Human Milk Oligosaccharides (HMOs)
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Abstract

It is a great success that biotechnological means are available today to produce amounts of single human milk oligosaccharides (HMOs) in a purity which allows performing metabolic and functional studies even in humans. As recent data indicate that there is a link between the Lewis blood group and the secretor status of an individual and certain inflammatory diseases, this review will also focus on the metabolic fate of secretor- and Lewis blood group-specific components. We conclude that there is no simple urinary or fecal excretion pattern of HMOs, although the pattern in urine often reflects the mother’s secretor/nonsecretor status. However, there are deviations for single HMOs which deserve special attention. In feces, the variation in excretion is much higher than in urine, which may be caused by variations in the infant’s intestinal microbiota. A gradual decrease in HMO excretion with time as proposed earlier does not take place as even after 7 months of exclusive breastfeeding often intact HMOs can be detected in feces and urine. In addition, we found that whenever oligosaccharides were detected in feces, LNT, the major core structure of HMOs, was present. Hence, our data do not support speculations that LNT is a preferable source for the microbiota.

Introduction

Already around 1900, concomitant with the discovery of lactobacilli and bifidobacteria and their relevance for health and disease, pediatricians realized that the microbial composition of stool samples from breastfed and bottle-fed infants differed. Observations indicated that this difference was particularly linked to the milk carbohydrate fraction. This was the starting point of research on human milk carbohydrates. In the middle of the last century, the first human milk oligosaccharides (HMOs) were identified by Richard Kuhn in Heidelberg (Germany) [1]. At the same time, Paul György’s studies in Heidelberg and later in Philadelphia (USA) focused on the identification of various HMOs as the “bifidus factor” in human milk (Fig. 1) [2]. Meanwhile about 150–200 single HMOs have been characterized.

In recent years, there has been a tremendous increase in our knowledge regarding specific effects of HMOs [3–10]. Even the first human studies on infant formula supplemented with single HMOs have already been published [11, 12]. In order to determine which compound(s) would be most suitable for supplementation, in which concentrations or combinations, and how long they should be given, studies are needed regarding the metabolic fate of HMOs as well as their local and systemic effects.

A straightforward strategy to investigate the metabolism of HMOs is to determine the presence or absence of natural milk oligosaccharides in feces, blood, urine, or tissue. However, some prerequisites hamper this simple approach as, for example, tissue and blood are not available or rather difficult to obtain from human infants. Also the methodological handling of a large number of samples is still a difficult task and requires standardized methods.

In the following, we briefly address the difficulties of compositional analysis before we present a summary of metabolic aspects from various studies which focus on the excretion of oligosaccharides in feces and urine.

Fig. 1. Pioneers in HMO research

Discovery of HMOs
Heidelberg (1950+)
- First structural identification of HMOs by classical chemical methods (2'FL, LNT, LNnT, LNFP I-III, 3'SL, 6'SL etc.)

In collaboration with R. Kuhn
Heidelberg and Philadelphia (1950+)
- Combination of structural and functional studies

Identification of HMOs as growth factors for Bifidobacteria
Compositional Analysis of Human Milk Oligosaccharides – Need for Standardized Methods

Standardized methods for routine analysis are not available yet, which is one major reason for the discrepancy between reported qualitative and quantitative data. For the identification of HMOs, various methods such as paper chromatography, HPLC coupled with mass spectrometry (MS), HPAEC-PAD (high-performance anion exchange chromatography), HPLC chip MS, capillary gel electrophoresis/laser-induced fluorescence techniques, or liquid chromatography-HPLC-MS have recently been used. All these methods provide detailed insights into the oligosaccharide pattern of individual milk samples, frequently paired with further information about the relative amount of single isomers. However, most of these techniques require a sophisticated and time-consuming sample preparation, which is a drawback for the analysis of large sample sets.

With regard to quantitative aspects, data from different laboratories also vary significantly [13–17]. Frequently, various MS methods are applied, and concentrations are given as “relative data”. However, MS can hardly be recommended for quantitative purposes, and interpretation of those data should be done with caution [18]. In addition, there is the need for using laborious derivatization techniques, such as permethylation or labeling with fluorescence reagents. As a consequence of quantitative considerations, it would be necessary to prove the efficiency of derivatization after each single step. Also, it has to be kept in mind that often a time-consuming sample preparation with various centrifugation and/or extraction steps using different cartridges has to be performed before applying the sample to an MS instrument. The efficiency of those requirements may vary, for example, between individuals or laboratories, issues which should certainly be solved as soon as possible.

**Figure 2:** Intake, metabolism, and potential functions of HMOs. Numbers indicate biological functions, e.g., prevention of pathogen adhesion (1), direct effects on epithelial cells (2), influence on the intestinal microbiota (3), and systemic effects (4) [Modified according to quantitative data from 16, 30].

**Metabolism of Human Milk Oligosaccharides**

The high structural diversity of HMOs complicates acquiring a sophisticated knowledge on “what structures are important for infants’ health” despite the recent methodological developments to characterize minute amounts of HMOs and their degradation products in biological samples.

Metabolic studies can help to identify if HMOs are absorbed and utilized by the infant’s organism. Moreover, fecal oligosaccharide profiling might give an indication of the intestinal health of infants due to the known relationship between microbiota and the abundance of complex glycans in the infant’s gut. Urinary excretion can support observations from studies addressing systemic effects of oligosaccharides.

Only recently, studies trying to prove a direct link between HMO structures and their prebiotic function in vivo in infants have been published. A recent proof-of-concept study indicated an association between the fecal HMO composition and gut microbiota of 2 breastfed infants over time, though both parameters differed substantially between both infants studied [19]. Another study showed a relationship between the fecal microbiome of exclusively breastfed infants and the HMO composition of the milk that was fed [20].

However, since the variation in both (HMOs and microbiota composition) is vast, unequivocal structure-function studies are inevitable for a better understanding of their association with health and disease.

First studies on fecal oligosaccharides and possible metabolites of HMOs in infants have already been reported by Lundblad’s group in the 1970s [21]. Recent studies using new technologies either included only one or a low number of infants, or showed data from premature infants whose intestinal function may be very different from healthy term infants [22, 23].

Current data have so far led to the conclusion that the major portion of HMOs reaches the lower gastrointestinal tract where HMOs might be used as nutritive factors by various microorganisms or influence their activities. The remainders are excreted intact or in metabolized form in feces or urine, indicating that there is no uniform metabolic process for all HMOs [5, 24, 25].

Figure 2 shows a summary of the intake, metabolism, and examples for potential functions of HMOs currently being discussed.
Content of Human Milk Oligosaccharides in Term and Preterm Milk

To further improve infant formulas in order to gain more benefit from HMOs, a question often raised is how much of an individual component should be supplemented. In this context, some studies indicated that one has to differentiate between term and preterm milk, as the latter is considered to have either a higher content or a different pattern than term milk [15, 23].

As we recently discussed, we found no difference in the total amount of HMOs between term and preterm milk, neither in colostrum nor in transitory or mature milk, which is exemplified for mature milk in Figure 3 a [16].

Often, however, no distinction is made between secretor and nonsecretor milk among the pool of samples analyzed, which will be further discussed in the following.

Intake of Human Milk Oligosaccharides – Differentiation between Lewis Blood Group and Secretor/Nonsecretor Milk Status

It has been established for a long time that the HMO pattern depends on the genetic background of the mother, i.e., on the Lewis blood group and the secretor status (Table 1) [26, 27]. This aspect is particularly important as feeding infants with a different HMO pattern might reduce or even increase the risk for certain diseases.

Grouping the same sample set of term and preterm milk (Figure 3 a) according to the secretor status and the Lewis blood group of the mother, it is obvious that there are large differences between milk samples depending on the secretor and Lewis blood group specific pattern (Fig. 3 b, c). Differences between individual HMOs in both groups and in Lewis a, Lewis b, and Lewis-negative milk samples are further discussed by Kunz et al. [16].

Comparison of Human Milk Oligosaccharides in Milk, Urine, and Feces in Mother-Child Dyads

We performed 2 stable isotope studies collecting milk, feces, urine, and breath samples at each suckling over up to 36 h after having applied an oral bolus of 13 C-labeled glucose or galactose given to lactating mothers [28–31]. In the following, we present some of these data together with those from 2 other cohort studies [16, 32].

Urinary Excretion Pattern

Our data so far support the general conclusion that the overall pattern of neutral oligosaccharides in the urine from breastfed infants reflects that of their mothers’ milk suggesting a strong association with the mothers’ Lewis blood group and secretor phenotype. The major difference between the milk from mothers with blood group Lewis a, Lewis b, and Lewis 0 is a rather complex pattern in “Lewis b milk” compared to “Lewis a milk,” and “Lewis negative milk” [25, 31, 32, 33]. Comparing HMOs from “Lewis a” or “Lewis b milk” with the urinary oligosaccharide pattern in the corresponding infants, for example, a similar pattern with the same molecular masses was observed [25, 30, 31].

Figure 3. HMO concentrations in mature human milk from mothers with term or preterm infants (a), secretor or nonsecretor status (b), and with blood group Lewis a, b, or 0 status (c). Concentrations are given as medians and interquartile ranges.

*p < 0.05; ** p < 0.01; *** p < 0.001 (adapted from Kunz et al. [16]).
Using MALDI-TOF (matrix assisted laser desorption ionization-time of flight)-MS, differences could be seen in the signal intensities for both, milk and urine, although the masses m/z 730 (LNT, LNnT), 876 (monofucosylated LNT/LNnT), or 1022 (difucosylated LNT/LNnT), as well as others, are present in all samples. As lacto-N-fucopentaose (LNFP) I is characteristic for “Lewis b milk,” one would expect that m/z 876, a major mass signal, represents this component; however, MS does not directly allow a clear assignment of various isomeric structures. Analyzing the fraction by HPAEC with pulsed amperometric detection enabling a separation of HMO isomers such as LNFP I and II, we were not able to detect LNFP I in any of the investigated urine samples from infants receiving “Lewis b milk” from their mothers [25, 31].

**Table 1. Presence of HMO structures according to the Lewis blood group and secretor (sec.) status**

<table>
<thead>
<tr>
<th>HMO structure</th>
<th>Blood group (% of the population)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lewis b</td>
</tr>
<tr>
<td>name</td>
<td>sec. (70)</td>
</tr>
<tr>
<td>2’FL</td>
<td>Fuc α1–2 Gal β1–4 Glc</td>
</tr>
<tr>
<td>LDFT</td>
<td>Fuc α1–2 Gal β1–4 (Fuc α1–3) Glc</td>
</tr>
<tr>
<td>LNFP I</td>
<td>Fuc α1–2 Gal β1–3 GlcNAc β1–3 Gal β1–4 Glc</td>
</tr>
<tr>
<td>LNFP II</td>
<td>Gal β1–3 (Fuc α1–4) GlcNAc β1–3 Gal β1–4 Glc</td>
</tr>
<tr>
<td>LNDFH II</td>
<td>Gal β1–3 (Fuc α1–4) GlcNAc β1–3 Gal β1–4 (Fuc α1–3) Glc</td>
</tr>
</tbody>
</table>

2’FL, 2’-fucosyllactose; LDFT, lactodifucotetraose; LNFP, lacto-N-fucopentaose; LNDFH, lacto-N-difucohexaose.  

| Traces may be present. |

Fecal Excretion Patterns – Changes with Time

Oligosaccharides known from human milk can be detected in many fecal samples from exclusively breastfed infants [26, 33]. Sometimes the feces of infants displayed a profile distinguishable from milk, where certain HMOs predominated and common, especially larger HMOs, were clearly diminished, and, often, no HMOs were detected, especially after 6–7 months of breastfeeding.

Some infants had reduced abundances of larger structures and a remarkably high proportion of difucosylated LN(n)T (isomers) at m/z 1022, which, in contrast, could not be detected in other infants. The predominance of the signal at m/z 1022 was also reported by Albrecht et al. [34] in 1 infant. In other cases, we observed lower fecal HMO excretions in earlier samples (2 months of age) compared to follow-up samples (7 months of age). This observation is contradictory to the reports of Albrecht et al. [34], who claimed a gradual decrease in HMO abundance with age, hypothesizing an association with gut (microbiota) maturity [22].

Furthermore, one would expect that secretor-specific HMOs would only be present in feces or urine of infants fed milk from “secretor mothers” or “Lewis b mothers.” However, we identified secretor-specific oligosaccharides 2’FL, DFLT, and LNDFH I not only in urine and feces from infants fed “secretor milk,” but often also in those fed “nonsecretor milk” [25]. This finding is in line with reports by Lundblad’s group [21] for feces and the study by De Leoz et al. [35] for urine.

In addition and contradictory to other publications, we detected LNT and LNnT, 2 structures commonly present in human milk, in many infants’ urine, and, if HMOs were present, in all fecal samples. In other reports on fecal samples, however, LNT was absent in samples from breastfed infants at different time points [22, 35].
Perspectives

It can be concluded that there is no simple urinary or fecal excretion pattern of HMOs, although the pattern in urine often reflects the mother’s secretor/non-secretor status. However, there are obviously deviations for some single HMOs which deserve special attention. In feces, the variation in excretion is much higher than in urine, which may be caused by variations in the microbial composition within the gastrointestinal tract. A simple gradual decrease in HMO excretion with time as proposed earlier does not take place, as even after 7 months of exclusive breastfeeding intact HMOs can be detected, although in the majority of infants no oligosaccharides are present. In addition, we found that whenever oligosaccharides were detected in feces, LNT, the major core structure of HMOs, was present. Hence, our data do not support previous speculations that LNT is a preferable background to improve the benefit of HMO supplementation.

Disclosure Statement

There are no conflicts of interest.

References

Human milk oligosaccharides (HMOs) are the third most abundant component of human milk. So far, more than 150 different and structurally distinct HMOs have been identified. HMO composition varies substantially between women, but remains fairly constant over the course of lactation in the same woman. Which maternal genetic and environmental factors drive the interindividual variations in HMO composition remains poorly understood, and it is currently unknown whether or not a woman’s characteristic HMO composition has evolved to match her own infant’s specific needs. A combination of preclinical, cohort and clinical studies is required to fully assess the many effects, functions and potential claims associated with HMOs.

In some cases, individual HMOs exert a certain effect and, while there might be some redundancy, the effects are often highly structure-specific. In other cases, a combination of different HMOs in specific ratios to each other is required to be effective, and future research needs to assess whether or not the administration of individual HMOs alone may be counterproductive and potentially harmful to the infant’s short- and long-term health. Overall, the personalized complexity of HMOs cannot be mimic in artificial infant formula and provides yet another powerful reason to protect, promote and support breastfeeding.

What are human milk oligosaccharides (HMOs)?

One Liter of mature human milk contains 5-15 g of complex carbohydrates that are collectively called human milk oligosaccharides (HMOs). Their high concentration makes HMOs the third most abundant solid component of mature milk, only exceeded by the concentration of lactose and total lipids and often exceeding the concentration of total proteins [1]. In comparison, the concentration of oligosaccharides in bovine milk is 100- to 1,000-fold lower and many of the bovine milk oligosaccharides are structurally distinct from and less complex than HMOs. While more than 150 different HMOs have been identified so far, their composition follows a basic blueprint that connects the five building blocks glucose (Glc), galactose (Gal), N-acetylgalactosamine (GlcNAc), fucose (Fuc), and N-acetylneuraminic acid (Neu5Ac) in specific linkages (Figure 1A) [2]. All HMOs carry lactose (Galβ1-4Glc) at the reducing end. Lactose can be elongated in the C3 position of Gal with either lacto-N-biose (Galβ1-3GlcNAc, type 1 chain) or N-acetyllactosamine (Galβ1-4GlcNAc, type 2 chain) (Figure 1B). This ostensibly minor difference in the linkage between Gal and GlcNAc might be important for HMO digestion and bioavailability. While the β1-3 linkage in lacto-N-biose (type 1) cannot be cleaved by the infant’s intestinal β-galactosidase (lactase), the β1-4-linkage in N-acetyllactosamine (type 2) is likely a substrate for the enzyme, suggesting that type 2 chain HMOs like lacto-N-neotetraose (LNnT) are partially digested in the infant’s proximal small intestine, do not reach the infant’s distal small intestine and colon intact, and are therefore not available for absorption or microbial utilization. In contrast, type 1 chain HMOs like lacto-N-tetraose (LNT) cannot be cleaved by lactase, resist degradation in the infant’s small intestine, reach the colon intact, and are available for absorption or microbial utilization. In addition to chain elongation at the C3 position of Gal, the disaccharides lacto-N-biose or N-acetyllactosamine can also be added at the C6 position of Gal, leading to chain branching (Figure 1C).

The repeated addition of disaccharides to the growing chain leads to more complex linear or branched HMOs (Figure 1D). Moreover, HMOs can be modified by the addition of Fuc in α1-2-, α1-3-, and/or α1-4-linkage and/or the addition of Neu5Ac in α2-3- and/or α2-6-linkage. Each Neu5Ac contains a carboxyl group and introduces a negative to an HMO. Fuc and/or Neu5Ac can be added to lactose to yield the small HMO trisaccharides 2'-fucosyllactose (2'FL), 3'-fucosyllactose (3'FL), 3'-sialyllactose, or 6'-sialyllactose (Figure 1E). One or more Fuc and/or Neu5Ac can also be added to the growing linear or branched HMO chain to form complex HMOs with potentially multiple negative charges if more than one Neu5Ac are added (Figure 1F). Neu5Ac-containing HMOs are often referred to as “acidic” HMOs.

Although HMO composition follows a basic blueprint and more than 150 different HMOs have been identified so far, it is important to note that every woman synthesizes and secretes a distinct HMO composition profile that varies substantially between different women, but remains fairly constant over the course of lactation for the same woman.
What drives the variation in HMO composition?

Some of the variation in HMO composition can be explained by maternal genetics [1]. HMO fucosylation is mainly catalyzed by two different fucosyltransferases, enzymes that facilitate the addition of Fuc to the HMO backbone in different linkages [3,4]. Fucosyltransferase 2 (FUT2) adds Fuc to Gal in an α1-2-linkage [5]. Mutations in the Secretor gene, which encodes FUT2, lead to an inactivation of the enzyme, and, as a consequence, milk samples of so called nonsecretor women have very low concentrations of α1-2-fucosylated HMOs, e.g. 2'-FL or lacto-N-fucopentaose 1 (LNFP1). Fucosyltransferase 3 (FUT3) adds Fuc to Gal or GlcNAc in an α1-3- or α1-4-linkage, depending on whether the substrate is a type 1 or type 2 HMO chain [6]. Mutations in the Lewis gene, which encodes FUT3, lead to an inactivation of the enzyme, and, as a consequence, milk samples of so called Lewis-negative women have very low concentrations of α1-3/4-fucosylated HMOs.

The permutation of active/inactive FUT2 and FUT3 enzymes lead to four distinct HMO groups [4]: i. Lewis-positive secretors (FUT2 and FUT 3 active), ii. Lewis-positive nonsecretors (FUT2 inactive, but FUT3 active), iii. Lewis-negative secretors (FUT2 active, but FUT3 inactive), and iv. Lewis-negative nonsecretors (both FUT2 and FUT3 inactive). These four groups can be distinguished by the presence or near absence of specific fucosylated HMOs, which is almost an all-or-nothing phenomenon. For example, 2'FL is either present and one of the dominant HMOs in the milk of secretor women (active FUT2), or it is almost completely absent in the milk of nonsecretor women (inactive FUT2). In contrast, the variation of HMOs that do not depend on FUT2 or FUT3 activity is subtler. In fact, it remains poorly understood which enzymes other than FUT2 and FUT3 are involved in HMO biosynthesis in the human mammary gland epithelial cell.

In addition to maternal genetics and potentially epigenetics, maternal environmental factors may also affect HMO composition. Our recent work with mice has shown that milk oligosaccharide concentrations decrease when lactating...
Dams are fed a high fat diet and increase when dams are exercising (unpublished data). How diet, exercise, and other lifestyle factors impact HMO composition in humans is currently under investigation. Maternal health may also affect HMO composition. Initial preliminary data suggest that obesity or chronic inflammatory diseases impact HMO composition (unpublished data). In addition, cross-sectional data from the Canadian Healthy Infant Longitudinal Development (CHILD) national birth cohort [7] indicate that parity increases overall HMO concentration, but maternal age, method of delivery, or infant gender showed no association with HMO composition (unpublished data).

What happens to HMOs after ingestion?

Once ingested, HMOs resist the low stomach pH as well as degradation through pancreatic and brush border enzymes in the small intestine [8,9], with the potential exception of type 2 chains in which the terminal β1-4-linked Gal may be cleaved off by the enzyme lactase. Approximately 1% of the ingested HMOs are absorbed and can be measured in the systemic circulation as well as in the urine [10-13], indicating that HMO effects extend to tissues and organs other than the intestine. Most HMOs reach the distal small intestine and colon intact where they are either metabolized by microbes or excreted with the feces.

What are potential HMO functions?

HMOs serve as metabolic substrates for specific and potentially health-promoting bacteria in the infant’s intestine [14-16], making them the first prebiotics that humans are exposed to in life – when the infant is breastfed (Figure 2A). However, recent data from ex vivo studies suggest that the prebiotic effects of different HMOs are not interchangeable and are indeed structure-specific (unpublished data). The composition of microbial communities isolated from infant fecal samples and cultured under anaerobic conditions changes over time depending on what specific HMOs are added to the culture. For example, the composition of a microbial community looks very different when exposed to either to a mixture of HMOs that were isolated from pooled human milk or to individual HMOs like 2’FL or LNT. Since the differential composition and activity of microbial communities has been linked to diseases like obesity, diabetes, inflammatory bowel disease or autism, exposing infants to individual HMOs in formula instead of a complex HMO mixture in human milk may increase disease risks. Long-term follow-up studies are required to rule out this potential risk and avoid potential harm to the infant’s short- and long-term health.

However, HMOs are more than just “food for bugs”. HMOs can have direct bacteriostatic or bacteriocidal antimicrobial effects (Figure 2B). For example, HMOs halt the growth of Streptococcus agalactiae (group B Streptococcus; GBS) [17], a leading cause of invasive bacterial infections in newborns, typically acquired vertically during childbirth secondary to maternal vaginal colonization. GBS transmission to the newborn is associated with risk of pneumonia, septicemia, and meningitis [18-20]. Multidimensional chromatography revealed that the bacteriostatic effect is structure dependent and that LNT is most effective. GBS transposon mutant library screening identified a GBS glycosyltransferase as the HMO target. Most intriguingly, the effects of HMOs synergize with common antibiotics like vancomycin and ciprofloxacin, with high relevance to the emerging antibiotic-resistance crisis [17].
HMOs also have antiadhesive effects (Figure 2C). Many pathogens need to attach to epithelial surfaces in order to proliferate and potentially invade host tissues. The attachment is often facilitated by pathogen surface molecules that bind to glycan structures on the epithelial cell surface, also known as the glycocalyx. HMOs and glycans on epithelial cell surfaces share many structural features, allowing HMOs to mimic epithelial surface glycans and serve as soluble decoy receptors. Instead of pathogens binding to epithelial surfaces and causing disease, they bind to the soluble HMO decoys and are washed out with attaching and without causing disease. Examples for antiadhesive effects of HMOs include bacterial pathogens like *Campylobacter jejuni* [21] or enteropathogenic *E. coli* (EPEC) [22] as well as protozoan parasites like *Entamoeba histolytica* [23].

HMOs may also have direct effects on epithelial cells, independent of, but indirectly affecting microbes (Figure 2D). For example, bladder epithelial cells that had been exposed to HMOs are significantly more resistant to uropathogenic *E. coli invasion* and cytotoxicity [24]. HMOs may also alter immune cell responses, either locally in the gut or systemically (Figure 2E).

In conclusion, there are indirect effects of HMOs on the infant that are mediated through changes in microbial communities, e.g. by serving as prebiotics, antimicrobials or antiadhesives, and there are direct effects on the infant, e.g. by modulating epithelial or immune cell responses. In addition, there may be multiple other mechanisms of HMO action that have not yet been discovered. Overall, the arsenal of HMO effects is likely relevant in a variety of health and disease contexts.

**How do we interrogate the effects of HMOs?**

Accumulating data from tissue culture, animal or human cohort studies suggests that HMOs benefit the human milk-fed infant. However, results from many of these studies raise additional questions. How relevant are results generated from *in vitro* or animal models? How similar are cell lines in culture compared to tissues in the infant body? How comparable are animal models to human anatomy, physiology, and pathophysiology? How meaningful are associations between HMOs and infant outcome measures in human cohort studies? Are the observed associations true cause-and-effect relationships? No one single study alone will likely provide a comprehensive answer. Instead, a thoroughly designed interrogative strategy that combines preclinical studies in tissue culture and animal models with clinical cohort analyses and eventually clinical intervention studies will be required to fully assess the many effects, functions and potentials claims associated with HMOs. Our recent work on necrotizing enterocolitis (NEC) serves as one example of how the combination of preclinical studies and human cohort studies can inform clinical intervention studies to test the hypothesis that HMOs contribute to the beneficial effect of human milk feeding.

NEC is one of the most common and fatal gastrointestinal disorders in preterm infants [25]. About 5% of all very-low-birth-weight (VLBW) infants develop NEC with a mortality rate of over 25%. While formula-fed infants are at a 6- to 10-fold higher risk to develop NEC [26], the protective mechanisms of feeding human milk remain poorly understood. In contrast to formula, human milk is an abundant source of HMOs. In addition, the NEC-preventing benefits for infant health. Clinical observations led to the hypothesis that HMOs contribute to the beneficial effect of human milk feeding.

**Figure 3. Combining preclinical studies and human cohort studies to inform clinical intervention studies with the goal to investigate HMO functions**

Necrotizing Enterocolitis (NEC) is one example of how a combination of preclinical studies and human cohort studies led to the identification one specific HMO with potential benefits for infant health. Clinical observations led to the hypothesis that HMOs contribute to a lower risk for preterm infants to develop NEC when they are given human milk. The preclinical model in neonatal rats confirmed the hypothesis and also identified one specific HMO that was most effective in reducing pathologic HMO analysis in milk that was fed to human preterm infants revealed associations between HMO composition and NEC and matching the preclinical data. The same HMO that was protective in the neonatal rat model was found in significantly lower concentration in milk fed to infants who developed NEC compared to control infants who did not develop NEC. The combined results from preclinical studies and human cohort studies identified one specific HMO that is likely responsible for the protective effect and informed clinical intervention studies. Continued work in preclinical models is going to help elucidate the underlying mechanism.
effect of human donor milk persists even after pasteurization, a process that destroys and inactivates many human milk bioactives, but keeps HMOs intact and active. Based on these observations we hypothesized that HMOs contribute to the lower NEC incidence in human milk-fed infants, and tested the hypothesis in a neonatal rat model [27]. Newborn rat pups were either left with the dam to serve as “breast-fed” control or removed from the dam and received formula by oral gavage. While all of the dam-fed pups survived the first 4 days of life, only ~75% of the formula-fed pups survived. However, 95% of the pups survived when they received a formula that was supplemented with HMOs that were isolated from pooled human milk. This significant increase in survival coincided with decreased pathological observations, both macroscopically as well as microscopically. We then applied a multidimensional chromatography approach to separate the different HMOs first by charge and later by size and identified one specific HMO, disialyllacto-N-tetraose (DSLNT), which contains two Neu5Ac, to be responsible for the beneficial effects. Enzymatic removal of just one of the Neu5Ac led to a complete loss of function, indicating that the effect is highly structure-specific [27].

While the data was encouraging, the validity of available preclinical NEC models in rodents or piglets is limited [28]. Animals are exposed to external hypoxic and/or hypothermic insults that are rather artificial, and the use of animals itself is a limitation due to interspecies differences in gastrointestinal development, anatomy and physiology. Thus, advancing a potential therapeutic like DSLNT from controversial preclinical models to clinical treatment trials carried a tremendous risk of failure. To help close the gap between animal models and clinical intervention studies, we used an intermediate approach and conducted a multicenter clinical prospective cohort study with mothers and their VLBW infants fed predominantly human milk [29]. The study was based on the observation that some infants still develop NEC despite receiving predominantly human milk. Since HMO composition varies between women it led us to hypothesize that infants who develop NEC received milk with less DSLNT than infants who do not develop NEC. We recruited 200 mothers and their very low-birth-weight infants that were predominantly human milk-fed. We analyzed HMO composition in human milk fed to infants over the first 28 days post partum, matched each NEC case with five controls, and used logistic regression and generalized estimating equation to show that DSLNT concentrations were significantly lower in almost all milk samples in all 8 NEC cases when compared to controls. In fact, DSLNT abundance could identify NEC cases prior to onset. Aggregate assessment of DSLNT over multiple days enhanced the separation of NEC cases and control subjects, making DSLNT content in human milk a potential non-invasive marker to identify infants at risk of developing NEC.

While the cohort association data alone would raise questions about cause-and-effect, the combination with data from the preclinical model substantially increases the confidence that the observed effects are indeed due DSLNT, lowering the risk threshold for a clinical intervention study to fail.

Overall, the NEC project is an example for how the combination of preclinical data and clinical cohort data can inform clinical intervention studies (Figure 3). A similar approach has been used in the past to relate HMOs to a reduction of *Campylobacter jejuni* infection and diarrhea [21, 30] and is currently used to study the effects of HMOs on infant body composition and childhood obesity risk as well as on allergies and asthma risk.

What we’ve learnt so far from this combined approach is that sometimes specific HMOs are effective and those effects are usually highly structure-specific and dose-dependent. The underlying mechanisms are likely mediated by specific receptors on host (infant) tissues or on microbes. In other cases, a combination of different HMOs in specific ratios to each other is required to be effective. The underlying mechanisms are likely mediated indirectly through shaping microbial communities or directly through a coordinated interaction of different HMOs with multiple different receptors or even different cell types, for example in the immune system. In situations where relative abundances of many different HMOs matter more than individual HMOs alone, it raises the question whether or not a woman’s characteristic HMO composition has evolved to match her own infant’s specific needs. In that case, HMOs can be seen as another example of personalized nutrition early on in life, which comes in addition to other personalized components of human milk like antibodies, milk microbiota, immune cells, and progenitor cells. In fact, the personalized complexity of HMOs provides yet another powerful reason to protect, promote, and support breast-feeding.

**Disclosure Statement**

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.
References


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Key Messages

- Human milk oligosaccharides (HMO) are a predominant component of human milk and are comprised of diverse structures that are neutral or acidic and some forms are sialylated or fucosylated, which contributes to their biological functions.
- HMO protect the infant from pathogenic infections, facilitate the establishment of the gut microbiota, promote intestinal development, and stimulate immune maturation.
- Some types of HMO are now commercially available and are being added to infant formula alone or in combination with other prebiotics.

Keywords

Human milk oligosaccharides, Immunity, Infant

Abstract

The immune system of the infant is functionally immature and naïve. Human milk contains bioactive proteins, lipids, and carbohydrates that protect the newborn and stimulate innate and adaptive immune development. This review will focus on the role human milk oligosaccharides (HMO) play in neonatal gastrointestinal and systemic immune development and function. For the past decade, intense research has been directed at defining the complexity of oligosaccharides in the milk of many species and is beginning to delineate their diverse functions. These studies have shown that human milk contains a higher concentration as well as a greater structural diversity and degree of fucosylation than the milk oligosaccharides in other species, particularly bovine milk from which many infant formulae are produced. The commercial availability of large quantities of certain HMO has furthered our understanding of the functions of specific HMO, which include protecting the infant from pathogenic infections, facilitating the establishment of the gut microbiota, promoting intestinal development, and stimulating immune maturation. Many of these actions are exerted through carbohydrate–carbohydrate interactions with pathogens or host cells. Two HMOs, 2'-fucosyllactose (2'FL) and lacto-N-neotetraose (LNnT), have recently been added to infant formula. Although this is a first step in narrowing the compositional gap between human milk and infant formula, it is unclear whether 1 or 2 HMO will recapitulate the complexity of actions exerted by the complex mixture of HMO ingested by breastfed infants. Thus, as more HMO become commercially available, either isolated from bovine milk or chemically or microbially synthesized, it is anticipated that more oligosaccharides will be added to infant formula either alone or in combination with other prebiotics.

Background

The human infant enters the world with a functionally naïve immune system affecting both adaptive and innate immune responses [1], which leaves the newborn at high risk for common infections. Postnatal immune maturation is stimulated by antigenic exposures and host-microbe interactions [1, 2]. How and what the infant is fed influences the development and competence of the immune system [3–5]. Human milk protects the infant during this vulnerable period by providing bioactive components that protect the infant from pathogenic infection, support intestinal development, promote barrier function, stimulate immune development, facilitate immune tolerance, and feed gut microbes [2–5]. Thus, human milk supplies multiple layers of protection for the infant (Fig. 1).

Breastfeeding, particularly exclusive breastfeeding for 6 months or more, relative to formula-feeding, decreases the incidence and/or severity of infectious diseases [6]. Many diseases with infectious and immune components in their etiology, including diarrhea, respiratory and urinary tract infections, otitis media, bacteremia, and necrotizing enterocolitis occur less often in breast than formula-fed infants [6, 7]. Breastfeeding has also been implicated in reducing the incidence of other diseases involving the immune system and immune tolerance, such as inflammatory bowel disease, celiac disease, asthma, allergy, type 1 diabetes, as well as acute lymphoblastic and acute myeloblastic leukemias [6, 8]. These benefits may be mediated in part through effects of breastfeeding on the intestinal microbiota [8, 9], which in turn stimulates maturation and specificity of the neonatal mucosal and systemic immune systems [2].
The immune benefit of breastfeeding has been attributed in part to the diverse bioactive components in human milk [2–5]. A strong case can be made for a key role of human milk oligosaccharides (HMO) in neonatal immune defense and maturation. As will be described below, HMO are present in high concentrations in human milk, exist in an incredible structural diversity [10–13], and confer host protection and mediate immune responses through a number of mechanisms [14, 15].

HMO Content and Composition

HMO are complex soluble glycans that are predominantly present in free form in milk. These glycans are synthesized from 5 basic monosaccharides: galactose, glucose, N-acetylgalactosamine, fucose, and the sialic acid derivative N-acetylneuraminic acid [10, 11]. With few exceptions, all HMO carry lactose (Galβ1–4Glc) at the reducing end, which can be elongated in β1–3 or β1–6 linkage by 2 different disaccharides, either Galβ1–3GlcNAc (type 1 chain) or Galβ1–4GlcNAc (type 2 chain) [11].

The HMO content has been reported in the range of 1–10 g/L in mature milk and 15–23 g/L in colostrum [10–13]. In term breast milk, ~35–50% of HMO are fucosylated, 12–14% are sialylated, and 42–55% are nonfucosylated neutral HMO [10–13] (Table 1). However, HMO composition is influenced by maternal genetics, including secretor and Lewis Blood Group status [10, 11]. HMO fucosylation is mediated by the 2 fucosyltransferases FUT2 (secretor gene) and FUT3 (Lewis gene). Nonsecretor mothers, who lack a functional FUT2 enzyme and represent about 30% of women worldwide, produce milk lacking in α1-2-fucosylated oligosaccharides like 2’α-fucosyllactose (2’FL) and lacto-N-fucopentaose (LNFP) I [10, 11]. The absence of these compounds may have functional consequences. For example, infants consuming milk produced by women who are nonsecretors exhibit delayed colonization of bifidobacteria, higher abundance of Streptococcus taxa, and have functional differences in the metabolic activity of their microbiota [16]. Infants fed milk from nonsecretor mothers are at higher risk for diarrheal diseases [17].

Table 1. Concentrations of major HMO in human milk [10 – 13]

<table>
<thead>
<tr>
<th>Categories of HMO (% total)</th>
<th>Oligosaccharide</th>
<th>Mean concentration (range), g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fucosylated (35–50%)</td>
<td>2’FL</td>
<td>2.7 (1.88–4.9)</td>
</tr>
<tr>
<td></td>
<td>3’FL</td>
<td>0.5 (0.25–0.86)</td>
</tr>
<tr>
<td></td>
<td>LNFP I</td>
<td>0.122 (0.106–0.145)</td>
</tr>
<tr>
<td></td>
<td>LNFP II + III</td>
<td>0.156 (0.120–0.161)</td>
</tr>
<tr>
<td>Sialylated (12–14%)</td>
<td>3’SL</td>
<td>0.2 (0.1–0.3)</td>
</tr>
<tr>
<td></td>
<td>6’SL</td>
<td>0.5 (0.2–1.22)</td>
</tr>
<tr>
<td>Nonfucosylated neutral (42–55%)</td>
<td>LNnT</td>
<td>0.3 (0.17–0.43)</td>
</tr>
</tbody>
</table>

2’FL, 2’-fucosyllactose; 3’SL, 3’-sialyllactose; 6’SL, 6’-sialyllactose; HMO, human milk oligosaccharides; LNFP, lacto-N-fucopentaose; LNnT, lacto-N-neotetraose.
HMO and the Microbiome

The development of the infant gut microbiota is a sequential process that begins in utero and continues during the first 2–3 years of life. Microbial composition and diversity is shaped by host genetics and multiple environmental factors, of which diet is a principal contributor [8, 9]. Studies conducted over the past decade have shown that specific Bacteroides and Bifidobacterium species that commonly colonize breastfed infants efficiently utilize HMO as carbon sources. This is particularly true of B. longum ssp. infantis (B. infantis), which is a predominant gut microbe in most breastfed infants [18]. The discovery of a genomic island in B. infantis in the infant gut [10, 18]. B. infantis produces peptides that encode specific enzymes for the metabolism of HMO supports an adaptation of this species to the intestinal milieu of the breastfed infant [18, 19]. Indeed, a recent study in human infants fed formula supplemented with 2'FL (1 g/L) and LNnT (0.5 g/L) demonstrated that the global microbiota composition of infants fed formulae with 2'FL and LNnT was significantly different to that of infants fed nonsupplemented formula (p < 0.001) at the genus level and closer to that of breastfed infants at 3 months of age [20]. In addition, Bifidobacterium was more abundant (p < 0.01); whereas Escherichia and unclassified Peptostreptococcaceae were less abundant in infants fed formula with 2'FL and LNnT compared to infants fed nonsupplemented formula, and these levels were closer to those observed in breastfed infants [20]. Furthermore, the concentrations of several important metabolites in stool (propionate, butyrate, and lactate) in infants fed the HMO-supplemented formula were more similar to those of breastfed infants [20].

Previously, we have shown that HMO fermentation by neonatal pig microbiota produced short-chain fatty acids and promoted the growth of beneficial bacteria in vitro [21] and in vivo [22]. Gut bacteria and the immune response, particularly the gastrointestinal immune response, are tightly interrelated [23]. Thus, in this animal model, HMO-induced changes in the gut bacterial populations of the pigs could alter the course of an intestinal infection [24] which in turn would alter the immune response [22]. Alternatively, the change in the gut bacteria could directly affect the immune system of these animals [2]. Additional ways whereby HMO may mediate neonatal immunity are summarized in the following section.

HMO as Immune Modulators

Summarized in Figure 2 are the results of an accumulating body of evidence showing that HMO indirectly and directly influence infant mucosal and systemic immune function. In general, intestinal health and barrier function are considered a first line of defense in innate immunity. Cell proliferation takes place in the crypts, and cells differentiate as they migrate up the villus-crypt axis, with the exception of Paneth cells, which migrate down to the base of the crypt. HMO reduce intestinal crypt cell proliferation [25, 26], increase intestinal cell maturation [26], and increase barrier function [26] (indicated by 1–3 in Fig. 2). A layer formed by mucus glycoproteins or mucins produced by goblet cells acts as a lubricant and a protective physical barrier between the intestinal epithelium and the luminal contents. This is particularly true for galacto-oligosaccharides (GOS) [27]. HMO affects epithelial immune gene expression both directly [28–30] and indirectly through the microbiota [31] (indicated by 5 and 6 in Fig. 2, respectively). As noted above, HMO serve as prebiotics to promote the growth of healthy bacteria, including Bifidobacteria and Bacteroides genera [32] (indicated by 7 in Fig. 2), and HMO inhibit infections by bacteria and viruses by either binding to the pathogen in the lumen or by inhibiting binding to cell-surface glycancceptors [14–15, 22] (indicated by 8 in Fig. 2). Additionally, dietary oligosaccharides decorate the intestinal lining contributing to the intestinal glycan repertoire [33]. HMO also contribute to epithelial barrier function by supporting the growth of B. infantis in the infant gut [10, 18]. B. infantis produces peptides that have been shown to normalize intestinal permeability through enhanced tight junction protein expression in a mouse model of colitis [34]. It is likely that HMO support other bacterial species that are important for the maintenance of gut integrity. These changes in intestinal barrier function would, in turn, alter both the local and systemic immune system [35]. HMO affect immune cell populations and cytokine secretion [22, 36] (indicated by 9 in Fig. 2). Some HMO are also absorbed into the blood stream [37–39] (indicated by 10 in Fig. 2), where they exert systemic effects by binding of monocytes, lymphocytes, and neutrophils to endothelial cells [40] (indicated by 11 in Fig. 2) and formation of platelet-neutrophil complexes [41] (indicated by 12 in Fig. 2). Readers are referred to a recent review by Kulinch and Liu [15] for additional discussion of this topic.

Carbohydrate Binding as a Potential Mechanism of HMO in the Immune System

Carbohydrates and carbohydrate-binding proteins play an important role in immune responses. Cells have unique glycan signatures made from combinations of specific glycan motifs that are engaged when a cell contacts another cell or other components of its environment [42, 43]. However, many of the glycan motifs found on mammalian cells are also found on microbes and in foods, including human milk. These similarities provide opportunities for host-microbe-HMO interactions.

Lectins are carbohydrate-binding proteins on the surfaces of mammalian cells that translate recognition of specific motifs and the spatial presentation of those motifs into action. Lectins are grouped according to their carbohydrate recognition domains (CRD) [42, 43].
Potential mechanisms whereby human milk oligosaccharides (HMO) influence host immune function. HMO affect innate immunity through the epithelial barrier: HMO reduce intestinal crypt cell proliferation (1), increase intestinal cell maturation (2), increase barrier function (3), and may influence goblet cell function (4), as has been shown for galacto-oligosaccharides. In addition, HMO affect epithelial immune gene expression both directly (5) and indirectly through the microbiota (6). HMO serve as prebiotics to promote the growth of healthy bacteria, including Bifidobacteria and Bacteroides species (7), and HMO inhibit infections by bacteria and viruses by either binding to the pathogen in the lumen or by inhibiting binding to cell-surface glycan receptors (8). HMO affect immune cell populations and cytokine secretion (9). HMO are also absorbed into the blood (10), where they affect binding of monocytes, lymphocytes, and neutrophils to endothelial cells (11) and formation of platelet-neutrophil complexes (12).
There are at least a dozen CRD identified in mammals, but 3 classes of lectins related to the influence of HMO on immune responses are C-type lectins, siglecs (sialic acid-binding Ig-like lectins), and galectins.

C-type lectins require calcium to function and include selectins, mannose-binding lectin, and dendritic cell-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN). C-type lectin receptors on the surface of dendritic cells (DC) determine whether the cell will induce tolerance rather than lymphocyte activation [44]. DC-SIGN is of particular interest with regard to mechanisms by which HMO can influence immunity because it has a CRD specific for fucose units. Furthermore, DC-SIGN is expressed by cells in the gastrointestinal tracts of infants [45]. These intestinal cells are likely antigen-presenting cells as DC-SIGN is expressed by antigen-presenting cells, specifically DC [43]. Although interactions between fucosylated ligands and DC-SIGN contribute to immune tolerance, the cellular response ultimately depends upon the other ligand-receptor reactions occurring simultaneously [43].

Siglecs are sialic acid-binding lectins most commonly found on subsets of immune cells [46]. There are at least 16 siglecex expressed by different leukocyte populations, which include sialoadhesin (siglec-1), CD22 (siglec-2), myelin-associated glycoprotein (MAG, siglec-4), siglec-15, and CD33-related siglecs. Siglec specificity derives from differences in secondary binding sites [43]. Siglecs are endocytic cell surface receptors that carry cargo between the cell surface and intracellular vesicles; these receptors are mainly expressed on cells involved in antigen processing and presentation [43]. Furthermore, sialic acid-containing molecules can gain entry to macrophages by binding to siglecs on the cell surface [46]. On mammalian cells, some sialic acid-containing glycans function as self-associated molecular patterns and prevent immune responses to nonpathogenic stimuli. Ligation of particular siglecs stimulates the production of the immunoregulatory cytokine interleukin (IL)-10 [47].

Galectins are important for cell turnover and immune regulation. The CRD of galectins is specific for β-galactosides. When cells are desialylated, the density of exposed galactose moieties on the cell surface increases. For example, naïve T cells express CD45 with an α-2,6-linked sialic acid. The amount of α-2,6-linked sialic acid is reduced following T-cell activation. The decrease in α-2,6-linked sialic acid renders the activated T cells susceptible to galectin-1-mediated apoptosis [48]. Thus, binding of sialylated HMO to cells may prevent apoptosis.

**HMO as Modulators of Mucosal Immunity**

Intestinal cell lines have been used to determine effects of HMO on immune-related gene expression and protein production. These cells have been co-incubated with oligosaccharides [28], bacteria [48], or lipopolysaccharides (LPS) to model a bacterial infection [29]. Co-incubation of Bifidobacterium with cells of the Caco-2 intestinal cell line and HMO resulted in downregulation of intestinal cell genes related to chemokine activity compared to co-incubation with glucose or lactose [29]. Conversely, in the absence of a bacterial co-stimulant, HMO increased expression of several chemokines by the HT-29 cell line [28]. Additional work in T84 and HCT8 intestinal cell lines showed that complex mixtures of HMO as well as 2′ FL reduced signatures of intestinal inflammation [29].

HMO have been demonstrated to affect the course of a gastrointestinal viral infection. In an acute rotavirus (RV) infection model where a 21-day-old piglet’s ileum was isolated in situ, intestinal loops co-treated with HMO and RV had reduced non-structural protein-4 (NSP-4) mRNA expression indicating that HMO can reduce RV replication [49]. Intestinal cytokine and chemokine expression, however, was not affected. Both neutral and acidic HMO decreased NSP4 intestinal mRNA expression in the in situ model, whereas only acidic HMO effectively inhibited RV infectivity in an in vitro model [49].

**HMO as Modulators of Systemic Immunity and Protection from Infection**

HMO are detected in the plasma of infants fed human milk at concentrations of 1–133 mg/L [37, 39], suggesting the potential for dietary HMO to directly affect immune cells circulating in the blood. As discussed above, many immune receptors recognize the oligosaccharide structures of their glycoprotein ligands [14, 15]. Because a subset of HMO is structurally similar to selectin ligands [14], it is likely that HMO can bind directly to immune cells and trigger signaling that results in changes to immune cell populations and functions. For instance, the P- and E-selectins recognize sialyl-Lewis x (sLeX), a glycan moiety of several HMO [11]. Additionally, fucosylation and sialylation, 2 enzymatic modifications common to HMO, enable binding to selectins [50]. HMO-induced disruption of immune protein-carbohydrate interactions reduced neutrophil rolling [40] and activation [41]. HMO directly affect immune cell proliferation and cytokine production in ex vivo experiments with peripheral blood mononuclear cells (PBMC) from neonatal pigs [36]. Stimulation with isolated HMO stimulated production of the regulatory cytokine IL-10 [36]. Others observed that the acidic HMO induce IL-10 production; additionally, they found that acidic HMO induce IFN-γ from ex vivo stimulated human cord blood mononuclear cells [45]. Isolated HMO enhanced proliferation of PBMC stimulated with a T-cell mitogen, phytohemagglutinin (PHA), and sialylated HMO enhanced proliferation of PBMC stimulated with the B-cell mitogen LPS [36]. In contrast, 2′ FL inhibited proliferation of unstimulated PBMC cultured for 3 days. Thus, the response to HMO may depend on the state of the infant. In the unstimulated state, HMO dampen proliferation, whereas HMO enhance proliferation in response to a mitogenic stimuli.
Table 2. Immune-related outcomes of HMO feeding studies

<table>
<thead>
<tr>
<th>Species</th>
<th>Study design</th>
<th>Major findings</th>
<th>Ref.</th>
</tr>
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<tbody>
<tr>
<td>Human infants</td>
<td>Healthy singleton infants enrolled by 5 days of life and fed formulae to 4 months of age</td>
<td>Breastfed infants and infants fed either formula with 2’FL were similar and had lower plasma inflammatory cytokines than infants fed the control formula. In ex vivo RSV-stimulated PBMC cultures, cells from breastfed infants were not different from those of either of the groups fed formula with 2’FL, but secreted less TNF-α and IFN-γ and tended to have lower IL-1Ra, IL-6 and IL-1β than cells from infants fed the control formula.</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td>- Breastfed</td>
<td>- Formula + 2.4 g/L GOS</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Formula + 2.2 g/L GOS + 0.2 g/L 2’FL</td>
<td>- Formula + 1.4 g/L GOS + 1.0 g/L 2’FL</td>
<td></td>
</tr>
<tr>
<td>Human infants</td>
<td>Healthy singleton infants enrolled by 14 days of life and fed experimental formulae to 6 months of age, and standard follow-up formula to 12 months</td>
<td>Infant fed HMO-supplemented formula had significantly fewer parental reports of: - Bronchitis through 4, 6, and 12 months - Lower respiratory tract infection through 12 months - Antibiotics use through 6 and 12 months</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td>- Formula</td>
<td>- Formula + 1.0 g/L 2’FL + 0.5 g/L LNnT</td>
<td></td>
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<tr>
<td>Colostrum-deprived piglets</td>
<td>15-day feeding study</td>
<td>HMO resulted in shorter duration of diarrhea and higher ileal IFN-γ and IL-10 mRNA expression than formula, but similar concentrations of RV-specific IgG and IgM as formula.</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>- Formula</td>
<td>- Formula + 4 g/L HMO (40% 2’FL, 35% LNnT, 10% 6’SL, 5% 3’SL, 10% free SA)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Infected with OSU strain RV on day 10 and analyzed on day 15</td>
<td>Infants fed 2’FL prevented body weight loss and reduced AIEC colonization, colonic inflammation, crypt cell CD14 expression, and IL-6, IL-17, and TNF-α production in response to AIEC infection.</td>
<td>29</td>
</tr>
<tr>
<td>Adult female C57BL/6 mice</td>
<td>E. coli infection model</td>
<td>2’FL and 6’SL attenuated diarrhea and hypothermia induced by OVA challenge, reduced intestinal mast cell numbers and passive cutaneous anaphylaxis, and increased Peyer’s patch T regulatory cells and CD11c+CD103+ DC. 6’SL increased OVA-specific IgG2a and MLN T regulatory cells. Splenocytes from 6’SL-treated mice produced more IFN-γ and IL-10 but less TNF. Splenocytes from 2’FL-treated mice produced less IFN-γ.</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td>- 0.25% DSS orally for days 0–3</td>
<td>- 2’FL (100 mg or vehicle by oral gavage days 0–4)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- 20 mg streptomycin by oral gavage on day 4</td>
<td>- Day 28 oral challenge with OVA (50 mg) every 3 days until day 43</td>
<td></td>
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</tbody>
</table>

2’FL: 2’-fucosyllactose; 3’S, 3’-sialyllactose; 6’SL, 6’-sialyllactose; AIEC, adherent-invasive E. coli; DC, dendritic cells; DSS, dextran sodium sulfate; GOS, galacto-oligosaccharides; HMO, human milk oligosaccharides; IFN, interferon; IP, intraperitoneal infection; Ig, immunoglobulin; IL, interleukin; LNnT, lacto-N-neotetraose; MLN, mesenteric lymph nodes; OSU, Ohio State University; OVA, ovalbumin; PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; RSV, respiratory syncytial virus; RV, rotavirus; SA, sialic acid; TNF, tumor necrosis factor.

To date, very few studies have fed HMO and analyzed immune outcomes [22, 29, 30, 51–54] (Table 2). Piglets [55] have been fed 2’FL, but only growth and toxicological outcomes were reported. A recently published paper described immune outcomes in human infants fed formula containing 2.4 g/L GOS, 2.2 g/L GOS + 0.2 g/L 2’FL, or 1.4 g/L GOS + 1.0 g/L 2’FL compared to a breastfed reference control [53]. Infants were fed the formula from 5 days to 4 months of age, and blood samples were obtained at 6 weeks of age for cytokine analysis, immune cell phenotyping, and ex vivo stimulation of isolated PBMC. Breastfed infants and infants fed either formula with 2’FL were similar and had lower plasma inflammatory cytokines than infants fed the control formula. In addition, cytokine secretion by PBMC from breastfed infants and infants fed either 2’FL-containing formula that were stimulated ex vivo with respiratory syncytial virus was similar and secreted less tumor necrosis factor-α and interferon-γ and tended to have lower IL-1RA, IL-6, and IL-1β than cells from infants fed the control formula [53].

Another recent study in human infants evaluated the effect of formula supplemented with both 2’FL (1.0 g/L) and LNnT (0.5 g/L) compared to an unsupplemented formula. Infants were fed the formulae from 14 days to 6 months of age, after which they were switched to a starchy-based follow-on formula and followed until 12 months of age. Infants fed the HMO-supplemented formula had significantly fewer parental reports ($p = 0.004–0.047$) of: bronchitis through 4 months...
been reported that human infants who were fed human milk to mice, the milk oligosaccharides LNFP III and LNnT are effects of dietary compounds on the immune system. In identifying an appropriate challenge model to assess the 6'SL affected intestinal T regulatory cells when administered to mice treated with vehicle [29]. Mice fed 2’ FL and subjected to ileocecal resection gained more weight and had greater crypt depth and villus height at the site of transection than nonsupplemented mice [30]. The 2’ FL-fed mice also had upregulated mucosal immune response genes in the distal small bowel [30]. The studies where pigs and human infants were fed the HMO have focused on 2’ FL, which is readily available in large quantities at reasonable cost, and fucosylated oligosaccharides have been shown to feed specific beneficial classes of bacteria during intestinal inflammatory events [56]. Given what is known about the effects of other HMO, these compounds also should be used in feeding studies when available in sufficient quantities.

Several studies in animal models support the reduced incidence of infection in human infants fed formula with HMO. In mice infected with *Escherichia coli*, once daily oral gavage with 100 mg, 2’ FL prevented body weight loss and reduced colonization with adherent-invasive *E. coli*, colonic inflammation, crypt cell CD14 expression, as well as IL-6, IL-17, and tumor necrosis factor-α production in response to adherent-invasive *E. coli* infection compared to mice treated with vehicle [29]. In a mouse model of food allergy, 2’ FL and 6’ SL administered via oral gavage reduced symptoms in mice sensitized toovalbumin, an egg protein [51]. Specifically, ovalbumin-stimulated splenocytes from mice treated with 6’ SL produced more IL-10 and less IFN-γ than those from un-treated mice. Furthermore, 2’ FL- or 6’ SL-treated mice had more regulatory immune cells in their intestinal immune tissues than untreated mice. Interestingly, neither 2’ FL nor 6’ SL affected intestinal T regulatory cells when administered to nonsensitized mice [51]. This exemplifies the necessity of identifying an appropriate challenge model to assess the effects of dietary compounds on the immune system. In mice, the milk oligosaccharides LNFP III and LNnT are Th2-biasing and suppress Th1 responses [57]. Recently, it has been reported that human infants who were fed human milk with low LNFP III concentrations (<60 μM) were 6.7-times (95% CI 2.0–22) more likely to become affected with cow’s milk allergy when compared to infants receiving milk with high LNFP III concentrations [58].

Another approach using knockout mice showed that SL-containing compounds can directly affect gastrointestinal mucosal immunity [52, 59]. In one study, the presence of 3’SL in the milk increased the number of immune cells infiltrating the gut in IL-10 null mice [52]. Furthermore, supplementation with 3’SL increased colitis severity in newborn IL-10 and St3gal4 (the enzyme that synthesizes 3’SGL null mice, and cross-fostering wildtype mice to deficient dams reduced colitis severity. One caveat of this work is that it was conducted in the absence of endogenous IL-10 production, whereas other in vivo studies have demonstrated that some HMO increase intestinal IL-10 [22, 51]. 3’ SL is a product of several pathogenic bacteria [60] and the conformation (α2,3-link between sialic acid and galactose) on pathogenic bacteria and in human milk is the same. 3’ SL is recognized by DC and generates an immune response through the TLR4 signaling pathway [61]. These results suggest that the presence of 3’ SL increases the inflammatory response through direct effects on DC. When TLR4 was absent, 3’ SL was less effective at inducing DC activation. However, those DC also demonstrated a minimal increase in CD40 expression suggesting that at least one other 3’ SL-sensing mechanism, albeit much less efficient than the TLR4 pathway, exists on DC. TLR4 is the receptor for *E. coli* LPS. Another link between 3’ SL and TLR4 is ex-plained in a newer paper, where it is demonstrated that 3’ SL stimulates the proliferation of the intestinal *E. coli* population and that this overgrowth of *E. coli* is responsible for exacerbation of dextran sulfate sodium colitis through release of proinflammatory cytokines from intestinal DC [62].

These examples demonstrate the complexity of the relationships between oligosaccharides, the gut bacteria and the immune system.

**Conclusion**

The rich diversity of HMO has the potential to modulate both innate and adaptive neonatal immunity. Findings from in vitro experiments and animal models show that HMO directly interact with gastrointestinal epithelial cells as well as with mucosal and systemic immune cells to modulate immune function. HMO also beneficially shape the microbiome of the breastfed infant. The increased availability of HMO from commercial sources as well as accumulating evidence demonstrating that formula supplemented with HMO is safe and may confer benefits for human infants have led to the recent addition of 2’ FL alone or in combination with LNnT to infant formulas. In addition, due to their beneficial effects on immune function and host defense, HMO may also be beneficial for other segments of the population who are...
immune compromised or at high risk for infection. There are limited studies in which animals or humans have been fed HMO. Additionally, few studies have assessed the effects of feeding complex mixtures of HMO on the immune response. Thus, future research is needed to delineate mechanisms and to fully realize the potential for HMO to benefit infant immune function.

References


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Abstract

Human milk oligosaccharides (HMOs) are elongations of the milk sugar lactose by galactose, N-acetylglucosamine, fucose and sialic acid. The HMOs composition of breastmilk is strongly influenced by polymorphisms of the maternal fucosyltransferases, FUT2 and FUT3, and by stage of lactation. Clinical observational studies with breastfed infant-mother dyads associate specific HMOs with infant gut microbiota, morbidity, infectious diarrhea and allergies. Observational and basic research data suggest that HMOs influence the establishment of the early life microbiota and mucosal immunity and inhibit pathogens, thereby contributing to protection from infections. Clinical intervention trials with infant formula supplemented with the single HMO, 2′-fucosyllactose (2′FL) or with 2 HMOs, 2′FL and Lacto-N-neotetraose (LNnT), demonstrated that they allow for age appropriate growth and are well tolerated. A priori defined exploratory outcomes related feeding an infant formula with 2 HMOs to fewer reported lower respiratory tract illnesses and reduced need for antibiotics during the first year of life, compared to feeding a control formula. In parallel, the early life microbiota composition shifted towards that of breastfed infants. Together, HMOs likely contribute to immune protection in part through their effect on the early life gut microbiota, findings that warrant further clinical research to appreciate our understanding of HMO biology and significance for infant nutrition.

Introduction

What are human milk oligosaccharides (HMOs)? What is their importance for infant nutrition? These questions have intrigued scientists and pediatricians alike for over a century. Advances in analytics as well as large-scale synthesis technologies stimulated great progress in recent years. These provided the materials and tools that enabled the detailed and accurate measurement of HMO quality and quantity, and the study of HMOs in basic research models, and through clinical observational studies and intervention trials.

“HMOs are not HMOs”, meaning that one specific HMO is not equal to another HMO, especially when considering structure function relations. Chemically, HMOs are elongations of the milk-specific sugar lactose in different linkages by one or a combination of the following monosaccharides: L-fucose (Fuc), D-galactose (Gal), N-acetyl-D-glucosamine (GlcNAc) and N-acety neuraminic acid (sialic acid, SA). Gal and GlcNAc generally elongate lactose as a disaccharide Gal-GlcNAc. The numerous and diverse HMOs produced might be categorized by specific structural features brought about by different glycosyltransferases involved in their synthesis. However, many HMOs combine different structural features.

Breastmilk, the recommended and naturally adapted nutrition for infants, is associated with a reduced risk for infection-related illnesses and possibly for diabetes and overweight, while the situation for allergies is less clear [1]. This suggests that breastmilk-specific components such as HMOs and other bioactives, may contribute to such benefits.

Due to their structural similarity with mucosal glycans and their non-digestible nature, HMOs expectedly affect numerous glycan-mediated processes like the colonization of the early life microbiota and the infectivity of pathogens (Figure 1A). Based on clinical observational and basic research data, HMOs, act in a structure-function specific way helping the (i) establishment of mucous adapted microbiome, (ii) resistance to pathogens and (iii) reactivity of the mucosal barrier and immunity, thereby contributing to immune protection.

Here, we briefly review genetic and environmental factors affecting HMO composition in breastmilk and the physiological role of HMOs as supported by clinical observation studies, preclinical research on mode of action and insights from clinical intervention trials.

Maternal glycosyltransferase polymorphisms affect HMOs composition.

HMOs resemble the blood group antigens and further sialylated glycans that cover the human mucosa. The same glycosyltransferases are generally involved in the synthesis of mucosal cell glycans and mammary gland expressed HMOs. The fucosyltransferases FUT2 (Secretor gene) and FUT3 (Lewis gene) are the best described due to their natural polymorphisms in humans [2] (Figure 2A). Specific genetic
polymorphisms abolish their respective enzyme activity. Thus, specific HMOs structures that depend on FUT2 or FUT3 can be identified. FUT2-dependent HMOs all contain alpha-1,2 linked fucose, for example 2’FL, lactodifucosyllactose (LDFT), Lacto-N-fucosyllactose-I (LNFP-I). Interestingly, trace amounts of 2’FL were found in breast milk of presumed FUT2 negative mothers in Asian populations [3, 4], indicating that the nature of these inactivating polymorphisms and thus the HMO profile may be population-specific [5]. Typical FUT3 dependent HMOs are LNFP-II, lacto-N-difucosylhexose-I (LNDFH-I) containing alpha-1,4 linked fucose and to a lesser extent 3-fucosyllactose (3FL) and LDFT containing alpha-1,3 linked fucose. In breastmilk that does not contain any detectable LNFP-II, reduced amounts of HMOs with alpha-1,3 fucose on glucose and increased amounts of those with an alpha-1,3 fucose on GlcNAc are found. Hence, another FUT (e.g. FUT4, 5, 6, 7 or 9) is also involved in the formation of HMOs with an alpha-1,3 linked fucose on GlcNAc and glucose.

The absence of a functional FUT2 or FUT2 and FUT3 affects the concentration of total HMOs in milk, when expressed as the sum of all quantified HMOs [2] (Figure 2). While some HMOs increase when FUT2 is missing (e.g. LNT, 3FL), in the absence of fucosylation additional larger non-fucosylated HMOs might also be produced.

To date, no common genetic polymorphisms for sialylated HMOs are described, indicating that if inactivating polymorphisms in sialyltransferase genes exist, they are extremely rare. From mouse studies, the sialyltransferases ST6Gal1 and ST3Gal4 are involved in the synthesis of 6’-sialyllactose (6’S) and 3’-sialyllactose (3’S) respectively, with a further sialyltransferase, probably ST3Gal1, also making 3’S [6].

Another mechanism affecting HMO composition is probably the donor and acceptor substrate availability as suggested by the increase of 3FL when the major fucosyl-HMO 2’FL decreases in concentration [3]. Interestingly, HMO concentrations change during stage of lactation with different HMOs showing different dynamics [3]. HMOs like 6’S or LNT decrease more rapidly during the first weeks of lactation, while 2’FL and 3’SL, for example, decrease more slowly over a longer time period and again others, like 3FL, actually increase in concentration with time of lactation (Figure 2B).

Such compositional changes due to the genetic background of mothers and stage of lactation can confound observations relating HMOs to clinical parameters in the breastfed infants and, therefore, need to be considered.

HMOs composition and infant gestational age, maternal diet and physiological state.

HMOs concentrations in colostrum, transitional and mature milk seem not to change between mothers giving birth to preterm (n=18; gestational age <37 weeks) and term (n=14; gestational age ≥37 weeks) infants [2]. Further, fucosylated and sialylated HMOs were reported to be similar between preterm and term milk, although preterm milk seemed more variable in the expression of fucosylated HMOs [7].

Today, we do not know whether and how maternal diet might influence HMO composition. A recent observational study with 33 breastfeeding mothers and their infants from The Gambia, Africa, reported a significantly higher HMO content in milk at 20 weeks of lactation in the dry season (n=21) compared to the wet season (n=12) [8]. The authors propose a possible link to the higher energy intake during the dry
season. In two other African mother-infant cohorts from Malawi (n=88 and n=215), total HMOs and also sialyl-HMOs and fucosyl-HMOs, were lower at 6 months postpartum in breastmilk of mothers having severely stunted infants compared to those with normal size infants [9]. These studies suggest that maternal nutritional and health status may affect HMO composition.

By analogy, higher maternal body mass index and gestational weight gain, generally reflecting an altered metabolic physiology, might affect HMO composition. Studies to this end are currently ongoing [10] (Binia et al. 2017 Abstract at FASEB SRC). Suitable studies are warranted to investigate possible alterations of HMOs composition due to maternal energy and specific nutrient intake.

The HMO composition is associated to the establishing gut microbiota in infants.

The early life microbiome has a major impact on the developing immune defenses, itself being an important element by providing pathogen colonization resistance, for example. Interestingly, the establishing intestinal microbiota also contributes, via an innate lymphoid cell-mediated process, to improved protection against respiratory tract infection [11]. The pioneers of human milk and breastfeeding research observed a strong link between breastfeeding and immune protection to infectious morbidity and mortality. Breastfed infants were recognized to harbor an early gut microbiota dominated by bifidobacteria, not seen in formula fed infants, and a human milk specific “bifidofactor” was identified in the HMO fraction of breast milk [12].

From research on early life microbiota, we know that bifidobacteria can utilize and grow on different individual HMOs in a strain-specific way [13, 14]. Several studies...
observed an increased bacterial metabolic activity upon growth on HMOs, exemplified by the formation of the short chain fatty acid acetate [15, 16]. Noteworthy, numerous potentially pathogenic bacteria from the Enterobacteriaceae group were shown not to grow on individual HMOs as the sole carbon source [17], while growth of other pathogens, like *Streptococcus agalactiae* (group B *Streptococcus*, GBS) was shown to be inhibited by HMOs [18, 19].

Recently, LNNt in breastmilk was associated with *Bifidobacterium longum* ssp *infantis* abundance [8]. In bi-associated gnotobiotic mice harboring only one Bacteroides and one B. longum ssp infants strain, LNNt lead to bifidobacteria dominance although both bacteria could actually use LNNt in vitro [20]. In gnotobiotic mice humanized with seven human microbes, *B. longum* ssp *infantis* also showed higher abundance when these mice were fed 2′FL combined with LNNt as compared to LNT alone (Sprenger N. et al. unpublished observation), although *B. longum* ssp *infantis* is able to grow on many different HMOs, including LNT, as substrate [13].

Genomic and glycomic analyses in infants provided further evidence for a role of HMOs in shaping the early gut microbiome, revealing associations between individual HMOs and bacterial genera in infant stool [21-23]. A bifidobacteria dominated gut microbiota in breastfed infants (n=105) at 4 months of age was associated to breastmilk containing FUT2-HMOs [24]. The FUT2 status of the infants and its possible confounding effects on the infant microbiota profile were not assessed, despite earlier data proposing the FUT2 status itself can influence the gut microbiota at least in adults [25]. In another cohort, the analysis of a relatively small subgroup of 4 month exclusively breastfed infants (n=14) showed an association of maternal FUT2-positive status with higher bifidobacteria abundance up to 2-3 years of age [26]. However, no statistically significant HMO effects on global bifidobacteria shifts were reported in another recent study of 33 Gambian mothers and infants [8], while the abundance of individual bifidobacteria like *B. longum* ssp *infantis* still correlated with LNNt concentrations in breastmilk. These first reports reveal the need for larger observational studies of similar design, including comprehensive breastmilk HMO analysis and infant FUT2 phenotyping to gain a more robust understanding of the link between HMO and infant gut microbiome composition.

Today, clinical observations in conjunction with basic research data suggest that FUT2-HMOs, like 2′FL and LNFP-I, but likely other non-FUT2 dependent HMOs, like LNNt for example, are involved in the establishment of a bifidobacteria dominated early life gut microbiota. In vitro studies help to understand HMO-related microbial metabolic capacities and strain specificities, while animal and human observational studies indicate that the interaction between bacteria and the gut mucosa reflect a more complex picture. Hence, with infant health in mind, it is central to gain a better understanding of HMO effects on the microbiome dynamics in their natural ecosystem through a holistic and ecology inspired approach.

**HMO composition is linked to infection risk in infants**

HMOs were studied in relation with infectious diarrhea incidence in a cohort of Mexican mothers and infants (n=93) [27, 28]. Higher breastmilk concentrations of α1-2 fucosylated HMOs were associated with lower incidence of all causes of moderate-to-severe diarrhea. The most frequently identified cause of diarrhea in the cohort was *Campylobacter jejuni* followed by *Escherichia coli*. Specifically, higher concentrations of 2′FL and LNFP-I in breastmilk related with a lower incidence of *C. jejuni* and *Escherichia coli* diarrhea respectively. These observations during the breastfeeding period did not persist in the post-breastfeeding period, indicating a possible transient HMO effect in the protection from infectious diarrhea. This fits their presumed role as antiadhesive antimicrobials. Experimental data from preclinical models also show protective effects of 2′FL from *C. jejuni* [29] and aggregating invasive *E. coli* [30]. From these data, 2′FL and other FUT2-HMOs seem to act as soluble ligands blocking *C. jejuni* from adhering to gut epithelial cells, while the protection from *E. coli* might rather be due to an anti-inflammatory effect, possibly combined with the modulation of the gut microbiota composition.

Glycans containing α1-2-linked fucose expressed on epithelial cells of FUT2 positive infants could act as receptors for pathogen binding, conferring a risk to specific infectious diseases for this population [31]. Genetic studies have shown that infants and children with a non-functional FUT2 gene have strain-specific protection against norovirus and Rotavirus [32, 33]. For specific rotavirus strains, susceptibility depends on FUT2, but also on FUT3 status [34]. Experimentally, infectivity of some rotavirus strains was reduced by the FUT2-HMO 2′FL, while other viral strains were affected by sialylated HMO, namely 3′SL and 6′SL [35]. Similarly, 2′FL also bound to specificity norovirus strains [36].

Besides interfering with pathogen attachment to the host mucosa, HMOs were recently reported to exert bacterial growth inhibitory activities on pathogenic group B *Streptococcus* (GBS) [18, 19, 37] a major cause of sepsis in preterm infants. Growth of GBS was specifically inhibited by LNT and LNFP-I, while sialylated HMOs or galactooligosaccharides (GOS) had no effect [19]. Experimental data suggests a putative glycosyltransferase of GBS to be involved [19]. Possibly pointing to a similar mechanism, HMOs from milk of a FUT2 negative mother were shown to have bacteriostatic properties via an alteration of biofilm formation [18]. In an observation study of 183 Gambian infant mother pairs, FUT3 positive mothers were reported to be less likely carriers of GBS, as were their infants.
at birth [37]. Interestingly, infants of FUT3 positive mothers were also more likely to clear GBS colonization from birth to 2-3 months of age compared to infants of FUT3 negative mothers.

In a pilot study of 49 mother-infant pairs, higher breastmilk concentrations of the FUT3 HMOs LNFP-II at 2 weeks were associated with a lower risk of respiratory and gastrointestinal illnesses at 6 and 12 weeks in infants [38]. This association was no longer significant past the breastfeeding period. Similarly, in a nested case cohort study of 143 HIV exposed uninfected children from Zambia, higher concentrations of fucosylated HMOs in breastmilk at 1-month post-partum related to a lower risk of mortality up to 2 years of age [39]. In another small mother-infant cohort from The Gambia (n=33), higher relative breastmilk concentrations of fucosylated HMOs (sum of LNFP-I and LNFP-III) and concomitant lower relative abundance of LNT was associated with lower risk of sickness up to 4 months of age [8].

For respiratory pathogens, direct exposure to HMO would appear less evident and thus any putative HMO-related protection may be mediated by the intestinal microbiome [11, 40]. Yet, experimentally, direct exposure of *Streptococcus pneumoniae* to LNNt and sialyl-LNnT and subsequent infection effectively blocked its colonization in the lung of a rabbit model [41]. In a cell based assay, LNnT and 2-FL dose dependently reduced Influenza and Respiratory Syncytial Virus (RSV) concentrations within respiratory tract cells [42].

Observational studies together with findings from preclinical models have provided first evidence for an association between HMOs and the risk of infections, mostly in a structure function specific way. Mechanistically, HMOs may act through multiple functions, although preclinical models highlight specific individual functions. The current studies also provide directions to be considered in future observational studies such as timing of milk sampling and breastmilk intake, etiology of infections, quantitative versus categorical analysis of HMOs and finally mother and infant genetics.

**HMO composition might be linked to allergy in infants**

Numerous environmental, including nutrition, and genetic factors affect allergies. Among them are breastmilk bioactives, and possibly HMOs. In a cohort of 266 Finnish mother infant pairs with a hereditary allergy risk, 2'FL concentrations in early breastmilk associated with a lower risk to manifest IgE-associated eczema at 2 years of age only in C-section born infants [43]. This observation suggests that 2'FL may influence IgE-associated eczema through the modulation of the early life gut microbiota, known to be different in C-section born infants compared to vaginal born infants. A possible relation of HMOs with cow milk allergy (CMA) was studied in another cohort of 39 mothers with infants who developed CMA by 18 months of age and 41 mothers with infants without CMA [44]. An association was seen between the milk concentration of several individual HMOs (LNFP-III, 6'SL, LNFP-I, DSLNT) and HMO clusters with reduced risk of CMA, with LNFP-III providing the strongest association. Breastmilk sampling varied over the first 6 months after birth and therefore might have introduced a bias, because HMO concentrations change dramatically during this period. Mechanistically, the authors speculate that LNFP-III might act on the immune system via dendritic cells and DC-SIGN. In a preclinical food allergy model, 2'FL and 6'SL were tested and both reduced symptoms involving mast cell activity [45].

The observational studies to date have their limitations, but still provide valuable preliminary data on possible relationships between specific HMOs and risk of allergies. To appreciate such a proposed link requires replication in larger cohorts with harmonized milk sampling, stratification for mode of delivery and evaluation of infant FUT2 and FUT3 genotypes.

**Insight from clinical intervention trials with specific HMOs.**

Recent progress in industrial biotechnology has made available few individual HMOs, namely 2'FL and LNnT. Preclinical safety toxicity tests established their safety and both obtained approval as novel foods in the European Union and were generally recognized as safe in the USA. In adults, both 2'FL and LNnT were studied alone or in combination at different doses from 5 to 20 g/day in a placebo controlled, blinded and randomized trial (n=100). Both HMOs were well tolerated and increased bifidobacteria abundance [46].

In infants, two placebo controlled, blinded and randomized clinical intervention trials showed the growth-safety and tolerance of 2'FL combined with either GOS or fructooligosaccharides (FOS) [47, 48] ([Kajizer et al. 2016 FASEB J]. Infants fed with an infant formula supplemented with 2'FL (0.2 or 1 g/L) combined with GOS or GOS alone showed similar growth as breastfed infants up to 4 months of age (n=314). In a subgroup of infants, immune markers were measured in plasma at baseline and upon stimulation of blood cells with RSV. Globally the immune profile resembled that of breastfed infants when the infant formula was supplemented with 2'FL at the lower or higher dose [48]. Another randomized controlled infant trial showed that an infant starter formula supplemented with 2 HMOs, 2'FL and LNnT, (n=88) allowed for age appropriate growth of term born infants, and was well tolerated when compared to the same infant formula without HMO (n=87) [49]. Interestingly, secondary exploratory findings showed an association between feeding the 2-HMO infant formula and less reported lower respiratory tract illnesses and medication use (especially antibiotics and antipyretics) during the first year of life and beyond the 6 months feeding period. At 3 months, the global microbiota profile shifted in the 2-HMO formula-fed infants away from the control formula fed infants and towards that observed in breastfed reference infants. This shift was mainly due to increases in *Bifidobacterium*...
concomitant with decrease in *Escherichia* and Peptostreptococcaceae [50]. A significantly higher number of infants who were fed the 2-HMO supplemented formula showed a microbiota community structure typical for breastfed infants compared to control formula fed infants, who had primarily a different microbiota community structure. Interestingly, infants with a microbiota community structure typical for control formula fed infants had a 2 times higher risk to use antibiotics during the first year of life compared to those with a microbiota community typical for breastfed infants [51].

These first clinical intervention trials with specific HMOs demonstrate their growth-safety and digestive tolerance. Additionally, as suggested from basic research and observational data, 2'FL and LNnT might contribute to protection from infection-related illnesses and reduced need for antibiotics, possibly through the modulation of the establishing early life gut microbiota.

**Conclusion**

HMOs composition is affected most notably by the maternal FUT2 and FUT3 status. This is likely due to an evolutionary selective pressure imposed by pathogens or the microbiome at large. Stage of lactation alters HMO composition possibly indicating different infant needs at different extra uterine developmental stages. However, giving birth to a preterm or term infant, who are at different developmental stages, seems not to affect the HMO composition of breastmilk. Clinical observations corroborated by preclinical data and clinical intervention trials support a role for specific HMO in immune protection, primarily from infection related morbidity and use of antibiotics. Further clinical studies, well-designed observational and especially placebo-controlled interventions, are warranted to further substantiate and grow our understanding of the HMO biology and significance for infant nutrition.

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Abstract

Human milk oligosaccharides are key components of human milk and appear in various compositions and concentrations in all human milks. In regulatory sense human milk oligosaccharides are classified as novel foods or novel food ingredients requiring safety assessment. In addition, if any health messages are intended to be used also health claim regulations apply. This chapter reviews the regulatory settings and studies human milk oligosaccharides are required to fulfill to be able to enter markets in European Union or United States or elsewhere. Examples include Lacto-N-neotetraose and 2-fucosyllactose with safety assessment in European Union and United States.

Introduction

Human milk oligosaccharides (HMOs) are a group of sugars which are structurally diverse unconjugated glycans. They are found in human milk with a composition unique to each lactating mother [1]. They have not been used in any other foods previously, and, therefore, the supplementation of HMOs to our food requires both safety assessments and most likely assessment of potential health benefits, too [2–5].

While HMOs have been shown to have an impact on the development of infant gut microbiota, it is not well known if HMOs also already affect human milk microbiota composition or microbiota in oral, nasopharyngeal, gastrointestinal, and urinary tract areas [6, 7]. However, they do have a role in the early colonization of infant gut, and also a modifying and potentially protecting role specific to the mother and infant during pregnancy and breastfeeding [8].

HMOs are currently regarded as new ingredients for infant formulas, other foods, and food supplements. Such consideration involves specific regulatory steps, which are in process in several countries and regions.

Issues pertaining to novel HMOs have increased rapidly due to the fast-paced research on the impact HMOs and the possibility of their larger-scale production. HMOs represent novel tools to modulate the gut microbiota and thus potentially provide health benefits for infants, children, and adults. Such food ingredients are in demand as the importance of the gut microbiota on health has been stressed. HMOs could be considered as prebiotics, and specific components targeted toward unique outcomes and functionalities can be expected to emerge.

Regulatory Framework

In the majority of countries, HMOs are characterized as novel foods in the food regulation, and, therefore, several food authorities require a mandatory safety assessment.

The regulations governing the introduction of novel foods vary by geographical region. In some cases, confusion can result in differentiating novel foods from functional foods, or, as in the European Union, the regulatory category of functional foods is formed by foods with European Commission-approved health claims. The fundamental difference between these two categories of foods is that novel foods must be evaluated based on their safety, whereas foods with health claims need to be evaluated for any desired nutritional, functional, or disease risk reduction claims. Figure 1 demonstrates that the terms are distinct, but sometimes foods and food ingredients fall in both categories, which then necessitate evaluation for both safety and efficacy. The 2-category evaluation in Europe appears most likely for HMOs.

The views and challenges of current legislative framework in the USA, Europe, and some other countries are summarized regarding the assessment of HMOs and their novel food status from the regulatory and scientific viewpoint. Additionally, the possibility of future health claims is discussed.

HMOs are intrinsic components to manipulate the gut microbiota by providing an energy source for beneficial intestinal microbiota and potentially acting as decoy molecules to inactivate potential and opportunistic pathogens in the mucosal surfaces for improving the health of the host. Health-promoting outcomes are in demand as the importance of the gut microbiota in human health has been revealed. The regulations governing the introduction of HMOs vary by geographical region. Novel foods and foods with health claims fall under specific regulations in several countries. The main requirements of the regulations in the EU and USA are similar, but the approval processes differ. There are a number of areas that need to be addressed in any safety assessment of novel food ingredients (NFI).
European Union

Worldwide, the regulations governing novel foods, functional foods, and traditional foods vary. In the EU, the introduction of novel foods that have not been used in the EU prior to May 15, 1997, is currently governed by the Novel Food Regulation (EC) 285/97 [5]. This Regulation clearly defines the risk assessment steps required for any authorization of a novel food prior to the introduction into the EU market. It also defines the concept “substantial equivalence” to commonly used foods, in which case a simplified notification procedure applies. It also defines the role of EU Member States in the approval process. One Member State is responsible for the initial safety assessment of the novel food. Other Member States often challenge the initial assessment, or they ask additional questions related to the safety of novel foods. In these cases, concerns are usually discussed and answered by the European Food Safety Authority (EFSA) committee evaluations. Finally, the authorization decision is made either by the Member State in lead or the European Commission with all Member States involved. In both cases, the authorization is valid all over the EU.

The Novel Food Regulation from 1997 has been under revision and the new regulation steps into force fully from January 2018 onwards. The most relevant change is the transfer to a fully centralized system in the EU. Dossiers are submitted to the European Commission, the EFSA is responsible for the safety assessment, and all authorizations are general, i.e. applicable for everybody without further notification. Changes in the update of the regulation cover also traditional foods from third-world countries. Currently, there is a transition period of 2 years during which the earlier mechanisms are still operable.

Current Regulation until 2018

Based on the current regulation, competent authorities of the member states and the European Commission assess if a food or food ingredient has no history of safe use prior to 1997 in Europe and hence is to be identified as “novel.” The regulation then requires an extensive safety assessment of the food or ingredient prior to acceptance to the EU market [4]. A list of authorized novel foods and NFIs is available in the public registry by the EC. The decisions are explained in an inventory specifying the uses and restrictions for each novel food or novel food component, process, or NFI (http://ec.europa.eu/food/safety/novel_food/authorisations/list_authorisations/index_en.htm). For bacteria added to foods, which could also be considered novel, there is an annually updated list of microbes intentionally added to foods (QPS, Qualified Presumption of Safety of Microorganisms in Food and Feed), and this list forms the basis of organisms at the species level which are considered safe for foods and feeds in the EU (EFSA 2016 update). Due to their impact on microbiota, human milk oligosaccharide safety assessment may partly be related to microbiota as well.

A novel prebiotic can potentially be a component of conventional foods, food supplements, or foods for particular nutritional uses. Foods incorporating novel ingredients

Figure 1: European regulatory areas on novel foods and foods with health claims related to potential HMO-containing foods.
comprise also those designed for specific dietary requirements and may include infant and follow-on formulas, processed cereal-based food, food for special medical purposes, and total diet replacement for weight control.

Current Human Milk Oligosaccharides with Regulatory Decision in the European Union

Until now, 2 HMOs have gone through the novel food safety assessment in Europe with a positive assessment outcome and approval by the European Commission.

2'-O-Fucosyllactose (2'-FL) is a synthetic trisaccharide consisting of L-fucose, D-galactose, and D-glucose, which is produced by using L-fucose and D-lactose as starting raw material. The NFI is intended by the applicant to be used in infant and follow-on formulae, foods for special medical purposes for infants and young children, and other foods for infants and young children. The NFI is also intended to be used in foods or food supplements for adults [3].

Lacto-N-neotetraose (LNnT) is a synthetic tetrasaccharide consisting of D-galactose, N-acetyl-D-glucosamine, D-galactose, and D-glucose, which is produced by using D-lactose as a starting raw material. The NFI is intended by the applicant to be used in infant and follow-on formulae, foods for special medical purposes for infants and young children, and other foods for infants and young children. The NFI is also intended to be used in foods or food supplements for adults [3].

A third safety assessment evaluation has been made by EFSA for the 2'-O-fucosyllactose in combination with lacto-N-neotetraose as a food supplement for children [5].

What Did the Member States Question in Case of Human Milk Oligosaccharides?

Several questions were raised by the EU member states following initial safety assessment. These included for example the following:

2'-O-Fucosyllactose [4]

- The intake of large quantities of indigestible carbohydrates can have laxative effects. As there are no human studies on 2'-FL, apart from experience with infants who have been breastfed, nothing can be said about the quantities of 2'-FL which may produce laxative effects.

- The potential for genotoxicity was only assessed in 2 in vitro tests for mutagenicity. A test for potential clastogenic activity is lacking (in vitro micro-nucleus test or in vitro chromosome aberration test). The absence of geno-toxicity for this 2'-FL preparation would only be demonstrated sufficiently upon obtaining a favorable outcome in such study.

Lacto-N-Neotetraose [5]

- Request confirmation that the solvents used are acceptable for food use.

- The estimated intake for distinct population groups, based on food consumption data from the UK, ranges from 26 to 329 mg/kg body weight per day for the 95th percentile. However, these intake estimates do not include the potential additional intake of LNnT from food supplements, as proposed in the application.

- The intake of large quantities of indigestible carbohydrates can have laxative effects. Since there are not yet any human studies on LNnT other than those with breastfed infants, no statement can be made on what levels of LNnT intake could potentially lead to laxative effects.

Nutrition and Health Claims

The EC regulation on nutrition and health claims 1924/2006 requires that such claims are based on scientific evidence and acceptability. EFSA has provided scientific and technical guidance for presenting applications for health claims on food [9]. Recently, an EFSA guidance document [10] or document on PAR-NUTS Directive 2009/39/EC or EU Regulation 609/2013 indicates that there are no escape routes to circumvent the Health Claims Regulation 1924/2006. Hendriksen and Verhagen [11] have developed a decision tree to discern PARNUTS foods from ordinary foods (with health claims). Following the publication of the Health Claims Regulation 1924/2006, the EFSA has evaluated around 3,000 health claims for scientific substantiation.

United States

In the United States, all foods and food ingredients are regulated under the Food Drug and Cosmetic Act (FDCA). In the USA, the safety of new and novel foods (including HMOs) is primarily the responsibility of the food manufacturer. The regulation states that:

any substance that is intentionally added to food is a food additive that is subject to premarket review and approval by the Food and Drug Administration (FDA), unless the substance is generally recognized, among qualified experts, as having been adequately shown to be safe under the conditions of its intended use.

In the USA, HMOs intended for use in foods other than dietary supplements are regulated under the same regimen as all other food ingredients – that is, they may be introduced as food additives or as GRAS (Generally Recognized as Safe) substances, at the discretion of the manufacturer. There are two other conditions that pertain to GRAS substances. First,
the information demonstrating safety must be generally available to the scientific community, usually regarded as requiring publication in the peer-reviewed scientific literature. Second, there must be general acceptance of the safety of the substances throughout the scientific community – there cannot be significant dispute regarding safety.

The FDA has no fundamental role in GRAS determinations except in an advisory capacity. The law that established GRAS (the 1958 Food Additive Amendment to the FDCA) specifically excluded GRAS substances from requiring FDA review and approval prior to entry into the food supply. The GRAS status of the intended use of a HMO is determined by a panel of qualified scientists who render the opinion that there is a “reasonable certainty of no harm” from the intended use, and further that they believe that other equally qualified scientists would reach the same conclusion. This process may be internal to the company and maintained as confidential to the company and disclosed only to customers under confidentiality.

In 1997, the law was amended to provide for a GRAS notification whereby companies could submit it to the FDA. The submission usually consists of an assessment of existing data by a group of recognized experts. Such safety assessment through scientific procedures requires the same quantity and quality of scientific evidence as is required to obtain approval of the substance as a food additive and ordinarily is based on published studies, which may be corroborated by unpublished studies and other data and information. This is a voluntary submission whereby the FDA reviews the information and the favorable FDA response is “FDA has no questions at this time.”

HMOs have a long history of use in food for infants in human milk. However, the compositions vary, and HMOs are not available in other foods. For new and novel HMOs, the route to market as a dietary supplement is to have GRAS self-affirmed for use in food and then to use it in a dietary supplement in the same form. Such notifications have been provided for 2'-O-fucosyllactose, 2'-fucosyllactose, and lacto-N-neotetraose:

2’-O-Fucosyllactose (Glycom A/S). Intended for use in term infant formula at a maximum level of 600 milligrams/Liter. Also intended for use in baked goods and baking mixes, beverages and beverage bases, coffee and tea, dairy product analogs, infant and toddler foods, grain products and pastas, milk (whole and skim), processed fruits and juices, processed vegetables and juices, and sugar substitutes at maximum levels ranging from 0.02 to 1.2 grams/serving [14].

Lacto-N-Neotetraose (Glycom A/S). Intended for use in term infant formula at a maximum level of 600 milligrams/Liter. Also intended for use in baked goods and baking mixes, beverages and beverage bases, coffee and tea, dairy product analogs, infant and toddler foods, grain products and pastas, milk (whole and skim), processed fruits and juices, processed vegetables and juices, and sugar substitutes at maximum levels ranging from 0.02 to 1.2 grams/serving [14].

Japan

In Japan, the assessment of novelty is based on both the source and the traditional use of foods or food ingredients in Japan. Details on novel HMOs are not available currently.

Australia

No applications of HMOs have been handled as of yet (August 2016).

Potential Areas for Health Claim Documentation

Several in vitro and even human studies have been conducted to foresee the potential of HMOs for health claims in Europe and the USA. An example of health claim substantiation is given for probiotics and prebiotics [15]. It is important to remember that the single HMOs and their combinations all form unique products for testing, and thus careful consideration and adjustment are necessary prior to conducting human intervention trials.

The areas of interest have included HMOs and necrotizing enterocolitis, different microbial challenges in the gut, traveller’s diarrhea, and even long-term potentiation of learning capabilities in a rodent model [16–20].

Summary and Future Perspectives

What is known of HMOs in the regulatory area in the United States and the European Union:

- In the EU, HMOs are considered novel foods/NFIs with 2 oligosaccharides, and their combination passed through safety assessment.
- In the USA, 3 HMOs have been passed through according to the GRAS notification system.
- In the EU, the Novel Food Regulation is updated and will be fully operational in 2018; the approval system will be revised, and new guidance for applications has been set by EFSA.
- No health claims exist for HMOs in the EU or USA and if applied for these need to be documented for efficacy.
- The regulatory framework of HMOs varies by geographic region, thus different interpretations on both safety and potential claims could be foreseen.

Disclosure Statement

No conflicts of interest.
References

9 EFSA Panel on Dietetic Products, Nutrition and Allergies: General scientific guidance for stakeholders on health claim applications. EFSA J 2016; 14: 4567.
10 EFSA Panel on Dietetic Products, Nutrition and Allergies: Guidance on the scientific requirements for health claims related to the immune system, the gastrointestinal tract and defence against pathogenic microorganisms. EFSA J 2016; 14:4569.
Human milk contains bioactive components that confer protection on the newborn. These include complex carbohydrates called Human Milk Oligosaccharides (HMOs). Research is revealing the full extent of the beneficial properties of HMOs.

**The benefits of Human Milk Oligosaccharides on immunity**

The immune system of the infant is functionally immature and naive.

HMOs give newborns multiple layers of protection:
- Protect against pathogenic infections
- Stimulate the maturation of the immune system
- Promote the development of the intestine
- Help establish the gut microbiota

Human milk contains a higher concentration and greater structural diversity of oligosaccharides...

Through their actions on the gut, HMOs positively influence the infant’s mucosal and systemic immunity.

HMOs are a predominant component of human milk with the potential to modulate the immune function of the infant.

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