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Human milk oligosaccharides: Shaping the infant gut microbiota and supporting health

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ABSTRACT

Human milk oligosaccharides (HMO) are complex sugars which are found in breast milk at significant concentrations and with unique structural diversity. These sugars are the fourth most abundant component of human milk after water, lipids, and lactose and yet provide no direct nutritional value to the infant. Recent research has highlighted that HMOs have various functional roles to play in infant development. These sugars act as prebiotics by promoting growth of beneficial intestinal bacteria thereby generating short-chain fatty acids which are critical for gut health. HMOs also directly modulate host-epithelial immune responses and can selectively reduce binding of pathogenic bacteria and viruses to the gut epithelium preventing the emergence of a disease. This review covers current knowledge related to the functional biology of HMOs and their associated impact on infant gut health.

1. Introduction

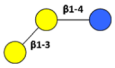
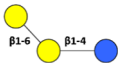
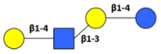
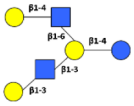
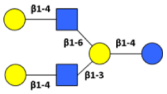
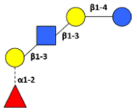
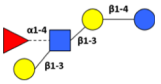
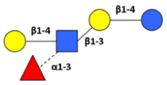
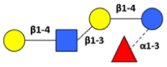
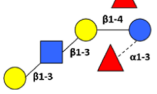
Breast milk is the optimal nutrition for infants, and its composition has been shaped by thousands of years of human evolution in order to provide tailor-made nutrition and protection to the developing neonate, all at an energetic cost to the mother (Ballard & Morrow, 2013; Garwolińska, Namieśnik, Kot-Wasik, & Hewelt-Belka, 2018). Recent advances in analytical tools and the development and integration of "-omics" technologies have offered valuable insights into the composition of human breast milk, while reinforcing our understanding of the health benefits associated with breast feeding (O'Sullivan, Salcedo, & Rubert, 2018). One of the most remarkable features of breast milk is the diversity and abundance of complex sugars (glycans) including human milk oligosaccharides (HMOs), which are indigestible to the infant and for this reason reach the colon intact (Kunz, Rudloff, Baier, Klein, & Strobel, 2000). For decades, the presence of this indigestible material challenged scientists since milk was unlikely to have retained superfluous content that did not benefit the infant. A prebiotic function for these human milk glycans was first described by Gyrogy and co-workers in 1954 (Gyorgy, Norris, & Rose, 1954), and in the subsequent 60-plus years, investigators have proposed numerous biological, physiological and protective functions which can be ascribed to HMOs (Bode, 2015). The abundance and structural diversity of these compounds would certainly allow for more than one function (Smilowitz, Lebrilla, Mills, German, & Freeman, 2014).

Major innovations in the field of glycomics have enabled the identification of over 200 HMO structures, with the concentration of oligosaccharides in human milk reaching levels 100–1000 times higher than in the milk of any domesticated farm animal (Bode, 2019; Oliveira, Wilbey, Grandison, & Roseiro, 2015; Urashima, Hirabayashi, Sato, & Kobata, 2018). Table 1 outlines the structure of the core HMOs found in human milk. Concentrations of HMO are highest in colostrum (20 g/L) and decrease across lactation, with 16 g/L detected in 30 day mature milk (Coppa et al., 1999). HMOs vary in size, structure, and complexity and are composed of 5 monosaccharide building blocks. Units of D-glucose (Glc), D-galactose (Gal), *N*-acetylglucosamine (GlcNAc), L-fucose (Fuc), *N*-acetylneuramanic acid are conjugated via glycosidic linkages to form a myriad of different HMO structures. All HMOs contain a lactose core at the reducing end, which is elongated via the addition of GlcNAc units (German, Freeman, Lebrilla, & Mills, 2008). Elongation occurs through the addition of GlcNAc units via β1-3 linkages (linear structures) or β1-6 structures (introduces branching). Type 1 HMOs contain lacto-*N*-biose subunits, while type II HMOs contain *N*-acetyllactosamine disaccharide units at their core (Table 1). Further structural diversity is brought about by the addition of fucose and sialic acid residues at the terminal positions. Composition of HMO varies between lactating mothers, and is impacted significantly by maternal genetics. This variability is largely driven by polymorphisms in the

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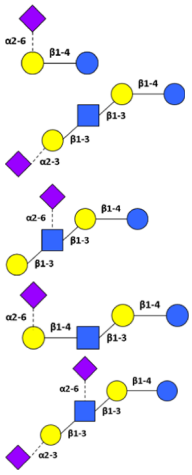
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Table 1
Nomenclature and structures of Core HMOs.

	Name	Abbreviation	Structure/Symbol
HMO Backbone	D-Glucose	Glc	
	D-Galactose	Gal	
	N-Acteylglucosamine	Neu5Ac	
	L-Fucose	Fuc	
	N-acetylneuraminic acid (sialic acid)	Neu5Ac	
	Lactose	Lac	
	Lacto-*N*-biose	LNB	
	N-acetyllactosamine	-	
Colostrum HMOs	Galactosyllactose	3′GL	
		4′GL	
		6′GL	
Neutral HMOs	Lacto-*N*-tetraose	LNT	
	Lacto-*N*-neotetraose	LNnT	
	Lacto-*N*-hexaose	LNH	
	Lacto-*N*-neohexaose	LNnH	
Neutral Fucosylated HMOs	2′-Fucosyllactose	2′-FL	
	3-Fucosyllactose	3-FL	
	Difucosyllactose	DFL	
	Lacto-*N*-fucopentaose	LNFP I	
		LNFP II	
		LNFP III	
		LNFP IV	
		LNFP V	
Sialylated HMOs	3′Sialyllactose	3′-SL	

(*continued on next page*)

Table 1 (*continued*)

	Name	Abbreviation	Structure/Symbol
	6′Sialyllactose	6′-SL	
	Sialyllacto-*N*-tetraose	LST a	
		LST b	
		LST c	
	Disialyllacto-*N*-tetraose	DSLNT	

fucosyltransferases *FUT2 (Se)* and *FUT3 (Le)*. If both genes are active then all fucose-borne HMO species can be synthesised. Mothers with an inactive *FUT2* gene cannot synthesise α1-2 fucosylated HMOs (such as 2′-fucosyllactose [2′-FL] and lacto-*N*-fucopentaose I [LNFP I]) while mothers with an inactive *FUT3* gene cannot synthesise α1-3 or α1-4 fucosylated HMOs (such as LNFP II and LNFP III). Based on these fucosyltransferase polymorphisms, four milk groups can be defined: 1) Lewis positive secretors (Le^+Se^+) 2) Lewis negative secretors (Le^-Se^+) 3) Lewis positive non-secretors (Le^+Se^-) 4) Lewis negative non-secretors (Le^-Se^-) (Thurl et al., 2010).

The differences in gut microbiota composition observed between formula-fed and breastfed infants have, at least partially, been attributed to the absence (until recently) of HMOs in infant formula milk (Guaraldi & Salvatori, 2012; van den Elsen, Garssen, Burcelin, & Verhasselt, 2019). A solution for narrowing the compositional gap between human milk and formula milk is the supplementation of infant formula with commercially available oligosaccharides (Reverri, Devitt, Kajzer, Baggs, & Borschel, 2018). Until recently, research in this field has been hampered by the lack of HMOs on pre-commercial and commercial scale. However, the (bio)technology for large-scale production of complex carbohydrates has advanced greatly, and although technical challenges remain, more and more structures are being manufactured at industrial scale (Faijes, Castejón-Vilatersana, Val-Cid, & Planas, 2019). These advancements have paved the way for the first infant safety, growth, and tolerance studies with particular HMOs, and have resulted in regulatory approval and commercialization of two major HMOs: 2′-FL, and lacto-*N*-neotetraose (LNnT) (Vandenplas et al., 2018). The commercial availability of these HMOs has allowed for a deeper appreciation of the functions of HMOs, many of which are believed to be exerted through interactions with the gut microbiota (Sela & Mills, 2010). It is expected that microbiome modulation will become a major translational asset in the near future, establishing the microbiome as a malleable and versatile therapeutic target (McBurney et al., 2019). It is important, therefore, that large-scale clinical studies are conducted to fully elucidate the mechanistic link between oligosaccharide structural variation and infant intestinal health. Such studies should aim to characterise the role of HMOs in gut microbiota composition and function, protection against pathogens, and support of barrier and immune functions. Determining how to standardize and integrate large multi-omic, annotated data sets will likely require multi-disciplinary approaches between chemists, glycobiologists, food scientists, microbiologists, bioinformaticians, and paediatricians. In this review, the importance of HMOs in the developmental physiology of infants will be discussed. The review highlights the milestones in HMO research, from early research to our current understanding of their biological role in infant health.

2. Pioneers in HMO research

The discovery of HMOs in the mid-1900s was the result of accumulative and collaborative efforts between paediatricians, microbiologists, and chemists. It was noted at the beginning of the 20th century, that infant mortality rates were lower in breastfed infants when compared to those of bottle-fed infants, with decreased occurrences of infectious disease. Pioneers in the field, such as Escherich, Moro, and Tissier, noticed variances in the bacterial composition between breast-fed and bottle-fed infant faeces, substantiating claims that feeding regime and composition of intestinal bacteria were linked to infant health (Escherich, 1886; Moro, 1900). Moro, at the time, postulated that human milk contained a factor which stimulated bacterial growth in the gut. However, it took chemists another 50 years to identify and characterize this growth-promoting factor. Interest in HMOs from a chemical perspective had sparked in 1888 when Eschbach had found “a different type of lactose” in human milk (reviewed in (Montreuil, 1992)), and in the 1930s Polonowski and Lespagnol designated this carbohydrate fraction “gynalactose” (Polonovski & Montreuil, 1954; Polonowski & Lespagnol, 1930, 1931). The real breakthrough in HMO research came when paediatrician Paul Gyorgy, and chemist Richard Kuhn hypothesised a link between “gynalactose” and Moro’s “growth factor”. In 1954, thanks to this partnership between a paediatrician and chemist, the bifidus factor in human milk responsible for enrichment of bacteria in breastfed infants was identified as *N*-acetylglucosamine (GlcNAc) (Gauhe et al., 1954; Gyorgy, Kuhn, Rose, & Zilliken, 1954; Gyorgy et al., 1954).

Since 1954, analytical tools and technologies which allow for structural characterisation of human milk glycans have been developed. Through the employment of these methods, investigators have identified over 200 HMOs (Bode, 2019; Oliveira et al., 2015; Urashima et al., 2018). As a result of four decades of research and development, it is now possible to precisely differentiate and quantify HMOs in complex matrices. This bestows opportunities upon researchers to examine the structure-function relationships between HMOs consumed in early life and infant intestinal development.

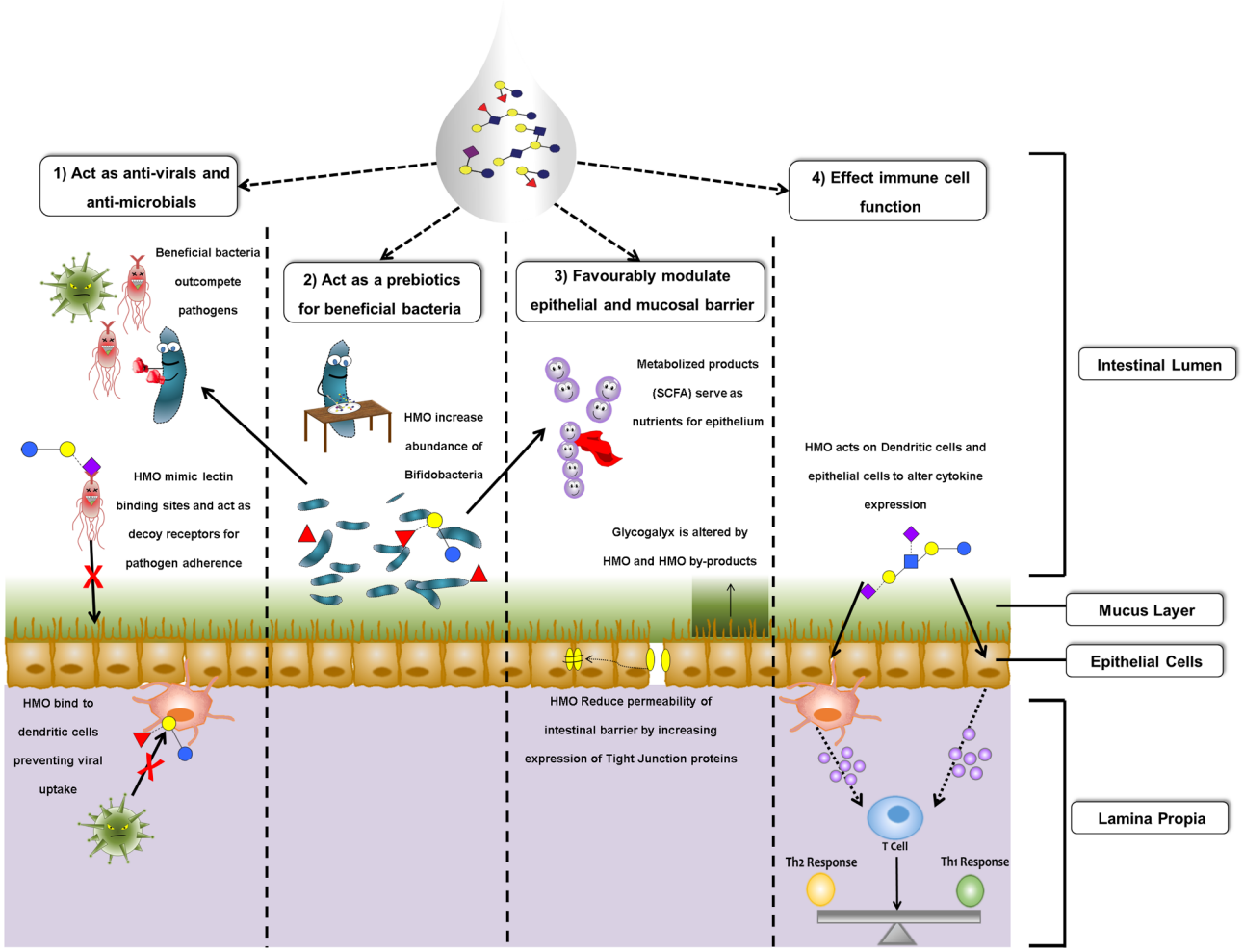


Fig. 1. Schematic summary of the beneficial roles human milk oligosaccharides (HMOs) play in the digestive tract of breast fed infants. 1) HMOs have been shown to act as antivirals and antimicrobials by binding to pathogens and toxins and by directly binding to epithelial receptors. Adhesion and infection by invading pathogens is subsequently blocked. HMOs also prevent viral uptake by binding C-lectin receptors on dendritic cells (DCs) 2) HMOs serve as prebiotic agents, selectively enriching bifidogenic gut bacteria. This confers a selective advantage to bifidobacterial species over pathogens, providing further protection against infectious disease. Metabolites produced during microbial fermentation of HMO have a role to play in developmental physiology of infants. 3) Short chain fatty acids (SCFAs), which are the major metabolites of HMO fermentation, have been shown to influence maturation of intestinal epithelial cells (IECs). HMOs improve barrier function by modulating expression of tight junction proteins, thereby reducing permeability of the intestinal barrier. Expression of proteins at the glycocalyx and mucus layers is also altered by HMOs and HMO metabolites. 4) HMOs interact with immune cells (DCs, T Cells, B Cells) and affect expression of pro/ anti-inflammatory cytokines. HMOs thereby play a role in maintaining immune homeostasis.

3. Functions of human milk oligosaccharides

Data from *ex vivo* and *in vivo* studies, including recent intervention studies in humans, highlight the importance of HMOs in the infant gastrointestinal tract (summarised in Fig. 1). Many infant gut bacteria, particularly members of the genus *Bifidobacterium*, possess the genetically encoded ability to consume HMOs, providing rationale for their predominance in the gut of breast-fed infants (Milani et al., 2017). HMOs serve as prebiotic components supplying metabolic substrates necessary for beneficial bacteria to thrive (Bode, 2009; Gibson et al., 2017). Genetic adaptations of these health-promoting bacteria represent an intriguing example of host-microbe coevolution. However, in recent years, thanks to accumulating evidence from cell culture studies, animal studies, and mother-infant association studies, it has become apparent that HMOs are more than just “food for bugs” (Bode, 2012). HMOs also function as soluble decoy receptors, preventing pathogen attachment to mucosal surfaces and thereby reducing infections (Laucirica, Triantis, Schoemaker, Estes, & Ramani, 2017). It has also been shown that HMOs improve gut barrier function (Maciej Chichlowski, De Lartigue, German, Raybould, & Mills, 2012), promote immune development and tolerance (Donovan & Comstock, 2016), modulate intestinal cell responses (Kong et al., 2019), and lower the risk of contracting necrotizing enterocolitis (NEC) (Autran et al., 2018; Good et al., 2016). In addition, HMOs are believed to provide the infant with a source of sialic acid, an essential nutrient in brain development and cognition (Jacobi et al., 2016; Tarr et al., 2015). Thus, HMOs afford multiple layers of protection and support for the functionally naïve newborn (Fig. 1). The functions that HMOs impart on a developing infant are as diverse as their structural composition, and these molecules are major contributors to breast milk being considered the ‘the gold standard in infant nutrition’ (Walker, 2010). While the future for this field is bright, there is much yet to explore. In the sections below, the biological, physiological and protective roles associated with these molecules will be discussed and the mechanisms by which HMOs are thought to exert these functions.

3.1. HMOs as growth factors for beneficial bacteria

Bifidobacterial strains are frequently found as the most abundant colonizers in the gut of breastfed infants, generally accounting for

50–90% of the total bacterial population detected in the faeces of breast-fed infants (Hao, Zhu, & Faden, 2019; Milani et al., 2017). This is believed to be due to their remarkable capacity to metabolize and consume prebiotic components in human milk (Fig. 1). Genomic analyses of particular infant-derived bifidobacterial strains have revealed an assortment of transporters, carbohydrate binding proteins, and glycosyl hydrolases (GHs), all of which are involved in the import and processing of HMOs (Garrido, Barile, & Mills, 2012; Thomson, Medina, & Garrido, 2018). These HMO-degrading enzymes are essentially limited to *B. breve, B. bifidum, B. longum* subsp. *longum (B. longum), B. longum* subsp. *infantis (B. infantis),* and, in some cases, *B. pseudocatenulatum* (Bottacini, Ventura, van Sinderen, & O'Connell Motherway, 2014; Gotoh et al., 2018). Recently, a genetically-encoded pathway for the metabolism of fucosyllactose has also been described in a *B. kashiwanohense* isolate (James et al., 2019). The presence of these genetic loci suggests a coevolution between the human host, HMOs, and the microbes enriched during the lactation period (Chichlowski, German, Lebrilla, & Mills, 2011). The underlying molecular mechanism involved in HMO utilization remains elusive since it is a complex process involving multiple gene clusters. Moreover, the catabolic processes involved vary depending on the species and between strains of the same species (Lawson et al., 2019).

While HMO metabolism is observed in various members of the *Bifidobacterium* genus, it is by no means a characteristic observed in all bifidobacterial isolates. In fact, it is misleading to refer to HMOs as bifidogenic given that they promote growth of some, but certainly not all, bifidobacteria. For example, Ward, Niñonuevo, Mills, Lebrilla, and German (2007) assessed the growth of various *Bifidobacterium* isolates on HMOs purified from breast milk or individual HMO structures and found that non-infant-borne strains such as *B. adolescentis, B. dentium*, and *B. animalis* subsp. *lactis* were incapable of digesting HMOs. In contrast, *B. infantis* and *B. bifidum* are considered avid HMO consumers, containing all the GHs required for catabolizing a whole spectrum of HMO linkages (Underwood, German, Lebrilla, & Mills, 2015). Generally, individual strains of *B. longum* subsp. *longum* and *B. breve* display poor growth on HMOs, but are capable of utilizing more simple structures such as lacto-*N*-tetrose (LNT) and lacto-*N*-neotetrose (LNnT) (James, Motherway, Bottacini, & van Sinderen, 2016; Ruiz-Moyano Seco de Herrera et al., 2013).

Two distinct HMO degradation strategies have been described in infant-borne bifidobacteria. The first involves importation of intact HMOs into the cytoplasm through ATP-Binding cassette (ABC) transporters, whereupon the oligosaccharides are hydrolysed by intracellular glycosidases. The second approach relies on cell wall-anchored secretory GHs which hydrolyse HMOs extracellularly releasing mono- and di-saccharides (Garrido et al., 2015; Kitaoka, 2012). Through genomic and phenotypic analysis it evident that *B. infantis* and *B. breve* use oligosaccharide transporters, while *B. bifidum* utilizes extracellular hydrolases and processes the liberated lacto-*N*-biose (LNB - the core structure of type 1 HMO- Table 1) intracellularly (Thomson et al., 2018). Interestingly, the strategy used by *B. longum* is strain dependent and reliant on the presence of the gene encoding lacto-*N*-biosidase (LnbX). LnbX-positive *B. longum* use extracellular hydrolases, while LnbX-negative utilize oligosaccharide transporters to assimilate HMO derivatives internally (Yamada et al., 2017). While the overall mechanistic model varies, all four infant gut-associated species encode a gene cluster which is dedicated to the assimilation of LNB. Genes within this GNB/LNB cluster are upregulated several fold during growth on HMO (Garrido et al., 2015). The gene cluster contains an ABC transporter and cytoplasmic phosphorylase which are linked to internalisation and metabolism of LNB (Nishimoto & Kitaoka, 2007).

Although divergent mechanisms are used for catabolism of HMO in each species, the HMO-derivatives enter the same central fermentative pathway, termed the 'bifid shunt'. The bifid shunt is a *Bifidobacteriaceae*-specific metabolic pathway and encompasses the Leloir, fructose-6-phosphate phosphoketolase, and amino-sugar metabolising pathways (Pokusaeva, Fitzgerald, & van Sinderen, 2011). Proteomic analysis by Kim et al. indicated upregulation in the activity of enzymes involved in the bifid shunt pathway in the presence of HMO, resulting in the release of large amounts of acetate and lactate (Kim et al., 2013). For every 2 mol of hexose that enter in this pathway five moles of ATP, three moles of acetate and two moles of lactate are produced. Flux through the bifid shunt pathway allows bifidoabacteria to produce more energy in the form of ATP than levels produced through fermentative pathways in lactic acid bacteria (LAB) (reviewed in detail by (Pokusaeva et al., 2011). Production of organic acids via bifid shunt pathways offers potential links between bifidobacterial physiology and infant nutritional and health outcomes (Alexander, Swanson, Fahey, & Garleb, 2019; Hidalgo-Cantabrana et al., 2017). The organic acids produced reduce intestinal pH, and can thereby inhibit pathogenic bacteria. It has also been suggested that these metabolites improve intestinal barrier function and contribute to immune system development (Tan et al., 2014).

The various catalytic mechanisms used by bifidobacterial strains for the consumption of HMOs may be linked to niche partitioning among bifidobacteria within the developing infant gastrointestinal tract (Pereira & Berry, 2017; Sela & Mills, 2010). Interestingly, the ability of a bifidobacterial strain to consume HMOs does not necessarily correlate with their dominance in the gut of breast-fed infants. Despite being recognised as avid HMO consumers, *B. bifidum* and *B. infantis* are detected in low numbers in the faeces of breast-fed infants, while *B. longum* and *B. breve* are often found as the dominant species in infant stools (Turroni et al., 2012), even though they demonstrate limited HMO assimilation *in vitro*. Thus, simply understanding the HMO assimilation pathways does not provide a full picture of how bifidobacteria strains utilize HMO to colonize the nursing infant colon. In order to fully appreciate the role HMOs play in the establishment of a healthy infant microbiome, consideration must be given to the multifaceted syntrophic relationships that exist between members of the infant microbiota (Gotoh et al., 2018; Milani et al., 2015).

Besides infant gut-associated bifidobacteria, HMO assimilation pathways have been described for a number of gut microbes belonging to other taxa. Numerous *Bacteroides* species were shown to metabolise 2′-FL, 3-FL, 3′-SL, 6′-SL, and LDFT (Yu, Chen, & Newburg, 2013) while *Lactobacillus casei* utilizes the HMO-derivatives LNB and lacto-*N*-triose II (Bidart, Rodríguez-Díaz, Monedero, & Yebra, 2014). In fact, a specific phosphotransferase system for import of LNB has been identified in *Lactobacillus* species (Bidart et al., 2014). Although the capacity of these gut microbes to consume HMO is generally quite limited, these minority species play a role in the gut ecology of breast-fed infants until weaning and may potentially influence the microbiota population in adulthood. It is also interesting to note that in a recent study by Salli et al., 2019, using a semi-continuous colon simulator, only slight changes in the microbiota were caused by the commercially available and most prevalent HMO, 2′-FL. This was reflected by the intermediate production of short chain fatty acids (SCFAs) with lower production of acetate and lactate observed when compared with fermentations in the presence of lactose or galacto-oligosaccharides (GOS). The results highlight the specificity of 2′-FL as an energy source for only certain microbes over GOS and lactose in this simulated gut model. Indeed, it may be that 2′-FL is more important in directly interacting with the host cells and tissues (discussed below) rather than indirectly through its action on the gut microbiota.

3.2. Effect of HMOs on the colonisation of bifidobacteria

The ability of probiotic strains to adhere to the intestinal epithelium plays a key role in influencing the composition of the gut microbiota. Microbes in surface-associated populations can out-compete other species solely by being more adhesive (McLoughlin, Schluter, Rakoff-Nahoum, Smith, & Foster, 2016). In recent years, a number of studies have indicated that HMOs could enhance the adhesion ability of

Table 2
Studies with HMO against enteric pathogenic infections.

Pathogen	HMO	Model	Findings	Reference
Campylobacter jejuni	α1-2 fucosylated HMO	Murine Model & *ex-vivo* human intestinal mucosa	H-2 antigen necessary for campylobacter binding, which is blocked by α1,2-fucosylated HMO	(Ruiz-Palacios et al., 2003)
	2′-FL	Hep-2 & HT-29 epithelial cells	2′-FL inhibited invasion of *C. jejuni* which was associated with a reduction in IL-8 expression	(Yu et al., 2016)
	Breast-milk samples	Observational clinical study in mother-infant dyads	Inverse relationship between concentrations of α1,2-fucosylated HMO and suscepitibility to *C. jejuni* caused diarrhea	(Morrow et al., 2004)
Candida albicans	Pooled HMO	H4 premature intestinal epithelial cells (pIECs)	Pooled HMO reduced invasion of pIECs by *C. albicans*	(Gonia et al., 2015)
Entamoeba histolytica	Pooled HMO	HT-29 epithelial cells	HMO-enriched fractions reduced adherence and cytotoxity of *E. histolytica in-vitro*	(Jantscher-Krenn et al., 2012)
Enteropathogenic *E. coli* (EPEC)	Pooled HMO	Murine model & HeLa and T84 epithelial cells	Reduced adhesion of EPEC in epithelial cells, which was reflected in reduced EPEC infection in mice	(Manthey et al., 2014)
	HMO fractions	Caco-2 epithelial cells	All HMO fractions (acidic, low MW neutral, and high MW neutral) reduced adhesion of *E. coli* O119 to epithelial cells	(Coppa et al., 2006)
	2′-FL and LNFP I	*In-vitro* binding assay	2′-FL and LNFP I bind extesntively to heat labile enterotoxin type 1	(El-Hawiet et al., 2015)
Helicobacter pylori	Acidic HMOs	HuTu-80 human duodenal cells	3′-SL demonstrated strongest inhibition of *H. pylori* adherence. 6′-SL less active in reducing *H. pylori* colonisation of HuTu-80 cells	(Simon et al., 1997)
Listeria monocytogenes	Pooled HMO	Caco-2 epithelial cells	Pre-incubation of Caco-2 cells with HMO reduced *L. monocytogenes* adherence by 50%	(Chen et al., 2017)
Norovirus	Breast-milk samples	Saliva samples	Human milk blocked genogroup I genotype 1 (GI.1) and genogroup II genotype 4 (GII.4) binding to saliva	(Jiang et al., 2004)
	HMO Structures	Surface plasmon resonance imaging (SPRi) of glycan microarrays	GI.1 (Norwalk) and GII.4 (VA387) capsids show greater affinity for fucosylated over non-fucosylated HMOs	(Shang et al., 2013)
	Fucosylated HMOs	Porcine gastric mucin or saliva samples	2′-FL binds GII.17 variants, while 2′-FL and 3-FL block GII.10 noroviruses.	(Koromyslova et al., 2017)
Rotavirus (RV)	Breast-milk fractions	Human kidney epithelial cells (MA-104) and mouse model	A sialic acid-containing human milk component inhibited RV replication *in-vitro* and reduced RV gastroenteritis *in-vivo*	(Yolken et al., 1992)
	Pooled HMO fractions	Human kidney epithelial cells (MA-104) and piglet animal models	Sialic acid containing HMO inhibited RV infection *in-vitro*. Neutral and acidic HMO reduced RV replication *in-vivo*	(Hester et al., 2013)
	2′-FL, 3′-SL, 6′-SL, GOS	Human kidney epithelial cells (MA-104)	All HMOs reduced infectivity of RV strains. G1P[8] infectivity was inhibited most significantly by 2′-FL. Combination of 3′-SL + 6′-SL had the greatest effect on G2P[4] variants	(Laucirica et al., 2017)
Salmonella fyris	2′-FL/ 3-FL & pooled breast milk	Caco-2 intestinal epithelial cells	Low MW fractions of HMO were most effective for inhibiting adhesion of *S. fyris*. 3FL and 2′-FL reduced infectivity of *S. fyris*	(Weichert et al., 2013)
Group B *Streptococcus* (GBS)	Breast-milk samples	Observational clinical studying mother-infant dyads	Infants born to Lewis positive mothers had lower incidences of GBS infection. Branched chain HMOs reduced GBS growth *in-vitro*	(Andreas et al., 2016)
	Neutral HMO (LNT, LNFP I)	*In-vitro* growth	LNT and LNFP I significantly inhibited growth of GBS	(Lin et al., 2017)
	Pooled HMO	*In-vitro* growth	HMO inhibits growth and alters biofilm formation of GBS, in a milk-group dependent manner	(Ackerman et al., 2017)

probiotic strains by influencing the expression of bacterial adhesins (Morrin & Hickey, 2020). This enhanced adhesion may prolong gut transit time of the probiotic and increase host-microbe and/or microbe-microbe interactions, thereby enabling greater health benefits to the host (Monteagudo-Mera, Rastall, Gibson, Charalampopoulos, & Chatzifragkou, 2019). HT-29 and Caco-2 cell lines are routinely employed to mimic the adhesion of probiotic bacteria to intestinal epithelial cells (Chichlowski et al., 2012). An *in vitro* study by Chichlowski et al. demonstrated that cultivation of *B. infantis* ATCC 15697 on HMOs significantly increased adherence of the strain to intestinal cells (Chichlowski et al., 2012). Likewise, incubation of the same strain (ATCC 15697) in the presence of 3′-SL and 6′-SL substantially increased *B. infantis* adherence to HT-29 cells (Kavanaugh et al., 2013). Subsequent transcriptomic analysis revealed that this increased adherence is associated with upregulation of chaperone proteins, transcription factors, adhesion-related proteins and glycosyl hydrolases (Kavanaugh et al., 2013). Additional studies which incorporate transcriptomic and proteomic analyses will further increase our understanding of the HMO-related factors which promote adherence of beneficial microbes to the mucosal surface. Nevertheless, it is plausible that HMO-containing nutritional products will be used in the future as a tool to increase the residence time of probiotic bacteria in the gut.

3.3. Anti-infective properties of HMO

By acting as prebiotic agents, HMOs confer a selective advantage to beneficial microbes over pathogens, thereby providing protection to the infant from infectious diseases (Fig. 1). Since pathogenic bacteria are less capable of metabolising HMO species, symbionts can grow and outcompete harmful invaders through competitive exclusion (Hoeflinger, Davis, Chow, & Miller, 2015). Moreover, bifidobacterial HMO metabolism results in the production of organic acids which creates an acidic environment wherein growth of pathogenic bacteria is hindered (Tan et al., 2014). In addition to indirect pathogenic deflection, HMOs also directly block pathogenic entry by acting as soluble receptor decoys (Fig. 1). Many viral, bacterial and protozoan pathogens must adhere to the 'glycocalyx' in order to invade the host and cause disease (Costerton, Irvin, & Cheng, 1981). The glycocalyx is the term given to the carbohydrate-rich layer which coats epithelial cells, and consists of glycans conjugated to proteins or lipids (Kavanaugh et al., 2015). The chemical structures of HMOs are similar to the glycans pathogenic bacteria exploit to attach to the epithelial cell surface (Newburg & Grave, 2014). Pathogens and toxins that recognise and bind to HMOs rather than cell-surface glycans will therefore pass harmlessly through the gastrointestinal tract without causing infection (Craft & Townsend, 2018). Oligosaccharides can also inhibit pathogens by competitive binding with the host cell surface receptor (Morrow et al., 2005). HMOs have been shown to provide stereospecific protection against a wide spectrum of pathogens, including those associated with diarrheal, respiratory, and urinary infections and even human immunodeficiency virus (HIV). As outlined in Table 2, a significant number of *in vitro* and *in vivo* studies have been carried out in recent years, the findings of which demonstrate the anti-bacterial, anti-viral and anti-amoebic properties of HMOs. These studies provide insight into their potential as valuable candidates for future therapeutic developments against pathogenic infection (Craft & Townsend, 2019).

3.3.1. Bacterial infections

One example of a pathogenic bacterium which is inhibited by oligosaccharides from human milk is *Campylobacter jejuni,* which is recognised as one of the most common causes of diarrhoea worldwide. HMOs have been shown to inhibit *C. jejuni* pathogenesis in mice and in *ex vivo* cultures of human intestinal mucosa (Ruiz-Palacios, Cervantes, Ramos, Chavez-Munguia, & Newburg, 2003). Subsequent *in vitro* studies demonstrated that α1-2 fucosylated carbohydrate moieties inhibit adherence of *C. jejuni* to the H-2 antigen on intestinal cells (Ruiz-Palacios et al., 2003). A more recent investigation found that 2′-FL not only inhibits binding to the mucosal surface, but also reduces invasion of *Campylobacter* by 80% (Yu, Nanthakumar, & Newburg, 2016). Anti-Campylobacter effects have also been observed in murine models, with 2′-FL reducing invasion and intestinal inflammation associated with *Campylobacter* infection (Yu et al., 2016). The findings of these studies align with results from a prospective study of 93 Mexican breastfeeding mother-infant dyads (Morrow et al., 2004). Researchers here, found there was an inverse relationship between concentrations of α1-2 fucosylated HMOs in maternal milk and susceptibility to *C. jejuni*-caused infant diarrhea, and concluded that α1-2 fucosyloligosaccharides are key components in human milk which contribute to protection against infectious pathogens (Morrow et al., 2004).

Oligosaccharides present in human milk also function to protect against *Escherichia coli* pathogenesis. Enteropathogenic *Escherichia coli* (EPEC) cause serious diarrheal disease with high mortality rates in infants. Pre-incubation of EPEC with pooled HMO fractions significantly reduced pathogenic colonization of cultured epithelial cells (Manthey, Autran, Eckmann, & Bode, 2014) while HMO-treated mice demonstrate significantly less EPEC infection than control or galacto-oligosaccharides (GOS)-treated pups (Manthey et al., 2014). These findings are consistent with work from Coppa et al. where infectivity of *E. coli* serotype O119 was shown to be inhibited by acidic HMO fractions as well as the high- and low-molecular-weight HMO fractions (Coppa et al., 2006). Additional to reducing adhesion of whole pathogens, 2′-FL and Lacto-*N*-fucopentaose-I (LNFP-I) may also reduce infectivity by binding to heat-labile enterotoxin type 1 (El-Hawiet, Kitova, & Klassen, 2015). Fucosylated HMO structures are also effective in mitigating adhesion and infectivity of enteropathogenic *Salmonella* (Weichert et al., 2013), while sialylated structures such as 3′-SL exhibit strong inhibition of *Helicobacter pylori* adhesion to gastrointestinal epithelial cells (Simon, Goode, Mobasseri, & Zopf, 1997).

Aside from reducing adhesion and invasion of pathogenic bacteria, HMOs have also been shown to reduce infection by modifying gene expression on the epithelial surface. Pre-incubation of Caco-2 cells with HMO alters gene expression of intestinal cells, resulting in a significant reduction in *Listeria monocytogenes* adhesion (Chen, Reiter, Huang, Kong, & Weimer, 2017). HMO pretreatment of IECs reduced infectivity of *L. monocytogenes* through induction of the unfolded protein response and eIF2 signalling (Chen et al., 2017). HMOs can also elicit anti-infective effects by directly inhibiting growth of pathobionts such as Group B Streptococci (GBS) (Ackerman, Doster, Weitkamp, & Aronoff, 2017). Considerable inhibitory effects against GBS were demonstrated for the HMO structures LNT and LNFP-I (Lin et al., 2017).

3.3.2. Viral infections

HMO-mediated protection has also been described for many viruses such as rotaviruses or noroviruses, which are some of the leading causes of gastroenteritis outbreaks worldwide (Widdowson, Steele, Vojdani, Wecker, & Parashar, 2009; Robilotti, Deresinski, & Pinsky, 2015). HMOs function as antivirals by mimicking receptor sites and blocking entry into the cell, but also by preventing viral replication within the cell. One of the earliest studies demonstrating the potential of HMOs to reduce infectivity of Norovirus found that human milk blocked genogroup I genotype 1 (GI.1) and genogroup II genotype 4 (GII.4) binding to saliva (Jiang et al., 2004). Several studies have identified histo-blood group antigens (HBGA) as key binding sites for norovirus adhesion (Schroten, Hanisch, & Hansman, 2016). Some HMOs demonstrate homology to HBGA, with Norovirus binding most avidly to high-mass HMO rich in α-fucose (Hanisch, Hansman, Morozov, Kunz, & Schroten, 2018). Surface plasmon resonance imaging of glycan microarrays revealed that affinity of Norovirus genotypes to HMO structures is strain specific. The results show both GI.1 (Norwalk) and GII.4 (VA387) capsids have greater affinity for fucosylated glycans over non-fucosylated HMOs (Shang et al., 2013). GII.4 capsids demonstrated preferential binding to glycans with H type 2 (Gal β1-4GlcNAc) or H type 6

(Galβ1-4Glc) moieties (such as LNFP-III and 2′-FL). In contrast, GI.1 capsids were shown to display broader binding-specificity, recognising H type 1 (Galβ1-3GlcNAc) linkages (such as LNFP-I or LNDFH-I) as well as H type 2/6. Moreover, GI.1 variants were shown to display affinity for glycans in the form of glycoproteins, but also in the form of free oligosaccharides, while GII.4 variants bound preferentially to glycans conjugated to proteins. The results of this study suggest that fucosylated HMOs mimic HBGA binding sites and thereby reduce adherence of norovirus (Shang et al., 2013). Studies from Hansman's group at the Univeristy of Heidelberg employed X-ray crystallography techniques to reveal that 2′-FL binds to GII.17 variants, while both 2′-FL and 3-FL were capable of blocking GII.10 noroviruses to HBGA, verifying that fucosyllated glycans function as antiviral agents against Norovirus by binding GI and GII HBGA sites (Koromyslova, Tripathi, Morozov, Schroten, & Hansman, 2017; Weichert et al., 2016).

Milk oligosaccharides have also been shown to function as antiviral agents against Rotavirus (RV) infection. A sialic acid-containing component in human milk was shown to inhibit virus replication *in vitro* and to reduce symptoms of gastroenteritis in animal models (Yolken et al., 1992). The results of this study were consistent with subsequent experiments which found that 3′-SL and 6′-SL act as antivirals against porcine RV (Hester et al., 2013). *In vivo* studies in piglet models revealed that both acidic and neutral fractions significantly reduced infectivity of RV (Hester et al., 2013). Most recently, HMOs were demonstrated to reduce infectivity of 2 dominant RV strains in monkey kidney epithelial cells, with notable strain-specific differences. G1P[8] infectivity was inhibited most significantly by 2′-FL while a combination of 3′-SL and 6′-SL had the greatest effect on G2P[4] variants (Laucirica et al., 2017).

In addition to the protective effects which have been demonstrated for HMOs against intestinal viruses, studies have also shown that human milk glycans can serve as protective agents against pathogenesis caused by respiratory viruses (Zevgiti et al., 2012). Sialylated oligosaccharides have been shown to reduce infectivity of influenza viruses, while 2′-FL reduced viral load of respiratory syncytial virus *in vitro* (Duska-McEwen, Senft, Ruetschilling, Barrett, & Buck, 2014). Moreover, intervention of 2′-FL in an influenza-specific murine model resulted in improved adaptive and innate immunity (Xiao et al., 2018). It is plausible therefore, to speculate that HMOs could act as anti-viral agents against members of the coronavirus family including severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), the causative pathogen of the COVID-19 pandemic. Many mammalian viruses have evolved to use glycans as host cell receptors (Thompson, de Vries, & Paulson, 2019) and potential associations between SARS-CoV-2 and glycans expressed on the cell surfaces cannot be disregarded. Investigating the potential of HMOs to act as soluble decoy receptors for the COVID-19 virus may be of great interest for future research.

3.3.3. Fungal infection

Although only a small number of publications are available which assess the anti-fungal effects of HMOs, the findings from these studies suggest that pooled HMOs can reduce infection by fungal species such as *Candida albicans*. In an *in vitro* study using H4 premature IECs, Gonia et al. found that HMOs altered hyphal induction in *C. albicans*, thereby preventing invasion of the intestinal epithelium (Gonia et al., 2015).

3.3.4. Protozoan parasite infections

Beyond anti-viral and anti-bacterial roles, studies are limited which detail the activity of HMOs against protozoan parasites. One example, however, is *Entamoeba histolytica*, an anaerobic amoebozoan which causes an estimated 55,000 deaths a year globally (Shirley, Farr, Watanabe, & Moonah, 2018). Jantscher-Krenn et al. demonstrated that isolated HMO-enriched fractions reduced adherence and cytotoxity of *E. histolytica* in an *in vitro* model (Jantscher-Krenn et al., 2012). By resembling Gal/GalNAc lectin binding sites, non-fucosylated HMOs act as soluble decoy receptors against amoebic infection.

3.4. HMOs modulating epithelial cell responses

The intestinal epithelium lining in the small intestine and colon is considered the first line of defence in innate immunity, since it acts as a physical, rate-limiting barrier between the gut lumen and the circulatory system (Maldonado-Contreras & McCormick, 2011). Intestinal crypts and villi are covered by a single layer of absorptive enterocytes and secretory cells (including goblet, paneth and enteroendocrine cells). Multipotent stem cells found in the basal portion of the intestinal crypt divide to form daughter cells, which differentiate as they migrate along the villus-crypt axis (Umar, 2010). Permeability of the epithelium is dependent primarily on the structure and expression of tight junctions. The absorption of nutrients through this epithelium occurs transepithelially (through cells) or paracellularly (between tight junctions). Perturbations in barrier integrity result in irregular immune responses including gastrointestinal infections, inflammatory bowel diseases, and allergies (Vancamelbeke & Vermeire, 2017). Reliable measurements of intestinal barrier function during clinical intervention studies are rare. Therefore, *in vitro* studies using cultures of HT-29, Caco-2, or other intestinal cell lines are commonly used to understand the mechanisms by which nutrients and microbial metabolites affect epithelial integrity. Researchers have demonstrated that HMOs promote tight junction protein expression (Fig. 1), and increase differentiation along the crypt-villus axis (Holscher, Davis, & Tappenden, 2014). By modelling the varying differentiation patterns along the crypt-villus axis with pre-confluent HT-29 and Caco-2Be cells, and post-confluent Caco-2Be cells, Holscher et al. assessed the effects of three HMO structures (LNnT, 2′-FL, and 6′-SL) on epithelial cell differentiation, apoptosis, and proliferation (Holscher et al., 2014). The results demonstrated that 2′-FL significantly increases differentiation in HT-29 cells when alkaline phosphatase was used as a marker of differentiation. The authors observed reduced cell proliferation after exposure to LNnT and 6′-SL, with 2′-FL demonstrating weak anti-proliferative effects. LNnT was the only HMO structure capable of reducing permeability across the epithelial barrier (Holscher et al., 2014). The results, which are consistent with previous work by Kuntz and colleagues, signify that the function of HMOs is stereospecific (Kuntz, Rudloff, & Kunz, 2008).

Grabinger et al. recently demonstrated that 2′-FL reduced the progression of colitis in an interleukin-10 null ($IL10^{-/-}$) murine model. Intestinal permeability was determined by measuring serum FITC-dextran levels and it was shown that supplementation with 2′-FL significantly increased epithelial integrity (Grabinger et al., 2019). These effects were associated with increased abundances of the commensal *Ruminococcus gnavus* and elevated levels of propionate in the cecum. This implies that by promoting the growth of beneficial bacteria, HMOs function to favourably modulate the epithelium barrier by creating a healthier balance of SCFAs (Fig. 1).

The glycocalyx acts as a skeleton of proteoglycans which provides binding sites for microorganisms and can function as a physical barrier preventing invasion of toxins. Defective maturation of the glycocalyx layer can therefore result in various gastrointestinal conditions. It has been shown through *in vitro* studies that HMOs support intestinal barrier function by altering expression of various glycocalyx components (Fig. 1). For example, it was revealed through the use of microarray glycan profiling that HMOs can alter patterns of glycosylation on the epithelial cell surface (Angeloni et al., 2005). Again, these effects were found to be structure-dependent with exposure to 3′-SL significantly reducing the number of sialic acid residues found on the surface of Caco-2 cells. Alterations in glycocalyx were caused by reduced expression of the sialytransferases ST3Ga11, ST3Ga12, and ST3Ga14, and was associated with a reduction in adhesion of enteropathogenic *E. coli* to the Caco-2 cells (Angeloni et al., 2005). More recently, Kong and colleagues demonstrated that fucosylated HMOs significantly enhanced glycocalyx development in Caco-2 cells (Kong et al., 2019). The study revealed that 2′-FL and 3-FL significantly enhanced the thickness of absorbed albumin, while 3-FL increased the area coverage of albumin,

Table 3
Receptors involved in biological recognition of HMO.

Type	Expression	Function	Name	HMO related ligand	Reference
C-type lectins	Antigen presenting cells	Internalisation of antigens which contain saccharide epitopes. Play a role in IgA transcyotosis and interact with various pathogens	DC-SIGN	2′-FL, 3-FL, LNFP-III, LNFP IV, LNDFH-I	(Noll et al., 2016)
Galectins	T cells/ Intestinal Epithelial cells	Regulation of immune cell homeostasis and inflammation. Particpate in regulation of T-cell function	Galectin-9	scGOS	(de Kivit et al., 2013)
			Galectin-3	3′-SL, LNFP I, II, III, LNDFH	(Kitova, El-Hawiet, & Klassen, 2014)
Selectins	Leukocytes/ Endothelial cells	Cell adhesion molecules that facilitate leukocyte trafficking.	P- selectin	HMO with sialyl	(Wright & Cooper, 2014) (Rudloff, Stefan, Pohlentz, & Kunz, 2002)
			E-selectin	Lewis X epitopes	
Siglecs	Neutrophils, monocytes, dendritic cells	Inhibit cell activation and proliferation, modulate macrophages/ dendritic cell production of cytokines. Regulate B-lymphocytes and T-cell fucntion	Siglec-4	2,3-sialic acid	(Koliwer-Brandl et al., 2011)
			Siglec-2	2,6-sialic acid	(Koliwer-Brandl et al., 2011)
			Siglec-5	Sialyl-Lewis c	(Noll et al., 2016)
			Siglec-9	Numerous sialylated HMO	(Noll et al., 2016)
TLRs	Macrophages and dendritic cells,	Activate NF-kB and interferon regulatory factor pathways, which results in secretion of pro-inflammatory factors.	TLR2	3-FL, 3′-SL, 6′-SL	(Cheng, Kiewiet, Groeneveld, Nauta, & de Vos, 2019) (Asakuma et al., 2010)
			TLR3	3′-, 4′-, 6′-GL	(He et al., 2014)
			TLR4	LNFP I, III, 2′-FL	(He et al., 2014) (Asakuma et al., 2010)

heparan sulphate, and hyaluronic acid in the glycocalyx of the Caco-2 cells. Increased adsorption of albumin is associated with improved stability of the glycocalyx and decreased adherence of pathogenic bacteria (Kong et al., 2019). Heparan sulphate and hyaluronic acid form the major glycosaminoglycan chains in the glycocalyx and are linked to the core proteins of proteoglycans. Gastrointestinal disorders such as inflammatory bowel disease (IBD) are associated with alterations in the expression of these glycosaminoglycan chains. Improved expression of heparan sulphate and hyaluronic supports enhanced colonic epithelial repair and homeostasis of innate immunity (Kong et al., 2019). HMOs also have a positive effect on mucin glycoproteins expression in the mucus barrier. Narrowing of this mucus layer is linked to increased permeability of the intestine and a predisposition to gastrointestinal disorders such as NEC (Hodzic, Bolock, & Good, 2017).

Recently, fractions of HMOs isolated from breast milk were shown to increase the expression of *MUC2* and decrease intestinal permeability in a neonatal mouse model of NEC (Wu et al., 2019). HMOs induced expression of mucins through direct action on chaperone proteins such as protein disulphide isomerase (Wu et al., 2019). Results of this animal study are in line with findings from an *in vitro* study (Cheng, Kong, Walvoort, Faas, & de Vos, 2019). Here, the effects of HMOs on expression of goblet cell secretory genes in a colorectal cell model were assessed. 2′-FL enhanced *MUC2* expression during IL-13 exposure, while 3-FL up regulated both *MUC2* and *TFF3* gene expression during TNFα and IL-13 exposure (Cheng, Kong, et al., 2019). Further studies, particularly intervention studies in infants, are required to fully elucidate the role of HMOs in the maturation of intestinal cells and mucosal surfaces. Results from *in vitro, ex vivo* and *in vivo* studies, however, suggest that HMOs positively influence gut barrier function, and thereby may prevent gut dysfunction and NEC through improved expression of tight junction proteins, glycoclayx components, and mucins.

3.5. HMO effects on immune cell populations and cytokine expression

While the vast majority of HMOs pass undigested into the colon, a small proportion is absorbed intact and excreted in the urine. The presence of intact HMO in the urine of breast-fed infants was first described in 1996 by Rudloff and colleagues (Rudloff, Pohlentz, Diekmann, Egge, & Kunz, 1996), with subsequent clinical studies reporting absorption rates of 1–5% HMO from the GI tract to the circulatory system (Goehring, Kennedy, Prieto, & Buck, 2014; Rudloff & Kunz, 2012; Ruhaak, Stroble, Underwood, & Lebrilla, 2014). These findings suggest that, in addition to their role in an infant's intestinal lumen, HMO may also contribute to the development and physiological functions of other organs and systemic cell systems. Studies have shown that HMOs modulate immune cell-cell interactions, thereby facilitating maintenance of balanced inflammatory cell responses (Fig. 1) (Donovan & Comstock, 2016). This HMO signalling is mediated by receptor molecules which are expressed predominantly on the surface of immune cells (Triantis, Bode, & Van Neerven, 2018). Several classes of lectins and toll-like receptors (TLRs) with HMO binding specificity have been described in the literature and are summarised in Table 3.

In the period directly after birth, a neonate's cellular immune system undergoes rapid development. Multiple immune factors, such as neutrophils, macrophages, monocytes, natural killer (NK) cells, T cells, and dendritic cells (DCs), play a critical role in fighting against invading pathobionts (Kelly & Coutts, 2000). At birth, an imbalance between Th1/17 and Th2 phenotypes is noted in T-helper cell populations. A neonate's immune system is directed more towards the Th2 phenotype which promotes humoral immunity and confers protection against extracellular pathogens. On the other hand, Th1/Th17 pathways target intracellular pathogens and are less prevalent in the developing neonate (Adkins, 2000). The immature immune system is also characterized by overexpression of inflammatory markers and inadequate feed-back regulation of immune signalling. It is hypothesised, therefore, that breast-milk oligosaccharides favourably modulate neonatal innate immune responses by controlling expression of inflammatory markers involved in cell trafficking and affecting cytokine and chemokine networks that regulate Th1/Th2 lymphocyte balance (Fig. 1) (Kulinich & Liu, 2016). However, information regarding the role of HMOs and specificities of their action in inflammation is limited and is largely dependent on findings from *in vitro* and *ex vivo* studies.

Several studies have focussed on assessing the immunomodulatory effects of HMO mixtures isolated from human milk. For example, He et al. assessed the impact of colostral HMOs on intact immature human intestinal mucosa (He, Liu, Leone, & Newburg, 2014). The authors found that colostral HMO reduced levels of pro-inflammatory cytokines (such as IL-1β, IL-6 and IL-8) while stimulating expression of cytokines associated with tissue repair and homeostasis. The group also found that exposure to HMOs elevated expression of Th1 polarization, and shifted the balance of Th1/ Th2 cytokines towards that of more mature tissues (He et al., 2014). More recently, isolated HMOs were found to stimulate semi-maturation of human monocytes-derived DCs which was associated with elevated levels of anti-inflammatory cytokines such as IL-6, IL-10, and IL-20 (Xiao et al., 2019). HMOs were also found to reduce LPS-induced production of pro-inflammatory cytokines such as IL-12p70 and TNF-α, by preventing the interaction of LPS and TLR4 (Xiao et al., 2019). Zhang et al. provided further mechanistic insight by demonstrating that a neutral HMO fraction influenced inflammatory cell populations via nuclear factor (NF)-κB and mitogen-activated

protein-kinase pathways (Zhang et al., 2019). Interestingly, a recent animal model study has demonstrated that HMOs may be protective against the development of type-1 diabetes (T1D) (Xiao et al., 2018). Dietary supplementation of HMOs was shown to reduce T1D incidence and to suppress development of pancreatic insulitis in non-obese diabetic (NOD) mice. These effects were associated with changes in cytokine profiles and microbiota composition (Xiao et al., 2018). These findings suggest that HMO-supplementation may hold therapeutic potential in the treatment of gut-related autoimmune diseases.

Certain fucose-containing HMO species have been shown to alter expression of inflammatory cell populations, with the majority of studies indicating that fucosylated-HMOs play a role in Th2-promoting activities. The anti-inflammatory effects of 2′-FL were demonstrated in T84 and H4 intestinal epithelial cells, wherein 2′-FL quenched LPS-induced inflammation during infection with type I pili *E.coli* strains (He et al., 2016). Exposure to 2′-FL reduced CD14 expression, a factor which mediates interaction of LPS and TLR4 (He et al., 2016). Similar effects for Fuc-borne HMO were observed by Sotgiu at al. wherein LPS-activated mononuclear cells taken from multiple sclerosis patients were exposed to 2′-FL and LNFP I. The oligosaccharides were found to reduce proliferation of the mononuclear cells, which was associated with a reduction in IL-12 and IFN-γ, while expression of IL-10 increased significantly (Sotgiu et al., 2006). Other fucosylated HMO have been shown to play a role in stimulating Th2 responses including LNFP III, which stimulates macrophage and NK cell activity *in vitro*, while increasing secretion of IL-10, TNFα, and prostaglandin E2 (Atochina & Harn, 2005). Immunomodulation by fucosylated HMO has also been demonstrated in animal studies. In a murine model of food allergy, 2′-FL reduced food allergy symptoms and induced significant increases in $IL10^+CD4^+CD25^+$ Tregs from Peyer's patches (Castillo-Courtade et al., 2015). Similar effects were observed for 6′-SL albeit through different mechanisms to 2′-FL. A limited number of observational studies in breast-fed infants have found associations between fucosylated HMO and the development of allergy. For instance, Seppo et al. evaluated associations between concentrations of HMO and the development of cow's milk allergy (CMA). The researchers here found that the concentration of LNFP III was lower in milk consumed by infant's who developed CMA, suggesting that HMO structures may play a protective role against the development of certain food allergies (Seppo, Autran, Bode, & Järvinen, 2017).

In contrast to fucosylated HMOs, the effects of sialylated HMOs are bidirectional with studies demonstrating stimulation of both Th1 and Th2 responses in *in vitro* models. In a 2010 study by Eiwegger et al., cord-blood derived mononuclear cells were exposed to pooled sialylated HMO (Eiwegger et al., 2010). Exposure to acidic HMO fractions promoted the production of IFN-γ and IL-10, resulting in down-regulation of Th2 responses which shifted the immune response towards a more balanced Th1-Th2 profile. In this same investigation, sialylated HMOs were also shown to reduce IL-4 production in lymphocytes derived from adults with peanut allergy (Eiwegger et al., 2010). Moreover, 3′-SL has been shown to exhibit proinflammatory attributes by stimulating mesenteric lymph node $CD11c^+$ dendritic cells, releasing cytokines that increase Th1 and Th17 immune cell populations (Kurakevich, Hennet, Hausmann, Rogler, & Borsig, 2013). In contrast to these findings, anti-inflammatory effects for 3′-SL were reported during an Caco-2 cell *in vitro* study with exposure to 3′-SL resulting in reduced expression of IL-8, IL-12, and TNFα (Zenhom et al., 2011). In addition to *in vitro* findings, results from clinical observational studies have identified associations between concentrations of certain sialylated HMO species in breast milk and development of NEC in preterm infants (Autran et al., 2018). In particular, low concentrations of DSLNT in breast milk were associated with development of NEC with authors suggesting that the DSLNT content in breast milk could serve as a potential non-invasive marker to identify infants at risk of developing NEC (Autran et al., 2018; Bode, 2018). Despite increasing evidence that HMOs contribute to balanced inflammatory responses, it remains to be seen whether individual HMOs will become novel prophylactic and therapeutic agents for the treatment of allergy, auto-immune, and inflammatory diseases in the future. Further comprehensive studies, particularly large placebo controlled infant studies, are required to elucidate the potential of HMO in immunomodulation.

4. Conclusions

HMOs are thought to play a central role in the development of the neonatal immune system by promoting healthy microbial diversity, preventing pathogen attachment, stimulating maturation of intestinal epithelial surface and by modulation of immune cells. Further research is required however to reveal whether HMOs may also have a therapeutic rather than a protective effect in infant gut health. Numerous *in vitro* and animal experiments have been performed with HMO but to date; these bioactives have not been tested in many placebo controlled infant studies. In addition, HMOs have mostly been studied in isolation, yet they exist as a diverse pool and interact in the mammary gland and the infant gut. Novel combinations of methodologies will be essential in future studies to unravel the multifaceted interactions, specific or otherwise, between HMOs and the infant gut microbiota and their collective impact on immune function and prevention of multiple disease states.

5. Ethics statements

This is a review article. It has not involved any human subjects and animal experiments.

CRediT authorship contribution statement

Clodagh Walsh: Writing - review & editing. **Jonathan A. Lane:** Writing - review & editing. **Douwe van Sinderen:** Writing - review & editing, Supervision. **Rita M. Hickey:** Writing - review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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