Food Research International 151 (2022) 110884

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

Review

Recent understanding of human milk oligosaccharides in establishing infant gut microbiome and roles in immune system

Ravindra Pal Singh [a,*,1], Jayashree Niharika [a,1], Kanthi Kiran Kondepudi [b], Mahendra Bishnoi [b], Jagan Mohan Rao Tingirikari [c]

[a] Laboratory of Gut Glycobiology, Food and Nutritional Biotechnology Division, National Agri-Food Biotechnology Institute (NABI), SAS Nagar, Punjab 140306, India
[b] Healthy Gut Research Group, Food and Nutritional Biotechnology Division, National Agri-Food Biotechnology Institute (NABI), SAS Nagar, Punjab 140306, India
[c] Department of Biotechnology, National Institute of Technology Andhra Pradesh, Tadepalligudem, Andhra Pradesh 534101, India

ARTICLE INFO

Keywords:
Human milk oligosaccharides
Bifidobacterium
Enzymes
Immune system
Decoy
Pathogen

ABSTRACT

Human milk oligosaccharides (HMOs) are complex sugars with distinctive structural diversity present in breast milk. HMOs have various functional roles to play in infant development starting from establishing the gut microbiome and immune system to take it up to the mature phase. It has been a major energy source for human gut microbes that confer positive benefits on infant health by directly interacting through intestinal cells and generating short-chain fatty acids. It has recently become evident that each species of *Bifidobacterium* and other genera which are resident of the infant gut employ distinct molecular mechanisms to capture and digest diverse structural HMOs to avoid competition among themselves and successfully maintain gut homeostasis. HMOs also directly modulate gut immune responses and can decoy receptors of pathogenic bacteria and viruses, inhibiting their binding on intestinal cells, thus preventing the emergence of a disease. This review provides a critical understanding of how different gut bacteria capture and utilize selective sugars from the HMO pool and how different structural HMOs protect infants from infectious diseases.

1. Introduction

Human milk is a mixture of bioactive compounds that contain glycans (carbohydrates), proteins, and fat globules (Ballard & Morrow, 2013). Amid them, human milk oligosaccharides (HMOs) are highly complex structures of unconjugated glycans (McGuire et al., 2017), ranging from 7 g/L in mature breast milk to 23 g/L in colostrum (Coppa et al., 1993; Gabrielli et al., 2011). In the current stage, there are over 200 different HMOs present in the human milk, of which structures of about 150 are characterized (X. Chen, 2015; Ninonuevo et al., 2006; Porfirio et al., 2020), varying in size from 3 to 22 monosaccharide units-mainly consisting of *N*-acetylglucosamine (GlcNAc), fucose, sialic acid, galactose, and glucose. The core structures of HMOs generally exhibit a lactose moiety at the reducing end. This lactose is often branched with L-fucose (α-1,2 or α-1,3 linkages) or sialic acid (α-2,3 or α-2,6 linkages), which corresponds to trisaccharide (fucosyllactose or sialyllactose). In the case of complex HMOs, lactose core has been further extended in branched or a linear fashion with repetitions of N-acetyl-lactosamine (LacNAc; β-D-Gal- β -(1,4)-D-GlcNAc, type 2 chain) or lacto-*N*-biose, (LNB; β -D-Gal-(1,3)-D-GlcNAc, type 1 chain), and these can be further adorned with L-fucose and/or sialic acid residue/s (German, Freeman, Lebrilla, & Mills, 2008; Ninonuevo et al., 2006; Prudden et al., 2017). This gold mine of human milk exhibits several benefits, for example, they act as glycan decoys (anti-adhesion) (Craft & Townsend, 2018; Ruiz-Palacios, Cervantes, Ramos, Chavez-Munguia, & Newburg, 2003), possess antibacterial properties (Lin et al., 2017), interact with immune cells that shape the host responses (Triantis, Bode, & van Neerven, 2018) including modulating factors for the human gut microbiome (Barratt, Lebrilla, Shapiro, & Gordon, 2017; German et al., 2008), and stimulate gut barrier function (Suligoj et al., 2020). HMOs act as prebiotics via promoting the growth of gut commensals and probiotics, especially *Bifidobacterium* and *Lactobacillus* in breast-fed infants (Underwood, German, Lebrilla, & Mills, 2015). HMOs (especially 3-fucosyllactose- 3-FL, and 6′-sialyllactose- 6′-SL) promote adhesion of *B. longum* subsp. *infantis* ATCC 15697 on HT-29 and Caco-2 cell lines as compared to cells grown on glucose and lactose (Kavanaugh et al., 2013; Wickramasinghe,

* Corresponding author.
E-mail address: r.p.singh@nabi.res.in (R.P. Singh).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.foodres.2021.110884
Received 30 May 2021; Received in revised form 19 November 2021; Accepted 6 December 2021
Available online 9 December 2021

Pacheco, Lemay, & Mills, 2015). Additionally, HMOs are involved in brain development by the enteric nervous system (known as the gut-brain axis) and protect against obesity and type- 2 diabetes by maintaining gut microbial homeostasis (LeMay-Nedjelski et al., 2021; Saben, Sims, Abraham, Bode, & Andres, 2021; Savino, Benetti, Liguori, Sorrenti, & Cordero Di Montezemolo, 2013).

Synthesis of HMOs depends on the expression of different glycosyltransferases such as β-1,3-N-acetylglucosaminyltransferases, and β-1,4-galactosaminyltransferases in mammary glands of humans, which glycosylates lactose to produce different HMOs. α-1,2- fucosyltransferases (encoded by FUT 2 gene) and β-galactoside sialyltransferases (SGalT) are accountable for fucosylation and sialylation of a terminal galactose residue(s) respectively (Kellman BP et al., 2020). Notwithstanding, it should be noted that the extension and branching of HMOs can potentially be catalyzed by multiple isozymes belonging to these families. It has been reported that lactating mothers health conditions (obesity, malnourishment, or hyperglycemia) and diet have a significant impact on the production and structural diversity of HMOs (Smilowitz, Lebrilla, Mills, German, & Freeman, 2014). In addition to those, the dietary intake of glucose, fucose, galactose, GlcNAc, sialic acid, fucosyllactose and lacto-N-tetraose (LNT) significantly contribute to their incorporation in HMOs (Elison et al., 2016; Orczyk-Pawilowicz & Lis-Kuberka, 2020; Seferovic et al., 2020; Sprenger & Duncan, 2012).

Particularly two fucosyltransferases, the FUT2 (encoding the secretor locus) and FUT 3 (encoding the Lewis blood group locus) greatly influence the variability of HMOs composition (Lefebvre et al., 2020). The FUT3 and the FUT2 transferases transfer α1,3/4- and α1,2-fucose group to the sub-terminal (GlcNAc) and terminal (Gal) sugars of the type I chain. The FUT2 enzyme specificity produces HMOs like 2′-fucosyllactose (2′-FL) and lacto-N-fucopentaose- I (LNFP-I), and the presence or absence of these sugars define the secretor or non-secretor milk status respectively. Whereas the FUT3 enzyme specificity produces HMOs like lacto-N-fucopentaose II (LNFP-II) and the presence or absence of the sugar defines Lewis status of milk (A. Wang et al., 2020). The FUT3 also adds fucose on to LNFP I and 2′-FL to form lacto-N-difucohexaose I (LNDFH I) and lactodifucotetraose (LDFT) respectively. The FUT3 can directly transfer fucose to lacto-N-neotetraose (LNnT), and lactose to form lacto-N-fucopentaose III (LNFP III) and 3FL, respectively (Mank, Hauner, Heck, & Stahl, 2020). It has been identified that secretor milk samples were predominant with LNFP I, LNDFH I, and 2′-FL, whereas non-secretor milk was characterized by LNFP II, 3FL and LNDFH II (Cabrera-Rubio et al., 2019; Gu, Wang, Beijers, de Weerth, & Schols, 2021). Conversely, levels of the above sugars in HMOs greatly depend on the type of mammalian enzymes involved in the synthesis of HMOs (Lefebvre et al., 2020). Among all the HMOs present in mother's milk, 2′-FL is reported to be the most abundant, followed by 3-FL (Christensen, Skov, Lendal, & Hornshoj, 2020; Thurl et al., 2010). Thus, a large number of scientific studies have mainly used 2′-FL and 3-FL for *in vivo* and *in vitro* analyses.

The importance of HMOs came into limelight when breast fed neonates were found to have high numbers of *Bifidobacterium* (György, Norris, & Rose, 1954; Tissier, 1900) Bestowed with prebiotic properties, HMOs promote a club of bacteria associated with wellbeing (Casaburi et al., 2021; Ma et al., 2020). Given the complexity of HMOs, they are resistant to digestive enzymes produced by human beings and reach both small and large intestines in their intact form, and hence act as a food source for the developing gut microbiome and maintain homeostasis. Increasing evidences from early microbial colonization suggested that it begins with *Staphylococcus*, *Klebsiella*, *Streptococcus,* and *Enterococcus* (present at vaginal skin) when a fetus reaches the lower uterus (Laforest-Lapointe & Arrieta, 2017), and the development of microbiota takes place after birth and converges toward an adult-like form by the end of first 3–5 years of infant's life (Rodriguez et al., 2015). Especially, the space of infant's gut is dominantly occupied by *Bifidobacterium* within a week after birth, and they remain dominant until weaning starts. How infants get *Bifidobacterium* is fairly now known. Some studies isolated the same strains from mother–infant pairs in vaginal born as compared to caesarean born infants (Makino et al., 2013; Mueller, Bakacs, Combellick, Grigoryan, & Dominguez-Bello, 2015; Palmer, Bik, DiGiulio, Relman, & Brown, 2007). It confirms that mother-to-infant (vertical) transmission takes place in the case of several Bifidobacterium species. In addition to delivery mode, the diet has been very instrumental in establishing early gut microbiota —several studies have found a higher abundance of *Bifidobacterium* in the gut microbiota of breast-fed infants relative to formula-fed infants (Balmer & Wharton, 1989; Harmsen et al., 2000; Yoshioka, Iseki, & Fujita, 1983). Growing literature suggests that *Bifidobacterium* are specialized in HMO utilization; thus, breast-feeding encourages *Bifidobacterium* population in the early life of infants (Garrido, Barile, & Mills, 2012; Sela & Mills, 2010).

Furthermore, The Environmental Determinants of Diabetes in the Young (TEDDY) study proposed the three distinct phases of infant gut microbiome development that include (1) a developmental phase (during months 3–14), (2) a transitional phase (during months 15–30), and (3) a stable phase (during months 31–46) (Stewart et al., 2018). During the early development of *Bifidobacterium* in the gut microbiome, *B. longum, B. breve, B. bifidum* are highly abundant as compared to *B. adolescentis, B. animalis* and *B. dentium* (Stewart et al., 2018). Afterwards, these populations start to decline and the populations of dietary fibers utilizing microbes increase such as *Bacteroides* and *Prevotella* in later stages of infant gut development. However, bacterial communities capable of HMOs utilization are present in the gut microbiome throughout the span of life (Suligoj et al., 2020). Interestingly, Pichler et al. (2020) suggested that species of *Roseburia* and *Eubacterium* play a vital role in the transition of gut microbiota from suckling to the weaning period. Nevertheless, the dynamic transition of bacterial communities from infant to adult-like gut microbiota is not yet elusive, especially concerning how *Bacteroides* and *Prevotella* occupy the space in the gut environment on the onset of the weaning period.

It has been recognized in recent years that understanding the molecular mechanistic functions of each HMO would be crucial for appropriately improving an infant's gut health through fortifying the infant formula milk. Therefore, this review will provide a critical overview of the functions of HMOs in the modulation of gut health and the defensive roles of HMOs against infectious pathogens.

2. Functions of HMOs in the modulation of gut health

A growing body of literature over several decades has identified diverse implications of HMOs in the health of infants including recruitment of initial intestinal microbiota (Pacheco, Barile, Underwood, & Mills, 2015). Given the importance of HMOs in the early life of infants, biochemists are currently trying to co-relate the role of each HMO structure in promoting the growth of beneficial microbes, improving immune systems, and understating their functions in pathogen suppression (Le Doare, Holder, Bassett, & Pannaraj, 2018; Tamburini, Shen, Wu, & Clemente, 2016). Whilst, analytical chemists are trying to elucidate the structural composition of HMOs present in human milk (Mank et al., 2020). Despite the huge benefits of HMOs, human milk is not always available, which forces many newborns to consume industrially produced infant formula. Analytical characterization of these infant formulas clearly indicates that they lack typical structures of HMOs (Ninonuevo & Bode, 2008). This piece of statement is undoubtedly supported by multiple studies, especially highlighting that formula-fed infants are vulnerable to increased risk of gastroenteritis, diarrhea, and various metabolic diseases including type- 2 diabetes (O'Sullivan et al., 2013; A. Stuebe, 2009; A. M. Stuebe & Schwarz, 2010). In other words, HMOs show great potential in the suppression of these diseases and promoting a healthy gut ecosystem. It is worth mentioning that most of the biologically relevant activities of HMOs are observed during the development of infants, especially thanks to fucosylated oligosaccharides (OSs) rather than non-fucosylated OSs that give paramount importance to HMOs (Table 1).

Table 1
Impact of human milk oligosaccharides on modulating gut microbiome.

Type of HMO	Model and type of analysis	Modulation of gut microbiome	Physiological function related to gut microbiota	Reference
2′-FL, 3-FL, LDFT, 3′-SL, 6′-SL	*In vitro* fermentation with bacterial species, Real-time PCR	↑*Bifidobacterium*, ↑*Bacteroides* ↓*Clostridium*, ↓*Lactobacillus*, ↓*Enterococcus*, ↓*Streptococcus*, ↓*Staphylococcus*, ↓*Enterobacter*, ↓*Escherichia*	Fucosylated HMOs strongly promote colonization and maintain a beneficial symbiotic microbiome.	Yu, Chen, Kling, et al. (2013)
3′-SL and 6′-SL	Selected strains	↑*B. longum* JCM 7007, 7009, 7010, 7011, 1272, 11347, ATCC 15708, ↑*Bacteroides vulgatus* ATCC 8482, ↑*B. thetaiotaomicron* ATCC 29148	Specific *Bacteroides* and *Bifidobacterium* differentially digest specific types of HMOs. Major fucosylated HMOs most strongly stimulate key species of mutualistic symbionts.	Yu, Chen, and Newburg (2013)
2′-FL and LNnT and their different combination	Clinical trial, healthy adults 16S rRNA	↑*Actinobacteria,* ↑*Bifidobacterium*, ↓*Firmicutes*, ↓*Proteobacteria*	Provides the first set of data on safety, tolerance and impact of HMOs on adult gut microbiota	Elison et al. (2016)
Pooled HMOs	Selected strains	↑*B. longum* ATCC 15697 ↑*Lactobacillus acidophilus* NRRL B-4495. ↓*Campylobacter jejuni* S107 ↓*Escherichia coli* K12	Highlight importance of glycan structures	J. Wang et al. (2017)
Pooled HMOs	Fecal sample, 16S rRNA sequencing,	↑*Bifidobacterium* species. ↑*Lactobacillus* species	Fucosylated HMOs can provide a solid foundation for development of “personalized” nutrition.	Bai et al. (2018)
LNFP II, lacto-N-difucohexaose II (HMOs found in non-secretor mothers)	Quantitative PCR and 16S gene pyrosequencing	↓*Lactobacillus,* ↓*Enterococcus,* ↓*Streptococcus,* ↓*Bifidobacterium,* ↓*Actinobacteria,* ↑*Lactobacillaceae,* ↑*Enterobacteriaceae,* ↑*Staphylococcaceae.*	Maternal secretor status influences the human milk microbiota composition	Cabrera-Rubio et al. (2019)
2′-FL and LNnT	Phase II, parallel, double blind, randomized, placebo-controlled study.	↑*Bifidobacterium*	Modulate gut microbiota of IBS patients toward improving infant’s health.	Iribarren et al. (2020)
HMO disaccharides (LNB, 3-FN and 6-FN)	Real-time PCR Stool samples	LNB-↑*Bifidobacterium* (especially- *B. breve* and *B. bifidum*), ↓*Blautia coccoides* 3FN- ↑ *B. breve* and *Lactobacillus*, ↓*B. coccoides* 6FN-↓ *Enterobacteriaceae* family	Evaluated disaccharides may show specific beneficial effects on the infant gut microbiota.	Rubio-Del-Campo et al. (2020)
2′-SL, 3′-SL, 6′-SL and lacto-N-triose	Selected strains	↑*B. longum* subsp. *infantis*, ↓*B. longum* subsp. *longum* and ↓*B. adolescentis*.	Impact on stimulation of gut microbiota is HMO-specific	L. Cheng et al. (2020)
Pooled HMOs	Metagenomic and flow cytometric assay	↑*Bifidobacterium*	The study highlights that genomic ability of a *Bifidobacterium* strain is not directly correlated with abundance of overall *Bifidobacterium* population.	Lugli et al. (2020)
2′-FL, LNnT	SHIME® model, Caco2 cell lines, and human intestinal gut organoid-on-chips	↑*Bifidobacterium,* ↑*B. coccoides/ E. rectale*,	Increase in SCFA production, mainly butyrate, which is related to increase in the gut barrier function through upregulation of claudin-5 and 8 genes	Suligoj et al. (2020)

2′-FL-2′-fucosyllactose, 3-FL-3-fucosyllactose, 6′-SL-6-′-sialyllactose, LNB- lacto-N-biose, 3-FN-fucosyl-α1,3-GlcNAc, 6-FN- fucosyl-α1,6-GlcNAc.

In the absence of adequate amount of HMOs, composition of the microbiome is altered where mucus-derived glycan degrading microbial and pathogenic bacterial populations (such as *Salmonella, Vibrio* and *Akkermansia muciniphila*) increase, leading to dysbiosis in the gut (Fig. 1A). Kostopoulos et al. (2020) identified a few enzymes that allow *A. muciniphila* to cleave HMOs (2′-FL, 3′-SL, LNT and lacto-N-triose II, LNT II) and help in the development of a healthy gut microbiome during the early life of the infants as well as establish a syntropy relationship with other bacteria, particularly *Bifidobacterium*. *A. muciniphila* can become detrimental as they have the capability to cleave mucus in the absence of HMOs. In the event of mucus degradation, microbial populations can invade the inner mucosal space, and free sulphate (SO_4^{2-}) can be generated from the degradation of glycosaminoglycan that is readily converted into thiosulphate via intermediate production of hydrogen sulfide (H_2S) (Furne, Springfield, Koenig, DeMaster, & Levitt, 2001). During inflammation the thiosulphate is converted into tetrathionate via reactive oxygen species, which leads to expansion of *Salmonella enterica* subsp. *enterica* serovar Typhimurium (Winter et al., 2010). Thus, gut homeostasis is disturbed leading to dysbiosis conditions (Fig. 1A). In this condition, loss of intestinal barrier integrity and leaky gut symptoms can arise due to a decrease in the production of short chain fatty acids (SCFAs), like- butyrate, acetate and propionate (Parada Venegas et al., 2019). Loss of intestine integrity can lead to translocation of pathogenic bacteria, lipopolysaccharides and toxic compounds into lamina propria, which induce inflammation and systemic immune dysregulation. In the altered gut, the innate immune cells can get activated and produce inflammatory cytokines, including type I interferons (IFNs) and interleukin (IL) (Lousberg, Fraser, Tovey, Diener, & Hayball, 2010). On the contrary, adequate quantities of HMOs increase health-promoting microbial communities including *Bifidobacterium* and *A. muciniphila* that lead to significant production of SCFAs, which in turn improve gut barrier integrity and increase activation of anti-inflammatory immunocytes and cytokines, such as regulatory T (Treg) cells (Fig. 1B) (Geirnaert et al., 2017; C. H. Kim, 2021). These beneficial microbes can outcompete harmful ones for nutrients and colonization of gut space. Thus, HMOs promote the gut environment by reinstating microbiota-mediated physiological functions (De Leoz et al., 2015).

In spite of hundreds of studies showing the beneficial impact of HMOs on infant’s health, recently only a few HMOs (such as 2′-FL and LNnT) have been introduced in the market as ingredients of infant formula, follow-on formula and young child formula, following their thorough mechanistic and clinical studies (Vandenplas et al., 2018). These are incorporated in the infant formula to mimic the nutritional contents of human milk as closely as possible. To further develop the next generation infant formula, there is a significant requirement to understand the mechanistic function of each HMO in order to woo their diverse benefits in terms of supporting the growth of beneficial bacteria, immune-modulating effects, anti-pathogenic effects, and stimulating intestinal barrier. Nevertheless, the current understanding of some HMOs is summarized below.

3. Mechanism for utilizing HMOs by human gut bacteria

HMOs exert a crucial impact on an infant’s gut microbiota (Table 1) and are a vital factor for development of the early microbiome (Tamburini et al., 2016). HMOs are resistant to gastric pH and reach the intestines without any change in their structures; thus, acting as a major

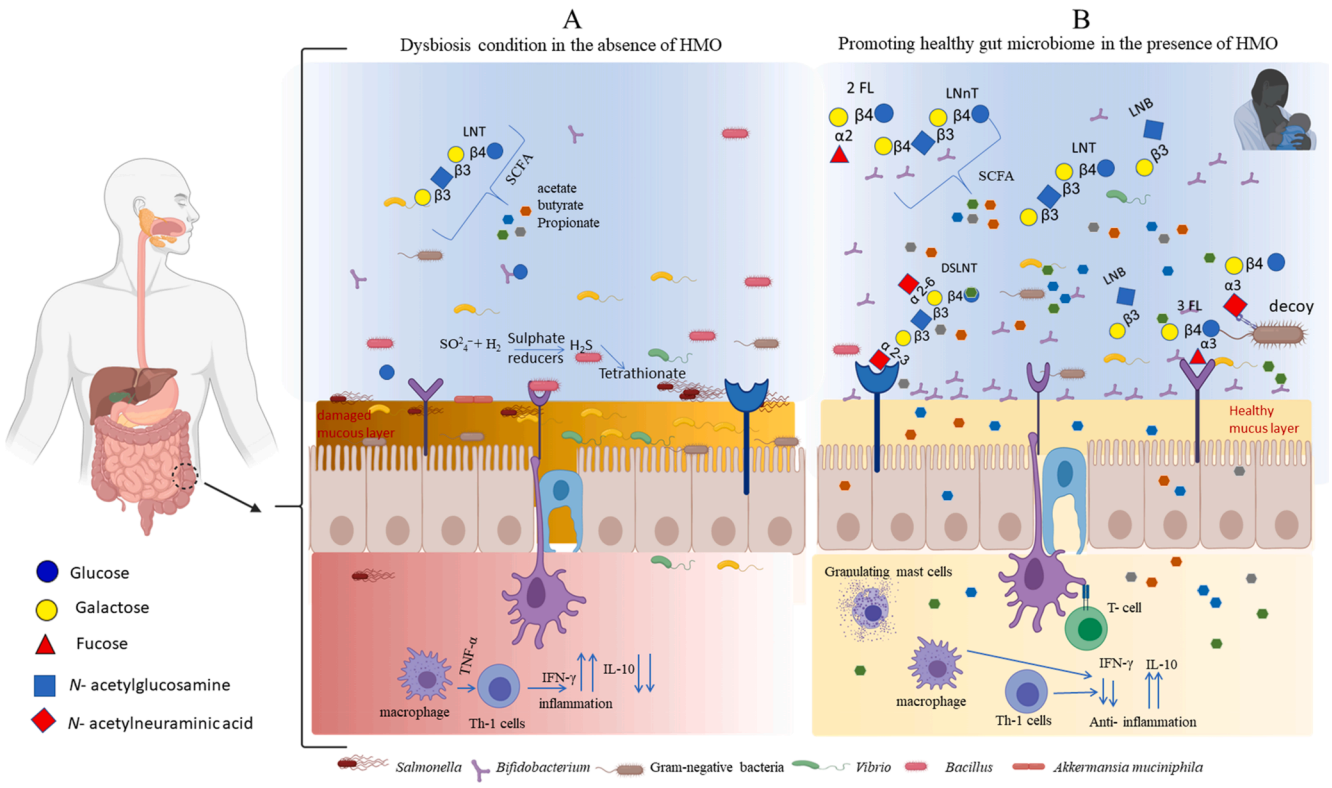

Fig. 1. Schematic overview of imperative roles of human milk oligosaccharides (HMOs) on the health of infants. (A) In lack of adequate amount of HMOs, microbiome modulation takes place where mucus-derived glycan degrading pathogenic (such as *Salmonella, Vibrio,* and *Akkermansia muciniphila*) populations increases. Because of mucus degradation, microbial populations inhabit inner mucosal space and generate free sulphate (SO_4^{2-}) that is readily converted into thiosulphate via intermediate production of hydrogen sulfide (H_2S) (Furne et al., 2001). In the inflammatory condition, thiosulphate transmutes into tetrathionate via reactive oxygen species, which leads to expansion of *Salmonella enterica* subsp. *enterica* serovar Typhimurium (Winter et al., 2010). Therefore, gut homeostasis is disturbed, resulting in dysbiosis. Loss of intestinal barrier integrity and leaky gut symptoms can be seen due to a decrease in the production of short chain fatty acids (SCFAs), alike- butyrate, acetate and propionate. Loss of intestinal integrity can lead to translocation of pathogenic bacteria, lipopolysaccharides and toxic compounds into lamina propria, which induce inflammation and systemic immune dysregulation. In the altered gut, innate immune cells can get activated and produce inflammatory cytokines, including type I interferons (IFNs) and interleukins (ILs). **(B)** Adequate quantity of HMOs increase health-promoting microbial communities including *Bifidobacterium* that lead to significant production of SCFAs, resulting in improvement of gut barrier integrity, and increase activation of anti-inflammatory immunocytes and cytokines, such as regulatory T (Treg) cells. Thus, it promotes gut environment by reinstating microbiota-mediated physiological functions (De Leoz et al., 2015). Beneficial microbes can compete with harmful microbes for nutrients and colonization of gut space. Furthermore, HMOs can act as a decoy for microbe-associated molecular patterns for inhibiting microbial adhesion on the intestinal gut lineage (Weichert et al., 2013) and inhibit the growth of *Streptococcus agalactiae* (group B *Streptococcus*) (Lin et al., 2017). 2′-FL: 2-fucosyllactose, 3-FL: 3-fucosyllactose, DSLNT: disialyllacto-N-tetraose, LDFT: lactodifucotetraose, LNnT: lacto-N-neotetraose, LNT: Lacto-N-tetraose, and LNB: Lacto-N-biose. Image is created with BioRender.

carbon source for infant gut microbiota. How these communities utilize the available carbon source is summarized in two sections, i.e. mechanism of utilizing HMOs by *Bifidobacterium* and non-*Bifidobacterium*.

3.1. Mechanism of utilizing HMOs by Bifidobacterium

Aforementioned, an infant's gut microbiome is predominantly abundant with *Bifidobacterium*. In particular, *Bifidobacterium infantis* harbors several proteins that are specialized in binding and transporting all sorts of HMOs into its cytoplasm (Garrido, Kim, German, Raybould, & Mills, 2011), and then digest them internally by a pool of GHs (J. H. Kim et al., 2013; Sakanaka et al., 2019). Other *Bifidobacterium* species are capable of digesting HMOs externally either by secretory or surface tethered GHs, for example, *B. bifidum* (Fig. 2). In addition, diverse mechanisms of GHs for utilizing HMOs are found in different species of *Bifidobacterium* (Sakurama et al., 2013; Wada et al., 2008; Yamada et al., 2017).

Glycome analysis of the *B. longum* subsp. *infantis* ATCC15697 revealed that it has the ability to use HMOs with a degree of polymerization ≤ 7 due to extraordinary activities of sialidase and fucosidase (LoCascio et al., 2007). Strikingly, a 43 kbp locus of this *bacterium* encodes extracellular solute binding proteins (SBPs), transporters (11 types) and several catabolic genes including GHs that are active on HMOs (Sela et al., 2008). SBPs and transporters in the *B. longum* subsp. *infantis* ATCC15697 are unique from other species of *Bifidobacterium* as they specialise in importing fucosylated HMOs in cytoplasm such as 2′-FL, 3-FL, LDFT and LNFP I. Sakanaka et al. (2019) characterized two fucosyllactose transporters (FL transporter-1 and −2) from this bacterium and concluded that FT-transporter-2 can import complex HMOs (2′-FL, 3-FL, LDFT and LNFP I) whereas FT-transporter-1 is responsible for importing short fucosylated HMOs (2′-FL and 3-FL). Upon checking the sequence homology of fucosyllactose –binding protein among gut microbes, it was observed that these are highly prevalent in *B. longum* strains, suggesting a unique adaptation strategy towards utilization of HMOs in the infant's gut. Indeed, LNT (type 1), lactose and type 2 specific (LNnT) cytoplasmic β-galactosidases and β-N-acetylglucosaminidases are solely observed in infant gut-associated *B. longum* subsp. *infantis*, which are responsible for efficient utilization of all sorts of HMOs (Yoshida et al., 2012). Hydrolysis of LNnT is successively achieved via *β*-galactosidase and β-*N*-acetylglucosaminidase to release Gal and GlcNAc, respectively (Miwa et al., 2010). A β-N-acetylglucosaminidase homologous gene is also present in the *B. breve* UCC2003 that may digest LNT and LNnT in the cytoplasm, but the strain could not import fucosylated HMOs (James, Motherway, Bottacini, & van

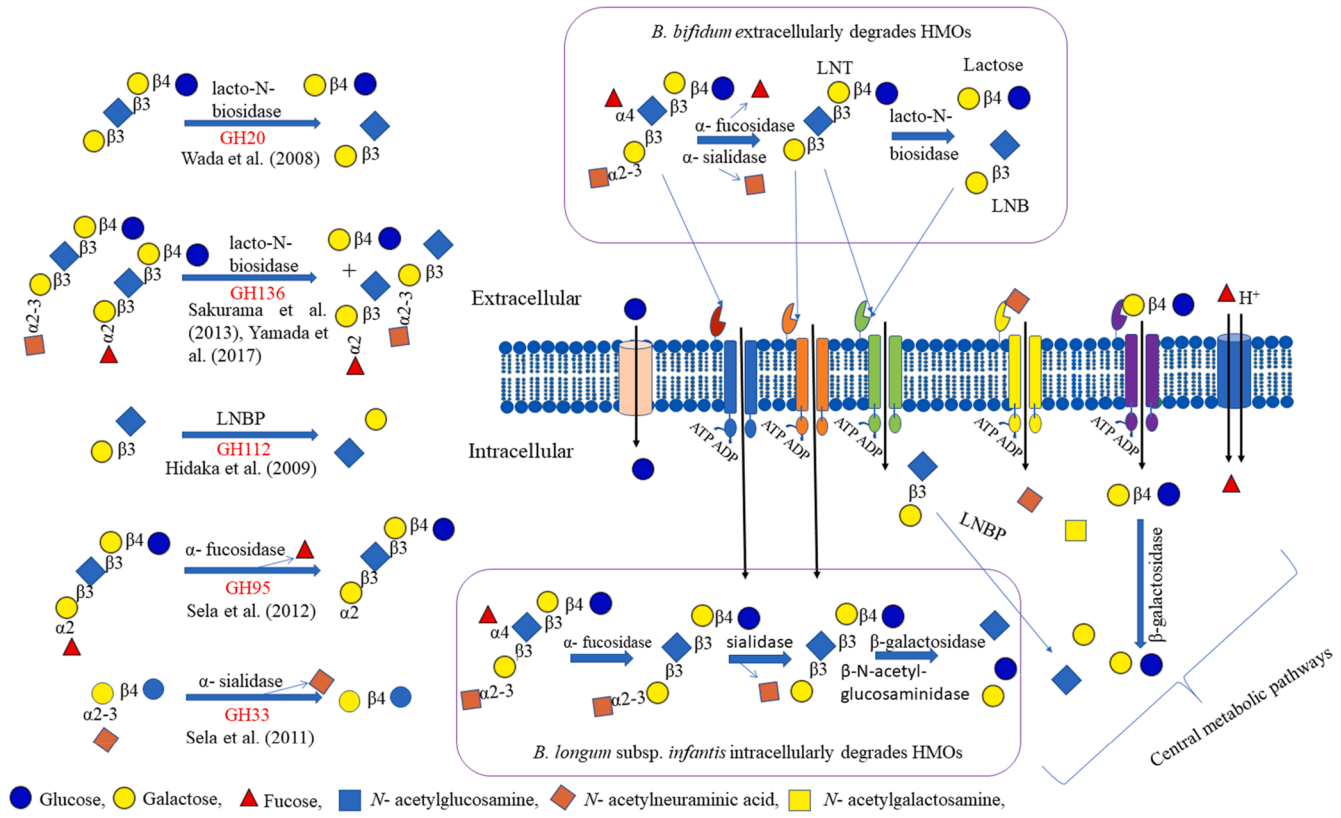

Fig. 2. Illustration for utilizing human milk oligosaccharides (HMOs) by different strains of *Bifidobacterium*. It is known that *Bifidobacterium bifidum* (Wada et al., 2008) and *Bifidobacterium longum* subsp. *infantis* (Sela et al., 2008) utilize predominant HMOs extracellularly and intracellularly respectively. The lacto-N-biosidase (GH136) of the *B. longum* subsp. *infantis* is quite different from GH20 in hydrolyzing fucosylated and sialylated HMOs, particularly in cleavage of lacto-N-fucopentaose I (Fucα1–2Galβ1–3GlcNAcβ1–3Galβ1–4Glc), and sialyl-lacto-N-tetraose (Neu5Acα2–3Galβ1–3GlcNAcβ1–3-Galβ1–4Gal) (Sakurama et al., 2013; Yamada et al., 2017). In many strains, generated lacto-N biose is further cleaved by lacto-N-biose phosphorylase (LNBP, GH112), which converts them into galactose-1-phosphate (Gal-1-P) and corresponding N-acetylhexosamine sugars (Hidaka et al., 2009). De-fucosylation and de-sialylation occur before LNBP by bespoke α-sialidase (Sela et al., 2011), and α-fucosidase (Sela et al., 2012). These unique molecular mechanisms for utilizing HMOs by *Bifidobacterium* highlight that they play a major role during infancy and stimulate the establishment of a *Bifidobacterium*-rich microbiota in the neonatal gut (Sakanaka et al., 2019).

Sinderen, 2016).

In the intracellular (or cytoplasmic) environment of many *Bifidobacterium,* the mechanism for degrading HMOs is mediated by lacto-N-biosidases (belonging to GH136 and GH20). The molecular mechanism highlights that GH136 (such as present in *B. longum* subsp. *longum*) is different from GH20 (reported in the *Bifidobacterium bifidum*) in terms of amino acid sequence, maturation process and substrate preference (Wada et al., 2008; Yamada et al., 2017). The GH136 is crucial for hydrolysing LNFP I and sialyllacto-N-tetraose, which are not substrates for GH20 (Sakurama et al., 2013). *B. bifidum* produces several GHs including 1,2-α-L-fucosidase, 1,3–1,4-α-L-fucosidase, and sialidase in an extracellular environment for efficient utilization of fucosylated and sialylated HMOs. These GHs initially remove sialic acid and fucose from the core structure of HMOs. Then, undecorated LNT is hydrolysed to lacto-N-biose (LNB) and lactose by GH20. These products are then imported into the cytoplasm by LNB transporter (Suzuki et al., 2008) for further degradation via lacto-N-biose phosphorylase (LNBP, GH112), which converts them into galactose-1-phosphate (Gal-1-P) and corresponding N-acetylglucosamine as shown in Fig. 2. Overall, it can be purported that imported type 1 and 2 chain HMOs are cleaved by bespoke GHs based on strain type. For instance, LNT is a preferred substrate by lacto-N biosidase (GH136) encoded by the *B. longum* subsp. *longum* as compared to a homologous enzyme (GH20) produced by the *B. bifidum* because *B. longum* subsp. *longum* lacks α –L- fucosidases in the extracellular environment, and uses 1,2- and 1,3–1,4-α-L-fucosidases secreted by *B. bifidum* for generation of LNT from LNFP I and lacto- N-difucohexaose I (the main HMO species) (Ashida et al., 2009; Kiyohara et al., 2011; Sela et al., 2012). Indeed, the GH136 of the *B. longum* subsp. *longum* has a 3-fold higher k_{cat} value towards LNT than GH20 of the *B. bifidum* (Ito et al., 2013; Sakurama et al., 2013). Asakuma et al. (2011) observed that *B. bifidum* ignores the degraded products (such as LNT) of HMOs in an extracellular environment even in the log phase, which suggests that *B. longum* subsp. *longum* and *B. breve* can preferentially utilize LNT. Whilst, *B. longum* subsp. *infantis* escape the direct competition by taking up all sorts of HMOs in their intact forms into the cytoplasm (Sakanaka et al., 2019). In a recent study, Thongaram, Hoeflinger, Chow, and Miller (2017) observed that *Lactobacillus acidophilus* NCFM produces a *β*- galactosidase in an extracellular environment that cleaves terminal Gal from LNT and leaves lacto-N-triose II in the medium. Therefore, it can be purported that lacto-N-triose II could be utilized by other strains of gut bacteria. These distinct HMO utilizing mechanisms among *Bifidobacterium* highlight the co-evolutionary adapted strategies in the infant's gut microbiome. This statement is well demonstrated by Lawson et al. (2020), whose study characterized 19 strains of *Bifidobacterium* and concluded that they have varied capabilities of HMO utilization. These variabilities in HMO utilization are an example of cooperative nature among *Bifidobacterium,* and are likely to act as foundation species for the development of post-birth gut microbiome.

Differences in functional properties of GH20 and GH136 can be understood from their crystal structures. The catalytic domain of GH20 is typically made of $(\beta/\alpha)_8$ barrel fold, with a narrow active site, which fits perfectly to the lacto-N-biose unit of LNT (Ito et al., 2013). During the interaction, GH20 uses the 2-acetamido group at the reducing-end of

GlcNAc unit as a nucleophile for the formation of an oxazoline intermediate- a very similar mechanism has been reported for β-*N*-acetylhexosaminidases (Mark et al., 2001). Contrary to this, the GH136 expressed in *B. longum* requires a chaperone for the activity of the enzyme (Sakurama et al., 2013) and 2-acetamido group at the reducing-end of GlcNAc unit does not take part in the catalytic mechanism. Additionally, O2 and O3 groups of Gal unit of LNT make little contact with the catalytic domain of the GH136 (Yamada et al., 2017). Owning to the distinct action mechanisms of these lacto-N-biosidases, it can be concluded that they have independently emerged through evolution due to high selective pressure for utilizing HMOs under a nutrient competitive environment.

As summarized above, different *Bifidobacterium* strains possess distinct mechanisms for utilizing predominant HMOs and also show cooperative behavior for maintaining gut homeostasis. For example, James et al. (2019) identified a fucose utilization pathway in the *B. kashiwanohense* APCKJ1 and *B. breve* for fucosyllactose (2′-FL and 3-FL). In this pathway, fucosyllactose eventually gets converted into acetate, formate, lactate and /or L-1,2 propanediol. Furthermore, this study has selected four genes identified in APCKJ1 related to intracellular metabolism of fucosyllactose and/or L-fucose for comparative analysis among *Bifidobacterium* genus. Their analysis reflected that fucose metabolism related loci are predominant across infant-associated *Bifidobacterium* (*B. breve*, *B. longum* subsp. *infantis* and *B. pseudocatenulatum*), with the exception of *B. bifidum.* Latter species is known to produce extracellular fucosidases and do not digest the released L-fucose, which is likely to be utilized by other gut bacteria (Turroni et al., 2014). Similarly, *B. kashiwanohense* DSM 21,854 and PV20-2 possess three and one α-fucosidases respectively, but does not utilize L-fucose (Bunesova, Lacroix, & Schwab, 2016). Thereafter a study by Schwab et al. (2017) demonstrated a cooperative behavior among *B. breve* and *Eubacterium hallii* (butyrate producers in an infant's gut). In this association, *B. breve* specialized in consumption of L-fucose and their fermented products (acetate, lactate and 1,2-propanediol) are consumed by the *E. hallii*. Furthermore, ecological importance of 1,2-propanediol produced by the *B. breve* has been empirically established by C. C. Cheng et al. (2020). This study used gnotobiotic mice model system to demonstrate that 1,2-propanediol increases ecological competitive fitness of *Limosilactobacillus reuteri* ATCC PTA 6475 to maintain the trophic interaction. These studies provide evidence about ecological importance of cross-feeding relationships based on bacterial metabolites, and we need more studies to understand the interactive functions of the infant's gut microbiome system.

3.2. Mechanism for utilizing HMOs by non– Bifidobacterium

Lacticaseibacillus casei utilises LNB by a specific mechanism that involves importing it with phosphorylation via phosphoenolpyruvate: a sugar phosphotransferase system. Phosphorylated LNB is then cleaved by phospho-β-galactosidase to release galactose-6-phosphate and *N*-acetylhexosamine (Bidart, Rodriguez-Diaz, Monedero, & Yebra, 2014). A study performed with disaccharides of HMOs and mucus constituents, such as LNB, galacto –N- biose (GNB), fucosyl-α1,3-GlcNAc (3′FN) and fucosyl-α-1,6-GlcNAc (6′FN), revealed that the growth of *Lactobacilli* is significantly promoted by 3′FN whereas LNB, GNB and 3′FN support the growth of *Bifidobacterium* species (Rubio-Del-Campo, Alcantara, Collado, Rodriguez-Diaz, & Yebra, 2020). However, the precise mechanism of their utilization pattern is not known yet.

It was predicted that other taxonomic groups of bacteria may colonize infant gut during the early weaning period. Strikingly, a recent study has focused on two strains of *Roseburia* and a strain of *Eubacterium* that have GH136, and established HMOs utilization capability of these strains (Pichler et al., 2020). *Roseburia hominis* DSM 16839 and *R. inulinivorans* DSM 16841 grew well on LNT, GNB and LNB, and GNB and LNB respectively, whereas *Eubacterium ramulus* DSM 15684 grew only on LNT. None of these strains grew on fucosylated HMOs (2′-FL and 3-FL). Surprisingly, sialylated HMOs (3′-SL and 6′-SL) were greatly utilized by *E. ramulus* DSM 15684, and *R. inulinivorans* DSM 16841; however, they were distinguished by their growth on free sialic acid-*R. inulinivorans* DSM 16841 can use free sialic acid. Similar to bifidobacterial strains, *R. inulinivorans* DSM 16841 produces extracellular GH136 and their digested products are then imported to cytoplasm where they are first de-fucosylated by bespoke GH95 and GH29 before being subjected to LNBP (GH112) as shown in Fig. 3. Identified GH136 from *R. inulinivorans* DSM 16841 is the first ever reducing end-acting enzyme reported to date (Pichler et al., 2020). In contrast to bifidobacterial and other *Roseburia* strains, *R. hominis* DSM 16839 possesses intracellular GH136 and acts only on type 1 chain of HMOs that can be imported (Fig. 3). The SBP of ATP transporter of the *R. hominis* DSM 16839 does not bind to decorated LNT but has affinity for the LNT, GNB and LNB. On the other hand, the high affinity of SBP of ATP transporter for GNB and presence of a separate transporter for sialic acid in the *R. inulinivorans* DSM 16841 imply that some metabolic interplays are going between different bacteria that can degrade O-glycan from glycolipid and glycoprotein. The capability of hydrolyzing O-glycan derived fragments is likely to provide competition against human gut pathogenic bacteria such as *Salmonella typhimurium* or *Clostridium difficile* (Ng et al., 2013). The above studies clearly indicate that *Bifidobacterium* and *Clostridia* have evolved in the infant gut to deliver a ton of benefits during suckling and early weaning periods respectively.

Salmonella, C. difficile, Clostridium perfringens, Vibrio, Staphylococcus, Klebsiella, Streptococcus, and *Enterococcus* do not have great potential for utilizing HMOs. Marcobal et al. (2010) screened various beneficial and pathogenic bacteria for utilization of HMOs and observed that *C. difficile*, *Enterococcus faecalis*, *Veillonella parvula,* and *Escherichia coli* EC100 did not thrive on HMOs as a substrate. Two commensal strains of *Streptococcus* (*S. mitis and S. oralis*) have been found to show selective growth on 2-'FL as compared to other strains of the genus (Seferovic et al., 2020). Contrary to this, fucosylated HMO does not support growth of the two most common *Staphylococcus* species, such as *S. epidermidis,* and *S. aureus.* It suggests that expansion of the population of commensal strains of *Streptococcus* during early colonization of infants gut may outcompete potential pathobionts such as *S. aureus.* Two antibiotic-associated pathogens, *C. difficile* and *Salmonella* can access freely available sialic acid, and fucose and sialic acid within the gut of infants respectively (Bäumler & Sperandio, 2016; Ng et al., 2013). Thus, the antibiotic intervention of early gut microbiota provides an opportunity for these pathogens to expand their population to sufficient densities where they induce self-promoting host inflammation. In a healthy gut environment, many beneficial bacteria can utilize freely available fucose and sialic acid, and provide a competitive environment to keep their population under control.

4. Immuno-modulatory properties of HMOs

HMOs are excellent modulators of the immune system. They interact with receptors (also called lectins) expressed on various cells of the immune system and epithelial cells (Fig. 4), thereby modulating the neonatal immune system in the infant's gut. A variety of lectins have been identified, including galectins, sialic acid-binding immunoglobulin (Ig)-like lectins (Siglecs), selectins, and C-type lectins. These are expressed on the cell surface of different immune cells such as macrophages, dendritic, neutrophils, eosinophils, monocytes, and natural killer (NK) cells. HMOs have been found to bind with these lectins.

Galectins specifically bind to β-galactoside sugars, such as N-acetyllactosamine (Galβ1-3GlcNAc or Galβ1-4GlcNAc) and fucosylated or sulfated sialylated galactose containing moieties (Rapoport, Kurmyshkina, & Bovin, 2008). Galectins can be secreted or freely mobile within the cells (Lippert et al., 2007). Secreted galectins can bind either to microbe-associated molecular patterns (Nio-Kobayashi, Takahashi-Iwanaga, & Iwanaga, 2009) or type 1 chain as LNT and type 2 chain as LNnT (El-Hawiet et al., 2017). Galectins (such as Gal-3) can also bind

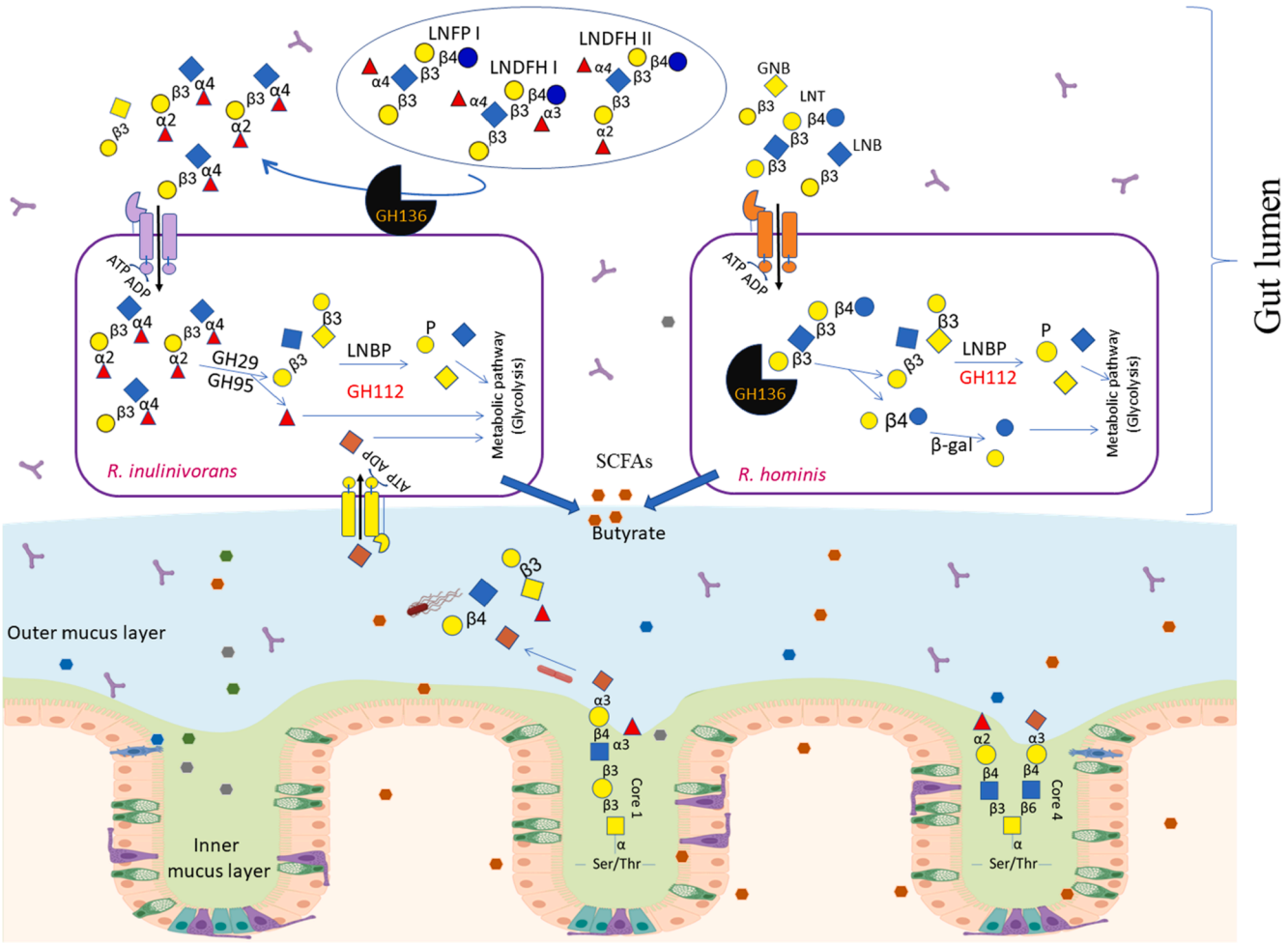

Fig. 3. Utilization of human milk oligosaccharides (HMO) by other than *Bifidobacterium*. Similar to *Bifidobacterium,* different modes of HMO utilization are present in *Roseburia hominis* DSM 16839 and *Roseburia inulinivorans* DSM 16841. *R. inulinivorans* expresses two enzymes belonging to glycoside hydrolases family 136 (GH136), which are present at the extracellular membrane and processes the fucosylated HMOs (LNFP I, LNDFH I, and LNDFH II) extracellularly before importing them into the cytoplasm via ABC transporter (Pichler et al., 2020). Decorated fucosylated HMOs are first de-fucosylated by bespoke GH95 and GH29 and then lacto-N-biose phosphorylase (LNBP, GH112) converts de-fucosylated HMOs into galactose-1-phosphate (Gal-1-P) and corresponding N-acetylhexosamine sugars. In case of *R. hominis*, GH136 is present in the cytoplasm, which converts LNT into lactose and LNB. LNB is further subjected to GH112 and lactose break-down into glucose and galactose by β-galactosidase. All generated monosaccharides and Gal-1-P enter into metabolic pathways for energy generation and production of small chain fatty acids such as butyrate (Anand, Kaur, & Mande, 2016; Pichler et al., 2020). These strains also cross-feed on mucus-derived glycan together with *Akkermansia muciniphila* (Pichler et al., 2020). Particularly, *R. inulinivorans* notably grows on sialic acid and GNB that is likely to be released from different core types of mucus glycans by actions of the sialidases and other enzymes, produced from the *A. muciniphila* (Derrien, Vaughan, Plugge, & de Vos, 2004) and other gut commensal anaerobes, like *Bacteroides thetaiotaomicron* (Martens, Chiang, & Gordon, 2008). Scavenging of the released sialic acid from glycans of mucus by the *Roseburia* is likely to provide anti- infective strategy against human pathogenic bacteria such as *Salmonella typhimurium* or *Clostridium difficile* (Ng et al., 2013). LNT: Lacto-N-tetraose, LNB: Lacto-N-biose, GND: Galacto-N-biose, LNDFH: Lacto-N-difucohexaose and LNFP: lacto-N-fucopentaose. Image is created with BioRender.

to various viruses and assist them in entering the human host (Ayona, Fournier, Henrissat, & Desnues, 2020) as well as metastasization of cancer cells (Vladoiu, Labrie, & St-Pierre, 2014). Thus, HMOs play not only vital roles in early life, but also in other fields, such as virus and cancer treatments. Several types of galectins have been reported in humans (https://www.genenames.org/data/genegroup/#!/group/629), but specific interactions are not yet unrelieved.

The Siglec family comprises 15 members of sialic acid binding protein in human being and is primarily expressed on the surface of immune cells of macrophages, monocytes, neutrophils, dendritic cells, basophils, eosinophils, and NK cells (Angata, Nycholat, & Macauley, 2015). Distinctive features of Siglec family members include the number of intracellular immunoreceptor tyrosine-based inhibitory motifs, extracellular immunoglobulin domains, loss of sialic acid recognition (Siglec-12), and the presence of a positively charged intramembrane residue (Siglecs-14–16) (Angata et al., 2015). 3′-SL, is one of the abundant components of HMOs, binds to Siglec-1 (Bhunia et al., 2004), and Siglec-3. In the case of Siglec-3, 3′SL provokes megakaryocyte differentiation and subsequent apoptosis in the human chronic myeloid leukemia K562 cells by lipid raft-dependent endocytosis (Ha et al., 2020). 3′-SL is also suggested to bind with Siglec-5, 7 (Alphey, Attrill, Crocker, & van Aalten, 2003), Siglec-9 and Siglec-10 (N. Li et al., 2001); though, the affinity of 3′-SL for these Siglecs is relatively low and a strong ligand is yet to be found.

Selectins are a family of cell adhesion molecules interceding selective leukocyte recruitment in inflammation (Ley, 2001). Blood group antigens, such as sialyl-Lewis a (sLe a), sialyl-Lewis × (sLe x), Lewis × (Le x) and Lewis y (Le y), are a portion of cell-surface glycoconjugates present on leukocytes (Elwakiel et al., 2018) and are vital for leukocyte extravasation and mucosal infiltration by various selectins (Ivetic, Hoskins Green, & Hart, 2019). These sialyl-Lewis mimic the structures of HMOs (especially, DSLNT) and are capable of inhibiting necrotizing enterocolitis (NEC) (Tables 2 and 3). Additionally, other sialylated HMOs (3′-SL, and 3′-sialyl-3-fucosyl-lactose) have been proposed to

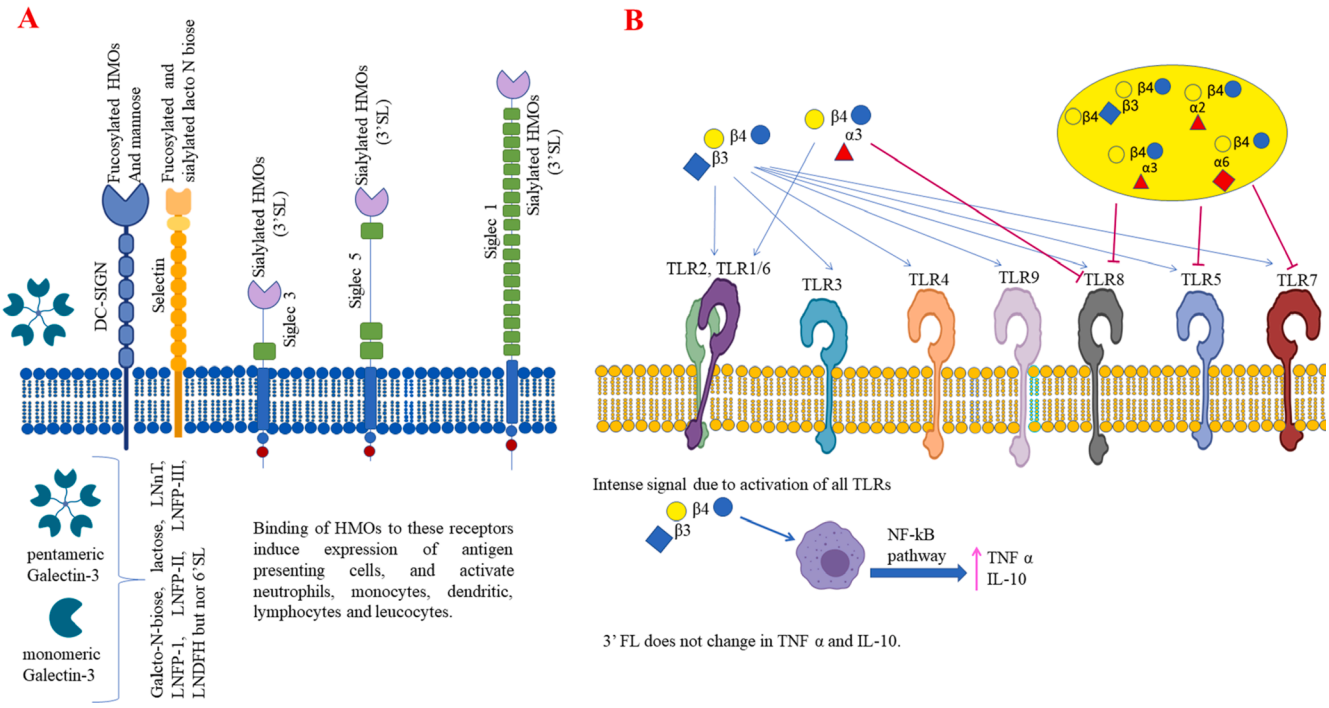

Fig. 4. Suggested immunological actions of the HMOs. Different structural HMOs are excellent modulators of the immune system by interacting with cell surface receptors (A) with carbohydrate binding protein called lectins, and (B) toll-like receptors (TLRs). The GlcNAcβ1-3Galβ1-4Glc recognizes and activates various TLRs and can increase production of TNF-α and IL-10 whereas other HMO may inhibit TLRs or does not alter the expression, particular 3′FL (Cheng et al., 2019). 3′FL: 3-fucosyllactose, LNFP III: lacto-N-fucopentaose III, LNFP I: lacto-N-fucopentaose I, LNFP II: lacto-N-fucopentaose II, LNnT: lacto-N-neotetraose, LNDFH: lacto-N-difucohexaose and 6′SL: 6′-sialyllactose.

inhibit adhesion of the monocyte, lymphocyte, and neutrophil to endothelial cells in the area of inflammation (Bode et al., 2004; Rudloff, Stefan, Pohlentz, & Kunz, 2002); thus, may interfere in leukocyte-platelet interactions. However, these studies suggest great potential of HMOs in the suppression of inflammation in infants by blocking activities of selectins, proper utilization of HMOs in this area is not yet elusive.

C-type lectin receptors, such as DC-SIGN (dendritic cell-specific intercellular adhesion molecule-3), are the most studied receptor and present on the surface of both macrophages and dendritic cells (Fig. 4). DC-SIGN has an EPN (Glu-Pro-Asn) tripeptide motif that is known for especially binding to fucose and mannose, and can recognize *N*-acetyl-glucosamine (GlcNAc) and glucose with little or no preference for larger oligosaccharides containing these sugar moieties (Noll et al., 2016). Conversely, it has a preference for a certain types of oligosaccharides, such as Le × trisaccharide over fucose (Pederson, Mitchell, & Prestegard, 2014). In the absence of a common galactose interacting motif (Gln-Pro-Asp), DC-SIGN may interact with GlcNAc (Lee et al., 2011). Glycan microarrays analysis has detected that the DC-SIGN can bind to α- linked fucosylated HMOs, such as 2′FL and 3-FL, but not to LNT (Noll et al., 2016). Given the physiological quantities [1–5 mM (0.5–2.5 g/L)] of 2′FL and 3-FL present in the secretor-positive HMOs, it can be said that they are modulating the immune system of infants as DC-SIGN works best at these concentrations. The DC-SIGN binds to various microbes including HIV and hepatitis-C due to the presence of high mannose containing glycoproteins on their envelopes; thus, HMOs could be employed as antiadhesion therapy against such deadly viruses (Lozach, Burleigh, Staropoli, & Amara, 2007). Nevertheless, this should be further evaluated by *in vivo* studies. Particularly, it was reported that sialylated HMOs (3′-SL and 6′-SL) interact with DC-SIGN present on neutrophils, monocytes and dendritic cells, leading to its activation (Noll et al., 2016). The importance of interaction of DC-SIGN with HMO mix (2′-FL, 3′-SL, 6′-SL, sialic acid, and LNnT) is further strengthened through an *in vivo* study that was performed on colostrum deprived newborn piglets.

The binding of HMOs to these receptors and production of bacterial metabolites, in particular acetate, butyrate, and propionate upon fermentation of HMOs, highlight their anti-inflammatory property and involvement in the maintenance of biological functions including infant's gut barrier preservation. Many of these functions are attributed to anti-inflammatory actions by HMOs and are summarized here.

4.1. *Anti-inflammatory property*

HMOs exhibit anti-inflammatory activity by regulating the production of interleukins (ILs) and lymphocyte activation (Chleilat et al., 2020). HMOs exhibit tolerogenic factors by inducing semi-maturation of human immune cells, thereby influencing the development of the neonatal immune system. For instances, *in vitro* studies performed on the human epithelial and the HeLa cells demonstrated that HMOs significantly decrease levels of inflammatory cytokines (IL- 1β, IL- 6, and IL- 8), and the monocyte chemoattractant proteins 1/2 (Wicinski, Sawicka, Gebalski, Kubiak, & Malinowski, 2020). Whilst, studies on T cells derived from umbilical cord blood in presence of sialylated HMOs implied that it enhances levels of the $CD3^+/CD4^+$ and $CD3^+/CD8^+$, and $CD3^+/CD8^+$ cells producing interferon γ, and IL-13 respectively, involving in allergic inflammation (Eiwegger et al., 2010; Eiwegger et al., 2004). It has also been reported that sialylated HMOs help in the maturation of lymphocytes and maintain the balance between T helper (Th)-1 and Th2 cytokines production (Eiwegger et al., 2010; Rudloff et al., 2006). Moreover, some sialylated HMOs have been postulated in the regulation of allergic responses by reducing the production of the IL-4 in the lymphocyte subgroup in case of a peanut allergy (Eiwegger et al., 2010; Eiwegger et al., 2004).

Human milk as a whole is strongly anti-inflammatory in action, and holds abundant apparatus that suppress toll-like receptor (TLR) signaling pathways. For example, a recent study that was looking at determining HMOs impact on the dendritic cells (DCs) revealed that it

Table 2
Function of human milk oligosaccharides against various pathogenic microorganisms.

HMOs	Pathogens	Disease caused	Model	Findings	Action	Reference
2′-FL, 3-FL, 3′-SL, 6′-SL, LNT and LNFP1	*Entamoeba histolytica*	Amoebiasis	Human colon adenocarcinoma HT-29 cell line	HMOs reduce *E. histolytica*-induced destruction of intestinal epithelial cells and prevent *E. histolytica* induced cytotoxicity.	Physiological and supraphysiological concentrations of LNT rescued HT-29 cells LNT but not LNFP1 rescued *E. histolytica* induced HT-29 cell destruction. 2′-FL did not rescue the cells but lactose did. The study highlighted that terminal Gal on structures of HMOs is required for their cytoprotective effect.	Jantscher-Krenn et al. (2012)
Bioengineered 2′FL and 3′FL	*Pseudomonas aeruginosa*	Opportunistic pathogen	Human respiratory epithelial cell line A549	Attachment is inhibited by 2′FL and 3′FL.	Act as anti-adhesion molecule	Weichert et al. (2013)
Pooled HMOs	Enteropathogenic *Escherichia coli* (EPEC)	Serious diarrheal illness with potentially high mortality in infants	Human epithelial cell lines HeLa (ATCC CCL-2) and HEp-2 (CCL-23), human colon epithelial cell line T84 (ATCC CCL-248), Wild-type C57BL/6J mice	Reduce EPEC attachment to HeLa, Hep2 and T84. HMO-treated C57BL/6J showed significantly lower EPEC colonization than control-treated mice.	HMOs act as decoy to block attachment.	Manthey, Autran, Eckmann, and Bode (2014)
HMOs	Uropathogenic *Escherichia coli* (UPEC)	Urinary tract infections	Human bladder epithelial cells (5637 ATCC HTB-9)	HMO reduced UPEC binding to epithelial cells and reduced expression of MAPK and NF-κB.	Sialic acid containing HMOs fraction reduces cytotoxic and proinflammatory effects of UPEC infection.	Lin, Autran, Espanola, Bode, and Nizet (2014)
Pooled HMO	*Candida albicans*	Causes the majority of fungal disease in premature infants	Human premature intestinal epithelial cells (pIECs)	HMOs reduced invasion of pIECs from *C. albicans* in a dose-dependent manner.	Hyphal morphogenesis at both cellular and transcriptional levels are affected with addition of HMOs. Expression of the gene encoding a *C. albicans* hyphal-specific adhesin, ALS3, was reduced in the presence of HMOs. HMOs physically block sites on the *C. albicans* cell surface that are needed for the fungus to associate with pIECs.	Gonia et al. (2015)
2′-FL	*Campylobacter jejuni*	Intestinal inflammation and diarrhoea	Human epithelial cells HEp-2 and HT-29	Decline in *C. jejuni* invasion	Subdue the release of proinflammatory molecules IL-1β, IL8, and chemokines MIP-2.	Yu et al. (2016)
			Four-week-old male wild-type C57BL/6 mice	Reduction in colonization of *C. jejuni* strain 81–176, acts as a prebiotic to restore healthy gut microbiota, decreasing histologic features of intestinal inflammation.	Suppression of inflammatory signaling molecules of the acute-phase mucosal immune response and no induction of induce IL-17 (adaptive T cell response)	
2′-FL		NEC	7–10 days old wild type mice	2′-FL protects against NEC. 2′-FL found in human milk may be mediating some of the protective benefits of breast milk	Protective effects occurred via restoration of intestinal perfusion through increased expression of the vasodilatory molecule eNOS.	Good et al. (2016)
Pooled HMOs	*Listeria monocytogenes*	Febrile gastroenteritis, septicaemia, meningitis	Caco-2 cell lines	HMO pre-treatment Caco-2 led to a 50% decrease *in Listeria* association,	Bacterial clearance in Caco-2 cells is likely to happen via induction of the unfolded protein response and eIF2 signaling. It is an example of host-intrinsic protective responses.	P. Chen, Reiter, Huang, Kong, and Weimer (2017)
2′-FL, 3′-SL, 6′-SL and GOS	Human rotaviruses G1P[8] and G2P[4]	Dehydrating gastroenteritis in children < 5 years of age	African green monkey kidney epithelial cells (MA104 cells)	The mixture of 3′-SL + 6′-SL at the same ratio used as is present in breast milk. Ratio was more potent in reducing rotavirus infectivity than when compared with 3′-SL or 6′-SL individually.	All oligosaccharides substantially reduced the infectivity of both human rotavirus strains *in vitro*, primarily through an effect on the virus.	Laucirica et al. (2017)
Neutral HMO (LNT, LNFPI)	Group *B Streptococcus* (GBS)		*In vitro*	LNT and LNFPI have growth inhibitory activity but do not possess bactericidal ability. While type 2 chain (LNnT) does not inhibit the growth.	GBS susceptibility to HMOs is attributed with some specificity to the gbs0738-encoded glycosyltransferase	Lin et al. (2017)
Pooled HMOs		Necrotizing enterocolitis (NEC)	C57BL/6 mouse pups	HMO treatment effectively prevented ileum collapse	HMO treatment directly down-regulated TLR4 expression and inhibited NF-κB activation in	C. Wang et al. (2019)

(*continued on next page*)

Table 2 (*continued*)

HMOs	Pathogens	Disease caused	Model	Findings	Action	Reference
				and intestinal flatulence in all EC models	intestinal epithelial cells during NEC. The study revealed that HMO showed anti-inflammatory effects of HMOs against NEC.	
			Caco-2, T84 and Madin Darby canine kidney I (MDCK-1) cells, C57BL/6 mice	Protection against NEC via upregulation of intestinal cell proliferation and differentiation. HMOs intensify expression of the intercellular tight junction proteins zona occluden 1 and claudin 1.	HMOs alter gene expression of target genes related to cell proliferation and differentiation, including upregulation of stem cell differentiation marker HMGCS2. Activate mTOR and PPAR signalling in enterocytes.	B. Li et al. (2020)
Sialylated HMOs		NEC	Neonatal rat, Caco-2 cells	Provides clinical evidence of involvement of NLRP3 inflammasome in NEC pathology.	The protective actions of HMOs might be owing to the suppression of TLR4/NF-κB/ NLRP3-mediated inflammation in NEC.	Zhang, He-Yang, Tu, and Zhou (2021)

2′-FL- 2-′ fucosyllactose, 3-FL- 3-fucosyllactose, 3′-SL- 3′-sialyllactose, 6′-SL- 6′-sialyllactose, eNOS- endothelial nitric oxide synthase, GOS- galacto-oligosaccharides, IL- interleukin, LNFP1-lacto-N-fucopentaose I, LNnT- lacto-N-neotetraose, LNT- lacto-N- tetraose, MIP-2-macrophage inflammatory protein 2, mTOR- mechanistic target of rapamycin, NF-κB- Nuclear factor kappa B, NLRP3- Nod-like receptor pyrin domain-containing 3, PPAR- peroxisome proliferator-activated receptors, TLR- toll-like receptor.

induced partial maturation of human monocytes derived DCs, and elevated levels of IL-6, IL-10, and IL-27 but not IL-12p70 and tumor necrosis factor-alpha (TNF-α) (Xiao et al., 2019). He et al. (2016) studied the effect of 2′-FL on suppression of IL8 induced by pathogenic enterotoxigenic *Escherichia coli* from intestinal epithelial cells, and concluded that 2′-FL directly inhibits the LPS-mediated inflammation through attenuation of CD14 induction. The CD14 induction is associated with LPS-TLR4 stimulation of macrophages.

Available literature suggests that 2′-FL, 3-FL, 6′-SL and LNnT have been intensively studied among all available HMOs. For instance, *in vitro* studies conducted on the human acute monocyte leukemia (THP1) and the human embryonic kidney (HEK) cell lines in the presence of 2′-FL, 6′-SL, and LNnT clearly displayed anti-inflammatory properties by suppressing the expression of TLR 5, 7 and 8 (Fig. 4B). However, 3-FL and 6′-SL activated the expression of TLR 2 and inhibited TLR 8 due to difference in structural arrangement of L-fucose from 2′-FL (Cheng, Kiewiet, Groeneveld, Nauta, & de Vos, 2019). In a randomized controlled trial, breastfed infants and infants fed infant formulas with 2′-FL showed 29–83% lower concentrations of inflammatory cytokines [interleukin (IL) receptor antagonist (IL-1ra), IL-1α, IL-1β, IL-6, and TNF-α] as compared to infants fed the control formula (absence of 2′-FL) (Goehring et al., 2016). In the full-thickness wound mice model system, LNnT enhanced the anti-inflammatory, type 2 immune response where the expression rate of IL-10, IL-4, and IL-13 was higher in the LNnT group as compared to the control (supplied with PBS) (Farhadihosseinabadi et al., 2020). LNnT has been reported to suppress Type Th 1 that produces IFN-γ, IL-2, and IL-2 induced TNF-β involved in inflammatory and immune reactions (Terrazas, Walsh, Piskorska, McGuire, & Harn, 2001). The above studies have clearly proven that 2′-FL, 3′SL, LNnT and 6′SL display greater anti-inflammatory activities among all the HMOs reported so far.

Other environmental factors also modulate the anti-inflammatory properties of HMOs. For example, a study conducted on the Sprague Dawley rats fed with 2′-FL, 3′-SL and their mix minimized the level of IL-18 in serum and affected the production of TNF-α and IL-5 in male rats (Chleilat et al., 2020). On the other hand, female Dawley rats fed with HMO mix (2′-FL and 3′-SL) enhanced the levels of anti-inflammatory cytokine (IL-10). This study hints that gender is one of the key factors to be considered while examining the effect of HMOs on gut microbiota and immune system along with other components. A synbiotic combination of *Bifidobacterium* strains along with HMOs on the Caco-2 cells has been carried out to demonstrate their combined impact on anti-inflammatory action. The study is mainly performed by culturing *B. infantis* ATCC 15697, *B. breve* SC95 and *B. bifidum* ATCC29521 on the Caco-2 cells in presence of HMOs. It amplified the expression of anti-inflammatory cytokine including IL-10 and diminished the expression of pro-inflammatory cytokines (IL-6 and IL-8) in the presence of HMOs as compared to glucose and lactose (Wickramasinghe et al., 2015).

Until now more than 200 HMOs have been identified; though, very few have been examined for their biological assay. Given this lacuna, there is a great requirement that other HMOs should be considered for further studies to determine their biological importance at the mechanistic level to efficiently use them as nutraceutical therapy in the long term goal.

5. Anti-adhesion and anti-microbial properties of HMOs

Breast milk has evolved over millennia to fulfill the nutritional requirements and provide protection against microbial diseases to the newborn. HMOs display antiadhesive and antimicrobial properties that they do so either by indirectly modulating the gut microbiome of the host and altering immune cell responses in the neonate or acting directly as soluble decoy receptors to reduce the binding affinity of various pathogenic microbes to receptors of various cells. Studies have shown that HMOs act as decoy receptors to reduce attachment of enteric pathogens like *Escherichia coli, Vibrio cholerae,* and *Salmonella fyris* (Coppa et al., 2006), *Campylobacter jejuni* (Ruiz-Palacios et al., 2003), and rotavirus (Laucirica, Triantis, Schoemaker, Estes, & Ramani, 2017) on gut lumen; thus, decreasing the mortality rate of infants (Table 2). For example, a clinical study using 2′-FL revealed that it plays a role in protection against diarrhea caused by *Campylobacter* and *Calicivirus* (Morrow et al., 2004).

Neutral high MW HMOs, such as LNT and LNnT, were found to reduce adhesion of *Streptococcus pneumoniae* on the buccal epithelial cells (Andersson, Porras, Hanson, Lagergard, & Svanborg-Eden, 1986). Later, Barthelson, Mobasseri, Zopf, and Simon (1998) observed that 6 sialyl-derivatives of LNnT are most effective against *S. pneumoniae*. Coppa et al. (2006) fractionated HMOs into high and low molecular weight (MW) neutral, and acidic oligosaccharides. The study has provided evidence that neutral high-MW fraction significantly inhibited adhesion of the *E. coli* O119 (mainly LNFP) and *V. cholera,* whereas neutral low-MW fraction was effective toward *E. coli* O119 and *S. fyris* (mainly 3-FL). Acidic fraction (mainly 3′-SL) was active against *V. cholera, E. coli* O119 and *S. fyris*. With the knowledge that fucosylated antigens are receptors for *Pseudomonas aeruginosa* adhesion (Scharfman et al., 2001), fucosylated HMOs were screened for blocking the adhesion

Table 3
Human population studies against different disease conditions with respect to HMOs and their outcomes.

HMOs	Disease against which study was done	Design	Location of study	Sample size	Outcomes	Reference
Pooled HMOs (secretor/ nonsecretor) with respect to DSLNT	Necrotizing enterocolitis (NEC), HIV	Clinical trial	Cape Town, South Africa	HIV infected mothers (n = 41 of which 22 secretor, 19 non-secretor) and non-infected mothers (n = 41 of which 20 secretor, 21 non-secretor). Both cases NEC incidence: 4 infants.	Low concentrations of DSLNT in breastmilk increased infant's risk of NEC.	Van Niekerk et al. (2014)
Secretor/non-secretor	Rotavirus	Multicenter case-control observational study	US	1564 children younger than 5 years with acute gastroenteritis, 818 healthy controls	Children with the non-secretor FUT2 polymorphism appeared statistically protected. Differential impact of HMOs in rotavirus might be determined on the basis of genomic specificity of FUT2.	Payne et al. (2015)
2′-FL, 3-FL, LNFP1, and LNFP II/III	HIV	Nested case-cohort analysis	Lusaka, Zambia,	HIV-infected women (n = 958), 2 y of age among 103 HIV-infected and 143 HIV-exposed uninfected children (HEU)	HMOs were protective against mortality only in HEU children.	Kuhn et al. (2015)
Formula supplemented with 2′-FL and GOS	Growth	Prospective, randomized, controlled, growth and tolerance study	28 sites throughout the United States	Infants exclusively formula-fed in 3 groups: (1) n = 101 control formula GOS, (2) n = 104 formula high GOS and low 2′-FL, (3) n = 109 medium GOS and medium 2′-FL, (4) breastfed (n = 106). Enrolment for 4 month	Formula supplemented with 2′FL is well tolerated without any difference from HMO fed infants.	Marriage, Buck, Goehring, Oliver, and Williams (2015)
Formula supplemented with 2′-FL and GOS	Immune parameters	Randomized controlled trial	28 sites throughout the United States	Infants exclusively formula-fed (n = 229) or breastfed (n = 86). Formula-fed infants were randomly assigned to 3 formulas, having GOS (n = 75) and GOS + 0.2 g 2′-FL/L (n = 76), or 1.0 g 2′-FL/L 2; n = 78. Enrolment for 4 month	Infants fed formula supplemented with 2′-FL exhibited lower levels of inflammatory cytokines including tumor necrosis factor-α and interleukins IL-1α, IL-1β, and IL-6.	Goehring et al. (2016)
Infant formula supplemented with both 2′-FL and LNnT	Respiratory infection (bronchitis)	Multicenter, randomized, double-blind trial	Belgium and Italy	≤14 days of age till 6 months infants, control, n = 87, test, n = 88	Reduced incidence of bronchitis and inclusion of these HMOs in infant formula is safe, well-tolerated, and supports age-appropriate growth.	Puccio et al. (2017)
FUT2-dependent HMOs	Allergic disease in infants with high allergy risk	Prospective cohort-randomized placebo-controlled trial	Finland	266 mother-infant pairs	Reduced the risk to manifest IgE-associated eczema at 2 years of age infants, but not in 5 years of age	Sprenger et al. (2017)
Pooled HMOs	Cow's milk allergy (CMA)	Observational cohort study		Infants with CMA (n = 39) and children without CMA (n = 41)	Infants receiving breast milk with low LNFP III levels are more likely to become affected with CMA	Seppo, Autran, Bode, and Jarvinen (2017)
Pooled HMOs	Morbidity	Sub-study embedded within a randomized trial	Gambia	Mother/infant pairs (n = 33, of which 21 secretors and 12 non-secretors). HMO composition were analysed at 4, 16, and 20 weeks postpartum	Higher LNFP I was associated with decreased infant morbidity. 3′-SL indicator of infant weight-for-age. Higher breast milk levels	Davis et al. (2017)
α- 2-Fucosylated HMOs. Such as 2′-FL	Acute respiratory infections (ARIs)	Community-based longitudinal cohort study, from birth to 2 years of age.	Bangladesh	Mothers, n = 221 and infants, n = 219	Fucosylated HMOs decrease the incidence of ARIs in breastfed babies aged 0 to 6 months by decreasing the risk of colonization with *Haemophilus influenzae*.	Binia et al. (2019)
2′-FL/LNnT	Irritable bowel syndrome	Phase II, parallel, double-blind, randomized, placebo-controlled study	Gothenburg, Sweden	60 patients	10 g dose of 2′FL/LNnT improved irritable bowel symptoms or/and no anxiety, or depression were detected.	Iribarren et al. (2020)
HMOs	NEC	Longitudinal metagenomic sequencing, HMO profiling of MOM	Newcastle upon Tyne, UK	Infants with NEC (n = 33) and controls (n = 37)	Study suggested that low levels of DSLNT can be used as a marker to predict NEC infection in human milk.	Masi et al. (2020)
Pooled HMOs	Obesity.	Clinical study	USA (Los Angeles County)	Hispanic mother-infant pairs (n = 157)	Higher LNFP-II in HMOs may decreases obesity risk across all infants, whereas higher LNnT and	Berger et al. (2020)

(*continued on next page*)

Table 3 (*continued*)

HMOs	Disease against which study was done	Design	Location of study	Sample size	Outcomes	Reference
					DSLNT may increase obesity risk in infants of nonsecretors only	
2′-FL, LNFP I, and DFLNT in secretors; LNT and LNFP II in nonsecretors.	Late-onset neonatal sepsis	Randomized clinical trial, retrospective cohort study	Peru	Mothers and their infants with birth-weight < 1500 g (n = 153)	FDSLNH which is correlated to factor 3 in colostrum might protect infants with very low birth weight from late-onset neonatal sepsis.	Torres Roldan et al. (2020)
2′-FL and LNnT	Irritable bowel syndrome	Multicenter, open-label trial	USA	245 patients	Administration of HMOs improved IBS symptoms substantial side effects.	Palsson et al. (2020)
HMOs	Maternal overweight and obesity, infant growth	longitudinal cohort studies	USA	Mother infant pair (n = 155)	Maternal BMI showed positive correlation with LNnT, 3-FL, 3′-SL and 6′-SL; negative correlation with DSLNT, DSLNH, FDSLNH and total acidic HMOs concentrations at 2 months. Infant intakes of 3-FL, 3′-SL, 6′-SL, DSLNT, DSLNH, and total acidic HMOs were positively associated with infant growth	Saben et al. (2021)

2′-FL- fucosyllactose, 3-FL-3-fucosyllactose, 3′-SL- 3-sialyllactose, 6′-SL- 6-sialyllactose, DFLac- difucosyllactose, DFLNH- difucosyllacto-N-hexaose, DFLNT- difucosyllacto-N-tetrose, DSLNH- disialyllacto-N-hexaose, DSLNT- disialyllacto-N-tetraose, FDSLNH- fucodisialyllacto-N-hexaose, FDSLNH- Fucosyl-disialyllacto-N-hexose, FLNH- fucosyllacto-N-hexaose, GOS- galactooligosaccharide, LNFP- lacto-N-fucopentaose, LNH- lacto-N-hexaose, LNnT- lacto-N-neotetrose, LNT- lacto-N-tetrose, LSTb- sialyl-lacto-N-tetraose b and LSTc- sialyl-lacto-N-tetraose c.

of this pathogen in the human intestinal and respiratory cell lines. It was noted that 2′-FL and 3-FL inhibit adhesion to epithelial cells (Weichert et al., 2013), and 3′-SL and 6′-SL are involved in the reduction of internalization in pneumocytes (Marotta, Ryan, & Hickey, 2014).

It is well known that breastfed infants experience decreased instances of *C. jejuni* and calicivirus-associated diarrhea compared to their formula-fed counterparts. Ruiz-Palacios et al. (2003) observed that *C. jejuni* uses H(O) antigen (Fuc α 1, 2-Gal β 1, 4GlcNAc) to bind on intestinal mucus, whereas fucosylated HMOs inhibit this binding. In a subsequent study, it was identified that 2′-FL and LDFH-I inhibit *C. jejuni* and *Calicivirus* binding to intestinal mucus respectively (Morrow et al., 2004). The 2′-FL also suppressed the *C. jejuni* induced inflammation in human epithelial cells HT-29 and HEp-2, and in mouse intestinal mucosa (Yu, Nanthakumar, & Newburg, 2016). Overall, these studies highlight that sialylated and fucosylated HMOs are gold boons for infant's health, and several recent clinical studies supporting this statement are mentioned in Table 3.

According to the World Health Organization (WHO), diarrheal disease is the second leading cause behind the death of children below 5 years. Every year nearly 1.7 billion cases of childhood diarrheal disease are reported and are responsible for the deaths of around 525,000 children. Among the causative agents of diarrhea, rotavirus is the leading cause of severe dehydration gastroenteritis in this age group worldwide (Tate, Burton, Boschi-Pinto, & Parashar, 2016). Feed intolerance and necrotizing enterocolitis in newborns are also linked to rotavirus infection (Ramani et al., 2013). Contrary to enteric infections in older children where multiple strains are responsible for diarrhea, neonatal rotavirus infections are clinically and epidemiologically distinct as they are mostly asymptomatic and are related to geographically restricted unusual strains (Haffejee, 1991; Ramani et al., 2013).

Two vaccines against rotavirus RotaTeq® and Rotarix® (live and attenuated viruses) were approved in 2006 and 2008 by the Food and Drug Administration (FDA) for use among infants in the USA and their usages were further allowed globally after WHO recommendation. However, the lower efficacy of these vaccines proves to be a major challenge in developing countries, where most cases of severe rotavirus occur (Jiang, Jiang, Tate, Parashar, & Patel, 2010). Vaccines have to be administered orally at 6, 10 and 14 weeks of age when the infant is still feeding on breast milk. It was initially presumed that HMOs were likely to increase the efficacy of vaccines. Subsequently, epidemiologic studies have shown a 2-fold increased risk of rotavirus-induced diarrhea in children who are not breastfed (Plenge-Bonig et al., 2010). Indeed, Laucirica et al. (2017) demonstrated that 2′-FL and a combination of 3′-SL + 6′-SL have reduced infectivity of the rotavirus strains G1P[8] and G2P[4] by 62% and 73% respectively. A recent study highlighted that biological concentration of LNT, LNFP, 2′-FL, LNnT and 6′-SL does not actually act as decoy receptors for G10P[11] rotavirus but they increase G10P[11] Rotavac® infectivity in the presence of 2′-FL and LNFP I (Ramani et al., 2018). Norovirus uses histo-blood group antigens for infection; however, 2′-FL acts as decoy molecules against different strains of norovirus (GI.1, GII.4 and GII.17) (Koromyslova, Tripathi, Morozov, Schroten, & Hansman, 2017). Thus, 2′-FL should be explored as antiviral drugs against norovirus. Overall these findings reflect on the fact that HMOs could promote the performance of live and attenuated rotavirus vaccines; however, further studies should be considered to see the possible impact of HMOs on COVID-19 infectivity.

Streptococcus agalactiae (Group B *Streptococcus*, GBS) is a causative agent of pneumonia, septicemia, and meningitis in infants (Edwards, 2006; Thigpen et al., 2011) and it is vertically transmitted to offspring during vaginal birth. Although intrapartum antibiotic prophylaxis is allowed to protect from early-onset neonatal sepsis from the GBS infection, the emergence of new antibiotic-resistant GBS strains has greatly hampered this treatment and forced us to find an alternative way. In this regard, Lin et al. (2017) observed that HMOs can inhibit the growth of the GBS without showing bactericidal effect, and demonstrated that chain 1 fucosylated and nonfucosylated LNT are prominent in its growth inhibition. GBS susceptibility to HMOs is credited to conserved putative glycosyltransferase (gbs0738), belonging to the GT-8 family. The enzyme could be involved in transferring glycan moiety onto peptidoglycan; nevertheless, it could pick HMO components if present in sufficient quantity. The binding of HMO components to the catalytic site might lead to inactivation of the enzyme. Concurrently, Ackerman et al. (2017) divided human milk into 5 distinct groups based on Lewis blood types and screened them with GBS. They discovered that Lewis blood, a + B- and a-b- have altered morphology and reduced biofilm development of GBS respectively. Though these studies are observatory and lack the molecular basis understanding, it is clear that fucosylated and sialylated HMOs along with glycosidic bond types are crucial factors for their biological activities. Molecular mechanisms for inhibiting pathogens by using HMOs should be explored by further studies.

6. Future prospective

In modern science, various tools ranging from analytical instruments to deep protein and genomic sequencing using omics approaches are available to decipher the inter-relationship between gut microbiome, their development and role in the evolution of human lifespan from fetus to infant, child and adult concerning various HMO structures. Until now, we have understood that *Bifidobacterium* are paramount for HMOs utilization where different strains evolutionarily adopted unique strategies to catch and utilize different HMOs to maintain gut microbiome homeostasis. The metabolic products (for example acetate and propionate) formed upon fermentation of HMOs have proven themselves to be life-savers as they protect against various metabolic and pathogenic diseases including improving gut barrier functions and bowel related disorders. Nevertheless, we still need to emphasize on various studies that can interrogate which factors, for example, bacterial cell wall components and their metabolites, nutrients or host genetics, have the optimal consequence on infants health. It is clear that *Bifidobacterium* will be introduced as probiotic or as synbiotic along with HMOs in the near future into infant formula or in the form of different drinks to promote infant health. In order to woo this possibility, further studies should provide molecular understanding of each strain of *Bifidobacterium* so that they can be exploited along with HMOs as one of the main ingredients.

HMOs structurally resemble epithelial cell surface glycan receptors; thus, function as decoys to pathogens. They act as competitive inhibitors to pathogen binding onto epithelial cells, particularly *viruses*. From a clinical point of view, using such molecules for disease prevention may be safer when compared with current approaches such as the use of preventative antibiotics. Antibiotics predictably cause collateral damage to the host microbiome and put selective pressure on pathogens to develop their resistance potential to concur over given antibiotics. The emergence of antimicrobial resistance strains is indeed a great public health challenge, and it has always forced us to develop new antibiotics. Owing to the decoy nature of HMOs against receptors of pathogens that do not kill them but just make them non-infective, less selection pressure is produced and in turn lessen the chance for an emerging resistant strain of the pathogen to given oligosaccharides. After all, when it comes to clinical impact quality is better than quantity. HMOs seem to be a promising new strategy that may have new modes of action and structural-based glycoconjugates of HMOs should be developed to achieve this goal in near future. Additionally, it emphasizes that we need to determine the structure of all identified HMOs of the human milk, and then each of them should be independently evaluated for their roles in maintaining the infant gut microbiome and immune system. It is important because we learned that each characterized HMO has different benefits (Tables 1–3), and understanding the activity of each of them will greatly enhance the value of HMOs at the clinical level.

We believe in the upcoming years more synthetic HMOs will be supplied to infants globally in the form of infant formula such as synthetic 2′-FL developed by the DuPont Nutrition & Health and Inbiose. It has already been approved in the European market in 2018. A wide number of clinical and preclinical studies have clearly stated the role of 2DFL, LNT, LNnT, 3′-SL, 6′-SL and LNFP-1 in improving the desired microbiota in the gut by serving as a food source for the beneficial bacteria. These HMOs should be involved in infant formula to support the immunity and gut health of the neonate after proper regulatory approval. Furthermore, if we are to predict that various probiotic and prebiotic formulations will be developed in near future for improving infants' health and longevity, we will need to understand the integrated interactions of HMOs, gut microbiota and immune system at molecular mechanistic levels.

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.

Acknowledgements

R. P. Singh would like to thank Department of Biotechnology, Ministry of Science & Technology, Government of India for providing Ramalingaswami Re-entry Fellowship and Grant In aid number: BT/PR32876/PFN/20/1471/2020. The Executive Director of NABI is greatly appreciated for his continuous support for providing infrastructural facilities.

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