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REVIEW



Does the contribution of human milk oligosaccharides to the beneficial effects of breast milk allow us to hope for an improvement in infant formulas?

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ABSTRACT

Human milk is a source of nutrients and contains many distinct bioactive components. Among these, human milk oligosaccharides (HMOs) have attracted considerable attention and are being investigated as a “novel foods”. Human milk is unique in its oligosaccharide composition. Recent research has focused on the complexity of HMOs by highlighting their diversity, structural variability, concentration variance, and structure-function relationships. In vitro and in vivo studies have demonstrated that HMOs drive infant gut microbiota, improve intestinal barrier functions, and modulate cell receptor signaling, thereby contributing to the development of infant immunity. These studies, combined with epidemiological data, indicate that some HMO may confer health benefits by preventing infections and diseases such as necrotizing enterocolitis and allergies. However, randomized controlled trials are restricted to structurally simple compounds such as 2’ fucosyllactose and lacto-N-neotetraose. More controlled clinical trials are needed to justify routine supplementation of formula. It is felt that a better understanding of the role of HMOs leading to the development of inexpensive methods for large-scale HMO production is needed.

KEYWORDS

Functional food; microbiota; prebiotic; prevention of infant diseases

Introduction

Breastfeeding, which has been recognized as the natural means for ensuring the survival of mammals since ancient times, is considered the gold standard for feeding infants. Breastfeeding provides infants with nutrients needed for healthy growth and development and is therefore considered to be the preferred source of nutrition, with which health benefits can be conferred to infants worldwide (Dieterich et al. 2013; Donovan 2019; WHO 2013a, 2013b). The benefits of breastfeeding are not only confined to children but also extend to their mothers (Del Ciampo and Del Ciampo 2018). Global public health recommendations stipulate that infants should be exclusively breastfed for the first six months of their life (WHO 2009). Currently, scientists are engaged in the process of analyzing and clarifying these health benefits. Human milk (HM) is both a comprehensive source of nutrition and a potent adjuvant that protects against infections and inflammation (Donovan 2019; WHO 2013a, 2013b). It contributes to microbial colonization, gut development, and immune maturation. Apart from nutrients, HM contains many distinct bioactive components among which HM oligosaccharides (HMOs) have attracted considerable attention as “novel foods” leading to the initiation of several studies (Andreas, Kampmann, and Mehring Le-Doare 2015; Fernández et al. 2013; Williams et al. 2017). Since the beginning of the 19th century, infants who cannot be breastfed are fed an infant formula, the composition of

which is being constantly improved in an attempt to mimic the beneficial effects of breastfeeding. The last modification in the composition of some formulas was the addition of certain specific HMOs. The objective of this review is to briefly summarize current knowledge concerning HMOs in order to discuss the potential for supplementing infant formulas (IF) with HMOs. Although many previous studies have reviewed the structure, metabolism and role of HMOs, it is felt that a brief updated overview of key points may be needed in order to reach the intended goal. (Andreas, Kampmann, and Mehring Le-Doare 2015; Bode 2012; Kunz and Rudloff 2017).

The development of HMO research, from chemists to clinicians

Scientific interest in the composition of human milk and its positive effects on health can be traced back to the end of the 19th century, when clinicians observed that breastfed infants had a much higher chance of survival and a lower incidence of infectious diseases than non-breastfed infants. These observations have since been confirmed via meta-analysis (Sankar et al. 2015; Vitoria et al. 2016; WHO 2000). The earlier the breastfeeding, the greater the child’s chances of survival (Smith et al. 2017). In the year 1900, it was noted that breastfed children carried bifidobacteria in their stool and were less likely to have infectious diarrhea than non-

Table 1. Prominent human milk oligosaccharides expected in milk of mothers according to genotype and consequent fucosyltransferase expression (FUT2, FUT3).

Genotype	Secretor +	Secretor -
Lewis +	Se⁺/Le⁺ (FUT2 + FUT3) All HMOs, mainly 2'-FL, 3-FL, DFL, LNT, LNnT, LNFP I, LNFP II, LNFP III, LNDFH I, LNDFH II, 3'-SL, 6'-SL	Se⁻/Le⁺ (FUT3) 3-FL, LNT, LNnT, LNFP II, LNFP III, LNDFH II, 3'-SL, 6'-SL
Lewis -	Se⁺/Le⁻ (FUT2) 2'-FL, 3-FL, DFL, LNT, LNnT, LNFP I, LNFP III, 3'-SL, 6'-SL	Se⁻/Le⁻ (-) 3-FL, LNT, LNnT, LNFP III, LNFP V 3'-SL, 6'-SL

2'-FL, 2'-fucosyllactose; 3-FL, 3-fucosyllactose; 3'-SL, 3'-sialyllactose; 6'-SL, 6'-sialyllactose; DFL, difucosyllactose; LNDFH, lacto-N-difucohexaose; LNFP, lacto-N-fucopentaose; LNnT, lacto-N-neotetraose; LNT, Lacto-N-tetraose.

breastfed children, following which Tissier proposed a relationship between these two findings (Tissier 1900). Two reviews have detailed the history of HMO research (Bode 2012; Kunz 2012). Briefly, in 1931, two French biochemists reported the presence of a nonlactose carbohydrate which was referred to as « gynolactose » (Polonowski and Lespagnol 1931). In 1954, the same team separated “gynolactose” into individual oligosaccharides (Polonowski and Montreuil 1954). In the following years, research work conducted both in Germany and in France began to describe HMOs (Kuhn 1958; Montreuil 1960). During the same time period, a German team hypothesized that the bifidogenic factor in HM consisted of oligosaccharides (OS) containing N-acetylglucosamine and polysaccharides. This finding connected these studies with each other (György et al. 1954). During the past decade, research has been directed at defining the complexity of OS in HM. It is also beginning to delineate their diverse functions leading to recent clinical trials.

Diversity and variability in HMOs concentrations and profiles

Structures

HMOs constitute a heterogeneous mixture of bioactive glycans that vary per individual. More than 200 different HMOs have been identified so far (Akkerman, Faas, and de Vos 2019; Bode 2012; Kunz 2012), but only 50 structures are assumed to represent 99% of HMO abundance in HM (Smilowitz et al. 2014). Most HMOs consist of a lactose core, which is lengthened by the addition of one or more monosaccharides (L-fucose, D-glucose, D-galactose, N-acetylglucosamine and sialic acid) in various sequences and orientations (Bode and Jantscher-Krenn 2012; Morozov et al. 2018; Thurl et al. 2010). Other core structures, built by the addition of lacto-N-biose units, or lactosamine units, have been detected (Amano et al. 2009; Bode and Jantscher-Krenn 2012; Morozov et al. 2018; Thurl et al. 2010; Wu et al. 2011).

HMOs concentration in human milk

The HMO fraction is the third largest component of HM besides lactose and lipids, and apart from water, represent approximately 20% of the total carbohydrate content in earliest HM (Coppa et al. 1999; Newburg, Shen, and Warren

2000; Thurl et al. 2017; Xu et al. 2017). According to many studies, the HMO content of HM usually varies over the course of lactation between 20–24 g/L in the colostrum to 10–15 g/L in mature milk (or 1.5–2.3 g/100 kcal) (Andreas, Kampmann, and Mehring Le-Doare 2015; Bode 2012; Chaturvedi et al. 2001; Chen 2015; Coppa et al. 1993; Thurl et al. 2017; Xu et al. 2017), while according to others it varied from 9.15 g/L at day 3 to 6.38 g/L at day 42 of lactation (Nijman et al. 2018). Lower contents and/or little variation has been reported by others (Borewicz et al. 2019; Kunz et al. 2017). Therefore, a term infant consuming approximately 800 mL of HM daily would intake approximately 10 g/d of HMOs. Further, intrapersonal variation which occurs over the course of lactation and inter-personal variations related to fucosylation also affect HMO composition, depending on the genetic background of the mother.

Factors affecting HMOs pattern variation

Wide variability found in the concentration of HMOs between different women, as well as in the same woman, during lactation, due to polymorphism in the Lewis and Secretor genes, has been discussed extensively (Thurl et al. 2017). These factors determine both the quantity and pattern of HMOs in milk. The total HMO content has been shown to vary by 3.7-fold and individual HMOs concentrations to vary by 20- to >100-fold (Azad et al. 2018). The presence of different neutral oligosaccharides in human milk depends on the activity of specific enzymes, fucosyltransferases (FUT), which catalyze the addition of fucose to HMOs in the lactating mammary gland (Bode 2012; Kunz et al. 2017). The Secretor gene encodes the enzyme, α [1,2]-fucosyltransferase (FUT2), responsible for linking fucose in a α 1-2 linkage to lengthen the HMO chain. The Lewis blood group gene encodes α 1-3/4-fucosyltransferase (FUT3) creating fucosylated oligosaccharides with a α 1-3/4 linkage (Bode 2012; Bode 2015; Coppa et al. 2011; Donovan and Comstock 2016; Kunz et al. 2017; Rudloff and Kunz 2012; Thurl et al. 1997, 2010, 2017). Due to differences in the expression of these enzymes, 4 main phenotypes are possible in relation to HMO profiles: Se⁺/Le⁺, Se⁻/Le⁺, Se⁺/Le⁻ and Se⁻/Le⁻ (Table 1) (Ayechu-Muruzabal et al. 2018; Bode 2015; Coppa et al. 2011; Kunz et al. 2017; Rudloff and Kunz 2012; Thurl et al. 1997, 2017). On average, 70% of mothers in the general population express both FUT2 and FUT3 (Se⁺/Le⁺), where 20% are Se⁻/Le⁺, 9% Se⁺/Le⁻ and 1% Se⁻/Le⁻ (Azad et al. 2018; Bai et al. 2018; Chen 2015; Erney et al. 2000;

McGuire et al. 2017). Moreover, sialylation contributes to increased variability of HMO structures in milk. On average, 35–50% of HMOs are neutral fucosylated, 42–55% are neutral nonfucosylated, and 12–14% are acidic sialylated in term breast milk (Donovan and Comstock 2016). Non-Secretor mothers produced significantly less HMOs than Secretor mothers. HMOs composition of HM is strongly influenced by glycosyltransferases polymorphisms (Ferrer-Admetlla et al. 2009). The concentration and diversity of HMOs genotypes vary depending on geographical location and ethnicity (Azad et al. 2018; Erney et al. 2000; Ferrer-Admetlla et al. 2009; McGuire et al. 2017; van Leeuwen et al. 2018).

Regardless of genetic status, concentrations of different HMOs develop differently during lactation (Thurl et al. 2017). Over time, the total concentration of fucosylated HMOs decreased while its relative abundance, expressed as percentage of total HMOs, increased and the concentration and relative abundance of sialylated and non fucosylated neutral HMOs decreased (Xu et al. 2017). Several studies have indicated that milk of mothers delivering preterm contained higher HMOs concentrations (Austin et al. 2019; Gabrielli et al. 2011; Gidrewicz and Fenton 2014). However, this was contradicted by the findings of another study in which the median levels in preterm milk did not differ from those of term milk, either in the colostrum or in transitional or mature milk (Kunz et al. 2017). HMOs pattern are not affected by freeze-drying of donors' milk, which is an important finding for neonatal units and milk banks (Hahn et al. 2019). However, results following sterilization appear to be mostly dependent on the method used (Hahn et al. 2019; Marx et al. 2014; Meredith-Dennis et al. 2018).

Other factors such as maternal age, weight, body mass index, parity, urban or rural residency, season and lactation stage were related to HMO contents and profiles by some authors (Azad et al. 2018; Davis et al. 2017; McGuire et al. 2017), but not all (Tonon et al. 2019). Maternal diet did not have an impact on HMO content (Azad et al. 2018; McGuire et al. 2017). However, HMOs concentration was associated with certain nutritional conditions of the mother. Gambian mothers nursing in the dry season (harvest season) when food was plentiful and energy intake high, produced milk with higher HMOs concentrations, compared with those nursing during the wet season when food stores were depleted (Davis et al. 2017). Surprisingly, in the colostrum of mother supplemented with a mixture of probiotics, concentrations of 3-fucosyllactose as well as 3'-sialyllactose were significantly higher than those in the colostrum of nonsupplemented mothers (Seppo et al. 2019). Meanwhile, the levels of difucosyllacto-N-hexaose, lacto-N-tetraose, lacto-N-fucopentaose I, and 6'-sialyllactose were lower in the colostrum of mothers who received probiotic supplementation than in those of the control group, which explained the lower total concentration of HMOs.

HMOs are unique

All mammalian milk or colostrum contain a variety of neutral and acidic oligosaccharides, the concentration and

nature of which are extremely variable from one species to another, including nonhuman primates and domestic farm animals such as camels, cows, donkeys, ewes, goats, mares, mice, pigs and rabbits (Albrecht et al. 2014; Prieto 2012; Urashima, Messer, and Oftedal 2017). The structure and composition of HMOs were far more complex than those of the OS in the milk of other species, including cow's milk (CM) on which most IFs are based. Compared to HM, CM contains lower levels (0.05–0.1 g/L in mature milk and up to 1 g/L in colostrum) and a lower variety of OS with remarkable seasonal variation (Fong, Ma, and McJarrow 2001; Liu et al. 2017; Nakamura et al. 2003; Robinson 2019; Urashima, Messer, and Oftedal 2017) and inter-breed variability (Kuntz, Rudloff, et al. 2019; Sundekilde et al. 2012). Among HMOs, 2'-fucosyllactose and oligosaccharides, the cores of which are predominantly lacto-N-tetraose, and type I OS containing the Gal(β 1-3) GlcNAc (lacto-N-biose I_N, LNB) structure, predominate over type II saccharides containing Gal(β 1-4)GlcNAc (N-acetyllactosamine, LacNAc) structure (Urashima, Messer, and Oftedal 2017). This predominance of type I OS in HM is a uniquely human characteristic. Owing to these differences, standard unsupplemented IFs contain much less OS, and express lesser structural diversity (Nijman et al. 2018).

HMOs metabolism

Intervention pathways of HMOs are shown (Figure 1). HMOs that are seen to be present in high concentrations in the colostrum at the beginning of lactation, decline as milk matures (Andreas, Kampmann, and Mehring Le-Doare 2015; Bode 2012; Chaturvedi et al. 2001; Chen 2015; Coppa et al. 1993; Nijman et al. 2018; Thurl et al. 2017; Xu et al. 2017). In addition, HMOs were recently shown to be present in maternal serum and urine during pregnancy and in amniotic fluid at delivery, suggesting that the fetus is already exposed to HMOs in utero (Jantscher-Krenn et al. 2019; Wise et al. 2018). These data should be analyzed with those reporting the initiation of maternal-to-offspring microbial colonization in utero (Aagaard et al. 2014; Chong, Bloomfield, and O'Sullivan 2018; Collado et al. 2016). However, these data regarding prenatal microbiome need to be confirmed (Hornef and Penders 2017; Perez-Muñoz et al. 2017; Walker et al. 2017). Moreover, it appears that, the infant is already in contact with his mother's HMOs, regardless of the postnatal feeding mode.

Once ingested, a great majority of HMOs reach the distal small intestine and colon intact, where they serve as a substrate for specific microbes, mainly bifidobacteria, thus acting as a prebiotic. (Asakuma et al. 2011; Brand-Miller et al. 1998; Chaturvedi et al. 2001; Coppa et al. 2001; Gnoth et al. 2000; Jacobi et al. 2016; Kuntz, Rudloff, et al. 2019; Sela and Mills 2010). Certain smaller molecular weight HMOs, such as 2'-fucosyllactose (2'FL), 3 fucosyllactose and lacto-N-neotetraose (LNnT), are absorbed intact into the circulation and excreted in the urine without metabolic modification at levels that correlate with their dietary intake from breast milk (Goehring et al. 2014). Furthermore, 2'-FL, LNnT, and 6'-

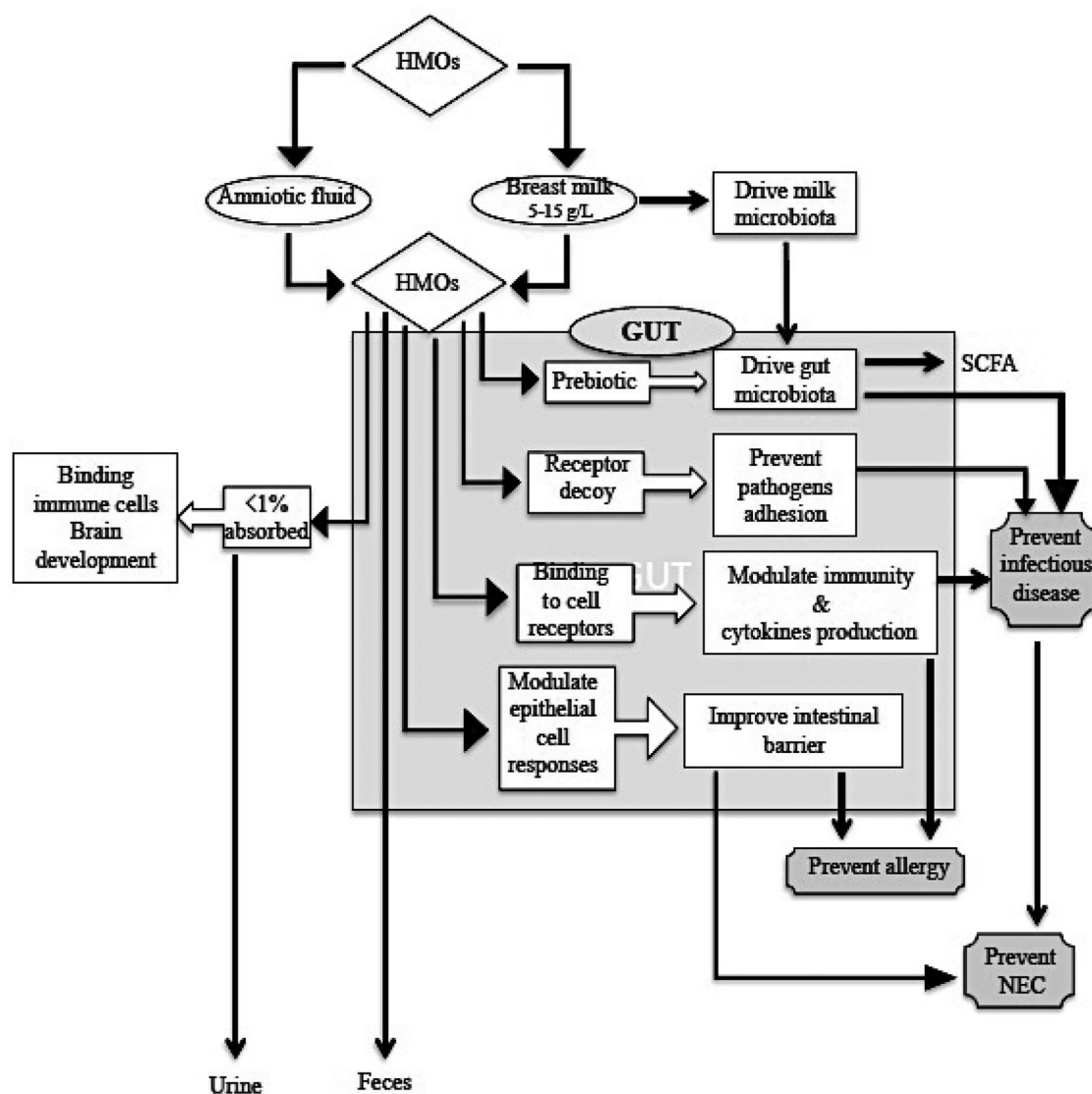


Figure 1. Overview of potential mechanisms whereby human milk oligosaccharides (HMOs) can have clinical benefits. (NEC, necrotizing enterocolitis; SCFA, short chain fatty acid).

siallylactose (6'-SL) have been shown to be quickly absorbed after being orally administered to rats (Vazquez et al. 2017). The relative fractions of the amounts ingested appear to be low, the resulting plasma concentration is 0.1% and the urinary concentration is 4% of the milk level. Therefore, the pattern in urine often reflects the mother's secretor/nonsecretor status (Goehring et al. 2014). Excretion of the remaining HMOs in the stool shows greater variation in relation to differences in the infant's intestinal microbiota (Dotz et al. 2015).

Demonstrated beneficial effects of HMOs

The high structural inter-individual diversity of HMOs complicates the acquisition of detailed information regarding structures that are particularly important for infant health (Kunz and Rudloff 2017), as well as the clinical impact of such diversity. On the other hand, the role of HMOs themselves must be discussed in relation to many other immune

and microbiota-modulating bioactive factors in HM, which also acts as a source of bacteria for the developing infant gut (Andreas, Kampmann, and Mehring Le-Doare 2015; Cacho and Lawrence 2017; Pacheco et al. 2015). HM microbiota also display great diversity and variability, depending on the individuality and health status of lactating women and their geographic location (Fernández et al. 2013; Kumar et al. 2016; Williams et al. 2017). The effects of HMOs are not limited to infant intestinal microbiota, and extend to the microbial composition of human milk, especially impacting bifidobacteria levels in the colostrum samples (Aakko et al. 2017).

Evidence demonstrating the beneficial effects of HMOs are essentially obtained from in vitro experiments and animal studies, combined with epidemiological data and other observational studies on the beneficial effects of HM. Even in cases where clear, specific structure-function relationships were established between several of these effects, the mechanisms involved remained far from being fully elucidated. Demonstrated beneficial effects attributed to HMOs which

are shown (Figure 1) have been briefly described (Akkerman, Faas, and de Vos 2019; Andreas, Kampmann, and Mehring Le-Doare 2015; Bode and Jantscher-Krenn 2012; Triantis, Bode, and van Neerven 2018).

Prebiotic effects of HMOs

HMOs act as prebiotics, which are nutritive factors that promote the growth of certain strains of beneficial bacteria, mainly genus *Bifidobacterium* and, to a lesser extent, some strains of *Bacteroides* and *Lactobacillus*, reducing the nutrient supply available to potentially harmful bacteria, thereby keeping their growth under control and helping to decrease susceptibility to bacterial and viral diseases (Aakko et al. 2017; Bidart et al. 2017; Borewicz et al. 2019; Craft and Townsend 2018; Marcobal et al. 2010; Newburg 2013; Thongaram et al. 2017; Thomson, Medina, and Garrido 2018; Underwood et al. 2015; Ward et al. 2006). HMOs use by bacteria depends on both HMO structure and the bacterial strain (Akkerman, Faas, and de Vos 2019; Thomson, Medina, and Garrido 2018). Some studies have contended that infant bacterial genera could be predicted using HMOs content in milk (Wang et al. 2015). By enhancing the development of infant microbiota, HMOs favor the production of postbiotic substances, mainly represented by short chain fatty acids (SCFA) (Perdijk et al. 2019).

Prevention of pathogen adhesion

Due to the nature of their structure, HMOs are able to act as receptor decoys for pathogens or toxins. This is made possible by the resemblance of HMO structure to various cell surface glycan receptors. As a result, pathogens bind to HMOs rather than to cell surface glycans, thereby preventing mucosal adherence of certain bacterial or viral pathogens, and contributing to a lower incidence of intestinal, respiratory and urinary tract infections (Craft and Townsend 2018; Donovan 2017; Morozov et al. 2018). However in vitro studies have shown that HMOs did not function as decoy receptors for the neonatal rotavirus strain, G10P. Population studies have indicated significantly higher levels of Lacto-N-tetraose (LNT), 2'-fucosyllactose (2'-FL) and 6'-sialyllactose (6'-SL) in the milk of mothers of rotavirus-positive neonates with gastrointestinal symptoms (Ramani et al. 2018).

Immunological effects

Depending on their structure, HMOs can bind different types of intestinal cell surface receptors (glycan-binding proteins, i.e. lectins) and some Toll-like receptors involved in cell signaling (Cheng et al. 2019; Kulinich and Liu 2016; Triantis, Bode, and van Neerven 2018). By acting on cells such as intestinal cells, antigen presenting cells, and leucocytes, HMOs may modulate intestinal epithelial cell responses and interfere with the regulation of T cell function, thereby modulating infant immunity. The combined effect of increased SCFA production and HMOs on

proliferation, differentiation and maturation of intestinal cells improved intestinal barrier functions (Cheng et al. 2019; Donovan and Comstock 2016; Holscher, Bode, and Tappenden 2017; Perdijk et al. 2019). Subsequently, HMOs contributed to the downregulation of a panel of inflammation-related genes, which decrease inflammatory cytokines and shift T-cell responses to a balanced Th1/Th2-cytokine production, promoting immune tolerance (He et al. 2014; Kulinich and Liu 2016; Xiao et al. 2019). HMOs may also exert systemic effects by binding monocytes, lymphocytes, and neutrophils to endothelial cells and via the formation of platelet-neutrophil complexes by some absorbed HMOs (Donovan and Comstock 2016).

Brain development

Absorbed HMOs and their metabolic products such as sialic acid have been shown to enhance brain development and cognition in animal models (Jacobi et al. 2016; Vázquez et al. 2015; Vazquez et al. 2017).

Clinical effects

Triantis, Bode and van Neerven reviewed studies indicating a correlation between HMOs and the decreased incidence of certain infectious disease, such as diarrhea, respiratory tract infections, sepsis and HIV transmission risk in young children (2018). Most of these studies emphasize the association of a favorable outcome with 2'-FL. Moreover, other experiments have demonstrated that HMOs may be considered as promising antivirals (Donovan 2017; Morozov et al. 2018). Specific HMOs (2'-FL, 3'-sialyllactose (3'-SL), 6'-SL) have been shown to inhibit human Rotavirus infectivity of specific strains, in a dose-dependent manner, whereas no effects have been demonstrated on the infectivity of neonatal G10P strains (Laucrica et al. 2017; Ramani et al. 2018). 2'FL, LNT, and LNFP I improved the infectivity of rotavirus vaccines (Ramani et al. 2018).

During the neonatal period, necrotizing enterocolitis (NEC) poses a grave threat to very low birth weight infants (VLBW), who, when fed HM were much less exposed to the risk for NEC (Patel, Panagos, and Silvestri 2017). The properties of HM are attributed to its microbiota and HMO content. The low secretor phenotype was associated with NEC, while the non-secretor genotype was associated with gram negative sepsis (Morrow et al. 2011). The potential of HMOs for preventing NEC was assessed in preclinical NEC animal models (Bering 2018; Bode 2018). A recent study investigated the correlation between concentration levels of 15 dominant HMOs in milk from 96 mothers and the incidence of NEC in 106 VLBW infants (<1000 g) (Wejryd et al. 2018). The results demonstrated that although low diversity of HMOs and low levels of a specific HMO, Lacto-N-difucohexaose 1 (LNDH1), were associated with NEC development, disialyllacto-N-tetraose (DSLNT) was not associated with NEC. Conversely, building on results obtained from animal studies, a new prospective multicenter investigation of 200 mothers and their VLBW offspring was

Table 2. Outcomes of randomized controlled clinical trials (RCT) in infants fed formulas supplemented by HMOs.

Authors (year)	Study design	Major findings
Marriage et al. (2015)	Multicentric, full-term infants enrolled by 5 d of life and fed 1 of 4 diets for 4 months: – HM (n = 106, non-randomized) • Control IF + 2.4 g/L GOS (n = 101) • IF + 2.2 g/L GOS + 0.2 g/L 2'-FL (n = 104) • IF + 1.4 g/L GOS + 1.0 g/L 2 FL (n = 109).	• No significant difference among any groups for weight, length, or head circumference growth, • No difference in average stool consistency, number of stools per day, and percent of feedings associated with spitting up or vomit.
Goehring et al. (2016)	Same population and groups as above. • PBMC isolated at 6 weeks of age.	• Breastfed infants and infants fed either of the experimental formulas with 2'-FL had 29–83% lower concentrations of plasma inflammatory cytokines than did infants fed control formulas [IL receptor antagonist (IL-1ra), IL-1α, IL-1β, IL-6, and TNF α] ($P \leq 0.05$). • In ex vivo RSV-stimulated PBMC cultures, breastfed infants and the two groups fed formula with 2'-FL had lower concentrations of TNF-α (31%) and IFN-γ (54%) ($P \leq 0.05$) and tended to have lower IL-1ra (25%) and IL-6 (38%) than did infants fed the control formula.
Kajzer, Oliver, and Marriage (2016)	Multicenter. Healthy term infants enrolled by 8 d of age and fed to 35 d of age: • HM reference group (n = 42) • Control IF without FOS (n = 36) • IF + 0.2 g 2'-FL/L + 2 g FOS/L (n = 41).	• No difference in anthropometric data or in stool consistency among groups, • The average number of stools/day for the HM group was greater than for both supplemented IF groups ($p < 0.0001$).
Puccio et al. (2017)	Multicenter, 2 parallel groups of healthy infants who were enrolled at ≤ 14 d of age and fed to 6 months of age: – Control IF (n = 87) • IF + 1.0 g/L 2'FL + 0.5 g/L LNnT (N = 88).	• No difference in weight gain, length, head circumference, and BMI; • No difference in flatulence, spitting-up, and vomiting incidence; • Infants fed HMOs-supplemented formula had softer stools at 2 months ($P = 0.021$) and fewer nighttime wake-ups ($P = 0.036$), but not at other times up to 12 months; • Infants fed HMOs-supplemented formula had significantly fewer parental reports of: • Bronchitis through 4, 6, and 12 months ($P < 0.01$), • Lower respiratory tract infection through 12 months ($P < 0.05$), • Lower antipyretics use through 4 months ($P < 0.05$), • Lower antibiotics use through 6 and 12 months ($P < 0.05$).
Berger et al. (2020)	Fecal DNA extraction and microbiota composition analysis from the above cohort.	• At 3 months, microbiota composition in the supplemented group appeared closer to that of the breastfed group by microbiota alpha and beta diversity analyses and distribution of fecal community types ($P < 0.001$); • More differences between formula groups were showed in the Cesarean-section delivered infants; • An increase of bifidobacteria ($P < 0.01$) and a decrease of <i>Escherichia coli</i> ($P < 0.01$) and unclassified Peptostreptococcaceae ($P < 0.05$) were observed. • At 12 months (6 months after cessation of the intervention), no significant difference in microbiota composition was observed.

2'FL, 2'-fucosyllactose; FOS, fructo-oligosaccharides; GOS, galacto-oligosaccharides; HM, human milk; HMOs, human milk oligosaccharides; IF, infant formula; IFN, interferon; IL, interleukin; LNnT, lacto-N-neotetraose; PBMC, peripheral blood mononuclear cells; RSV, respiratory syncytial virus; RV, rotavirus; TNF, tumor necrosis factor.

conducted, and showed that especially DSLNT (OR 0.84; 0.74–0.88; $p = 0.001$) and, to a lesser extent, lacto-N-fucopentaose (LNFP1) (OR 0.91; 0.84–0.97; $p = 0.006$) made a significant protective contribution to reducing NEC risk, whereas difucosyllacto-N-tetraose (DFLNT); (OR 1.15; 1.01–1.28; $p = 0.022$) increased NEC risk (Autran et al. 2018; Bode 2018). In this study, DSLNT concentrations between mothers of preterm infants varied greatly. DSLNT was considered the most effective HMO for reducing NEC, while 2'-FL exhibited a moderate effect on neonatal mouse and rat

models, but none on piglet models (Bode 2018). Amounts of DSLNT in donor milk batches stored in milk banks were found to be slightly lower, (Marx et al. 2014). This raised issues related to the possibility of supplementing pooled donor milk with DSLNT (Bode 2018).

Infants born via C-section and fed breastmilk containing FUT2-dependent HMOs, such as 2'FL, showed a lower incidence of eczema as well as allergic diseases such as Ig-E-mediated ones, after reaching the age of 2 y (Sprenger et al. 2017). An observational study indicated that the milk of

mothers of infants with CM allergies contained lower levels of LNFP I, LNFP III, 6'SL, and DSLNT than those in the milk of mothers of nonallergic infants (Seppo et al. 2017).

The effect of HMOs on infant growth may be either positive or negative depending on the structure and abundance of the HMOs concerned (Alderete et al. 2015; Davis et al. 2017). 2'-FL and LNnT were positively and negatively associated with weight velocity from 0 to 5 months, respectively (Larsson et al. 2019). However, others did not observe a significant difference in growth up to 4 months of age, in infants consuming HM with low or high fucosylated HMOs concentrations (Sprenger et al. 2017). The correlation between HMO concentrations and weight of infants fed with milk from Se⁺/Le⁺ mothers was found to be negative (Tonon et al. 2019). Sialylated HMOs were significantly less abundant in milk from Malawian mothers with severely stunted infants (Charbonneau et al. 2016).

Clinical trials on supplemented infant formula

Since the end of the 19th century, formula manufacturers have focused on delivering innovative products that more closely replicate the nutrient composition of human milk and its functional properties, in that order.

Trials with non-human OS

Since the production of synthetic HMO-like structures is challenging, many infant formulas were initially supplemented with different types of non-human oligosaccharides, including galacto-oligosaccharides (GOS), fructo-oligosaccharides (FOS), polydextrose or mixtures of these OS (Vandenplas, Zakharova, and Dmitrieva 2015). Although mixing GOS and FOS increased the number of bifidobacteria and lactobacilli, only limited evidence of significant clinical benefits was observed (Akkerman, Faas, and de Vos 2019; Kajzer, Oliver, and Marriage 2016; Vandenplas, Zakharova, and Dmitrieva 2015). While open trials indicated a decrease in the incidence of gastroenteritis, upper respiratory infections, fever episodes, and antibiotics usage (Arslanoglu et al. 2008; Bruzzese et al. 2009), randomized clinical trials (RCT) failed to confirm these results, because neither a decrease in the number of fever episodes (van Stuijvenberg et al. 2011) nor a decrease in the number of diarrhea episodes or prescriptions for antibiotics (Sierra et al. 2015) was observed. This last study, using only GOS, failed to show a decrease in the incidence of allergic manifestations up to 12 months of age in the supplemented group. However, a mixture of GOS/FOS was previously shown to significantly reduce plasma levels of total IgE, IgG1, IgG2, and IgG3 without affecting IgG4 (van Hoffen et al. 2009).

Infants fed a formula supplemented with bovine milk OS and probiotics (*Bifidobacteria lactis* or *Bifidobacteria longum*, and *Lactobacillus rhamnosus*) showed no difference in growth but frequently excreted softer stools, containing more bifidobacteria and lactobacilli than the controls (Meli et al. 2014; Radke et al. 2017; Simeoni et al. 2016). The

incidence of diarrhea and febrile infections was similar in both groups (Radke et al. 2017).

Trials with HMOs

At present, biotechnological means enable the production of commercially available HMOs which can be added to IFs (Mano et al. 2018; Zeuner et al. 2019). However, the composition and abundance of HMOs in HM have not yet been reproduced (Nijman et al. 2018). Considering all different aspects of the findings summarized above, including structure-function relationships, it appears that reproducing the beneficial effects of breast milk remains a considerable challenge.

Currently available HMOs are generally small (tri- and tetrasaccharides) and structurally simple compounds, which represent only a small portion of the HM glycome (Newburg 2013). The few clinical studies that have been conducted on HMO supplemented formulas were essentially focused on growth and tolerance (Table 2) and showed normal growth and no deleterious secondary effects associated with their use (Marriage et al. 2015; Puccio et al. 2017; Reverri et al. 2018). Two recent studies involved RCT using HMOs supplemented IFs (Table 2). The first investigated immune outcomes in human infants fed a formula containing 2'-FL. The results were similar to that for infants fed HM, while the group receiving a formula supplemented with only GOS showed a different result (Goehring et al. 2016). The other study described low level, parent-reported instances of bronchitis, respiratory tract infections and medication use in infants fed a formula supplemented with 2' FL and lacto-N-neotetraose (LNnT) (Puccio et al. 2017). More recently, the microbiota changes of the supplemented infants enrolled in this study were characterized (Table 2) (Berger et al. 2020).

On the basis of the above reports, these 2 HMOs, 2' FL and LNnT, are considered as safe by the European Union as well as by the US and approved for use as food (Salminen 2017). These have already been added to some IFs in several countries.

Conclusion and future perspectives

The abundance and complexity of HMOs in human milk are unique. Findings of in vitro experiments and animal model trials as well as observational studies on breastfed infants, reported so far, strongly suggest that HMOs may exert specific benefits on infants via various mechanisms based on the specific structures of these HMOs. HMOs play a key role in promoting healthy microbial diversity, preventing pathogen adhesion and replication, inducing maturation of intestinal mucosa, binding specific receptors, modulating immune cells and producing cytokines, leading to the development of neonatal immune systems. Interpretation of these actions in the absence of HM, which is a dynamic source of other bioactive factors and cells, remains a matter for debate. Large differences in the total amounts and patterns of individual HMOs that could be attributed to differences

in the genetic backgrounds of the mothers, are present. This raises the issue of whether all milks are equivalent in terms of beneficial effects on infant health. Nevertheless, the beneficial effects of HMOs provide a powerful rationale for women to breastfeed their infants.

The beneficial effects of HM are obviously not shared by infants fed with unsupplemented IFs. So far, attempts made using GOS/FOS or bovine milk OS have not been convincing at a clinical level. Recent clinical studies have demonstrated a great potential for biotechnological oligosaccharide production. Only two neutral HMOs, 2'-FL and LNnT, representing a very small portion of the HM glycome, have been subjected to clinical intervention studies. The number of RCTs that evaluated the effect of these HMOs on infant health are even scarcer, generating relatively limited evidence of the potential preventive effects of supplemented IFs with one or both of these HMOs against infectious diseases. Although studies highlighting the prophylactic effects of DSLNT on NEC occurrence in VLBW infants are promising, trials testing the benefits of adding DSLNT to formulas have not yet been conducted. Industrial scale production of DSLNT is probably more complicated than that of simpler HMOs. This emphasizes the need for developing inexpensive methods for large-scale production of HMOs, via chemical, enzymatic, or cell-based systems. It is felt that further studies, especially RCTs, are needed to substantiate the claim that HMO supplementation is a step forward in the direction of improving IFs in such a manner as to mimic the beneficial effects of breastfeeding. Future HMO research should also be focused on elucidating the structure and functions of complex and long chain HMOs. Once the best HMOs, or combinations of HMOs, are determined, the possible utility of adding probiotics, in particular bifidobacteria, to IFs may need to be investigated. The prospect of conducting such studies would be fascinating as well as exciting and fulfilling to researchers and clinicians.

Disclosure statement

No potential conflict of interest was reported by the authors.

Abbreviations

2'-FL	2'-fucosyllactose
3-FL	3-fucosyllactose
3'-SL	3'-sialyllactose
6'-SL	6'-sialyllactose
CM	Cow's milk
DFL	Difucosyllactose
DFLNT	Difucosyllacto-N-tetraose
DSLNT	Disialyllacto-N-tetraose
FOS	Fructo-oligosaccharides
FUT	Fucosyltransferases
GOS	Galacto-oligosaccharides
HM	Human milk
HMOs	Human Milk Oligosaccharides
IF	Infant formula
LNDFH	Lacto-N-difucohexaose
LNFP	Lacto-N-fucopentaose
LNnT	Lacto-N-neotetraose
LNT	Lacto-N-tetraose
NEC	Necrotizing enterocolitis

OS	Oligosaccharides
RCT	Randomized clinical trials
SCFA	Short chain fatty acid
VLBW	Very low birth weight infants

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