Early Life Nutrition
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Human Milk Oligosaccharides

Inspired by Nature, Supported by Science

dsm-firmenich

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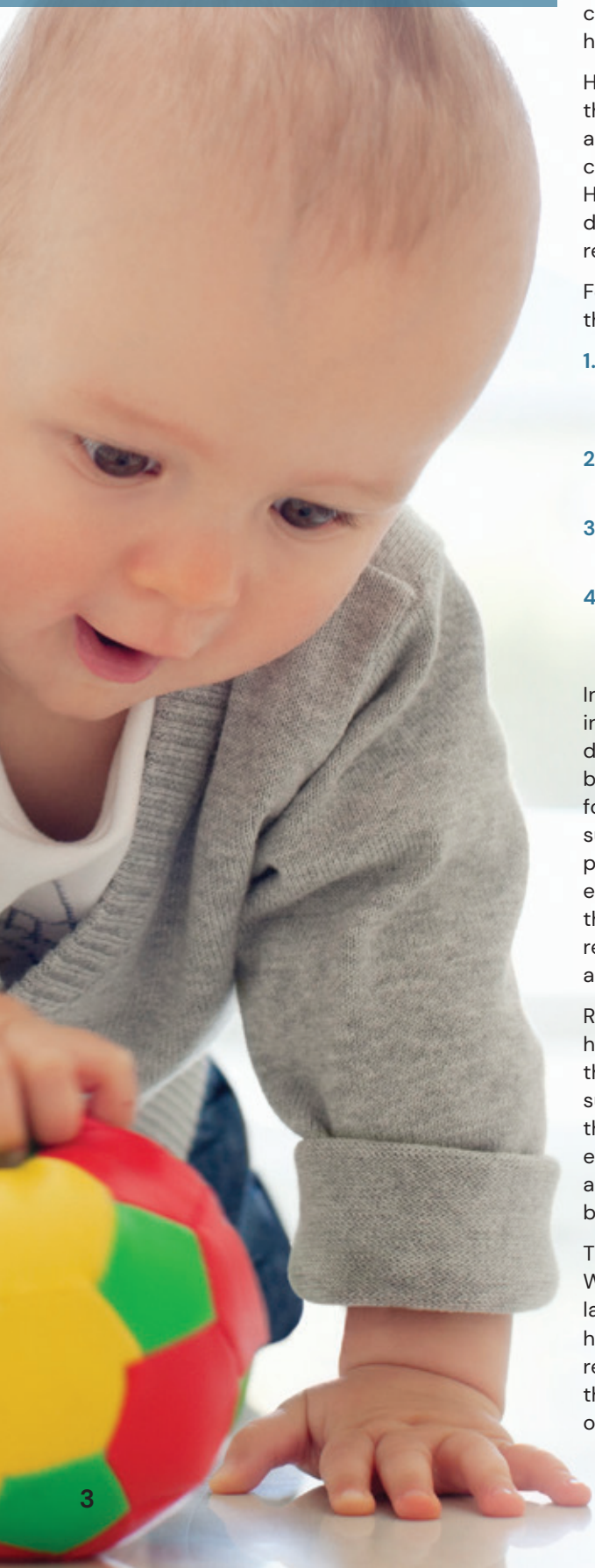
1 | Executive Summary

Human milk is the gold standard in infant nutrition, providing ideal nourishment to support an infant during the early stages of life.[1-4]



Aside from the ability of human milk to promote healthy growth and development, it also provides bioactive compounds which are believed to influence the development of the immune system, gastrointestinal tract (GI) tract, microbiome, and the brain.[5-8] Human milk oligosaccharides (HMOs) are the most abundant non-digestible bioactive compounds in human milk and the third largest solid component of human milk after lipids and lactose.[8-11]

HMOs are made up of various combinations of five monosaccharides, though all structures begin with lactose.[12] There is incredible structural and functional diversity across HMOs, and each mother's HMO composition is unique.[13,14] Both genetic and non-genetic factors influence HMO composition. While more than 200 HMOs have been identified to date,[12,15] a recent study found that a relatively small number of HMOs are responsible for nearly 90% of the total HMO fraction in human milk.[16]

Four key mechanisms of HMOs are proposed and explored throughout this document:

1. **Support the growth of beneficial bacteria via a prebiotic effect and exert a positive influence on the development and maintenance of the gut microbiota[17-19]**
2. **Positively influence gut health and support intestinal barrier function[20,21]**
3. **Deflect adhesion of undesirable microorganisms to cell surfaces by acting as decoy receptors[22-24]**
4. **Impact the immune system systemically, directly modulating immune cell populations and impacting the inflammatory response[9,25,26]**

Infant formula is a breast milk substitute that undergoes continuous innovation. As the knowledge of the science of human milk evolves, so does infant formula, with the goal of bringing it closer to breast milk – both compositionally and functionally. Technology has recently allowed for HMOs to be added to infant formula, and research on formulas supplemented with HMOs is underway to identify how they might positively influence infant health. Clinical trials in infants to date offer evidence for the safety of HMOs,[17,27-30] their ability to positively impact the microbiome,[17,29] and their potential to support the immune response.[28,30,31] The concrete safety record of HMOs has led to regulatory approvals for their use in infant formula around the globe.

Research on the potential health benefits of HMOs has expanded beyond healthy, full-term infants as scientists seek to uncover the full range of their potential application. HMOs are being explored for their potential to support gut health in premature infants.[32-34] There is increasing interest in their roles in neurodevelopment and brain health.[35-37] HMOs are being explored for a possible role in allergy management in children.[38-40] In adults, HMOs are being studied for their ability to support gut health in both healthy adults and those with gastrointestinal diseases.[41-43]

There is still much to learn about the potential health benefits of HMOs. While the combination of preclinical and clinical studies have resulted in a large body of evidence describing their mechanisms of action, possible health benefits, and safety and efficacy, more research is needed to fully realize how best to leverage these uniquely human oligosaccharides. In the meantime, early and emerging data show great promise for the ability of HMOs to positively impact the health of infants, children, and adults.

2 | HMOs: Created by Nature

2.1 | Breastmilk: The Standard in Infant Nutrition

Human milk is the undisputed gold standard in infant nutrition, providing ideal nourishment to support an infant during the early stages of life.[1-4] It promotes healthy growth and development and provides many distinct bioactive compounds that help to support the developing immune system and contribute to a beneficial community of microbes in the gut.[6,8,44] The bioactives in human milk may partially explain the association between breastfeeding and lower rates of infectious diseases and mortality during infancy, as well as a reduced risk for the development of conditions such as obesity, inflammatory bowel disease (IBD), and type 2 diabetes in adulthood.[45-47] Leading global organizations, including the World Health Organization (WHO) and the United Nations Children's Fund (UNICEF), and scientific expert bodies such as the American Academy of Pediatrics (AAP) and European Society for Pediatric Hepatology, Gastroenterology and Nutrition (ESPGHAN), encourage that infants are fed human milk exclusively for the first six months of life, followed by continued breastfeeding with appropriate complementary foods for up to two years and beyond, as mutually desired by the mother and infant.[2-4,48]

Human milk has intrigued and inspired scientists for over a century. While the nutrient composition of human milk has been characterized over the last several decades, attention has recently turned to the biologically active substances in human milk that are believed to influence the development of the immune system, the gastrointestinal tract (GI) tract, the gut microbiome, and the brain.[5-8] HMOs are the most abundant non-digestible bioactive compounds in human milk.[8-10] The discovery of their dominance in breastmilk has generated a scientific pursuit to understand their role in human health.

2.2 | HMOs are Uniquely Human

HMOs are a group of non-digestible carbohydrates and the third largest solid component of human milk after lipids and lactose.[11] More than 200 HMOs have been identified to date.[12,15] The distinctive structure, concentration, and variety of oligosaccharides in human milk sets them apart from those found in the milk of other species.[15,49,50]

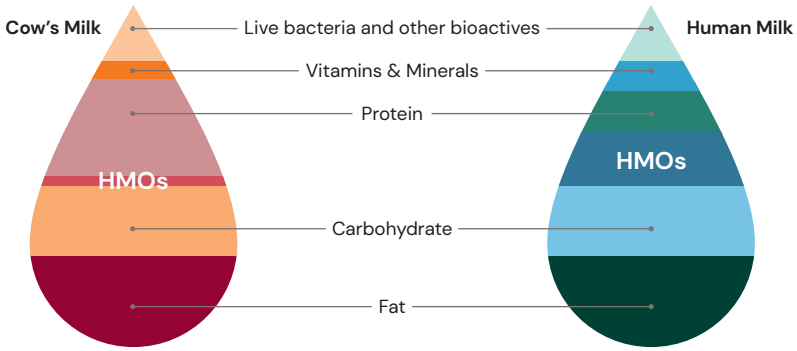


Figure 1: **Composition of cows milk vs. human milk**[12,15]

Human milk oligosaccharides are the third largest solid component of human milk.

It has been estimated that the production of HMOs by the mammary gland requires a considerable amount of the total energy expended for milk production.[51] The human body's investment in creating HMOs along with the prevalence of HMOs in breastmilk suggests these bioactive components likely play significant roles in infant development and overall health.[9]

Until recently, HMOs were only available to breastfed infants. As such, differences in certain health outcomes between formula-fed and breastfed infants – such as different microbiota composition, higher rates of infectious disease, and higher risk of diseases associated with inflammation such as asthma and atopy – may partly be explained by this key differentiating feature.[7,9,52,53] Technological advances have recently allowed for the addition of HMOs to infant formula. As clinical trials have been done to evaluate infant formulas supplemented with HMOs, evidence is accumulating that HMOs can positively influence infant health.[17,27,28,30]

2.2.1 | HMOs Contribute Substantially to Breastmilk Composition

Mature breastmilk – or milk produced between the second week to the third month of lactation – contains an average of 11.3 ± 2.6 g/L of HMOs.[16] Each mother's HMO concentration and composition is unique.[13] Variation between individuals occurs according to maternal genetics, geographical location, ethnicity, across the stages of lactation, and even within individuals over discrete breastfeedings.[49,54-56]

The concentration of HMOs in breastmilk is generally highest during the early stages of lactation and gradually declines with time, though there are exceptions.[57-60] For example, the HMO 3-fucosyllactose (3-FL) increases with time, rising 10-fold between the first and twenty-fourth month of lactation.[16,61]

2.2.2 | HMOs Offer Several Potential Functional Benefits

HMOs have been reported to positively influence infant health through a variety of mechanisms. These include positively modulating the microbiome, promoting intestinal barrier integrity, supporting immune system function, and reducing the incidence of infection.[12,17,28]

Once ingested, HMOs largely resist digestion and reach the intestines intact.[12,62] There, they serve as prebiotics, selectively supporting the growth of beneficial bacteria and supporting a community of healthy microbes in the gut.[17,63] Some preclinical and clinical data suggest the potential for HMOs to positively impact gut barrier function and help maintain a healthy immune system.[20,26,31,51] Emerging data also suggest HMOs may deflect the adhesion of undesirable microorganisms to cell surfaces by mimicking cell surface receptors.[22-24,64,65]

Scientists are also uncovering potential roles for HMOs in brain development and cognition.[36,37,66] Additionally, HMOs are being studied to explore how they might benefit children and adults.[41,43,67,68] Taken together, this wide variety of possible functional benefits opens the door for future innovations.

Key Takeaways:

1. HMOs are the most abundant indigestible bioactive molecules in human milk. The discovery of their high levels in breastmilk – the gold standard in infant feeding – has generated a pursuit around understanding their contributions to infant and human health.
2. As the third largest solid component of human milk, the prevalence of HMOs in breastmilk suggests this bioactive component likely plays a significant role in infant development and overall heath.
3. HMOs are thought to positively influence infant health through a variety of mechanisms, including supporting a healthy gut microbiome, contributing to immune system function, reducing the risk of infection, and positively impacting gut barrier function.

2.3 | The History and Discovery of HMOs

The scientific exploration of HMOs began over 130 years ago, starting with the recognition of breastmilk's unique benefits.[62,69] Late in the 19th century, a carbohydrate fraction specific to human milk was isolated. In parallel, a growth-promoting factor for *Bifidobacterium* was identified in human milk and referred to as the "bifidus factor."[12] Whether or not there was a connection between these two areas of research was yet unknown.

In the early 1930s, scientists were able to characterize the unique carbohydrate fraction that had previously been isolated in human milk. With this, the first individual HMOs were distinguished, initiating the beginning of real interest in these compounds.

Then, in the mid-1950s, a critical discovery was made – scientists were able to determine that HMOs promoted the growth of bifidobacteria, confirming that the "bifidus-factor" discovered earlier and HMOs were one in the same.[62,70] Subsequently, in the mid-to-late 20th century, some of the potential mechanisms of HMOs began to emerge from preclinical studies: promoting the growth of beneficial microbes, contributing to immune system function and reduced infection, and supporting gut health.[71]

The availability of HMOs for addition to infant formula and food products is a recent innovation, largely due to technological advances allowing for the commercial development of these unique substances. As research on HMOs continues, a growing body of evidence enhances our understanding of the health benefits of these oligosaccharides and shapes our modern understanding of HMO functionality.[9,52,62,72,73]

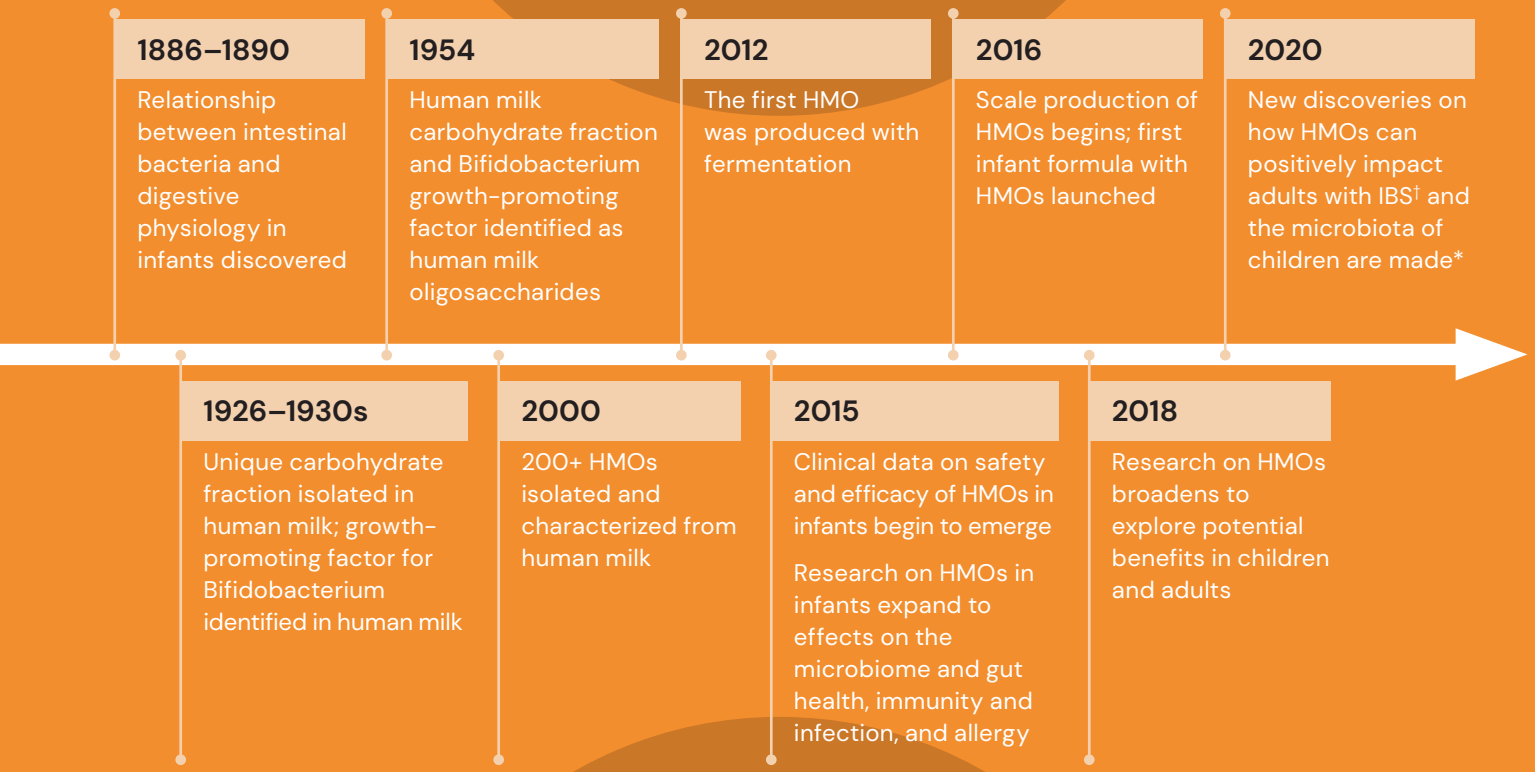


Figure 2: **Key events in the identification of HMOs and their functions**[12,17,27,28,42,43,62,67]

† Irritable bowel syndrome; * Overweight or obese children

3 | HMOs: an Overview of their Structure, Composition, and Complexity

3.1 | The Structure of HMOs

There are five monosaccharides that comprise the structure of HMOs: glucose, galactose, N-acetylglucosamine, fucose, and sialic acid.[12,74]

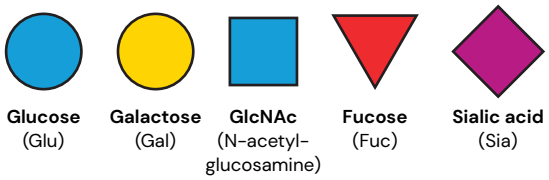


Figure 3: **The five building blocks of HMOs**

All HMOs begin with lactose, which consists of the monosaccharides galactose and glucose joined by a β-1,4-glycosidic linkage.

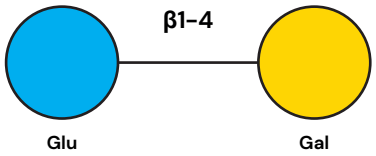


Figure 4: **The structure of lactose**

The lactose backbone is then extended or elongated by one or more of the four monosaccharides: galactose (Gal), N-acetylglucosamine (GlcNac), fucose (Fuc), or sialic acid (Sia).[11,74] The variety of monosaccharide(s) linked to the lactose backbone, the quantity and type of linkages used to build the structure, and the number of monosaccharide units added determine the specific HMO that is formed.[11,74,75]

There is remarkable structural diversity across the oligosaccharides in human milk.[14] The wide variety of HMOs can be grouped into three broad categories:[76]

- **Fucosylated (neutral) HMOs**
- **Sialylated (acidic) HMOs**
- **Neutral core HMOs**

Fucosylated HMOs make up the majority – approximately 70% – of the total HMO fraction in human milk. Sialylated HMOs (including both fucosylated and non-fucosylated acidic HMOs) make up about 20% of the HMO fraction, while neutral core structures make up the remaining 10%.[14,16,49]

The HMOs 2′-fucosyllactose (2′FL) and 3-FL are examples of fucosylated HMOs, 3′-sialyllactose (3′SL) and 6′-sialyllactose (6′SL) are among those belonging to the sialylated group, and lacto-N-neotetraose (LNnT) and lacto-N-tetraose (LNT) are two of the non-fucosylated neutral HMOs.

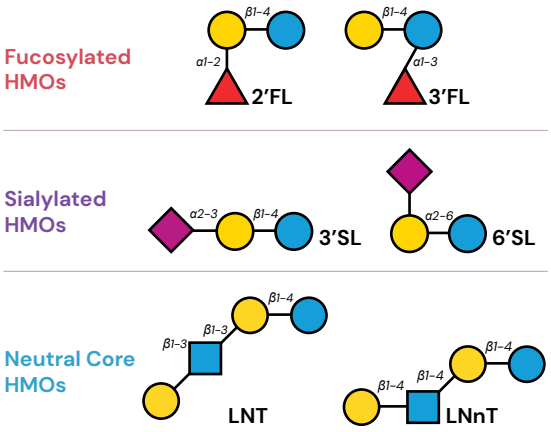


Figure 5: **Structure of HMOs from each of the three main HMO categories**

As stated above, the concentrations of HMOs in breastmilk vary according to their structural grouping. Figure 5 illustrates how the distribution of these varies in breastmilk according to the stage of lactation.[16]

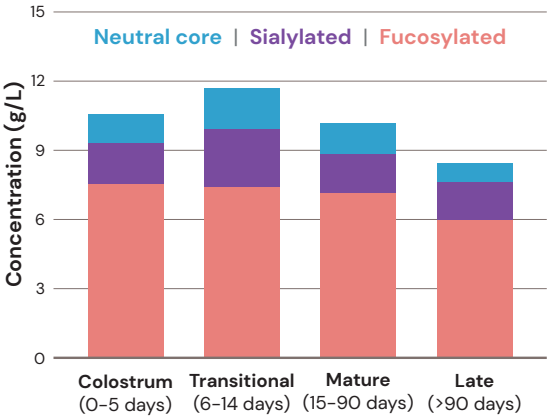


Figure 6: **Proportions of fucosylated, sialylated, and neutral core HMOs in human milk throughout lactation periods[16]**

Scientists are increasingly recognizing a fourth category of HMOs, acidic fucosylated HMOs. While the three broad categories named above are universally accepted for grouping HMOs, the suggestion that an additional category may be relevant reflects the increasing acknowledgment of their complexity and diversity.[49,77]

3.2 | HMO Composition: an Overview

3.2.1 | Genetic and Non-Genetic Factors Influence HMO composition

Genetics plays a significant role in HMO composition. Variety in HMO structures is largely determined by the expression of specific glycosyltransferases, or enzymes that catalyze the building of oligosaccharides by establishing glycosidic linkages.[12,14]

The documented variations in HMOs are partly dependent on a woman's Secretor (Se) and Lewis (Le) blood group status.[12,14,78] These characteristics influence the activity of two gene loci encoding for the α1-2 fucosyltransferase (FUT2, encoded by the Se gene) and the α1-3/4-fucosyltransferase (FUT3, encoded by the Le gene). The expression of Se and Le genes, and thereby activity of FUT2 and FUT3, are important factors in determining the structure and abundance of specific HMOs in a woman's milk.[12,14,16,25,72]

Women with an active Se locus express FUT2 and are classified as secretors. Worldwide, FUT2 is active in approximately 70-80% of mothers, with some variation depending on geography, race, and ethnic background.[56,79,80] The milk of secretor women contains high concentrations of α 1-2-fucosylated HMOs; 2'FL and lacto-N-fucopentaose (LNFP) I are some of the most abundant HMOs in the milk of secretor women.[58,72,75]

Women who are considered non-secretors do not express an active FUT2 enzyme, and their milk has low or undetectable levels of α1-2-fucosylated HMOs like 2'FL.[14,72] Both secretor and non-secretor women express FUT3, which catalyzes the addition of fucose in α1-3/4

linkages. Non-secretor mothers produce higher levels of Lacto-N-tetraose (LNT), LNFP II, LNFP III, and lacto-N-difucohexaose (LNDFH) II.[14,75] Interestingly, 3-FL can be produced by both secretors and non-secretors, however, levels tend to be higher in non-secretor women.[61]

Women can be separated into four milk groups, classified according to their Se/Le blood group and expression of FUT2 and FUT3 enzymes, as summarized in Table 1. While this classification system may be an oversimplification of HMO variety and complexity, it offers a framework for the general patterns of how HMOs appear in human milk.[12,14,81]

As stated earlier, factors aside from genetics also influence a woman's HMO composition, yet the mechanisms for how these factors are involved are not yet clearly understood. Nevertheless, observational studies have identified that both fixed and modifiable parameters are associated with HMO composition. Those that have been identified and explored include maternal environment, age, health status and diet, lactation period, pregnancy gestation, and the use of medications or probiotic supplements during pregnancy and lactation.[13,82-84] While intriguing, the science behind these associations is not well-established and further exploration is needed.

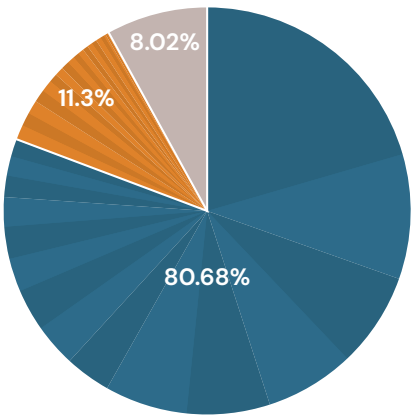
Table 1: **Milk Groups According to Se (FUT2) and Le (FUT3) Blood Group and Secretor Status**[12,14,16,81]

Milk Group	Classification		Se	Le	Fucose Linkages	Main HMOs Secreted	Estimated Frequency (globally)
1	Secretors	Lewis Positive	+	+	α1-2, α1-3, α1-4	2'FL, 3-FL, DFL, LNT, LNnT, LNFP-I, LNFP-II, LNDFH-I, LNDFH-II	70%
2	Non-Secretors	Lewis Positive	-	+	α1-3, α1-4	3-FL, LNT, LNnT, LNFP-II, LNFP-III, LNDFH-II	20%
3	Secretors	Lewis Negative	+	_	α1-2, α1-3	2'FL, 3-FL, DFL, LNT, LNnT, LNFP-I, LNFP-III	9%
4	Non-Secretors	Lewis Negative	-	-	α1-3	3-FL, LNT, LNnT, LNFP-III, LNFP-V	1%

3.2.2 | HMO Variability and Composition

A recent review reveals noteworthy findings related to the variability and dynamic nature of HMOs throughout the course of lactation.[16] Researchers assessed and ranked concentrations of HMOs from healthy mothers across the globe, with the goal of achieving worldwide representative means of individual HMOs in term milk. As previously stated, a variety of factors – both genetic and non-genetic in nature – impact HMO composition in individual women. However, in this study, these variables were pooled based on the objective to illustrate the most abundant HMOs observed globally, regardless of secretor status or other factors.[16]

The findings from Soyyilmaz *et al.* reflect the dynamic quantitative distribution of the approximately 200 identified HMOs. 2'FL is the most abundant HMO, as it constitutes approximately 20% of total HMOs. After 2'FL, the next five most abundant HMOs (based on quantitative measure or molar concentration) are LNDFH I, LNFP I, LNFP II, LNT, and 3-FL. These top six HMOs comprise over half of the overall HMO composition in human milk. Further, the research team was able to identify that 80% of the total HMO fraction is made up by only the 15 most abundant individual HMOs, as illustrated in Figure 7.[16]



Total = 11.3 g/L

Top 15 HMOs 80.68%

20.5%	2'-FL	3.35%	LNnT
9.91%	LNDFH-I	2.64%	LDFT
7.5%	LNFP-I	2.55%	FDS-LNH
7.1%	LNFP-II	2.33%	LNFP-III
6.61%	LNT	1.71%	3'-SL
6.56%	3'-FL	1.53%	LST c
3.63%	6'-SL	1.35%	TF-LNH
3.41%	DSLNT		

Further 11.3%

1.22%	DS-LNH	0.44%	F-para-LNH-I
1.08%	F-LNH-III	0.41%	DF-LNH c
1.08%	F-LNH-II	0.41%	LNnH
1.01%	DF-LNH a	0.39%	DF-pLNnH
0.91%	LST b	0.33%	LST a
0.7%	LNFP-V	0.15%	LNFP-VI
0.69%	LNDFH-II	0.11%	FSL
0.68%	LNH	0.08%	pLNH
0.55%	SKNFP II	0.05%	6'SLN
0.5%	F-LNH-I	0.05%	pLNnH
0.46%	DF-LNH b		

All others 8.02%

Figure 7: **Composition of the HMO fraction of human milk[16]**

3.3 | Other Oligosaccharides in Infant Formula

Aside from HMOs, there are other prebiotics, such as galactooligosaccharides (GOS) and fructooligosaccharides (FOS) that have been added to infant formula. Research on these non-digestible carbohydrates (NDCs) has identified their ability to positively impact the gut microbiome.[85] NDCs were added to infant formula to mimic the functionality of HMOs during a time when HMOs were not commercially available. However, these other prebiotic fibers are structurally quite different than HMOs and cannot fully replicate the specific activity of HMOs.[73,85]

GOS and FOS are oligomers of galactose or fructose, respectively, and are elongated from lactose and sucrose. Given their structural make-up, they contain a different matrix of oligosaccharides compared to HMOs. They lack the branching and sidechains common in HMOs, as well as N-acetylglucosamine, sialic acid, and fucose. Since most of the functional benefits of HMOs are structure specific, GOS and FOS are not an equal substitution for HMOs and their biological impacts.[12,72]

It is important to understand that GOS and FOS do not naturally occur in human milk.[72] Perhaps one of the most enlightening consequences of this relates to how these prebiotics support the microbiome. GOS and FOS do support the growth of beneficial bacteria like *bifidobacteria* and *lactobacilli*, however, *in vitro* data suggest they are less specific in the bacteria they support compared to HMOs. Salli *et al.* illustrated this difference between NDCs and HMOs, showing that only beneficial bacteria such as bifidobacteria – and specifically, *B. infantis* – and certain *Bacteroides* species utilized HMOs as their fuel source, while these beneficial microorganisms plus pathogenic bacteria were able to utilize GOS.[86]

Key Takeaways:

1. Based on their structural characteristics, HMOs can be classified into three broad categories:
   - Fucosylated (neutral) HMOs
   - Sialylated (acidic) HMOs
   - Neutral core HMOs
2. Not every woman synthesizes the same oligosaccharides; different women produce different types and combinations of oligosaccharides. Genetics play a significant role in HMO composition. The documented variations in HMOs are dependent on a woman's Secretor (Se) and Lewis (Le) blood group status.
3. While over 200 HMOs have been identified so far, a small number of HMOs represent the majority of HMO content in human milk. 80% of the total amount of HMOs is made up by only the 15 most abundant individual HMO structures.

4 | Potential Health-Related Benefits of HMOs

As knowledge is gained around how HMOs work, scientists have been able to identify key mechanisms by which HMOs are thought to exert potential health benefits. Through preclinical, observational, and randomized controlled trials (RCTs), research has identified that the potential health benefits of HMOs present through four primary modes of action:

1. **By exerting a positive influence on the development and maintenance of the gut microbiota via a prebiotic effect, supporting the growth of beneficial bacteria**[17–19]
2. **By supporting gut health, modulating intestinal barrier function, and producing favorable metabolites for a well-functioning GI tract**[20,21]
3. **By deflecting the adhesion of undesirable microorganisms to cell surfaces, acting as decoy receptors and mimicking cell surface receptors**[22–24]
4. **By impacting the immune system systemically, directly modulating immune cell populations and cytokine secretion and impacting the inflammatory response**[9,25,26]

Each of these mechanisms will be explored further below.

4.1 | HMOs Act as Prebiotics and Stimulate the Growth of Beneficial Bacteria

HMOs are a group of carbohydrates that are known to possess prebiotic properties. A prebiotic is defined as "a substrate that is selectively utilized by host microorganisms conferring a health benefit".[87] Ultimately, a prebiotic serves as a fuel source for potentially helpful bacteria.

It is well known that HMOs resist digestion and reach the large intestine intact.[12,62] Once there, they are utilized by bacteria within the intestinal microbiota, selectively fueling the growth of favorable bacteria and limiting the nutrients available for harmful bacteria.[25,51,53] Based on this ability, HMOs can play a fundamental role in guiding the development of a balanced microbiome.[12,63,73,88]

The development of the infant gut microbiota begins in utero, followed by a much larger microbial exposure and colonization that takes place during delivery and early feeding.[89,90] The initial colonization – as well as the further development – of an infant's microbiota is affected by a wide variety of factors, including the mode of delivery, exposure to antibiotics or probiotics, feeding type, and family environment and lifestyle.[91,92]

There is evidence for the impact of early feeding type on infant gut microbiota. A recent meta-analysis compared the gut microbiota from exclusively breastfed (EBF) and non-EBF infants.[93] Infants who were exclusively breastfed for a longer duration had higher concentrations of bifidobacteria and a more stable bacterial composition, as well as reduced gut microbiota dysbiosis associated with diarrhea. Non-EBF infants had higher abundances of Bacteroidetes and Firmicutes compared to EBF infants, as well as a microbial community that appeared closer to that of an adult. These findings illustrate the powerful impacts of EBF on the infant gut microbiota.[93]

Bifidobacteria dominate the breastfed infant's gut and can account for up to 80–90% of their microbiota composition.[63,94] Bifidobacteria have been shown to be beneficial to infant health through protection against infections and contributing to the maturation of the immune system.[95,96] The dominance of bifidobacteria in breast milk is well recognized by numerous studies, and there is evidence that the *Bacteroides* and *Bifidobacterium* species that commonly colonize breastfed infants early in life efficiently utilize HMOs as carbon sources.[95–98]

Bifidobacterium longum subsp. infantis (B. infantis) has been shown to widely consume HMOs; similar findings exist for *B. bifidum* and other Bifidobacterium species.[12,63,99–101] Yu and colleagues have demonstrated the prebiotic activity of 2'FL, as well as difucosyllactose (DFL) and 3-FL for multiple *Bifidobacterium longum* species plus several Bacteroides species, a group of bacteria also known to commonly colonize breastfed infants early in life.[102]

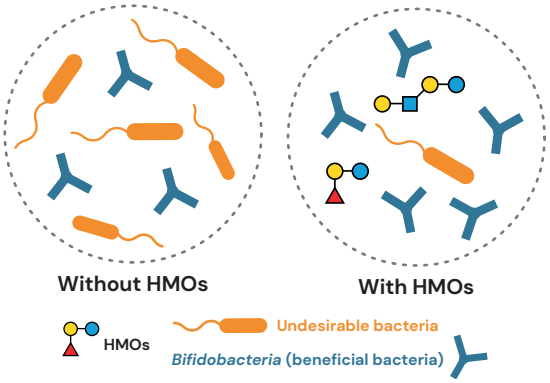


Figure 8: **HMOs stimulate the growth of beneficial bacteria**

Other preclinical work – focused on several *Bifidobacterium breve (B. breve)* strains – has illustrated the widespread growth ability of these strains with the HMOs LNT and LNnT, and to a lesser degree, with 2'FL, 3-FL, 3'SL, and 6'SL.[103]

The understanding that there is selective utilization of certain HMOs by specific bacteria is growing. Discoveries in this area reinforce the knowledge that HMO functionality is structure-specific – not all HMOs serve the same purpose – and further, that the growth of beneficial bacteria can be encouraged while the growth of harmful pathogens might be discouraged.[72,88]

A recent preclinical study adds to our understanding of this topic. Fifty-seven bacterial strains – both commensal and pathogenic – were evaluated for their ability to utilize three HMOs: 2'FL, 3-FL, and DFL. In addition to HMOs, other carbon sources were assessed as well, i.e., GOS, glucose, lactose, and fucose. Interestingly, the HMOs were utilized by probiotic strains typically dominant in the gut of the breastfed infant – as well as other commensal species found in the gut of healthy humans – such as *B. infantis*, *B. bifidum*, *Bacteroides fragilis*, and *Bacteroides vulgatus,* but not by pathogenic bacteria. A similar group of species were also grown by GOS, however, GOS – plus lactose and glucose – additionally supported the growth of potentially undesirable and pathogenic microorganisms. The authors concluded that HMOs selectively support the growth of species and strains of bacteria seen in the breastfed infant.[86]

There is also some evidence that the secretor status of a mother influences an infant's microbiome, and specifically, their abundance of bifidobacteria. In one study, Lewis *et al.* found that infants of secretor mothers had bifidobacteria established earlier and at higher absolute levels compared to infants of non-secretor mothers, providing some insight into the interplay of milk groups and an infant's microbial community.[19] In related findings, Korpela and colleagues illustrated that infants from non-secretor mothers born via caesarean-section – who classically have less diversity and lower amounts of bifidobacteria vs. vaginally born infants – had more pronounced characteristics of a caesarean birth than infants of secretor mothers born by caesarean section, including depleted bifidobacteria and increased amounts of undesirable microorganisms like enterococci.[104] These findings suggest that the negative effects of caesarean-section birth on the microbiome may be further pronounced in infants whose mothers are non-secretors. This presents the concept that screening for secretor status at birth might identify infants who would benefit from a supplemental source of HMOs.[104]

HMOs have been shown in other human observational studies to selectively feed helpful bacteria to support a healthy microbiome.[19,104] Clinical evidence for the ability of HMOs to positively modulate the microbiome has also been generated, which will be further explored in Sections 5 and 6.[17,41,42]

4.2 | HMOs Play a Role in Gut Integrity and Support Gut Health

In addition to supporting a healthy microbial community in the GI tract, HMOs are thought to support gut health through other mechanisms as well. Emerging evidence suggests HMOs may benefit the GI tract by positively impacting the mucosal barrier, decreasing epithelial permeability, and supporting local immunity in the gut.[20,105]

The GI tract is the largest surface area of the body that is exposed to antigens and microbes. As such, it must act as both a gatekeeper and barrier, selectively allowing nutrients to enter the epithelium, while preventing the translocation of bacteria and other undesired substances.[20,106]

Research has shown that by-products of HMO metabolism are short-chain fatty acids (SCFAs), produced by the gut microbiota during HMO fermentation.[51,107] SCFAs help to lower intestinal pH and thus increase acidity; this is believed to help inhibit potentially pathogenic bacteria. Additionally, SCFAs support mucus production and mitigate local inflammation, important elements of gut barrier function.[26,108–111] The HMOs 2′FL, 3-FL, 6-SL, LNnT, and DFL have been observed to generate SCFAs when digested by various desirable microbes *in vitro*, and thus work to support a community of healthy bacteria in the gut.[51,107]

Preclinical data also illustrate that HMOs aid in tight junction protein expression, which strengthens the intestinal epithelium and in turn, supports the immune system.[20,21,112] *B. infantis*, when grown on HMOs *in vitro*, was able to bind to intestinal cells to enhance tight junction protein expression while also supporting transepithelial resistance – a measure of gut barrier function.[20,21,26] In an animal model, intestinal permeability was observed to significantly decrease in rats supplemented with 2′FL and 3′SL compared to control animals.[113] A recent *in vitro* investigation yielded similar findings: individual HMOs and HMO blends that included combinations of fucosylated (2′FL, DFL), sialylated (3′SL, 6′SL), and/or non-fucosylated neutral HMOs (LNnT, LNT) positively impacted the intestinal barrier, with 2′FL appearing to have the greatest impact on gut barrier function.[114]

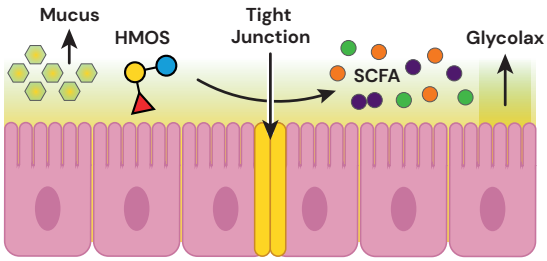
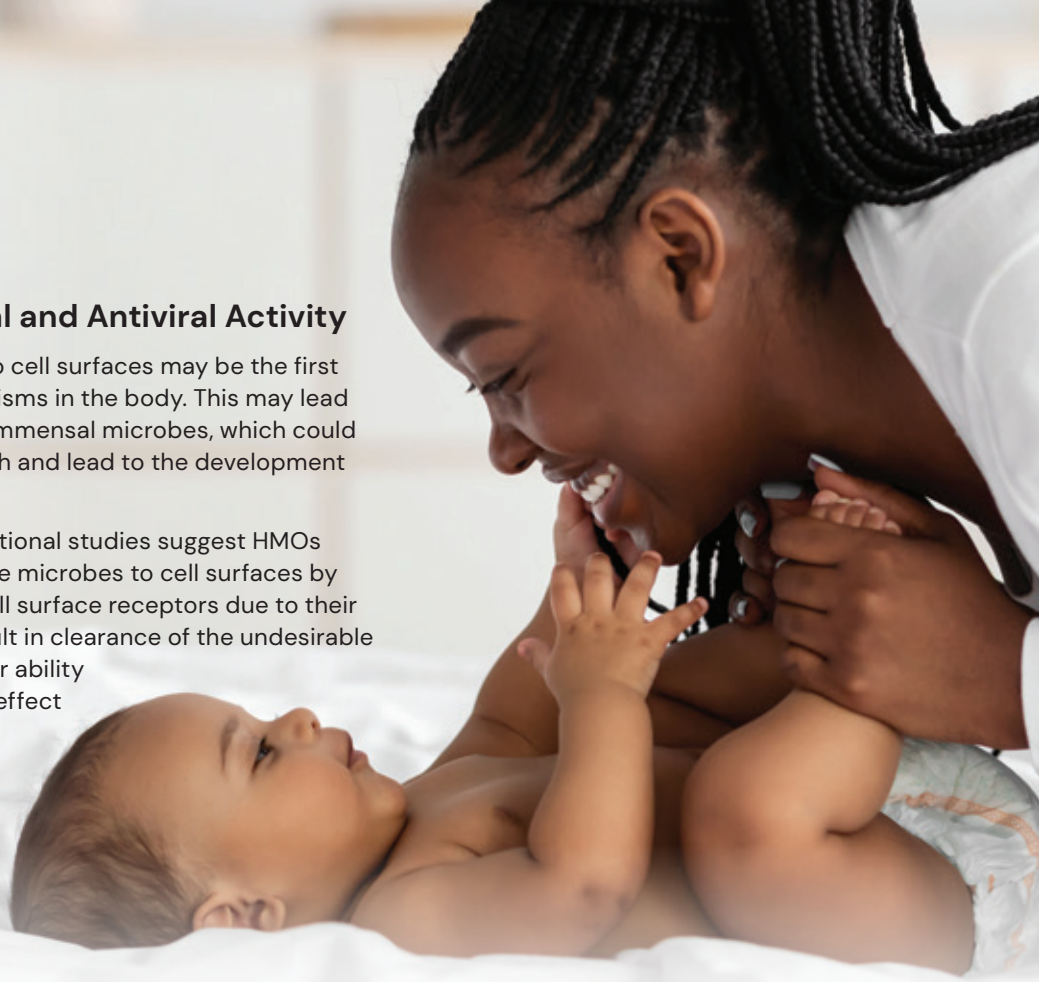


Figure 9: **HMOs support gut health**

HMOs contribute to intestinal barrier function by assisting with the growth of short-chain fatty acids, aiding in tight junction protein expression, and supporting glycocalyx and mucus formation. Adapted from Walsh 2020b, Ayechu-Muruzabal 2018, and Rousseaux 2021.[71,76,182]

Other *in vitro* studies contribute to our understanding of HMOs' influence on gut health. Mechanistic studies have illustrated the ability of 2′FL and LNnT to increase intestinal cell maturation and barrier function.[115–117] 2′FL and 3-FL have been shown to contribute to the intestinal barrier by supporting glycocalyx formation – a component of the epithelium that acts as an adhesion site for beneficial bacteria while simultaneously discouraging adhesion of undesirable microorganisms.[118] Further, HMOs have been found to significantly stimulate the production of MUC2 – an intestinal-type secretory mucin and major component of the gut's mucus layer and gut barrier function[119] – and assist with regulation of gut motor contractions in *in vitro* models.[120]



4.3 | HMOs Have Antimicrobial and Antiviral Activity

The adhesion of undesirable microbes to cell surfaces may be the first step in colonization of these microorganisms in the body. This may lead to an unfavorable abundance of non-commensal microbes, which could disrupt certain elements of human health and lead to the development of disease.[91,121]

Preclinical evidence and human observational studies suggest HMOs may help deflect adhesion of undesirable microbes to cell surfaces by acting as decoy receptors, mimicking cell surface receptors due to their structural similarity.[22-24,64,65] This can result in clearance of the undesirable microorganisms, which may prevent their ability to disrupt health. This unique beneficial effect of HMOs is highly dependent on their structure.[12,88]

Important findings related to HMOs' ability to deflect undesirable microorganisms come from preclinical studies. Ruiz-Palacios *et al.* investigated this mechanism against *Campylobacter jejuni* (*C. jejuni*), one of the major causes of bacterial diarrhea worldwide.[122] The study team demonstrated that HMOs inhibited campylobacter adherence to epithelial cells *in vitro*; specifically, fucosylated human milk oligosaccharides inhibited campylobacter binding.[122]

Yu *et al.* tested the ability of 2'FL to deflect *C. jejuni* from adhering to the intestinal epithelium, as well as to decrease *C. jejuni*-associated mucosal inflammation. 2'FL attenuated 80% of *C. jejuni* adherence and decreased mucosal inflammatory signals in an *in vitro* model. Similarly, in a mouse model, ingestion of 2'FL reduced *C. jejuni* colonization by 80%, as well as decreased intestinal inflammation (shown by histologic features) by 50-70%.[65]

Recently, the antipathogenic activity of 2'FL and LNnT (individually and in combination) was assessed *in vitro* against *Clostridioides difficile* (*C. difficile*), an undesirable microorganism that causes *C. difficile* infection (CDI).[123] After induction of dysbiosis and CDI, 2'FL and LNnT were introduced into the gut model. A significant decline in *C. difficile* levels with exposure to the HMOs, individually and in combination, was observed. Further, this occurred in parallel to increasing SCFA and *Bifidobacteriaceae* levels. Finally, when CDI was exposed to the antibiotic vancomycin in conjunction with the HMOs, the combination of interventions seemed to assist in preventing *C. difficile* recurrence over time and in boosting desired microbial diversity following antibiotic therapy. Together, these findings suggest a role for HMOs in mitigating the impact of *C. difficile* and illustrate their prebiotic potential after the unfavorable consequences of antibiotic therapy.[123]

Coppa *et al.* evaluated the ability of pooled and individual HMOs to decrease the growth of *Escherichia coli* (*E. coli*), *Salmonella fyris* (*S. fyris*), and *Vibrio cholerae (V. cholerae)*, common pathogenic bacteria implicated in diarrheal infections, from binding to intestinal cells. Overall, pooled HMOs significantly reduced pathogen adhesion for *E. coli* and *V. cholerae*, but not of *S. fyris*. When the inhibitory effect was analyzed for a collection of neutral low-molecular-weight HMOs that included 2'FL and 3-FL, bacterial adhesion was significantly reduced for *E. coli* and *S. fyris*. These results illustrate a desirable effect of HMOs on bacteria that are common sources of intestinal infection in infants.[23]

Group B Streptococcus (GBS) is an undesirable microbe that colonizes approximately 15-30% of pregnant women, and in turn, roughly half of the babies born to these mothers will be colonized.[124,125] Studies have demonstrated the ability of the HMOs LNT and LNFP I to hinder the growth of GBS *in vitro* by exerting antimicrobial and antibiofilm effects. Indeed, up to 89% inhibition of GBS growth and 90% inhibition of biofilm formation have been demonstrated in such studies.[24,126]

In addition to the effects observed with bacterial microorganisms, the effects of HMOs on viral infections in lung cell lines have been observed. A study exploring the effects of 2'FL and LNnT on human respiratory epithelial cell lines *in vitro* has shown that the viral load of respiratory syncytial virus (RSV) was significantly decreased with exposure to 2'FL. Further, LNnT and 6'SL significantly decreased influenza A viral load. The results suggest these HMOs may support the innate immune response to undesired viruses.[127]

While much more scientific exploration around this mechanism of HMOs is needed, the findings described above are promising for the potential role HMOs could play in modulating and supporting the immune response. Whether or not these observations – which are largely *in vitro* – can consistently translate to human infants requires more exploration in clinical trials.

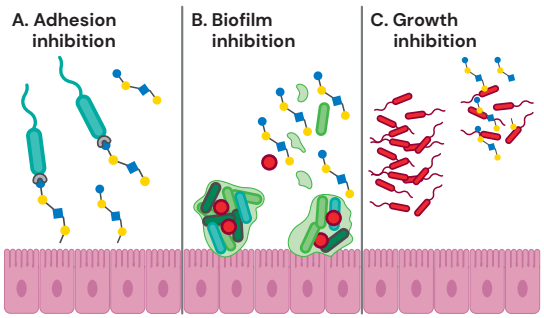


Figure 10: **HMOs exhibit various antimicrobial and antiviral mechanisms**

A) HMOs can act as decoy receptors, deflecting undesirable microbes from adhering to cell surfaces. B) HMOs may inhibit the activity of the biofilms of undesirable microorganisms; C) HMOs have been shown to inhibit the growth of undesired bacteria and viruses. Adapted from Asadpoor 2021.[188]

4.4 | HMOs Modulate the Immune Response

An increasing number of *in vitro* studies suggest that HMOs directly modulate immune responses by affecting immune cell populations and cytokine secretion.[12] It has been postulated that HMOs may act as an immune-modulator to guide the postnatal maturation of the immune system, potentially shifting T-cell responses towards a more balanced Th1 and Th2 cytokine production.[12,88] HMOs may achieve this by acting locally on cells of the mucosa-associated lymphoid tissues or on a systemic level.[12]

Furthering the knowledge of how HMOs interact with the immune system, other work has uncovered the ability of HMOs to signal toll-like receptors (TLRs) and thus influence cytokine production. In a preclinical study, both 2'FL and 3-FL were found to influence TLR signaling, in some cases activating and in other cases inhibiting TLRs. These results suggest a structure-specific response from HMOs in immunomodulation.[128]

2'FL has been reported to help enhance antibody responses to vaccines in a preclinical model. A study that investigated the effect of 2'FL on vaccination responsiveness in a murine influenza vaccination model found dietary 2'FL could significantly enhance vaccine specific delayed-type hypersensitivity responses, accompanied by increased serum levels of vaccine-specific IgG1 and IgG2a in a dose-dependent manner. The authors suggested that the direct effects of 2'FL on immune cell differentiation may be in part responsible for the improvement of both humoral and cellular immune responses to vaccination.[129]

In a human intervention study, healthy, full-term infants were fed with either a control formula containing 2.4 g/L GOS (n=39) or one of two experimental formulas with either 0.2 g/L 2'FL and 2.2 g/L GOS or 1.0 g/L 2'FL and 1.4 g/L GOS; a breastfed reference group was also included.[31] The infants fed the formulas containing 2'FL displayed statistically significantly lower levels of plasma inflammatory cytokines – between 29% and 83% lower – including interleukin (IL) receptor antagonist IL-1ra, IL-1α, IL-1β, IL-6, and tumor necrosis factor α (TNF-α) compared to the control formula.[31] Furthermore, in *ex vivo* RSV-stimulated PBMC cultures, cytokine levels were not different between breastfed infants and those consuming formulas with 2'FL, but each of these groups displayed lower levels of cytokines than the control formula, and 31% lower TNF-α and 54% lower IFN-γ were observed.[31]

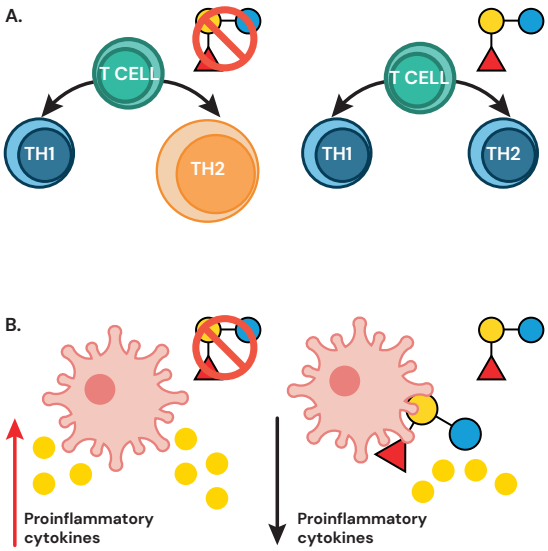


Figure 11: **HMOs modulate the immune response**

HMOs have shown the potential to impact the immune response by A) encouraging a more balanced Th1/Th2 response, and by B) influencing cytokine production and thus, the inflammatory response. Adapted from Bode 2012a and Carr 2021.[8,12]

The importance of HMOs in human milk cannot be disputed and the evidence available to date confirms this. Early data from RCTs in infants does support an immunomodulating role for HMOs; these will be explored in Section 5. Still, many questions remain on just how HMOs work to support the immune response. Continued research will help the scientific community answer these questions and guide HMO innovation to support the immune system and its development in early life.

Key Takeaways:

1. Research has identified that the potential health benefits of HMOs present through four primary modes of action:
   - By selectively feeding the beneficial gut bacteria and enhancing the growth of beneficial bacteria like bifidobacteria
   - By supporting gut health and gut integrity
   - By acting as decoy receptors and deflecting adhesion of undesirable microorganisms to cell walls
   - By modulating the immune response directly and indirectly
2. Preclinical and observational studies have demonstrated the ability of HMOs to effect each of these modes of action, yet clinical data are still needed to confirm and further explain the proposed mechanisms.
3. The existing knowledge around the mechanisms for how HMOs might impact human health can drive new explorations around other functional benefits of these substances.

5 | Clinical Evidence for HMOs' Ability to Support Infant Health

Technological advances over the last decade have allowed for the production of certain HMOs, moving the pursuit of deeper knowledge of these complex compounds from the laboratory to human clinical trials. The ability to synthesize HMOs has given scientists the opportunity to substantiate their safety, as well as to explore potential health benefits.

Considering HMO's prominence in breastmilk, some of the most exciting discoveries around HMOs have come from research centered in infant nutrition. Clinical trials in infants to date offer evidence for the safety of HMOs,[17,27-30] their ability to positively impact the microbiome,[17,29] and their potential to support the immune response.[28,30,31] An overview of some of the key clinical studies on HMOs is summarized below.

5.1 | The Safety of HMOs

Overall, the administration of infant formula supplemented with HMOs – including 2'FL alone or with LNnT, or a combination of 5 HMOs (2'FL, 3-FL, LNT, 3'SL, and 6'SL) – has been shown to be safe and well tolerated at the levels tested, as reviewed by Reverri *et al.* and shown in several clinical studies.[17,27-30,130-133] The ability of an infant formula to support adequate growth is central to the determination of its safety. Across these studies, adverse events, as well as gains in weight, length and head circumference in infants receiving formula with HMOs were comparable to those of infants receiving formulas without HMOs. These data provide evidence for the safety and suitability of supplemental HMOs in infant formulas designed for use early in life. The robust safety record of HMOs when fed to infants has translated to regulatory approvals for use of HMOs in infant formula around the globe.

5.2 | Highlights of Clinical Studies in Infants

2'FL and LNnT are the most clinically explored HMOs to date, and in addition to demonstrating their safety during infancy, studies have shown them to be well-tolerated, help establish the microbiome, and influence immune-related parameters. With continued research and innovation, the ability to study additional HMOs has grown. An infant formula supplemented with a combination of five HMOs (2'FL, 3-FL, LNT, 3'SL, and 6'SL) was recently evaluated in a growth and tolerance study, and the results add to the clinical evidence around the use of HMOs in infancy.[131] Table 3 summarizes the RCTs conducted with HMOs in infants.

GI tolerance of infant formulas with HMOs is another important metric in the assessment of their overall safety. Available data from clinical trials have shown that infant formulas with HMOs are tolerated equivalently to infant formulas without HMOs, with the exception of one desirable difference observed across two studies. In one, an infant formula with 2'FL and LNnT resulted in significantly softer stools at two months compared to the control formula without HMOs.[28] In another, an infant formula with a blend of five HMOs produced softer stools at a higher frequency compared to the control formula without HMOs.[131] Otherwise, differences in stool consistency or frequency, or incidence of spit-up or vomit were not seen for infant formulas with 2'FL compared to infant formulas without HMOs.[27,130] Another RCT found a partially hydrolyzed infant formula with 2'FL and *B. lactis* was well tolerated based on stool parameters, the frequency of spit-up and vomit, and duration of crying and fussing.[133] Overall, the totality of data across all published studies with HMOs in infant formula support their tolerance and nutritional suitability.

A sub-study within the Marriage *et al.* RCT was conducted to assess the impact of infant formula with 2'FL on markers of immune function in healthy term infants. In this study, infants were randomized to receive one of three different formulas, each with a total of 2.4 g/L of oligosaccharides comprised of different combinations of 2'FL and/or GOS. Two of the three formulas contained 2'FL with GOS (0.2 g/L with GOS, or 1.0 g/L with GOS) and the third contained only GOS. A breastfed reference group was included. Breastfed infants and infants fed either formula with 2'FL had 29-83% lower concentrations of plasma and *ex vivo* inflammatory cytokines vs. infants fed the control formula, suggesting a role for HMOs to impact the developing immune system.[31]

Puccio and colleagues assessed healthy, full-term infants fed a formula supplemented with 1.0 g/L 2'FL and 0.5 g/L LNnT for six months. This study demonstrates the safety of an infant formula supplemented with two HMOs and gives a first indication of the potential health benefits of 2'FL and LNnT.[28] In addition to documenting adequate growth and tolerance in infants fed a formula with the two HMOs, the study also assessed morbidity based on adverse events (AE) reported by the infant's caregivers. Infants fed the HMO-supplemented formulas had significantly fewer parental reports of:

- **Bronchitis through 4, 6, and 12 months of age**
- **Lower respiratory tract infections through 12 months of age**
- **Use of antipyretics (anti-fever medications) through 4 months of age**
- **Use of antibiotics through 6 and 12 months of age**

These results suggest HMOs impact the developing immune system and may influence immune-related outcomes.[28]

Stool microbiota of the same group of enrolled infants was analyzed at 3 and 12 months of age.[17] At three months, microbiota composition in the infants fed formula with HMOs appeared closer to that of the breastfed group with a fecal community type highly abundant in *Bifidobacteriaceae*. Specifically, abundance of the genera *Escherichia*, *Bifidobacterium*, unclassified *Peptostreptococcaceae*, and *Streptococcus* in infants fed formula with HMOs was closer to that of the breastfed reference group. However, at 12 months (6 months after infants stopped consuming the formula with HMOs), significant differences in microbiota composition were not observed between the two formula groups, indicating that the effect of HMO supplementation on microbiota composition is not sustained after intake of HMOs is discontinued.[17] This same study also assessed antibiotic usage between groups and found that infants with a fecal community type high in *Bifidobacteriaceae* at three months were less likely to require antibiotics through 12 months of age.[17] The results from this study are important – they provide detailed insight into how HMOs can change the microbiota in formula-fed infants and, in turn, link that to real-world health outcomes.

In another study, an extensively hydrolyzed whey-based formula (EHF) supplemented with the two HMOs 2'FL (1.0 g/L) and LNnT (0.5 g/L) and with reduced protein content (2.2 g/100 kcal vs. 2.47 g/100 kcal) was studied in infants with cow's milk protein allergy (CMPA) with the goal of assessing growth and other anthropometric parameters, tolerability, safety, infections and medication use during the first year of life.[30] Weight gain in the test group was non-inferior to control group, and there were no significant differences in anthropometric parameters between groups. Both formulas were safe and well-tolerated. At 12 months of age, infants fed the formula with HMOs had a significant reduction in the frequency of upper respiratory tract infections. They also had a lower incidence of ear infections, translating to a 70% relative risk reduction of ear infections in the first year of life. Infants fed the formula with HMOs had a 30-40% lower risk of lower respiratory tract infections and GI infections, yet this did not reach statistical significance. This study provides further evidence for the ability of HMOs to support the immune response and positively impact the occurrence of common infections in infancy.[30]

Finally, studies to further our understanding of how HMOs interact with probiotics have recently been done. Colonization with the probiotic *L. reuteri* has been shown to be beneficial in infants in several clinical trials. It has demonstrated the ability to positively shift the microbiome of infants delivered via caesarian-section toward the composition observed in vaginally-delivered infants.[134] Further, two meta-analyses revealed reducing crying and fussing time in breastfed infants who received *L. reuteri*.[135,136] The safety and efficacy of a routine starter formula with *L. reuteri* dsm-firmenich 17938 and 2'FL was recently studied; the objectives were to assess growth, GI tolerance, the gut microbiome, and intestinal maturation in infants fed the experimental formula compared to the same formula without 2'FL. There were no differences between groups for markers of growth or parent-reported stooling characteristics or GI symptoms and associated behaviors.[29] Infants fed the formula with *L. reuteri* and 2'FL had a different microbiota composition compared to infants fed the formula without 2'FL at two and three months, with the pattern observed approaching that of the breastfed control group for phylogenic diversity and relative abundance of *Bifidobacterium*.[29] The authors conclude that the positive effects of 2'FL are incremental to the those of *L. reuteri*, and together, these substances help shift the microbial pattern of formula-fed infants to be closer to that of the breastfed infant.[29]

Innovation around HMOs is underway and will continue for years to come. The anticipated ability to synthesize additional HMOs will expand HMO research and broaden our knowledge of the potential health benefits of these oligosaccharides. As discussed above, novel formulas and formulas with an increased variety of HMOs are already being produced and studied; this is reflection of the continuous effort to evolve infant formula to bring it closer to human milk in composition and functionality, and to offer optimal nutrition during a critical period of development. Additional formulations are anticipated, using the complex composition of breastmilk – which includes neutral, fucosylated and sialylated HMOs – as inspiration. As research continues, promising new findings are likely to emerge; these will fuel the goal of fully realizing the benefits of HMOs and bringing them to as many infants as possible.

Table 3: **Randomized Controlled Trials of HMOs in Infants**

Study	Population	Study groups	HMO, dose, duration	Outcomes
Marriage 2015[27]	Healthy, full-term infants <5 days old N=424	Test formula 1: infant formula with 0.2 g/L 2'FL and 2.2 g/L GOS Test formula 2: infant formula with 1.0 g/L 2'FL and 1.4 g/L GOS Control formula: infant formula with 2.4 g/L GOS Breastfed reference group	Infant formula with 2'FL: 0.2 g/L or 1.0 g/L Formulas fed for 4 months	Infant formulas with 2'FL were well-tolerated and resulted in growth equivalent to that of infants fed formula without HMOs and to breastfed infants No differences among groups for stool consistency or frequency, or percent of feedings associated with spit up/vomit. Absorption of 2'FL from supplemented infant formula was similar to that of breastfed infants
Goehring 2016[31]	Healthy, full-term infants <5 days old N=315	Sub-study of Marriage 2015; same as above	Sub-study of Marriage 2015; same as above	Breastfed infants and infants fed either formula with 2'FL had 29-83% lower concentrations of plasma and *ex vivo* inflammatory cytokines vs. infants fed control formula (IL-1ra, IL-1α, IL-1β, IL-6, and TNF-α, IFN-γ)
Kajzer 2016[130]	Healthy, full-term infants <8 days old N=119	Test formula: Infant formula with scFOS and 2'FL Control formula: infant formula without oligosaccharides Breastfed reference group	Infant formula with 2'FL (0.2 g/L) and scFOS (2.0 g/L) Formulas fed until 35 days of age	No significant differences in average number of stools per day between experimental and control formula groups No significant differences between three groups for: anthropometric data, stool consistency, formula intake, or percent of feedings associated with spit-up/vomit.

Table 3: **Randomized Controlled Trials of HMOs in Infants** *continued*

Study	Population	Study groups	HMO, dose, duration	Outcomes
Puccio 2017[28]	Healthy, full-term infants ≤ 14 days of age N=175	Test formula: infant formula with 1.0 g/L 2'FL and 0.5 g/L LNnT Control formula: infant formula without HMOs Breastfed reference group	Infant formula with 1.0 g/L 2'FL and 0.5 g/L LNnT Formulas fed to 6 months of age	Infant formula with HMOs was well-tolerated and resulted in growth equivalent to that of infants fed formula without HMOs, and less parent-reported illness (bronchitis and LRTI) and medication use (antibiotics) through 12 months of age‡ GI tolerance of infant formula with HMOs was not different compared to control formula (flatulence, spitting-up and vomiting) Infant formula with HMOs resulted in softer stools and fewer nighttime wake-ups at 2 months of age
Storm 2019[133]	Healthy, full-term infants ≤14 days of age N= 79	Test formula: 100% whey partially hydrolyzed infant formula with 0.25 g/L 2'FL and $1x10^6$ CFU *Bifidobacterium animalis* ssp. *lactis* strain Bb12 Control formula: 100% whey partially hydrolyzed infant formula with $1x10^6$ CFU *Bifidobacterium animalis* ssp. *lactis* strain Bb12	Infant formula with 0.25 g/L 2'FL Formulas fed for 42 days	Infant formula with 2'FL + Bb12 was well-tolerated, as assessed by the Infant Gastrointestinal Symptom Questionnaire (IGSQ) score which measures stooling, vomiting/spit-up, flatulence, crying, and fussing. IGSQ scores were similar between groups.
Berger 2020[17] (same cohort as Puccio 2017)	Healthy, full-term infants ≤ 14 days of age N=175	Test formula: infant formula with 1.0 g/L 2'FL and 0.5 g/L LNnT Control formula: infant formula without HMOs Breastfed reference group	Infant formula with 1.0 g/L 2'FL and 0.5 g/L LNnT Formulas fed to 6 months of age	At 3 months of age, the microbiota of infants fed formula with HMOs was closer to that of the breastfed reference group with a fecal community type highly abundant in Bifidobacteriaceae. Specifically, abundance of the genera Escherichia, Bifidobacterium, unclassified Peptostreptococcaceae, and Streptococcus in infants fed formula with HMOs was closer to that of the breastfed reference group Formula fed infants with an abundance of bifidobacteria at 3 months of age were significantly less likely to require antibiotics during the first year of life.
Parschat 2021[131]	Healthy, full-term infants < 14 days N=225	Test formula: formula with blend of 5 HMOs (5HMO-Mix) Control formula: formula without HMOs Breastfed reference group	Blend of 5 HMOs, 5.75 g/L, fed via infant formula 52% 2'FL 26% LNT 13% 3-FL 5% 6'-SL 4% 3'-SL Formulas fed for 16 weeks	Gains in weight, length, and head circumference were not different between the two formula groups. Formula with 5HMO-Mix was well tolerated; infants fed 5HMO-Mix and breastfed infants had softer stools at higher frequency compared to control infants. Infant formula with 5HMO-Mix at 5.75g/L is safe and well-tolerated.

Table 3: **Randomized Controlled Trials of HMOs in Infants** *continued*

Study	Population	Study groups	HMO, dose, duration	Outcomes
Vandenplas 2021[30]	Full-term infants, 0-6 months of age with physician-diagnosed CMPA N=194	Test formula: 100% whey EHF with 1.0 g/L 2'FL and 0.5 g/L LNnT and reduced protein content (2.2 g/100 kcal) Control: 100% whey EHF without HMOs; 2.47 g protein/100 kcal	Infant formula with 1.0 g/L 2'FL and 0.5 g/L LNnT Formula fed for 4 months; outcome parameters collected through 12 months of age	Weight gain in the test group was non-inferior to control group. There were no significant differences in anthropometric parameters between groups. Both formulas were safe and well-tolerated. At 12 months of age, infants fed formula with HMOs had significant reduction in frequency of URTI and a lower incidence of ear infections. Infants fed formula with HMOs had a 30-40% lower risk of LRTI and GI infections, yet this did not reach statistical significance.
Alliet 2022[29]	Healthy, full-term infants, <14 days old N=289	Test formula: Bovine milk-based formula containing L. reuteri DSM 17938 and 1.0 g/L 2'FL Control: Bovine milk-based formula containing L. reuteri DSM 17938 Breastfed reference group	1.0 gm/L 2'FL Formulas fed for 6 months	Weight gain in test group was non-inferior to control group. Anthropometric z-scores, parent reported stooling characteristics, GI symptoms and associated behaviors, and adverse events were similar between groups. Microbiota composition – including Bifidobacterium abundance – in the test group was significantly different from the control group at two and three months and approached that of the breastfed infant.

‡Secondary outcomes; 2'FL: 2-fucosyllactose; 3-FL: 3-fucosyllactose; 3'SL: 3-sialyllactose; 6'SL: 6-sialyllactose; EHF: extensively hydrolyzed formula; GI: gastrointestinal; GOS: galactooligosaccharides; HMOs: human milk oligosaccharides; IFN: interferon; IL: interleukin; LNT: lacto-N-tetraose; LNnT: lacto-N-neotetraose; lcFOS: long chain fructooligosaccharides; LRTI: lower respiratory tract infection; RA: receptor antagonist; scFOS: short chain fructooligosaccharides; TNF: tumor necrosis factor; URTI: upper respiratory tract infection

Key Takeaways:

1. The totality of data across all published studies with HMOs in infant formula support their safety, tolerance, and nutritional suitability during a critical stage of early development.
2. Clinical trials in infants to date have demonstrated the ability of HMOs at their tested levels to positively impact the microbiome and support the immune response.
3. Innovation around the use of HMOs for infants continues and has recently allowed for the expansion of the number of HMOs available for addition to infant formulas. As the application of HMOs in infant formula expands, further study is needed to more fully understand the breadth of their health benefits and ability to support early development.

6 | The Future of HMOs: New Learnings on the Horizon

Research on HMOs continues to grow and evolve. New areas of exploration are emerging as scientists seek to fully leverage these complex compounds for the full spectrum of health benefits they have the potential to offer. HMOs are being explored for their potential to reduce the incidence of necrotizing enterocolitis (NEC) and improve outcomes in premature infants.[32-34] Their roles in neurodevelopment and brain health are under investigation.[35-37] New discoveries are being made related to HMOs and allergy management in children.[38-40] In adults, the role of HMOs in gut health continues to be researched.[41-43] As research progresses, the scientific community will learn more about how HMOs can impact our health across the lifespan.

6.1 | Exploring the Ability of HMOs to Support Premature Infants

Premature infants miss several weeks to months of development *in utero*. Many of their physiological functions are immature at birth, placing them at greater risk for several health and developmental challenges. Premature birth is a major health concern, and a leading cause of neonatal morbidity and mortality.[137,138]

6.1.1 | HMOs may play a role in reducing the risk of NEC development

Preterm infants are predisposed to gut immaturity and associated gastrointestinal disorders, including NEC. NEC is the most common gastrointestinal emergency in the neonatal intensive care unit (NICU), characterized by inflammation and injury to the gut wall barrier. Its clinical presentation ranges from feeding intolerance, abdominal distention, blood in stools, and respiratory distress to bowel necrosis, perforation, sepsis, and shock in advanced cases. Extreme cases can lead to death.[139]

A definitive explanation of the pathophysiology of NEC remains obscure; however, colonization of the GI tract that deviates from what is observed in healthy, term infants is thought to play a role.[140,141] Studies have shown that premature infants who develop NEC have higher proportions of undesirable microorganisms like *Proteobacteria* and *Firmicutes*, and a decreased abundance of *Bifidobacteria* and *Bacteroidetes*.[142,143] Further, GI tract immaturity – which includes decreased gut barrier function – is thought to predispose preterm infants to NEC.[144]

Based on this knowledge – as well as the evidence for HMOs to positively influence the microbiome and support gut health – preclinical and observational studies have been done to test the hypotheses that HMOs could contribute to a decreased risk of NEC and may decrease the severity of the disease if it does develop.

In animal trials using a NEC model, rat pups who were exposed to formulas with HMOs had decreased severity of NEC based on pathology scores, as well as improved survival. The HMOs disialyllacto-*N*-tetraose (DSLNT) and 2'FL were identified to be responsible for the NEC-protective effects observed.[145,146] Sodhi *et al.* demonstrated the ability of 2'FL and 6'-SL to positively and significantly modulate markers of NEC pathogenesis and NEC severity in mouse and piglet models of the disease.[33] Other preclinical studies have attempted to identify mechanisms by which HMOs might offer protection from NEC. Li and colleagues showed that HMOs support expression of tight junction proteins and improved gut barrier function in epithelial cells, suggesting a role in supporting gut health and function.[147]

Finally, observational studies have provided valuable insights to the potential role of HMOs in mitigating the development of NEC. The same group that studied DSLNT for a NEC-protective effect in animals analyzed HMO composition in mothers of premature infants to assess whether a similar association exists in humans. DSLNT concentrations were indeed significantly lower in infants who developed NEC compared to those who did not, suggesting a protective mechanism is involved.[34] The findings from two other studies – Masi *et al.* and Van Niekerk *et al.* – are in parallel to this outcome, showing a similar inverse relationship between DSLNT concentrations in human milk and the risk of NEC.[148,149] Other relationships between HMOs and NEC have been identified: Wejryd *et al.* discovered a link between low levels of LNDH I and the development of NEC in preterm infants on day of life 14 and 28 and at 36 weeks post-menstrual age. An association between NEC development and low levels of sialyl-lacto-*N*-tetraose a (LSTa) and LNnT at 28 days was also identified. Additionally, a trend towards less NEC in infants with Secretor and Lewis-positive mothers was observed, suggesting a role for HMO composition and diversity in the risk of developing NEC.[150]

While there is limited evidence for a role of HMOs in NEC, the early findings discussed here suggest more research is warranted. Clinicians and scientists will continue pursuing ways to improve clinical outcomes of infants born prematurely; HMOs may contribute to that goal.

6.1.2 | Early evidence for a connection between HMOs and infections in preterm infants

Due to immune system immaturity, premature infants are at increased risk for systemic infection, including late-onset sepsis (LOS), defined as an infection that develops after 72 hours of life. LOS is a common complication of prematurity and affects roughly 20-30% of very low birth weight infants (VLBW).[151,152] A research group led by Torres Roldan explored whether there were associations between maternal HMO composition and LOS in VLBW infants in Peru. After assessing 153 infant-mother pairs and their milk samples, a link between higher levels of the HMO Fucosyl-disialyllacto-*N*-hexose (FDSLNH) and a reduced risk of developing LOS was found.[153] While promising, additional studies are needed to guide the clinical application of specific HMOs for reducing infection in premature infants.

6.2 | Emerging Science: HMOs May Influence Brain Health and Cognitive Development

Infancy is a critical period of brain growth and development, and the nutrition delivered during this time plays a foundational role in brain maturation and cognitive outcome.[154] Breastfed infants show higher intelligence test scores, language development, and intelligence quotient (IQ), among other markers of cognitive functioning.[155,156]

While evidence exists for an association between breastfeeding and improved performance on intelligence tests, the mechanism for this association is not clearly understood. Multiple factors may be involved, including the social interaction between mother and baby during breastfeeding or the many nutritive and non-nutritive bioactives factors in breastmilk.[157-159]

It has been established that human milk is a rich source of sialic acid, an essential compound for optimal brain development and cognition. Exogenous sialic acid has been shown to increase brain ganglioside and glycoprotein sialic acid concentrations, parameters that are associated with brain growth and maturation.[35] Indeed, the highest concentration of sialic acid in the human body is found in the brain.[160] Studies have identified that sialic acid levels are significantly higher in the grey matter of breastfed vs. formula-fed infants. As a marker of total ganglioside content, higher sialic acid levels are thought to correlate with brain development and cognition.[35] In piglets, dietary supplementation with sialic acid resulted in increased memory and learning.[161]

Emerging science indicates that sialylated HMOs – like 3'SL and 6'SL – may be utilized as building blocks for the brain and might play a nutritional role in brain development in early infancy.[35,36] Since cow's milk formulas naturally contain small amounts of sialic acid,[162] lower consumption of this compound may be a differentiating factor between formula-fed and human milk-fed infants. Animal studies have shown that supplementation with 3'SL and 6'SL can increase sialic acid in the brain, influence structural brain development, positively impact gut microbiota, and support cognition, memory, and the behavioral responses to stress exposure.[36,66,163-165]

Other HMOs have been investigated in animal trials on brain health and development as well; 2′FL in combination with oligofructose supported improved recognition memory and cognitive performance,[166] and 2′FL and LNnT influenced recognition memory as well as overall brain structure.[167] 2′FL has been shown to improve rodents' performance on cognitive tests and long-term potentiation – central to learning and memory.[168,169]

Finally, human observational studies have further explored this association between HMOs and brain health and cognition. Berger *et al*. studied the impact of 2′FL via breastmilk on cognitive development at 24 months of age and in relation to maternal obesity and breastfeeding frequency.[37] Breastmilk was analyzed at one and six months of age for HMO composition. The number of breastmilk feedings per day and 2′FL levels at one month proved to be predictors of cognitive development as assessed by the Bayley-III Scale. Interestingly, associations to cognitive development and HMOs were not seen at six months of age, suggesting a critical window of time during which 2′FL may influence brain development.[37] In another observational study, a positive association was found for human milk concentrations of 2′FL at one month and motor scores at six months of age. Additionally, positive associations were observed for concentrations of 6′SL at one month and cognitive and motor scores on developmental assessments at 18 months of age.[170]

This exciting area of HMO research will continue as scientists continue to seek a deeper understanding of how HMOs influence brain health and development. There is also great interest in exploring whether HMOs might influence the gut-brain axis, or the bi-directional communication pathway between the GI tract and the brain.[66,171]

6.3 | Discoveries on the Benefits of HMOs for Children

There are few studies of HMOs in children to date, but early findings are encouraging. The beginnings of an evidence base for the application of HMOs in children is being built around their safety, tolerability, and their effects on growth and metabolism. Scientists are also working to identify other areas where HMOs may positively influence health outcomes in children.

Fonvig *et al*. evaluated the effect of supplementing 2′FL, either alone or in combination with LNnT, on gut microbiota in 6- to 12-year-old children with overweight and obesity. Participants received a total of 4.5 g HMO per day, either solely as 2′FL, or in a 4:1 mass ratio if receiving both 2′FL and LNnT. The study found that HMO supplementation led to a significant increase in bifidobacteria abundance, similar to the effects that have been observed in infants and adults. Additionally, this study demonstrated that HMO supplementation in children is safe and well-tolerated.[67]

Another RCT in 461 healthy Chinese children aged 1 to 2½ years demonstrated that a young child formula (YCF) with 2′FL was well-tolerated and did not result in growth or tolerance differences compared to children receiving other formulas without HMO in the study.[172] In support of the addition of HMOs to formulas intended for use beyond infancy, a recent study demonstrated the presence of six HMOs (2′FL, 3-FL, 3′SL, 6′SL, LNT, and LNnT) in breastmilk through 400 days postpartum, confirming a role of HMOs into childhood.[173]

An observational study investigated the association between maternal HMO composition and infant growth during the first five years of life. The results illustrate a connection between HMO exposure in infancy and childhood outcomes. Height and weight gains through five years were associated with maternal HMO composition three months after delivery in secretor mothers. Specifically, inverse associations were observed for HMO diversity and height z-scores at five years of age, and for LNnT concentration and weight and length z-scores through five years of age. Conversely, the concentration of 2′FL was positively associated with 12 month weight z-scores and five year height z-scores.[174] Further, maternal pre-pregnancy BMI – a factor known to influence infant body composition – was directly associated with HMO composition.[174] This study adds to the accumulating evidence that HMOs can influence growth patterns for years beyond their exposure in the early life stage.[83] More study is needed to further understand this potential role of HMOs in mediating child growth.

The findings from the studies being done in children support the theory that HMOs have a role in health beyond infancy. Further study will reveal if and how the impacts of HMOs change from infancy to childhood.

6.4 | HMOs May Play a Role in Allergy Management

With the knowledge that HMOs influence the microbiome, gut barrier function, and the immune response, interest in whether these compounds may have application for children with allergies has increased.[175] Cow's milk protein allergy (CMPA) – one of the major food allergies experienced by infants and young children[176] – is associated with an unfavorable gut microbiota and GI tract permeability.[40] In an effort to understand the potential application of HMOs to allergy, several studies have been conducted to date.

For a formula to be indicated for the management of CMPA, it must meet clinical hypoallergenicity criteria: ensure with 95% confidence that 90% of infants and/or children with CMPA would not react to the formula in a double-blind placebo-controlled food challenge (DBPCFC).[177] Nowak-Wegrzyn and colleagues tested a whey-based EHF supplemented with 2'FL and LNnT in children with diagnosed CMPA aged two months to four years to assess whether it would indeed meet hypoallergenicity criteria and thus be indicated for use with cow's milk allergy. The tested formula did meet the criteria, confirming the hypoallergenicity of an infant formula with HMOs and allowing it to be recommended for the management of CMPA.[38]

Early evidence for a potential role of HMOs in allergy management comes from a prospective, observational study. Infants zero to 60 days of age with suspected food protein allergy, persistent feeding intolerance, or symptoms associated with CMPA who were already receiving an EHF formula were switched to a similar EHF, but with added 2'FL, to assess growth, tolerance, and safety. This study showed that the addition of 2'FL to an EHF formula was well-tolerated, supported normal growth, and was safe. Further, the majority of parents reported improvements in eczema, vomiting, constipation, and spit-up/gagging 60 days after switching to an EHF with 2'FL, suggesting the HMO may play a role in diminishing symptoms associated with allergy.[39]

Building on the possibility there may be an association between the HMO content in human milk and later development of allergy, researchers found that infants who received human milk with low LNFP III concentrations were more likely to develop CMPA compared to infants who consumed human milk with high levels of LNFP III.[178] Other studies have also documented an association

between HMO profiles and either increased or decreased risk of later allergic development[179] or food sensitization.[180] Sprenger and colleagues observed that infants born via cesarean section and at high hereditary risk for allergies had a lower risk for manifestation of IgE-associated eczema at two years of age when fed breastmilk from Secretor mothers.[181]

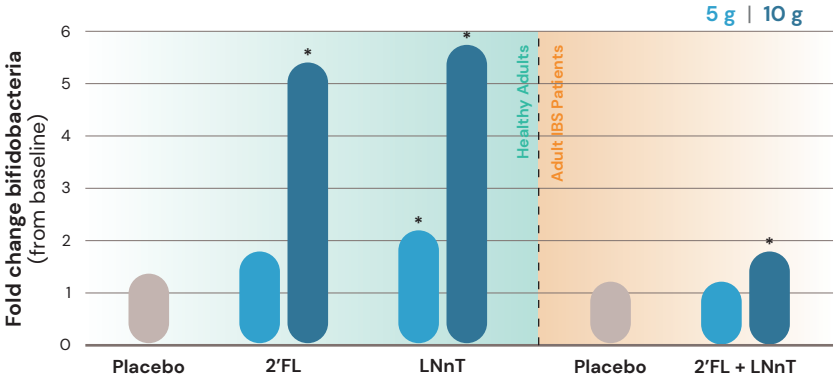
Recognizing the emerging data, a group of pediatric gastroenterology and allergy-immunology experts published a statement proposing a link between HMOs and prevention of allergy.[40] They speculate an association based on the ability of HMOs to positively impact the gut microbiota and intestinal barrier function and support the immune response, mechanisms which are thought to be related to the underlying etiology of allergy development.[175,182] Thus, the authors concluded HMOs' mechanisms of action may play a beneficial role in allergy prevention, while also recognizing the need for more research around this hypothesis.[40]

6.5 | HMOs and Gut Health in Adults

Emerging clinical data suggest that supplementation with select HMOs – 2'FL and LNnT – in adults can positively impact the gut microbiota.[41,42,67,183] As intestinal dysbiosis has been described as a common element of the physiology of some GI conditions, studies providing preliminary evidence that HMOs might positively impact GI health and wellness at later stages of life are of interest. Early studies have also shown that HMOs may reduce the severity of symptoms related to irritable bowel syndrome (IBS) and associated quality of life,[183] – and in some cases, even across various types of IBS.[43]

6.5.1 | Randomized, Controlled Trials in Adults

Scientific evidence confirms that the gut microbiome plays an essential role in the overall health of the GI tract.[184] RCTS have shown 2'FL and LNnT can beneficially modulate the intestinal microbiota, primarily by increasing the abundance of bifidobacteria in both healthy adults[41] and adults with IBS.[42]



2'FL: p < 0.05 for 10 g **LNnT:** p < 0.05 for 5 g and 10 g **2'FL + LNnT:** p < 0.01 for 10 g

* Significant compared to placebo

Figure 12: **Changes in bifidobacteria from baseline after HMO intervention in healthy adults and adults with IBS**

Healthy adults: Change in bifidobacteria from baseline after 14 days of intervention; n=100. Adapted from Elison 2015.[41] Adult IBS Patients: Change in bifidobacteria from baseline after 4 weeks of intervention; n=60. Adapted from Iribarren 2020.[42]

6.5.2 | Open-label Trial in Adults

In a recent open-label trial,* HMOs helped reduce symptom severity for patients with IBS.[43] The 12-week, open-label, multi-center study enrolled 317 adults with IBS.[43] Supplementation with 5 g of a mixture of 2'FL and LNnT (4:1 ratio) demonstrated significant improvements in stool consistency, IBS symptom severity†, and quality of life based on the following parameters:

- **There was an over a 4.6-fold increase in normal stools.‡ Similar improvements in stool consistency were reported across IBS subtypes (predominant constipation [IBS-C], predominant diarrhea [IBS-D], fran or mixed bowel habits [IBS-M]).**
- **A 54% reduction in overall severity of IBS symptoms over the 12-week study period was reported based on the IBS Symptom Severity Score (IBS-SSS). A similar pattern was seen in all IBS subtypes.**
- **Eighty-two percent of participants reported a clinically significant improvement in their IBS-SSS total score by week 12.**

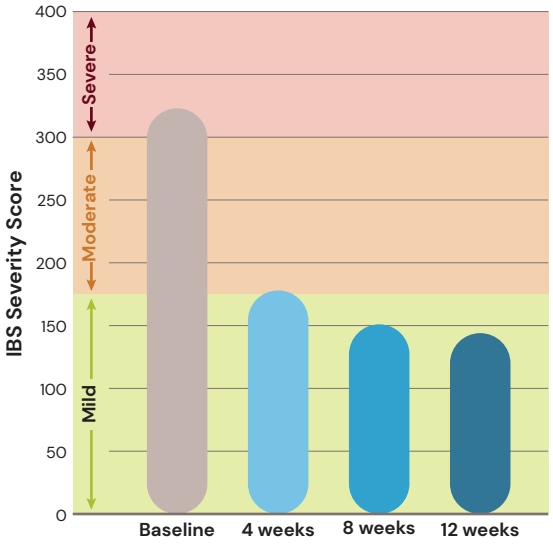


Figure 13: **Overall IBS Severity Score**

A reduction of 50 points or more in the IBS-SSS total score is considered clinically significant.[189] The average reduction of symptoms in this study was 150 points after four weeks. Adapted from Palsson 2020.[43]

* Study participants had visibility to the product being tested in the trial; † Based on the IBS Symptom Severity Score (IBS-SSS); ‡ As measured by the Bristol Stool Form Scale (BSFS)

Ninety-six percent of participants considered the HMO supplement tolerated, well-tolerated, or extremely well-tolerated. Finally, participants reported an improved quality of life, with health-related quality of life (IBS-QOL) scores rising 48% from baseline to week 12.[43]

6.5.3 | Pre-Clinical and Pilot Studies in Adults

In a preclinical study, Wang and colleagues investigated the impact of 2′FL on *E. coli* O157 in healthy adult mice. 2′FL was found to reduce intestinal *E. coli* colonization by 90%, while also reducing intestinal inflammation and increasing SCFA – benefits that were attributed to the deflection of *E. coli* from cell surfaces. Further, 2′FL was found to increase the abundance of a potential probiotic, *Akkermansia*. Based on these findings, the research team postulates that 2′FL may play a role in reducing the risk of foodborne illness in humans.[185]

Ryan et al. conducted an *in vitro* experiment to assess the bifidogenic and butyrogenic effects of 2′FL on the stool of healthy adults as well as the stool of those with IBS or ulcerative colitis (UC).[183] *Bifidobacterium* and *Eubacterium rectale-Clostridium coccoides* counts increased after fermentation of 2′FL in the samples from all groups, a desirable impact due to the bifidogenic and butyrate-producing characteristics of these bacteria, respectively. Additionally, the production of SCFA butyrate was positively impacted by fermentation of 2′FL.[183] Subsequently, an open-label trial was conducted in 12 adults with IBS or UC to assess the impact of 2′FL on Quality of Life assessments for those with GI conditions. Consuming a supplement with 2′FL was linked to improvements in intestinal symptoms and scores on the GI Quality of Life Index and the IBD Questionnaire, demonstrating a practical benefit to HMO supplementation in adults with GI conditions.[183]

Further study is needed to expand on these early findings, however, preliminary data are promising for the ability of HMOs to support gut health in adults. The application of HMOs to populations other than infants is expected to grow as new research on their benefits beyond early life emerges.

6.6 | Summary

There are many exciting areas of HMO research underway, which will certainly lead to new findings and new applications. Given the presence of HMOs in human milk, the potential health benefits of HMOs for infants are anticipated to a certain degree. The discovery that HMOs may influence health across the lifespan is exciting and encouraging, and illustrates that HMOs lend themselves to countless innovations.

Key Takeaways:

1. HMOs are being explored for their ability to support gut health in premature infants, including a potential role in mitigating the risk of NEC.
2. Emerging science indicates that HMOs – such as 2′FL, 3′SL and 6′SL – may support cognitive development, either by serving as building blocks for the brain or by playing a nutritional role in brain health and development.
3. The beginnings of an evidence base for the application of HMOs in children is being built around their safety and effects on growth. Evidence to date does indeed support the conclusion that HMOs are safe and well-tolerated in children. Additionally, data are growing around the bifidogenic impact of HMOs in children as well.
4. Early evidence for a potential role of HMOs in allergy management exists. Further, some physicians believe HMOs could have a role in allergy prevention. The rationale is based on the ability of HMOs to positively impact the gut microbiota and intestinal barrier function and support the immune response, mechanisms which are thought to be related to the underlying etiology of allergy development.
5. Emerging clinical data suggest that supplementation with select HMOs – 2′FL and LNnT – in adults can positively impact the gut microbiota and may reduce the severity of symptoms related to IBS, leading to an increased quality of life.

7 | Key Conclusions

Human milk is the undisputed gold standard for feeding infants. While many nutritional aspects of human milk have been identified and characterized over the last several decades, attention has turned recently to the bioactive components of human milk, and specifically, HMOs. The discovery that HMOs are the third most abundant solid component of human milk has triggered research focused around advancing the understanding of their various roles in human health.[9,52,186,187] As the mechanisms of HMOs are being uncovered, key health benefits are emerging:

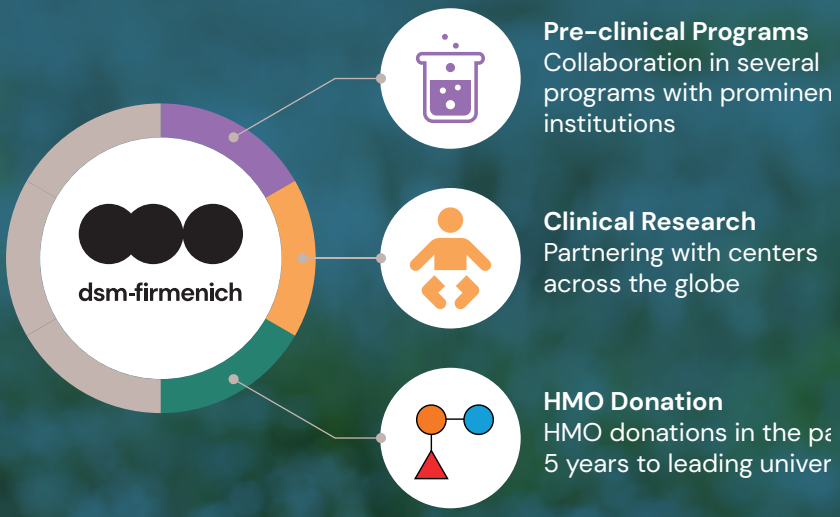
- **Acting as prebiotics and promoting the predominance of a gut microbiota rich in beneficial bacteria such as bifidobacteria**
- **Supporting short-chain fatty acid production and thus increasing acidity – important elements of a functional gut barrier, the immune system, and a healthy gut ecosystem**
- **Serving as decoy receptors for undesirable microorganism, deflecting their adhesion to cell surfaces**
- **Supporting the immune response**
- **The potential for influencing brain development and cognitive function**

The scientific community is early in the journey to fully understanding the potential health benefits of HMOs. With breastmilk always the gold standard and a guide for infant nutrition, there is still much to learn about the many roles HMOs likely play in our overall health and wellness. In the meantime, early and emerging data show promise for the ability of HMOs to support the health of infants, children, and adults.

8 | Our Commitment to HMO Research

Pioneering the Future of HMOs with Innovation and Research

dsm-firmenich's leadership in HMO innovation is complemented by their dedication to advancing scientific study on the benefits of HMOs. Their research activities are enhanced by their collaborations with some of the world's premiere scientists who lead HMO research. dsm-firmenich's three-pillar research strategy is built on a strong foundation of clinical partners, leading universities, and external labs.



Pre-clinical Programs
Collaboration in several programs with prominen institutions

Clinical Research
Partnering with centers across the globe

HMO Donation
HMO donations in the pa 5 years to leading univer

Together with their research partners, dsm-firmenich advances scientific discoveries of HMOs through pre-clinical and clinical studies. dsm-firmenich facilitates on-going HMO research through its HMO Donation Program. The objective of this innovative program is to promote further research around HMOs to gain a deeper understanding of the health benefits, modes of action, and potential applications of HMOs. The HMO Donation Program also seeks to broaden the network of scientists and experts within the field of HMO research.

To date, dsm-firmenich has supported over 100 research projects through this program. These projects span 55 different research centers all around the globe. The HMO Donation Program accesses dsm-firmenich's HMO library, which contains nearly 20 different HMO structures.

These efforts have led to over 44 publications around how HMOs impact human health.

dsm-firmenich's Regulatory Approvals Lead to Global Access to HMOs

The ability to synthesize HMOs at commercial scale remains one of the most exciting developments in infant nutrition, closing the gap in one of the largest compositional discrepancies between cow's milk-based infant formulas and human breast milk. One of the most important results of this innovation is the increased availability of HMOs for infants all around the world. As such, a commitment to working with regulatory bodies worldwide is necessary to ensure broad access to the benefits of HMOs.

dsm-firmenich leads in the number of regulatory approvals for HMOs worldwide. At date of publication, six HMO products have secured market authorization as new ingredients in foods and supplements in the European Union and the United States. dsm-firmenich holds the exclusive authorization of LNT, 6'SL, 3'SL, and a mixture product 2'FL/DFL in the European Union and holds exclusive market access of 2'FL and LNnT in Australia/New Zealand.

The close collaboration of dsm-firmenich's global and local regulatory teams recently led to an effective strategy for navigating the HMO approval process in China. The Chinese Ministry of Agriculture and Rural Affairs, MARA, recently confirmed the safety of six different manufacturing strains to produce HMOs, opening the door to the next steps in the regulatory process of bringing products with HMO innovation to the Chinese market. This achievement is due to dsm-firmenich's steadfast commitment to purposeful advancements in Early Life Nutrition in China and around the world.

As scientific and consumer recognition of the importance of HMOs in human nutrition accelerates, the number of new HMO products is expected to increase, and similarly, the number of regulatory authorizations will continue to grow. These efforts will continuously expand the availability of HMOs to more and more infants around the world, furthering the goal of enhancing Early Life Nutrition globally.

dsm-firmenich: your innovative end-to-end partner in HMOs

- **dsm-firmenich has one of the broadest portfolios of commercially available HMOs for applications across the lifecycle**
- **Our extensive history as the market pioneer of HMOs provides credibility and assurance of quality as a highly trusted source for infants, children, and adults**
- **GlyCare HMOs feature dry blend powder properties, the longest shelf-life (5 years), and the highest purity of all HMOs on the market**
- **The only vertically integrated manufacturer of HMOs with both straight, premix capabilities and market ready solutions under one roof**
- **Available for use in more than 160 countries, the largest market access for HMOs worldwide, and in supply with the world's largest HMO facility**
- **Our strong end-to-end innovation program is developing the largest pipeline of straight and blended HMOs for a healthier future**

YOUR END-TO-END PARTNER

PRODUCTS CUSTOMIZED SOLUTIONS EXPERT SERVICES

9 | References

1. Lessen R, Kavanagh K. Position of the academy of nutrition and dietetics: promoting and supporting breastfeeding. *J Acad Nutr Diet.* 2015;115(3):444-449.
2. Johnston M., Landers S., Noble L., Szucs K., L. V. Breastfeeding and the use of human milk. *Pediatrics*. 2012;129(3):e827-841.
3. World Health Organization. *Guiding Principles for Complementary Feeding of the Breastfed Child*. Geneva: WHO Press;2001.
4. UNICEF. Breastfeeding - A Mother's Gift, for Every Child. Nutrition Section. 2018.
5. Ballard O, Morrow AL. Human milk composition: nutrients and bioactive factors. *Pediatr Clin North Am*. 2013;60(1):49-74.
6. Andreas NJ, Kampmann B, Mehring Le-Doare K. Human breast milk: A review on its composition and bioactivity. *Early Hum Dev*. 2015;91(11):629-635.
7. Le Doare K, Holder B, Bassett A, Pannaraj PS. Mother's Milk: A Purposeful Contribution to the Development of the Infant Microbiota and Immunity. *Front Immunol*. 2018;9:361.
8. Carr LE, Virmani MD, Rosa F, et al. Role of Human Milk Bioactives on Infants' Gut and Immune Health. *Front Immunol*. 2021;12:604080.
9. Cheng L, Akkerman R, Kong C, Walvoort MTC, de Vos P. More than sugar in the milk: human milk oligosaccharides as essential bioactive molecules in breast milk and current insight in beneficial effects. *Crit Rev Food Sci Nutr*. 2020:1-17.
10. Morrow AL, Newburg DS. Chapter 4 - Human Milk Oligosaccharide. In: Neu J, Poindexter B, eds. *Gastroenterology and Nutrition (Third Edition)*. Philadelphia: Elsevier; 2019:43-57.
11. Kunz C, Rudloff S, Baier W, Klein N, Strobel S. Oligosaccharides in human milk: structural, functional, and metabolic aspects. *Annu Rev Nutr*. 2000;20:699-722.
12. Bode L. Human milk oligosaccharides: every baby needs a sugar mama. *Glycobiology*. 2012a;22(9):1147-1162.
13. Thum C, Wall CR, Weiss GA, Wang W, Szeto IM, Day L. Changes in HMO Concentrations throughout Lactation: Influencing Factors, Health Effects and Opportunities. *Nutrients*. 2021;13(7).
14. Walsh C, Lane JA, van Sinderen D, Hickey RM. From lab bench to formulated ingredient: Characterization, production, and commercialization of human milk oligosaccharides. *Journal of Functional Foods*. 2020a;72:104052.
15. Urashima T, Taufik E, Fukuda K, Asakuma S. Recent advances in studies on milk oligosaccharides of cows and other domestic farm animals. *Biosci Biotechnol Biochem*. 2013;77(3):455-466.
16. Soyyılmaz B, Mikš MH, Röhrig CH, Matwiejuk M, Meszaros-Matwiejuk A, Vigsnæs LK. The Mean of Milk: A Review of Human Milk Oligosaccharide Concentrations throughout Lactation. *Nutrients*. 2021;13(8).
17. Berger B, Porta N, Foata F, et al. Linking Human Milk Oligosaccharides, Infant Fecal Community Types, and Later Risk To Require Antibiotics. *mBio*. 2020a;11(2).
18. Korpela K, Salonen A, Hickman B, et al. Fucosylated oligosaccharides in mother's milk alleviate the effects of caesarean birth on infant gut microbiota. *Sci Rep*. 2018;8(1):13757.
19. Lewis ZT, Totten SM, Smilowitz JT, et al. Maternal fucosyltransferase 2 status affects the gut bifidobacterial communities of breastfed infants. *Microbiome*. 2015;3.
20. Smilowitz JT, Lebrilla CB, Mills DA, German JB, Freeman SL. Breast milk oligosaccharides: structure-function relationships in the neonate. *Annu Rev Nutr*. 2014;34:143-169.
21. Chichlowski M, De Lartigue G, German JB, Raybould HE, Mills DA. Bifidobacteria isolated from infants and cultured on human milk oligosaccharides affect intestinal epithelial function. *J Pediatr Gastroenterol Nutr*. 2012;55(3):321-327.
22. Weichert S, Jennewein S, Hüfner E, et al. Bioengineered 2'-fucosyllactose and 3-fucosyllactose inhibit the adhesion of Pseudomonas aeruginosa and enteric pathogens to human intestinal and respiratory cell lines. *Nutr Res*. 2013;33(10):831-838.
23. Coppa GV, Zampini L, Galeazzi T, et al. Human milk oligosaccharides inhibit the adhesion to Caco-2 cells of diarrheal pathogens: Escherichia coli, Vibrio cholerae, and Salmonella fyris. *Pediatr Res*. 2006;59(3):377-382.
24. Lin AE, Autran CA, Szyszka A, et al. Human milk oligosaccharides inhibit growth of group B Streptococcus. *J Biol Chem*. 2017;292(27):11243-11249.
25. Plaza-Díaz J, Fontana L, Gil A. Human Milk Oligosaccharides and Immune System Development. *Nutrients*. 2018;10(8).
26. Triantis V, Bode L, van Neerven RJJ. Immunological Effects of Human Milk Oligosaccharides. *Front Pediatr*. 2018;6:190.
27. Marriage BJ, Buck RH, Goehring KC, Oliver JS, Williams JA. Infants Fed a Lower Calorie Formula With 2'FL Show Growth and 2'FL Uptake Like Breast-Fed Infants. *J Pediatr Gastroenterol Nutr*. 2015;61(6):649-658.
28. Puccio G, Alliet P, Cajozzo C, et al. Effects of Infant Formula With Human Milk Oligosaccharides on Growth and Morbidity: A Randomized Multicenter Trial. *J Pediatr Gastroenterol Nutr*. 2017;64(4):624-631.
29. Alliet P, Vandenplas Y, Roggero P, et al. Safety and efficacy of a probiotic-containing infant formula supplemented with 2'-fucosyllactose: a double-blind randomized controlled trial. *Nutr J*. 2022;21(1):11.
30. Vandenplas Y, Żołnowska M, Berni Canani R, et al. Effects of an Extensively Hydrolyzed Formula Supplemented with Two Human Milk Oligosaccharides on Growth, Tolerability, Safety and Infection Risk in Infants with Cow's Milk Protein Allergy: A Randomized, Multi-Center Trial. *Nutrients*. 2022;14(3).
31. Goehring KC, Marriage BJ, Oliver JS, Wilder JA, Barrett EG, Buck RH. Similar to Those Who Are Breastfed, Infants Fed a Formula Containing 2'-Fucosyllactose Have Lower Inflammatory Cytokines in a Randomized Controlled Trial. *J Nutr*. 2016;146(12):2559-2566.
32. Morrow AL, Meinzen-Derr J, Huang P, et al. Fucosyltransferase 2 non-secretor and low secretor status predicts severe outcomes in premature infants. *J Pediatr*. 2011;158(5):745-751.
33. Sodhi CP, Wipf P, Yamaguchi Y, et al. The human milk oligosaccharides 2'-fucosyllactose and 6'-sialyllactose protect against the development of necrotizing enterocolitis by inhibiting toll-like receptor 4 signaling. *Pediatr Res*. 2020.
34. Autran CA, Kellman BP, Kim JH, et al. Human milk oligosaccharide composition predicts risk of necrotising enterocolitis in preterm infants. *Gut*. 2018;67(6):1064-1070.
35. Wang B, McVeagh P, Petocz P, Brand-Miller J. Brain ganglioside and glycoprotein sialic acid in breastfed compared with formula-fed infants. *Am J Clin Nutr*. 2003b;78(5):1024-1029.
36. Jacobi SK, Yatsunenko T, Li D, et al. Dietary Isomers of Sialyllactose Increase Ganglioside Sialic Acid Concentrations in the Corpus Callosum and Cerebellum and Modulate the Colonic Microbiota of Formula-Fed Piglets. *J Nutr*. 2016;146(2):200-208.
37. Berger PK, Plows JF, Jones RB, et al. Human milk oligosaccharide 2'-fucosyllactose links feedings at 1 month to cognitive development at 24 months in infants of normal and overweight mothers. *PLoS One*. 2020b;15(2).
38. Nowak-Wegrzyn A, Czerkies L, Reyes K, Collins B, Heine RG. Confirmed Hypoallergenicity of a Novel Whey-Based Extensively Hydrolyzed Infant Formula Containing Two Human Milk Oligosaccharides. *Nutrients*. 2019;11(7).
39. Ramirez-Farias C, Baggs GE, Marriage BJ. Growth, Tolerance, and Compliance of Infants Fed an Extensively Hydrolyzed Infant Formula with Added 2'-FL Fucosyllactose (2'-FL) Human Milk Oligosaccharide. *Nutrients*. 2021;13(1).
40. Sekerel BE, Bingol G, Cullu Cokugras F, et al. An Expert Panel Statement on the Beneficial Effects of Human Milk Oligosaccharides (HMOs) in Early Life and Potential Utility of HMO-Supplemented Infant Formula in Cow's Milk Protein Allergy. *J Asthma Allergy*. 2021;14:1147-1164.
41. Elison E, Vigsnaes LK, Rindom Krogsgaard L, et al. Oral supplementation of healthy adults with 2'-O-fucosyllactose and lacto-N-neotetraose is well tolerated and shifts the intestinal microbiota. *Br J Nutr*. 2016;116(8):1356-1368.
42. Iribarren C, Törnblom H, Aziz I, et al. Human milk oligosaccharide supplementation in irritable bowel syndrome patients: A parallel, randomized, double-blind, placebo-controlled study. *Neurogastroenterol Motil*. 2020;32(10):e13920.
43. Palsson OS, Peery A, Seitzberg D, Amundsen ID, McConnell B, Simrén M. Human Milk Oligosaccharides Support Normal Bowel Function and Improve Symptoms of Irritable Bowel Syndrome: A Multicenter, Open-Label Trial. *Clin Transl Gastroenterol*. 2020;11(12):e00276.
44. Oddy WH. The impact of breastmilk on infant and child health. *Breastfeed Rev*. 2002;10(3):5-18.
45. Hennet T, Borsig L. Breastfed at Tiffany's. *Trends Biochem Sci*. 2016;41(6):508-518.
46. Victora CG, Bahl R, Barros AJ, et al. Breastfeeding in the 21st century: epidemiology, mechanisms, and lifelong effect. *Lancet*. 2016;387(10017):475-490.
47. Le Huërou-Luron I, Blat S, Boudry G. Breast- v. formula-feeding: impacts on the digestive tract and immediate and long-term health effects. *Nutr Res Rev*. 2010;23(1):23-36.

48. Agostoni C, Braegger C, Decsi T, et al. Breast-feeding: A commentary by the ESPGHAN Committee on Nutrition. *J Pediatr Gastroenterol Nutr.* 2009;49(1):112-125.

49. Thurl S, Munzert M, Boehm G, Matthews C, Stahl B. Systematic review of the concentrations of oligosaccharides in human milk. *Nutr Rev.* 2017;75(11):920-933.

50. Ninonuevo MR, Park Y, Yin H, et al. A strategy for annotating the human milk glycome. *J Agric Food Chem.* 2006;54(20):7471-7480.

51. Yu ZT, Chen C, Newburg DS. Utilization of major fucosylated and sialylated human milk oligosaccharides by isolated human gut microbes. *Glycobiology.* 2013b;23(11):1281-1292.

52. Chouraqui JP. Does the contribution of human milk oligosaccharides to the beneficial effects of breast milk allow us to hope for an improvement in infant formulas? *Crit Rev Food Sci Nutr.* 2020:1-12.

53. Vandenplas Y, Berger B, Carnielli VP, et al. Human Milk Oligosaccharides: 2'-Fucosyllactose (2'-FL) and Lacto-N-Neotetraose (LNnT) in Infant Formula. *Nutrients.* 2018;10(9).

54. Asakuma S, Urashima T, Akahori M, et al. Variation of major neutral oligosaccharides levels in human colostrum. *Eur J Clin Nutr.* 2008;62(4):488-494.

55. Gómez-Gallego C, Morales JM, Monleón D, et al. Human Breast Milk NMR Metabolomic Profile across Specific Geographical Locations and Its Association with the Milk Microbiota. *Nutrients.* 2018;10(10).

56. McGuire MK, Meehan CL, McGuire MA, et al. What's normal? Oligosaccharide concentrations and profiles in milk produced by healthy women vary geographically12. *Am J Clin Nutr.* 2017;105(5):1086-1100.

57. Austin S, De Castro CA, Bénet T, et al. Temporal Change of the Content of 10 Oligosaccharides in the Milk of Chinese Urban Mothers. *Nutrients.* 2016;8(6).

58. Thurl S, Munzert M, Henker J, et al. Variation of human milk oligosaccharides in relation to milk groups and lactational periods. *Br J Nutr.* 2010;104(9):1261-1271.

59. Wu J, Wu S, Huo J, et al. Systematic Characterization and Longitudinal Study Reveal Distinguishing Features of Human Milk Oligosaccharides in China. *Curr Dev Nutr.* 2020;4(8):nzaa113.

60. Xu G, Davis JC, Goonatilleke E, Smilowitz JT, German JB, Lebrilla CB. Absolute Quantitation of Human Milk Oligosaccharides Reveals Phenotypic Variations during Lactation. *J Nutr.* 2017;147(1):117-124.

61. Plows JF, Berger PK, Jones RB, et al. Longitudinal Changes in Human Milk Oligosaccharides (HMOs) Over the Course of 24 Months of Lactation. *J Nutr.* 2021;151(4):876-882.

62. Kunz C. Historical aspects of human milk oligosaccharides. *Adv Nutr.* 2012;3(3):430s-439s.

63. Asakuma S, Hatakeyama E, Urashima T, et al. Physiology of consumption of human milk oligosaccharides by infant gut-associated bifidobacteria. *J Biol Chem.* 2011;286(40):34583-34592.

64. Morrow AL, Ruiz-Palacios GM, Jiang X, Newburg DS. Human-milk glycans that inhibit pathogen binding protect breast-feeding infants against infectious diarrhea. *J Nutr.* 2005;135(5):1304-1307.

65. Yu ZT, Nanthakumar NN, Newburg DS. The Human Milk Oligosaccharide 2'-Fucosyllactose Quenches Campylobacter jejuni-Induced Inflammation in Human Epithelial Cells HEp-2 and HT-29 and in Mouse Intestinal Mucosa. *J Nutr.* 2016;146(10):1980-1990.

66. Tarr AJ, Galley JD, Fisher SE, Chichlowski M, Berg BM, Bailey MT. The prebiotics 3'Sialyllactose and 6'Sialyllactose diminish stressor-induced anxiety-like behavior and colonic microbiota alterations: Evidence for effects on the gut-brain axis. *Brain Behav Immun.* 2015;50:166-177.

67. Fonvig CE, Amundsen ID, Vigsnæs LK, et al. Human Milk Oligosaccharides Modulate Fecal Microbiota and Are Safe for Use in Children With Overweight: A Randomized Controlled Trial. *J Pediatr Gastroenterol Nutr.* 2021;73(3):408-414.

68. Šuligoj T, Vigsnæs LK, Abbeele PVD, et al. Effects of Human Milk Oligosaccharides on the Adult Gut Microbiota and Barrier Function. *Nutrients.* 2020;12(9).

69. Kunz C, Egge H. Chapter 1 - From Bifidus Factor to Human Milk Oligosaccharides: A Historical Perspective on Complex Sugars in Milk. In: McGuire MK, McGuire MA, Bode L, eds. *Prebiotics and Probiotics in Human Milk*. San Diego: Academic Press; 2017:3-16.

70. Wiciński M, Sawicka E, Gębalski J, Kubiak K, Malinowski B. Human Milk Oligosaccharides: Health Benefits, Potential Applications in Infant Formulas, and Pharmacology. *Nutrients.* 2020;12(1).

71. Walsh C, Lane JA, van Sinderen D, Hickey RM. Human milk oligosaccharides: Shaping the infant gut microbiota and supporting health. *Journal of Functional Foods.* 2020b;72:104074.

72. Bode L, Jantscher-Krenn E. Structure-function relationships of human milk oligosaccharides. *Adv Nutr.* 2012b;3(3):383s-391s.

73. Akkerman R, Faas MM, de Vos P. Non-digestible carbohydrates in infant formula as substitution for human milk oligosaccharide functions: Effects on microbiota and gut maturation. *Crit Rev Food Sci Nutr.* 2019;59(9):1486-1497.

74. Bode L. Human milk oligosaccharides: prebiotics and beyond. *Nutr Rev.* 2009;67 Suppl 2:S183-191.

75. Bode L. The functional biology of human milk oligosaccharides. *Early Hum Dev.* 2015;91(11):619-622.

76. Ayechu-Muruzabal V, van Stigt AH, Mank M, et al. Diversity of Human Milk Oligosaccharides and Effects on Early Life Immune Development. *Front Pediatr.* 2018;6:239.

77. Kirmiz N, Robinson RC, Shah IM, Barile D, Mills DA. Milk Glycans and Their Interaction with the Infant-Gut Microbiota. *Annu Rev Food Sci Technol.* 2018;9:429-450.

78. Kobata A. Structures and application of oligosaccharides in human milk. *Proc Jpn Acad Ser B Phys Biol Sci.* 2010;86(7):731-747.

79. Castanys-Muñoz E, Martin MJ, Prieto PA. 2'-fucosyllactose: an abundant, genetically determined soluble glycan present in human milk. *Nutr Rev.* 2013;71(12):773-789.

80. Erney RM, Malone WT, Skelding MB, et al. Variability of human milk neutral oligosaccharides in a diverse population. *J Pediatr Gastroenterol Nutr.* 2000;30(2):181-192.

81. Mank M, Hauner H, Heck AJR, Stahl B. Targeted LC-ESI-MS(2) characterization of human milk oligosaccharide diversity at 6 to 16 weeks post-partum reveals clear staging effects and distinctive milk groups. *Anal Bioanal Chem.* 2020;412(25):6887-6907.

82. Azad MB, Robertson B, Atakora F, et al. Human Milk Oligosaccharide Concentrations Are Associated with Multiple Fixed and Modifiable Maternal Characteristics, Environmental Factors, and Feeding Practices. *J Nutr.* 2018;148(11):1733-1742.

83. Bode L. Human Milk Oligosaccharides: Structure and Functions. *Nestle Nutr Inst Workshop Ser.* 2020;94:115-123.

84. Seppo AE, Kukkonen AK, Kuitunen M, et al. Association of Maternal Probiotic Supplementation With Human Milk Oligosaccharide Composition. *JAMA Pediatr.* 2019;173(3):286-288.

85. Verkhnyatskaya S, Ferrari M, de Vos P, Walvoort MTC. Shaping the Infant Microbiome With Non-digestible Carbohydrates. *Front Microbiol.* 2019;10:343.

86. Salli K, Hirvonen J, Siitonen J, Ahonen I, Anglenius H, Maukonen J. Selective Utilization of the Human Milk Oligosaccharides 2'-Fucosyllactose, 3-Fucosyllactose, and Difucosyllactose by Various Probiotic and Pathogenic Bacteria. *J Agric Food Chem.* 2021;69(1):170-182.

87. Gibson GR, Hutkins R, Sanders ME, et al. Expert consensus document: The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics. *Nat Rev Gastroenterol Hepatol.* 2017;14(8):491-502.

88. Jantscher-Krenn E, Bode L. Human milk oligosaccharides and their potential benefits for the breast-fed neonate. *Minerva Pediatr.* 2012;64(1):83-99.

89. Rodríguez JM, Murphy K, Stanton C, et al. The composition of the gut microbiota throughout life, with an emphasis on early life. *Microb Ecol Health Dis.* 2015;26:26050.

90. Wassenaar TM, Panigrahi P. Is a foetus developing in a sterile environment? *Lett Appl Microbiol.* 2014;59(6):572-579.

91. Altveş S, Yildiz HK, Vural HC. Interaction of the microbiota with the human body in health and diseases. *Biosci Microbiota Food Health.* 2020;39(2):23-32.

92. Milani C, Duranti S, Bottacini F, et al. The First Microbial Colonizers of the Human Gut: Composition, Activities, and Health Implications of the Infant Gut Microbiota. *Microbiol Mol Biol Rev.* 2017;81(4).

93. Ho NT, Li F, Lee-Sarwar KA, et al. Meta-analysis of effects of exclusive breastfeeding on infant gut microbiota across populations. *Nat Commun.* 2018;9(1):4169.

94. Turroni F, Peano C, Pass DA, et al. Diversity of bifidobacteria within the infant gut microbiota. *PLoS One.* 2012;7(5):e36957.

95. Arboleya S, Watkins C, Stanton C, Ross RP. Gut Bifidobacteria Populations in Human Health and Aging. *Front Microbiol.* 2016;7:1204.

96. Underwood MA, German JB, Lebrilla CB, Mills DA. Bifidobacterium longum subspecies infantis: champion colonizer of the infant gut. *Pediatr Res.* 2015;77(1-2):229-235.

97. Marcobal A, Barboza M, Sonnenburg ED, et al. Bacteroides in the infant gut consume milk oligosaccharides via mucus-utilization pathways. *Cell Host Microbe.* 2011;10(5):507-514.

98. Sela DA, Li Y, Lerno L, et al. An infant-associated bacterial commensal utilizes breast milk sialyloligosaccharides. *J Biol Chem.* 2011;286(14):11909-11918.

99. Bunesova V, Lacroix C, Schwab C. Fucosyllactose and L-fucose utilization of infant Bifidobacterium longum and Bifidobacterium kashiwanohense. *BMC Microbiol*. 2016;16(1):248.

100. Marcobal A, Barboza M, Froehlich JW, et al. Consumption of human milk oligosaccharides by gut-related microbes. *J Agric Food Chem*. 2010;58(9):5334-5340.

101. LoCascio RG, Ninonuevo MR, Freeman SL, et al. Glycoprofiling of bifidobacterial consumption of human milk oligosaccharides demonstrates strain specific, preferential consumption of small chain glycans secreted in early human lactation. *J Agric Food Chem*. 2007;55(22):8914-8919.

102. Yu ZT, Chen C, Kling DE, et al. The principal fucosylated oligosaccharides of human milk exhibit prebiotic properties on cultured infant microbiota. *Glycobiology*. 2013a;23(2):169-177.

103. Ruiz-Moyano S, Totten SM, Garrido DA, et al. Variation in consumption of human milk oligosaccharides by infant gut-associated strains of Bifidobacterium breve. *Appl Environ Microbiol*. 2013;79(19):6040-6049.

104. Korpela K, Salonen A, Hickman B, et al. Fucosylated oligosaccharides in mother's milk alleviate the effects of caesarean birth on infant gut microbiota. *Sci Rep*. 2018;8.

105. Nolan LS, Rimer JM, Good M. The Role of Human Milk Oligosaccharides and Probiotics on the Neonatal Microbiome and Risk of Necrotizing Enterocolitis: A Narrative Review. *Nutrients*. 2020;12(10).

106. Vancamelbeke M, Vermeire S. The intestinal barrier: a fundamental role in health and disease. *Expert Rev Gastroenterol Hepatol*. 2017;11(9):821-834.

107. Vester Boler BM, Rossoni Serao MC, Faber TA, et al. *In vitro* fermentation characteristics of select nondigestible oligosaccharides by infant fecal inocula. *J Agric Food Chem*. 2013;61(9):2109-2119.

108. Gibson GR, Wang X. Regulatory effects of bifidobacteria on the growth of other colonic bacteria. *J Appl Bacteriol*. 1994;77(4):412-420.

109. Schwab C, Gänzle M. Lactic acid bacteria fermentation of human milk oligosaccharide components, human milk oligosaccharides and galactooligosaccharides. *FEMS Microbiol Lett*. 2011;315(2):141-148.

110. Tan J, McKenzie C, Potamitis M, Thorburn AN, Mackay CR, Macia L. The role of short-chain fatty acids in health and disease. *Adv Immunol*. 2014;121:91-119.

111. Gao Y, Davis B, Zhu W, Zheng N, Meng D, Walker WA. Short-chain fatty acid butyrate, a breast milk metabolite, enhances immature intestinal barrier function genes in response to inflammation in vitro and in vivo. *Am J Physiol Gastrointest Liver Physiol*. 2021;320(4):G521-g530.

112. Okumura R, Takeda K. Roles of intestinal epithelial cells in the maintenance of gut homeostasis. *Exp Mol Med*. 2017;49(5):e338.

113. Chleilat F, Klancic T, Ma K, Schick A, Nettleton JE, Reimer RA. Human Milk Oligosaccharide Supplementation Affects Intestinal Barrier Function and Microbial Composition in the Gastrointestinal Tract of Young Sprague Dawley Rats. *Nutrients*. 2020;12(5).

114. Natividad JM, Rytz A, Keddani S, Bergonzelli G, Garcia-Rodenas CL. Blends of Human Milk Oligosaccharides Confer Intestinal Epithelial Barrier Protection in Vitro. *Nutrients*. 2020;12(10).

115. Holscher HD, Davis SR, Tappenden KA. Human milk oligosaccharides influence maturation of human intestinal Caco-2Bbe and HT-29 cell lines. *J Nutr*. 2014;144(5):586-591.

116. Holscher HD, Bode L, Tappenden KA. Human Milk Oligosaccharides Influence Intestinal Epithelial Cell Maturation In Vitro. *J Pediatr Gastroenterol Nutr*. 2017;64(2):296-301.

117. Donovan SM, Comstock SS. Human Milk Oligosaccharides Influence Neonatal Mucosal and Systemic Immunity. *Ann Nutr Metab*. 2016;69 Suppl 2(Suppl 2):42-51.

118. Kong C, Elderman M, Cheng L, de Haan BJ, Nauta A, de Vos P. Modulation of Intestinal Epithelial Glycocalyx Development by Human Milk Oligosaccharides and Non-Digestible Carbohydrates. *Mol Nutr Food Res*. 2019;63(17):e1900303.

119. Cheng L, Kong C, Walvoort MTC, Faas MM, de Vos P. Human Milk Oligosaccharides Differently Modulate Goblet Cells Under Homeostatic, Proinflammatory Conditions and ER Stress. *Mol Nutr Food Res*. 2020;64(5):e1900976.

120. Bienenstock J, Buck RH, Linke H, Forsythe P, Stanisz AM, Kunze WA. Fucosylated but not sialylated milk oligosaccharides diminish colon motor contractions. *PLoS One*. 2013;8(10):e76236.

121. Saavedra JM, Dattilo AM. Early development of intestinal microbiota: implications for future health. *Gastroenterol Clin North Am*. 2012;41(4):717-731.

122. Ruiz-Palacios GM, Cervantes LE, Ramos P, Chavez-Munguia B, Newburg DS. Campylobacter jejuni binds intestinal H(O) antigen (Fuc alpha 1, 2Gal beta 1, 4GlcNAc), and fucosyloligosaccharides of human milk inhibit its binding and infection. *J Biol Chem*. 2003;278(16):14112-14120.

123. Vigsnaes LK, Ghyselinck J, Van den Abbeele P, et al. 2'FL and LNnT Exert Antipathogenic Effects against C. difficile ATCC 9689 In Vitro, Coinciding with Increased Levels of Bifidobacteriaceae and/or Secondary Bile Acids. *Pathogens*. 2021;10(8).

124. Borghesi A, Stronati M, Fellay J. Neonatal Group B Streptococcal Disease in Otherwise Healthy Infants: Failure of Specific Neonatal Immune Responses. *Front Immunol*. 2017;8:215.

125. Rao GG, Khanna P. To screen or not to screen women for Group B Streptococcus (Streptococcus agalactiae) to prevent early onset sepsis in newborns: recent advances in the unresolved debate. *Ther Adv Infect Dis*. 2020;7:2049936120942424.

126. Ackerman DL, Doster RS, Weitkamp JH, Aronoff DM, Gaddy JA, Townsend SD. Human Milk Oligosaccharides Exhibit Antimicrobial and Antibiofilm Properties against Group B Streptococcus. *ACS Infect Dis*. 2017;3(8):595-605.

127. Duska-McEwen G, Senft AP, Ruetschilling TL, Barrett EG, Buck R. Human milk oligosaccharides enhance innate immunity to respiratory syncytial virus and influenza in vitro. *Food and Nutrition Sciences*. 2014;5:1387-1398.

128. Cheng L, Kiewiet MBG, Groeneveld A, Nauta A, de Vos P. Human milk oligosaccharides and its acid hydrolysate LNT2 show immunomodulatory effects via TLRs in a dose and structure-dependent way. *Journal of Functional Foods*. 2019;59:174-184.

129. Xiao L, Leusink-Muis T, Kettelarij N, et al. Human Milk Oligosaccharide 2'-Fucosyllactose Improves Innate and Adaptive Immunity in an Influenza-Specific Murine Vaccination Model. *Front Immunol*. 2018;9:452.

130. Kajzer JA, Oliver J, Marriage BJ. Gastrointestinal Tolerance of Formula Supplemented with Oligosaccharides. Paper presented at: Experimental Biology2016; San Diego.

131. Parschat K, Melsaether C, Jäpelt KR, Jennewein S. Clinical Evaluation of 16-Week Supplementation with 5HMO-Mix in Healthy-Term Human Infants to Determine Tolerability, Safety, and Effect on Growth. *Nutrients*. 2021;13(8).

132. Reverri EJ, Devitt AA, Kajzer JA, Baggs GE, Borschel MW. Review of the Clinical Experiences of Feeding Infants Formula Containing the Human Milk Oligosaccharide 2'-Fucosyllactose. *Nutrients*. 2018;10(10).

133. Storm HM, Shepard J, Czerkies LM, et al. 2'-Fucosyllactose Is Well Tolerated in a 100% Whey, Partially Hydrolyzed Infant Formula With Bifidobacterium lactis: A Randomized Controlled Trial. *Glob Pediatr Health*. 2019;6:2333794x19833995.

134. Garcia Rodenas CL, Lepage M, Ngom-Bru C, Fotiou A, Papagaroufalis K, Berger B. Effect of Formula Containing Lactobacillus reuteri DSM 17938 on Fecal Microbiota of Infants Born by Cesarean-Section. *J Pediatr Gastroenterol Nutr*. 2016;63(6):681-687.

135. Sung V, D'Amico F, Cabana MD, et al. Lactobacillus reuteri to Treat Infant Colic: A Meta-analysis. *Pediatrics*. 2018;141(1).

136. Schreck Bird A, Gregory PJ, Jalloh MA, Risoldi Cochrane Z, Hein DJ. Probiotics for the Treatment of Infantile Colic: A Systematic Review. *J Pharm Pract*. 2017;30(3):366-374.

137. Behrman RE, Butler AS. *Institute of Medicine Committee on Understanding Premature, Birth Assuring Healthy*, Outcomes. Washington (DC): National Academies Press (US) Copyright © 2007, National Academy of Sciences.; 2007.

138. Callaghan WM, MacDorman MF, Rasmussen SA, Qin C, Lackritz EM. The contribution of preterm birth to infant mortality rates in the United States. *Pediatrics*. 2006;118(4):1566-1573.

139. Shulhan J, Dicken B, Hartling L, Larsen BM. Current Knowledge of Necrotizing Enterocolitis in Preterm Infants and the Impact of Different Types of Enteral Nutrition Products. *Adv Nutr*. 2017;8(1):80-91.

140. Collado MC, Cernada M, Neu J, Pérez-Martínez G, Gormaz M, Vento M. Factors influencing gastrointestinal tract and microbiota immune interaction in preterm infants. *Pediatr Res*. 2015;77(6):726-731.

141. Brehin C, Dubois D, Dicky O, Breinig S, Oswald E, Serino M. Evolution of Gut Microbiome and Metabolome in Suspected Necrotizing Enterocolitis: A Case-Control Study. *J Clin Med*. 2020;9(7).

142. Torrazza RM, Ukhanova M, Wang X, et al. Intestinal microbial ecology and environmental factors affecting necrotizing enterocolitis. *PLoS One*. 2013;8(12):e83304.

143. Morrow AL, Lagomarcino AJ, Schibler KR, et al. Early microbial and metabolomic signatures predict later onset of necrotizing enterocolitis in preterm infants. *Microbiome*. 2013;1(1):13.

144. Halpern MD, Denning PW. The role of intestinal epithelial barrier function in the development of NEC. *Tissue Barriers*. 2015;3(1-2):e1000707.

145. Jantscher-Krenn E, Zherebtsov M, Nissan C, et al. The human milk oligosaccharide disialyllacto-N-tetraose prevents necrotising enterocolitis in neonatal rats. *Gut*. 2012;61(10):1417-1425.

146. Autran CA, Schoterman MH, Jantscher-Krenn E, Kamerling JP, Bode L. Sialylated galacto-oligosaccharides and 2'-fucosyllactose reduce necrotising enterocolitis in neonatal rats. *Br J Nutr*. 2016;116(2):294-299.

147. Li B, Wu RY, Horne RG, et al. Human Milk Oligosaccharides Protect against Necrotizing Enterocolitis by Activating Intestinal Cell Differentiation. *Mol Nutr Food Res*. 2020:e2000519.

148. Masi AC, Embleton ND, Lamb CA, et al. Human milk oligosaccharide DSLNT and gut microbiome in preterm infants predicts necrotising enterocolitis. *Gut*. 2021;70(12):2273-2282.

149. Van Niekerk E, Autran CA, Nel DG, Kirsten GF, Blaauw R, Bode L. Human milk oligosaccharides differ between HIV-infected and HIV-uninfected mothers and are related to necrotizing enterocolitis incidence in their preterm very-low-birth-weight infants. *J Nutr*. 2014;144(8):1227-1233.

150. Wejryd E, Martí M, Marchini G, et al. Low Diversity of Human Milk Oligosaccharides is Associated with Necrotising Enterocolitis in Extremely Low Birth Weight Infants. *Nutrients*. 2018;10(10).

151. Shane AL, Sánchez PJ, Stoll BJ. Neonatal sepsis. Lancet. 2017;390(10104):1770-1780.

152. Adams M, Bassler D. Practice variations and rates of late onset sepsis and necrotizing enterocolitis in very preterm born infants, a review. *Transl Pediatr*. 2019;8(3):212-226.

153. Torres Roldan VD, Urtecho SM, Gupta J, et al. Human milk oligosaccharides and their association with late-onset neonatal sepsis in Peruvian very-low-birth-weight infants. *Am J Clin Nutr*. 2020;112(1):106-112.

154. Beluska-Turkan K, Korczak R, Hartell B, et al. Nutritional Gaps and Supplementation in the First 1000 Days. *Nutrients*. 2019;11(12).

155. Victora CG, Horta BL, Loret de Mola C, et al. Association between breastfeeding and intelligence, educational attainment, and income at 30 years of age: a prospective birth cohort study from Brazil. *Lancet Glob Health*. 2015;3(4):e199-205.

156. Belfort MB, Rifas-Shiman SL, Kleinman KP, et al. Infant feeding and childhood cognition at ages 3 and 7 years: Effects of breastfeeding duration and exclusivity. *JAMA Pediatr*. 2013;167(9):836-844.

157. Lechner BE, Vohr BR. Neurodevelopmental Outcomes of Preterm Infants Fed Human Milk: A Systematic Review. *Clin Perinatol*. 2017;44(1):69-83.

158. Horta BL, Loret de Mola C, Victora CG. Breastfeeding and intelligence: a systematic review and meta-analysis. *Acta Paediatr*. 2015;104(467):14-19.

159. Kramer MS, Aboud F, Mironova E, et al. Breastfeeding and child cognitive development: new evidence from a large randomized trial. *Arch Gen Psychiatry*. 2008;65(5):578-584.

160. Wang B, Brand-Miller J. The role and potential of sialic acid in human nutrition. *Eur J Clin Nutr*. 2003a;57(11):1351-1369.

161. Wang B, Yu B, Karim M, et al. Dietary sialic acid supplementation improves learning and memory in piglets. *Am J Clin Nutr*. 2007;85(2):561-569.

162. Wang B, Brand-Miller J, McVeagh P, Petocz P. Concentration and distribution of sialic acid in human milk and infant formulas. *Am J Clin Nutr*. 2001;74(4):510-515.

163. Clouard C, Reimert I, Fleming SA, Koopmans SJ, Schuurman T, Hauser J. Dietary sialylated oligosaccharides in early-life may promote cognitive flexibility during development in context of obesogenic dietary intake. *Nutr Neurosci*. 2021:1-18.

164. Mudd AT, Fleming SA, Labhart B, et al. Dietary Sialyllactose Influences Sialic Acid Concentrations in the Prefrontal Cortex and Magnetic Resonance Imaging Measures in Corpus Callosum of Young Pigs. *Nutrients*. 2017;9(12).

165. Hauser J, Pisa E, Arias Vásquez A, et al. Sialylated human milk oligosaccharides program cognitive development through a non-genomic transmission mode. *Mol Psychiatry*. 2021;26(7):2854-2871.

166. Fleming SA, Mudd AT, Hauser J, et al. Dietary Oligofructose Alone or in Combination with 2'-Fucosyllactose Differentially Improves Recognition Memory and Hippocampal mRNA Expression. *Nutrients*. 2020a;12(7).

167. Fleming SA, Mudd AT, Hauser J, et al. Human and Bovine Milk Oligosaccharides Elicit Improved Recognition Memory Concurrent With Alterations in Regional Brain Volumes and Hippocampal mRNA Expression. *Front Neurosci*. 2020b;14:770.

168. Oliveros E, Ramirez M, Vazquez E, et al. Oral supplementation of 2'-fucosyllactose during lactation improves memory and learning in rats. J *Nutr Biochem*. 2016;31:20-27.

169. Vazquez E, Barranco A, Ramirez M, et al. Dietary 2'-Fucosyllactose Enhances Operant Conditioning and Long-Term Potentiation via Gut-Brain Communication through the Vagus Nerve in Rodents. *PLoS One*. 2016;11(11):e0166070.

170. Oliveros E, Martin MJ, Torres-Espinola FJ, et al. Human Milk Levels of 2′-Fucosyllactose and 6′-Sialyllactose are Positively Associated with Infant Neurodevelopment and are Not Impacted by Maternal BMI or Diabetic Status. J *Nutr Food Sci*. 2021;4.

171. Al-Khafaji AH, Jepsen SD, Christensen KR, Vigsnæs LK. The potential of human milk oligosaccharides to impact the microbiota-gut-brain axis through modulation of the gut microbiota. *Journal of Functional Foods*. 2020;74:104176.

172. Leung TF, Ulfman LH, Chong MKC, et al. A randomized controlled trial of different young child formulas on upper respiratory and gastrointestinal tract infections in Chinese toddlers. *Pediatr Allergy Immunol*. 2020;31(7):745-754.

173. Liu S, Cai X, Wang J, et al. Six Oligosaccharides' Variation in Breast Milk: A Study in South China from 0 to 400 Days Postpartum. *Nutrients*. 2021;13(11):4017.

174. Lagström H, Rautava S, Ollila H, et al. Associations between human milk oligosaccharides and growth in infancy and early childhood. *Am J Clin Nutr*. 2020;111(4):769-778.

175. Järvinen KM, Martin H, Oyoshi MK. Immunomodulatory effects of breast milk on food allergy. *Ann Allergy Asthma Immunol*. 2019;123(2):133-143.

176. Sampson HA. Food allergy. Part 2: diagnosis and management. J Allergy Clin Immunol. 1999;103(6):981-989.

177. AAP. American Academy of Pediatrics. Committee on Nutrition. Hypoallergenic infant formulas. *Pediatrics*. 2000;106(2 Pt 1):346-349.

178. Seppo AE, Autran CA, Bode L, Järvinen KM. Human milk oligosaccharides and development of cow's milk allergy in infants. *J Allergy Clin Immunol*. 2017;139(2):708-711.e705.

179. Lodge CJ, Lowe AJ, Milanzi E, et al. Human Milk Oligosaccharide profiles and allergic disease up to 18 years. *Journal of Allergy and Clinical Immunology*. 2020.

180. Miliku K, Robertson B, Sharma AK, et al. Human milk oligosaccharide profiles and food sensitization among infants in the CHILD Study. *Allergy*. 2018;73(10):2070-2073.

181. Sprenger N, Odenwald H, Kukkonen AK, Kuitunen M, Savilahti E, Kunz C. FUT2-dependent breast milk oligosaccharides and allergy at 2 and 5 years of age in infants with high hereditary allergy risk. *Eur J Nutr*. 2017;56(3):1293-1301.

182. Rousseaux A, Brosseau C, Le Gall S, Piloquet H, Barbarot S, Bodinier M. Human Milk Oligosaccharides: Their Effects on the Host and Their Potential as Therapeutic Agents. *Front Immunol*. 2021;12:680911.

183. Ryan JJ, Monteagudo-Mera A, Contractor N, Gibson GR. Impact of 2'-Fucosyllactose on Gut Microbiota Composition in Adults with Chronic Gastrointestinal Conditions: Batch Culture Fermentation Model and Pilot Clinical Trial Findings. *Nutrients*. 2021;13(3).

184. Illiano P, Brambilla R, Parolini C. The mutual interplay of gut microbiota, diet and human disease. *Febs j*. 2020;287(5):833-855.

185. Wang Y, Zou Y, Wang J, Ma H, Zhang B, Wang S. The Protective Effects of 2'-Fucosyllactose against E. Coli O157 Infection Are Mediated by the Regulation of Gut Microbiota and the Inhibition of Pathogen Adhesion. *Nutrients*. 2020;12(5).

186. Moukarzel S, Bode L. Human Milk Oligosaccharides and the Preterm Infant: A Journey in Sickness and in Health. *Clin Perinatol*. 2017;44(1):193-207.

187. Pacheco AR, Barile D, Underwood MA, Mills DA. The impact of the milk glycobiome on the neonate gut microbiota. *Annu Rev Anim Biosci*. 2015;3:419-445.

188. Asadpoor M, Peeters C, Henricks PAJ, et al. Anti-Pathogenic Functions of Non-Digestible Oligosaccharides In Vitro. *Nutrients*. 2020;12(6).

189. Francis CY, Morris J, Whorwell PJ. The irritable bowel severity scoring system: a simple method of monitoring irritable bowel syndrome and its progress. *Aliment Pharmacol Ther*. 1997;11(2):395-402.

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