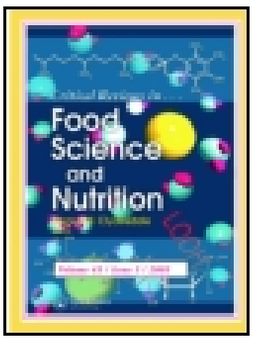

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Non-digestible carbohydrates in infant formula as substitution for human milk oligosaccharide functions: Effects on microbiota and gut maturation

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ABSTRACT

Human milk (HM) is the golden standard for nutrition of newborn infants. Human milk oligosaccharides (HMOs) are abundantly present in HM and exert multiple beneficial functions, such as support of colonization of the gut microbiota, reduction of pathogenic infections and support of immune development. HMO-composition is during lactation continuously adapted by the mother to accommodate the needs of the neonate. Unfortunately, for many valid reasons not all neonates can be fed with HM and are either totally or partly fed with cow-milk derived infant formulas, which do not contain HMOs. These cow-milk formulas are supplemented with non-digestible carbohydrates (NDCs) that have functional effects similar to that of some HMOs, since production of synthetic HMOs is challenging and still very expensive. However, NDCs cannot substitute all HMO functions. More efficacious NDCs may be developed and customized for specific groups of neonates such as pre-matures and allergy prone infants. Here current knowledge of HMO functions in the neonate in view of possible replacement of HMOs by NDCs in infant formulas is reviewed. Furthermore, methods to expedite identification of suitable NDCs and structure/function relationships are reviewed as *in vivo* studies in babies are impossible.

KEYWORDS

Human milk oligosaccharide; infant formula; non-digestible carbohydrates

1. Introduction

1.1. Human milk

Breast milk is the golden standard for nutrition of newborn infants (Walker 2010). It is evolutionary optimized and provides newborns with all essential nutrients needed in the first months of infancy (Breastfeeding and the Use of Human Milk 2012; Oftedal 2012). The composition of human milk (HM) is highly variable in time and changes in response to many factors to meet the infant's requirements according to its age (Ballard and Morrow 2013). HM is therefore considered to be a dynamic complex bio fluid and contains a wide range of varying amounts of proteins, lipids and carbohydrates. It is widely believed that the composition of HM is specifically tailored by individual mothers to meet the requirements of the neonate (Walker 2010). Especially human milk oligosaccharides (HMOs) have gained considerable attention in that respect. HMOs are known to exert prebiotic effects and thereby play an essential role in guidance and the development of a healthy microbiome (Smilowitz et al. 2014).

1.2. The effect of human milk oligosaccharides on microbiota development

Although highly regulated, colonization of the neonatal gut by the microbiota is a very vulnerable period (Rautava et al. 2012). Usually the infant gut is first colonized by bacteria derived from the mother. The first microorganisms invading the infant include aerobic or facultative anaerobic bacteria, which create a new environment that promotes the colonization of strict anaerobes such as *Bifidobacteria, Clostridia* and *Bacteriodes* (Adlerberth and Wold 2009; Morelli 2008). This colonization is influenced by many factors such as the mode of delivery, hygienic circumstances and feeding practice (Figure 1) (Toole and Claesson 2010). Breastfeeding is involved in maturation of microbiota by providing stimulating components that serve as feed for specific bacterial species to ensure an enrichment of key members of the gut microbiota. Specific HMOs have been shown to stimulate the growth of *Bifidobacteria* (Karav et al. 2016). As reduced numbers of *Bifidobacteria* species are associated with the development of atopic diseases in later life, HMOs are relevant components of HM (Penders et al., 2006).

1.3. Effects of HMOs on gut health and immune system development

As indicated above, healthy colonization of the gut is of great importance for general health of the infant, but it is especially important for the development of an effective gut barrier function and the establishment normal immune responses (Kleessen et al. 2001; Rautava et al. 2012) and HMOs play a big

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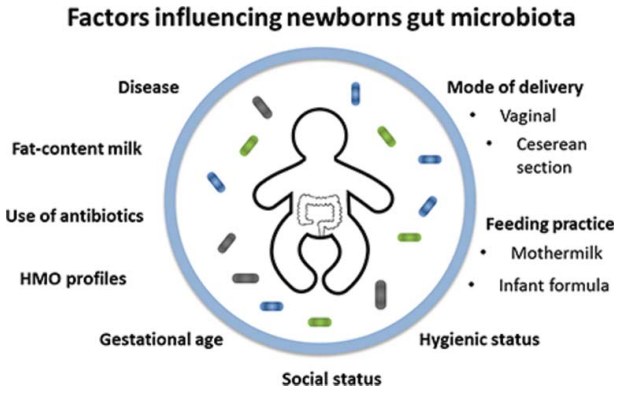

Figure 1. Factors influencing the composition and colonization of newborns gut microbiota.

role in this. The gut barrier consists of the resident microbiota, a mucus layer, epithelium and resident or recruited immune cells, to provide defense against pathogens and their toxins secreted in the lumen. However, immune responses in newborns are immature. This immaturity is characterized by a decreased ability of fetal monocytes and granulocytes to respond to lipopolysaccharide, while the immature fetal adaptive immune response is characterized by a dominant Th2−type response and a lack of a Th1 type immune response and a relative lack of immunological memory (Simon et al. 2015). HMOs play a role in the maturation of the infant immune system: in addition to the effects on the maturation through microbiota, as described above, HMOs also exert direct immune modulating effects (Kulinich and Liu 2016).

1.4. NDCs to replace HMOs in infant formula: Effects on microbiota and the immune system

HMOs are unique for HM and not found in the same composition and diversity in animal milk (Kunz et al. 2000). This imposes an issue for infants for whom consumption of mother milk is not an option, and cow milk derived infant formulas have to be fed. During recent years, much effort has been directed to find carbohydrates that might substitute some of the HMO functions. HMO supplementation of cow milk infant formulas is still too expensive for broad application. Nowadays cow-milk derived infant formulas are therefore often supplemented with affordable non-digestible carbohydrates (NDCs) such as galacto-oligosaccharides (GOS) and fructo-oligosaccharides (FOS) to substitute for some of the HMO functions (Vandenplas et al. 2015). Although beneficial effects of these carbohydrate supplementations have been reported on prevention of allergy and atopic dermatitis (Hoffen et al. 2009; Rijnierse et al. 2011), it remains largely unclear by which mechanisms these effects are accomplished. Furthermore, there are more potentially beneficial NDCs available which might be suitable for the supplementation of infant formulas.

This review is focusing on current knowledge of HMO-functions and its structures. Different beneficial effects of HMOs are discussed. Identification and understanding HMO-functions is important for proposing NDCs that might serve as substitute. The functional effects of these HMOs should be partly or completely substituted by NDC in infant formula. Currently applied NDCs are discussed, but as better NDCs might become available for babies with health issues, also test systems representative for effects in babies are presented and discussed.

2. Human milk oligosaccharides

2.1. HMO structures and compositions

HMOs constitute a heterogeneous mixture of glycans that vary per individual (Chaturvedi et al. 2001). The amount of HMOs in HM are dependent on the stage of lactation and varies from around 20.9 g/L in colostrum to 12.9 g/L in mature milk (Coppa et al. 1993). HMOs contain 3 to 22 saccharide units per molecule and are made up of 5 building blocks (Figure 2a): glucose (Glc3), galactose (Gal), N-acetylglucosamine (GlcNAc), fucose (Fuc) and sialic acid (Neu5Ac) monosaccharides (Bode 2015). Although the combinatorial potential is immense, only about 200 different compositions of HMOs have been characterized (Wu et al. 2012). These are classified in 13 groups according to their core structures. All HMOs consist of a lactose core (Galβ1–4Glc) (Figure 2b), which forms the reducing end (Kunz et al. 2000). The core HMO structures can be further elongated enzymatically by β1–3 or β1–6 linkage to either Galβ1–3GlcNAc (type-1 chain) or to Galβ1–4GlcNAc (type-2 chain) (Figure 2c) (Bode 2015). The core structures can be decorated with fucose (Fuc) and sialic acids residues. Fuc can be attached by α1–2, α1–3, or α1–4 linkages and/or sialic acids are connected with α2–3 or α2–6 linkages at the terminal positions (Figure 2d) (Bode 2015).

All HMOs are synthesized in the mammary gland. The qualitative and quantitative composition of HMOs depends on the expression of specific transferases enzymes in lactocytes (Bode 2015). Secretor (Se) and Lewis (Le) blood group genes are two important gene groups for HMO profiles (Johnson and Watkins 1992; Kumazaki and Yoshida 1984; Stahl et al. 2001). The Se gene encodes for the α1–2-fucosyltransferase enzyme (FUT2) and is expressed in so-called 'secretors' (Kumazaki and

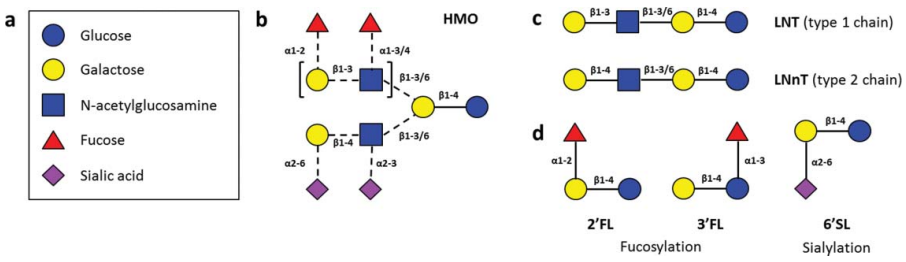

Figure 2. HMO structures. (A) HMO building blocks, (B) Possible linkages of HMO building blocks, (C) type 1 and type 2 chains and (D) structures of 2′FL, 3′FL and 6′SL HMOs.

Yoshida 1984). FUT2 is responsible for α1–2 linkage of Fuc to the terminal Gal of the type 1 chain of HMOs. Therefore, secretor mothers can produce α1–2-fucosylated components such as 2′-fucosyllactose (Fuca1–2Galß1–4Glc) and lacto-N-fucopentaose (Fuca1–2Galß1–3GlcNAcß1–3Galß1–4Glc). Activation of the Le-gene results in expression of a1–3/4-fucosyltransferase (FUT3), which is responsible for α1–4 linkage of Fuc to a subterminal GlcNAc of the type 1 chain of HMOs (Johnson and Watkins 1992). Fuc attachment to a subterminal GlcNAc of the type 1 chain by an α1–4 linkage through FUT3 results in the presence of Le a-sugars in nonsecretor milk (Le a+b−) and Le b-sugars in secretor's milk (Le a-b+) (Stahl et al. 2001). In Le-negative women (Le a-b-) this leads to an absence of these sugars, in both secretors and nonsecretors. As a result there are four different groups of mothers: Se+Le+, Se-Le+, Se+Le- and Se-Le-. There might be other factors influencing HMO biosynthesis as well, as a recent study by McGuire et al. (McGuire et al. 2017) showed that HMO concentrations and profiles vary geographically, even when secretor (Se) and Lewis (Le) blood group genes were taken into account.

2.2. Currently known HMO functions

Many different HMOs functions have been identified, including functions such as selectively enriching gut bacteria and thereby influencing microbiota composition (Garrido et al. 2011), preventing pathogen adhesion to epithelial cells (Jantscher-Krenn et al. 2012; Ruiz-Palacois et al. 2003), enhancing intestinal epithelial barrier function (Holscher et al. 2014) and preventing infection and supporting immunity (Naarding et al. 2005). These effects are all potentially beneficial for the neonate (Figure 3). In the next sections the functions are briefly reviewed.

2.2.1. Effects of HMOs on microbiota composition

Because of a lack of glycoside hydrolases and intestinal membrane transporters, HMOs are not digested in the upper part of the gastrointestinal tract of infants (Engfer et al. 2000; German et al. 2008). As a consequence the majority of HMOs reach the colon, where they serve as a substrate for specific microbes,

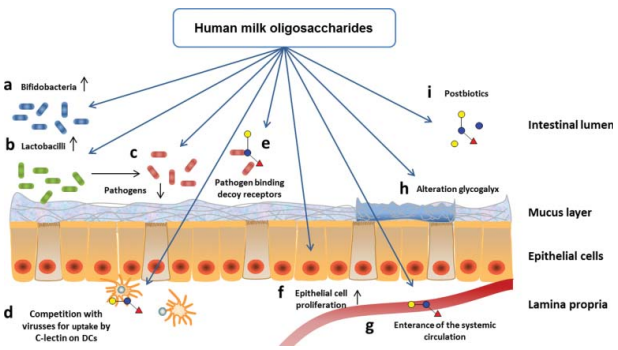

Figure 3. Beneficial effects of human milk oligosaccharides (HMOs). HMOs can specifically stimulate growth of (A) Bifidobacteria and (B) Lactobacilli, competing with and resulting in (C) a lower number of pathogens. Furthermore, HMOs can (D) compete with viruses for uptake by C-lectin receptors on dendritic cells and (E) act as pathogen binding decoy receptors to prevent binding of pathogens to glycan structures on epithelial cells. HMOs can also (F) influence epithelial cell proliferation, (G) enter the systemic circulation, (H) alter the glycogalyx and fermentation products of HMOs and (I) post-biotics for other microbiota species.

influencing both the composition and activity of the gastrointestinal microbiota (Garrido et al. 2011). HMOs are specifically known to influence populations of beneficial microorganisms, such as *Bifidobacterium* (Garrido et al. 2011), a dominant species in the intestine of breast-fed infants. These bacteria have the ability to utilize HMOs, depending on their equipment with dedicated glycoside hydrolases, transporters and other molecules contributing to degradation (Goh and Klaenhammer 2015). As certain bacteria such as *Bifidobacteria* and *Lactobacilli* specifically express sialidases to cleave Sia and fucosidases to cleave Fuc, it is believed that these species co-evolved with HMOs (Garrido et al. 2016; LoCascio et al. 2007; Sela et al. 2012; Ward et al. 2006).

Although there are multiple *Bifidobacteria* species that can utilize HMOs, there are also *Bifidobacterica* species that utilize HMOs to a lesser extent (Sela et al. 2012). This ability to utilize HMOs depends on the enzymes the *Bifidobacteria* are equipped with. *Bifidobacterium longum* supsp. *Infantis* (*B. infantis*), a key infant gut microbe, possesses a large cassette of genes associated with HMO consumption, enabling this subspecies to grow to a high density on a broad array of HMOs as sole carbon source (Marcobal et al. 2010). Other subspecies may only be able to consume structures of limited diversity (Sela et al. 2012). It is believed that subspecies *B. infantis* ATCC 15697 imports intact HMOs and subsequently deconstructs them with intracellular glycoside hydrolases, such as α-fucosidases, α-sialidases, β-galactosidases and *N*-acetyl- β-D-hexosaminidases (Sela et al. 2012). Other species of *Bifidobacterium*, such as *B. bifidum*, degrate HMOs using extracellular glycosidases and transport mono-, di-, or oligosaccharides rather than whole HMO complexes (Garrido et al. 2011; Marcobal et al. 2010; Yoshida et al. 2012). *Bifidobacteria* can selectively grow and thrive in the infant gut, since they have the advantage of being able to utilize HMOs over other bacteria that cannot utilize HMOs. The species that cannot utilize HMOs have a disadvantage and do not readily colonize. However, HMOs do not only serve as substrate for bacteria but they can also enhance *Bifidobacteria* persistence in the gastrointestinal tract by increasing their ability to bind to epithelial cells (Chichlowski et al. 2012; Kavanaugh et al. 2013). In the study of Wickramasinghe and collegues (Wickramasinghe et al. 2015) it was found that both strain and carbon source affect binding affinity of *Bifidobacteria* to epithelial cells. Furthermore they found that HMO-fed *Bifidobacteria* have more anti-inflammatory effects in human gut intestinal Caco-2 cells, when compared to *Bifidobacteria* grown on other carbon sources, such as glucose and lactose.

HMO metabolites, or so-called 'post-biotics', derived from fermentation of HMOs by *Bifidobacteria* can have beneficial effects as well, as they stimulate growth of other members of the gut microbiota. In a study by Asakuma et al. (Asakuma et al. 2011) different *Bifidobacteria* species were grown on media containing a main neutral oligosaccharide fraction. They found that *B. bifidum* used secretory glycosidases to degrade HMOs, but degraded HMOs were left outside the cell, indicating that different species can share produced sugars. Fermentation of those sugars by other species can result in the production of the short chain fatty acids (SCFAs) such as butyrate and propionate. Both butyric acid and propionate are important for gut health as they can interact with host epithelium to stimulate mucin release,

increase mucosal blood flow and modulate the immune system (Plöger et al. 2012; Reichardt et al. 2014). Schwab and colleagues (Schwab et al. 2017) found a trophic interaction between *Bifidobacteria* and *Eubacterium hallii* (*E. hallii*), a common member of the adult gut microbiota, during L-fucose degradation. In a single culture *E. hallii* was not able to grow on L-fucose. In co-cultures *B. Longum* subsp. *Infantis* was able to utilize L-fucose and produced 1,2-propanediol (1,2-PD), which was used by *E. hallii* to produce propionate.

2.2.2. HMOs and prevention of pathogen adhesion

HMOs can prevent adherence of bacterial and protozoan-parasitic pathogens to gut epithelial cells by acting as soluble decoy receptors (Jantscher-Krenn et al. 2012; Ruiz-Palacios et al. 2003). They block a crucial step in the binding of pathogens to cell surface sugars (glycans), also known as the glycocalyx, present on gut epithelial cells (Springer and Gagneux 2013). These glycans are conjugated to lipids or proteins (Huang et al. 2016). HMOs resemble some of the glycan structures of glycocalyx sugars, allowing competition with pathogen receptors. Pathogens that recognize and bind to HMOs will pass the intestinal tract without binding to epithelial cells preventing infection of the neonate (Bode 2015). An example is the pathogenic microorganism *Campylobacter jejuni* (*C. jejuni*). Infections with *C. jejuni* can have significant impact on infant health as it can cause diarrhea, the most common cause of infant mortality (Liu et al. 2012). 2′-fucosyllactose (2′FL) HMOs serve as a soluble decoy receptors for *C. jejuni*, reducing binding and colonization of the pathogen (Ruiz-Palacios et al. 2003). This results in a lower incidence of diarrheal episodes in breast-fed children. Jantscher-Krenn *et al.* (Jantscher-Krenn et al. 2012) found that Lacto-N-tetraose HMOs also prevent the attachment of the protozoan parasite *Entamoeba histolytica*, confirming the anti-adhesives properties of HMOs.

2.2.3. Effects of HMOs on epithelial cell responses

HMOs do not only impact microbes or can serve as decoy receptor for pathogens, but can also directly influence host cell responses against pathogens and modify e.g. the glycogalyx (Angeloni et al. 2005). A study of Kuntz *et al.* (Kuntz et al. 2008) showed that both neutral and acidic HMO fractions modulate intestinal epithelial cell apoptosis, proliferation and differentiation and Angeloni et al. (Angeloni et al. 2005) found that HMOs can alter epithelial cell gene expression, leading to a different expression of cell surface glycocalyx. A different expression of glycocalyx molecules changes the ability of certain pathogens to bind to the cell surfaces (Bode 2006). The HMO 3′SL specifically reduces the expression of sialyltransferase ST3Ga11, ST3Ga12 and ST3Ga14, leading to less binding of sialic acid groups to glycans (Angeloni et al. 2005). As a result, species that specifically bind to glycans containing sialic acid groups, such as *E. Coli*, will have lower ability to bind to epithelial cells (Bode 2006). Recently, Holscher *et al.* (Holscher et al. 2014) found that HMOs play a role in maturation of epithelial cells as well. In this study, HT-29 and Caco-2Bbe cell lines were used to build a model of the crypt-villus axis and the effects of lacto-N-neotetraose (LNnT), 2′FL, and 6′- sialyllactose (6′SL) on differentiation of epithelial cells were tested. All three HMOs tested reduced cell proliferation, suggesting a specific role for HMOs in the maturation of the gastrointestinal tract (Holscher et al. 2014).

2.2.4. Effects of HMOs on prevention of infection and support of immunity

HMOs can also prevent infection of viruses by competing for binding to receptors. Glycans are recognized by the carbohydrate receptors C-type lectins on DCs, specific tissue macrophages and other myeloid antigen presenting cells (Cummings and McEver 2009). C-type lectins belong to the family of antigen uptake receptors as they recognize glycans on the cell surface of many viruses, bacteria and parasites (Cummings and McEver 2009). Many viruses are known to use this receptor to infect the host (Huang et al. 2016). Upon binding viruses can hide in DCs for several days in order to escape from the immune system (Hong et al. 2009). HMOs can prevent infection via this pathway. A well-characterized pathogen pattern recognition-receptor (PPR) C-type lectin involved in infection is dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN). This receptor recognizes mannose-containing glycol-conjugates, but has an even higher affinity for fycosylated structures such as Lewis antigens. HM contains monomeric Lewis epitopes that bind to DC-SIGN and thereby compete with pathogen binding, resulting in lower infection rates of for example HIV-1 (Naarding et al. 2005).

DC-SIGN binding by HMOs might also be an important mechanism by which HMOs contribute to immune maturation in infants as this receptor induces adaptive immune responses (Engering et al. 2002). The DC-SIGN receptor internalizes antigen after activation and presents it to other immune cells, thereby initiating various immune responses. The outcome of the response depends on the interplay between DC-SIGN and Toll like receptor (TLR) signalling and the type of glycan it interacts with (Van Vliet et al. 2008). DC-SIGN is considered to play an important role in the induction of immune tolerance and maintenance of homeostasis by modification of T cell responses (Garcia-Vallenjo and van Kooyk 2009).

HMO effects are not only seen locally in the gut, but can also be systemic as HMOs can pass the gut-barrier (Rudloff et al. 2012). Presence of HMOs in urine of infants indicates that HMOs are absorbed into the bloodstream (Rudloff et al. 2012). Blood absorbed HMOs have been shown to reduce invasion of uropathogenic *Escherichia coli* (*E. coli*) by interacting with ureter epithelial cells, making them more resistant against *E. coli* invasion by a suppression of intracellular signaling of apoptotic pathways (Lin et al. 2014). As a result epithelial cells cannot respond to *E.coli* bacteria. These effects were only observed with the HMO 3′-sialyllactose (3′SL) (Lin et al. 2014). 2′FL HMOs can directly influence inflammatory reactions as well (He et al. 2014). Recently, He *et al.* (He et al. 2014) found that HMOs and 2′FL HMOs can also directly inhibit LPS-mediated inflammation during invasion of enterotoxigenic *E. coli* in T84 and H4 intestinal epithelial cells.

3. Use of non-digestible carbohydrates in infant formula

For a variety of reasons not all infants can be fed with HM. Infants that do not receive HM are routinely fed with cow-milk

derived infant formulas, which do not contain HMOs. Since the production of synthetic HMO-like structures is challenging and still very expensive due to the complexity of HMOs, many cow-milk derived infant formulas are nowadays supplemented with non-human oligosaccharides. In the next section, we will discuss similarities in functional effects between HMOs and NDCs. As galacto-oligosaccharides (GOS), fructo-oligosaccharides (FOS) and pectin-oligosaccharides (POS) are the most commonly applied, the focus will be on these molecules.

3.1. Galacto-oligosaccharides

Galacto oligosaccharides (GOS) are comprised of galactose units with one glucose unit at the reducing end. The length of the chain typically ranges from 2 to 10 units with variations in branching and glycosyl linkage (Ackerman et al. 2017). Glycosyl linkages include β1,3, β1,4 and/or β1,6. The type of linkage is determined by the enzyme source used to link the different units (Ackerman et al. 2017; Intanon et al. 2014). Like HMOs, GOS can reach the large intestine, where they can act as prebiotics (Macfarlane et al. 2008). For this reason, multiple studies focused on the effect of GOS in infant formula on microbiota composition, as well as on the direct effects GOS can have on epithelium or immune cells in the large intestine.

3.1.1. Effects of GOS on microbiota composition

GOS has many beneficial effects on microbiota. In a study of Ben et al. (Ben et al. 2004) infant formula substituted with 2.4 g/l GOS was fed to term infants. Human milk and infant formula without GOS were used as a positive and negative controls respectively. After 3 and 6 months, intestinal *Bifidobacteria* and *Lactobacilli* numbers were significantly increased in infants fed with GOS supplemented infant formula compared to infants fed with infant formula without GOS. There were no significant differences between mother milk and GOS supplemented infant-formula. Another study by Fanaro et al. (Fanaro et al. 2009) also showed that addition of 5 g/L GOS to infant formula increased numbers of *Bifidobacteria*. In this study 4–6 month old infants were fed with formula with or without GOS and numbers of *Bifidobacteria* were studied at 6 and 12 weeks after the start of the study (Fanaro et al. 2009). Watson *et al.* (Watson et al. 2013) also showed *in vitro* that GOS and lactulose supported favorable growth of *Bifidobacteria* and *Lactobacilli* strains when cultured as single strains under anaerobic conditions. More beneficial effects of GOS on microbiota composition have been extensively reviewed by Macfarlane *et al.* (Macfarlane et al. 2008).

3.1.2. GOS and prevention of pathogen adhesion

Like HMOs, GOS can also act as soluble decoy receptors to prevent the adhesion of pathogens to gastrointestinal epithelial cells. A study by Shoaf et al. (2006) investigated the effect of GOS on the adherence of enteropathogenic Escherichia coli (EPEC) in an *in vitro* study using HEp-2 and Caco-2 cell lines (Shoaf et al. 2006). Purified GOS could reduce adherence of EPEC to HEp-2 and Caco-2 cell by 65 and 70% respectively. Adherence inhibition of pathogens was dose dependent and reached a maximum at 16 mg/ml (Shoaf et al. 2006).

3.1.3. Effects of GOS on epithelial cells responses

GOS also has effects on intestinal epithelial cells and has been reported to stimulate gut barrier function (Bhatia et al. 2015). An *in vitro* study by Bhatia et al. (Bhatia et al. 2015) showed that GOS enhanced intestinal barrier function through modulation of the secretory function of human goblet cells by up regulation of related genes and protein expression (Bhatia et al. 2015). GOS can also stimulate *in vitro* tight junction assembly in gut intestinal human Caco-2 cells, which is beneficial for the integrity of the epithelial barrier (Akbari et al. 2015). Furthermore, GOS can prevent deoxynivalenol-induced histomorphologic alterations in the villi of the mouse intestine, indicating that it has protective effects on villi as well, which is important for undisturbed adsorption of nutrients (Akbari et al. 2015). In another study by Akbari et al. (2017) it was found that GOS could beneficially influence barrier integrity of Caco-2 cells *in vitro*. Incubation with GOS had a protective effect on deoxynivalenol-induced impairment of the monolayer integrity by inducing acceleration of the tight junction reassembly. Also, GOS had reducing effects on release of the inflammatory marker CXCL8 by the cells (Akbari et al. 2017).

3.1.4. Effects of GOS on prevention of infection and support of immunity

Alizadeh and coworkers (2016) studied the effect of GOS supplementation on the intestinal immune system using a neonatal piglet model. Piglets were used as the maturation of the intestines in piglets are considered to closely resemble that of human neonates and infants (Alizadeh et al. 2016). In this study, piglets were fed with milk formulas with or without GOS for 3 or 26 days. It was found that GOS substitution supported intestinal development. Also, mRNA expression levels of porcine β-defensin-2, and sIgA levels in saliva were increased in animals fed with GOS, suggesting an improvement of mucosal immune responses (Alizadeh et al. 2016).

3.2. Fructo-oligosaccharides

Fructans comprise a heterogeneous group of polymers composed of a linear chain of fructose units terminated by a glucose residue with variation in glycosidic linkage and degree of polymerization. Chain lengths can vary from 2 to up to 200 residues. In this section we will discuss effects of short chain FOS (scFOS), which have between 3 and 5 residues per chain and oligofructose (or long chain FOS (lcFOS)) that have 6 to 10 residues. scFOS are naturally present and can be extracted from sources like chicory. Both scFOS and lcFOS can also be produced enzymatically from inulins obtained from natural sources such as chicory and sugar beet (Singh et al. 2016)

3.2.1. Effect of FOS on microbiota composition

Like GOS and many HMOs, scFOS reaches the colon where it might selectively stimulate the growth of *Bifidobacteria* (Macfarlane et al. 2008). *In vitro* studies have shown that strains of *Bifidobacterium animalis* and *Bifidobacterium longum* are able to utilize scFOS (Valdés-Varela et al. 2017) as a carbon source. Bouhnik et al. (Bouhnik et al. 1996) studied the effect of a mixture of FOSs on fecal *Bifidobacteria* numbers. Healthy adults received 12.5 g/day FOS for a period of 12 days.

FOS ingestion led to a significant increase in *Bifidobacteria* numbers compared to placebo groups. Another study by Bouhnik and colleagues (Bouhnik et al. 2004) also demonstrated the bifidogenic effects of scFOS. However, the bifidogenic effect of scFOS seems to be dose dependent (Bouhnik et al. 1999). A study performed by Paineau and coworkers (Paineau et al. 2014) demonstrated that scFOS also has a bifidogenic effect in infants fed with formula supplemented with scFOS. In those infants, fecal concentrations of *Bifidobacteria* increased at the age of 2 and 3 months compared to placebo groups (Paineau et al. 2014). Fermentation of lcFOS seems to be more selectively than scFOS. Marx and colleagues (Marx et al. 2000) found that many different *Bifidobacterium* strains are capable of utilizing scFOS during *in vitro* fermentation, but *B. adolescentis* was the only strain that successfully metabolized lcFOS and fructans of other chain lengths. This implies that lcFOS can selectively stimulate growth of *B. adolescentis*. scFOSs do not only influence *Bifidobacteria* numbers. A study by Respondek et al. (Respondek et al. 2013) evaluated the effect of scFOS on microbiota composition and metabolic parameters in a mice model harboring the human-type microbiota and with diet-induced obesity. Addition of scFOS to a high-fat diet did not only increase fecal *Bifidobacteria*, but also *Clostridium coccoides*, whereas *Clostridium leptum* numbers decreased. The changes in numbers of *Clostridium bacteria* could be correlated to metabolic changes. This indicates scFOS can induce profound metabolic changes by modulation of the intestinal microbiota (Respondek et al. 2013).

3.2.2. Effects of FOS on prevention of infection and support of immunity

ScFOS has anti-pathogenic and immune stimulating functions. Paineau et al. (2014) studied the effect of scFOS substitution on fecal antipoliovirus IgA levels in infants at 4 months of age. Although the enhancing effect was not significant, specific IgA levels tended to be higher in the group of infants fed with formula with scFOS compared to the placebo group. This might indicate that scFOS can stimulate mucosal immune development (Paineau et al. 2014). Other studies involving animal experiments support this. Swanson and colleagues (Swanson et al. 2002) studied FOS effects on gut health in healthy adult dogs by examining microbial populations and fermentation products. In this study, FOS appeared to enhance parameters of gut health by positively altering gut microbial ecology and fecal protein catabolites (Swanson et al. 2002). Hosono and coworkers (Hosono et al. 2003) investigated the immunological influences of FOS in mice. In this study, mice received 0–7.5% FOS for 6 weeks. Mice that received 2.5% FOS had a significant higher fecal IgA levels. IgA secretion by Peyer's patch cells was upregulated in a dose dependent manner in response to FOS. $CD4^+$ T-cells from the Peyer's patches showed a dose-dependent increase in production of interferon-ϒ and interleukin IL-10. In addition, a high response in IL-5 and IL-6 production was found. Together, these data suggest that FOS can change the intestinal immune environment (Hosono et al. 2003). Furthermore, Le Bourgot et al. (Le Bourgot et al. 2016) found that a scFOS supplementation could stimulate *influenza* vaccine responses in weaning pigs. The pig diets were supplemented with 0.15% scFOS for 7 weeks. Post weaning, the scFOS diet increased anti-*influenza* IgA levels in pig serum and feces.

A mechanism by which FOS can possibly influence mucosal immunity is by reducing intestinal inflammation. Zenhom et al. (2011) investigated the effect of FOS on the expression of the peptidoglycan recognition protein 3 (PGlyRP3) in an *in vitro* experiment using Caco-2 cells. Activation of this receptor reduces the expression of proinflammatory cytokines. FOS could induce PGlyRP3 in a dose and time dependent manner. However, when PPARγ was antagonized, PglyRP3 production was inhibited. When Caco-2 cells were transfected with specific small interfering RNA targeting PGlyRP3, the anti-inflammatory effects of FOS, measured by cytokine release and expression and NF-κB translocation, were abolished. This might indicate that FOS exerts an anti-inflammatory effect by inducing nuclear PPARγ, which regulates the anti-inflammatory PGlyPR3 (Zenhom et al. 2011). FOS also has direct effects on gut barrier function. Akbari et al. (2017) investigated the effect of FOS on epithelial integrity using an *in vitro* Caco-2 monolayer cultured on transwells. Incubation with 2% FOS could significantly modulate deoxynivalenol-induced epithelial barrier disruption measured by transepithelial electrical resistance and paracellular flux of luciferase yellow (Akbari et al. 2017).

3.3. Mixtures of galacto-oligosaccharides and fructo-oligosaccharides

Mixtures of GOS and FOS are already widely added to infant formulas distributed in Europe. Currently, GOS and FOS are added in a ratio of 9:1. This ratio was chosen to mimic the molecular size distribution of HMOs (Knol et al. 2005).

3.3.1. Effects of GOS/FOS mixtures on microbiota composition

Knol et al. (Knol et al. 2005) found that infant formula supplemented with a mixture of GOS and FOS (in a ratio of 9:1, oligosaccharide formula, OSF-group) had stimulating effects on the growth of *Bifidobacteria* and the metabolic activity of the total intestinal flora (Knol et al. 2005). Fermentation profiles of infants fed with infant formula supplemented with GOS/FOS were closer to that observed in breast-fed infants compared to infants fed with control formula lacking the NDCs.

3.3.2. GOS/FOS mixtures and prevention of pathogen adhesion

There are no specific studies investigating the effect of a GOS/FOS mixture on the prevention of pathogen adhesion to epithelial cells. However, because GOS can prevent pathogen adhesion (Shoaf et al. 2006), it is likely that GOS in a mixture with FOS can also prevent pathogen adhesion.

3.3.3. Effects of GOS/FOS mixtures on epithelial cell responses

To the best of our knowledge, there are no studies describing the effect of GOS/FOS mixtures on epithelial cell responses. However, since both GOS and FOS do have direct effects on epithelial cells (Akbari et al. 2015, 2017), we also expect mixtures of GOS and FOS to exert beneficial effect on epithelial barrier integrity.

3.3.4. Effects of GOS/FOS mixtures on prevention of infection and support of immunity

The immune system can be modulated by GOS/FOS mixtures, but the majority of these effects are likely to be the result of the effect of NCDs on microbiota. Establishing a balanced microbiome is important to establish normal immune responses as imbalances, or dysbiosis, can cause disease (Kau et a. 2011). It has been shown that infant formula substituted with a mixture of scGOS/lcFOS (9:1) reduced the incidence of atopic dermatitis during the first six months of life (Moro et al. 2006). A mixture of oligosaccharides also had beneficial effects on mucosal immunity in low-atopy-risk infants (Gruber et al. 2010) and even after five years a protective effect on the incidence of certain allergic manifestations was observed (Arslanoglu et al. 2012). Scholtens et al. (2008) performed an *in vivo* study to investigate the effect of GOS/FOS supplementation on the development of the fecal secretory IgA (sIgA) response. Supplementation of infant formula with 6g/L scGOS/lcFOS (ratio 9:1) resulted in higher concentrations of fecal sIgA after 26 weeks in infants exclusively fed with formula, suggesting a positive effect on mucosal immunity (Scholtens et al. 2008).

3.4. Pectin oligomers

Pectins are polysaccharides rich in galacturonic acid molecules. These galacturonic acid molecules form the backbone of the molecule (Brejnholt 2009). The backbone structure can have side chains of neutral sugars e.g., arabinose, rhamnose, and galactose (Brejnholt 2009). Some carboxylgroups on the galacturonic acid backbone can be esterified by methyl groups (Brejnholt 2009). The percentage of methyl-esterified galacturonic acid units in the pectin chain is known as the degree of methylation (DM) and is used to classify the pectins into different categories (Brejnholt 2009) with different physicochemical properties. These different pectins have different in health promoting effects (Vogt et al. 2016).

3.4.1. Effects of POS on microbiota composition

Although there are no commercially available pectin-supplemented infant formulas yet, pectin and/or modified pectins have been shown to have several beneficial effects in experimental studies related to infant formula (Jeurink et al. 2013). This may make these NDCs suitable for the supplementation in infant formula in the near future. Recently Di et al. (Di et al. 2017) studied the potential prebiotic effects of 5 different pectin oligosaccharides (POS) structures, which can be obtained from pectin by enzymatic treatment and acid hydrolysis (Di et al. 2017). It was found that POS exhibited prebiotic activity in *in vitro* batch fermentation studies and enhancing effects on numbers of *Bifidobacteria* over time. However, the bifidogenic effect was not the same for all POSs tested and seems to be dependent on structure. Especially the arabinose-rich rhamnogalacturonic acids were responsible for the prebiotic effects (Di et al. 2017).

3.4.2. POS and prevention of pathogen adhesion

POS can serve as decoy receptors to neutralize toxins produced by pathogens. Olano-Martin and coworkers (Olano-Martin et al. 2003) evaluated pectins and POS fractions and their ability to interfere with the toxicity of Shiga-like toxins from Escherichia coli O157:H7 in an *in vitro* experiment using the HT29 cell line. Medium supplemented with different concentrations of pectins or POS fractions was preincubated with Shiga-like toxins and subsequently added to the cell culture. After 48 hours, cytotoxicity was measured and compared with cells incubated with only Shiga-like toxins. Addition of POS fractions could protect cells from damage induced by Shiga-like toxins. Pectin polymers protected cells in a lower extend compared to POS fractions (Olano-Martin et al. 2003). There are no specific studies investigating the direct pathogen-binding abilities of POS.

3.4.3. Effects of POS on epithelial cell responses

Pectin molecules have the ability to directly influence epithelial cell responses. In a study by Vogt et al. (2016), lemon pectins originated were found to stimulate barrier function in an *in vitro* experiment with a T84 epithelial cells. Incubation with lemon pectins had a protective effect on barrier function measured by transepithelial electrical resistance (Vogt et al. 2016).

3.4.4. Effects of POS on prevention of infection and support of immunity

There are also many studies demonstrating immune modulatory effects of POS and of oligosaccharide mixtures containing POS (Stam et al. 2011; Vos et al. 2010). In the study of Vos et al. (Vos et al. 2010) the kinetic effects of immune modulation by a mixture of oligosaccharides and the correlation between microbiological and immunological parameters were studied in a murine vaccination model. In this study C57BL/6 mice were supplemented with scGOS and lcFOS (ratio 9:1) in combination with pectin derived acidic oligosaccharides (pAOS). It was found that the early phase of the vaccine-specific immune response could be influenced by this supplementation i.e. it could increase the delayed-type hypersensitivity (DTH) responses. Analysis of the microbiological parameters showed there was a correlation between the increased percentage of cecal lactobacilli and the increased DTH responses, suggesting that microbiota might play a role in this time-specific immune modulating effect. Stam et al. (Stam et al. 2011) also used a mixture of oligosaccharides (scGOS/lcFOS ratio 9:1 and pAOS) to study specific immunoglobulin responses to Haemophilus influenza type b (Hib) and tetanus immunization in healthy non-atopic infants during the first year of life. Although there are many studies reporting modulating effects of prebiotics on the immune responses in infants at risk of allergy, in this study with healthy infants no effects of supplementation of food formulas with the oligosaccharide mixture were found on antibody specific levels when compared with children on control diets. Therefore, the authors hypothesized that the oligosaccharide mixture might promote T-helper 1 (Th1) and T-regulatory (Treg) immune responses and downregulate of IgE-mediated allergic responses, but it does not affect the normal vaccine-specific serum antibody response (Stam et al. 2011).

4. *In vitro* systems to test NDC effects?

Several studies have shown that other, more effective NDCs might become available if we had faster and more predictive means to unravel the structure/function relationships of HMOs

and NDCs and their effects on health parameters described above (Bermudez-Brito et al. 2015a; Bermudez-Brito et al. 2015b). Processes that directly after birth should be enforced are not only the prevention of pathogen adhesion, but for example also the transition from a Th2 environment in utero towards a more Th1 environment to allow the baby to respond to pathogens (Nussbaum and Sperandio 2011). How and which HMOs have such an effect is unknown but it is an important phenomenon in immune maturation (Nussbaum and Sperandio 2011). Also, it is predictable that infant formulas should be different for healthy and disease-prone infants. For example, prematures are born with a rather proinflammatory, Th1 like phenotype and should be fed with NDC supplements preventing overt immune responses to avoid necrotizing enterocolitis (Martin and Walker 2006). Also, allergic prone infants with the risk to develop a too strong Th2 response might benefit from specific infant formulas to manage the allergy and priming with potential allergens (Moro et al. 2006; Stam et al. 2011). Invasive experiments in infants obviously have great restrains and as a consequence, the number of *in vivo* studies is limited and such studies can only be done when safety is guaranteed. The field would greatly benefit from a physiological relevant *in vitro* model of the infant intestine for testing efficacy of NDCs. Such an *in vitro* model of in infant intestine should meet a number of prerequisites. It should contain a polarized epithelium cell layer, microbiota and presence of (immature) immune cells. In this way utilization of NDCs and effects on immunity and microbiota communities as well as its cross-talk with the immature immune cells can be studied ex vivo and serve at least as selection tool for infant formula applicable NDCs.

4.1. Challenges for developing a new in vitro model

A major challenge for creating a physiological relevant *in vitro* model is the creation of a microenvironment in which both aerobic epithelial cells and aerobic as well as anaerobic microbiota species can survive and exchange information (Ulluwishewa et al. 2015). About 90% of the commensal microbiota are obligate anaerobes and therefore should be cultured in an anaerobic environment. For example, certain *Bifidobacteria* species show different growth patterns when they come in contact with oxygen (Ruiz et al. 2011). To overcome this problem, many studies use ultraviolet (UV)-killed bacteria or bacterial components. This, however, might not reflect the physiological situation as there are studies reporting that live bacteria exert greater beneficial effects than non-viable bacteria (Ulluwishewa et al. 2015). Furthermore, the absence of living bacteria in an *in vitro* system leads to the absence of components secreted by bacteria.

For co-culturing human eukaryotic cells and living bacteria, an anaerobic compartment should be created in an *in vitro* system mimicking the infant gut intestine. Currently there are two models published in which cell-cultures are combined with anaerobic bacteria-cultures. Sadabad *et al.* (Sadabad et al. 2015) developed a coculture system called the "Human oxygen-Bacteria anaerobic (HoxBan) coculture system' in which coverslip attached Caco-2 cells in 10 mL DMEM medium were placed on top of *F. Prausnitzii* bacteria grown in YCFAG-agar in 50mL culture tubes. This was done in order to study interactions between *F. Prausnitzii* and epithelial cells. In another study, Ulluwishewa *et al.* (Ulluwishewa et al. 2015) used a unique apical anaerobic model of the intestinal epithelial barrier, which enabled co-culturing of living obligate anaerobes with Caco-2 cells. In this model a trans well insert with a human intestinal epithelial Caco-2 cell monolayer was fitted on a chamber lid. This sealed of a chamber filled with aerobic cell culture medium from the anaerobic environment. The insert was filled with anaerobic cell culture medium (Ulluwishewa et al. 2015). In this way, the Caco-2 cells were exposed to an aerobic environment on the basal side, while the apical side was exposed to an anaerobic environment, making it able to co-culture human intestinal epithelial Caco-2 cells with anaerobic bacteria in the apical compartment (Ulluwishewa et al. 2015).

Although both research systems were successful in combining cell-cultures with anaerobic bacteria-cultures, a more specific model is needed to test NDC effects, since the model of Sadabad et al. (Sadabad et al. 2015) applied agar for growing bacteria. Although this is a simple and logical way to create an anaerobic compartment, it will not be possible to study NDC fermentation, since agar will be fermented as well and interfere with studies on fermentation and utilization of the NDCs. The model of Ulluwishewa et al. (Ulluwishewa et al. 2015) only used a single bacteria strain in the anaerobic compartment. To test NDC effects, whole microbiota samples should be applied.

5. Concluding remarks

In the past decades, more knowledge has become available about the functions HMOs serve. Unique features of HMOs include the possibility to modulate and manage microbiota composition in a beneficial way by stimulating growth of *Bifidobacteria*, modulate immune reactions, defend against infections and change gut epithelial cell responses (Bode 2006). By doing so HMOs are very important for guiding immune barrier development and maintenance of health in infants. As HMOs are unique for HM, these beneficial effects are lost in neonates fed with cow's-milk derived infant formula. Substitution of infant formula with adequate NDCs to substitute HMO functions is therefore very important and effects might not only influence health in early but also in later life. For example, an adequate colonization of microbiota in early-life helps to maintain health by reducing the incidence of inflammatory, autoimmune and atopic diseases (Martin et al. 2010).

Although currently the substitution of infant formula with NDCs is mainly focused on the prebiotic effects, more attention should be given to the direct beneficial immune effects which are independent of microbiota. There are many immune modulating effects of NDCs (Bermudez-Brito et al. 2015a; Bermudez-Brito et al. 2015b; Vogt et al. 2016). An important function of the NDCs in infant formula is to guide the transition from a Th2 environment in utero towards a more Th1 environment to allow the baby to respond to pathogens. Disturbance of this transition, as seen in prematures, could cause the immune system to react with proinflammatory cytokine production and secretion of antibacterial peptides, increasing the risk of development of (allergic) disease in later life. Recent work of Bermudez-Brito et al. (Bermudez-Brito et al. 2015b) showed that not all NDCs currently described for infant formula do support the

induction of Th1 responses. Depending on its composition the NDCs either promote Th1, Th2 or Treg responses. For this reason, more research is needed to understand the specific effects of NDCs. When specific NDCs can be linked to beneficial health effects this information can be used to better mimic the functional activities of HM components and more specific of HMOs. Furthermore, it might be possible to design NDC mixtures that support the development of healthy immune responses in a controlled and intelligent way, as this composition is probably different for healthy and disease-prone infants.

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