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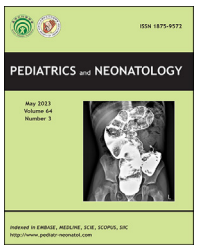

Review Article

Human milk oligosaccharides as prebiotics

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Based on its richness in immune-related components such as human milk, human milk oligosaccharides (HMOs), milk proteins, and lipids, breast milk can be considered the first functional food that humans encounter in their lifetime. According to WHO recommendations breast milk has to be the only food in an infant's diet in the first six months of age which is then continued up to two years of age with the suitable complementary foods. Regarding breast milk balanced composition, it is considered as the best food of infants thus many studies have been carried out to determine the benefits of breast milk. Based on numerous studies breast milk have a tendency to reduce the risk of type 2 diabetes, obesity, allergies, celiac disease, necrotizing enterocolitis (NEC), gastrointestinal tract infections and some type of cancers. The benefits of breast milk can be explained by its special combination which includes; macronutrients, micronutrients and bioactive components such as immunoglobulins, hormones, growth factors and oligosaccharides. One of the essential bioactive compounds of breast milk is known as human milk oligosaccharides (HMOs). HMOs are unique, bioactive carbohydrates which are identified as the most significant components of breast milk. Since they have structural complexity and multifunctional properties, they are one of the most wondered components of breast milk. HMOs promote the development of the neonatal intestinal immune, and nervous systems. This article briefly describes the history, complex structure and different functions of HMOs and highlight the importance of maternal diet for HMO biosynthesis.

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1. Introduction

Human milk is considered to be an excellent food for infants.[1,2] According to the World Health Organization (WHO), breast milk must be the sole nutrition source for newborns up to six months of age, and then breastfeeding has to be continued until two years of age while appropriate complementary foods are included in the infant's diet.[3] Since breast milk has balanced composition and energy, it is considered to be the ideal food for infants. In this context, human breast milk was found to have an inverse association with the development of type 2 diabetes and obesity in later life. Also, it has been stated that human milk reduces the risk of developing allergies, celiac disease, necrotizing enterocolitis (NEC), gastrointestinal tract infections and childhood cancers.[4,5] The first two years of a child's life are particularly important, as optimal nutrition during this period lowers morbidity and mortality, reduces the risk of chronic disease, and fosters better development overall. Optimal breastfeeding is critical, for it may save lives, especially among those who are under five years of age. Based on WHO data, many infants and children do not receive optimal feeding. For example, only about 44% of infants aged between zero to six months (0—6 months) worldwide were exclusively breastfed over the period of 2015—2020.[3]

Positive outcomes of breastfeeding are attributed to the milk's unique ingredients. Human milk contains macronutrients and micronutrients such as carbohydrates, proteins, lipids, vitamins and minerals, and all these compounds are in acceptable amounts for infants. Furthermore, breast milk contains bioactive components such as immunoglobulins, hormones, growth factors and oligosaccharides.[1,2,6] Bioactive factors are components which affect body function and health while they impact biological processes in the body.[6] The bioactive molecules of breast milk support immune system development, and breast milk also increases intestinal barrier function nd balances microbiota composition in infants.[7] One group of the essential bioactive compounds of breast milk are known as human milk oligosaccharides (HMOs). HMOs are unique, bioactive carbohydrates which are identified to be the most significant components of breast milk. Since they have structural complexity and multifunctional properties, these are one of the most effective components of breast milk.[1] Breast milk contains high amounts of HMOs whereas cow milk HMOs are at trace levels. While the number and precise composition of HMOs vary depending on time of lactation, the genetic makeup of mothers and potential environmental exposures, human breast milk contains an average of 5—15 g of oligosaccharides per liter, making HMOs the third most abundant solid component of breast milk after lactose and lipids.[8] Approximately 100 different types of HMOs have been identified in human milk; however, fewer than 50 are present in significant amounts. The most abundant HMO in the majority of mothers' breast milk is 2′-fucosyllactose (2′-FL), a trisaccharide consisting of glucose, galactose, and fucose. Up to date, the two large international analyses about HMOs in human milk have been conducted. A study which included 400 lactating women from 10 countries concluded that 85% of human milk samples had detectable 2′-FL at concentrations of 0.06—4.65 g/L.[9] The other large study found similar results from 410 lactating women from 11 international cohorts with 65—98% of human milk samples having 2′-FL with mean concentrations ranging from 0.702 to 3.440 g/L.[9] HMOs were not included in the past due to the expense and difficulty of synthesis.[10] However, 2′-FL HMO is now available in some commercial infant formulas.[1]

HMOs have been proven to have additional benefits for the developing neonate. HMOs can regulate neonatal immunity by altering host epithelial and immune cell responses in the infant gut, alter immune responses systemically, or act as soluble decoy receptors to block the attachment of various microbial pathogens to cell surface receptors. HMOs are therefore a very significant component of breast milk. They contribute to the development of the infants' microflora and immune system. Acting with various mechanisms, they offer protection against many infections and alleviate their course. They have been shown to have anti-bacterial, anti-viral and anti-inflammatory effects. Up to 6 months, intake of only breast milk is vital as it protects the health and life of infants (Fig. 1).[1] The benefits of HMOs may extend beyond infancy to development of allergies or cognitive functions, making them the focus of intense current scientific research, with a growing number of studies revealing their role in human physiology.[8]

This review summarily explains the complex structure and the beneficial effect of HMOs; their prebiotic properties and relation to maternal nutrition are particularly emphasized.

2. Concentration, structure and variation of HMOs

Compared to other mammalian milk, the oligosaccharides in human breast milk occur in more complicated structure and in a greater quantity.[11] After lactose and lipids, the third most largest biomolecules of human breast milk are HMOs, which represent roughly 20% of whole carbohydrate concentration of breast milk.[12] The highest HMOs concentration is found in colostrum, or the syrup-like, yellowish fluid that is secreted before and a few days after parturition. Colostrum contains 20—25 g/L of HMOs and as milk maturates the HMOs content of breast milk reduces to 5—20 g/L.[10] However, despite the reduction in the HMOs caused by maturation, HMOs concentration still eclipses concentration of total milk proteins.[10]

Differences can be observed in terms of the size, structure and complexity of HMOs.[13] Besides the structural variations, there are five main monosaccharide building blocks which HMOs are composed of. The five monosaccharide building blocks of HMOs are as follows: D-glucose (Glc), D-galactose (Gal), N-Acetylglucosamine (GlcNAc), L-fucose (Fuc) and sialic acid (Neu5Ac).[14] By different glycosidic linkages, diverse types of HMOs come from those monosaccharide blocks. Lactose core is carried at the attenuated end of all HMOs, which is elongated by insertion of one of two disaccharides, either Lacto-N-biose (Galβ1-3GlcNAc-) or N-acetyllactosamine (Galβ1-4GlcNAc-) via a β1-3 (linear chain structure) or β1-6-linkage (branched-chain structure).[10,14] Lacto-N-biose addition to the end of the lactose core is considered type-1-chain elongation

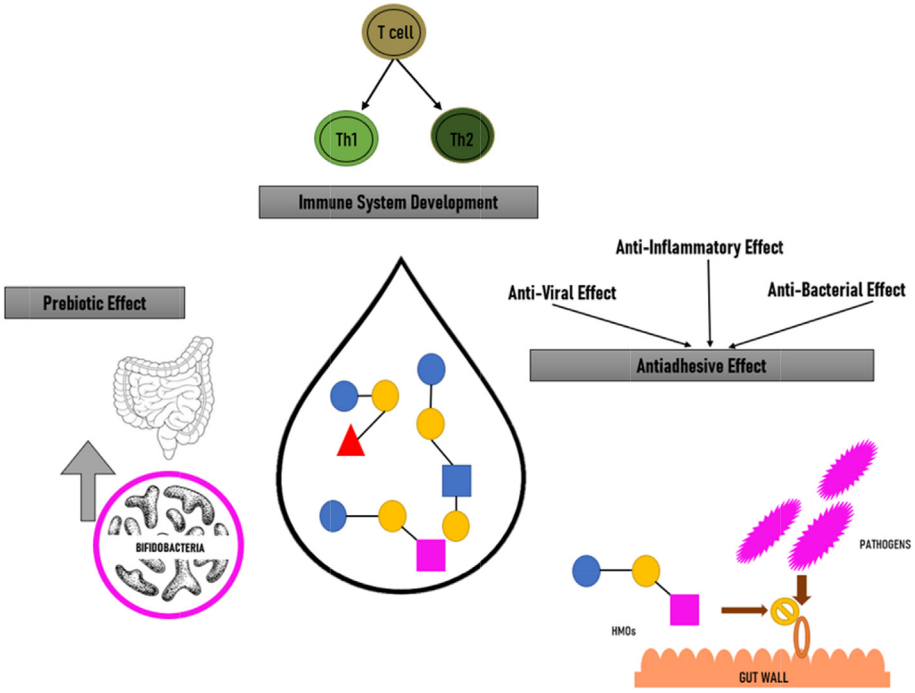

Fig. 1 Physiological health benefits of HMOs.

whereas N- acetyllactosamine extension is considered type-2-chain elongation. Type-2-chain can be further elongated by the addition of one of these two disaccharide units by β1-3 or β1-6. Furthermore, another structural diversification of HMOs occurs by fucosylation or sialylation. Lactose or the extended oligosaccharide chain can be fucosylated by the addition of fucose residue via α1-2, α1-3 or α1-4 linkage or/and sialylated by the addition of sialic acid residue via α2-3 or α2-6 linkage to the terminal points.[10]

In mature breast milk, 35–50% of HMOs are fucosylated, 12–14% are sialylated, and 42–55% are nonfucosylated neutral HMOs.[15] Composition of HMOs is based on maternal genetics, so mother's milk may individually contain different types of HMOs. This alteration is based on the polymorphisms in two genes: Fucosyltransferases Secretor (FUT2) and Lewis (FUT3). All fucose-borne HMO types can be synthesized by a woman when both FUT2 and FUT3 genes are active. On the other hand, a woman with passive FUT2 gene is not able to synthesize α1-2 fucosylated HMOs (for instance; 2′-fucosyllactose [2′-FL] and Lacto-N-fucopentaose I [LNFP I]) and a woman with inactive FUT3 is unable to synthesize α1-3 or α1-4 fucosylated HMOs (such as Lacto-N-fucopentaose II and III). As a result of these polymorphisms, four milk groups can be specified:1) Lewis positive secretors (Le + Se+); 2) Lewis negative secretors (Le−Se+); 3) Lewis positive non-secretors (Le + Se−); and 4) Lewis negative non-secretors (Le−Se−).[13] Different milk groups are shown in Table 1.[1]

Table 1 Group of mothers with different fucosylated HMO types.[1]

Lewis Positive Secretors (Se+Le+)	FUT2 Active	FUT3 Active
Lewis Negative Secretors (Se+Le−)	FUT2 Active	FUT3 Inactive
Lewis Positive Nonsecretors (Se−Le+)	FUT2 Inactive	FUT3 Active
Lewis Negative Nonsecterors (Se−Le−)	FUT2 Inactive	FUT3 Inactive

A study which included 410 healthy, lactating women from 11 different cohorts concluded that geographical differences could cause variations in HMOs content among healthy women.[16] Nevertheless, maternal genetics is not the only aspect which causes variations in the HMOs content among women.[17] A prospective observational study which included 427 women determined that multiple factors, such as the stage of lactation, parity, ethnicity, city of residence, breastfeeding exclusivity and milk collection season, could cause changes in HMOs concentration.[18]

3. HMOs as prebiotics

The first prebiotic definition was made by Glenn Gibson and Marcel Roberfroid in the 1990s as "a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health."[19] However, based on this explanation, limited types of structures such as lactulose, galactooligosaccharides (GOS) and short/long-chain β-fructans (Fructo-oligosaccharides (FOS) and inulin) are classified as prebiotics.[19]

In 2008, the new definition of prebiotics was agreed in the Sixth Meeting of the International Scientific Association of Probiotics and Prebiotics (ISAPP). ISAPP explained "dietary prebiotics" as "a selectively fermented ingredient

that results in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health". There are 3 main criteria that a compound should meet to be considered as a prebiotic: (i) it has to be resistant to the gastric acidity, mammalian enzymes and gastric absorption; (ii) should be fermented by microbiota in the intestines; and (iii) it improves host's health indirectly by having a beneficial effect on the growth and/or activity of intestinal bacteria.[19]

According to the definition of prebiotics, HMOs should be considered as one of the most significant prebiotics since they acted as prebiotics before the recognition of that term. There was a mandatory criterion to accept HMOs as prebiotics to the effect that HMOs must be a non-digestible type of carbohydrate.[20] HMOs are resistant to gastrointestinal digestion in host infants, and so the majority of HMOs reach the colon, where they may serve as prebiotics to form a healthy gut ecosystem by stimulating the growth of beneficial microorganisms and by acting as receptor analogues to inhibit binding of pathogens and toxins to epithelial cells.[21]

An *in vitro* study was done to ascertain whether HMOs were resistant to gastrointestinal system hydrolysis.[22] In that study, maltodextrin, which hydrolyses rapidly, was used as the control substrate since HMOs resist hydrolysis in pancreatic juice and brush border membrane. In the study findings, most HMOs reached the large intestine as substrate for bacterial metabolism and they were selectively fermented by intestinal microflora.[18,22] It is clear that the majority of HMOs are resistant to acidic pH and that they cannot be hydrolysed by enzymes in the pancreas and brush margins.[23] Following gastrointestinal absorption, up to one percent of HMOs become available in systemic circulation at a level (ranging 10—100 mg/mL) which is sufficient for interaction with cell receptors.[23] When all these features are evaluated, HMOs are to be considered as prebiotics.[24]

HMOs are essential to construct and preserve the infant's gastrointestinal microbiota, which is important for general health.[25,26] Compared to non-breastfed infant gut, *Bifidobacterium* species are the most dominant colonizers in breast-fed infants and they commonly make up 50—90% of the bacterial population in faeces. Higher amounts of *Bifidobacterium* species population in breasted infants are attributed to their ability to consume the prebiotics in human milk, which is to say HMOs.[13] According to the literature, HMOs have numerous functions, including: selectively enriching gut bacteria and thereby promoting healthy microbiota composition; preventing pathogen adhesion to epithelial cells; enhancing maturation of intestinal mucosa and intestinal epithelial barrier function; modulating immune cells, pathogen recognition receptors and signalling pathways related to maturation of lymphoid tissue; influencing cytokine and chemokine networks that regulate T helper 1/T helper 2 (Th1/Th2) lymphocyte balance; and preventing infection and supporting immunity.[1,14,27—29]

As prebiotics, HMOs are essential for infant health because they promote the growth of *Bifidobacterium* species.[13] The consumption of HMOs by *Bifidobacteria* in the infant gastrointestinal tract increases the benefit of *Bifidobacteria* to overcome other pathogenic microorganisms, so infants can be protected against infectious diseases.[30] As Asakuma et al. demonstrated, some but not all species/subspecies of Bifidobacterium are equipped with genetic and enzymatic sets dedicated to the utilization of HMOs, and can grow on HMOs; however, the ability to metabolize HMOs has not been directly linked to the actual metabolic behavior of the bacteria.[21] Regarding the type of *Bifidobacteria,* as shown by an *in vitro* study, *Bifidobacterium longum* subsp. *infantis, Bifidobacterium bifidum,* and *Bifidobacterium breve* utilize a high percentage of purified HMOs,[21,31] whereas *B. longum* subsp. *longum* and *B. longum* subsp. *suis* strains were found to exhibit low HMO utilization.[31,32] Also, *Bifidobacterium adolescentis* and *Bifidobacterium animalis* were incapable of HMO utilization. Moreover, it has been shown that HMO usage by other gut microbiota members such as *Clostridium, Enterococcus, Escherichia, Eubacterium, Lactobacillus, Staphylococcus, Streptococcus, and Veillonella species pluralis* is limited or nil.[32]

It is unclear which HMO type best affects the growth of Bifidobacteria species. Several studies have shown that individual HMOs may have different health effects according to their composition.[33,34] For instance, Cheng et al. reported that since 3-FL and 2′-FL have the same molecular composition *B. longum* subsp. *infantis* can grow quickly on 3-fucosyllactose (3-FL) and reach greater cell densities on 2′-FL.[33]In contrast, *B. breve-M-16V* was reported to be a good consumer of lacto-*N*-tetraose (LNT) and a poor consumer of 3-FL, 2′-FL, 3′-siallylactose (3′-SL), 6′-siallylactose (6′-SL), and lacto-*N*-neotetraose (LNnT).[34] Since small quantities and few types of HMOs can be synthesized and added to infant formulas, it is essential to determine which type of HMOs supports the growth of individual gut bacteria. Further studies are needed to specify which HMO type is optimal for the growth of specific Bifidobacteria.[33]

4. Impact of maternal diet on HMOs composition

It is known that maternal diet affects breast milk composition.[35] Particularly fatty acid and vitamin contents of breast milk were found to be influenced by maternal diet. However, the direct effect of a single nutrient on the composition of human milk has not been studied, so the exact impact of mother's diet on breast milk is unknown.[31] The knowledge gap regarding maternal diet and HMOs has attracted study. The impact of maternal diet on HMOs has attracted particular interest.[6] As shown in Table 2, studies which examined the maternal nutrition and the type of HMOs in breast milk yielded different results. Two separate single-blinded cross-over dietary intervention studies were performed to evaluate the impact of maternal diet on human milk.[36] In the first study (Glucose/Galactose Cohort), lactating women eight to eleven weeks postpartum were randomly assigned to receive a liquid, isocaloric, isonitrogenous drink containing either glucose or galactose as the sole carbohydrate source for a total of 30—57 h. The sugar treatment provided 60% of their daily estimated energy requirement, and a standardized mixture of protein and essential fatty acids was provided to supplement treatment. Milk samples were collected, followed by a one-to two-week washout period for each woman. In the second study (Carbohydrate/Fat Cohort), seven different lactating women nine-to twelve-weeks

Table 2 Studies which examined the maternal nutrition and the type of HMOs in breast milk.

Reference	Aim of the study	Study Design	Population and sample number	Main Outcomes
Seferovic MD et al.[36]	To directly examine the effect maternal diet on HMO composition.	A single-blided cross-over controlled feeding	n = 7 Glu/Gal Cohort, n = 7 Carb/ Fat Cohort.	A high fat diet resulted in a decrease in the concentration of HMO-bound sialic acid, and a higher glucose versus galactose diet affected the concentration of HMO-bound fucose.
Quin C et al.[37]	How maternal diet affects HMO biosynthesis and how diet-induced HMO changes affect infant gut microbiome and immunity.	A prospective cohort clinical study	n = 16 breast milk samples from healthy Euro-Canadian mothers, separated into two groups classified as milk- or almond beverage-consumers (1 sample per mother).	Maternal diet affects the biosynthesis of HMOs and in turn can affect the bacterioma and immune development of their infants. Fruit intake and unsaturated fatty acids in breast milk positively affect the abundance of several HMOs, including 16 sulfonated HMOs.
Azad et al.[12]	To detect modifiable (ex. multivitamin intake) and nonmodifiable factors linked with HMO concentrations	Prospective observational study	n = 427 breast milk samples from each Canadian multi-ethnic mothers with healthy term infants (1 sample per mother).	Few of the modifiable factors examined were found to be linked with the concentration of HMOs. Multivitamin intake was found to be associated with higher DSLNH levels.
Jorgensenet al.[39]	To determine whether lipid-based nutrient supplements (LNSs) affect milk bioactive protein and HMO concentrations at 6 months of postpartum.	Randomised, single blind, parallel group controlled supplementation trial.	n = 645 breastmilk samples from Malawian women.	The study findings concluded that nutrient supplementations during pregnancy and postpartum would not increase HMO or bioactive milk proteins at 6 months of postpartum.
Seppo et al.[40]	To observe the relationship between maternal probiotic supplementation during pregnancy and HMOs composition	A double-blinded placebo-controlled study	n = 81 colostrum samples from pregnant women carrying children at great risk for allergy from the Helsinki suburban area. (1 sample per mother)	Two types of HMOs were found to be higher in the probiotic supplemented group (3-Fucosyllactose and 3′-Sialyllactose). The level of some HMO types was found to be lower in the probiotic supplemented group including Difucosyllacto-N-Hexaose, LNnT, LNFP, 6′-Sialyllactose
Qiao Y et al.[41]	To determine the potential effect of vitamin A intake on breast milk sialic acid levels	cross-sectional observational study	n = 90 breast milk samples from healthy Chinese women (1 sample per mother)	Higher dietary consumption of vitamin A was found to be associated with higher milk sialic acid levels

postpartum were randomly assigned to receive either a high fat (55% fat, 30% carbohydrate, 15% protein) or carbohydrate (25% fat, 60% carbohydrate, 15% protein) diet for eight days consisting of packed meals with isocaloric, isonitrogenous nutrient compositions. The milk samples collected at the completion of each dietary treatment were used for analysis. The mothers' consumption of different carbohydrate sources did not change the energy and macronutrient content of breast milk, but the high-fat diet was related to an increase in the fat and energy content of breast milk. Correspondingly, both dietary interventions resulted in a significant change in the HMO profile across paired samples. In the Glucose/Galactose Cohort, the concentration of total HMO-bound fucose was reduced in the glucose diet relative to the galactose diet, while no single fucosylated HMO was statistically different between diets; this was expected given the high numbers of individual HMO species and the relatively small cohort size. In general, individual HMO increased as much as 50% with galactose and 30% with high fat diet, while other HMOs were remarkably stable. In the Carbohydrate/Fat Cohort, the concentration of total HMO-bound sialic acid was reduced in the high fat diet relative to the carbohydrate diet, though no individual sialylated HMOs were statistically different between diets following in the subjects paired samples which were available for analysis.[36] Another study was conducted to investigate the effect of maternal diet on HMOs biosynthesis and determine how diet-induced HMO changes affect infant gut microbiome and immunity.[7] HMOs measured from breast milk samples correlated with maternal dietary consumption obtained through validated 24-h diet recall questionnaires and breast milk fatty acids. The study found that fruit intake and unsaturated fatty acids in breast milk were positively associated with increased absolute abundance of numerous HMOs, including the 16 sulfonated HMOs that we first identified in humans here. The diet-derived monosaccharide 5-N-glycolyl-neuraminic acid was clearly detected in all samples. To gain insight into the potential impact of Neu5Gc on the infant microbiome, the study used a constrained coordination approach, and Neu5Gc levels and Bacteroides spp. in infant faeces. However, Neu5Gc was not associated with significant changes in infant immune markers, unlike sulfonated HMOs, the expression of which correlated with suppression of two major Th2 cytokines, interleukin-10 and interleukin-13. The findings of this study highlight the importance of maternal diet for HMO biosynthesis and provide as yet unexplored targets for future studies investigating interactions between HMOs and the gut microbiome and immunity in infants.[37]

Three studies have been carried out to investigate the association between dietary supplements (nutrient or probiotic) and HMO composition.[38–40] Azad et al. showed that only Disialyl-lacto-N-hexaose (DSLNH) was significantly higher in breast milk of mothers reporting taking multivitamin supplements.[38] Jorgensen et al., in their large-scale interventional study related to nutrient supplementation and HMOs, hypothesized that nutrient supplementation during late pregnancy would influence HMO levels, which might be related to improved overall nutritional status. The same study also found that African mothers with suboptimal nutritional intake given lipid-based nutrient supplements demonstrated no effect on HMO levels. In that study, 647 pregnant women had either micronutrient capsules containing 18 micronutrients or a 20g-dose of a high-energy, micronutrient-fortified lipid-based nutrient supplement with the same 18 micronutrients and 4 additional minerals (2.6 g protein, and 10 g fat to provide 118 kcal of total energy). Neither intervention resulted in an increase in HMO levels at 6 months postpartum. However, no data were collected about the adequacy or quality of the mothers' diet, so effects of other dietary factors could not be excluded. Since the participants in this study generally had lower socioeconomic status and lower BMIs, it is thought that the effects of supplementation may still be likely in energy-replete, well-nourished individuals.[39] Furthermore, the relationship between maternal probiotic supplementation during pregnancy and HMOs composition was examined in a double-blinded placebo-controlled study. Eighty-one women were randomly selected for colostrum test. According to the results, two types of HMOs were found to be higher in the probiotic-supplemented group (3-Fucosyllactose and 3′-Sialyllactose). On the other hand, the level of some HMO types was found to be lower in the supplemented group including Difucosyllacto-N-Hexaose, LNnT, LNFP, and 6′-Sialyllactose.[40] Moreover, in a cross-sectional observational study, the potential effect of vitamin A intake on breast milk sialic acid levels was verified. According to the results, higher dietary consumption of vitamin A was found to be associated with higher sialic acid levels.[41] Overall, studies evaluating maternal dietary intake or supplementation and association with HMO volume and profiles are scarce. The variation in assessment methods is too great to allow for overall conclusions. Dietary intake and quality during pregnancy do not appear to have a significant effect, whereas some individual dietary components and supplements may be effective in the short-term or postpartum period. Most of the included studies had small samples, which also limits the ability to draw any significant conclusions.[42]

5. Conclusion

It can be determined that HMOs are key components in breast milk with diverse functional benefits. Prebiotic function is one of HMOs most emphasized issues. HMOs are considered prebiotics because they resist acidic pH and enzymatic degradation and reach the large intestine intact to support microbial growth. As prebiotics, HMOs promote the growth of some specific species of Bifidobacteria necessary to overcome pathogens thereby preventing infectious diseases. One reason why breast milk plays an important role in the first two years of life of babies is oligosaccharides. The health benefits of HMOs may be greater than currently thought, so more studies should be conducted by multidisciplinary teams of chemists, paediatricians, nutritionists, and microbiologists. Also, research should focus on the effect of maternal diet on HMOs composition. When the factors affecting the composition of HMOs are fully understood, it will be possible to facilitate the synthesis of HMO-like structures to enrich infant formulas.

Transparency declaration

The lead author affirms that this manuscript is an accurate and transparent account of the study being reported.

Declaration of competing interest

None declared.

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