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Impact of dietary fibers in infant formulas on gut microbiota and the intestinal immune barrier

Chunli Kong, Marijke M. Faas, Paul de Vos and Renate Akkerman

Human milk (HM) is the gold standard for the nutrition of infants. An important component of HM is human milk oligosaccharides (hMOs), which play an important role in gut microbiota colonization and gut immune barrier establishment, and thereby contribute to the maturation of the immune system in early life. Guiding these processes is important as disturbances have life-long health effects and can lead to the development of allergic diseases. Unfortunately, not all infants can be exclusively fed with HM. These infants are routinely fed with infant formulas that contain hMO analogs and other non-digestible carbohydrates (NDCs) to mimic the effects of hMOs. Currently, the hMO analogs 2'-fucosyllactose (2'-FL), galacto-oligosaccharides (GOS), fructo-oligosaccharides (FOS), and pectins are added to infant formulas; however, these NDCs cannot mimic all hMO functions and therefore new NDCs and NDC mixtures need to become available for specific groups of neonates like preterm and disease-prone neonates. In this review, we discuss human data on the beneficial effects of infant formula supplements such as the specific hMO analog 2'-FL and NDCs as well as their mechanism of effects like stimulation of microbiota development, maturation of different parts of the gut immune barrier and anti-pathogenic effects. Insights into the structure-specific mechanisms by which hMOs and NDCs exert their beneficial functions might contribute to the development of new tailored NDCs and NDC mixtures. We also describe the needs for new in vitro systems that can be used for research on hMOs and NDCs. The current data suggest that "tailored infant formulas" for infants of different ages and healthy statuses are needed to ensure a healthy development of the microbiota and the gut immune system of infants.

1. Introduction

Human milk (HM) is the gold standard for infant nutrition. It contributes to a healthy development of infants after birth. The composition of HM is highly variable and is believed to be evolutionary optimized to meet the needs of infants at different ages and stages of development. HM therefore varies in composition during lactation and provides infants with all essential nutrients they need in their first months of life. HM is composed of various amounts of proteins, lipids and digestible and indigestible carbohydrates. Indigestible carbohydrates are predominantly human milk oligosaccharides (hMOs), which are the third most abundant solid component in HM after lactose and lipids. hMOs are important bioactive components of HM and have recently gained a considerable amount of attention due to the prebiotic effects they exert and their ability to guide the development of a stable microbiota community and a healthy mucosal immune system in infants.

The colonization of infants' gastrointestinal tract (GIT) by microbiota is a critical and very vulnerable period in life and is influenced by various genetic and environmental factors. The development of a stable microbiota composition contributes to the development of an effective gut barrier and a balanced mucosal immune system. Any disturbance in the microbiota colonization, for example, by the use of antibiotics or diet changes may not only lead to immune-related diseases such as allergies but also to inflammatory bowel disease (IBD). This illustrates the importance and intertwined relationship between the microbiome and the host immune system. As hMOs are resistant to digestion in the upper GIT, they can reach the large intestine and stimulate the growth of key members of the microbiota such as Bifidobacteria and Lactobacillus.

In addition to the microbiota stimulating effects, hMOs also directly influence the development of the infants' mucosal immune system and gut barrier maturation. The gut barrier, consisting of resident microbiota, a firm mucus layer, the epithelium, and the underlying immune system together provide protection against various pathogens and toxins in the
lumen of the gut.\textsuperscript{18} Infants are born with a relatively immature immune system, which is characterized by a loose epithelial barrier and a weak response to pathogenic stimuli.\textsuperscript{19} hMOS are known to exert direct immune modulatory effects on different cell types in the infants’ GIT and thereby contribute to the maturation of the gastrointestinal immune system.\textsuperscript{20,21} Furthermore, hMOS can improve the barrier function by modulating mucus secretion and interaction with the epithelial lining of the gut.\textsuperscript{22,23} In this way, hMOS ensure a healthy and efficient development of the infants’ gut immune system.\textsuperscript{2}

hMOS are unique for HM and not found in the same compositions or complexity as milk of other species.\textsuperscript{24} However, in specific cases, infants cannot be fed with HM, for example, when milk production is not sufficient to feed an infant, or when the mother has a disease that can possibly be transmitted through HM. Even though it was recently demonstrated that the currently emerging SARS-CoV-2 (COVID-19) is not transmitted through HM,\textsuperscript{25} there are studies showing that in case of other viral infections like \textit{M. tuberculosis}, \textit{T. pallidum}, and \textit{B. burgdorferi}, breastfeeding is not advisable.\textsuperscript{26,27} In these cases, and for many other valid reasons, infant formulas are the first option for the nutrition of newborn infants. Infant formulas, which are often cow milk derived, are nowadays often supplemented with hMO analogs or other non-digestible carbohydrates (NDCs) to substitute for some hMO functions.\textsuperscript{28} Galacto-oligosaccharides (GOS) and fructo-oligosaccharides (FOS) are already broadly added to infant formulas, either individually or combined in a typical ratio of 9:1.\textsuperscript{29,30} Pectins are also of interest for supplementation in infant formulas.\textsuperscript{31} The production of synthetic hMOS is still challenging and very expensive; however, the hMO analog \textit{2’-fucosyllactose (2’-FL)} can be produced in sufficient quantities and therefore there are already infant formulas containing 2’-FL on the market. Addition of these NDCs to infant formulas has been proven effective for the prevention of allergic diseases and the development of a stable and breastfed-like microbiota composition.\textsuperscript{3,7,2} However, there are also other NDCs and NDC combinations available that are of potential interest as supplements for infant formula. Insight into the mechanisms by which NDCs exert their beneficial effects on microbiota and immunity might contribute to the development of new and more tailored mixtures of NDCs for specific groups of infants like preterm or disease-prone infants.

In this review, we review and discuss the current insights into the development of microbiota compositions in differently delivered neonates, the composition of the gastrointestinal immune barrier and subsequently how hMOS and currently applied NDCs can support maturation and differentiation of the gut microbiota and immune barrier. We will give an update on currently known hMO functions and the effects of the hMO analog 2’-FL and various NDCs on the regulation and modification of gut microbiota compositions, as well as their effects on the gut epithelium and gut immune barrier regulation. This knowledge might contribute to the development of new NDC interventions in infants to support their early life development.

2. The colonization of the infants’ gastrointestinal tract by gut microbiota

The human GIT is colonized by a large diversity of microorganisms, collectively called the gut microbiota, which is mainly dominated by the gut bacteria, but also includes protozoans, viruses, and fungi.\textsuperscript{31} The first 2–3 years of life are considered to be a critical window for the colonization of the GIT.\textsuperscript{34,33} It is widely believed that this colonization starts right after birth.\textsuperscript{36} However, there are also studies available suggesting that colonization already starts \textit{in utero}.\textsuperscript{37} Most studies investigating the colonization period focus on colonization by bacterial species, which is therefore the main focus of this review. There are many factors identified that influence the bacterial colonization of the GIT.\textsuperscript{38} The mode of delivery is one of those factors that highly impacts the colonization by the initial bacterial species of the gut microbiota.\textsuperscript{39}

Vaginally delivered infants are first colonized by bacteria from the mothers’ vaginal, gastrointestinal, and skin microbiome. This is illustrated by a high abundance and diversity of bacteria from the genera \textit{Bifidobacterium}, \textit{Lactobacilli}, and \textit{Bacteroides}, which belong to the phyla Actinobacteria and Bacteroidetes.\textsuperscript{40,41} In contrast, infants delivered through a caesarean section (c-section) are first colonized by the bacteria from the skin and oral microbiota, as well as by species from the surrounding environment.\textsuperscript{10} During the first 3 months of life, different bacteria of the phylum Firmicutes, such as \textit{Clostridiaeae}, \textit{Veillonella}, and \textit{Klebsiella}, are abundantly present in the GIT of infants delivered through c-section. In addition, these infants are found to have a low abundance and diversity of Actinobacteria and Bacteroidetes.\textsuperscript{42} Notably, many \textit{Bifidobacterium} spp. and \textit{Bacteroides} spp. that are commonly found in vaginally delivered infants are not, or only in a very low abundance, present in c-section delivered infants.\textsuperscript{43}

The diversity in microbiota compositions between vaginally born infants and infants born via c-section gradually disappears after 4 to 12 months as a consequence of nutritional intervention.\textsuperscript{40} In a recent longitudinal study, in which children were followed from 5 weeks of age until 6–11 years of age, fecal microbiota analysis illustrated that diet is the most important determining factor for the microbiota composition from 13 weeks onwards.\textsuperscript{44} In this study, babies that were fed with mother milk developed a different gut microbiota composition compared to babies fed with infant formulas.\textsuperscript{45} Infants born via vaginal delivery and breastfed afterwards tend to have a lower incidence of immunological disorders and a healthier microbiota composition with a high abundance of the beneficial bacteria \textit{Bifidobacterium} spp. and \textit{Lactobacillus} spp., as compared to c-section or infant formula fed infants.\textsuperscript{39,44,45} In a Swedish cohort study, babies fed with mother milk were found to have a high percentage of \textit{Bifidobacterium} and \textit{Lactobacillus} until 12 months of age, while for infant formula fed babies, species belonging to \textit{Clostridia} were more prevalent.\textsuperscript{46} This difference in the early life has large effects on the adult disease, \textit{i.e.} low levels of \textit{Bifidobacterium} and high abundance
of *Staphylococcus aureus* in early life are associated with a high risk of overweight by the age seven.  
This illustrates the demand for improving infant formulas to achieve a similar gut microbiota to breastfed babies in early life.

The bacteria present in the infant’s GIT play many important beneficial roles in infants. First of all, they play a role in the digestion of food components that are not absorbed in the stomach and in the small intestine like NDCs. This is realized through special enzymes including glycoside hydrolases, glycosyltransferases, polysaccharide lyases, and carbohydrate esterases secreted by the gut bacteria, which can catalyze specific molecular linkages in the NDCs. This process results in the formation of short-chain fatty acids (SCFAs) like acetate, propionate, and butyrate that can serve as immune regulatory molecules or as nutrients and an energy source for other beneficial bacteria. SCFAs also have beneficial effects on intestinal epithelial cells, for example by stimulating their regeneration. In addition, commensal bacteria can synthesize a range of other molecules including vitamin K and vitamin B, which are also involved in host immune cell development.

As the microbiota compositions in newborn infants are rapidly changing over time, the ability of the gut microbiota to ferment and digest hMOs and NDCs is dependent on the infant’s age and the bacterial species present in the GIT. As the complexity of microbiota compositions usually increases upon ageing, the ability of the microbiota to ferment and digest larger and more complex hMOs and NDCs also increases over time. This was recently illustrated by a study of Logtenberg *et al.*, which showed that both fermentation capacity by infant fecal inoculum and SCFA production increased between 2 and 8 weeks of age in an *in vitro* batch fermentation of fructan-type NDCs. For this reason, also the infant’s age should be taken into account for the formulation of new NDC mixtures.

### 3. Gut immune barrier

The gut immune barrier is formed by the commensal microbiota, the gut mucus layer, the epithelial cell layer, and the resident and recruited immune cells in the lamina propria (Fig. 1). This barrier not only mediates food digestion and nutrient absorption, but it also forms a physical and immune barrier that prevents luminal pathogenic microorganisms, antigens, and detrimental luminal substances from entering the body and provides tolerance to commensal microbiota. In healthy adults almost 70% of the body’s immune cells reside in the GIT, making it the largest immunologically active organ of the body. hMOs and NDCs can impact the gut immune barrier at different levels, but before discussing their impact in the next sections, here the composition of the gut immune barrier will be explained in more detail.

The organization of the gut immune barrier differs between the small and large intestine (Fig. 1). In the small intestine, the immune barrier is highly organized with the presence of the majority of the immune cells in the lamina propria of the GIT, the epithelial cell layer, and a thin mucus layer and a low abundance of bacteria in the lumen, which allows the direct crosstalk of the food antigens and microbiota with the epithelium and the underlying immune system. The epithelial cells in the small intestine form many finger-like structures, known as the villi, which largely extend the surface area for nutrient absorption and immune sampling. The so-called crypts, containing intestinal stem cells and Paneth cells, are located at the base of the villi. Stem cells continuously generate new epithelial cells to replenish the cells lining the villi. Paneth cells secret defensins in response to the invasion of pathogens ensuring a healthy balance in the gut microbiota population.

The immune components of the gut-associated lymphoid tissue (GALT) in the small intestine are organized into specialized globle-like structures called Peyer’s patches (PPs) and mesenteric lymph nodes (MLNs) as well as the lamina propria. The PPs in the small intestine are small lymphoid tissues that harbor many immune cells. They are covered by specialized epithelial cells, the microfold cells (M-cells), that sample antigens from the lumen and present them to the immune cells residing in the PPs. Dendritic cells present under the epithelial lining are also able to sample the luminal content by protruding their dendrites into the lumen. Because of their antigen presenting function, PPs are considered to be important regulators of immune responses. B-cells are located in the follicles of the PPs and can secrete IgA, which can bind and neutralize pathogens and food antigens. The antigen presenting cells (APCs) in the PPs, like the dendritic cells, can migrate to the MLNs where they can also activate and instruct naive lymphocytes. Depending on the antigens presented to the naïve T-cells in PPs, they skew into different subsets, for example into T-regulatory (Treg) cells, which provide tolerance against food antigens and microbiota, or T-helper (Th) cells, that can be induced in response to pathogenic antigens.

The organization of the large intestine is different in several aspects. The epithelial layer of the large intestine has no villi, only crypts and is covered with a thick and firm mucus layer. As a consequence, there is less direct contact between the luminal content and the mucosal immune system. The mucus layer of the large intestine is composed of two layers: the relatively loose outer mucus layer that harbors the commensal bacteria and the germ-free inner mucus layer. The outer mucus layer is usually made up of the secreted mucins, of which mucin-2 (MUC2) is the major component which is a highly glycosylated mucin produced by the goblet cells that are integrated in the epithelial lining. Mucins contain similar molecular structures to hMOs and can serve as a substrate for bacterial fermentation and thereby stimulate the growth and colonization of specific bacterial strains. In addition, the mucus layer provides protection against intrusion of pathogenic bacteria.

The inner germ-free mucus layer is tightly attached to the intestinal epithelium and is mainly dominated by transmembrane mucins produced by intestinal epithelial cells with
various glycoproteins. These glycoproteins form the backbone of the various glycans that build up a glycocalyx layer on the apical side of the brush border covering the intestinal epithelium. A fully developed and intact glycocalyx layer is of significant importance for the mediation of the interaction between the gut microbes and the host immune system. The intestinal genes responsible for mucin production are already expressed before birth, but the development of a mature and protective mucus layer starts after birth and is of high importance for the establishment of a healthy immune system. Techniques such as glycomics are being explored to unravel the glycan reservoir of the glycocalyx layer to better understand the glycocalyx profiles, functions, and responses to the gut microbiota for giving dietary advice or improved medical treatment.

The intestinal epithelial cells are structurally assembled by intercellular tight junctions (TJ), adherens junctions, and desmosomes, of which TJ proteins are located at the most apical site of the epithelial cells and act as regulators of epithelial barrier integrity. They are also known as targets of immune regulation, as disruption of the TJ interactions will decrease barrier integrity and allow antigens, microbes, and toxins to enter the lamina propria and subsequently induce serious immune responses and gut inflammatory disorders. Many studies have demonstrated that microorganisms and dietary components influence the regulation of the TJ protein expression. Pattern recognition receptors (PRRs), which are widely expressed by the intestinal epithelial cells and intestinal immune cells, play an important role in the regulation of barrier integrity and TJ expression.

Toll-like receptors (TLRs) are the most well-studied PRRs, among which TLR2 and TLR4 are the best studied due to their interaction with gut bacteria and dietary components and their ability to mediate epithelial barrier integrity.

The gut immune barrier in newborn infants is relatively immature and has to develop into the structures described above. A characteristic of an infant is having a lower barrier function which is containing a looser epithelial barrier structure but also by an immature immune response and a microbiome with low diversity in species. Maturation processes of microbiota, barrier and immunity are considered to be under tight control by mother milk components such as hMOs and should therefore also be supported by hMO analogs or NDCs that are added to infant formulas. In the next section, after describing the diversity of hMOs in mother milk, we will review the current insight into how hMO and NDCs in infant formulas contribute to the development of different parts of the gut immune barrier and how it prevents pathogenic infections.

4. Human milk oligosaccharides (hMOs)

hMOs comprise a large family of over 200 different oligosaccharides that are composed of 5 main sugar building blocks: D-glucose (Glc), D-galactose (Gal), N-acetylglucosamine (GlcNAc), L-fucose (Fuc) and sialic acid (NeuAc) (Fig. 2). All hMOs are built up based on a backbone of lactose (Galβ1–4Glc) as the reducing end, which can be further
The core structure of hMOs can also be decorated by Fuc (via α1–2, α1–3, or α1–4 linkage) or NeuAc (via α2–3 or α2–6 linkage), which forms the fucosylated (FL) or sialylated (SL) hMOs.77 hMO profiles are determined by the enzymes expressed in the lactocytes of the mother.78 For fucosylated hMOs, α1–2 linked Fuc is added through α1–2-fucosyltransferase (FUT2), whereas Fuc linked through an α1–3 or α1–4 linkage is added through α1–3/4-fucosyltransferase (FUT3). The FUT2 enzyme is encoded by the Secretor (Se) gene and the FUT3 enzyme is encoded by the Lewis (Le) gene. With the possibility of either a positive (+) or a negative (−) expression of the Se and Le genes, secretion patterns can be divided into four groups: Se+Le+, Se−Le+, Se+Le−, and Se−Le−.78

The amounts and compositions of hMOs in mother milk change over time, from around 20 to 25 g L−1 in the colostrum to 5 to 20 g L−1 in mature milk.77 There are studies showing that the hMO profiles in mother milk are influenced by environmental factors and by the feeding practice of a mother. For example, a study by Davis et al., in which hMO profiles of 33 Gambian mothers were analyzed, showed that the amount of hMOs in mother milk increased during the dry season, which could be related to the availability of food.79 A Canadian study also showed that other environmental factors including sunlight, climate and allergen exposure influence hMO synthesis.80 In the following sections, we describe the beneficial effects of hMOs on the different layers of the GIT in infants (Fig. 3).

4.1 Effects of hMOs on gut microbiota compositions

As mentioned before, hMOs are resistant to digestion in the upper GIT and can reach the large intestine intact. There they can modulate the gut microbiota composition and metabolism of infants in a structure-dependent manner.81 In general, the
The different ability to utilize different hMO structures is dependent on the sets of enzymes bacteria are equipped with. Some bacteria are able to internalize hMOs and subsequently deconstruct them using intracellular GHs. Examples of such bacteria are *B. infantis* species. These species were shown to abundantly express enzymes including α-sialidase, α-fucosidase, β-galactosidases, and β-N-acetylhexosaminidases to degrade a wide spectrum of hMOs including α-sialidase, α-fucosidase, β-galactosidases, and β-N-acetylhexosaminidases. This can result in the formation of monosaccharides that can be released into the cytoplasm of the bacteria as well as in the formation of SCFAs as end products. There are also strains that utilize hMOs using extracellular enzymes. For example, in a study by Nishiyama et al., it was found that *B. bifidum* species could selectively utilize fucosylated or sialylated hMOs using extracellular enzymes, including lacto-N-bioside (LNBase) and galacto-N-biose (GNB), which are responsible for catalyzing the phosphorolysis of Galβ1–3GalNAc and Galβ1–3GlcNAc structures in these hMOs. The degradation of hMOs by this strain results in the formation of monosaccharides that can stimulate the growth of other species that cannot degrade hMOs themselves through cross-feeding.

Bacterial cross-feeding between hMO utilizing species and other species can contribute to a more diverse microbiota composition. There are various studies available showing the effect of hMO utilization by *Bifidobacteria* strains on the growth of other species. For example, Salli *et al.* found that 2′-FL promoted the growth of *Bifidobacteria* and increased the production of SCFAs and lactic acid in a human study. Lactic acid can be further utilized as a carbon source by secondary strains like *Eubacterium hallii*, *Faecalibacterium prausnitzii*, and *Clostridium butyricum* to produce butyrate. In another recent study by Lawson *et al.*, *Bifidobacterium* strains isolated from breastfed infants were analyzed for their cross-feeding abilities. It was found that *B. longum* LH206 could partially degrade 2′-FL into fucose and lactose, and the conditioned media of *B. longum* LH206 could stimulate the growth of *B. longum* LH659 indicated by an increased acetate production. In the same study, *B. pseudocatenulatum* LH13 degraded 2′-FL into...
acetate, ethanol, formate, and 1,2-propanediol, which could be further utilized by the non-degrader *B. longum* LH12.\(^5^0\)

### 4.2 hMOs and prevention of pathogen adhesion

hMOs are structurally similar to intestinal mucosal glycans, and can therefore serve as decoy receptors that bind pathogenic bacteria in the gut lumen and prevent them from adhering to the intestinal epithelial cells. There are various studies available demonstrating the decoy effects of hMOs. For example, fucosylated oligosaccharides were shown to effectively inhibit the adhesion of *Campylobacter jejuni*,\(^8^9,^9^0\) *Listeria monocytogenes*,\(^9^1\) and enteropathogenic *Escherichia coli* (EPEC)\(^9^2\) to the gut epithelium. On the other hand, the sialylated oligosaccharide 3′-SL was shown to inhibit the adhesion of *Helicobacter pylori* in a rhesus monkey model.\(^9^3\)

Coppa *et al.* compared the effects of single hMOs with pooled hMO fractions on the inhibition of the adhesion of enterotoxigenic *Escherichia coli* (ETEC), *Vibrio cholerae*, and *Salmonella* *fyris* to epithelial Caco-2 cells.\(^9^4\) Among the single hMOs only 3-FL, but not 2′-FL, 3′-SL, or 6′-SL, showed an inhibitory effect on the adhesion of ETEC and *S. fyris*, while the pooled hMO fractions showed a wider and also stronger inhibitory effect. This suggests that pathogens adhere to the epithelium of the host through multiple glycan structures, and specific oligosaccharide structures decor the groups of pathogens that adhere through the same structures. However, this study did not provide mechanistic insight into the interference of hMOs and the binding of pathogens to specific receptors. A study by Ruiz-Palacios *et al.* further investigated the decoy abilities of hMOs. They found that α1–2 fucosylated moieties of hMOs inhibited the adhesion of *C. jejuni* to intestinal mucosa and demonstrated that binding to intestinal H2 antigen is essential for *C. jejuni* infection, which could be specifically inhibited by fucosyl α1–2 linked oligosaccharides.\(^9^0\) More recently, Behrouz demonstrated that the hMO LNnT could prevent the adherence of *Staphylococcus pneumoniae* to chinchilla tracheal epithelial cells by blocking the GaIb1–4GlcNacβ1-3Gal receptor.\(^9^5\)

### 4.3 Effects of hMOs on the epithelial cell barrier

Nowadays, there are some studies available demonstrating the direct effect of hMOs on the different cells of the intestinal epithelial barrier. For example, Cheng *et al.* tested the effects of various individual hMOs including 2′-FL, 3-FL, and LNT2 on the gut mucus barrier function using a LS174 T goblet cell line in the presence of different stressors. They showed that 3-FL and LNT2 could improve MUC2 gene expression during challenges with both IL-13 and TNF-α. 3-FL and LNT2 also stimulated MUC2 secretion at the protein level.\(^2^2\) There are also studies showing the effects of hMOs on the epithelial glycocalyx layer. Angeloni *et al.* showed that hMOs influence the expression of different glycocalyx molecules that might have an effect on the ability of certain pathogens to bind to epithelial cells.\(^9^0\) In another study by Kong *et al.*, the effects of 2′-FL and 3-FL on the average thickness and area of the intestinal epithelial glycocalyx components, hyaluronic acid (HA) and heparan sulfate (HS), in Caco-2 cells were quantified using immunofluorescence staining. It was found that 3-FL, but not 2′-FL, could enhance albumin absorption and increase HA and HS, stimulating the development of the glycocalyx layer.\(^9^7\) In addition to their mucus and glycocalyx modulating properties, hMOs can also stimulate epithelial cell maturation. Holscher *et al.* found that individual or combinations of 2′-FL, 3-FL, and 6′-SL reduced cell proliferation, and increased differentiation of both intestinal epithelial HT-29 and Caco-2Bbe cells, which were associated with the maturation of the intestinal epithelial cells.\(^2^3\)

### 4.4 Effects of hMOs on infection prevention and mucosal immune responses

Besides modulating the mucus protein and glycan expression, hMOs also exert immune regulating effects, for example via Toll-like receptors (TLRs).\(^2^1\) Cheng and colleagues tested the stimulatory effects of the individual hMOs 2′-FL, 3-FL, 6′-SL, LNT2, and LNnT on multiple TLRs, including TLR2, TLR3, TLR4, TLR5, TLR7, TLR8, and TLR9. They demonstrated that the activation and inhibition of specific TLRs are highly dependent on the hMO type, e.g. 3-FL activated TLR2 and inhibited TLR5, TLR7, and TLR8, while LNT2 activated all TLRs and led to a subsequent increase of IL-10 and TNF-α secretion in THP-1 macrophages.\(^2^1\) It was hypothesized that the unique N-acetylgalacosamine end of this hMO was responsible for the observed effects.

Studying the TLR activation and inhibitory properties of hMOs is a relevant topic, as many infant diseases can be linked to specific TLR interactions. For example, TLR4 activation is involved in the pathogenesis of necrotizing enterocolitis (NEC), a neonatal disease with a high mortality. Upon TLR4 activation, necroptosis is activated by the intestinal epithelium, leading to the development of NEC.\(^9^8\) hMO supplementation is beneficial for infants suffering from NEC, as it increases the epithelial cell differentiation and it accelerates the crypt cell turnover to protect the intestinal epithelial cell injury from NEC.\(^9^9\) Importantly, hMOs also activate epidermal growth factor receptors and reduce the TLR4 expression and inhibit the downstream NF-κB signaling, resulting in a lower expression of the inflammatory cytokines IL-6 and IL-8.\(^9^9\) More specifically, Sodhi *et al.* showed that the supplementation of 2′-FL and 6′-SL, but not of lactose, to infant formulas prevents NEC in both mice and piglet models, and attenuates NEC-associated inflammation in human ileum samples, which is realized through the inhibition of TLR4.\(^1^0^0\) Above all, hMOs have been listed by the FDA as nutritional supplements for gut disease treatment, i.e. irritable bowel syndrome. These functions have been more extensively reviewed by Cheng *et al.* \(^2\)

## 5. Non-digestible carbohydrates (NDCs) in infant formulas to substitute hMO functions

Infants who cannot be fed with HM are routinely fed with infant formulas, which are often cow milk derived. As these
infant formulas do not contain hMOS they are supplemented with NDCs that partially resemble hMO effects. Nowadays, there are more infant formulas coming to the market containing different NDCs. Until January 2020, there are 17 isolated or synthetic NDCs listed by the FDA (Food and Drug Administration), among which GOS, FOS, inulin, and polydextrose have been marketed in Europe and are under extensive clinical research in the United States. To date, GOS and FOS are commonly used in infant formulas as well as the hMO analog 2′-FL. An increasing amount of research is dedicated to the selection of other NDCs and NDC mixtures that can be used in infant formulas to support the development of similar gut microbiota composition and gut immune responses found in breastfed infants. For example, it can be hypothesized that the mixtures of neutral NDCs like inulin-type fructans and acidic NDCs like pectin-derived oligosaccharides can resemble natural hMO mixtures more closely and therefore cover a wider range of beneficial effects. An important finding of this research is that the NDC type, structure, and dose influence the impact of NDCs on the gut microbiota and gut immune barrier functions. To optimize NDC formulations for infant formulas, insight into the mechanisms by which NDCs exert their beneficial effects is crucial. Therefore, we will review the current knowledge on the hMO analog 2′-FL as well as various NDCs that are available for the supplementation of infant formulas in the following sections, with a specific focus on the hMO effects they resemble (Fig. 3).

5.1 2′-Fucosyllactose (2′-FL)

Nowadays, it is possible to produce some small hMO molecules in sufficient quantities that allow application in infant formulas. 2′-FL is one of the commercially available hMO analogs that is not isolated from HM but is produced as a fermentation product of genetically engineered microorganisms which include the strains of E. coli and yeast. 2′-FL is composed of a lactose molecule decorated with one α1–2 linked fucose unit (Fig. 2d). 2′-FL reaches the large intestine and can therefore exert direct and indirect effects by stimulating gut microbiota fermentation processes in the large intestine. In the past few years, 2′-FL has been added to infant formulas as an hMO analog and has been tested in several clinical studies to determine the optimal dosage that can be regarded as safe and which will be well tolerated in infants (Table 1). In contrast to hMOS in mother milk, 2′-FL is an individual molecule, not a mixture of various hMO structures. As a consequence, the dosage of 2′-FL differs from the total amount of hMOS found in human milk. In recent years, a growing number of studies have become available on the effect of supplementation of infant formulas with individual 2′-FL, which will be described in the next sections.

5.1.1 Effects of 2′-FL on microbiota compositions.

Nowadays, there are various studies available showing the effect of supplementation of infant formulas with 2′-FL on the gut microbiota compositions of infants. As described before, Bifidobacteria can selectively utilize hMO mixtures and are a dominant species in the intestine of breastfed infants. The maternal fucosyltransferase 2 status has been proven to directly affect the gut bifidobacterial communities in the gut of breastfed infants. Children who are fed with breast milk of non-secretor mothers, who do not express α1–2-fucosyltransferase responsible for making α1–2 fucosylated hMOS, are shown to be delayed in the establishment of a bifidobacterial-laden microbiota, which is probably related to a difficulty in acquiring the species of Bifidobacteria that are able to consume the specific hMOS present in the mother milk. This illustrates the direct link between α1–2 fucosylated hMOS like 2′-FL and the colonization by Bifidobacteria. Supplementation of infant formulas with 2′-FL might therefore also increase the abundance of Bifidobacteria in the infant’s intestine. This is corroborated by an in vitro study by Van den Abbeele et al. who demonstrated that 2′-FL increased the relative abundance of Bifidobacterium adolescentis and butyrate-producing bacteria in a validated gut model using the microbiota of 6-month-old formula-fed infants. There are no clinical studies available yet that confirm the direct link between 2′-FL supplementation and an increase of Bifidobacteria in vivo.

5.1.2 2′-FL and prevention of pathogen adhesion. In an in vitro study by Weichert et al., biotechnologically synthesized 2′-FL could inhibit the adhesion of Campylobacter jejuni, enteropathogenic E. coli (EPEC), S. fyris, and Pseudomonas aeruginosa to the intestinal human cell line Caco-2 by 26%, 18%, 12% and 17%, respectively. 2′-FL could also inhibit the adherence of Pseudomonas aeruginosa to the human respiratory epithelial cell line A549 by 24%. Another study showed that 2′-FL could also significantly reduce the adhesion of E. coli to Caco-2 cells, while it did not have an effect on the adherence of S. firis. Yu et al. found that 2′-FL could attenuate C. jejuni invasion by 80% and decreased the release of mucosal pro-inflammatory signals IL-8 by 60–70%, IL-1β by 80–90% and MIP-2 by 50% in an in vitro model using HEp-2 and HT-29 cells. Additionally, in a mouse model, 2′-FL also attenuated C. jejuni invasion by 80% and reduced weight loss by 5%. Histologic features of intestinal inflammation were also reduced by 50–70%.

5.1.3 Effects of 2′-FL on epithelial cell responses. Next to its effect on microbiota and pathogen adhesion, there are also studies showing the direct effects of 2′-FL on epithelial cells. Kong et al. showed that 2′-FL, as well as 3-FL, significantly increased the thickness and coverage area of absorbed albumin in the glycoalyx layer on Caco-2 cells and thus possibly contributes to the development of the glycoalyx layer. Zehra et al. found that 2′-FL modulated human epithelial cell responses related to allergic diseases by the selective inhibition of CCL20 release in response to the antigen–antibody complex, TNF-α or PGE2. The effect was PPARy dependent. This might be an important finding as 2′-FL can be used as a therapeutic in food allergy.

5.1.4 Effects of 2′-FL on the prevention of infection and mucosal immune responses. Various in vitro studies show the immune modulating effects of 2′-FL. Cheng et al. showed that 2′-FL, among other hMO analogs, could specifically inhibit the immune receptor TLR5 and 7 and thus serve as an antagonist
for these receptors and prevent too strong immune activation.29 He et al. found that 2′-FL can also directly inhibit LPS-mediated inflammation during the invasion of ETEC in T84 and H4 intestinal epithelial cells by attenuating IL-8 production through decreasing CD14 induction.20 There are also various in vitro studies on the effect of 2′-FL on the prevention of virus infection. Duska-McEwen et al. showed that 2′-FL could enhance innate immunity to airway infections by decreasing respiratory syncytial virus (RSV) viral load in airway epithelial cells.119 They also showed that 2′FL could attenuate RSV-induced inflammatory cytokines IL-8 and TNF-α production in airway epithelial cells, and in RSV-infected peripheral blood mononuclear cells (PBMCs).119 2′-FL, as well as 3′-SL and 6′-SL, could also reduce the infectivity of human rotavirus G1P and G2P in African green monkey kidney epithelial cells (MA104 cells). As breastfed children are directly protected against human rotaviruses, this indicates that the supplementation of infant formulas with 2′-FL may confer these benefits to formula-fed infants.120 Koromyslova et al. found that 2′-FL can block the binding of noroviruses to histo-blood group antigens, preventing the viruses from infecting the cells.121 There are also in vivo studies showing the beneficial effects of the hMO analog 2′-FL in infant formulas. For example, in a study where healthy term infants were fed with formulas supplemented with 2′-FL, infants had similar levels of plasma inflammatory cytokines to breastfed infants which were 29–83% lower compared to those of children fed with the breastfeeding group.112

### 5.2 Galacto-oligosaccharides (GOS)

Galacto-oligosaccharides (GOS) are a mixture of NDCs that have already been widely applied to infant formulas.103 They are composed of galactose units with one glucose unit at the reducing end, and the degree of polymerization (DP) of GOS typically ranges between 2 and 10 (Fig. 2e).122 GOS are enzymatically synthesized using galactosyl transfer reactions.
which link the galactose units via β1–3, β1–4, or β1–6 linkage, depending on the source of the enzymes. Like hMOs, GOS are resistant to digestion in the upper GIT and can therefore reach the large intestine, where they influence microbiota compositions and exert direct effects on the intestinal epithelium and immune reactions.

5.2.1 Effects of GOS on gut microbiota. The beneficial effect of GOS on gut microbiota in infants has been shown widely in studies that tested GOS as a supplement in infant formulas (Table 1). Matsuki et al. studied the effect of infant formulas supplemented with GOS at a concentration of 3 g L⁻¹ on microbiota composition in 35 healthy term infants of 0–2 months. After 2 weeks of feeding, fecal samples were collected and examined. GOS supplementation stimulated the abundance of indigenous *Bifidobacteria* in the infants’ gut and reduced the α-diversity of the gut microbiota. Other fecal characteristics related to the gut microbiota metabolism such as SCFA production were not influenced by GOS. In another study, Sierra et al. studied the impact of a higher GOS concentration of 4.4 g L⁻¹ in a study with 365 healthy term infants of 0–2 months. GOS were administered until 12 months of age and the effects on the gut microbiota composition and metabolism were studied at 4 months of age. GOS-containing infant formulas increased the *Bifidobacterium* counts, but a lower butyrate concentration was found in the fecal samples. In addition, it was found that GOS could also attenuate the adverse effects from the environment on the gut microbiota development of the infants. In a study among Kenyan infants aged 6.5–9.5 months, GOS could attenuate the adverse side effects on the microbiota caused by the use of iron-containing infant formulas to treat anemia. Supplementation of these infant formulas with GOS resulted in a higher abundance of *Bifidobacterium* and *Lactobacillus* and a lower abundance of virulent and toxic genes of the pathogens. Taken together, these studies show that GOS, like hMOs, can specifically stimulate beneficial strains in infants.

5.2.2 Effects of GOS on pathogen adhesion. Many studies have shown that GOS can also act as decoy receptors that block the adhesion of pathogenic bacteria to the intestinal epithelium. In a study by Shoaf et al., the effect of purified GOS on the inhibition of the adherence of *EPEC* was investigated using an *in vitro* assay with epithelial HEp-2 and Caco-2 cells. The adherence was reduced by 65% and 70%, respectively, when compared to non-treated cells. Furthermore, it was proved that the inhibitory effects of GOS were mediated via the adherence factor BfpA, a bundle-forming pilus protein of *EPEC*. Another study that investigated the protective effect of GOS on the adhesion of *ETEC* in neonate piglets demonstrated the binding ability of porcine albumin-conjugated GOS to various *ETEC* strains. *ETEC* K88 was found to have the greatest affinity, and the adhesion to the intestinal mucins was partially inhibited by GOS. The inhibition effect of GOS on the adhesion of other pathogens including *EPEC*, *Salmonella*, and *C. rodentium* to the epithelial cells was also extensively reported. GOS can stimulate the gut barrier function by directly interacting with the gut epithelial cells. Akbari et al. evaluated the effects of Vivinal GOS syrup and purified GOS fractions with different DPs on the barrier integrity of epithelial cells during a challenge with the fungal toxin deoxynivalenol (DON). Pre-incubation of the epithelial Caco-2 cells with GOS for 24 hours could prevent the disruption of the barrier integrity induced by DON. Vivinal GOS showed the most pronounced protective effect with accelerated reassembling of the tight junction and the production of Interleukin-8 (IL-8). In another study, exposure of Caco-2 cells to GOS of DP2–7 (1.4% w/v) also showed the ability to improve the integrity of the tight junction via increasing the trans-epithelial electrical resistance (TER). More recently, the transcriptome of epithelial Caco-2 cells was fully characterized after stimulation with GOS and the hMO sialyllactose (SL) and the effects on the epithelial barrier function were studied by investigating cellular pathways involved in cell proliferation, differentiation, and re-epithelialization. GOS modulated 63 pathways of the epithelial barrier transcriptomes, among which the top 4 most significantly modulated pathways were influenced in the same way by the hMO SL, indicating a similarity in function between GOS and SL. In another study, Cheng et al. showed that GOS could stimulate the gene expression of MUC2 and TFF3 in epithelial goblet cells in a dose- and time-dependent manner. In this study, medium containing GOS could also significantly stimulate MUC2 at the protein level.

5.2.4 Effects of GOS on gut mucosal immune responses. The gut immune system can be modified by GOS as well, as illustrated by various studies. Bermudez-Brito et al. used an *in vitro* system to test the effects of GOS on T-cell populations. In this study, GOS selectively increased the Treg cytokine IL-10 and Th1 cytokine INF-γ, while it decreased the secretion of the Th1 cytokine TNF-α, indicating that GOS modulates the gut immune responses and thereby contributes to a tolerogenic milieu. Recently, the effect of GOS on mucosal immune homeostasis was evaluated in a suckling piglet model. In this study, newborn piglets were fed with GOS (1 g per kg body weight) for one week. The animals fed with GOS had a higher production of SCFAs, which subsequently reduced IL-8 and increased IL-10 measured in the large intestinal mucosa of piglets. In addition, the expression of the tight junction proteins ZO-1 and Claudin-1 was increased through suppressing the phosphorylation of NF-κB and activating the AMPK signaling pathway.

5.3 Fructo-oligosaccharides (FOS) and inulin

Fructo-oligosaccharides (FOS) and inulin are composed of linear chains of fructose units that are terminated by a single glucose unit with variations in glycosidic linkages and different DPs. FOS and inulin are categorized according to their chain length. Short-chain FOS (scFOS) are composed of 3 to 5 residues per chain, while long-chain FOS (lcFOS) have 6 to 10 residues. FOS can be enzymatically obtained from natural inulin. Inulin usually refers to fructans with a higher chain length, usually containing up to 200 fructose residues and can be derived from different plant-based sources like chicory, dahlia, or Jerusalem artichoke.
5.3.1 Effects of FOS and inulin on gut microbiota. Supplementation of infant formulas with FOS and inulin has been widely shown to improve gut microbiota compositions in infants by exerting an enhancing effect on the abundance of *Bifidobacteria* species.\textsuperscript{104,135} Even though there are also studies reporting the minimal adverse effects of FOS supplementation on the gut microbiota by increasing the counts of *C. difficile*, *E. coli*, and *Clostridium* (Table 1),\textsuperscript{136,137} a higher abundance of *Bifidobacteria* in infants fed with FOS and inulin supplemented formulas will result in a closer resemblance of the microbiota compositions found in breastfed infants. This contributes to a lower incidence of fever, diarrhea, and infections during the antibiotic treatment.\textsuperscript{138–140} Over the last few years, there have been many studies showing the beneficial effects of FOS and inulin on the regulation of the gut microbiota composition, as listed in Table 1.\textsuperscript{135,141} For example, in a randomized controlled double-blind study with 164 healthy formula fed infants by Oswari et al.,\textsuperscript{137} it was found that the supplementation of infant formulas with 4 g inulin per L increased the numbers of *Bifidobacteria* and *Lactobacillus* in the fecal microbiota as compared to the infant formula supplemented with 2 g inulin per L or without inulin. Even though FOS and inulin are well degraded by *Bifidobacteria*, their degradation by *Lactobacilli* is only limited to a few strains of *Lactobacillus casei* and *Lactobacillus paracasei* subsp.\textsuperscript{142,143} The inulin-degrading strain *Lactobacillus paracasei* I321 isolated from infant fecal microbiota was shown to resist the competitive environment of the fecal microflora in vitro and showed an anti-salmonella effect through the secretion of antibacterial agents and competitive adhesion mechanisms.\textsuperscript{144} Notably, an increased intake of inulin is associated with a higher production of butyrate, which is believed to prevent colon cancer and inflammation.\textsuperscript{145} However, Nielson showed that *Bifidobacteria* can proficiently degrade inulin, but did not contribute to the butyrogenic effect.\textsuperscript{88}

5.3.2 Effects of FOS and inulin on pathogen adhesion. FOS and inulin induce a strong inhibitory effect on the adhesion of pathogens. For inulin, this inhibitory effect seems to be dependent on the degradation of inulin by commensal bacteria rather than by the direct effects of inulin on the pathogens. This was illustrated by a study of Van Den Abbeele et al., in which an *in vitro* assay was developed to evaluate the effects of inulin in PBS and inulin in intestinal fluid, which contained abundant commensal bacteria, on the adhesion of mucin-adhered *Clostridia* to the mucin agar. The adhesion of *Clostridia* was significantly inhibited in intestinal fluid; however, this inhibitory effect of inulin was not detected in PBS.\textsuperscript{146} In a more recent study, Piotrowski et al. tested the effect of inulin on the adhesion of 12 *Clostridium difficile* strains to HT-29, mucus-secreting HT-29 MXT and CCD 841 human epithelial cell lines. The direct effects of inulin on the adhesion of *C. difficile* were not observed.\textsuperscript{147} A study by Kanjan et al. showed that inulin was able to promote the competitive adhesion of *Lactobacillus paracasei* I321 isolated from infant fecal microbiota to mucin when co-cultured with *Salmonella typhimurium* and thereby reduced the adhesion of *S. typhimurium*.\textsuperscript{144} In another study, Shoaif et al. showed that FOS and inulin directly inhibited the adhesion of EPEC to Caco-2 cells by 32% and 42%, respectively.\textsuperscript{125}

5.3.3 Effects of FOS and inulin on gut epithelial cell responses. Inulin has been shown to exert direct effects on gut epithelial cells and can contribute to the development of a stronger defense line against pathogen-induced disruptions of the epithelial integrity. In an *in vitro* study by Wu et al.,\textsuperscript{146} pre-incubation of inulin was found to protect the barrier integrity of Caco-2Bbe1 cells in a challenge with enterohemorrhagic *Escherichia coli* (EHEC), illustrated by a rescue of transepithelial electrical resistance (TER) levels. Pre-incubation of the cells with inulin alone also increased TER levels. The alteration of barrier integrity was further examined by measuring the expression levels of tight junction proteins and mRNA. It was found that inulin promoted the epithelial barrier function through a directly increased tight junction protein expression of occludin.\textsuperscript{148} In another study, a mixture of inulin, containing molecules ranging from DP3 to 10, showed beneficial effects on the glyocalyx layer of Caco-2 cells.\textsuperscript{97} It increased the amount of heparan sulfate in the glyocalyx layer. In the same study, a mixture of long-chain inulin and DP30–60 could specifically increase the amount of hyaluronic acid.\textsuperscript{57}

5.3.4 Effects of FOS and inulin on gut mucosal immune responses. Inulin can directly influence mucosal immune responses in a chain length and structure-dependent way. Vogt et al. found that short-chain inulin could induce an anti-inflammatory balanced IL-10/IL12 ratio in human peripheral blood mononuclear cells, *i.e.* more IL-10 compared with IL-12, while the long-chain length inulin induced a pro-inflammatory cytokine profile, *i.e.* more IL-12 compared with IL-10.\textsuperscript{149} In a mouse model, long-chain inulin induced an anti-inflammatory cytokine production profile, promoted T-cell responses by increased regulatory T-cells, and enhanced tight junction proteins and anti-microbial peptides as well as the SCFA production.\textsuperscript{114} Long-chain inulin (DP10–60) also supported the immune system and provided protection against pathogenic antigens induced by hepatitis-B vaccination through increasing Th1-cells, and stimulating a Th1-type cytokine profile and stronger TLR2 activation than the short-chain inulin (DP2–25).\textsuperscript{149} These findings demonstrated that inulin can support different immune responses in a structure-specific way.

5.4 Pectins

Pectins are complex hetero-polysaccharides composed of an α1–4 linked galacturonic acid backbone, which can be interrupted by the α-rhamnose unit with the side chains of neutral sugars including arabinose and galactose.\textsuperscript{150} The carboxyl group of galacturonic acid on the galacturonic acid backbone can be esterified with methyl groups (Fig. 2f).\textsuperscript{152} The percentages of the carboxyl groups of galacturonic acid that are methyl-esterified determine the degree of methyl esterification (DM).\textsuperscript{49} According to differences in DM, pectins are classified as low DM pectins (25–50%) and high DM pectins (50–80%).\textsuperscript{151} Pectin is a common polymer in the cell wall of...
plants, including vegetables and fruits, such as potatoes, lemon, and hawthorn. A major characteristic of pectin is its gel-forming ability, for which it is widely applied in the food and pharmaceutical industries. Pectin-derived acidic oligosaccharides are of great interest for the supplementation of infant formulas to mimic the sialic acid found in HM to promote the beneficial gut microbiota.

5.4.1 Effects of pectin on the gut microbiota. Pectin has the potential to shape the gut microbiota to obtain a balanced gut microbiota. Pectins can be degraded by various Gram-negative Bacteroides species, and are often less degraded by the Gram-positive bacteria like Firmicutes bacterial species. However, Faecalibacterium prausnitzii and Eubacterium eligens belonging to the Firmicutes species have recently also been identified as pectin degraders. The growth of the species was tested on sugar beet pectin DP4 and DP5. E. eligens DSM3376 showed a rapid growth on both DP4 and DP5, F. prausnitzii A2–165 could utilize DP4, but not DP5, while F. prausnitzii S31/L3 grew better on DP5 than on DP4, and F. prausnitzii L2–6 showed a slow growth on both DP4 and DP5. In a study by Bianchi et al., citrus pectin was shown to enhance Bifidobacterium longum BB-46. In another study, it was found that the acidic hydrolysate from pectin used for the supplementation of infant formulas at a concentration of 2 g L$^{-1}$ did not affect the Bifidobacteria and Lactobacilli counts. However, when combined with neutral GOS/FOS (9 : 1, 6 g L$^{-1}$), it resulted in a higher abundance of Bifidobacteria and Lactobacilli (Table 1).

Pectin can also promote bacterial cross-feeding resulting in increased abundances of non-degrading bacteria. This was illustrated in a study by Bui et al., where lactate production resulting from pectin degradation by Bacteroides thetaiotaomicron stimulated the growth of Anaerostipes h寸anm solifornans resulting in a higher butyrate production. Bang et al. also reported that the production of SCFAs after the fermentation of pectin increased bacterial strains of Lachnospira, Dorea, Clostridium, and Sutterella, with a subsequent increase in butyrate levels.

5.4.2 Pectin and prevention of pathogen adhesion. As illustrated by a number of studies, pectin oligosaccharides can prevent pathogen adhesion to the host and serve as decoy receptors to pathogens. Rhoades et al. evaluated the ability of pectin to inhibit the adhesion of six strains of pathogenic EPEC in an in vitro study using the human gut epithelial HT-29 cell line. The culture medium with different concentrations of pectin was tested on the EPEC viable counts to determine the effect of pectin. It was found that the medium containing pectin at a concentration of 2.5 mg mL$^{-1}$ significantly decreased the adhesion of pathogenic bacteria by 70% when compared to the non-pectin-treated group. The anti-adhesive effect of five different pectins, which varied in the extraction method, molecular weight, and degree of esterification, was also studied on the adhesion of EHEC O157:H7 to HT-29 cells. Pectin concentrations used in this study ranged from 0.001 mg mL$^{-1}$ to 5 mg mL$^{-1}$ for all five pectin mixtures tested. Although all pectin samples showed anti-adhesive effects, it was found that higher concentrations and lower molecular weights showed the strongest anti-adhesive effects, up to 100% when compared to the control group without pectin. The above studies were performed by including pectins and the pathogens to the epithelial cells at the same time. In another study by Thöle et al., it was found that the pre-incubation of H. pylori with pectin-like okra extracts decreased their ability to adhere to human gastric cells. Among the extract fractions by ammonium sulfate precipitation with 30%, 60%, and 90% saturation levels, the 60% and 90% saturation fractions significantly decreased H. pylori adhesion. The pectin fractions were found to exert their decoy effects through binding to the bacteria cell membrane proteins BabA, SabA, and HpA.

5.4.3 Effects of pectin on gut epithelial cell responses. Pectin has been demonstrated to induce morphological changes of the small intestine in vivo, and the mechanism has been extensively explored in vitro. In a study conducted by Nishida et al., pectin from Prunus domestica L. was incubated with the human intestinal epithelial Caco-2 cell line for 24 h. Although the total amount of the glycolcalyx component HS was not affected, the disaccharide compositions of HS were markedly altered through the binding of pectin with fibronectin and α5β1 integrin at the Caco-2 cells. The structural change of HS was further found to promote the secretion of Wnt3a, a protein that strongly binds to HS in Caco-2 cells, which can stimulate the proliferation of the rat IEC6 cell, a model of intestinal crypt cells. In a more recent study, Kong et al. evaluated pectins from lemon with different DMs and their ability to influence the glycolcalyx compositions of Caco-2 cells. Consistently, neither the amount of HS nor HA was changed, but pectin significantly increased the absorbed albumin of Caco-2 cells. The direct regulatory effect was also observed in an in vitro study with the HT-29 cell line. Both high DM and low DM pectins stimulated the mucin secretion of HT-29.

5.4.4 Effects of pectin on gut mucosal immune responses. Pectin supplementation to infant formulas stimulates a Th1-type and regulatory T-cell immune response and attenuates the Th2-type immune response. Vos et al. demonstrated that this effect is achieved through selectively promoting Lactobacilli growth in a murine vaccination model. Pectin also exerts immune modulating effects that are independent of the gut microbiota. For example, Sahasrabudhe et al. found that pectins with different DMs (DM7, DM22, DM45, DM60, and DM75) could directly bind and block TLR2 and thereby inhibit the pro-inflammatory TLR2–TLR1 pathway. In this study, the low DM pectins showed the most pronounced inhibitory effect. The effect of low DM pectins (DM7) on the mucosal barrier function was also tested in an in vivo mouse model. It was shown that low DM pectins upregulated the gene expression of tight junction proteins (ZO-1 and occludin), the anti-microbial peptide β defensin-1, and increased SCFA production. These results are in line with the observation that low DM pectins are better digested than high DM pectins.
6. In vitro systems to evaluate the effects of dietary fibers

The development of more effective hMOs and NDC mixtures for the supplementation of infant formulas to mimic beneficial hMO functions has been hampered due to the lack of efficient in vitro test models. In vitro models are needed because testing in infants is ethically not approved and animal studies are very labor-intensive. In addition, the results from currently applied in vitro models are not always easy to translate to humans in vivo.\textsuperscript{174,175} Even for the animal studies, many factors can cause the ineffective translation to human trails, i.e. specific animal models are needed for humans of different age, sex, health status, and dose translation. Especially in some animal models, translation of the used dosage to clinical trials is not tolerated or even toxic in humans.\textsuperscript{176} Considering these issues, it is of essential importance to develop new in vitro systems that resemble the human gut conditions more closely. A few potential new in vitro systems will be discussed.

Research on the development of intestinal organoids from stem cells, progenitor cells, or tissue explants has increased rapidly over the past few years.\textsuperscript{72,99,177} These organoids might be of interest for studying new NDCs and NDC mixtures as they closely resemble the in vivo GIT. Intestinal organoids are epithelial 3D spheroids that are cultured on extracellular matrixes and represent either intestinal villi or crypts containing a mixture of complex intestinal epithelial cell populations mimicking the intestinal physiology and pathology (Fig. 4a).\textsuperscript{178,179} In the case of NDC research, these organoids might be used to study NDC effects on host-microbiota interactions, medicine and antibiotic interferences, effects on tissue repair, and regeneration of the intestine.\textsuperscript{180–182} The culturing techniques still need to be extensively optimized to improve organoid viability and quality control, as currently there are high batch-to-batch variations.\textsuperscript{183} Techniques that can be employed to measure NDC effects include immunohistochemistry, which is commonly applied to visualize and localize structures such as the villi, crypts, and certain proteins. In addition, the culturing medium can be collected to quantify the cytokine secretion, and the gene expression of different cell types in the organoids can also be measured using PCR techniques.\textsuperscript{180,184} However, due to the heterogeneity in size and shape, and generally the small size of the 3D organoids, analysis of the barrier integrity is very complicated.\textsuperscript{185} The closed configuration of the 3D organoid also makes it technically difficult to reach the apical lumen side.\textsuperscript{186} With the application of the transwell inserts, epithelial cells, for example grown as 3D organoids and later dissociated into the monolayers, make it possible to do barrier integrity analysis.\textsuperscript{187}

Another approach is the use of gut epithelial cells grown in transwells (Fig. 4b). The transwell system is built up around a porous transwell on which epithelial cells are grown. Insertion of these transwells into normal cell culture plates results in the creation of two separate culturing chambers, apically and on the basolateral side of the epithelial cell layer. By culturing immune cells in the basolateral compartment of the transwell system, it can be used to mimic the communication between intestinal epithelial cells and the immune cells in the lamina propria.\textsuperscript{130} The dietary fiber interventions in these interactions can also be investigated using this in vitro transwell co-culture system.\textsuperscript{188,189} Experiments using the transwell culture system are most often conducted under static conditions. However, food intake, gut microbiota, and the intestinal epithelium are continuously exposed to shear force generated by the intestinal peristalsis.\textsuperscript{190} Shear force has been shown to affect gut microbiota colonization,\textsuperscript{191,192} gut microbiota interaction with the host,\textsuperscript{193} and intestinal epithelium characteristics including the gut mucus layer and tight junctions.\textsuperscript{194,195} A wide range of studies have shown the effect of shear forces on the intestinal

![Fig. 4 Schematic overview of intestinal in vitro systems. (a) Intestinal organoids cultured in the Petri dish; (b) transwell system that is separated by a porous membrane on which the gut epithelial cells can grow, with the upper chamber to mimic the gut lumen where the gut microbiota and the dietary fibers can be cultured and the lower chamber to mimic the lamina propria where the endothelial cells and immune cells are present; (c1) the gut-on-a-chip system, which is established by introducing the shear force to the static transwell system on a polydimethylsiloxane chip with two separate chambers. The side vacuum chambers exert the cyclic stretching force across the chip to generate the intestinal peristalsis; (c2) vertical view of (c1) with the direction of flow; (d1) the gut-on-a-chip system with a gradual decreasing shear force by a gradual increasing cell culture area; (d2) vertical view of (d1) with the direction of flow; (e1) the ibidi system with a constant and continuous shear force; (e2) vertical view of (e1) with the direction of flow.](image-url)
epithelial cell behavior and the bacterial colonization, showing the need to mimic shear force in in vitro models to more closely mimic the in vivo situation.\textsuperscript{196,197}

Recently, a more advanced transwell system named micro-fluidic device was constructed to develop a gut-on-a-chip system involving the key elements of the in vivo environment, in which the gut lumen shear force effects were introduced to the normal transwell system, allowing the interactions of the gut microbiota in the gut lumen, the intestinal epithelial cells, and the immune cells or endothelial cells in the laminar propria to be investigated in vitro.\textsuperscript{190} It is established based on the polydimethylsiloxane (PDMS) chip with two separate chambers, resulting in the upper gut lumen flow chamber and the lower capillary flow chamber.\textsuperscript{190} The epithelial cells are seeded on the median porous membrane, and the immune cells or endothelial cells can be introduced in the lower chamber. The vacuum chambers on the two sides of the main transwell chambers make a physiological cyclic stretching force across the membrane to generate intestinal peristalsis (Fig. 4c1 and c2).\textsuperscript{198} Peristaltic shear force was recently proposed to stimulate the differentiation of the human-induced pluripotent stem cells to induce the polarized 3D villi structure forming the in vivo characteristic of intestinal epithelium with different cell types, i.e. absorptive enterocytes, goblet cells, and Paneth cells. This gut-on-a-chip system gives research opportunities to involve as many genetic risk factors as associated with the Celiac disease by establishing a Celiac disease-on-a-chip system (Fig. 4c1 and c2).\textsuperscript{198} As the shear forces vary in different parts of the intestine, another gut-on-a-chip model was made with a gradually increasing culturing area, which resulted in a gradually decreasing shear force ranging between 0.002 and 0.03 dyne cm\(^{-2}\) from one side of the device to the other (Fig. 4d1 and d2). With an increase of the shear force in this model, the organization of the cytoskeleton and the formation of microvilli were found to increase, and the mucus production and the tight junction of the epithelial cells also increased but not at the highest shear force, i.e. 0.026–0.03 dyne cm\(^{-2}\).

However, to date there is only one experimental set-up available that investigated the effects of dietary fibers on the intestinal epithelial cells and one gut commensal bacteria in the presence of peristaltic shear force. This study used a so-called ibidi system in which a constant and continuous shear force can be exerted on the gut epithelium (Fig. 4e1 and e2). In this study, the effects of three individual hMOs, i.e. 2'-FL, 3-FL, and LNT2, were investigated, combined with intestinal peristaltic shear force on the crossstalk of the intestinal epithelial Caco-2 cells and L. plantarum WCFS1. It was found that 3-FL and LNT2 supported the crossstalk between L. plantarum WCFS1 and Caco-2 cells, and shear force showed increasing effects on the modulating effects of hMOs.\textsuperscript{199} Including shear force as a factor in the current in vitro models would increase their translation to in vivo GIT and would make translation of the results generated using these models to the in vivo situation easier.

### 7. Concluding remarks

As outlined in this review, the beneficial functions of hMOs for the development of the gut microbiota and the gut immune system in early life have been extensively studied in both in vitro and in vivo studies.\textsuperscript{85,86} Despite these many identified health benefits, most of the hMOs are currently not applied in infant formulas, except for 2'-FL and LNnT,\textsuperscript{102} because the synthesis of the specific oligosaccharide structures is still challenging both technically and financially. Currently, it is difficult to generate more complex hMOs for basic research studies and clinical trials. On the other hand, research focuses on the functions of the individual hMO and NDC structures or only on the mixtures of well-defined composition is still scarce. Also, the number of studies exploring new NDC mixtures is still very limited and very focused on GOS-FOS.\textsuperscript{2,28}

In recent years, emerging research has demonstrated that the supplementation of NDCs to infant formulas can partially mimic hMO functions.\textsuperscript{123,129} Nowadays, especially the direct immune regulatory effects of NDCs are more recognized in addition to their conventional prebiotic effects.\textsuperscript{129,200} However, not all NDCs have the same regulatory effects on the host immune responses. Immune active NDCs often either exert Th1 or Th2 immune regulatory properties, depending on their molecular structures.\textsuperscript{110,201} Specific structures and specific Th1 or Th2 regulating NDCs might be needed as preterm infants are experiencing high proinflammatory Th1-prone immune response, and allergy-prone infants develop too strong anti-inflammatory Th2 immune responses.\textsuperscript{202} Tailoring specific infant formulas for these groups might benefit the development and long-term health in these babies.

Another important factor that might change the type and composition of NDCs applied in infant formulas is the novel insights into the needs of the gut; in early life some hMOs and also some NDCs seem to be poorly digested by the microbiota of newborn infants,\textsuperscript{53,203} which suggests that in the early phases of life other more simple NDCs and hMOs might be needed to support a healthy development of the gut microbiota.\textsuperscript{43} This is less important at increasing age as gut microbiota tend to develop and express more enzymes that facilitate the fermentation of hMOs and NDCs.\textsuperscript{7} The importance of the use of tailored NDCs for infants of different age classes was illustrated by a study of Logtenberg et al., in which microbiota of 8-week-old infants were shown to have an increased fermentation capacity of fructan-type NDCs compared to infants of 2 weeks old.\textsuperscript{51} Thus, understanding how and which hMO and NDC molecules can support microbiota development, modulate infant immune responses and gut barrier function, as well as investigating how hMO and NDCs can be utilized by the gut microbiota of infants of specific ages and health status is necessary for creating effective health supporting infant formula.
In addition, the most optimal dosage of hMO analogs and NDCs in infant formulas needs to be considered carefully. For example, it still remains to be determined whether 2′-FL in the applied dosage can achieve the same benefits as GOS and FOS which is used in higher concentrations. It is known that the amount of hMOs in mother milk decreases between 2 and 12 weeks of lactation, whereas the amount of NDCs added to infant formulas usually remains stable. Insight into specific molecular hMO structures that increase and decrease over time might provide insight into the needs of infants of specific ages as well. A recent study by Borewicz et al. showed that even though the total amount of hMOs was decreasing during the first weeks of lactation, the amount of specific hMOs, i.e. 3-FL and LNFP III, increased in this period. This might indicate a specific role of these molecules in infants of specific ages as well as the importance of proper dosage of NDCs for infants. Dosage of NDCs in infant formulas is of high importance and might therefore not always be directly translatable to the in vivo situation. Appropriate clinical trials to determine the dosages for specific groups of infants with different health statuses are therefore indispensable.

Our expectation is that formulas will be created for specific infant target groups. By reviewing current insight into the health benefits of hMOs and NDCs we hope to contribute to the design of “tailored infant formula” for infants of different age groups and different health statuses, to ensure a healthy development of the gut microbiota and immune system of infants.

Conflicts of interest

The authors declare no conflicts of interest.

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