pharmacogenomics, the identification of the positive, negative, or even adverse effects, impacts of a medication on diverse populations (Relling & Evans, 2015), nutrigenomics is a potential tool to understand the interactions between genome and dietary components (Neeha & Kinth, 2013). By this approach food intake can become more effective as nutrigenomics contributes to an understanding of the mechanisms that underlie individual variations in dietary requirements, as well as in the capacity to respond to food-based interventions. This can also apply to milk make-up, opening new venues to improve its composition or to prevent the

manifestation of gastrointestinal diseases (Gruber, Lichti, Rath, & Haller, 2012).

Important advances have been made in understanding individual efficacy of human milk after the discovery that secretor and Lewis status from an individual determine how the microbiota develops and how breast milk protects against disease. Blood genotype is very important for the composition and health benefits of the breast milk. Recently even more specific genotypes have been identified thanks to the improvements in the detection techniques. Such is the case of the Bombay and para-Bombay phenotypes in Asiatic or Indian populations. In these populations adverse reactions to NDCs in infant formula have been described (Chiang et al., 2012; Kaneko et al., 2014). It exemplifies that more knowledge is needed about the role of genetically determined blood variants and food component efficacy in supporting maturation of the intestinal immune barrier.

List of Abbreviations

2'-FL	2-fucosyllactose
5-HT	Serotonin
BMOs	Bovine milk oligosaccharides
CLCA1	Chloride channel accessory 1
CNS	Central nervous system
EECs	Enteroendocrine cells
ENS	Enteric nervous system
FOS	Fructo-oligosaccharides
FUT2	α 1,2 fucosyltransferase
FUT3/4	α 1,3/1,4 fucosyltransferase
GalNAc	N-acetyl-D-galactosamine
GOS	Galacto-oligosaccharides
hBD-1,-2, -3	Human beta defensin 1, -2, -3
HD5	Human alpha defensin 5
HD6	Human alpha defensin 6
HMGLs	Human milk glycolipids
HMGPs	Human milk glycoproteins
HMOs	Human milk oligosaccharides
IBD	Inflammatory bowel disease
IFN γ	Interferon gamma
LacdiNAc	N,N'-Diacetylglucosamine
LAcNAc	N-Acetyl-D-lactosamine
Le	Lewis phenotype
LNnT	Lacto-N-neotetraose
LPS	Lipopolysaccharide
MAGI3	Membrane Associated Guanylate Kinase
MFGM	Human milk fat globule membrane
MLCK	myosin light chain kinase
NDCs	Nondigestible carbohydrates
NEC	Necrotizing enterocolitis
Neu5Ac	N-acetylneuraminic acid
Neu5Gc	N-glycolylneuraminic acid
NOD2	Nucleotide-binding oligomerization domain-containing protein 2
PRRs	Pattern recognition receptors
RELM	Resistin-like molecule
SCFAs	Short-chain fatty acids
Se	Secretor phenotype
TFF3	Trefoil factor 3
TLRs	Toll-like receptors
TNF α	Tumor necrosis factor alpha
ZG16	Zymogen granule protein 16

Author Contributions

SFL and PDV constructed the layout of the review and drafted the manuscript. SFL scanned the literature, retrieved, and processed papers referenced in the review. PDV revised the work critically for intellectual content. All authors edited the manuscript and revised the submitted version.

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