HUMAN MILK COMPOSITION AND HEALTH OUTCOMES IN CHILDREN

EDITED BY: Daniel Munblit, Valerie Verhasselt and John O. Warner
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HUMAN MILK COMPOSITION AND HEALTH OUTCOMES IN CHILDREN

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Table of Contents

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EDITORIAL
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Editorial: Human Milk Composition and Health Outcomes in Children

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Keywords: allergy, breast milk, cytokines, fatty acids, human milk, human milk oligosaccharides, microbiota, milk fat globule

Editorial on the Research Topic

Human Milk Composition and Health Outcomes in Children

Human breast milk (HM) is the physiological nutrition during an infants' first few months of life, a crucial period for immune system development, metabolic and endocrine programming for growth, development, and lifelong health. HM is a complex and variable mixture with a multitude of constituents each contributing either singly or in combination to health outcomes (1). This Research Topic aims to present recent research findings on the complex relationships between HM composition and health outcomes in children, with state-of-the-art reviews, systematic analysis of published data, providing a complete presentation of current knowledge in the field.

Human infants are born with a physiologically relatively immature immune system which is partly compensated by immune active molecules in HM. Furthermore, many immune active molecules in HM have been linked with a variety of health outcomes, suggesting they may also influence the trajectory of immune development (2). Review from Rajani et al. focuses on variations in immune active components that have been reported to be associated with allergic risk in children, providing insight into potential mechanisms of action. Although cytokines and growth factors have been mostly investigated in relation to allergy development, some authors studied associations with growth patterns (3) but little is known of their impact on the growth of infants in the less affluent countries. Saso et al. found levels of cytokines in mature milk being weakly predictive of poor infant growth in Gambia, suggesting that milk compositional changes may be a consequence of suboptimal maternal health and nutrition.

Transforming growth factor beta (TGF-β) is one of the best-studied immune regulatory cytokines and often is investigated with regards to allergy development. A recent systematic review failed to find convincing evidence of associations between HM TGF-β and allergic outcomes (4). Most of the researchers have drawn conclusions based on the links between concentration of immunological markers in colostrum and/or mature milk and allergic diseases. Morita et al. used a more creative approach, looking at ratio (mature milk/colostrum) rather than single time-point concentration, reporting lower TGF-β1 ratio to be associated with the development of eczema. This indicates that innovative approaches to data analysis coupled with more extensive use of serial data could unveil links between the TGF-β and allergic diseases development.

To further understand how breastfeeding can influence immune development, Hsu and Nanan present a critical analysis of potential associations between breastfeeding and the development of thymus, playing a crucial role in T lymphocytes maturation and output.

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Human milk oligosaccharides (HMOs) are as a group the 3rd largest component of HM. More than 200 HMOs are present in HM, and diversity and complexity of HMOs are very specific to HM. Both, short-and long-chain oligosaccharides are found, at a unique ratio. HMOs play an important role in microbiome development and immune system maturation thereby having short- and long-term impacts on infant health. In this Research Topic, two reviews address HMOs importance for infant health and development. Ayechu-Muruzabal et al. provide an overview of the HMOs diversity and impact on early life immune development, while Triantis et al. discusses potential effects of HMOs on infectious and non-communicable diseases and inflammation in general. This paper emphasizes the role of HMOs in altering immune responses through binding to immune-related receptors. There were very few attempts to systematically review available evidence on human milk composition and they are needed to guide future research and highlight gray areas in need of further investigation (5). In view of this, a systematic review of available data on HMOs and associations with immune-mediated disease and infection in childhood is very timely. Doherty et al. reported low concentrations of lacto-N-fucopentaose (LNFP)-III being associated with cow's milk allergy and that higher fucosyl-oligosaccharide levels provides some protection against infectious disease, however, the evidence is sparse and more studies are required.

Although HM has been considered sterile this concept has changed in the last decades with extensive research of HM microbiome. In a recent systematic review of published evidence, a total of 820 microbe species were identified in HM, mainly consisting of Proteobacteria and Firmicutes (6). Human milk oligosaccharides and HM microbiome are acting in synergy, influencing immune system development. Moossavi et al. provide a nice overview review of the latest evidence, mechanisms, and hypotheses for the synergistic and/or additive effects of milk microbiota and HMOs in relation to asthma development in children.

Metabolomic studies increase in numbers and provide complex analysis, with recent data showing distinct patterns associated with maternal lifestyle and the environment (7). Complex, but intriguing interactions between nutrients, microbiome, and metabolites in HM is comprehensively reviewed in the work from Bardanzellu et al. They outline targets for the future research, suggesting a promising approach, of integrative assessment of metabolomics, microbiomics, and HM cellular composition, as the way toward better understanding the potential health promoting properties of HM.

Attention to HM polyunsaturated fatty acids (PUFAs) in relation to health outcomes is based on the hypothesis that the modern diet has led to changes in ratio of n-6 to n-3 fatty acids which may result in increase in allergic diseases prevalence in children. This concept resulted in a number of studies assessing HM PUFAs profile association with atopy/allergy development. Recent systematic review of 18 papers from 15 study populations reported heterogeneity among studies with insufficient evidence to suggest that HM PUFAs influence the risk of childhood allergic diseases (8). This was primarily explained by lack of standardized methodology, such as differences in stage of lactation, variations in definition of allergic outcomes as well as lack of adjustment for potential confounders or even over-adjustment in some studies. Logan and Genuneit discuss the results of the systematic review in their commentary. In light of their subsequent original research (9) they suggest avenues for future research, such as employment of the centered log ratio transformation to overcome spurious correlation, considerations for alternative ways of grouping fatty acids and reduction of selection bias being of particular importance.

Diversity of HM constituents, such as immune active factors, oligosaccharides, and microbes contribute to complex interactions within the HM and associated with gut barrier function, the gut microbiota, and oral tolerance induction (10). It was hypothesized that modulation of infant gut immunity and microbiome may facilitate tolerance development and thus allergy prevention. The impact of breastfeeding on shaping the neonate's gut microbiota and preventative effect on allergy development is reviewed by van den Elsen et al. Emerging data suggests that Milk Fat Globule Membrane (MFGM) may also play in important role in structural and functional maturation of infant gut. Comprehensive review of one of the less well-studied HM constituents, factors influencing its characteristics and potential role in gut immunity maturation presented by Lee et al.

Future of HM research is tightly linked with multi-disciplinary collaborations, aiming to approach HM as a complex and dynamic fluid, which will have various impact on the short and long-term of infant health. We hope that this compilation of articles accumulating existing evidence in the field of HM research will be of an interest to the readers and will inspire more state-of-the-art work on the topics described.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication. The views expressed in the paper are those of the authors and not those of the NIHR or Department of Health (UK).

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REVIEW
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Shaping the Gut Microbiota by Breastfeeding: The Gateway to Allergy Prevention?

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Evidence is accumulating that demonstrates the importance of the gut microbiota in health and diseases such as allergy. Recent studies emphasize the importance of the "window of opportunity" in early life, during which interventions altering the gut microbiota induce long-term effects. The neonate's gut microbiota composition and metabolism could therefore play an essential role in allergic disease risk. Breastfeeding shapes the gut microbiota in early life, both directly by exposure of the neonate to the milk microbiota and indirectly, via maternal milk factors that affect bacterial growth and metabolism such as human milk oligosaccharides, secretory IgA, and anti-microbial factors. The potential of breastmilk to modulate the offspring's early gut microbiota is a promising tool for allergy prevention. Here, we will review the existing evidence demonstrating the impact of breastfeeding on shaping the neonate's gut microbiota and highlight the potential of this strategy for allergy prevention.

Keywords: breastmilk, allergy, gut microbiota, neonate, prevention

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WHY BREASTFEEDING AS A POTENTIAL STRATEGY FOR ALLERGY PREVENTION BY MICROBIOTA SHAPING?

Most of the human body is heavily colonized by all kinds of microorganisms, including bacteria, viruses, fungi, protozoa, and parasites. The largest amount of microorganisms are found within the colon, although the presence in other parts of the gastrointestinal tract cannot be neglected (1, 2). The development of the human gut microbiome is a highly complex process (1). The order and timing by which the gut is colonized early in life has a lasting impact on the microbiome and contributes largely to the variation in microbiota observed between individuals (3). Over the first 3 years of life, the microbiota evolves from relatively simple but rapidly increasing in diversity to an adult state that is more complex and more stable (1). In addition to mode of delivery and antibiotic exposure, nutrition is a key factor in shaping the early microbiota composition and function [as reviewed in Tamburini et al. (4)]. Besides the important role of the gut microbiota in nutrient and bile acid metabolism and the production of vitamins (5), colonization and signaling by microbes plays a pivotal role in gut mucosal immunity as well as systemic immunity. The development of the microbiota ecology parallels that of the gut mucosal immune system. Accumulating evidence is showing that perturbations in the gut microbiota in early life, while the immune system is still developing, can have long-lasting effects on local and systemic immune health.

To date, a clear protective effect of breastmilk on allergy development has not been demonstrated (6–11). However, breastmilk contains factors that can affect key players in allergy development such as gut barrier function, the gut microbiota and oral tolerance induction (12). Therefore, we propose that the modulation of breastmilk composition could be a promising tool for allergy prevention. Here we will review the existing evidence demonstrating the impact of breastfeeding on the neonate's gut microbiota and highlight the potential of shaping the neonate's gut microbiome through breastmilk to decrease allergic disease risk. A literature search was performed in PubMed on original studies and review articles addressing (1) the association of the early life gut microbiota with allergic outcomes (2) immunological mechanisms for the early life gut microbiota to affect allergy development and (3) how breastfeeding shapes the gut microbiota. The primary focus of the review were recent studies addressing these topics in the early life window.

MODULATING EARLY LIFE GUT MICROBIOTA MAY REDUCE LONG-TERM ALLERGIC DISEASE RISK

A Window of Opportunity to Alter the Gut Microbiota for Allergy Prevention

Epidemiological and animal studies have linked perturbations in the infant gut microbiota, when the immune system matures and the gut is colonized with microbiota, with disease risk later in life (4, 13). This highlights the existence of a window of opportunity for disease prevention, including atopic disease, which matches the period in life of breastfeeding. Germ-free (GF) mice have elevated levels of serum immunoglobulin (Ig)E because B cells undergo more isotype class switching to IgE in Peyer's patches and mesenteric lymph nodes. Microbial colonization of GF pups starting between birth and 1 week after weaning, completely inhibits this induction of IgE if a diverse microbiota is used. This implies the need for a critical level of microbial diversity following birth to prevent IgE induction (14). Colonization of GF mice with gut microbiota in early life, but not in adults, is also sufficient to protect from mucosal invariant natural killer T (iNKT) cell accumulation in the lung and allergic airway inflammation (15). Antibiotic administration exacerbated allergic airway inflammation, reduced FoxP3+ regulatory T cells (Treg) in the colon and increased serum IgE only when treatment was initiated in early life (16). In humans, a link between gut microbial dysbiosis in the first 100 days of life and an increased risk of asthma was demonstrated (17). Also other studies have shown an association between the early intestinal microbiota and the risk of allergic sensitization (18–20). All provide evidence of a link between early gut microbiota dysbiosis and an increased risk to develop allergic disease.

Microbiota Richness, Diversity, Composition, and Metabolism in Early Life as Key Elements for Later Allergy Risk

It is still very preliminary to define a "healthy" or "normal" human gut microbiota. The most recent studies define a healthy microbiota as highly diverse, i.e., above 600.000 bacterial genes (21). Nutrition is certainly one way to reach such diversity (2, 22). In addition to diversity there is a core microbiota which is common the most human guts and which has been proposed as a "must have"-set of bacteria (23, 24). Their precise role still needs to be clearly defined but they are mostly involved in the control of inflammation and innate immunity. Although much less details are known in early life, more and more studies try to determine associations between alterations in neonatal microbiota and disease outcome. Microbiota richness, diversity, and composition all may play a role in shaping of the immune response by the gut microbiota. Gut microbial alterations are not limited to shifts in the abundance of certain microbes, they also include alterations in microbiota metabolism and changes in the production of microbial-derived metabolites such as short-chain fatty acids (SCFA) (25, 26).

Bisgaard et al. (18) demonstrated the inverse association between the early gut bacterial diversity and the risk of allergic sensitization, but not asthma or atopic dermatitis (18). However, another study showed that infants with cow's milk allergy had an increased diversity of the gut microbiota and an altered microbial composition dominated by Lachnospiraceae as compared to non-allergic infants (27). In a Chinese cohort, 20 key food allergy-associated bacterial genera, but no difference in fecal microbiota diversity, were observed. The specific microbiota signature detected, could distinguish between IgE-mediated and non-IgE-mediated food allergic infants. The genus *Clostridium sensu stricto* significantly correlated with antigen-specific IgE in infants with food allergy (28). A low gut microbiota richness, overrepresentation of Enterobacteriaceae and underrepresentation of Bacteroidaceae in early infancy were associated with food sensitization in a subset of the Canadian Healthy Infant Longitudinal Development (CHILD) study (20). In the same cohort, Canadian infants at risk of asthma showed a reduction in the relative abundance of the bacterial genera *Lachnospira, Veillonella, Faecalibacterium,* and *Rothia* in early life and had lower fecal concentrations of the SCFA acetate (17, 29). A causal role of these bacterial taxa was demonstrated in mouse experiments (17). The impact of microbial dysbiosis at 3 months of age was further confirmed in a non-industrialized population in rural Ecuador (30). Interestingly, different bacterial taxa were involved compared to Canadian infants. Some fecal fungal taxa were altered too and genes involved in carbohydrate and taurine metabolism were highly altered (30). Another birth cohort showed that neonates with a relatively lower abundance of bacteria such as *Bifidobacterium*, *Akkermansia,* and *Faecalibacterium*, along with higher abundance of the fungi *Candida* and *Rhodotorula* and pro-inflammatory fecal metabolites, had the highest risk of childhood atopy and asthma (31). Russian children at low risk for the development of allergic disease had higher proportions of *Bifidobacterium*, whereas Finnish and Estonian children with a higher risk of allergies had increased abundance of *Bacteroides* (32). Furthermore, early colonization with Lactobacilli was shown to decrease the risk of allergy (19) while early colonization with *Staphyloccocus aureus* and *Clostridium difficile* characterizes infants developing allergy later in life (33–35). Colonization with *Escherichia coli* was

associated with IgE-mediated eczema (36, 37). However, a study using early administration of *E. coli* as probiotic strategy found a reduction in allergy development, pointing toward strain-specific effects of *E. coli* (38). Recently it was also reported that the kinetic of development of the gut microbiome during the first year of life affects the risk of childhood asthma in children from asthmatic mothers. One-year-old children with an immature microbial composition had an increased risk of asthma at age 5 years compared to children with mature microbiota (39).

POTENTIAL MECHANISMS OF ALLERGY PREVENTION IN EARLY LIFE BY THE MICROBIOTA

To induce tolerance at mucosal surfaces, antigens are taken up by dendritic cells (DC) which migrate to the lymph nodes where the local production of factors like transforming growth factor beta (TGF-β) induces the differentiation of naïve T cells to antigen-specific Treg (40). Here, we will summarize the current observations in early life specifically, as this coincides with the period of breastfeeding, which demonstrate an effect of the microbiota on the maturation of the immune system (**Figure 1**). Various studies have demonstrated a role of the microbiota in early life on the development of FoxP3+ Treg. *Ex vivo* culturing of human adult peripheral T cells with sterile fecal water from children at high risk of developing atopic disease, reduced the percentage of FoxP3+ Treg cells (31). Neonatal colonization with a specific strain of the commensal *E. coli* lead to oral tolerance failure. It reduced tolerogenic DC and subsequently Treg populations (41). On the other hand, neonatal enrichment of mice with *Clostridium* species from human indigenous microbiota resulted in higher numbers of colonic FoxP3+ Treg in adulthood, likely induced by intestinal epithelial cell-secreted TGF-β, and lower allergy risk (42). Another study demonstrated the pivotal role of early life colonization with *Bacteroides fragilis* expressing polysaccharide A (PSA) for iNKT cell inhibition and Treg development in the intestine (43). Colonizing adult mice did not have this effect (43). Another study has emphasized a role for the gut microbiota in the modulation of IL-22 secretion and gut barrier function. Colonization of young mice with Clostridia induced IL-22 production by group 3 innate lymphoid cells (ILC3) and T helper 17 cells in the intestinal lamina propria. IL-22 was critical for sensitization to food allergen as it induces antimicrobial peptide production by Paneth cells and mucus production by goblet cells to strengthen the gut barrier. This prevents the transfer of dietary antigen across the barrier and therefore allergic sensitization (44). Recent studies have also linked the kynurenine pathway, involved in the breakdown of tryptophan by host cells, with the gut microbiota and allergy (45). In host immune and epithelial cells the enzyme indoleamine 2,3-dioxygenase (IDO) metabolizes tryptophan to kynurenine and downstream products (46). IDO can become activated in response to allergen-induced immune activation (45). Kynurenines regulate immune homeostasis and exhibit tolerogenic effects by causing T cell anergy and apoptosis and induce the generation of Treg, leading to attenuation of allergic responses (45). The gut microbiota plays an important role in stimulating IDO activity, as demonstrated in GF mice (46, 47), making this pathway dependent on early life gut microbiota development (45). Targeting the gut microbiota to modulate tryptophan metabolism could therefore have the potential to prevent allergic disease (45).

Shaping of the Immune System by the Microbiota

MAMP Signaling

The gut microbiota exerts direct effects on the immune system through microorganism-associated molecular pattern (MAMP) signaling. Bacterial as well as fungal and viral molecular patterns such as lipopolysaccharides (LPS), flagellin, peptidoglycans (PG), formyl peptides, and unique nucleic acid structures are sensed by pattern recognition receptors (PRR) including membrane bound Toll-like receptors (TLR) and cytoplasmic NOD-like receptors (NLR) (25).

LPS binds to a complex of membranous TLR4, CD14 and myeloid differentiation protein 2 (MD-2) or circulating sCD14 receptors. TLR4 is the receptor transmitting the MAMP's signal inside the cell to trigger genes of inflammation. LPS are complex molecules with a lipid A part containing different fatty acids esterified to a glucosamine structure. It can possess 4, 5, or 6 aliphatic chains of different length including cyclic fatty acids specific from bacteria. Hexa-acylated LPS, such as seen in *E. coli*, is considered highly inflammatory whereas LPS with a lower number of acylated fatty acids, such as those from the Porphyromonadaceae family, are less inflammatory or even anti-inflammatory (48). Variation in LPS immunogenicity can play a role in immune education and driving allergic disease. High exposure to *Bacteroides*-derived penta- or tetra-acylated LPS, which has immune inhibitory properties, was demonstrated in infants with high risk of allergic disease. This form of LPS does not induce endotoxin tolerance, leading to inflammatory responses later in life (32, 49). A similar observation was reported for PGs which can be pro- or anti-inflammatory according to the structure and molecular weight of the molecule. In addition, PG can bind to different TLRs, particularly TLR2, as well as the intracellular NLR NOD1 and 2, with different outcome (50, 51). Furthermore, TLR signaling by bacterial products induces a tolerogenic environment and Treg expansion in the intestine (52). These studies demonstrate that MAMPs can engage innate and adaptive immunity through the stimulation of different TLRs and NLRs. This suggests that the gut microbiota ecology at birth directs the specific crosstalk between MAMPs and the immune system and educates both innate and adaptive immunity. This could result in protection against the development of allergic diseases.

Metabolite Signaling

Besides the direct effects of gut microbes on the immune system, the production of metabolites by the fermentation of dietary fiber and other complex macronutrients that escape digestion in the small intestine, plays an important role. Molecules produced or derived from bacterial metabolism are drivers of cellular host functions and notably the intestinal immune, epithelial,

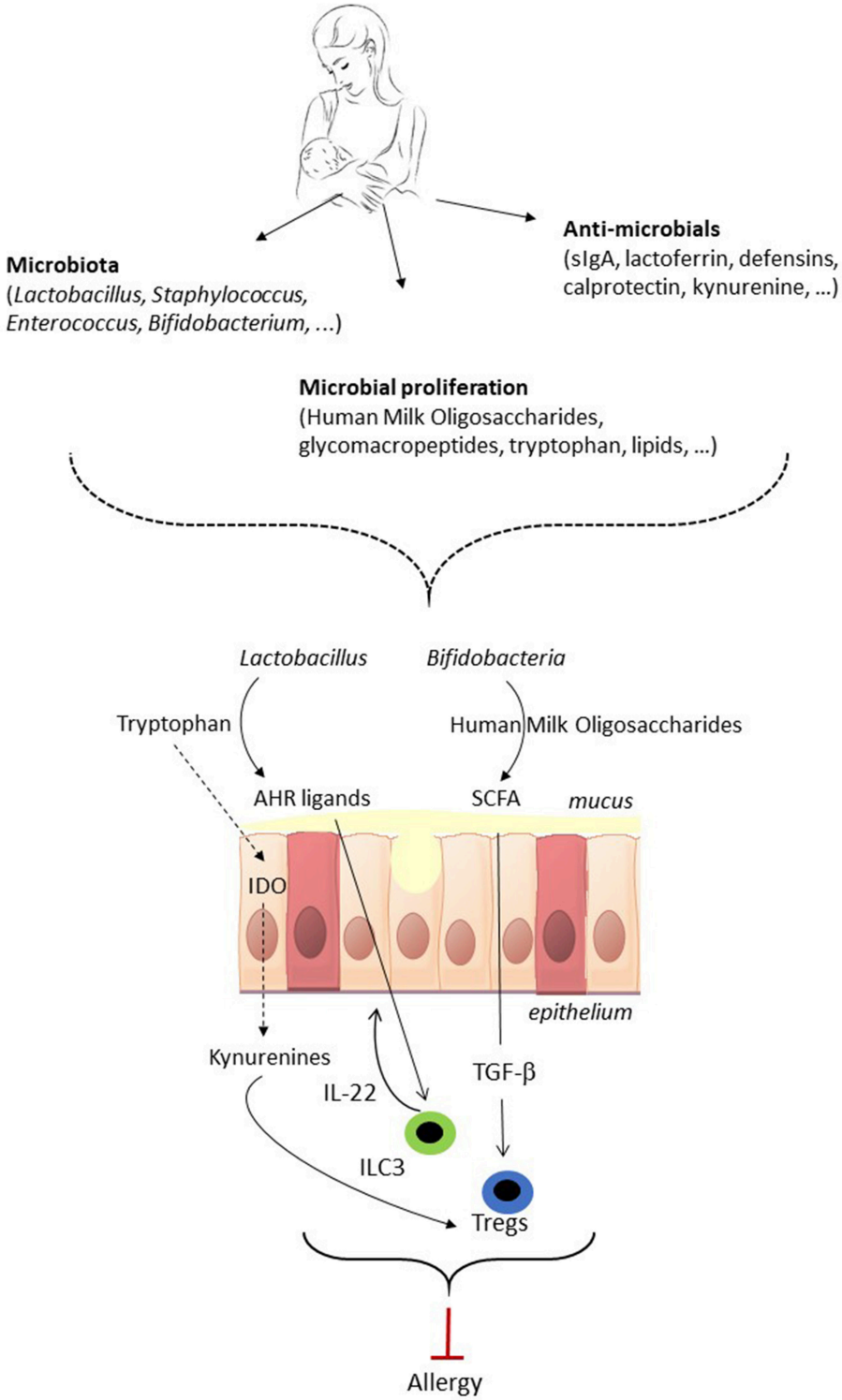

FIGURE 1 | The potential of breastmilk to prevent allergic disease by shaping of the neonatal gut microbiota. Breastmilk contains microbes as well as factors that indirectly shape the gut microbiota of the neonate. Breastmilk can direct the early microbiota composition, i.e., favor the growth of *Bifidobacteria* and *Lactobacillus*, and affect microbiota metabolic function, which subsequently can impact on immune development and maturation. The gut microbiota in early life impacts on immune maturation via microorganism-associated molecular patterns (MAMPs) signaling (not shown in the figure) and via microbiota metabolites such as short-chain fatty acids (SCFA) and aryl hydrocarbon receptor (AHR) ligands. The gut microbiota of the neonate can direct the immune system toward allergy prevention via the induction of FoxP3+ regulatory T cells (Treg), which contribute to oral tolerance induction, and IL-22 production by group 3 innate lymphoid cell (ILC3) which

(Continued)

FIGURE 1 | strengthens the gut barrier. As a result, shaping of the infant's gut microbiota by breastmilk has the potential to direct the immune system toward allergy prevention. TGF-β, Transforming Growth Factor beta; IDO, indoleamine 2,3-dioxygenase.

vascular, and neural systems (53). Bacterial metabolites can induce epigenetic changes such as chromatin modifications that allow the microbiota to exert long-lasting effects on immunity (54). Metabolites of microbial origin include choline metabolites, vitamins, and phenolic derivatives (53). Primary bile acids can be transformed into secondary bile acids by the metabolism of bacteria, which can differently activate the bile acid receptors farnesoid X receptor (FXR) and G protein-coupled bile acid receptor (TGR5) (55). Specific commensal bacteria, especially *Lactobacilli*, metabolize the essential amino acid tryptophan into indole derivatives that can bind to aryl hydrocarbon receptors (AHR) expressed by immune and epithelial cells. AHR signaling is important for ILC3 activation and intestinal barrier function (25). The role of AHR ligands on immune development during the neonatal phase specifically is largely unclear to date. Branched-chain fatty acids such as valerate, isobutyrate, and isovalerate are derived from bacterial amino acid metabolism. SCFA, predominantly butyrate, acetate and propionate, are produced upon breakdown of dietary fiber. Lactate and succinate are intermediate metabolites in the production of SCFA, but can exert immune modulating effects themselves too (56, 57). SCFA are an important energy source for intestinal epithelial cells. Furthermore, SCFA signal through G protein couples receptors such as GPR43, GPR41, and GPR109A present on epithelial and immune cells, and inhibition of histone deacetylases (25, 58). In adult mice it has been demonstrated that SCFA can enhance the intestinal barrier function, induce tolerogenic DC and promote anti-inflammatory Treg in the colon, all contributing to immune tolerance (25). In the lung SCFA impair the ability of DC to promote a T helper 2 response and reduce ILC2 proliferation and function (59, 60). Data on the effects of SCFA on the maturation of the gut and immune system in the neonate is currently largely lacking. However, there is limited evidence that higher acetate levels in infants might assist in the protection against allergic disease (17).

BREASTFEEDING SHAPES GUT MICROBIOTA COMPOSITION AND METABOLISM

A Role of Breastmilk in the Establishment of the Gut Microbiota in the Neonate

Recent studies suggest that microbial transfer from the mother to the fetus already occurs *in utero*. Microbes have been detected in the placenta, amniotic fluid, fetal membrane, umbilical cord blood, and meconium (4, 61). However, the first major exposure of the neonate to microbes happens during birth and is highly dependent on the mode of delivery (1, 4). Besides birth mode and/or antibiotics use just before or after delivery, early nutrition is a key factor directing the early microbiota composition and function as it provides nutrients for bacterial growth and dictates their production of metabolites (1, 2, 22). Recently, a large, multi-center study confirmed that breastfeeding status was the most significant factor associated with microbiome structure in early life (62). The first bacteria to establish in the neonatal gut are mostly aerobic or facultative anaerobic bacteria such as enterobacteria, enterococci, and staphylococci. During their growth they consume oxygen allowing the rise of anaerobic bacteria including bifidobacteria (63). The gut microbiome in breastfed infants is usually dominated by bifidobacteria and *Lactobacillus* species, while formula-fed infants harbor a more diverse gut microbiota that resembles that of older children (22, 64). Relatively small amounts of formula supplementation of breastfed infants, only during the first days of life, already resulted in shifts in microbiota composition (65). In addition to shaping microbiota composition, early feeding practice affects microbiota metabolism. The microbiomes of newborns and young infants are enriched in genes required for the degradation of sugars from breastmilk (human milk oligosaccharides, HMOs) (22). Compared with formula-fed children, breastfed infants have lower absolute concentrations of fecal SCFA, potentially due to the less diverse microbiota, and higher concentrations of lactate. However, the relative proportion of acetate was higher in exclusively breastfed children (56). The introduction of solid foods changes the metabolic function of the gut bacteria as genes involved in the degradation of sugars from breastmilk are less needed and utilized. Instead, the microbiota adapts to the available energy sources and functionally matures to be able to degrade complex sugars and starch found in solid food (22). Interestingly, the microbiota composition in African and European infants is very similar until the introduction of solid foods, indicating the dominant role of diet over other variables in shaping the microbial composition of the gut in early life (66).

Mechanisms of Breastmilk-Induced Neonatal Gut Microbiota Shaping

Breastmilk provides the neonate with its own microbiota as well as prebiotic, immunological and other microbiota-shaping compounds that indirectly can alter colonization patterns in the neonate (**Figure 1**). Therefore, a varied composition of breastmilk could be considered as a selective bioactor to reach gut microbiota diversity and hence good health.

Human Breastmilk Microbiota

Breastmilk contains 10^2-10^4 viable bacteria per mL (67), and thereby can directly affect the establishment of the neonatal microbiota (68). *Lactobacillus, Staphylococcus, Enterococcus*, and *Bifidobacterium* are transferred through breastfeeding (67). Milk bacterial communities are complex and vary between individuals. The breastmilk microbiota also evolves over the period of breastfeeding. Colostrum microbiota has a higher diversity than mature milk (69). In colostrum, *Staphylococcus,* lactic acid bacteria and *Streptococcus* are the most abundant (69). After 1 month, the *Staphylococcus* abundance is dramatically reduced,

while the lactic acid bacteria are still highly abundant (68, 69). The maturation of the breastmilk microbiota happens in parallel with the evolution of the neonate's microbiota. As soon as 3–4 days after birth, the gut microbiota of infants begins to resemble the colostrum microbiota (61), followed by a gut microbiota rich in bifidobacteria and lactobacilli (22).

The origin of bacteria in breastmilk is under debate. Some suggest that human milk bacteria are derived from the maternal skin as some bacterial phyla that are common in human milk, such as *Staphylococcus,* are usually present on adult skin (67). It has also been demonstrated that during suckling breastmilk flows back into the mammary ducts (67, 70), which provides a route for bacteria found in the infants oral cavity to enter the mammary gland (67, 69). However, most studies propose that the translocation of maternal gut bacteria to the mammary gland is the major pathway (2, 64). DC and macrophages can sample live commensal bacteria from the gut lumen and keep them in the mesenteric lymph nodes. From there, the bacteria can circulate to other locations in the body, including the mammary glands (67). Mothers having a cesarean section, show a more diverse milk microbiota with reduced frequency of bifidobacteria as compared to mothers after a vaginal delivery. This effect is most pronounced for infants from women undergoing an elective cesarean-section, suggesting that signals related to labor affect bacterial transfer to the mammary glands (69).

Human Milk Oligosaccharides

HMOs are structurally complex sugars unique to human breastmilk. They are indigestible and do not provide energy for the infant but serve as prebiotics, which are substrates for fermentation processes by intestinal microbes, inducing the growth or activity of beneficial bacteria (71). HMOs are highly abundant in human milk but absent in most formula nutrition and are believed to play a major role in the differences between the gut microbiota in breast- vs. formula-fed infants. HMO composition in maternal milk is regulated by genetic fucosyltransferase-2 (FUT2) secretor status and other factors including lactation stage, maternal health and ethnicity (72). HMOs act as antiadhesive agents that inhibit pathogen adhesion to mucosal surfaces, preventing colonization and as antimicrobials by preventing proliferation of certain bacteria (72, 73). Furthermore, HMO favor *Bifidobacterium* growth and are their preferred substrates for the production of SCFA and lactate in infancy (56, 74, 75). *Bifidobacterium* predominance in the stool is the main characteristic of breastfed infants (68) and the higher relative proportion of acetate in breastfed compared to formula-fed infants may be due to the absence of HMO in formula (56). Infants receiving breastmilk from non-secretor mothers, who lack the functional FUT2 enzyme, show a delay in the establishment of bifidobacteria highlighting the need for maternal milk HMOs in bifidobacteria growth (76). A recent study further demonstrates that HMOs in breastmilk can also modulate the transcriptional activity of certain bacteria such as *B. fragilis*, rather than modifying their relative abundance (26). Furthermore, another commensal, *E. coli,* that cannot directly degrade HMOs, benefits indirectly by consuming metabolites produced by *B. fragilis* upon HMOs degradation (26). An association between HMO profiles, but not individual HMOs, and food sensitization has been demonstrated in 1-year-old infants (77). A small clinical study further found that infants receiving maternal milk with low concentrations of the HMO lacto-N-fucopentaose III were more likely to develop cow's milk allergy (76). A likely explanation, besides the possibility of direct effects of HMO on immune cells, is the prebiotic modulation of the gut microbiota by HMOs influencing immune development and food sensitization (77).

Glycomacropeptide

Casein glycomacropeptide (GMP) is a small glycoconjugated peptide present in human milk. GMP has a prebiotic effect on bifidobacteria and lactic acid bacteria (78).

Secretory IgA

Secretory IgA (sIgA) are also important factors in shaping the gut microbiota in the neonate. IgA-producing plasma cells in the mammary gland originate from the maternal gut. The specificity of sIgA in breastmilk is therefore dictated by maternal exposure to pathogenic enteric bacteria and by commensal bacteria in the maternal gut. Maternal IgA produced in the mammary gland, are transported across the epithelial cells into the milk and acquire the secretory part from epithelial cells (79). As newborns produce only low levels of sIgA, breastmilk-derived sIgA prevent expansion and penetration of pathogenic bacteria while their intestinal immune system is developing (80, 81). IgA also has functions beyond pathogen exclusion. Experiments in mice using IgA-deficient dams demonstrated a long-lasting role of breastmilk-derived sIgA in shaping of the gut microbiota. Taxa from the family Lachnospiraceae were upregulated in the absence of sIgA from breastmilk (82). Milk sIgA are also necessary for the prevention of excessive expansion of pro-inflammatory segmented filamentous bacteria (SFB) by coating these bacteria (83). A recent birth cohort showed that IgA recognition patterns differed between healthy and allergic children. This was already visible at 1 month of age, when IgA is predominantly maternally derived in breastfed children. Interestingly, mainly butyrate-producing gut commensals such as *Faecalibacterium* were IgA free in children with allergic symptoms (84). IgA can also modulate the production of metabolites by the microbiota, as recently shown in an adult mouse model (85). In this study it was demonstrated that IgA has the capacity to modulate bacterial composition, gene expression, and metabolic function of the gut microbiota by antigen-independent binding to intestinal bacteria (85).

Antimicrobial Proteins and Peptides

Antimicrobial factors in breastmilk have the potential to shape the microbiota. Breastmilk is the main source of lactoferrin for the infant and protects them from bacterial invasion by sequestering iron from bacterial pathogens and direct interaction with bacteria (79). The ability to protect the infant against pathogenic microorganisms helps the development of a beneficial microbiota (79). The amount of fecal bifidobacteria and lactobacilli in newborns positively correlated with fecal lactoferrin (86). This suggests that lactoferrin promotes specific

microbial composition and might be critical for the microbiota to develop in early life. High levels of calprotectin (heterodimer of calcium binding proteins S100A8 and S100A9) also demonstrate antimicrobial properties that can be attributed to its metal ion chelation capacity. This results in growth inhibition of especially manganese sensitive bacteria such as *S. aureus* and group B streptococci. After birth, calprotectin concentrations are high in human milk (87). Defensins in breastmilk also have antimicrobial activity against common neonatal pathogens (88) and have been shown to affect the intestinal microbiota (89).

Tryptophan Metabolites

Tryptophan is a precursor of a large number of metabolites, including endogenous metabolites such as kynurenine and bacterial metabolites such as indole derivatives (46, 47). Tryptophan and its metabolites are present in breastmilk (90, 91) and can have profound effects on the gut microbial composition, metabolism and function in the infant (47). Kynurenines have antimicrobial properties, which can directly impact on the gut microbiota (47). AHR ligands in breastmilk originate from the maternal diet as well as from the maternal microbiota. Maternal milk immunoglobulins help in the transfer of these metabolites to the neonate (91). The AHR signaling pathway is also able to influence microbiota composition (92).

Components of the Innate Immune Response

Factors of the innate immune response that are present in breastmilk, but not infant formula, include soluble TLR2, TLR4 and their co-receptors CD14 and MD2, which are involved in binding LPS (89, 93, 94). These factors probably contribute to the composition of luminal and enterocyte surface bacteria (89, 95).

Lipids

Lipids impact on the gut microbiota ecology either directly by feeding some bacteria or indirectly by triggering the host to secrete hormones and bile acids (96). Bile acids are major regulators of the gut microbiota (55). They are detergents for bacteria explaining, at least in part, the low bacterial count in the duodenum where bile acids are mostly released. Human milk fatty acid profiles were associated with the relative abundance of five taxa (*Bacteroides,* Enterobacteriaceae, *Veillonella, Streptococcus,* and *Clostridium*) in the gut microbiota of breastfed neonates (97).

CONCLUSIONS AND PERSPECTIVES

The potential of breastmilk to alter the offspring's early gut microbiota is a promising tool for immune education and allergy prevention (**Figure 1**). This requires identifying (1) what a beneficial microbiota for allergy prevention in our modern environment is (2) which factors in breastmilk are necessary to guide the establishment of such a beneficial microbiota and (3) how to enrich breastmilk with the required factors. There have been major advances in the recent years in the identification of a beneficial microbiota for allergic disease prevention. In particular, the necessity of a diverse gut microbiota and the importance of the metabolism of the microbiota have been highlighted. The role of the breastmilk microbiota and HMOs in shaping of the neonatal gut microbiota has become more and more evident recently. There is however still poor knowledge on the possibilities to modulate breastmilk factors involved in the microbiota shaping. The administration of probiotics to lactating mothers has led to inconsistent results regarding the possibility to change the milk microbiota (98, 99) and there is currently no clue on the possibility to modify HMOs content in breastmilk. There is a need for randomized intervention trials that study the allergy preventive effects of supplementation of lactating mothers with milk-modulating factors such as pre- and probiotics, to shape the infants microbiota and subsequently program the immune response.

AUTHOR CONTRIBUTIONS

LvdE and VV proposed the topic to be covered in the manuscript and wrote the main content with the contribution of JG and RB.

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Conflict of Interest Statement: JG is employed by Danone, a company producing products for infant nutrition.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Breast Milk Cytokines and Early Growth in Gambian Infants

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Background: Breast milk provides nutrition for infants but also delivers other bioactive factors that have key protective and developmental benefits. In particular, cytokines are thought to play a role in immunomodulation, although little is known about their impact on health outcomes in early life.

Objective: The purpose of this pilot study was to evaluate the relationship between cytokines in breast milk and infant growth outcomes in a low-income setting.

Methods: 100 mother-infant pairs were followed up to 2–3 months postpartum as part of a prospective longitudinal cohort study in urban Gambia, West Africa. The concentrations of 9 pro-inflammatory cytokines (IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12, IL-13, IFN-γ, TNFα), IGF-1 and TGFβ2 were measured in colostrum within 12 h of birth and in breast milk at the final visit, scheduled between day 60 and 89 postpartum. Infant weight was recorded and converted to weight-for-age *Z*-scores (WAZ) at the same time points. Growth outcomes were defined in our study as (a) change in WAZ between birth and final visit (b) WAZ at final visit. Linear regression analysis was used to determine the ability of colostrum and breast milk cytokine concentrations to predict growth outcomes up to 2–3 months postpartum.

Results: Gambian infants demonstrated growth faltering across the first 2–3 months postpartum. There was no significant relationship between cytokines in colostrum and subsequent change in WAZ between birth and the final visit, in either unadjusted or adjusted models. However, cytokines in mature breast milk, TNFα, IFNγ, IL1β, IL2, IL4, and IL6, were weak negative predictors of WAZ scores at the final visit, in unadjusted models ($p < 0.05$). When adjusted for maternal anemia (as a proxy for maternal nutrition), TNFα and IL6 remained significant predictors ($p < 0.05$).

Conclusions: Variations in breast milk cytokine levels do not play a substantial role in the growth faltering observed across early infancy. The potential contribution of other factors, such as micronutrients, hormones or human milk oligosaccharides, must be elucidated. Cytokine levels in mature breast milk were weakly predictive of poor infant growth, possibly reflecting a "read-out" of suboptimal maternal health and nutrition.

Keywords: breast milk, colostrum, cytokine, growth, weight, infant, neonate, immunity

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INTRODUCTION

Human breast milk (BM) provides nourishment for infants but also delivers a range of non-nutritive bioactive factors that have key protective and developmental benefits, including actively shaping and priming the infant immune system (1–6). It is increasingly recognized that the complex effects of colostrum and BM on the diverse nature and function of the infant microbiome are key to coordinating these roles (7–10). Moreover, biologically active substances influence the infant's response to energy intake and metabolism (3, 11–13). BM composition, however, is very diverse, dynamic, varying between women and over the course of lactation, and sensitive to both maternal and environmental factors (14–16). The exact nature of these variations, their impact on infant morbidity and growth outcomes and the exploitative potential for future interventions has yet to be fully elucidated (10, 17–22).

Cytokines and growth factors have been identified as important bioactive components within BM and are found in differing concentrations (23, 24). It is hypothesized that the mammary gland is the principal source of these compounds in milk (6, 25). In addition, leukocytes isolated from expressed human milk have demonstrated the ability to secrete a small number of cytokines (26). Once ingested, milk-derived cytokines and growth factors pass through the infant's gastrointestinal tract, resisting proteolysis and retaining their bioactivity. They subsequently interact with specific gut epithelial receptors, enabling them to be absorbed from the gastrointestinal tract and thereby enter the infant circulation (12, 18, 19, 27).

Cytokines, particularly in colostrum, are thought to play a key role in immunomodulation, including promoting sIgA production, mediating infant immune responses, for example through B cell growth and differentiation, and boosting development of intestinal tolerance (4, 5, 12, 28, 29). However, the impact of these immune factors on early infant growth and body composition is less well-documented.

Growth faltering in sub-Saharan Africa, including The Gambia, has been well-established previously (30) and poor growth in infancy and early childhood has adverse consequences for child mortality, and on subsequent adult stature and health outcomes (31, 32). At the same time, the rate of breast feeding is high in these low-income populations and is the only sustainable feeding option for many. Despite intensive efforts aimed at achieving optimal nourishment for mother and child, substantial infant growth faltering remains, suggesting the potential importance of other non-nutritive factors (30, 33). A more thorough understanding of how natural variation in BM bioactive components influences infant development and may exacerbate or protect against growth failure would help guide the development of preventative and therapeutic strategies, particularly in low-income settings (21, 31).

Abbreviations: IL, Interleukin; IGF, Insulin-like growth factor; EGF, Epidermal growth factor; TGF, Transforming growth factor; HMO, Human milk oligosaccharide; Hb, Hemoglobin; BM, Breast milk; WAZ, Weight-for-age Z score; SD, Standard deviation; CI, Confidence interval.

The aim of this study was to evaluate the relationship between the cytokine and growth factor profile in human BM and infant growth outcomes in the first 3 months post-partum in The Gambia, a low-income country in West Africa.

MATERIALS AND METHODS

Study Setting, Population, and Ethics

Samples for this project were made available as part of a larger prospective longitudinal cohort study on *Group B Streptococcus* infection, conducted between 1st January and 31st December 2014, in an urban area of The Gambia, West Africa (34). The Gambia is a low-income country with an estimated 79,000 births annually and an infant mortality rate of 42/1,000 (2016) (35). Moreover, 47% of infants are exclusively breastfed under 6 months and 98% breastfed (mixed and exclusive) beyond the first year of life (2013) (35). The cohort in this sub-project consisted of 100 consecutive pregnant women and their infants followed up across the first 2–3 months postpartum in two government health centers offering antenatal care to women. These centers were considered representative samples of the standard of care available to Gambian women in urban settings, with a combined birth rate of ∼12,500 births annually(34). Women between the ages of 18–40 years who had a negative HIV test and low-risk, singleton pregnancy (no evidence of pre-eclampsia, cardiomyopathy, maternal gestational diabetes, placenta praevia, twin pregnancy) were recruited in the final trimester of pregnancy after giving informed consent. They were excluded if they did not plan to breastfeed or remain in the nearby area for the first 3 months postpartum. Infant exclusion criteria included marked prematurity (<32 weeks gestation), low birth weight (<2.5 kg), congenital abnormalities or requiring resuscitation at the time of delivery with subsequent transfer to a neonatal unit. Further demographic and recruitment details, including eligibility, inclusion and exclusion criteria and consent protocol, are summarized in **Figure 1** and have previously been published (34). The study was approved by the joint Gambian Government/Medical Research Council Research Ethics Committee, SCC 1350 V4.

Data Collection

Following birth, participants were monitored daily at home until day 6 and then asked to return to the clinic for a final visit which was scheduled between day 60 and 89 postpartum. Details were documented via standardized questionnaires and included maternal factors (age, ethnic origin, gravida, parity, weight, blood pressure, hemoglobin concentration, illnesses in pregnancy, use of antibiotics/medications/ traditional medicines/antimalarial prophylaxis) and infant factors (anthropometry, sex, morbidity, vaccinations, vital signs, feeding). Full details on data collection have been described previously (34). Anthropometric measures were performed by trained field nurses at birth and at the final visit; infant weight was measured using electronic baby scales (model 336; Seca, UK), to the nearest 0.01 kg. Weight-for-age Z score (WAZ) was calculated based on the WHO growth standards (36).

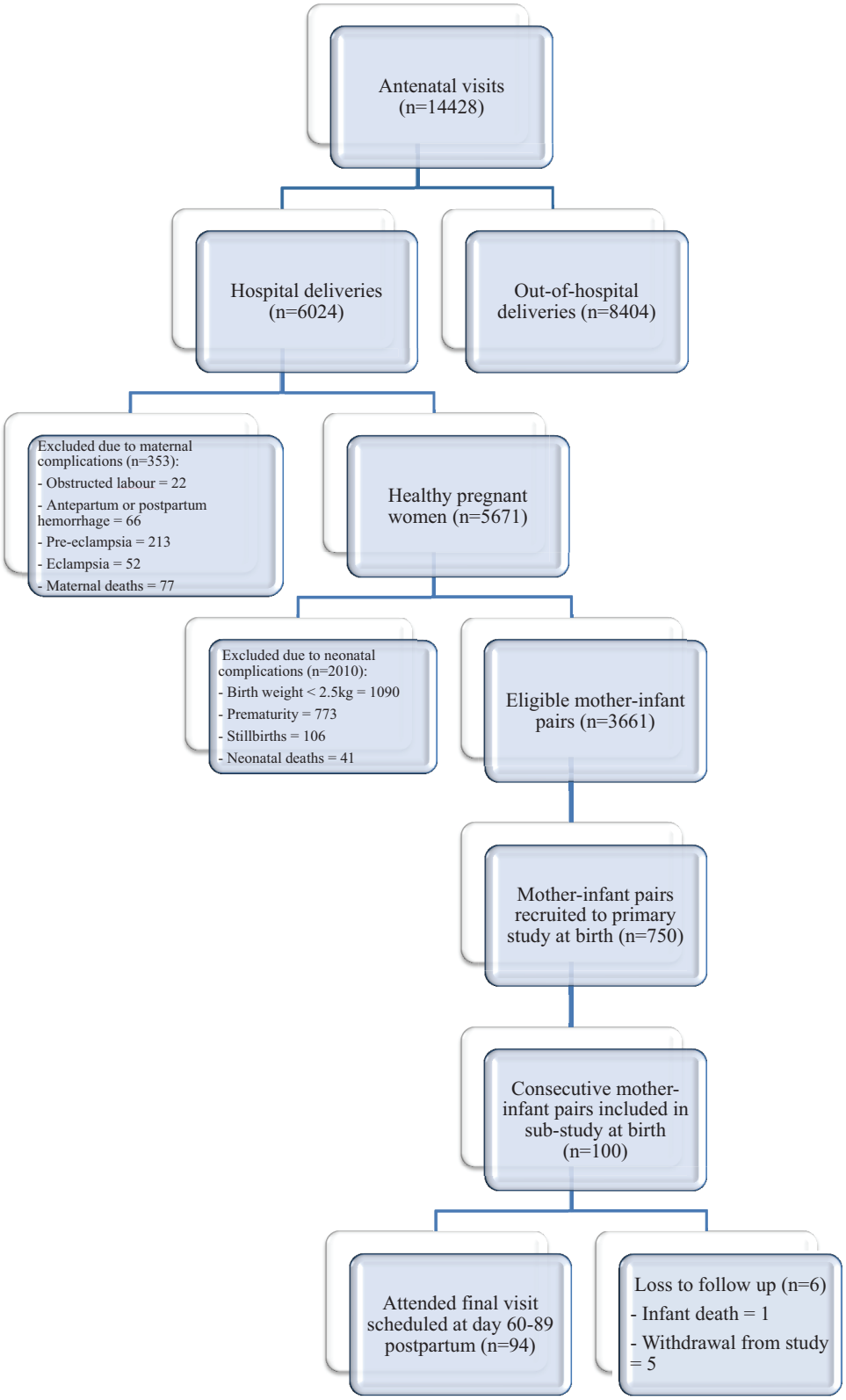

FIGURE 1 | Flow diagram to demonstrate recruitment, retention and loss to follow up (34).

Colostrum and Breast Milk Sample Collection

Colostrum was collected within 12 h of birth and mature BM was collected at the final visit. After washing their hands with soap, mothers wiped each breast with a 0.02% chlorhexidine wipe and then hand-expressed their milk prior to feeding their infant; the first 0.5 mls was discarded and then 2–3 mL of colostrum and 4–5 mL of BM was collected from both breasts into sterile plastic containers. Milk samples were immediately stored on cold packs at 4°C and transported to the laboratory within 6 h of collection (37, 38).

Laboratory Procedures

BM samples were centrifuged at 3,200 rpm for 30 min to separate whey and lipid layers. The lipid layer was removed with a scalpel and stored separately. The whey layer was stored in 1 mL aliquots and frozen at −70°C. It was transferred to 5 mm NMR capillary tubes for analysis (37, 38).

Cytokine Quantification

Assays were run in duplicate and concentrations of IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12, IL-13, IFN-γ, TNFα, IGF-1, and TGFβ2 in colostrum and BM were determined by electroluminescence using kits from Meso-Scale Discovery Multi-Spot Kit (MSD, Rockville, MD, United States). Laboratory experiments were run according to manufacturer's protocol, using an eight-point standard curve. Samples below the lower limit of detection were allocated a concentration for each cytokine of half the lower limit of detection to aid the statistical analysis and as per manufacturer's instructions (**Supplementary Table 1**). The coefficient of variance (CoV) for the assay was 25% (37, 38).

Growth Outcomes

Growth outcomes were defined in our study as (a) change in WAZ between birth and final visit b) WAZ at final visit. Children with Z scores below −2 were defined as underweight (WAZ ≤ −2) (36).

Statistical Analysis

The sample size was based on a sample of convenience for a pilot study of 100 mother-infant pairs as there are no published studies of expected cytokine values in milk and their association with growth outcomes on which to base our calculations. Descriptive statistics were calculated for maternal and infant demographics, infant growth parameters and cytokines in colostrum and mature BM. Shapiro-Wilk test was used to assess the normality of the distribution of cytokines. Because all measurements deviated significantly from normality, non-parametric tests were used in further analysis. Potential differences and associations between cytokines in colostrum and mature BM were calculated using Wilcoxon rank-sum and Spearman rank correlation tests, respectively. Differences in WAZ scores between male and female infants were calculated using Wilcoxon rank sum tests. All other univariate analysis was performed using simple linear regression on log transformed data. Multivariate linear regression (including stepwise regression) analysis was then used to determine the ability of cytokine concentrations to predict longitudinal growth outcomes in the first 2–3 months post-partum by analyzing the association between (i) colostrum cytokines and change in WAZ between birth and final visit (ii) BM cytokines and WAZ at final visit. Potential maternal and infant confounders found to be significant in variable univariate analysis with a $p < 0.1$ or known to be associated with infant growth (*a priori*) were included in the multivariate models. Maternal hemoglobin (Hb) was categorized as either Hb <11 g/dL (anemia) or Hb ≥11 g/dL (39). Maternal illness was categorized as either having none or ≥ 1 self-reported acutely unwell episodes recorded during pregnancy, regardless of severity or outcome. Maternal gravida was categorized as either nil or ≥1 previous pregnancies, regardless of outcome. Maternal parity was defined as either nil or ≥1 previous pregnancies carried to a viable gestational age.

All statistical analyses were completed using the statistical packages STATA version 12 (StataCorp 2013, California CA, United States), GraphPad Prism version 6.0 (GraphPad Softward Inc., La Jolla, CA, United States) and R package version 3.4.1. Differences were considered statistically significant at $p < 0.05$.

RESULTS

Maternal and Infant Characteristics

A total of 100 mother-infant pairs were included in the analysis. The mean ± 1 SD maternal age was 25.6 ± 5.7 years and mean ± 1 SD maternal weight at delivery was 63.4 ± 9.8 kg. The infants were born at mean gestational age of 39 weeks and followed up to a mean ± 1 SD of 61.6 ± 1.3 days postpartum; although scheduling allowed for final visits to occur up to 90 days postpartum, all final visits in this cohort took place between 60 and 66 days postpartum. All infants were exclusively breast fed throughout the duration of the study. Demographic characteristics of mother-infant pairs are summarized in **Table 1**.

Infants were born with a mean weight ± 1 SD of 3.36 ± 0.48 kg and WAZ ± 1 SD of 0.10 ± 0.99 (**Table 2A**). However, substantial growth faltering was seen across the first 2–3 months postpartum, equivalent to crossing over one major centile band (**Figure 2**). Of the 94/100 infants remaining in the study at the final visit, infants' WAZ had declined to a mean ± 1 SD of −0.59 ± 1.20; 71% of infants demonstrated a decline in their WAZ and 15% were underweight by 2–3 months postpartum (WAZ<-2) (**Tables 2A,B**). The difference between male and female infant WAZ scores was non-significant at birth ($p = 0.505$) and final visit ($p = 0.791$). Similarly, the odds ratio of being underweight and having a decline in WAZ by 2–3 months postpartum did not differ significantly between the sexes: for male infants, the odds ratio was 1.4 (CI 0.45–4.41, $p = 0.56$) and 1.69 (CI 0.68–4.1775, $p = 0.25$), respectively.

Cytokines in Colostrum and Breast Milk

83 samples of colostrum (from 100 women) and 90 samples of mature breast milk (from 94 women) were successfully collected. In the remaining cases, insufficient sample was available, either because the mother was unable to express milk, or due to insufficient sample remaining after the primary analysis was complete. Colostrum at birth contained significantly

TABLE 1 | Description of study population.

Variable	Value
Maternal age, years (mean ± SD)	25.6 ± 5.7
Maternal weight, kg (mean ± SD)	63.4 ± 9.8
Gestational age at birth, weeks (mean ± SD)	39.0 ± 2.5
Length of post-partum follow-up, days (mean ± SD)	61.6 ± 1.3
MATERNAL Hb CATEGORIES, *n* (%)	
Hb ≥ 11g/dL	33 (40)
Hb < 11g/dL	49 (60)
MATERNAL ILLNESS CATEGORIES, *n* (%)	
Illness during pregnancy	16 (18)
No illness during pregnancy	73 (82)
PARITY CATEGORIES, *n* (%)	
Primiparous	21 (21)
Multiparous	79 (79)
GESTATIONAL AGE CATEGORIES, *n* (%)	
≥37 weeks	76 (76)
< 37 weeks	24 (24)
INFANT SEX, *n* (%)	
Male	50 (50)
Female	50 (50)

Values are rounded to 1 decimal place.
Missing values: maternal weight =12, maternal Hb = 18, maternal illness = 11.
SD, standard deviation; Hb, hemoglobin.

TABLE 2 | Summary of infant growth data at birth and final visit.

(A) INFANT WEIGHT AND WAZ SCORES		
Infant growth indicator, mean ± SD	**Birth**	**Final visit**
Weight (kg)		
Male	3.45 ± 0.43	5.24 ± 0.68
Female	3.27 ± 0.42	4.84 ± 0.74
All	3.36 ± 0.48	5.04 ± 0.74
WAZ score		
Male	0.18 ± 0.85	−0.57 ± 1.05
Female	0.02 ± 1.12	−0.61 ± 1.34
All	0.10 ± 0.99	−0.59 ± 1.20
(B) INFANT WAZ CATEGORIES		
WAZ categories, n (%)	**Birth**	**Final visit**
≤ −3		
Boys	0	0 (0)
Girls	0	3 (100)
All	0	3
−3 < WAZ ≤ −2		
Boys	0	8 (72)
Girls	0	3 (27)
All	0	11
> −2		
Boys	49 (51)	39 (49)
Girls	48 (49)	41 (51)
All	97	80

All values are rounded to 2 decimal places; WAZ, Weight-for-age Z score; SD, standard deviation.

higher concentrations of nearly all cytokines than BM ($p < 0.001$) (**Supplementary Table 2**). Furthermore, the percentage of samples with detectable concentrations was significantly greater for the majority of cytokines in colostrum when compared to BM ($p < 0.05$), with the exception of TNFα, IGF1, TGFβ2, and IL6 (the latter was almost significant, $p = 0.06$). Of note, the levels of IGF1 in colostrum and BM were below the assay limit of detection in >75% of subjects and, therefore, this data has been removed from further analysis (**Supplementary Table 2**). There were minimal significant associations between the levels of cytokines in colostrum and in mature milk, with only a weak positive correlation found with IL12 ($r = 0.257$, $p = 0.023$) (**Supplementary Table 3**).

Associations Between Maternal/Infant Factors and Infant Growth Outcomes

Only maternal illness and infant gestational age at delivery were found to be significant or near significant predictors of change in WAZ between birth and final visit, in univariate analysis ($p < 0.05$) (**Table 3**). There were no significant maternal or infant predictors of WAZ at final visit.

Associations Between Cytokines and Infant Growth Outcomes

The association between colostrum cytokines and change in WAZ between birth and final visit was non-significant in both the unadjusted and adjusted models in this Gambian cohort.

Moreover, unadjusted models demonstrated that lower TNFα, IFN-γ, IL1β, IL2, IL4, and IL6 levels in BM were weakly associated with a higher WAZ at final visit ($p < 0.05$). Although significant, the β -coefficients were small. When these models were adjusted for maternal anemia, only TNFα and IL6 remained significant predictors of WAZ at final visit ($p < 0.05$) (**Table 4**). Other multivariate models were non-significant.

DISCUSSION

In many sub-Saharan African countries, infants are small at birth, show catch-up growth initially, but then rapidly demonstrate reduced growth velocity, leading to substantial growth faltering that becomes maximal by their second year of life (30, 33). Our findings show a similar pattern with clear growth faltering across the first 2–3 months postpartum. Identifying the factors that contribute to this pattern of growth—despite optimal feeding practices—is important, as developing interventions to optimize BM bioactive components could reduce the risk of poor growth and morbidity in early infancy, particularly in low-income settings (10, 13, 30, 31, 40). Using data from a cohort of mother infant pairs from urban Gambia, we have shown that variations in BM cytokine levels do not play a substantial role in the growth faltering observed across early infancy.

To date, there has been a paucity of studies in the literature reporting on cytokines and growth factors in BM and their role in

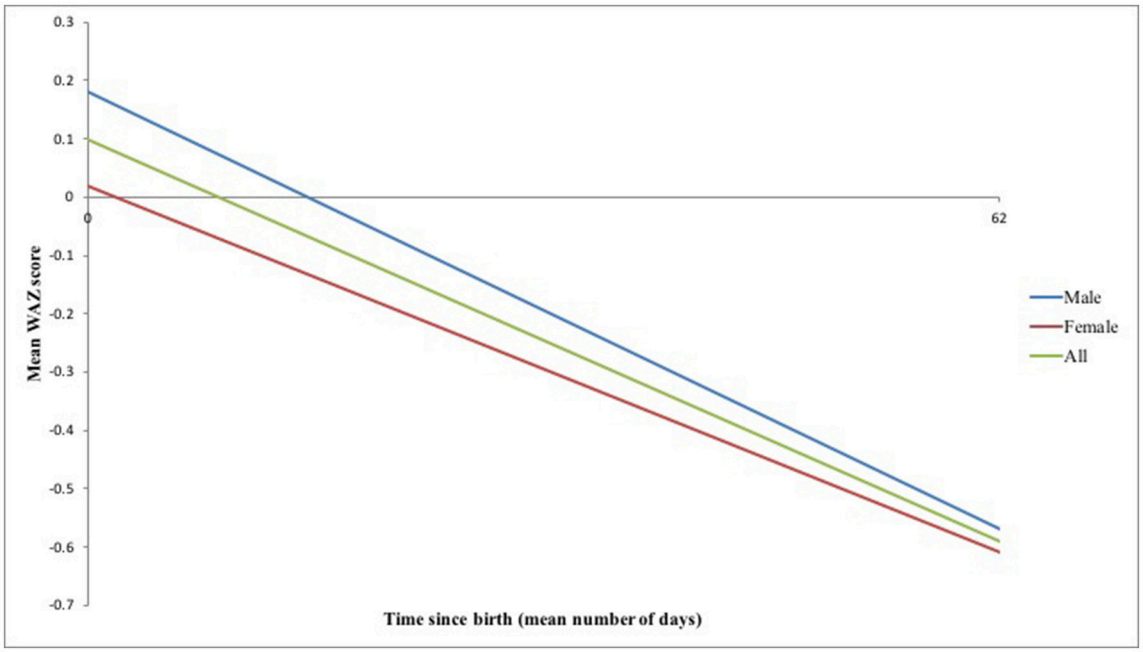

FIGURE 2 | Mean WAZ scores across the first 2–3 months postpartum.

TABLE 3 | Relationship between maternal/infant factors and difference in WAZ at birth and final visit.

Maternal/infant factor	*β*	*p-value*
Maternal illness	0.646	0.064
Gestation	0.095	0.077
Maternal age	0.018	0.423
Maternal weight	0.003	0.840
Maternal Hb	−0.200	0.449
Gravida	0.082	0.276
Parity	0.085	0.273
Infant sex	−0.388	0.140

All values are rounded to 3 decimal places.
Linear regression β coefficients for maternal/infant factors predicting difference in WAZ at birth and final visit.
WAZ, Weight-for-age Z score; Hb, hemoglobin.

growth and development in the early postnatal period. Previous studies have primarily focussed on weight gain, exploring the relationship between BM factors, particularly hormones, and infant adiposity and risk of obesity (13, 41–47). To our knowledge, we are the first to examine the relationship between BM cytokine profile and longitudinal growth outcomes, specifically WAZ, in the context of early growth faltering in a low-income setting.

The protective effects of bioactive factors in BM on infant health and developmental outcomes are thought to be mediated by immunomodulatory processes. More specifically, cytokines may play a role in enhancing sIgA production by neonatal leucocytes (e.g., TNFα, TGFβ2, IL6, IL10) (26, 48); in promoting infant gut maturity and barrier function (e.g., EGF, IGF1, TGFβ2) (23, 28, 40, 49); in boosting resistance to pathogen colonization (38); and in dampening inflammatory responses (e.g., IL2, IL10, TGFβ2) (6, 29, 48, 49). However, the exact mechanisms underlying growth patterns remain speculative. One hypothesis, proposed in the context of human milk oligosaccharides (HMOs), is that milk composition may shift toward a more protective profile, associated with a lower risk of infection and inflammation, thereby enabling the infant to invest energy in growth (7, 9, 10).

A cross-sectional preliminary study by Fields et al. suggested that BM concentrations of cytokines (IL6, TNF-α) and hormones/growth factors (insulin, glucose and leptin) may differentially influence weight gain and the development of fat and lean body mass in infants in the early postpartum period. WHO indicators of infant growth (Z scores), however, were not calculated or applied in their analysis (47). By contrast, our study focussed on an important WHO indicator of growth, WAZ, in the context of growth faltering; in our Gambian cohort, there was a decline in WAZ in over two-thirds of infants with 15% becoming underweight by 2–3 months postpartum, despite no infant being underweight at birth. Moreover, there was no significant correlation between colostrum cytokine levels and change in WAZ across the first 2–3 months post-partum, both in the adjusted and unadjusted models. The interrelationship of other colostrum factors, such as micronutrients or other bioactive analytes, including HMOs and hormones (adiponectin, insulin, ghrelin and leptin), may play a bigger role in variable growth outcomes and is currently under investigation (9–11, 13, 41–47, 50).

On the other hand, our findings suggest that cytokines in mature BM may predict infant growth measured at that same

TABLE 4 | Relationship between breast milk cytokine concentration and WAZ at final visit.

	Unadjusted analysis				Adjusted analysis*			
Breast milk cytokine	***β***	**95% CI**	***R^2***	***p-value***	***β***	**95% CI**	***R^2***	***p-value***
IlIβ	< −0.01	−0.003 to −0.001	0.10	< 0.01	< −0.01	−0.001 to 0.001	0.02	0.81
IL2	−0.01	−0.167 to −0.028	0.09	< 0.01	−0.02	−0.076 to 0.070	0.02	0.94
IL4	−0.48	−0.969 to 0.007	0.04	0.05	0.095	−0.401 to 0.592	0.02	0.70
IL6	< −0.01	−0.002 to 0.0003	0.09	< 0.01	< −0.01	−0.002 to 0.000	0.17	< 0.01
IL-10	−0.02	−0.058 to 0.013	0.02	0.21	0.01	−0.026 to 0.043	0.02	0.61
IL-12	−0.06	−0.188 to 0.065	0.01	0.34	0.02	−0.103 to 0.151	0.02	0.70
IL-13	−0.02	−0.066 to 0.019	0.01	0.28	0.02	−0.026 to 0.060	0.02	0.43
IFN-γ	−0.02	−0.025 to −0.005	0.09	< 0.01	< −0.01	−0.011 to 0.010	0.02	0.90
TNFα	< −0.01	−0.004 to −0.001	0.09	< 0.01	< −0.01	−0.004 to-0.001	0.17	< 0.01

*Unadjusted and adjusted linear regression β coefficients for breast milk cytokines predicting WAZ at final visit (*adjusted for maternal anemia).*
CI, Confidence interval; WAZ, Weight-for-age Z score; IL, interleukin; IFN, interferon; TNF, tumor necrosis factor; IGF, insulin-like growth factor.

point in time. Higher levels of specific pro-inflammatory BM cytokines were negatively associated with WAZ at the final visit in our cohort, although the relationship was weak and clinical significance remains as yet unclear. The relationship between a pro-inflammatory environment and poor weight gain, both in the fetus and infant, has been previously described (46, 51–54). More specifically, the importance of TNFα and IL6 to early infant growth has already been suggested by Fields et al. (47). Higher concentrations of BM IL-6 were significantly associated with lower relative weight, weight gain, percent fat, and fat mass in healthy term infants at 1 month of age; higher TNF-α significantly correlated with lower lean mass but not measures of adiposity (47). A subsequent longitudinal study by the same group, however, could not replicate these findings, demonstrating only non-significant associations with infant body composition (46).

Cytokines in mature BM may impact infant growth through immunomodulation, as discussed earlier. Equally, these associations may represent context-specific correlations, rather than causation. For example, the cytokine profile in BM may reflect maternal health at this specific time point, including nutritional and disease status, which in turn may play a key role in growth outcomes. Further elucidation of factors that shape mature BM cytokine profile and understanding of underlying mechanistic or causational processes is therefore needed. This may have translational implications, such as the identification of maternal groups with high risk of underweight infants, and initiation of appropriate interventions subsequently.

Our study has several limitations. Firstly, it is a pilot exploration of outcomes in a small maternal-infant cohort within a limited time period. Given that (a) growth faltering is thought to start in the first few months of life, reaching its peak by 2 years of age in poorly-resourced settings and (b) cytokine profiles vary within and between mothers, it would be worth analyzing BM composition and growth outcomes in a larger cohort, at multiple time points and beyond 3 months postpartum. This would enable a more accurate WAZ trajectory to be plotted and the longer-term impact of BM cytokine differences to be fully appreciated. Of note, infants are thought to demonstrate a non-linear weight gain over this early post-partum period, and so weight at the final time point could either reflect a change in recent infant mass/weight or infant size more globally. Moreover, analysis of different types of BM and at various time points not evaluated in our study (e.g., foremilk vs. hindmilk; timing of sample collection in relation to feeding, lactational stage, or seasonal changes) may be valuable and has previously been shown to be important (4, 12, 15, 16, 55).

Furthermore, our focus was on one primary growth outcome: weight and associated WAZ scores. The latter is a well-established WHO growth indicator, especially at birth (when length measures may be difficult) and throughout the early postnatal period. Weight-for-length (WLZ) and length-for-age (LAZ) Z scores are equally important beyond the first year of life; stunting in early childhood has been shown to predict future risk of morbidity and mortality (32, 56) although is unlikely in the first 90 days post-partum. Similarly, head-circumference-for-age Z score (HAZ) is thought to be a good indicator of more chronic poor growth and undernutrition (57–59). Other potential growth outcomes to be measured include body composition factors (e.g., lean and fat mass, skinfold thickness), currently being explored particularly in BM hormone and obesity studies (11, 13, 41, 60).

In addition, our study eligibility criteria were strict, for example, infants born very prematurely or in poor condition requiring resuscitation (with subsequent transfer to neonatal intensive care) or women with high risk conditions in pregnancy were excluded. Future studies focussing on these high-risk groups may provide further insight into variables that regulate BM profile and, subsequently, boost or protect against growth faltering.

Finally, our study did not measure the contribution to early postpartum growth of other sources of immune factors, including the ante-and perinatal environment (cord blood, maternal non-milk derived cytokines) and endogenous postnatal production (infant serum). In parallel, the interplay of other bioactive factors, such as hormones and human milk oligosaccharides, must be explored.

In conclusion, our findings suggest that the interactions of cytokines in both colostrum and BM do not play a substantial role in the early growth faltering observed in Gambian infants. Above all, this preliminary study is valuable as a strong foundation

to direct further investigation into the impact of bioactive factors in BM on infant growth and development, a topic with limited studies to date. Improved understanding of the early postnatal determinants of growth may be important for developing successful interventions to boost growth and health outcomes, particularly in infants at highest risk or in poorly resourced settings.

ETHICS STATEMENT

All subjects gave informed consent in accordance with the Declaration of Helsinki. Each participant signed an informed consent form or, in case of illiteracy, thumb-printed and the consent form was signed by an impartial witness. The study was approved by and carried out in accordance with the recommendations of the joint Gambian Government/Medical Research Council Research Ethics Committee, SCC1350V4.

AUTHOR CONTRIBUTIONS

AS was responsible for manuscript writing and had input into the data analysis. DM, SM, and KL had input into the manuscript writing. AF was involved in laboratory analysis. OB was responsible for statistical analysis. KL was responsible for the original study design, data and statistical analysis and original idea for manuscript. All authors had access to the data and input into the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fped.2018.00414/full#supplementary-material

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REVIEW
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Compositional Dynamics of the Milk Fat Globule and Its Role in Infant Development

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Human milk is uniquely optimized for the needs of the developing infant. Its composition is complex and dynamic, driven primarily by maternal genetics, and to a lesser extent by diet and environment. One important component that is gaining attention is the milk fat globule (MFG). The MFG is composed of a triglyceride-rich core surrounded by a tri-layer membrane, also known as the milk fat globule membrane (MFGM) that originates from mammary gland epithelia. The MFGM is enriched with glycerophospholipids, sphingolipids, cholesterol, and proteins, some of which are glycosylated, and are known to exert numerous biological roles. Mounting evidence suggests that the structure of the MFG and bioactive components of the MFGM may benefit the infant by aiding in the structural and functional maturation of the gut through the provision of essential nutrients and/or regulating various cellular events during infant growth and immune education. Further, antimicrobial peptides and surface carbohydrate moieties surrounding the MFG might have a pivotal role in shaping gut microbial populations, which in turn may promote protection against immune and inflammatory diseases early in life. This review seeks to: (1) understand the components of the MFG, as well as maternal factors including genetic and lifestyle factors that influence its characteristics; (2) examine the potential role of this milk component on the intestinal immune system; and (3) delineate the mechanistic roles of the MFG in infant intestinal maturation and establishment of the microbiota in the alimentary canal.

Keywords: milk fat globule, milk fat globule membrane, infant development, gut maturation, microbiota, immune system

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INTRODUCTION

Human milk has evolved to meet the unique requirements for infant growth and development and should be the sole source of nutrients for the developing infant during the first 6 months of life (1, 2). One component of milk is the milk fat globule (MFG), which is difficult to mimic in infant formulas due to its highly complex structure and variable composition. Beyond the traditional role of milk fat as a source of nutrients, which provides up to 50% of the total calories in milk, the functional importance of the MFG structure and composition on infant development is of increasing interest.

Biosynthesis of the MFG is energetically costly. Formation begins with the packaging of triacylglycerols (TAGs) into micro-lipid droplets that bud from the endoplasmic reticulum of mammary gland alveolar epithelial cells to form cytoplasmic lipid droplets (CLD) surrounded by a phospholipid monolayer. Migration of the CLD to the apical pole of the epithelial cell results in fusion with the plasma membrane and the addition of a peripheral bilayer that contains a variety of bioactive proteins and lipids (3). The fully-fledged MFG covered by the membrane (MFGM) is then secreted outside the cell to become part of the milk that provides nourishment for the infant (3, 4).

MFG composition varies considerably among individuals and is dynamic over the course of lactation, but also varies over a single breastfeed (5). These variations reflect maternal factors including diet, environment, maternal genetics, and body composition, as well as the changing needs of the infant over the period of lactation (**Figure 1**). Recent research has shown a protective effect of MFGM against infectious diseases (6, 7), in part through the modulation of the intestinal immune response and the gut microbiota (8–10). Although the underlying mechanism is not entirely clear, the MFGM harbors two forms of glycoconjugates (glycoproteins and glycolipids), which are thought to have antimicrobial, anti-inflammatory, and prebiotic functions in the gut (11, 12). These functions may be responsible, in part, for the aforementioned modulation of immune response and microbiota.

The focus of this review is on the role maternal and environmental factors play in mediating MFG lipid and protein composition, along with inter-species differences (e.g., bovine and human), and the biological significance of the human MFG in the infant intestine, with particular attention given to structural and immune system development. The importance of this component of milk on infant health outcomes as well as identified gaps in the research literature that have been under-explored are also discussed.

COMPOSITION OF MILK FAT GLOBULE (MFG)

MFG Lipids

Structural Components

MFGs are heterogeneous structures, varying in diameter, triglyceride content, and membrane and fatty acid composition (13–15). The diameter of the MFG varies between 0.2 and 15 μm, and its composition is observed to vary by size, adding more complexity to the study of their structure and function. Progress in lipids analysis has been slower than for other biomolecules, such as proteins and metabolites. This may be due partly to unsophisticated instruments, which inadequately capture the complexity of lipids, and partly to the limited amount of information that can be gleaned from genomic studies since lipid fingerprints are not directly linked to the genome (4). The core of the MFG is composed primarily of TAGs, which represent 98% of total milk fat and provides approximately half of the infant's energy intake in addition to essential fatty acids required for growth and development (16).

Milk fat contains over 400 different fatty acids, among which 15 constitute 90% of the total fatty acid pool (17). In mature human milk, the majority of TAGs in the MFG core consist of 18:1(n-9) oleic (20–35%), 16:0 palmitic (18–23%), and 18:2(n-6) linoleic (LA) (8–18%) acids **(Table 1)** (46). Medium chain fatty acids (MCFAs) comprise 12% of total fatty acids, and < 1% are short chain fatty acids (SCFAs) (49). Long chain polyunsaturated fatty acids (LC-PUFA), notably 20:4(n-6) arachidonic (ARA), 20:5(n-3) eicosapentaenoic (EPA), and 22:6(n-3) docosahexaenoic (DHA) acids, as well as one of the two essential fatty acids, 18:3(n-3) α-linolenic acid (ALA) are some of the least abundant, although a wide inter-individual variation exists that is dependent on maternal diet and genetics (**Table 1)** (50). The location of fatty acids on the glycerol backbone is highly conserved within species, but not between species, with saturated fatty acids typically occupying the *sn*-2 position of the TAG, and specifically palmitic acid occupying 50-60% of all fatty acids at the *sn*-2 position (defined as β-16:0) in human milk (51–53). The *sn*-1 and *sn*-3 positions are occupied primarily by unsaturated fatty acids, of which approximately half is oleic acid (54).

Surrounding the TAG core is the milk fat globule membrane (MFGM), which is derived from the mammary gland epithelium (55). The MFGM is a complex mixture of 60% proteins and 40% lipids (56) and functions to stabilize the globule as an emulsion. The major building components of the MFGM are the membrane phospholipids, i.e., glycerophospholipids (glycerol-based phospholipids), which are comprised of phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylserine (PS) and phosphatidylinositol (PI) (45), and sphingolipids, notably sphingomyelin (SM) (57). SM, the predominate sphingolipid in MFGM, is present in much higher quantity in human milk compared to milk from other species (e.g., bovine milk) (47). In general, PE, PC and SM are the most abundant phospholipids in the MFGM, while PS and PI are relatively minor components, although inter-individual variation does exist (47) (**Table 1**).

The MFGM exists as a polymorphic lipid phase with lipid-disordered domains rich in glycerophospholipids that are fluidic and lipid-ordered domains that are more rigid at body temperature (58). The lipid-ordered domains in the MFGM are called lipid rafts because cholesterol and sphingolipids interact to form circular assemblies in the outer leaflet (43). Lipid rafts, which contain approximately 80% of total milk cholesterol, play an important role in maintaining membrane structure (43), and are critical for many biological processes including compartmentalizing membrane proteins to modulate their functions (44).

Different lipid classes in the MFG exhibit distinct fatty acid profiles, with the MFGM phospholipids containing more unsaturated fatty acids than the MFG core (43). Although phospholipids in the MFGM only represent 0.5–1% of the total fat in milk, 15–20% of the total LC-PUFA (e.g., DHA and ARA) in milk is present in the MFGM phospholipids (59, 60). In contrast to MFGM phospholipids, MFGM sphingolipids are highly saturated, and are thought to maintain the lipid raft

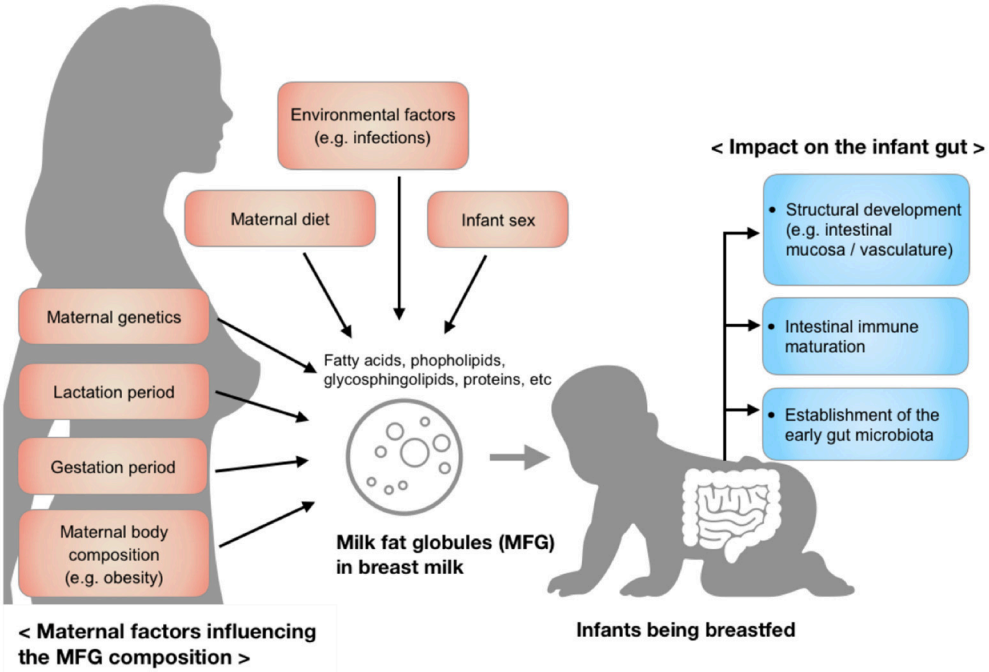

FIGURE 1 | Factors influencing the composition of milk fat globules in breast milk and the impact of those components on the infant gut during early development.

structure due to their tightly packed structure and higher melting temperature. This structure may be important in digestion, allowing for delivery of sphingosine and ceramides to the distal gut (57). The varied digestion and absorption kinetics of the MFG may be physiologically important for the infant. To date, most research has focused on understanding and modulating the overall fatty acid composition and lipid content in milk rather than studying structural function.

Glycosphingolipids (sphingolipids with a carbohydrate moiety) such as cerebrosides and gangliosides are present at low abundance (61, 62). Gangliosides are glycosphingolipids with one or more sialic acid residues, and are classified according to the number of sialic-acid residues on the molecular backbone (M = mono- or 1; D = di- or 2, as GM or GD), the number of residues attached to the sugar moiety, and the biosynthetic pathway from where they are derived (63). Human milk contains a much higher concentration of gangliosides than bovine milk (64). Supplementation of infant formula with a ganglioside-enriched dairy fraction has shown beneficial impacts on cognitive development in infants aged 0–6 months (35).

The size of the MFG is related to the TAG/phospholipid ratio, fatty acid composition (14), and cholesterol content (43). Argov et al. (65) demonstrated that smaller fat globules tend to have more phospholipids (65) that may partly result from a biosynthetic balance between phospholipids and neutral lipids coordinated in the milk-secreting epithelial cells (4). Independent of cellular TAG content, increasing intracellular phosphatidylethanolamine content has been shown to facilitate fusion between lipid droplets and hence increase the size of the MFG (66). It is also thought that the type of esterified fatty acids in the MFG core and membrane lipids contributes to globule size (67), as a higher content of LC-PUFA and medium chain fatty acids (MCFA) was observed in small fat globules in bovine milk (65, 68). Differences in digestion and fat release patterns between smaller and larger fat globules have also been described (69, 70), suggesting that varying sizes of the MFG may have distinct physiological effects. Interestingly, the mean diameter of human MFG appears to be largest in colostrum, followed by mature milk, and the smallest in transitional milk (71). Nano-sized particles termed lactosomes that are devoid of triglycerides and gangliosides but rich in phospholipids have been identified and isolated at a density equivalent to high-density lipoproteins (HDL) (higher density than native MFGs), which suggests that they may also have biological functions (65, 72).

Maternal Factors Influencing MFG Lipids

a. Lactation Period

The composition of milk fat is dynamic over the course of lactation and adapts to changes in the maternal environment, diet and physiological state. While fatty acids with 4 to 14 carbons can be made from *de novo* synthesis in the mammary gland, the 16 carbon fatty acids are derived either from circulation, body stores, or diet (73). As milk matures, the average fatty acid chain length decreases because the mammary gland increases its capacity to produce MCFAs (12-14 carbons) (74). The overall LCFA content remains similar throughout lactation, with the exception of stearic acid, which is higher in colostrum (74); however, wide variations among different populations exist likely due to dietary differences (75, 76). As lactation proceeds, TAG concentrations tend

TABLE 1 | Lipid composition in milk fat globules.

Fatty acids[a]	**% total fatty acids**	**Biological significance**
Palmitic acid (16:0)	18–23	Energy metabolism (18); used in the synthesis of other bioactive lipids (18)
Oleic acid (18:1)	20–35	Energy storage and metabolism (19); alters cell membrane fluidity (13)
Linoleic acid (n-6 18:2)	8–18	Skin barrier function (20); precursor to ARA; competes with n-3 fatty acid metabolism (21)
Linolenic acid (n-3 18:3)	0.43–1.33	Precursor to EPA and DHA (22)
Arachidonic acid (n-6 20:4)	0.36–0.49	Eicosanoid synthesis (23); neurodevelopment (24)
Eicosapentaenoic acid (n-3 20:5)	0.07–0.26	Precursor to eicosanoids (23); immune function (23)
Docosahexaenoic acid (n-3 22:6)	0.17–0.99	Cell signaling; neurodevelopment and vision (25)
Phospholipids[b]	**% of total phospholipids**	**Biological significance**
Phosphatidylinositol	4.6	Cell signaling; activation of Akt (26)
Phosphatidylcholine	25.2	Membrane structure; lipoprotein assembly and secretion (27)
Phosphatidylserine	5.9	Induction of apoptosis (28); carrier of DHA (29)
Phosphatidylethanolamine	28.6	Component of phospholipase D (30); cell proliferation and differentiation by regulation of pathways including MAPK and NF-kB (31)
Sphingomyelin	35.7	Metabolized to ceramide and sphingosine (32); vascular development (33); immune function (34)
Gangliosides[c]	14.8–26.8 mg/L in human milk	Cognitive development (35); altering membrane fluidity and function of enterocytes (36–39); cell-cell communication (40); gut maturation and immunity (41, 42)
Cholesterol[d]	90–150 mg/L in human milk	Structural maintenance of membranes (43); compartmentalization of membrane proteins to modulate functions (44); substrate for bile acids, vitamin D, hormones and oxysterols (45)

[a] *Fatty acid content represents mature milk collected in nine countries (46).*
[b] *Polar lipids were quantified using HPLC-ELSD (47).*
[c] *Total ganglioside content represent Malaysian mother's milk quantified using HPLC-MS (48).*
[d] *Total cholesterol content adapted from Koletzko (45).*
ARA, Arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

to increase for the first few weeks, whereas cholesterol and cholesterol esters gradually decrease (74). Although the concentration of sphingomyelin in human milk appears to remain constant, colostrum is observed to contain more total phospholipids (75, 77) and LC-PUFAs relative to transitional and mature milk (71, 77). Yet, in most studies, including those referenced above, fatty acids in the MFG core and those in the MFGM lipids have not been separately analyzed despite the reported differences in the two fractions (59, 60).

Levels of total gangliosides in human milk appear to be highest in colostrum (48). While the GD3 ganglioside is the predominant form in human colostrum, a shift toward GM3 predominance is observed in mature milk (64). Gangliosides contain significantly more LCFA and less MCFA in colostrum compared to mature milk (which is similar to the overall fatty acid trend in milk), as well as more monounsaturated fatty acids and less LC-PUFA (78). Distinct fatty acid esterification profiles have also been reported for human compared with bovine gangliosides (e.g., higher amounts of LCFAs longer than 20 carbons in bovine gangliosides) (79). Whether these differences translate into different health outcomes remains to be investigated.

b. Genetic Factors

Several maternal factors influence the lipid profile of human milk, and maternal genotype is a strong determinant. Within mammary epithelial cells, fatty acids activated by acyl-CoA synthase undergo a number of enzymatic reactions to produce other fatty acids, TAG, and phospholipids (4). Some of the most studied genes involved in milk lipid synthesis are those involved with the synthesis of LC-PUFA, likely due to the implication of LC-PUFA in immune responses and cognitive development in infants. These genes include fatty acid desaturase (*FADS*) genes (80–82) as well as members of the *ELOVL* family of genes that encode elongase enzymes (82, 83). However, fewer studies on maternal genetics regulating levels of phospholipid classes in the MFGM have been published. One study revealed that a polymorphism in diacylglycerol acyltransferase 1 (*DGAT1*) was associated with varying compositions of phospholipids and phospholipid/TAG ratios in bovine milk (84). Various enzymes are involved milk lipid synthesis and transport processes (4, 66, 73), which leaves considerable scope for further research on the genetic variants regulating milk lipid composition.

c. Diet

Many studies have focused on the effect of maternal diet or supplementation on milk fatty acids, and in particular LC-PUFA (16, 45, 85, 86), but how those influences are reflected in the MFG core and membrane lipids have not been clear. Milk ganglioside and phospholipid concentrations have been reported to differ by maternal geographic region within China, suggesting that diet may influence their amounts in human milk (75). Additionally, maternal socioeconomic

and nutritional status have been shown to be associated with variations in total lipid and phospholipid content (87). Supplementation with LC-PUFA was also shown to increase the concentrations of total phospholipids (+18%) and sphingomyelin (+30%), as well as alter phospholipid composition of milk (59). Total choline intake, including choline supplements, have been shown to be positively correlated with breast milk phosphatidylcholine, especially at lower maternal intakes (88).

d. Gestation
Infant sex may influence milk lipid composition. Milk from mothers who gave birth to boys appeared to have higher concentrations of SM, PC, PE, and PI relative to milk from mothers who gave birth to girls (89). Associations between milk fatty acids and infection (in mother, infants, or both) were observed (90, 91), but no studies have reported such associations with milk polar lipids. Further studies into the mechanisms behind the observed relationships are warranted.

The composition of breast milk lipids appears to shift depending on whether an infant is born full-term or prematurely, including total fat (92, 93), DHA (94) and MCFA content (95). Although phospholipid composition was reported to be comparable between term and preterm milk throughout lactation (61), one study in Japan showed higher SM and lower PC in preterm mature milk relative to term mature milk (96).

MFGM Proteins

Structural Components

The proteins of the MFG are located within the MFGM, and account for 1-4% of the total protein fraction in milk, and 1% of the total globule mass (97). Much attention has been given to the MFGM proteins, which have been extensively explored using proteomics. To date, approximately 500 proteins have been identified in human milk (98), some of which have been well characterized and include the glycosylated butyrophilin, mucins, xanthine oxidoreductase, lactadherin, CD proteins, the non-glycosylated adipophilin, and fatty acid binding proteins (**Table 2**). These proteins are the major proteins observed across all mammalian species, which suggests important biological functions (97, 98, 107). Interestingly, the quantities of those proteins greatly vary between species (101).

Human MFGM proteins were first separated by 2-demensional electrophoresis in 1997 (108) and besides the common MFGM proteins (mentioned above), the following proteins have been frequently identified in human MFGM in several proteomics studies: α-lactalbumin, lysozyme, β-casein, clusterin, lactoferrin, Immunoglobulins (e.g., IgA α-chain), tenascin, apolipoproteins (e.g., type A-I) and fatty acid synthase (1, 97, 108, 109) (**Table 3**).

BTNs are members of the immunoglobulin (Ig) superfamily, and it is the butyrophilin subfamily 1 member A1 (BTN1A1) that has been shown to be associated with human MFGM (97). The structures of the BTNs and their functions have been reviewed elsewhere (118). Another abundant class of proteins in human MFGM is the mucins (MUC), of which Mucin 1 (MUC1) and MUC4 are the most abundant. MUC proteins have highly glycosylated extracellular domains, which makes them resistant to digestion (119), and potentially available to act as decoys for pathogens (described below).

Xanthine oxidoreductase (XOR) is another major protein (120) with a critical role in milk fat secretion. XOR aids with the fusion of the apical plasma membrane onto the fat globules through structural interactions with BTN and adipophilin (ADPH) as a tripartite structure (121). XOR is a highly conserved molybdoenzyme that oxidizes a wide range of substrates (generally with low specificity) including purine nucleotides and has been suggested as an antibacterial component in the MFGM (122). Loss of XOR has been shown to result in less efficient milk fat secretion in mice (123).

Other glycosylated MFGM proteins include milk fat globule-epidermal growth factor 8 protein (MFG-E8; PAS VI/VII), also known as lactadherin (124). Human lactadherin was first identified in the MFGM as well as in the lactating mammary gland but was recently also found in other tissues such as the endometrial epithelium (125). It has been implicated in the autoimmune disease systemic lupus erythematosus (126), and may have a role in sepsis (127).

The well-described non-glycosylated MFGM proteins include adipophilin (ADPH), and fatty acid binding proteins (FABP). ADPH (also known as perilipin 2) is in the perilipin family of proteins that regulate lipolysis by controlling the access of proteins to the lipid droplet surface (128). A recent study using homology modeling suggested that the ADPH C-terminus forms a four-helix bundle motif which aids in formation of a stable membrane bilayer during lipid secretion (shown in mice and *in vitro*) (129). FABP is involved in the intracellular transport of fatty acids, which is a critical step in the synthesis of MFG lipid constituents such as TAG and phospholipids (4).

Compared to the aforementioned major MFGM proteins, hundreds of other proteins with lower abundance exist, which include the glycosylated enzymes carbonic anhydrase, milk alkaline phosphatase (AP), lactoferrin, osteopontin (OPN) and lysozyme (109, 130, 131), while the last three are present primarily in milk whey and to a lesser extent in the MFGM. Carbonic anhydrase, also present in saliva, serum and tissues, has a few proposed functions: acid neutralizer, antibacterial agent (130) and growth factor (132), yet clinical significance as a milk component has not been established. Milk AP is an enzyme derived from the membrane of the mammary gland epithelial cell and is covalently bound to phosphatidylinositol of the MFGM (133). This same enzyme was shown to be expressed in human liver, where zinc and magnesium are required for maximal activity (134). Lactoferrin, a member of transferrin family, was first identified in milk but is also found in most exocrine fluids of mammals (e.g., saliva, tears and bile) (135). Lactoferrin is a multifunctional protein and a key component in innate immunity (136, 137). It has also been shown to improve neurodevelopment in a piglet model (131). Charlwood et al. identified α-lactalbumin and β-casein in MFGM isolates (109), although incorporation of whey and casein proteins to the MFGM via sulfhydryl–disulfide interchange

TABLE 2 | Properties of major human milk fat globule membrane (MFGM) proteins.

Protein	Molecular weight (kDa)	Location in milk fraction	Function	Difference with bovine MFGM	Change over lactation	Glycosylated (Y/N)	Resistance to digestion
Butyrophilin subfamily 1 member A1 (BTN1A1)	56	MFGM	Milk fat globule secretion, immune system	Higher in human than in bovine MFGM[a]	Higher in mature MFGM than in colostrum MFGM[a,c]	Y	Rapidly digested in the infant stomach[f] but more resistant to pepsin compared to XOR[g]; well digested by trypsin and by pronase E[h]
Mucin 1 (MUC 1/PAS 0)	250-450	MFGM	Immune protection	Lower in human than in bovine MFGM, but not significant ($P > 0.05$)[b]	No significant change reported in human MFGM; but in bovine MFGM higher at d7 (7.7-fold) compare to colostrum[d]	Y	Significantly resistant to gastric digestion and may survive to the distal gut[f,i]; detected in feces of breastfed infants
Mucin 4 (MUC 4)	232	MFGM	Immune protection	Higher in human MFGM ($P < 0.05$); not detected in bovine MFGM[b]	No significant change reported[c]	Y	Not specified, but likely be resistant to digestion due to the heavy glycosylation as glycoproteins tend to be resistant to proteases relative to non-glycoproteins[j]
Xanthine oxidase (XDH/XO, XOR)	145	MFGM	Milk fat globule secretion, immune system	Lower in human than in bovine MFGM[e]; but not significant in another study ($P > 0.05$)[b]	Highest at 6 months during 12 months lactation[c]	Y	Resistant to hydrolysis by trypsin and partially attacked by pronase E[h]
Lactadherin (PAS VI/VII, MFG-E8)	43	MFGM	Immune system	Lower in human than in bovine MFGM ($P < 0.05$)[b]	No significant change reported[c]	Y	Resistant to human neonatal gastric juice digestion at pH 4 (bovine lactadherin)[i]; detected intact in gastric aspirate samples of preterm-infants[f]; resistant to hydrolysis by trypsin and partially attacked by pronase E[h]
Cluster of differentiation 14 (CD14)	40	MFGM	Immune system	Higher in human than in bovine MFGM ($P < 0.05$)[b] ; CD36 was dominant in bovine MFGM[d]	*Not specified*	Y	Resistant to pepsin[j]
Adipophilin (ADPH)	52	MFGM	Milk fat globule secretion	*Not specified*	No significant change reported in human MFGM; but in bovine MFGM 3.4-fold upregulated at day 7 compared to colostrum[d]	N	Well digested by trypsin and by pronase E[h]
Fatty-acid binding protein (FABP)	13	Whey and MFGM	Fatty acid transport, milk fat globule lipid synthesis,	Higher in human than in bovine MFGM *(no P value reported)*[e]	Higher at later lactation[c]	N	*Not specified*

[a] Yang et al. (99), [b] Hettinga et al. (1), [c] Liao et al. (97), [d] Reinhardt and Lippolis (100), [e] Yang et al. (101), [f] Peterson et al. (102), [g] Ye et al. (103), [h] Vanderghem et al. (104), [i] Chatterton et al. (105), and [j] Le et al. (106). Note that the study by [d] Reinhardt and Lippolis (100) used bovine MFGM.

reaction during the isolation could be the origin (138). OPN is present in the MFGM as a minor constituent (97). It makes a protein complex with lactoferrin *via* electrostatic interaction, potentially preventing it from being digested (139) as OPN is resistant to proteolysis by infant gastric juice *in vitro* (105). Several enzymes including a 5′-nucleotidase, an ATPase, and a nucleotide pyrophosphatase that are known to be localized in liver plasma membranes have also been identified in the MFGM (55).

Maternal Factors Influencing MFGM Proteins

a. Lactation Period

As the MFGM is derived from the mammary gland epithelial cell, mammary gland cell biology, which varies over the course of lactation, is captured in the MFGM proteome. From postpartum day 1–7, significant increases in bovine MFGM proteins related to lipid synthesis (e.g., acyl-CoA synthetase, lanosterol synthase, lysophosphatidic acid acyltransferase and FABP) and secretion (e.g., the

TABLE 3 | Properties of minor human milk fat globule membrane (MFGM) proteins.

Protein	Molecular weight (kDa)	Location in milk fraction	Function	Difference with bovine MFGM	Change over lactation	Glycosylated (Y/N)	Resistance to digestion
Carbonic anhydrase 6	35	MFGM	Acid neutralizer, antibacterial component, and growth factor	*Not specified*	No significant change reported[c], or 8-fold higher in colostrum MFGM than the MFGM in mature milk[e]	Y	*Not specified*
Milk alkaline phosphatase (AP)	86	MFGM	Immune system	*Not specified*	*Not specified*	Y	*Not specified*
Lysozyme	17	Predominantly in whey and to lesser extent in MFGM	Antibacterial component, immune system	Higher in human than in bovine MFGM ($P < 0.05$)[b]	Higher at later stages of lactation[a,c]	Y	Not detected in feces of breastfed infants[i]; resistant to pepsin, but susceptible to trypsin[j]
Lactoferrin	78	Predominantly in whey and to lesser extent in MFGM	Antibacterial component, immune system	Higher in human than in bovine MFGM ($P < 0.05$)[b]	No significant change reported[c]; however, higher in early milk than in mature milk[f]	Y	4-9 % of ingested lactoferrin detected in feces of breastfed infants[i]
Osteopontin (OPN)	41-75	Predominantly in whey and to lesser extent in MFGM	Antibacterial component, immune system	Lower in human than in bovine MFGM but not significant ($P > 0.05$)[b]	No significant change reported[a,c]; but in bovine milk, higher in early milk MFGM[d]	Y	Partially resistant to proteolysis when incubated with infant gastric juice *in vitro*[k]
α-Lactalbumin	16	Predominantly in whey and to lesser extent in MFGM	Antibacterial component, immune system	Higher in human than in bovine MFGM ($P < 0.05$)[a]	Higher in mature human MFGM than colostrum MFGM[a]; or no change reported[c].	Y	Digested in the small intestine, releasing bioactive peptides and essential amino acids[l]
Immunoglobulins (e.g., IgA α-chain C region)	37-38	Predominantly in whey and to lesser extent in MFGM	Antibacterial component, immune system	A wider range of Ig present in human MFGM; IgA is more enriched in human than in bovine MFGM[b]	IgG H chain, Ig heavy chain variable region, polymeric immunoglobulin receptor and immunoglobulin J chain were higher in colostrum[a]	Y	Resistant to digestion and survived intact to the stool[m]
Toll-like receptors (e.g.,TLR2, 4)	∼90	MFGM	Antibacterial component, immune system	Higher TLR2 in human than in bovine MFGM[g]	Higher TLR4 in mature milk MFGM than in colostrum MFGM[h]	Y	*Not specified*
Clusterin	52	MFGM	Antibacterial component	Higher in human than in bovine MFGM ($P < 0.05$)[b,g]; but another study reported lower quantity in human than in bovine MFGM[a]	No significant change reported[a]; but in bovine, colostrum MFGM has significantly higher quantity than mature MFGM[b]	Y	Resistant to gastric hydrolysis[n]
Tenascin	241	Whey and MFGM	Antibacterial component	*Not specified*	Significantly higher in colostrum MFGM than in mature MFGM ($P < 0.05$)[a,c]	Y	Resistant to gastric hydrolysis[n]

[a]Yang et al. (99), [b]Hettinga et al. (1), [c]Liao et al. (97), [d]Reinhardt and Lippolis (100), [e]Karhumaa et al. (110), [f]Rai et al. (111), [g]Lu et al. (107), [h]Cao et al. (98), [i]Davidson and Lonnerdal (112), [j]Hamosh (113), [k]Demmelmair et al. (114), [l]Layman et al. (115), [m]Demers-Mathieu et al. (116), and [n]Dallas et al. (117).
Note that the study by [d]Reinhardt and Lippolis (100) used bovine MFGM.

tripartite complex; BTN, APN and XOR), as well as mucins (MUC1 and 15) have been observed, suggesting a developmental shift to increase efficiency of milk lipid secretion (100). In contrast, apolipoproteins (e.g., A1, C-III, E, and A-IV) and immune-related proteins (e.g., immunoglobulin γ1 chain C region, clusterin and lactoferrin) have been observed to decrease (100) in bovine MFGM.

Analysis of human milk revealed that as lactation proceeds (from 0 to 6 months of exclusive breastfeeding), levels of proteins related to lipid synthesis and transfer (e.g., FABP, nonspecific lipid transfer protein, and proactivator polypeptide), intracellular folate uptake (e.g., folate receptor-α), actin filament organization (e.g., gelsolin and heat shock protein beta-1), antioxidant function (e.g., glutathione peroxidase 3), and antimicrobial function (e.g., lysozyme C)

were found to increase in the MFGM (97). BTN and XOR levels were also shown to increase over the first 6 months of exclusive breastfeeding but tended to decrease during partial weaning (from 6 to 12 months) (97). No significant changes have been observed for MUC4, lactadherin, carbonic anhydrase 6, and lactoferrin in human MFGM (97). However, another study showed significantly higher levels of carbonic anhydrase 6 in human colostrum compared to mature milk (110). In contrast, the levels of proteins with potential antimicrobial function, including human leukocyte antigen (HLA) II (which aids antigen presentation to T cells) (97) and AP (140), were found to be higher in colostrum compared to mature human milk, which may possibly compensate for immature neonatal immunity (discussed in section Intestinal Immune Maturation). Compared to bovine MFGM, the human MFGM proteome has been less explored (**Tables 2**, **3**).

b. Environmental Factors
Proteins in the MFGM have been associated with various health benefits, particularly in immune defense (97). In addition to gradual changes during lactation, temporal fluctuations in the immune-related proteins of the MFGM have been observed during immune challenges such as bacterial infection. For example, the infectious bacterium *Mycoplasma agalactiae*, which causes mastitis, initiates an immune response involving up-regulation of proteins involved in host defense, inflammation, and oxidative stress, and down-regulation of proteins involved in milk fat metabolism and secretion in lactating ewes (141). Similarly, in lactating cows infected with *Staphylococcus aureus,* neutrophil extracellular traps (NETs), which are known to amplify bactericidal properties of antimicrobial peptides, were observed to accumulate in the MFGM fraction to a greater extent than observed in the whey or milk exosome proteins (142). Although these were shown in other species, similar phenomena likely occur in human MFGM reflecting the mammary gland immune response. Whether those proteins will still be active in the intestine of breast-fed infants remains to be determined, and in this sense, their metabolic fates in the GI tract are worth investigating.

c. Species
So far, only a few studies have reported (1, 98, 99, 101, 107) or summarized (143) cross-species comparison of the MFGM proteome (**Tables 2**, **3**). One such study revealed that several MFGM proteins involved in lipid and fatty acid catabolism are higher in relative abundance or uniquely found in human milk compared with milk from other mammalian species. These proteins include peroxisomal acyl-coenzyme A oxidase 3, bile salt-stimulated lipase (BSSL), peroxisomal bifunctional enzyme, peroxisomal multifunctional enzyme type 2, hormone-sensitive lipase, lipoprotein lipase and sphingomyelin phosphodiesterase, all of which as isolates *in vitro* retain their lipolytic activity (107). BSSL has demonstrated roles in immune-modulation, intestinal growth (144), and antimicrobial action (145).

Variable expression of host defense proteins has also been observed across different species. Human MFGM is enriched with MUC4 and TLR2 relative to MFGMs from other mammals (bovine, goat and yak), which may improve innate immune response and protection against gram-positive pathogens (107). Compared to bovine MFGM, human MFGM is significantly enriched with lactoferrin, whereas cathelicidins (antimicrobial peptides) appear to be uniquely found in bovine MFGM (107). In another study, Yang et al. reported that human MFGM (pooled from 10 mothers between 3 and 8 months post-partem) was significantly more enriched with FABP but much less in XOR compared to pooled bovine MFGM (101). Importantly, bovine MFGM appears to exhibit a wider range of proteins with antibacterial properties (e.g., cathelicidins), whereas human MFGM was more enriched with the proteins involved in mucosal immunity (i.e., IgA, CD14, lactoferrin, and lysozyme) (1).

Commercially available MFGM isolates (e.g., used in infant formulas) are predominantly bovine sourced. It is therefore of great interest to understand the differences between human and bovine MFGM (and therefore the functions) to fully understand what functions are missing from bovine MFGM. Variations in isoforms and glycoforms of MFGM proteins exist within and/or between species, although less explored (146–148), which may contribute to differences in molecular functions such as binding, receptor activity, signaling, and enzyme activity. This aspect is beyond the scope of this review but further comparative studies would greatly benefit the field and promote its application.

FUNCTION OF MFG IN INFANT GUT MATURATION

In addition to its role in digestion and nutrient absorption, the gastrointestinal (GI) tract functions as a critical first defense immunological barrier. Gut maturation is stimulated by constant interactions between dietary components, endogenous secretions, host gastrointestinal cells, and microorganisms, all of which contribute to the development of intestinal morphology, immune function and composition of the gut microbiota. The "critical window" hypothesis postulates that events occurring early in life that disrupt the microbial ecology of the young gut, increase the risk of developing disease later in life (149). In view of this, the mechanisms through which breast milk guides early intestinal development while protecting against potentially harmful insults has been a key research question. The MFG may have a critical role in intestinal development. However, the MFG has been historically removed from breast milk substitutes (8). This section aims to review the primary roles of the MFG and its components in the development of: (1) the intestinal structure; (2) the intestinal immune system; and (3) the gut microbial community structure, with a focus on infancy.

Structural Development of the Intestine

Intestinal growth and maturation begin *in utero* and continues postnatally. During this period, intestinal development is characterized by active tissue growth and morphological changes (150). Human milk lipids contain numerous components that aid in the postnatal development of the intestinal mucosa, vasculature, and motility. Studies on human infants are limited

due to the invasive nature of the procedures involved, and therefore the bulk of available research utilizes animal models, and particularly the piglet model, which has physiological similarity with humans (151).

a. Development of the Small Intestinal Mucosa
Milk lipids appear to improve intestinal integrity by serving as essential building blocks for cellular membrane structure and as signaling messengers for cell growth, proliferation and migration (8). It was recently reported that the addition of bovine MFGM to a control formula in rat pups accelerated intestinal development and improved intestinal mucosal architecture by improving epithelial cell proliferation and differentiation, as well as expression of tight junction proteins to levels similar to mother-reared pups (152). These findings are consistent with another study that showed feeding an MFGM-rich post-weaning diet to mice strengthened the mucosal barrier by protecting against LPS-induced gut leakiness (153). Recently it was shown in a Caco-2 cell model that addition of polar lipids derived from bovine milk in the form of beta serum concentrate mitigated damage caused by a TNF-α challenge to the intestinal epithelial barrier (154). It is possible that MFGM aids in the maturation of the gut through both direct and indirect modulation of the gut microbiota, particularly since MFGM supplementation is observed to have the greatest effect on the colon (154), which is the site harboring the highest density of microbes.

The MFGM is a carrier of polar lipids, whose digestive products are essential for the morphological and functional development of the newborn intestine. The MFGM is an exclusive carrier of gangliosides to the neonatal gut (42), and a ganglioside-enriched diet has been shown to significantly increase total ganglioside and GD3 content, while decreasing GM3 and reducing the ratio of cholesterol to ganglioside in the enterocyte membrane of rat pups (36). These findings suggest that gangliosides can be incorporated into the intestinal mucosa, where they can alter membrane fluidity and enterocyte function (36).

Gangliosides are integral components in cell membranes, and the oligosaccharide residues that extend from the cell surface serve as surface markers in cell-cell communication (40). Gangliosides may also modify the brush border membrane of the GI tract. For instance, when dietary bovine gangliosides were provided to weanling rats, multiple changes in the intestinal epithelium were observed, including an increase in the content of ether phospholipids (a group of phospholipids with an alkyl or alkenyl bond at the *sn*-1 position) (39), greater incorporation of LC-PUFAs such as DHA and ARA (38), and enhanced LCFA uptake (37). In an *ex vivo* study, pre-exposure of infant bowel tissue to gangliosides reduced bowel necrosis and pro-inflammatory signals in response to LPS, implying an *in vivo* functional re-structuring of enterocytes (155).

Sphingolipids such as SM that are present in the MFGM and in the intestinal apical membrane are digested by brush border enzymes (expressed at birth) to generate the digestive products: ceramide, sphingosine, sphingosine-1-phosphate (S1P), and ceramide-1-phosphate (8). These metabolic products are known to mediate intracellular signaling pathways that are involved in cell growth, differentiation, apoptosis and immune cell migration in the neonatal mucosa (8, 156), and to facilitate enzymatic and morphological maturation of the intestine (157). The importance of sphingolipids and gangliosides in infant gut maturation, immunity, and neurological development has been reviewed elsewhere (42).

In newborns, PC and SM in MFGM are important sources of choline, which is an essential component of cell membranes, neurotransmitters (e.g., acetylcholine), and for neurogenesis and synaptogenesis (8). Phospholipids carry essential LC-PUFAs, critical molecules for membrane fluidity of the intestinal mucosa or neuronal tissues (57). The benefits of phospholipids in human milk have been broadly discussed (158).

b. Development of Intestinal Vasculature
During the first month of life, intestinal tissues expand and develop, and intestinal perfusion increases as a means to supply sufficient oxygen to accommodate these increases in metabolic activity. Vascular tone is mediated by endothelial nitric oxide synthase (eNOS) and the primary constrictor stimulus, endothelin-1 (ET-1), which promotes vasodilation and increases vascular resistance, respectively [reviewed in Nowicki (159)]. In addition to these endogenous regulators, SM and its metabolites (mentioned above) are able to alter infant vasculature, thereby influencing gut maturation. For example, S1P activates Akt signaling (a protein kinase with many regulatory functions in the cell), which initiates angiogenesis by invoking endothelial cell migration and morphogenesis (33). Additionally, S1P increases vascular barrier function by up-regulating adherence junctions (160, 161). Importantly, S1P levels are metabolically regulated by adiponectin, another component of milk, which increases turnover of ceramide to S1P by up-regulating ceramidase activity (162). In contrast to S1P, ceramide inhibits Akt signaling, which increases apoptosis and eNOS induction during vasculature remodeling in order to accommodate new growth prior to angiogenesis (161). This coordinated interplay of SM metabolites in the MFG establishes the architecture of blood vessels to meet the high metabolic demand of the expanding GI tract, aiding in gut maturation.

Other factors involved in small intestinal development derived from the MFGM have been suggested. For example, lactoferrin (Lf) bi-directionally stimulates proliferation and differentiation of the small intestinal tissue by interacting with Lf receptors located on the enterocytes and crypt cells (163). Expression of the plasma membrane Lf receptor was shown to be highest in the small intestine (163).

Intestinal Immune Maturation

When faced with an immune challenge, two key biological factors are involved in the intestinal defense response: the mucosal immune system and the gut microbiota (the latter will be discussed in the following section). A recent study reported that supplementation with bovine MFGM was able to enhance overall immunity and metabolism (10) which may explain the previously

reported reduction in infection-related diarrhea (6). This section discusses potential mechanisms whereby components in the MFGM are able to regulate cellular events to enable maturation of the mucosal immune system, thereby improving infant health (**Table 4**).

While the immune system begins to develop *in utero*, there is rapid development after birth. Immediately following delivery, newborns have a limited capacity to initiate immune responses as the adaptive immune system is still influenced by the active suppression that occurs *in utero* to prevent adverse immunological reactions from occurring between the mother and fetus (183). In addition, this suppression enables the infant to develop tolerance against antigens such as breast milk proteins and commensal microorganisms after birth (184). For example, fetal $CD4^+$ naïve T-cells tend to differentiate into $Foxp3^+$ $CD25^+$ regulatory T-cells (T_{reg}) in fetal lymphoid tissue (185, 186), which suppresses antigen-specific immune reactions and inflammation. This active suppression of the adaptive immune system, combined with low exposure to antigens prior to birth (little immunological memory) (187, 188), and a sudden flow of food and microorganisms entering the gut after birth increases the risk of infections in newborns.

Maturation of adaptive immunity involves antigen exposure, followed by T-cell differentiation in response to antigens presented by antigen presenting cells (APCs). Human MFGM contains human leukocyte antigen II (HLAII) (97), an antigen presenting complex typically expressed on the surface of APCs that may be derived from HLA-DR (a subgroup of the major histocompatibility complex (MHC) Class II) on the mammary gland epithelium (169). Notably, only secretory epithelial cells in the lactating mammary gland, but not non-lactating cells, were found to express HLA-DR (169). Moreover, milk exosomal MHC Class II is more abundant during early lactation and gradually decreases, whereas MHC Class I shows the opposite trend (171). These studies suggest that HLAII on the MFGM may be involved in presenting antigens encountered by mothers to $CD4^+$ T-cells in the infant gut, thereby supporting immune education during early life when tolerance is being established.

Some MFGM proteins may be involved in modulating properties of T-cells, the key regulator of the immune system. Butyrophilin (BTN) has been shown to negatively regulate T-cell proliferation and activity. When mouse $CD4^+$ T-cells were activated by immobilized anti-CD3 antibody in the presence of the recombinant Fc fusion proteins BTN1A1 and BTN2A2, T-cell proliferation as well as IL-2 and IFN-γ production were inhibited (168). Thus, BTN in the mammary gland epithelium and the MFGM may control the function of maternal T-cells in the mammary gland and milk, respectively. They may also impact neonatal T-cells, although human BTN1A1 is digested rapidly in the infant stomach (102).

Lactadherin, also known as MFG-E8, is another MFGM protein with T-cell regulatory function. Lactadherin supplementation of formula led to the differentiation of naïve $CD4^+$ T-cells to $CD3^+CD4^+CD25^+$ T_{reg} in Peyer's patches of rat pup ileum, an important segment of the intestine that is involved in the immune response (173). In the same study, expression of $OX62^+CD4^+SIRP^+$ dendritic cells (DCs) increased in Peyer's patches, which was coupled with an increase in production of the anti-inflammatory cytokine, IL-10 (173). These patterns in the T-cell population and cytokine production were shown to continue after weaning (173), suggesting a long-lasting effect of lactadherin supplementation. Previously, a positive correlation was observed between lactadherin concentrations in breast milk and protective effects against rotavirus infection (measured by morbidity) in Mexican infants, which was independent of the level of secretory IgA and other milk components such as BTN and mucin (119). This observation is supported by cell culture models showing that human lactadherin limits the infectivity of rotavirus in Caco-2 cells (174). Furthermore, lactadherin prevents tissue damage caused by prolonged inflammation by clearing apoptotic cells, thereby facilitating immune resolution (127, 172). To accomplish this, lactadherin binds to phosphatidylserine on the external membrane of apoptotic cells via its C-terminal V/VIII like domains, and its epidermal growth factor (EGF) domain contains the arginine-glycine-aspartate (RGD) motif that interacts with $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin receptors of phagocytes (172). The binding of lactadherin to integrin receptors leads to activation of the signaling cascade that enables macrophages to engulf apoptotic cells (127). Delivery of lactadherin by the MFGM may be critical because active mitosis, which occurs during intestinal maturation, is accompanied by a high rate of apoptosis due to the many errors made in DNA replication and the subsequent clearance of mutated cells by the p53 protein (189).

Another minor MFGM protein associated with the developing immune system is milk alkaline phosphatase (AP), whose anti-inflammatory activity in the gut may contribute to protection against inflammation that is induced by the presence of large quantities of lipids from a high-fat diet (190). Although the role of AP in MFGM is not entirely clear, AP is endogenously produced by enterocytes, which, as a host defense mechanism, dephosphorylates pro-inflammatory molecules such as lipopolysaccharide (LPS), inhibiting TLR-mediated NFκB signaling and subsequent inflammation in the gut (190). Milk phospholipids and fatty acids, particularly LCFA, are strong stimulators of intestinal AP activity (190).

Osteopontin (OPN) is another minor MFGM protein that is involved in the development of both the innate and adaptive immune system of newborns. OPN functions as an opsonin, binding directly to bacteria such as *Streptococcus agalactiae* and *S. aureus* to enhance phagocytosis by macrophages (191). OPN is also involved in balancing the Th1 and Th2 immune responses as a cytokine, where it induces the Th1 immune response through elevation of IL-12 production from macrophages, while suppressing the Th2 immune response through lowering IL-10 secretion (192).

Milk lipids have been shown to interact with milk proteins during digestion, altering the types of peptides remaining in the gut, which in turn affect their bioactivities. In a piglet model, a formula incorporating both milk fat and vegetable oil stabilized by a protein-MFGM fragment mixture inhibited digestion of β-casein and β-lactoglobulin, thereby increasing the proximal jejunum and ileum content of immunoreactive peptides derived from those proteins. This was not observed in animals fed a formula incorporating vegetable oil with the same emulsifier nor a formula with vegetable oil stabilized only with a protein mixture

TABLE 4 | MFGM proteins and lipids involved in the infant intestinal immune system.

MFGM protein	Functions
α-lactalbumin	• Proteolysis of α-lactalbumin generates peptides with bactericidal or immune-stimulatory activities (164, 165). • Protects against diarrhea caused by enteropathogenic *Escherichia coli* (166).
Butyrophilin subfamily 1 member A1 (BTN1A1) Human leukocyte antigen II (HLAII)	• Involved in the regulation of lipid secretion (167). • Involved in T-cell proliferation and metabolism (168). • May present maternal antigens to infant T-cells (169–171).
Lactadherin (PAS VI/VII, MFG-E8)	• Regulates apoptosis by phagocytes (127, 172). • Induces anti-inflammatory responses (173). • Regulates T-cell proliferation and cytokine production profile by dendritic cells (173). • Involved in the protective effect against rotavirus (119, 174).
Lysozyme	• Inhibits the growth of Gram-negative bacteria by disrupting the outer membrane and cooperating with lactoferrin (175).
Mucin-1 (MUC1)	• Binds to microorganisms and chemicals to prevent infection and inflammation (176). • Inhibits the growth of *Salmonella enterica* serovar Typhimurium (177), S-fimbriated *Escherichia coli* (178), and rotavirus (179). • Suppresses inflammation caused by *Pseudomonas aeruginosa* and its flagellin by down-regulating Toll-like receptor pathways (180, 181).
Osteopontin (OPN)	• Binds to *Streptococcus agalactiae* and *Staphylococcus aureus*, enhancing phagocytosis by macrophages (162). • Induces Th1 immune response (elevating IL-12 production from the macrophages) while suppressing the Th2 immune response (reducing IL-10 secretion) [162)].
Xanthine oxidoreductase (XOR)	• Generates reactive oxygen species with antibacterial properties (182).
Gangliosides	• Regulates activity and functionalities of immune cells including lymphocytes and dendritic cell, playing a role in developing immune tolerance (41).

(8, 9). This indicates that milk fat (mainly TAG) and differences in structural organization of molecules at the interface are the contributors for the observed modulatory effects.

Gangliosides in the MFGM are involved in multiple aspects of the mucosal immune system. Dietary gangliosides have been shown to modulate intestinal cytokine and IgA production (41) as well as lymphocyte activation (193). An inhibitory role of GM3 and GD3 on dendritic cell functionalities has also been reported (41), suggesting that in addition to promoting defense against aggressive antigens, milk gangliosides may promote tolerance against non-aggressive antigens, which is equally important during the first stages of life. GD3 levels in milk are higher in colostrum and GD3 has superior inhibitory activity against dendritic cell functions compared with GM3, indicating that immune modulation by gangliosides is more prominent during early infancy (41). This suggests that there may be a relationship between compositional changes in breast milk and infant gut maturation over the course of lactation.

Limitations in Studies of MFGM Functions in Infant Immune Development

There are some limitations in studies of MFGM and its roles in infant immune development. Most of the proposed immune mechanisms are based on studies focusing on isolated components of MFGM rather than intact MFGM. Indeed, the fate of MFGM through the neonatal and infant GI tract during digestion remains to be elucidated. In order to better understand how MFGM is able to modulate the intestine, future studies should examine how MFGM is digested, and in which part of the GI tract bioactive components of MFGM proteins are liberated from their complex structure. Furthermore, because the MFGM composition changes dynamically during lactation, and given that there is variability among individuals (102), identifying those components of the MFGM responsible for infant immune development would help move the field forward. Specifically, there is a lack of data concerning which components of MFGM in human milk are highly conserved and/or variable (not only in quality but also in quantity). This information may help us better understand the critical components of MFGM, and define which are important for maternal and/or infant health. For example, lactose exhibits very low variation (4% CV) among individuals, which suggests that this major osmotic component that regulates milk volume is highly conserved and may be important for the developing infant (194). Nonetheless, it is becoming more evident that MFGM proteins support the proper education and development of the immune system in infants.

IMPACT ON THE INFANT GUT MICROBIOTA

The human microbiota represents a community of commensal, symbiotic, and pathogenic microorganisms inhabiting the body

(195). From the earliest moments in life, the microbiome is progressively built on a foundation initiated by key events, including delivery method (196), gestational age (197), antibiotic use (198), and nutrition (199), and eventually stabilizes into an adult-like state by approximately 12 months of age (198). The gut microbiota is dynamic during early life and is critically important for the maturing infant GI tract and the immune system as it establishes the basis for long-term metabolic and immune health (200). Nutrition remains a crucial factor during early development as dietary components are in constant interaction with the nascent microbiome, and emerging evidence suggests that the MFG, or components thereof, may contribute toward its evolution at this early stage of life. Structural differences between human MFG, which are enveloped by the MFGM, and infant formulas, which contain homogenized vegetable fats emulsified with dairy proteins and/or emulsifiers, influence the extent to which nutrients are digested in the intestine and have downstream effects on the gut microbiota. The following sections summarize evidence for a modulatory role of MFG and its components on the gut microbiota of the developing infant.

MFG Core Lipids

The structure of human MFG and specific positional distribution of fatty acids may explain differences in the gut microbiota between breast-fed and formula-fed infants. Human milk contains β-16:0 (palmitic acid esterified on the *sn-2* position) in contrast to vegetable-sourced palmitic acid, which is esterified in the *sn*-1 or−3 positions (201). One study found that supplementing an infant formula with β-16:0 increased fecal abundance of *Lactobacillus* and *Bifidobacterium* genera in infants after 6 weeks of feeding compared to a control formula containing vegetable-sourced 16:0 (202). The mechanisms behind this observation need to be determined, but the position of palmitic acid on the TAG suggests that lipid structure may be important for gut microbiota. Similar bifidogenic results were shown with an infant formula supplemented with both β-16:0 and a mixture of prebiotic oligosaccharides (203) and formulas containing high amounts of β-16:0 with and without supplemented oligofructose (204).

In contrast to a formula containing only vegetable-derived lipids, a combination of milk fat and MFGM fragments altered fecal microbial composition in piglets by increasing *Proteobacteria* abundance at the expense of *Firmicutes* (which included the *Escherichia*/*Shigella*, and *Klebsiella* genera), as well as increasing *Bacteroidetes* (including members of the *Parabacteriodes* genus) (9). This may partly be explained by an increased intestinal content of immune modulatory peptides (as discussed earlier) as well as milk lipid-derived metabolites. However, studies on the gut microbial composition of breast-fed compared to formula-fed infants found the opposite effect, where a higher abundance of *Firmicutes* was observed in breast-fed infants, at the expense of *Proteobacteria* (205). Interestingly, differences have also been observed in the rate at which large and small MFG molecules are digested by pancreatic lipase, the former of which are hydrolyzed more slowly (206) likely affecting the accessibility of lipid products of the MFG core for bacterial metabolism.

Another study found that an infant formula high in MCFAs (coconut oil) emulsified with bovine MFGM (Lacprodan® MFGM-10) enriched the bacterial families *Bacteriodaceae*, *Desulfovibrionaceae*, *Rikenellaceae*, and *Porphyromonadaceae*, while formulas made with LCFAs emulsified with soy lecithin increased the abundance of *Enterobacteriaceae*, *Erysipelotrichaceae*, *Coriobacteriaceae*, and *Enterococcaceae* in the colon of germ-free mice (207). The effect of dietary fatty acids on the gut microbiota may also depend on the chain length and desaturation degree of the fatty acids (207). Human milk contains high amounts of medium chain fatty acids (207), such as C10:0 and C12:0, which have previously been shown to inhibit the growth of several strains of food-borne pathogens (208). Lipolysis of MFG core lipids is capable of causing cell lysis in undesirable microbes through the detergent-like characteristics of free fatty acids, and monoglycerides (113, 209).

An interesting feature of MFG lipids is that they can influence which protein digestion products enter the colon by altering the rate at which proteins are hydrolyzed in the small intestine (9) (as discussed earlier in section Intestinal Immune Maturation). A review on bacterial utilization of undigested luminal proteins and peptides was previously published (210). Overall, these results suggest that lipids derived from milk fat may indirectly enrich specific bacterial populations in the infant gut; however, additional studies are required to understand how this occurs.

MFGM Lipids

It is conceivable that remnants of the MFGM escaping digestion in the lumen proceed to the large intestine and support the colonization of microbial communities (15). Comparison of the colonic bacteria of germ-free mice fed two different types of emulsifiers, soy lecithin vs. MFGM phospholipids (Lacprodan® PL-20, Arla, Denmark), revealed that MFGM phospholipids tended to enrich *Porphyromonadaceae,* while soy lecithin tended to enrich *Enterobacteriaceae* and *Enterococcaceae*. Interestingly, mice fed MFGM phospholipids showed lower cecal concentrations of branched SCFAs (isovaleric acid, isobutyric acid), which are products of protein metabolism, compared to soy lecithin fed mice, suggesting a decrease in proteolytic activity in the ceca (207). In a separate study, rat pups fed a formula supplemented with bovine MFGM (Lacprodan® MFGM-10, Arla, Denmark) experienced an increased gut microbial species richness and evenness compared with rat pups fed a formula containing vegetable fat. At the phylum level, the microbiota of rat pups fed MFGM were more similar to the group reared on dam's milk, with similar levels of *Firmicutes* and *Proteobacteria*. In contrast, the control pups on regular formula experienced increased *Proteobacteria* and reduced *Firmicutes*. In the same study, *Lactobacilli* were determined to be the most abundant in dam-reared pups, present in pups supplemented with MFGM, but not detected in pups fed control formula (152).

In addition to supporting the growth of beneficial microbes, MFGM also exhibits antimicrobial activity that appears to protect against the development of infectious diseases. For example, a double-blind randomized controlled trial (RCT) showed that during the first 12 months of life, infants fed an experimental formula supplemented with bovine-derived

MFGM (Lacprodan® MFGM-10) from 2 until 6 months of age experienced fewer acute otitis media (AOM) infections, and less fever than those receiving a control formula (7). These findings are supported by the observation that breast-fed infants experience lower rates of AOM in comparison to formula-fed infants (211, 212), and parallel another RCT in pre-school children (mean age 4.4 ± 0.9 y), which showed that consuming 200 mL chocolate milk containing an MFGM concentrate derived from bovine milk enriched with phospholipids (INPULSE®) daily for 4 months resulted in fewer days with fever, a reduction in fever incidence, and improved behavioral outcomes compared to children consuming a non-enriched chocolate milk control (213).

MFGM polar lipids such as sphingophospholipids and gangliosides have been shown to exhibit antimicrobial activities and protect against lipopolysaccharide-induced inflammation associated with Gram-negative bacteria (153, 214) and the development of colitis after *Clostridium difficile* infection (152). Degradation products of sphingomyelin, such as sphingosine, have shown bactericidal activities against specific bacteria (208, 215). Adult mice fed a high-fat diet supplemented with sphingomyelin sourced from bovine milk resulted in lower fecal Gram-negative bacteria while enriching bifidobacteria (216). In preterm newborns, infants fed a formula supplemented with bovine gangliosides resulted in reduced *E. coli* fecal counts and enriched *Bifidobacterium* compared to a standard formula (193).

MFGM Proteins

The protective mechanism behind the anti-infection activities associated with the MFG may also be related to MFGM proteins. For example, xanthine oxidase and antimicrobial proteins such as lactoferrin, lysozyme, and secretory immunoglobulin A (sIgA) are known antibacterial and/or antiviral proteins. Bovine MFGM has demonstrated antimicrobial activity, whose *in vitro* digestive products have been shown to selectively suppress the growth of *Salmonella typhimurium* (217).

Xanthine oxidoreductase (XOR), a major oxidative enzyme present in the MFGM, generates reactive oxidative species (ROS) such as hydrogen peroxide and superoxide anion, as well as reactive nitrogen species (RNS), which may play a role in the antimicrobial defense of the GI tract (122). A decoy effect of surface carbohydrates of XOR has also been suggested (122). Indeed, a growth inhibitory activity of XOR was demonstrated against *E. coli* (218) and *S. enteritides* (182). One interesting feature of XOR associated in immune defense of the infant is that infant saliva contains hypoxanthine and xanthine, substrates for XOR, in concentrations that are 10-fold higher than in adults, resulting in the generation of sufficient quantities of hydrogen peroxidase to inhibit the growth of *S. aureus* and *Salmonella* spp. (219). Interestingly, the enzyme activity of XOR in human milk peaks during the first few weeks of lactation likely as a protective measure for the immature gut at this stage, diminishing thereafter despite constant levels of protein expression (220). XOR is located in the inner leaflet of the MFGM, and its release and activation in the oral phase may provide a first line of defense against pathogen invasion. Indeed, a study on the oral microbiota in infants (<2 mo) given formula supplemented with bovine MFGM found that *Moraxella catarrhalis*, one of the most common bacteria associated with otitis, was less prevalent in oral swabs at 4 months of age compared to infants fed standard formula (221).

α-Lactalbumin, a minor protein embedded in the MFGM, is digested by pepsin, trypsin, and chymotrypsin during passage through the GI tract (97), generating bactericidal peptides (e.g., Gly-Leu-Phe; GLF peptides) (165) that protect from infection by enhancing macrophage phagocytosis and stimulating oxidative metabolism in neutrophils (164). Supplementation of infant formula with α-lactalbumin has previously shown protection against diarrhea by enteropathogenic *E. coli* in infant rhesus macaques (166). Another minor component of the MFGM, lysozyme, has potent bactericidal properties against both Gram-positive and -negative bacteria due to the presence of 1,4-β-N-acetylmuraminidase which can degrade bacterial cell walls (222). This action by lysozyme is supported by lactoferrin, which sequesters iron and directly interacts with the negatively charged Lipid A moiety of LPS to damage the bacterial membrane (114).

These data together provide evidence of the supportive effect of MFGM on the mucosal immune system, and that MFGM serves as a key component in milk enhancing intestinal defense during early life, while establishing stable commensals in the gut.

MFGM Glycobiome and the Infant Gut Microbiota

The MFGM contains glycoconjugates (glycolipids and glycoproteins) harboring both *N*-linked and *O*-lined glycan moieties (12). About 70% of bovine glycolipids in milk are associated with the MFGM (223). The glycosylation patterns of these glycoconjugates in milk are tightly regulated by gene expression (98, 224), and determine resistance to digestive enzymes and functionality in the gut. The ability of certain bacteria to selectively bind to the intestinal mucosa through recognition of specific sugar moieties influences susceptibility to infection (11). The glycoproteins (MUC1, lactadherin) and gangliosides of the MFGM have the ability to interfere with pathogen recognition of, or attachment to, the intestinal mucosa, causing the pathogens to instead interface with the antimicrobial components embedded within the MFGM (225). For example, several glycoproteins derived from porcine MFGM were able to inhibit intestinal adhesion of *E. coli* F4ac (226). Mucin has been shown to inhibit the invasion of common enteric pathogenic bacteria such as the *Salmonella enterica serovar Typhimurium SL 1344* (177), S-fimbriated *Escherichia coli* (178), and rotavirus (179), which could partly explain the lower risk of *Salmonella* infection in breast-fed infants compared to formula-fed infants (177). The structural diversity of MFGM-bound oligosaccharides greatly differs between mammalian species (11, 146–148), and little is known about the functional differences. However, it is tempting to speculate that the differences have to do with the specificity of host-microbial interactions.

Probiotic/Prebiotic Effects of MFGM in the Infant Gut

It has been hypothesized that probiotic bacteria (such as members of the *Lactobacillus* and *Bifidobacterium* genera) are able to pass from the mammary gland through to the infant colon by adhering to components of the MFGM, suggesting that the MFGM may be a probiotic carrier (227). Recently, Pannaraj et al. (228) established that bacteria found naturally in breast milk are able to seed the infant gut during the early stages of gut development (228). Another study showed that select OTUs assigned to *Bifidobacterium*, specifically *B. breve*, *B. bifidum*, and *B. longum*, were identified in breast milk and infant feces of the same mother-infant pair, but not in the oral cavity, suggesting that breast milk provides an important inoculum of specific bacteria for seeding the infant gut (229). Bacteria (including *Lactobacillus*) present in milk have been shown to preferentially associate with the MFGM utilizing glycan adhesion factors that enable them to bind to mucin (227). In addition, bacteria with greater surface hydrophobicity, such as *L. reuteri*, are better able to adhere to the MFGM (230), a phenomenon that is related to properties of the bacterial cell surface. In this context, several patents have been published for utilizing the MFGM as a probiotic carrier, which have recently been reviewed with a focus on lactic acid bacteria (227).

Mucin and lactadherin have been detected intact in gastric aspirate samples of pre-term infants fed breast milk, which suggests that MFGM glycoproteins remain stable and are able to survive gastric digestion (102). The MFGM may therefore confer a prebiotic effect, potentially providing a source of carbon to support the growth of the colonic bacteria (231). Indeed, members of both the *Ruminococcus* and *Bifidobacterium* genera are capable of producing extracellular glycosidases to digest glycans and glycolipids (232). Interestingly, *Lactobacillus paracasei*, and *Bifidobacterium* spp. isolated from cheese products were shown to survive in carbohydrate-restricted media by utilizing membrane-bound sugars on the MFGM as an energy source (233). This prebiotic effect is likely due to the sialic acid residues (234) on the gangliosides, which can be utilized by *B. infantis* and *B. bifidum* (231). This may account for the increased SCFAs observed during *in vitro* incubation of fecal material with MFGM isolates (235).

CONCLUSIONS AND FUTURE PERSPECTIVES

MFGs are complex structures that are found in breast milk and growing evidence suggests a role for these important biomolecules in the early stages of human life. The observation that MFGs are heterogeneous in size and composition suggests that MFGs take on multiple roles in the developing neonate. It is known that the rate at which MFGs are digested is related to their diameter, and that proteins in the MFGM of some globules are able to resist pepsin hydrolysis better than others. Unique features of the MFGM, such as the lipid rafts, which are formed through the integration of cholesterol with highly saturated sphingolipids, create a rigid structure that enables minor components of the MFGM with bioactive properties to survive digestion. The extensive glycosylation of major and minor proteins found in the MFGM also appears to help them resist digestion, thereby enabling their passage to the colon intact. Thus, it is intriguing to consider that, in addition to their role as an energy-dense source of nutrition, the MFG may aid in the development of intestinal structure and the immune system, as well as the establishment of the intestinal microbiota in the neonate. Most studies published to date rely on data generated from animal models as the methods required to rigorously examine these research questions remain too invasive for human studies. However, important observations have already been made, including the finding that MFGM supplementation can accelerate intestinal development and improve intestinal integrity and vascular tone. Furthermore, several proteins associated with the MFGM are able to modulate the production and activity of immuno-modulatory components, such as T-cells, providing mechanistic evidence to support the role of MFG in development of the immune system. Finally, several animal studies and a growing number of studies involving infants show that the MFG and its components shift core microbial populations in the lower gut *via* multiple mechanisms, including the action of MFGM fragments that are resistant to digestion, through the unique distribution of fatty acids in the lipid core, and by antimicrobial activities associated with some MFGM components. Future studies should aim to elucidate the digestive fate of individual MFG and MFGM components to better understand their metabolic fate in different regions of the GI tract. As discussed, the MFG can be affected by maternal genetics, diet and environmental factors, and a deeper understanding of the connection between those factors, MFG composition, and downstream effects in the infant may improve dietary strategy of nursing mothers. Further, data on biological conservation as well as variations in MFG components observed among mammalian species (e.g., bovine vs. human) would provide guidelines for the development of infant formulas that meet the specific needs of human infants in cases where breastfeeding cannot be done. Overall, a growing body of literature continues to unravel the unique features of the MFG which suggest it plays important roles in preventing infection, supporting neurodevelopment, and shaping the maturing immune system and gut microbiota. These characteristics further underscore the importance of breast milk for the developing neonate.

AUTHOR CONTRIBUTIONS

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Does Breast Milk Nurture T Lymphocytes in Their Cradle?

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Breast feeding has been associated with improved infant outcomes in multiple aspects, including immune outcomes such as infections and potentially atopy and autoimmunity. However associations do not necessarily implicate cause and effect and at this point, exactly how breast feeding and components of breast milk may modulate the infant's immune compartment remains unclear, especially in humans. Some lines of evidence suggest that breastfeeding affects the development of the infant's thymus, a critical organ for T cell development. This may be a direct effect mediated by breast milk components or alternatively, a secondary effect from the impact of breast feeding on the infant's gut microbiome. Here we discuss the potential mechanisms and impact of this association between breast feeding and thymic development.

Keywords: breast feeding, thymus, immunity, breast milk microbiome, regulatory T cells

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INTRODUCTION

Thymus derived lymphocytes are one of the central players of the adaptive immune system. Adaptive immune responses in mammals are characterized by extreme specificity in terms of antigen recognition. Such highly tailored responses have evolved as a powerful defense strategy against rapidly changing pathogens resulting in improved host survival. A broad and effective development of adaptive immune responses is eminently important in the newborn period, when the offspring is exposed to a range of environmental pathogens it shares with its mother. Here mammals, in contrast to reptiles, are provided with the unique opportunity to shape thymic and T cell development in the offsprings for a prolonged period after birth by means of lactational immune programming.

Human breast milk contains a wealth of bioactive substances including proteins, oligosaccharides, polyunsaturated fatty acids, micronutrients, metabolites and microbial components. Many of these bioactive constituents can modulate the infant immune system in the critical first year of life. Whilst the protective effects of maternal IgA in breast milk for neonatal infections is well established (1), the role of the many other immunologically active components in breast milk is less clear. Notably despite the heterogeneity of various studies and the lack of randomized controlled trials, there is evidence that breastfeeding may influence long-term infant immune outcomes such as allergic and gastrointestinal diseases (2). Here we review the current evidence for the influence of breastfeeding on the infant immune system, with a focus on the thymus, a critical organ for T cell immune development.

BREAST FEEDING AND INFANT IMMUNE OUTCOMES

In addition to *in utero* immune programming, breast feeding offers an early postnatal route by which the mother may pass on protective factors to her infant and shape the developing immune system. Perhaps the best established data for the effect of breastfeeding on infant immunity is its protective effect for neonatal respiratory and gastrointestinal infections. It is thought that breast milk immunoglobulin A (IgA) secreted by activated resident switched memory B cells in mammary glands, is the main proponent for this effect, as it supplements the low neonatal IgA production (3). Additionally, secretory IgA from breast milk is also thought to promote infant immune tolerance to various antigens by reinforcing the gut epithelial barrier and reducing pro-inflammatory responses (4).

In addition to this short term protection, breast feeding may be associated with improved infant immune outcomes. These studies consist of observational and cohort studies and due to variation in study methodology and definitions, the results are more mixed. One prospective cohort study found no significant evidence that infant feeding practices in the first 6 months influenced the development of wheeze, asthma or atopic dermatitis (5). Another study found that increased duration of breast feeding was associated with protection against non-atopic asthma (6). A meta-analysis including studies up to 2014 suggested that breast feeding was protective for asthma and children who were exclusively breastfed for more than 3–4 months have a lower risk of developing eczema under 2 years of age, however potential recall bias and lack of confounder adjustments in some of the included studies may have influenced the analysis (7). Some studies also show that breast feeding may protect against food allergy (8, 9), however other studies suggest otherwise (10, 11). In terms of autoimmune disease, breast feeding appears to confer protection to the infant for both type I and II diabetes (12) and multiple sclerosis (13). However evidence for a protective effect on other autoimmune diseases such as arthritis and inflammatory bowel disease is controversial (14).

Several putative mechanisms have been put forward regarding the reason for the potential immune protective effect of breastfeeding. Reduced neonatal infections in breastfed infants is thought to contribute to the reduced rate of asthma in these children, as early and recurrent respiratory infections have been linked with development of asthma (15). In addition to IgA, there are a multitude of immune active substances including immune cells, cytokines and microbiome in the breast milk, which may be involved in infant immune programming (2). However due to the significant variability in breast milk components (16), it has been difficult to study or isolate the effect of these substances in humans. Mechanistic studies in mice showed that presence of allergen (via maternal exposure to allergen during lactation) with TGFβ, or IgG/antigen immune complex in breast milk led to protection against allergic asthma in the progeny via induction of antigen specific Treg cells (17, 18). This is supported by a systemic review of human studies, which revealed that two thirds of the studies showed an association between increased TGFβ levels in breast milk with reduced atopic outcomes (19). Soluble CD14 (sCD14), a co-receptor for bacterial cell wall components such as lipopolysaccharides (LPS) is also found in variable concentrations in human breast milk (20). High breast milk sCD14 level has been associated with protection against atopic disease (21), possibly by enhancing Th1 immune responses, though the exact immune mechanism is unclear.

Another significant influence of breast milk on the infant is the presence of breast milk microbiota and the effect of its colonization on the infant immune system. Indeed, the human breast milk contains a variety of viable bacteria (22) and there are early significant differences in the gut microbiota between exclusively breastfed and formula-fed infants (23), suggesting the gut microbiome may affect allergy development. However exactly how the breast milk microbiota may influence infant gut microbiome and immune outcome needs to be interrogated in future studies.

Many other immune active substances have been found in the breast milk including human milk oligosaccharides (HMO), which are thought to be protective against mucosal infections (24) and may be linked to reduced development of allergy in the infant (25).

THE THYMUS

The thymus is a critical center for T cell development. Developmentally, it is derived from the pharyngeal arches and is formed and functional at around 14–16 weeks of gestation (26). Lymphoid progenitors migrate from the bone marrow to the thymus, where they undergo a series of differentiation checkpoints. Briefly, the important processes include the development of formation and rearrangement of T cell receptor, acquisition of important surface markers such as CD3, CD4, CD8, and positive selection (where cells that bind inadequately to self MHC/antigen complex are deleted), then finally negative selection (where cells binding too strongly to MHC/self-antigens are deleted) (27). This results in the continual generation and emigration of naïve T cells from the thymus. Foxp3+ regulatory T cells (Tregs), a critical immune cell subset for immune tolerance, are also generated in the thymus in a similar manner, except their development requires a differential strength of signaling from self-antigen/MHC complexes, so that they are favored to suppress unwanted immune responses to self-antigens (28). The importance of the thymus is seen in cases of complete failure of thymic development (thymic aplasia) such as in of 22q11.2 deletion and CHARGE syndrome, which leads to severe combined immune deficiency with absent T cells and poor T cell dependent antibody responses (29).

Despite its important role in T cell generation, in all vertebrates the thymus involutes with age. Thymic stromal cells and architecture begin to regress as early as the first year of life, with disorganization of cortico-medullary junction and replacement of thymic tissue with adipocytes (30). The rate of regression increases to about 3% per year during adulthood

(31). This thymic involution is thought to be an important contributor to immunosenescence, which results in reduced naive T cell output, restricted T cell receptor repertoire, impaired responses to pathogens and vaccines, thereby leading to increased susceptibility to infections, autoimmune diseases and malignancy (32). A similar process is brought forward in infants who have early thymectomy for cardiac surgery, who show impaired thymic output with reduced T cell numbers and function, as well as immunoglobulin levels (33, 34). Several studies have attempted to examine the impact of thymectomy on clinical outcomes (35, 36). Whilst no significant morbidities were noted in the thymectomized population, it is important to note that thymic regrowth occurs in the majority of cases (37), making it difficult to assess the true impact of postnatal absence in thymic tissue. Furthermore the timing of thymectomy is quite variable, which may also influence the magnitude of the clinical impact.

A LINK BETWEEN BREASTFEEDING AND THYMIC DEVELOPMENT

A study by Hasselbalch et al. more than 20 years ago found that there was a correlation between breast feeding and thymic size (38). In a small cohort of 47 healthy infants, they assessed the thymic size at birth and 4 months of age using trans-sternal ultrasound measurement. The geometric thymic index was derived from the multiplication of the largest transverse and sagittal diameter on ultrasound. They found that the thymic index was significantly higher in babies who were exclusively breastfed compared to partially breastfed (infants who have breast milk at least once a day) and exclusively formula fed infants. However there was significant overlap between the groups. The authors concluded that the thymus is significantly larger breast-fed infants. A follow-up study from the same group recruited a different cohort of 50 partially breastfed infants at 8 months (39). The thymic index was derived by the same methodology. In this study they found that at 10 months of age, those infants who had continued to have some breast milk at 10 months had a slightly larger thymus compared to those who have stopped breast feeding. However the difference was very small with significant overlap. Notably these infants were all breast fed for at least 8 months, and therefore one might not expect a significant difference with extra 2 months of breast feeding. Importantly, both of these studies used trans-sternal ultrasound to assess the thymus size postnatally, which may be problematic due to echogenic shadow from the overlying bone. Additionally, how well thymic size correlates with thymic function and output is unclear and neither of these studies specifically examined thymic output.

In a subsequent paper, Jeppesen et al. examined the CD4 and CD8 counts and proportion in infants between 1 and 12 months of age (40), where they found some evidence of reduced CD8 and CD4 percentages in infants who had stopped breast feeding at 8 or 4 months of age respectively. They also found a modest correlation between the frequency of breast feeding and CD4 T cell counts, but not between thymic size and T cell counts. This study provides some evidence that breast feeding may influence the T cell compartment in infants, but fails to show that this influence is mediated through the thymus. Indeed other measures of thymic output or function would be needed to clarify this issue. A good candidate would be measurement of T cell receptor excision circles (TREC), which are genetic by products of T cell receptor recombination. TREC level is higher in naïve T cells and becomes diluted with T cell activation and division, therefore it serves as a good marker for thymic output of naïve T cells (41). Alternatively, measurement of recent thymic emigrants by their expression of CD45RA and CD31 with flow cytometry could also be used to monitor thymic output.

Additionally, in looking at immune outcomes in breast fed infants, one should also examine the effect of breast feeding on thymic output of Treg cells, since they are integral for controlling allergic and autoimmune responses. Unfortunately at this stage no study has examined this aspect in humans.

BREAST MILK COMPONENTS THAT MAY AFFECT THYMIC DEVELOPMENT

Several components in breast milk can potentially modulate the T cell mediated immunity by directly or indirectly shaping thymic development. An interesting study conducted in rural Gambia showed that infants born in the "hungry season" had smaller thymuses, associated with significantly higher adult mortality due to infectious diseases, compared to those born in harvest season (42). A later study showed that the reduced infant thymic size in the hungry season was associated with reduced thymic output as measured by TRECs. Furthermore, they showed that breast milk of mothers in the hungry season had significantly lower interleukin 7 (IL-7) levels compared to harvest season (43). These findings are correlative, but since animal studies have shown that breast milk cytokines can be passively transferred to the infant's systemic circulation (44), it does suggest that breast milk IL-7, an important factor for T cell thymopoeisis (45), may play a role in infant thymic development. Nevertheless, many other breast milk components may potentially differ in hungry and harvest season to influence infant thymic development.

Another interesting component in breast milk is the highly conserved RNA packaged in exosomal particles, known as microRNA (miR). These miRs are able to regulate immune cell development at the post-transcriptional level (46). In particular, murine models show that miR-155 plays an important role in regulating thymic Treg cell and Th2 cell development (47). Another miR, miR-449a appears to be important for regulating thymic medullary epithelial cell development in mice (48). Importantly, human breast milk contains abundant miRs (including miR-155) which may regulate infant immunity (49). Hence, transfer of miRs might be implicated in regulating infant thymic development and immunity.

In addition to bioactive molecules, data from a murine model indicates that viable maternal CD4+ immune cells are transmitted through breastmilk and home to the offspring's

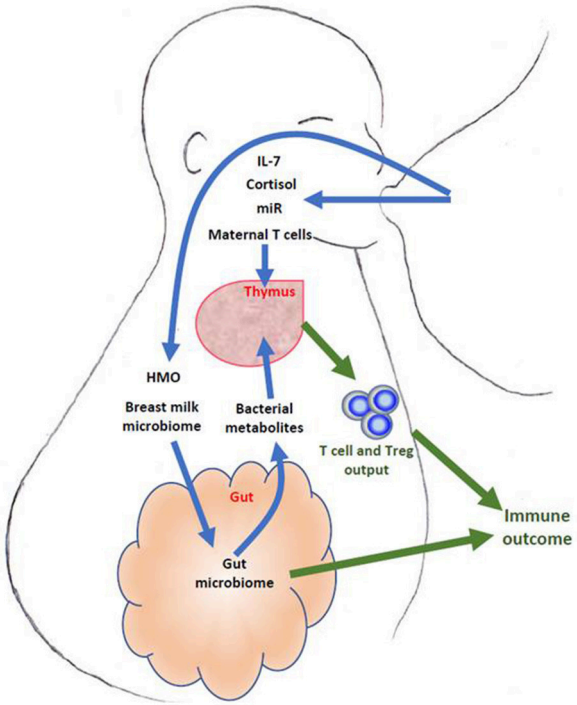

FIGURE 1 | Schematic description of breast milk components that may influence fetal thymic development and output. Some such as IL-7, micro-RNA (miR), hormones and maternal T cells may act directly through the thymus, whilst others such as breast milk microbiome, human milk oligosaccharides (HMO) may act via the gut microbiome and its metabolites.

thymus. Here they appear to play an important role in educating the CD8+ T cell compartment. This might be an important mechanism to prepare the offspring against pathogens like mycobacteria or other persistent intracellular pathogens, which the offspring might encounter in the shared maternal environment (50). Another murine study showed that maternal cytotoxic T lymphocytes in breast milk are able to localize to the Peyer's patch of the nursed infant (51). Overall it is conceivable that maternal immune cells, especially T cells, may be passively transferred to the infants via breast milk. There is some evidence at least in the animal model that these maternal T cells may traffic to the infant thymus, where they may educate and influence infant T cell repertoire, thereby potentially affect infant immune outcomes.

Hormonal levels in human breastmilk may also have an important potential to influence thymic development. For example, corticosteroids are known to regulate thymic epithelial differentiation and the formation of Hassel bodies (52). Cortisol level varies significantly in breastmilk depending on the stress levels of the mother and the circadian rhythm (53), therefore these factors may also influence the infant thymic development. In addition, the actual act of breastfeeding and nursing might also have a soothing effect on the suckling child and hence influence the infants own corticosteroid output impacting thymic function. Indirect evidence for this was provided in a mouse model where hand- but not brush-stroked mice demonstrated a significant increase in thymic and splenic T cell number (54).

EFFECTS OF BREAST MILK ON THE GUT MICROBIOME

Human breast milk itself contains a significant bacterial load which influences the colonization and development of the infant gut microbiome (55). In addition, human milk oligosaccharides (HMO) may help to establish a healthy gut microbiome in the infant by regulating the gut epithelium, immune cells and micro-organisms (56). Among the many metabolites produced by the gut microbiome, short chain fatty acids (SCFA) have been shown to exhibit powerful immunomodulatory effects especially on T cells. Most recently it has been shown that high fiber diet during pregnancy and lactation fuels the production of SCFA in mice, which may act directly via SCFA receptor in the thymic microenvironment to enhance thymic output of Treg cells (57). Therefore breast milk may potentially modulate infant thymic development by influencing infant microbiome composition and bacterial metabolite production.

SUMMARY AND FUTURE DIRECTIONS

Overall, there is significant evidence from animal models that breast feeding may influence infant thymus and hence T cell development. However there are currently no data regarding whether such thymic changes may influence long term immune outcomes. Further investigations into the possible influence of maternal breast milk on human thymic development are required. Many bioactive components of the breast milk including cytokines, hormones, micro-RNAs, oligosaccharides, as well as active maternal immune cells and mammary microbiome, may modulate the infant immune development through mechanisms both dependent and independent of the thymus. The main concepts are summarized in **Figure 1**. However most of the mechanistic studies have been performed in animal models, where milk components and their effects may be very different. Furthermore, there is a lack of direct evidence linking the effect of breast milk components to thymic output and infant immune outcomes. This is partially due to the lack of specific and systemic approaches in the measurement of thymic output and breast milk components in parallel with long term immune outcome follow-ups. Future well-designed longitudinal studies would be required to address these issues. These studies may enable us to design strategies to modulate lactational immune programming for the primary prevention of immunopathologies such as autoimmune disease and allergies.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Diversity of Human Milk Oligosaccharides and Effects on Early Life Immune Development

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One of the well-known features of human milk, is the capacity to protect against the risk and impact of neonatal infections, as well as to influence the onset of allergic and metabolic disease manifestations. The major objective of this review is to provide a detailed overview regarding the role of human milk, more specifically the diversity in human milk oligosaccharides (HMOS), on early life immune development. Novel insights in immune modulatory effects of HMOS obtained by *in vitro* as well as *in vivo* studies, adds to the understanding on how early life nutrition may impact immune development. Extensive description and analysis of single HMOS contributing to the diversity within the composition provided during breastfeeding will be discussed with specific emphasis on immune development and the susceptibility to neonatal and childhood infections.

Keywords: human milk oligosaccharides, mucosal immunity, tolerogenic dendritic cells, infections, early life nutrition

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THE PROTECTIVE EFFECT OF BREASTFEEDING AGAINST INFECTIONS

It has been long noted that breastfeeding protects newborns against infections. Infant formula has been developed over many decades into adequate nutrition for those infants who cannot receive human milk. However, even modern infant formulas lack many components tailor made by each mother for the immune imprinting of her baby, such as specific antibodies (based on the immunologic history of the mother) and human milk oligosaccharides (HMOS) (based on the mother's specific genetic makeup regarding e.g., Lewis (Le) blood group and secretor (Se) status). Exclusive breastfeeding until the age of 4 months followed by partial breastfeeding is associated with a reduction in respiratory and gastrointestinal infectious diseases (1). For example, infants admitted to the hospital with Respiratory Syncytial Virus infection (RSV) are less likely to have been breastfed (2, 3). Similarly, infants who are not exclusively breastfed at 6–8 weeks of age, have a higher risk of hospitalization in early life in relation to a wide range of common infections (2). On the contrary, specific prebiotic oligosaccharides [like short chain galacto- and long chain fructo-oligosaccharides (scGOS/lcFOS)] are already added in a 9:1 ratio to plain infant formula and have been shown to reduce the development of atopic eczema and allergies as well as reduce the impact of pediatric infections (4, 5). Therefore, further optimization of infant nutrition when breastfeeding is unavailable is a principal factor required to further support immune development in early life.

The protective effect of human milk is postulated to be achieved by several mechanisms including the provision of pathogen specific maternal antibodies. This will provide the infant with pathogen specific protection during the first months of life, in which infant's own B cell development has not reached its full potential. Specifically, within the first years of life, the B cell repertoire matures upon encounter of pathogens, eventually providing a full range of protection against the recurrent pathogens. The immunoglobulins in human milk possess a broad range of pathogen specificity, which mirrors the maternal antigenic state. In addition, the concentration of soluble IgA (sIgA) are remarkably high and variable, and correlate to levels of IgG and IgM detected within different regions (6). At birth (for example via vaginal delivery as well as during breastfeeding) the neonate encounters a large variety of microorganisms which are determinant in the establishment of the microbiome in adult-life (7, 8). The initial colonization follows successive steps and is altered through the first year of life by diverse factors including genetic as well as environmental factors such as, introduction of oral feeding. Human milk was shown to stimulate healthy intestinal microbial diversity which includes colonization of several Bifidobacteria and Lactobaccillus species, which in turn, will result in the development of a balanced metabolic response (8, 9). In addition, human milk provides direct support to further development of the immune system in the neonate (10, 11).

The immune system of the neonates needs to adapt and respond to diverse stimuli encountered in early life. Immune homeostasis is determined by the cross-talk between exposure to the mucosal surfaces encompassing cross talk between epithelial cells and underlying immune cells (12). Human milk contains diverse factors like HMOS, milk epidermal growth factor or vitamin A, which contribute to the development of the neonatal mucosa and thus, to the promotion of the neonatal immune system by counterbalancing the deficiencies in early life. These variations are designated as poor IgA production, defective antimicrobial peptide secretion, lack of epithelial chemokine secretion as well as increased permeability among others (6, 10, 13).

Beyond functional components in human milk also the intestinal microbiota can help to further develop these aspects of the mucosal immune system (2). This emphasizes the relevance of a healthy intestinal microbiome diversity for adequate immune development in the first years of life (9). Next to the various classes of pathogen specific immunoglobulins in human milk, the presence of antimicrobial molecules, including specific non-digestible free carbohydrate structures and other molecules like glycoconjugates in breast milk have been shown to bind to pathogens (14). The nutrient source in early life, in particular the non-digestible human milk oligosaccharides (HMOS) in case of breastfeeding are of importance for healthy neonatal microbial colonization (7, 8, 12), immune development (15–19), as well as B cell development (20). This review aims to reveal the current state of knowledge regarding the immunomodulatory properties of HMOS and its unique complexity with differences in short chain as well as long chain structures. Recently some of these structures have become available via manufacturing procedures, and it might be considered to apply these in future generations of infant milk formula (21).

Abbreviations: 2′-FL, 2′-Fucosyllactose; 3′-SL, 3′-Sialyllactose; 6′-SL, 6′-Sialyllactose; APC, Antigen-presenting cells; DC, Dendritic cell; DC-SIGN, Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin; FoxP3, Forkhead box protein 3; FUT 2, Fucosyltransferase 2; HMOS, Human Milk Oligosaccharides; lcFOS, Long Chain Fructo-oligosaccharides; MHC-I (II), Major Histocompatibility Complex Class I (II) molecules; SCFAs, Short Chain Fatty Acids; scGOS, Short Chain Galacto-oligosaccharides; tDC, tolerogenic dendritic cells; Th, T-helper cell; TJ, Tight-Junction; TLRs, Toll-Like Receptors; Tregs, Regulatory T cells.

DIVERSITY OF HUMAN MILK OLIGOSACCHARIDE COMPOSITION

HMOS are the third most abundant class of biomolecules found in human milk after lactose and lipids, reaching between 5 and 20 g/L in mature human milk (22). Up to 1% of HMOS are absorbed in the gastrointestinal tract and found available in systemic circulation (23). This diversity and abundance is unique in humans and not seen in other mammals (24). The concentration of total HMOS is subjected to variations dependent on lactational stage (25), maternal nutrition (26), genetic predisposition (27) or even geographic localization and socioeconomic environment of milk donors (28). Although HMOS are composed out of only 5 different monosaccharides, the structural complexity of HMOS encountered in human milk is unique (21). The monosaccharides which are used as building blocks for HMOS are glucose (Glc), galactose (Gal), N-Acetyl-Glucosamine (GlcNAc), fucose (Fuc), and sialic acid (Neu5Ac). These single monosaccharides are conjugated via several linkage types (i.e., glycosidic bonds). With only a few exceptions, HMOS structures do follow a strict building plan (**Figure 1**). Each HMOS structure starts with a lactose unit "Gal (β1-4) Glc" which results from formation of a β1-4 glycosidic linkage between galactose and glucose catalyzed by the lactose synthase protein complex (30). Several tri-saccharides can be synthesized by appending either galactose or fucose to the reducing or non-reducing end of the lactose residue, which is performed through galactosyl- or fucosyl-transferase activity. Resulting components are e.g., 3′-galactosyllactose (Gal(β1-3)Gal(β1-4)Glc), 4′-galactosyllactose (Gal(β1-4)Gal(β1-4)Glc), 6′-galactosyllactose (Gal(β1-6)Gal(β1-4)Glc), 2′-FL (Fuc(α 1-2)Gal(β1-4)Glc), and 3-fucosyllactose (3-FL) (Gal(β1-4)[Fucα1-3]Glc). If sialic acids are connected to the non-reducing end of lactose via sialyl-transferases, 3′-sialyllactose (3′-SL; Neu5Ac (α 2-3)Gal(β1-4)Glc) and 6′-sialyllactose (6′-SL) (Neu5Ac(α 2-6)Gal(β1-4)Glc) are formed. Further elongation of lactose via the free 3-OH group of galactose can occur by addition of Gal (β1-x)GlcNAc units of either type I (Gal (β1-3)GlcNAc, Lacto-N-biose) or type II (Gal (β1-4)GlcNAc, N-Acetyllactosamine). Up to now, 19 different human milk oligosaccharide core structures have been described. These core structures may be linear or branched and can be further decorated with fucoses or sialic acid residues. Which indicates a myriad of different HMOS structures produced in the human mammary gland. The

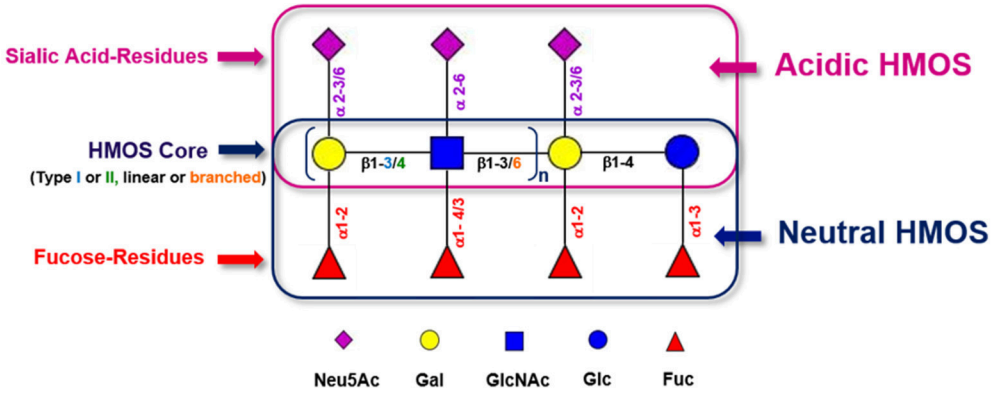

FIGURE 1 | Generic building scheme of HMOS. Lactose and Type I (Gal (β1-3)GlcNAc-R) or Type II (Gal (β1-4)GlcNAc-R) HMOS core structures can be further extended linearly by adding additional Gal-GlcNAc building blocks to terminal Galactoses via β1-3 glycosidic linkages or via β1-6 glycosidic linkages. In the latter case, branching of the HMOS structure occurs. The (elongated) HMOS core structures can be further decorated with Fucoses (Fuc) and/or Sialic Acid (Neu5Ac) residues following distinct rules. Symbolic representation of monosaccharides according to CFG guidelines (29).

cellular localization of HMOS synthesis in the mammary gland epithelium is believed to be the Golgi apparatus.

Among other early life factors, the individual maternal genetic disposition has a huge influence on the HMOS profile of human milk. More specifically, the individual expression pattern of Lewis (Le) and Secretor (Se) gene alleles codes for different fucosyl-transferases (FUTs), as shown in **Table 1** The activity of these FUTs can lead to fucosylation of lactose and various other human milk core structures as indicated.

An active Se gen codes for FUT2 which transfers fucose via an α 1-2 glycosidic linkage. Prominent HMOS resulting from FUT2 activity are e.g., 2′-FL and LNFP I. Glycans like LNFP I which are carrying the reducing terminus Fuc (α1-2)Gal (β1-3)GlcNAc belong to the group of Le^d or H type 1 antigens. H type antigens link the Le/Se system with the blood group ABH system (31). In contrast, an active Le gene codes for FUT3 which in turn enables fucosylation via either α1-3 or α1-4 glycosidic linkage. FUT3 related structures are e.g., LNFP II and LNFP III. LNFP III is also an example for an $Lewis^x$ (Le^x) structural motif, whereas LNFP II represents a $Lewis^a$ (Le^a) epitope. Le^a epitopes are characterized by the carbohydrate sequence Gal (β1-3)[Fuc (α1-4)]GlcNAc-R. Le^x-antigens contain type II structures with the following residue: Gal (β1-4)[Fuc (α1-3)]GlcNAc-R. If both, Se and Le genes are active, fucosylated HMOS structures bearing either one, two or all the possible types of fucosylation (i.e., via α 1-2, α 1-3, and α 1-4 glycosidic linkages) can occur. Lacto-N-difucohexaose I (LNDFH I) which also resembles a $Lewis^b$ epitope with the monosaccharide motif Fuc (α1-2)Gal (β1-3)[Fuc (α1-4)]GlcNAc-R, is a known metabolite of joined FUT2 and FUT3 activity. It is noteworthy to mention that also other, Le/Se-system independent fucosyl-transferases may contribute to formation of α 1-3-fucosylated HMOS such as 3′-FL or LNFP V.

The complexity and relative abundance of different HMOS contained in human milk can for instance be characterized by size exclusion chromatography (SEC) and coupled refractive index detection (RI). A resulting SEC-RI trace is shown in **Figure 2.** Even more detailed information about complexity and individual monosaccharide compositions of HMOS could be derived by a subsequent MALDI-MS (33) analysis of individual SEC HMOS fractions. The acidic sub-fraction adds a further dimension to the overall variety of HMOS. The total number of neutral and acidic HMOS structures based on the MALDI-MS analyses of total human milk carbohydrate SEC-fractions is estimated to exceed the number of 1,000 different structures (24).

Based on the Le/Se status of the mother and specifically the related fucosylated HMOS structures found in the respective human milks, milk group systems of 4 different milk types have been defined (27). Therefore, it is possible to determine individual human milk types by probing presence of specific fucosylated HMOS like 2′-FL, DFL, LNFP I, LNFP II, LNDFH I, and LNDFH II with suited analytical means. An overview of the relationship between maternal Le and Se genotype and some major HMOS structures present in the respective milk types is given in **Table 1**. A recent review has summarized most of the qualitative and quantitative approaches to characterize the diversity of HMOS structures present within human milk (21).

BIOLOGICAL FUNCTIONS OF THE DIFFERENT HMOS

The presence of the unique diversity of HMOS, suggests different biological functions and mechanisms by which they may influence the infant's microbiome and immune maturation and their susceptibility to infections as summarized below and shown in **Figure 3**.

The topics exemplified in **Figure 3** are further substantiated point by point in the following section.

Antimicrobial and Antiviral Effects of HMOS

HMOS play a role in the prevention of infections in breastfed infants by direct blockage of viral and bacterial cellular pathogens

TABLE 1 | Relationship between maternal genotype and exemplified Le- or Se- related major HMOS expected to be present in milks of respective milk types.

Maternal genotype		Frequency in France/Europe (31)	Prominent HMOS expected in milk group	Milk group
Secretor	Lewis			
Se/–	Le/–	69	2′-FL, 3-FL, DFL, LNT, LNnT, LNFP I, LNFP II, LNFP III LNDFH I, LNDFHII, 3′-SL, 6′-SL	Type I
se/se	Le/–	20	3-FL, LNT, LNnT, LNFP II, LNFP III, LNDFH II, 3′-SL, 6′-SL	Type II
Se/–	le/le	9	2′-FL, 3-FL, DFL, LNT, LNnT, LNFP I, LNFP III, 3′-SL, 6′-SL	Type III
se/se	le/le	1	3-FL, LNT, LNnT, LNFP III, 3′-SL, 6′-SL	Type IV

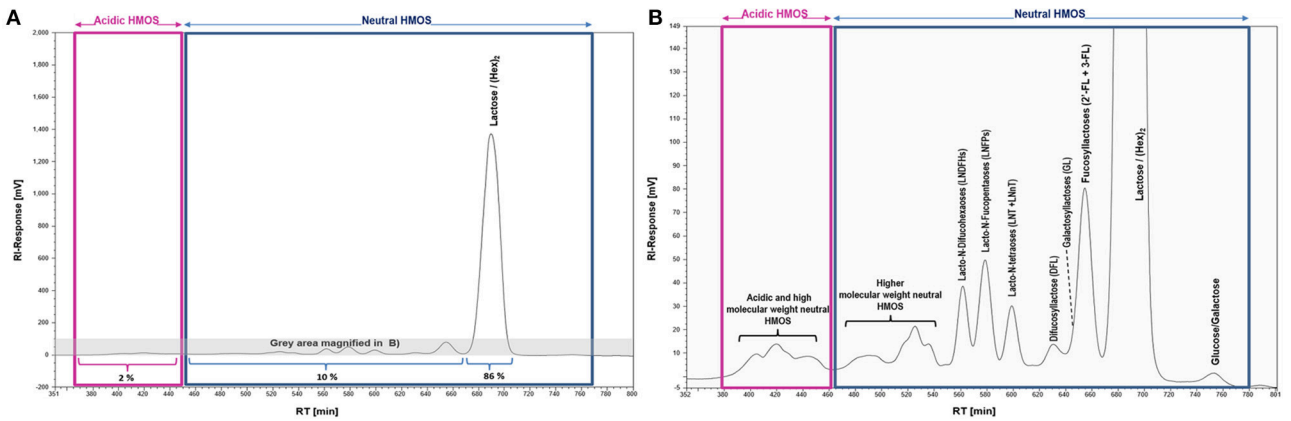

FIGURE 2 | SEC-RI profiles of the total HMOS and mineral fraction from pooled human milk; **(A)** Full SEC-RI Profile, 86% of compounds detected by RI consist of Lactose/$(Hex)_2$, 10% of other neutral HMOS and 2% of acidic HMOS; **(B)** Magnified section of **(A)** zooming into acidic and neutral HMOS; HM sampling, pooling, isolation of the total HMOS fraction and SEC-RI analysis have been performed as described earlier (32).

and toxins infection by mimicking cell entry receptors (34–36). The first mechanisms by which HMOS may exert their anti-infective properties are through the inhibition of virus binding to the host cells by mimicking viral receptors and/or by blocking virus entry into the cell, as well as intracellularly, by blocking viral replication. The anti-infective potential of HMOS has been demonstrated for both neutral as well as acidic HMOS, for example different strains of Norovirus have affinity for specific HMOS structures (35, 37). In addition, both sialylated and fucosylated milk oligosaccharides reduced the infectivity of rotavirus (38). Interestingly, HMOS with multiple Le^x epitopes were shown to inhibit HIV-1 transfer to CD4+ T lymphocytes more efficiently than other HMOS structures (39). HMOS may also block microbial pathogen entry, since HMOS from pooled human milk were shown to significantly reduce *Escherichia coli* attachment to cultured epithelial cells (40). Likewise, it has been shown *in vitro* that LNT, or its fucosylated derivative LNFPI, both can inhibit the growth of Group B Streptococci (41). Moreover, the presence of 3′-FL within the complex mixture of HMOS structures has been inversely correlated with Group-B Streptococci abundancy in infants (42). In addition, α(1-2)-fucosylated HMOS like 2′-FL, or LNDFH I may reduce of early life diarrhea incidence and severity, via their ability to block specific diarrhea inducing pathogens (43).

Prebiotic Effect of HMOS

Development of selective bacterial strains is subjected to their capacity to metabolize HMOS (44). The role of microbial modulation i.e., the prebiotic capacity of specific HMOS structures have in addition been subject of extensive studies. More specifically, secretor positivity of mothers, hence expressing FUT2 and therefore able to produce α(1-2)-glycosidic-fucosylated HMOS, have been shown to affect the gut bifidobacterial communities of breastfed infants (45). Bifidobacteria and Bacteroides species are known to metabolize HMOS with high efficiency in contrast to other bacterial species such as *E. coli, Clostridia, Eubacteria, Enterococci* (44). This appears strain specific and selective for specific HMOS structure (44, 46, 47). For example, *Bifidobacteria* exhibited strong growth stimulation while expansion of *Clostridium perfringens* and *E. coli* were suppressed within cultures using specific HMOS (like 2′-FL, 3-FL, and LDFT), whereas Enterobacteria could not grow on 2′-FL or 6′-SL cultures (48). In addition, utilization of fucosylated type human milk oligosaccharides by isolated human gut microbes was shown (49). These data indicate selective and specific prebiotic capacities of different functional HMOS structures, showing growth of commensal bacteria such as *Bifidobacteria* at the expense of pathogens, as shown in **Figure 3**. Hence beyond directly blocking viral and bacterial entrance to the host also these prebiotic capacities of

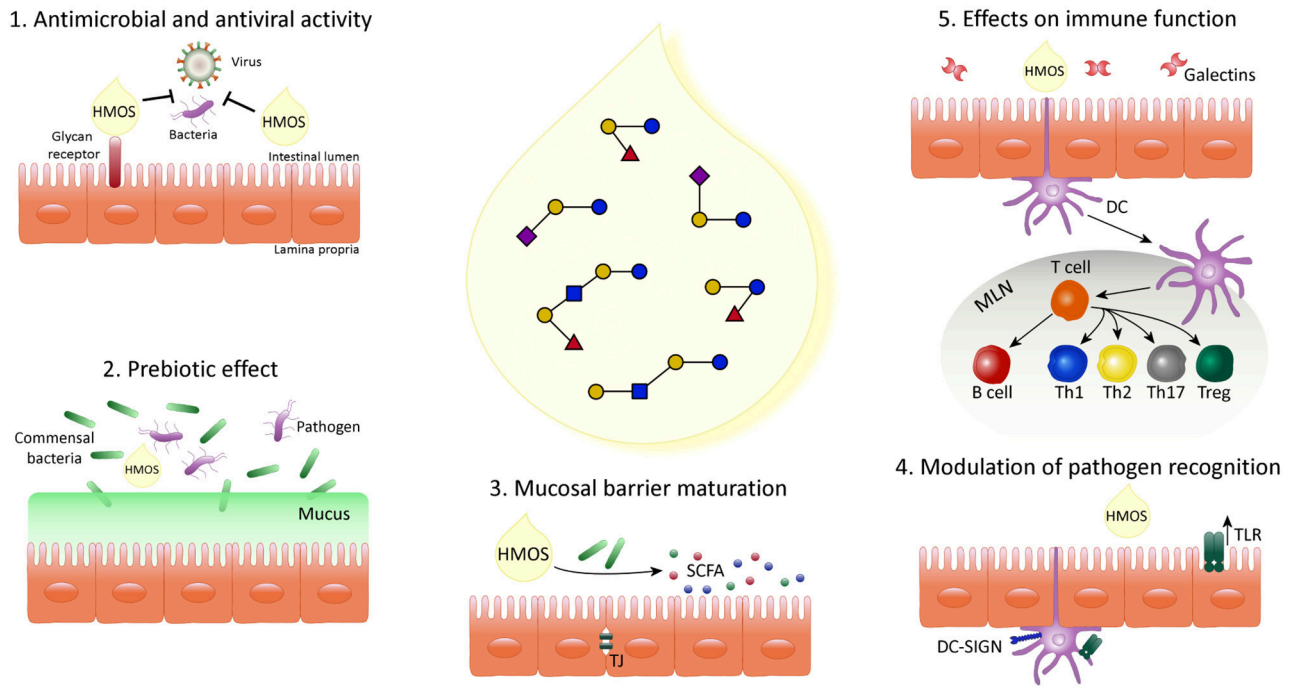

FIGURE 3 | Schematic overview indicating the diversity in structure and function of HMOS. HMOS are composed of a complex mixture of oligosaccharides. This diversity of structures results in various roles in the epithelial cell layer, surrounding mucosa and immune system composing the digestive tract of breastfed infants. (1) HMOS have shown antimicrobial and antiviral effects by binding to virus bacteria, toxins and/or eukaryotes reaching the mucosal surfaces as well as by direct binding to epithelial surface receptors and blocking the access of pathogens. Thus, avoiding their replication and subsequent infection. (2) Commensal bacteria, illustrated as Bifidobacteria, metabolize HMOS and thus, their growth is promoted while pathogens less able to metabolize HMOS will experience growth suppression. (3) HMOS and Short Chain Fatty Acid (SCFA), metabolites of HMOS, were shown to influence intestinal epithelial cell (IEC) maturation by promoting differentiation while suppressing proliferation as well as tight junction development, required for proper intestinal barrier function. (4) Expression of receptors involved in pathogen recognition, such as TLR as well as their signal transduction was increased after HMOS exposure which in turn impacts the immune homeostasis. (5) DC in close proximity to the intestinal epithelial barrier are involved in the immunomodulatory effects described for HMOS. DCs exposed to HMOS play a role in the DC/T cell interaction leading to T cell differentiation and/or T cell/B cell interaction which may occur in secondary lymphoid organs, depicted as mesenteric lymph nodes (MLN), subsequently promoting immune homeostasis.

HMOS may help to reduce the susceptibility to infection of the host.

Mucosal Barrier Maturation by HMOS

HMOS interact with glycans present in the surface of intestinal epithelial cells (IEC) or with dendritic cells (DC) which protrude to the gut lumen from lamina propria. This results in direct support of epithelial barrier maturation or an indirect effect on barrier integrity via modulation of the microbiota and consequent short chain fatty acid (SCFA) production (50). In this regard, beyond blocking pathogen invasion, HMOS may also promote mucosal barrier maturation by increasing the differentiation of IECs. Indeed, synthetic HMOS or HMOS isolated from human milk were shown to promote differentiation and reduce proliferation of various IEC cultures (HT-29 and Caco-2). Similarly, expression of mucosal maturation factors was promoted in fetal intestine cultures after exposure to HMOS isolated from colostrum. These findings suggest that some specific HMOS may be able to promote gut maturation and contribute to epithelial barrier integrity in the gastrointestinal tract of neonates (18, 50, 51).

Modulation of Pathogen Recognition by HMOS

Receptors involved in the recognition of microbes such as toll-like receptors (TLR) are suggested to be modulated by HMOS. Subsequently the response of the host cell to pathogens is altered (17, 37). *In vitro* studies to elucidate the receptors involved in HMOS effects have been performed mostly in cells isolated from adult individuals which might not translate directly to the neonatal situation. Specific HMOS structures have been postulated to modulate bacterial and viral signaling on epithelial cells and/or DC (19). For instance, 2′-FL modulates CD14 expression in human enterocytes, thereby attenuating LPS-induced inflammation *in vitro* (17). On the contrary, HMOS such as sialyllactoses, human galactosyllactoses and/or LNFP III may be ligands for toll like receptors (TLR). For example, TLR-3 signaling seems specifically inhibited by human milk

3′-galactosylactose (52). Moreover, it has been shown that the addition of human milk as well as HMOS interacts directly with DCs, through DC-SIGN, Siglecs and related glycan-binding proteins which are also essential in immune regulation (53–55). DCs are key in directing the adaptive immune response toward effective immunity identification and clearance pathogens. Alpha-fucosylated HMOS (2′-FL and 3-FL) showed specific binding to DC-SIGN (54). Effects of scGOS/lcFOS were suggested to be mediated by TLR-4 (56). Similarly, TLR-4 as well as TLR-3 have also been related to modulate the effects of HMOS. 3′-FL, 2′-FL were able to modulate TLR-3 and elicit an anti-inflammatory effect, while exposure to 2′-FL inhibited inflammation through TLR-4 (52). More specifically it has clearly been shown that the addition of scGOS/lcFOS ameliorates the microbial composition reducing the presence of clinically relevant pathogens (57). Selectins were also suggested as possible receptors for binding of HMOS due to their ability to block P-selectin (58). Several receptors are hypothesized to be involved in the recognition of HMOS. The diversity of HMOS structures present in human milk might determine HMOS-glycan receptor binding. HMOS target TLRs and C-type lectins which are vital in pathogen recognition, immune modulation and essential during development of the immune system in early life. Therefore, HMOS may contribute to the development of a balanced and effective immune response, hereby providing protection toward infections.

Effect on Immune System Development by HMOS

Specific HMOS, such as 2′-FL, 3′-SL, 6′-SL, and LNT have been detected within the intestine as well as in systemic circulation of breastfed infants (23, 59, 60). Increasing evidence collected during the past two decades suggesting a role of HMOS directly on immune cells. Despite all efforts, the effects described remain rather incomplete (19). Nevertheless, it is suggested that HMOS play a role in supporting the developing mucosal and systemic immune system (13, 16). HMOS derived from human colostrum can modulate intrinsic expression of inflammatory markers associated with cell trafficking and modulate signaling pathways related to maturation of lymphoid tissue and influence cytokine and chemokine networks that regulate Th1/Th2 lymphocyte balance. The anti-inflammatory effect of for instance 2′-FL is known. 2′-FL from pooled human milk showed the ability to dampen pro-inflammatory mediator IL-8 release from T84 IEC line after type 1 pili *E. coli* infection (17). Similarly, reduced IL-8 expression was measured in fetal intestinal human tissue when exposed to 3′-, 4- and 6′-galactosyllactoses from human milk colostrum (17, 52). 2′-FL was shown to inhibit the inflammatory mediators secreted after TNFα induced *in vitro*, possibly through the inhibition of NF-κB activation (61). Furthermore, *in vitro* data demonstrate 2′-FL and LNFP I to be able to reduce monocyte activation and to modulate the release of IFNγ, IL-12, and IL-10 (62).

In addition, specific prebiotic oligosaccharides have been demonstrated to be immune modulatory (63–65). Immunomodulatory effects have been demonstrated for 2′-FL, suggesting an additional function of specific oligosaccharides (66–68). However, if these effects also relate to improved infection susceptibility in infants remains to be established. From the *in vitro* based human milk immune cell interaction studies some specific anti-inflammatory effects have been identified.

Galectins are another class of lectins involved in the regulation of immune and inflammatory processes (55). Interestingly, HMOS are reported to bind to various recombinant human galectins like hGal-1, -3, -4, -7, -8, and -9 in a very structure dependent and selective way. Human milk glycans with terminal type I sequences (Galβ1-3 GlcNAc) preferentially bind to hGal-7, whereas hGal-2 did not bind to human milk glyco-types but to a human blood group A Type 2 determinant (55). Beyond serving as a glycan receptor, galectins can also be secreted as soluble mediators and affect immune function. In this regard, IEC derived galectin-9 was increased after exposure of IEC to a mixture of scGOS/lcFOS in combination with a TLR-9 ligand in an *in vitro* co-culture model of IEC and activated immune cells (69). Galectin-9 played a key role in enhancing IFNγ and IL-10 production by immune cells underlying the IEC in this model (55). Further research will reveal the specific role of galectins in immunomodulation after exposure to HMOS, as well as their similarities with the immunomodulatory properties seen by scGOS/lcFOS. However, it is important to realize that an efficient immune response remains to be mounted against the intruding pathogen. Providing efficient protection, in most cases, will go hand in hand with the induction of inflammation. If an anti-inflammatory response is beneficial in relation to the protection against pathogens, will be pathogen and host specific, and can only be elucidated *in vivo*.

HUMAN MILK OLIGOSACCHARIDE IMPACT *IN VIVO*

It is the unique complexity of human milk oligosaccharides which leads to the speculation that these abundantly available structures in human milk play a key role in providing protection against infections in neonates. From the limited *in vivo* studies, we know that specific HMOS structures can reduce the interaction of specific pathogens like *Salmonella*, *Shigella*, *Vibrio cholerae*, *E. coli*, Polioviruses, Rotavirus and Respiratory Syncytial virus (RSV) with the host (11, 70). Within some studies, levels of 2′-FL, lacto-N-difucohexaose (LNDFH I), (α 2-linked fucosyloligosaccharide) and ratios between 2-linked and 3-/4-linked oligosaccharide were associated, with presence of specific pathogens like *E. coli*, *Campylobacter* and *Norovirus* (35, 43). Interestingly, the provision of secretory type related complex mixtures of HMOS, have been associated with a direct protection against specific infections (71). Fucosyltransferase 2 non-secretor and low secretor status seems to associate with severe outcomes in premature infants. Meaning that within this study a low secretor phenotype was associated with the onset of NEC, and non-secretor genotype was associated with gram negative sepsis (71). In addition, it has been suggested that FUT2, the regulator of Lewis and ABO(H) antigens in the intestinal mucosa, could be a host genotypic feature affecting

susceptibility to ETEC infection (72). Several intervention studies have reported the functional benefit of adding prebiotic oligosaccharides to infant formula. More specifically, specific prebiotic oligosaccharides have been shown to ameliorate the development of allergies as well as reduce the impact of pediatric infections (4, 5, 73–75). In this regard, the immune modulating effect that seems to decrease the risk on developing atopy and allergy, also may lower the infection risk in neonates, which is suggestive for basic immune modulation early in life. The clinical consequences of specific individual HMOS structures however, remain to be further elucidated (76). The first clinical safety studies are now reported on the use of specific HMOS combinations i.e., 2′-FL and scGOS (60) or the combination of two single oligosaccharides 2′-FL and LNnT (68, 77). Although growth and 2′-FL uptake were similar between formula receiving infants and as seen in breastfed infants, the possible functional benefits regarding immune development and/or infection susceptibility related to a single oligosaccharide are however not extractable from these studies. Therefore, the identification and understanding of protective elements in human breast milk decreasing infant's susceptibility to infection remains limited (75).

In conclusion, components of breast milk (including HMOS) play a key role in the development of the neonatal immune system by preventing pathogen replication, promoting healthy microbial diversity, inducing maturation of intestinal mucosa and by modulation of immune cells as well as pathogen recognition receptors. Currently, there is little understanding about the role of the diverse HMOS structures in optimally inducing microbiome and immune development and consequently how they may provide protection against infections. Therefore, we postulate that HMOS are involved in regulation of mucosal immune and barrier function in multiple ways, although the specific mechanisms remain poorly understood and may be a compilation of the biological functions of individual structures and their interactions. Further investigation into the components of breast milk and their roles in providing protection to infants is required, irrespective of the mechanism by which specific HMOS structures can provide protection toward certain pathogens.

AUTHOR CONTRIBUTIONS

VA-M, AvS, and MM have written the review. LW, JG, and BvL supervised the program. BS made specific contributions to the program with regard to human milk and in particular functional oligosaccharides. All authors listed have approved it for publication.

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REVIEW
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Immunologically Active Components in Human Milk and Development of Atopic Disease, With Emphasis on Food Allergy, in the Pediatric Population

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Breast-feeding is currently recommended to prevent the development of allergic diseases; however, data are conflicting and mechanisms are unclear. The immunomodulatory composition of human milk is poorly characterized and varies between mothers. We and others have shown that high levels of human milk IgA and certain cytokines and human milk oligosaccharides are associated with protection against food allergy in the infant, but it is unclear whether they are responsible for or simply biomarkers of the vertical transfer of protection. Because human milk has pre- and probiotic properties, the anti-allergy protection afforded by human milk may be due to its control on the developing gut microbiome. In mice, murine milk IgA supports gut homeostasis and shapes the microbiota, which in turn diversifies the intestinal IgA repertoire that reciprocally promotes the diversity of gut microbiome; these mechanisms are poorly understood in humans. In addition, several human milk bioactives are immunostimulatory, which may in part provide protection against allergic diseases. The regulation of immunologically active components in human milk is incompletely understood, although accumulating evidence suggests that IgA and cytokines in human milk reflect maternal exposures. This review summarizes the current literature on human milk components that have been associated with protection against food allergy and related allergic disorders in early childhood and discusses the work relating to regulation of these levels in human milk and possible mechanisms of action.

Keywords: breast milk composition, breast feeding, atopic development, IgA, breast milk microbiome, cytokines, human milk oligosaccharides (HMOs), fatty acids

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INTRODUCTION

Breast-feeding is a natural process of providing nourishment to offspring. Human milk is the optimal source of nutrition for term infants during the first 6 months of life as it provides nutrients, antimicrobial factors, and exposure to important immunomodulatory factors infants need to grow, develop, and thrive (1). There are various studies showing that human milk provides defense against infections and development of allergic disease (2, 3). The first few months of life are a crucial window in which the still-developing infant immune system can be influenced, with

breast-feeding allowing for continued exposure to the mother's immune system. This can impact oral tolerance induction and development of allergy (**Figure 1**). However, the immunomodulatory composition of human milk is poorly characterized and varies between mothers.

Many studies have been published investigating the effect of breastfeeding on atopic diseases, though conclusions from these studies were conflicting, with some authors claiming a protective effect, some remaining undecided, and a few even suspecting that breastfeeding might promote the development of atopic diseases (4–6). Systematic reviews and meta-analyses have concluded an overall protective effect of breastfeeding against atopic dermatitis, wheezing/asthma, allergic rhinitis and cow's milk allergy (CMA) in early childhood (7–10), and breast-feeding is currently recommended to prevent allergic diseases (11). A multidisciplinary review of the literature from 1966-2001 by van Odijk et al. reviewed 132 articles discussing early feeding methods and outcome of atopic disease (7). Only 56 of these articles were conclusive and the conclusion of the reviewers was that breastfeeding is protective of atopic diseases (asthma, recurrent wheezing, atopic dermatitis), and the protective impact is stronger in children with atopic heredity. The review also concluded that exposure to small doses of cow's milk during first days of life predisposes to cow's milk allergy (CMA), and in children with atopic heredity, breastfeeding and extensively hydrolyzed formula protect against CMA. A meta-analysis by Gdalevich et al. in 2001 showed that at least 3 months of exclusive breastfeeding protected from eczema and asthma in children with a family history of atopy (12, 13). Development of food allergy was not assessed. This has been reproduced in various other observational studies from Australia, Sweden, and Denmark. (14–16). The Promotion of Breastfeeding Intervention Trial (PROBIT), a large randomized trial from Belarus, was able to promote breastfeeding duration and exclusivity of breastfeeding at 16 hospitals and found that at these sites, infants had fewer gastrointestinal infections and lower incidence of eczema in the first year of life (17). However, the follow-up at 6 years of this same cohort showed a lack of protective effect with this intervention on asthma, eczema, or hay fever (5). The American Academy of Pediatrics Committee on Nutrition and Section on Allergy and Immunology published a clinical report in 2008 concluding that there is evidence that breastfeeding until 4 months, compared with feeding formula made with intact cow's milk protein, prevents (or delays) the occurrence of atopic dermatitis, wheezing and cow's milk allergy in early childhood (9). Interestingly, Katz et al. reported in 2010 in a large-scale prospective population-based study that early exposure to cow's milk protein as supplementation to breastfeeding might prevent IgE-mediated cow's milk protein allergy (18). The Cochrane Database Systematic Review in 2012 by Kramer and Kakuma (3) concluded that with breastfeeding *beyond* 3-4 months, there is no significant reduction in risk of atopic eczema, asthma, or other atopic outcomes demonstrated in studies from Finland, Australia, and Belarus. This was confirmed to be the case for eczema in the retrospective ISAAC Phase Two Study of >51,000 children randomly selected in 21 countries (19). The most recent systematic review by Lodge et al. from 2015 showed the protective effect of more vs. less breastfeeding against risk of asthma in children 5–18 years, especially in lower income countries, and against allergic rhinitis in children ≤5 years (10). There was a

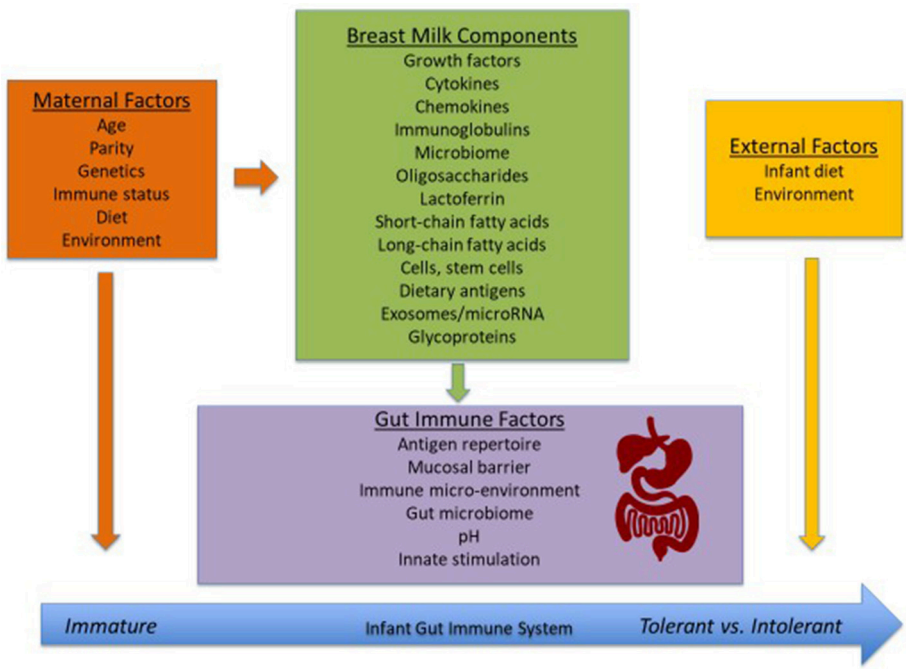

FIGURE 1 | Factors that influence the development of the neonatal immune system.

significant effect of protection against eczema for children ≤2 years by exclusive breastfeeding for 3-4 months. Estimate for an effect of breastfeeding on food allergy had high heterogeneity and low quality. Most recently, a retrospective study in 2016 from Japan noted that cow's milk formula exposure during the first 3 months of life may also have a protective effect on CMA (20). However, data are conflicting, especially given the lack of randomized controlled trials and varied definitions of breastfeeding and allergic outcomes. Unfortunately, most studies have been underpowered for food allergies or not assessed at all due to methodologic problems of making the firm diagnosis. However, among all the atopic diseases, breastfeeding may have the most impact on development of oral tolerance to foods, which develops in the gastrointestinal tract. Epidemiologic studies have not accounted for the human milk composition, which varies from one mother to another, and may be a remarkable confounder impacting its protective properties.

Human milk impacts the development of the infant gut microbiome, along with other maternal and environmental factors. At birth the infant transitions from a highly regulated maternal, microbiota-scarce environment to becoming colonized with *ex utero* microbiota (21). With vaginal birth, the infant microbiota originates mainly from the mother's intestine, vagina and skin, while the hospital environment and the mother's skin provide the first colonizing microbes with C-section birth (21–23). The bacterial colonization of the newborn intestine may contribute to development of the neonatal immune functions or susceptibility to immune-mediated disorders in early (and later) life (6, 24, 25). Evidence from both animal (26) and human studies (27–31) have reported that gut dysbiosis precedes the development of atopy, atopic eczema and food allergy/sensitization. In the past year, several studies have linked the importance of gut microbiome and food allergy. Kourosh et al. sought to better understand fecal microbiome in children with IgE mediated food allergy and were able to show that there were significant differences in microbial composition amongst food-allergic children, especially in the *Clostridia* class, compared with healthy siblings and healthy children (32). Fieten et al. looked for differences in fecal microbiome in children with or without food allergy in the setting of atopic dermatitis (33). Their pilot study showed significant differences in the microbiome profile between these two groups, specifically with *Bifidobacterium breve*, *Bifidobacterium pseudocatenulatum*, *Bifidobacterium adolescentis*, *Escherichia coli*, *Faecalibacterium prausnitzii*, and *Akkermansia muciniphila*. Finally, Fazlollahi et al. looked at the role of gut microbiota in egg allergic children and found a distinction in diversity of microbial flora compared to non-food allergic controls (34). While this data is important for our discovery of the end outcome of atopy, the specific human milk components on microbiome and atopy development are discussed in this review.

Human milk originates in the lactating mammary tissue. Milk lipid, lactose, and the majority of milk proteins are produced in the lactating cells (35). Human milk contains immune cells, immunoglobulins, cytokines, chemokines, growth factors, lactoferrin, oligosaccharides, enzymes (peroxidases, lysozymes), secretory components, and hormones, along with foreign food antigens, bacteria and viruses (6, 36). Several of these bioactive factors have been assessed in relation to development of allergies in the infant, and many of these immunologically active factors in human milk are missing in processed cow's milk and infant formulas, in which the whey to casein ratio is markedly lower than in human milk (37, 38). This review summarizes the current literature on human milk components that have been associated with protection against food allergy and related allergic disorders in early childhood and discusses the work relating to regulation of these levels in human milk and possible mechanisms of action.

CYTOKINES, CHEMOKINES, AND GROWTH FACTORS

Cytokines, which include chemokines, interleukins, interferons, and growth factors, are signaling molecules that function in cellular communication. Human milk is a rich source of immunostimulatory and immunoregulatory cytokines (6, 39). There is variation in the concentration of cytokines among mothers, and overall concentrations for several of those are relatively low in human milk, causing debate in the clinical significance of cytokine levels on health outcomes. Some of the variation in cytokine levels is thought to be due to varying maternal (microbial) exposures. Milk interleukin (IL)-10, interferon (IFN)-γ (40) and transforming growth factor (TGF)β (41) levels have been shown to vary depending on mothers' country of residence, and country of birth (42), and TGFβ as an example is in human milk at a biologically meaningful concentration.

TGFβ is an important regulatory cytokine involved in suppression of both Th1 and Th2 pathways, and is the molecule that has been most studied. The three isoforms of TGFβ combined make it the most prevalent cytokine in human milk, with the most abundant being TGFβ-2 (43, 44). Immunomodulatory cytokines in murine milk, including TGFβ have been shown to influence the development and maturation of the mucosal immune system in neonatal mice and to be associated with the protection against allergic asthma (45). Some studies have confirmed that milk TGFβ is immunologically active, and involved in the induction of oral tolerance, perhaps by inducing increased production of specific IgA (46, 47). Alternatively, TGFβ-2 has been shown to induce maturation of immature intestinal epithelial cells (48). Protection induced by human milk TGFβ has especially been noted in the development of atopic dermatitis (43). This was supported in a review in 2010 by Oddy and Rosales of twelve human studies that determined that 67% of the studies showed a positive association of TGFβ-1 or TGFβ-2 preventing atopic outcomes in infancy and early childhood (49). The study concluded that TGFβ is likely essential in the development of immune responses in infants and may provide protection against adverse immunological outcomes (49). Overall, however, there is conflicting data regarding the role TGFβ in the development of atopic disease in humans (41, 50–56). Most recently, a study by Morita et al. showed that lower concentration of TGFβ-1 in human milk at 1 month, but not TGFβ-2, may be linked to development of eczema (57).

In another study of food allergy, the concentration of TGFβ-1 in colostrum from mothers of infants with IgE-mediated cow's milk allergy was lower than from mothers of infants with non-IgE-mediated cow's milk allergy; however, the levels in healthy controls were found in between (58). The studies are summarized in **Table 1**. A recent study showed that human milk TGFβ was associated with increased richness, evenness and diversity of infant gut microbiome composition (61).

Emerging data regarding the role of other human milk cytokines and chemokines on allergic disease development has been variable. A summary of association between cytokines and the development of food allergy can be found in **Table 2**. Earlier studies using ELISA found that levels of IL-4 are lower and IL-8 and CCL5 (RANTES) are higher in human milk from atopic compared to non-atopic mothers (63, 64), though others found that cytokine levels were largely not related to maternal atopy (6, 65). Various studies report low to undetectable levels of other cytokines and chemokines including IFNγ, IL-2, IL-4, IL-5, IL-10, IL-12, IL-13, CCL5, CXCL8, CXCL10, and TNF-α and have found no association with development of atopic disease despite their involvement in immune and intestinal development (39, 50, 53, 55, 66, 67). Pro-inflammatory cytokines, including IL-1β, IL-6, and IL-8 are also found in human milk in low concentrations. IL-6, IL-8, CXCL8, and CXCL10 in human milk have been shown to be affected by factors such as gestational smoking, maternal race, and season (68) and exercise has been associated with elevated levels of IL-1β and IL-17 (69). There are studies showing that some of these cytokine levels in milk may impact allergic outcomes in offspring. Increased levels of IL-1β in human milk have been shown to be associated with protection against eczema (55). Soto-Ramirez et al. showed that IL-5 and IL-13 levels in milk, although extremely low, are risk factors for asthma at 12 months of age (67). CCL5 in milk was the strongest risk factor for development of atopic dermatitis in the study by Ochiai et al. (65). Because food allergy represents a failure in development of mucosal tolerance to foods, immune factors in human milk may have a more direct effect on development of food allergy (62). In fact, our study showed that a panel of pro-inflammatory and regulatory cytokines including IL-1β, IL-6, IL-10, and TGFβ-1 in human milk were associated with protection against CMA (6, 62). These cytokines promote IgA production, Th17 differentiation and microbiota-driven crosstalk between gut macrophages and RORγt$^+$ ILC-3 population (70). It is unclear whether these bioactive factors are directly related to protection or whether they are biomarkers of another protective mechanism (6).

Other growth factors have also been shown to be present in high concentrations in human milk, including vascular endothelial growth factor, hepatic growth factor, and epidermal growth factor, though the clinical importance is unknown (56, 62, 71). Most recently, a study was conducted by Munblit et al. in which 398 pregnant/lactating women in the United Kingdom, Russia, and Italy were followed prospectively to look for an association between levels of immune mediators in colostrum/mature human milk and allergic outcomes in infants during the first year of life (56). Hepatocyte growth factor (HGF) in mature human milk was protective against common cold incidence at 12 months. Other study outcomes in infants included eczema symptoms, parental-reported food allergy, and recurrent cough/wheeze at 6 and 12 months of age. Results showed higher levels of IL-13 in the colostrum and mature human milk were protective against parent reported food allergy and eczema respectively. IL-2, IL-4, IL-5, IL-10, IL-12, and IFNγ showed no significant association with eczema, wheeze or food allergy (56).

SOLUBLE CD14/TLR

Human milk may also influence neonatal microbial recognition by modulating Toll-like receptor (TLR)-mediated responses specifically and differentially (72). Necrotizing enterocolitis has been shown to be reduced in infants who are breastfed, mediated likely via the lipopolysaccharide (LPS) receptor TLR4 preventing mucosal injury and promotion of repair (73). CD14 is the soluble component (sCD14) of the TLR4, which has a role in innate immunity. It binds to LPS from gram-negative bacteria and intestinal enterocytes. The absence of sCD14 reduces the TLR4 response to LPS. Colostrum is rich in sCD14 with levels decreasing over time whereas neonates lack CD14. Soluble CD14 levels have been found to be lower in colostrum and human milk of mothers with children who develop atopy or eczema, sensitization (6, 74). Later studies, however, deny an association between levels of sCD14 and development of atopy (52, 54). In 2015, Savilahti et al. showed that elevated sCD14 in human milk 3 months post-partum was associated with development of IgE-mediated allergic disease by 5 years of age in children who had hereditary risk of atopy, suggesting that sCD14 in milk influences the emergence of allergy in children with atopic heredity (75). This study contrasted with a study by the same group from 2005 that showed sCD14 levels were lower in *colostrum* of mothers with infants developing atopic symptoms and IgE sensitization than of those of infants with no atopy (51). Studies regarding sCD14 in human milk are summarized in **Table 3**. The conclusions are mixed and there does not appear to be a clear relationship between sCD14 levels in human milk and development of atopic disease.

IMMUNOGLOBULIN A (IgA)

The predominant immunoglobulin in human milk is IgA, most of which is in the form of secretory IgA (SIgA), with smaller amounts of IgG and IgM (6, 76). An older study utilized human milk from a prospective birth cohort of 145 mother-infant dyads oversampled for high risk of food allergies and followed for 12-18 months for development of CMA. The study showed that high levels of human milk total (77) and cow's milk-specific IgA (78) were associated with protection against CMA, consistent with other reports (79, 80). While the exact function of IgA in human milk is unknown, it is thought to supplement infant IgA production, which only commences after birth (78, 81). Data from several studies support a role for maternal environment (geographic location, microbial pressure, exposure to farm animals and cats) in driving milk IgA levels and

TABLE 1 | Studies pertaining to TGFβ in human milk and development of atopic disease.

Study	Year	Location	Size	Duration/Age	Outcomes
Kalliomaki et al. (43)	1999	Finland	$n = 47$	Up to 12 months	Increased TGFβ-1 and 2 levels in colostrum were associated with higher post weaning-onset atopic disease
Saarinen et al. (58)	1999	Finland	$n = 6209$	Up to 12.7 months	Increased TGFβ-1 levels in colostrum are associated with infants who develop IgE-mediated cow's milk allergy versus non-IgE-mediated cow's milk allergy; healthy controls were found in between
Bottcher et al. (50)	2003	Sweden	$n = 53$	Up to 2 years	TGFβ-1 and 2 levels were not significantly associated with eczema, salivary IgA, or allergic sensitization
Oddy et al. (59)	2003	Australia	$n = 243$	Infancy	Increased TGFβ-1 is associated with lower risk of wheeze in infancy
Savilahti et al. (51)	2005	Finland	$n = 4674$	Up to age 4 years	TGFβ-1 and 2 levels were not significantly associated with atopy development
Snijders et al. (52)	2006	Netherlands	$n = 315$	Eczema (up to 12 months), Wheezing (up to 2 years), Allergic sensitization (up to 2 years)	No significant association of with TGFβ-1 and development of eczema, wheezing or allergic sensitization
Bottcher et al. (60)	2008	Sweden	$n = 54$ (*L. reuteri*) $n = 55$ (control)	Up to 2 yeas	Decreased TGFβ-2 in colostrum is associated with lower incidence of allergic sensitization and a trend of protective effect on eczema development
Kuitunen et al. (53)	2012	Finland	$n = 364$ (colostrum) $n = 321$ (BM)	At 2 years of age	Increased TGFβ-2 is associated with higher risk of allergic disease and eczema
Ismail et al. (54)	2013	Australia	$n = 79$	Up to 12 months	TGFβ-1 level was not significantly associated with eczema or allergic sensitization
Orivuori et al. (41)	2014	Finland, France, Germany and Switzerland	$n = 610$	Eczema (up to 4 years), asthma (up to 6 years), allergic sensitization (up to 6 years)	TGFβ-1 level was not significantly associated with eczema, asthma, or allergic sensitization
Jepsen et al. (55)	2016	Denmark	$n = 223$	Up to 3 years	TGFβ-1 level was not significantly associated with recurrent eczema or wheeze
Munblit et al. (56)	2017	United Kingdom, Russia and Italy	$n = 398$	Up to 6 months	Increased TGFβ-2 is associated with higher risk of eczema
Morita et al. (57)	2018	Japan	$n = 43$ (eczema) $n = 53$ (control)	Up to 6 months	Lower TGFβ-1 ratio (1-month milk/colostrum) is associated with higher risk of eczema

specificity (40, 41, 82). Some studies have shown a link between high IgA levels and protection for the development of atopic dermatitis (41, 51) while other studies show no link between sIgA and development of other atopic diseases (41, 50, 54).

Mucosal IgAs are produced by plasma cells in the gut lamina propria and are transported across epithelial cells by the polymeric immunoglobulin receptor (pIgR) (83). Human milk IgA is produced by mammary gland B cells that have migrated from the mother's intestine via the "enteromammary link" (84, 85), as shown in animal studies (86–89). This is controlled by the mucosal vascular addressin MadCAM-1 or mucosa-associated epithelial chemokine CCL28, which interacts with the gut homing receptor $\alpha_4\beta_7$ integrin (90) and mucosa-associated CCR10 (91). Consistent with this, in a rabbit model either oral or inhaled RSV resulted in RSV-IgA production in milk, bronchial and enteral secretions, whereas systemic immunization did not (92). Studies in humans (93) showed that oral immunization in women resulted in an increase in plasma cells in milk, but not in saliva or serum, (85). This forms the hypothesis that human milk IgA reflects the antigenic exposure of the mother's gut to dietary proteins as well. Using the cohort mentioned above, it was shown that a strict maternal diet restricting cow's milk was associated with lower levels of sIgA levels in human milk than cow's milk-containing diet (78). This implies that the antigenic stimulation encountered by the maternal gut directs the antibody specificity of human milk (85). In order to further understand the regulation of IgA in milk, epitope-specific binding of IgA in milk was compared to paired maternal serum samples (85). This revealed that IgA in human milk had partially different epitope specificity to cow's milk antigens than IgA in serum, suggesting different pools of antibody-producing lymphocytes controlling serum and human milk antibodies, respectively, and therefore supporting evidence for enteromammary milk. In summary, IgA levels expressed in human milk are influenced by many maternal factors, including diet, location, exposures, microbiota, and likely plays a protective role against development of cow's milk allergy.

TABLE 2 | Summary of association between cytokines and the development of food sensitization/allergy.

Study	Year	Location	Size	Duration/Age	Cytokines assessed	Food allergy development
Bottcher et al. (50)	2003	Sweden	$n = 53$	Up to 2 years	IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, IL-16, IFN-γ, TGFβ-1, TGFβ-2, RANTES, eotaxin	No significant association
Snijders et al. (52)	2006	Netherlands	$n = 315$	Up to 2 years	IL-12 or TGFβ-1 (IL-10 undetectable)	No significant association
Kuitunen et al. (53)	2012	Finland	$n = 364$ (colostrum) $n = 321$ (3 month HM)	At 2 years of age	IL-10, TGFβ-1	No significant association
Järvinen et al. (62)	2015	Finland	$n = 145$	Up to 2 years	IL-1α, IL-1β, IL-6, IL-10 PDGF-BB, CCL27, VEGF, TSLP, CCL11, CXCL10, and CXCL11, CCL22, TGFβ-1, (TNF-a and -b, CCL1, CCL17, IL-31, eotaxin 3, CXCL9, IL-5, GM-CSF, and IL-12p70 undetectable)	IL-1β, IL-6, IL-10, and TGFβ-1 in human milk showed association with cow's milk tolerance
Munblit et al. (56)	2017	United Kingdom, Russia and Italy	$n = 398$	Up to 6 months	IL-2, IL-4, IL-5, IL-10, IFNγ, IL-12, IL-13, HGF, TGFβ-1, TGFβ-2, TGFβ-3	IL-13 associated with protection, otherwise no significant association

HM, Human milk.

TABLE 3 | Studies pertaining to sCD14 in human milk and development of atopic disease.

Study	Year	Location	Size	Duration/Age	Outcomes
Jones et al. (74)	2002	United Kingdom	Varies (multiple cohorts)	At 6 months	Decreased sCD14 in 3 month HM is associated with higher eczema incidence
Oddy et al. (59)	2003	Australia	$n = 243$	Up to 12 months	sCD14 levels in 2 week HM showed no significant association with infant wheeze
Savilahti et al. (51)	2005	Finland	$n = 4674$	Up to 4 years	Decreased sCD14 levels in colostrum were associated with a higher incidence of allergic sensitization and eczema
Snijders et al. (52)	2006	Netherlands	$n = 315$	Eczema (up to 12 months), wheeze (up to 2 years), or allergic sensitization (up to 2 years)	sCD14 level in 1 month HM was not significantly associated with eczema, wheeze, or allergic sensitization
Ismail et al. (54)	2013	Australia	$n = 79$	Up to 12 months	sCD14 level in 1 and 4 week HM was not significantly associated with eczema or allergic sensitization
Savilahti et al. (75)	2015	Finland	$n = 260$	Up to 5 years	Increased sCD14 level in 3 month HM is associated with higher incidence of allergic sensitization and eczema

HM, Human milk.

MICROBIOME

Infant microbiome composition is influenced by breastfeeding (94, 95). Human milk can modify the infant microbiome directly through seeding from the maternal microbiome and through the other effects of human milk. Diversity of the infant gut microbiome develops in the first 2 years of life and *Bifidobacteria* dominate throughout the first year (96). Recent studies have shown that host genetics, prenatal environment and delivery mode can shape the newborn microbiome at birth [reviewed in (97)]. Following this, postnatal factors, such as antibiotic treatment, diet and environmental exposure, further modulate the development of the infant's microbiome and immune system. Living on farms, avoiding antibiotics, vaginal delivery, and other environmental factors leading to greater diversity in the microbiome have been associated with a major reduction in the risk of atopic diseases(98, 99). Several large studies have confirmed the role of breastfeeding in determining the gut microbiome. Initially there is lower microbiome diversity with breastfeeding, as human milk selects for a highly adapted intestinal microbiota, and when breastfeeding is ceased and complementary feeds start, *Lactobacilli*, *Bifidobacteria*, and *Enterobacteriaceae* are replaced with a microbiota dominated by *Clostridium* and *Bacteroides* species(100–103). The WHEALS birth cohort confirmed that together with the mode of delivery, breastfeeding is one of the most important factors impacting

infant microbiome (95). Interestingly, however, only 12–14% of variability was explained by maternal mode of delivery, exposure to pets, demographics and breastfeeding. This may be partly due to the fact that the human milk biologically active components such as IgA and HMOs, which can modulate microbial composition and function, were not specifically considered. Their concentrations vary between mothers, and this variation is not captured in a coarse definition of breastfeeding.

Several culture-dependent and–independent studies have revealed that colostrum and human milk contain a variety of bacterial communities that colonize the infant's gut. The initial studies demonstrated predominance of staphylococci, lactobacilli, streptococci and propionibacterium, and closely related gram-positive bacteria (104). Culture-independent molecular techniques, especially those utilizing 16S rRNA sequencing have confirmed a similar diversity of bacteria, but also presence of several others including Gram-negative bacteria (6, 105–109). Milk bacterial communities vary between mothers but are relatively stable within individuals (106). Human milk microbiota has been shown to act as a source of bacterial species that colonize the infant gut (110), to be but different from skin suggesting an endogenous route for human milk colonization (105, 111). The amount of bacteria ingested by an infant per 800 mL of milk consumed daily is estimated at 1×10^5-1×10^7, though this is likely an underestimation (112). Recently, it was shown that human milk provides a source of about one-fourth of infant gut microbiota (113).

HUMAN MILK OLIGOSACCHARIDES

Human milk oligosaccharides (HMOs) provide the main substrate for an infant's gut microbiota during exclusive breastfeeding, particularly promoting bifidobacteria and Bacteroides (114–116). Some HMOs have anti-inflammatory properties, and support maturation of the gut mucosal immune system (117). Some also have an inhibitory effect on intestinal cell growth (118), and some bind to dendritic cells through the lectin receptor DC-SIGN (119) inhibiting HIV transfer to T-cells. These oligosaccharides are not digestible by the infant and are extensions of lactose generated by the action of a series of glycosyltransferases. For fucose, two fucosyltransferases FUT2 (secretor gene) and FUT3 (Lewis gene) are implicated. Depending on the Lewis blood group and secretor status, different enzymes are available for the synthesis of HMOs. As a result, human milk from different mothers have significant variations in qualitative and quantitative composition of HMOs. HMO composition is relatively stable during the course of lactation, although it is not known whether minor daily variations are due to the mother's diet (120). This heterogeneity implies that some breast-fed infants are not being exposed to certain structures. Non-secretor mothers, lacking a functional FUT2 enzyme (FUT2−/−), represent 15-25% of mothers depending on their ethnic background (121, 122), and their milk is missing all alpha-2 linked fucose oligosaccharides (21). Infants fed by non-secretor mothers are delayed in establishment of bifidobacteria-laden microbiota (123). Differences in HMOs have also been associated with susceptibility to infectious gastroenteritis (124, 125) and HIV (126–128). In our previous studies, certain HMO profiles were associated with protection against cow's milk allergy (129). Infants who received human milk with low Lacto-N-fucopentaose (LNFP) III concentrations were more likely to become affected with CMA when compared to those receiving milk with high levels ($p = 0.00036$, odds ratio 6.7, 95% CI 2.0–22). Two other studies have assessed the association between HMO and atopic diseases. A study that followed 20 infants for the first 18 months for development of FA, and measured HMOs using HPLC was powered to only find major effects, and indeed did not find a significant difference in HMOs between mothers of allergic and non-allergic children (130). In a second study, infants fed by non-secretor mothers had delayed development of bifidobacteria-laden microbiota (123) and if also born via c-section had a higher risk to manifest IgE-associated eczema (21). However, development of food allergy or composition of individual HMOs were not assessed. These data support the role of HMOs in protection against CMA, possibly through their effect on infant gut microbiome. Most recently, the Canadian Healthy Infant Longitudinal Development (CHILD) study, compared HMO profiles with food sensitization at 1 year of age (131). The study found that lower risk for food sensitization was associated with higher concentrations of fucosyl-disialyllacto-N-hexaose (FDSLNH), lacto-N-fucopentaose II (LNFPII), lacto-N-neotetraose (LNnT), lacto-N-fucopentaose I (LNFPI), sialyllacto-N-tetraose c (LSTc), and fucosyllacto-N-hexaose (FLNH), and relatively lower concentrations of lacto-N-hexaose (LNH), lacto-N-tetraose (LNT), 2′-fucosyllactose (2′FL), and disialyllacto-N-hexaose (DSLNH). Further investigation into HMO composition is necessary to better understand the role of HMOs in pathophysiology and possibly future therapeutics for prevention of atopic disease.

FATTY ACIDS

Milk lipids are principal macronutrients in human milk and studies have shown that milk from atopic mothers varies in fatty acid content. Polyunsaturated fatty acids (PUFAs), more specifically the omega-3 (ω-3) fatty acids, e.g., docosahexaenoic (DHA) and eicosapentaenoic (EPA), have been recently shown to have anti-inflammatory effects in chronic inflammatory diseases, such as asthma (132). On a maternal fish oil supplementation trial, omega-3 PUFA levels were positively associated with IgA and sCD14 levels, suggesting a relationship between fatty acid status and mucosal immune function (133). Another study has shown that atopic mothers' milk has lower levels of n-3 long-chain PUFA at 1 month of lactation than non-atopic mothers (134). Overall, the studies examining the fatty acid profile in human milk as a risk factor for subsequent atopic disease have been mixed, though generally found that n-3 PUFAs in human milk possibly protect against atopic diseases (134–139). The conflicting findings may be due to the complex interactions between different fatty acids types and the divergent functions on immune system based on the dose (6, 140).

More recently, the short-chain fatty acids (SCFAs), including acetate, butyrate and propionate, have been demonstrated as possibly important mediators of allergic inflammation. Inflammation is likely a by-product of the metabolic activity of gut microbiota given that SCFAs are altered in children who are or become overweight or atopic (141). SCFAs are the first metabolites produced by the gut microbiota of newborns, with synthesis increasing rapidly after birth (142). As commensal microbiome has been shown to be protective against food sensitization in animal models (26), this may be due to the SCFAs produced by these commensal bacteria. In mice, experimental data has shown that increased SCFAs, especially acetate and butyrate, may prevent development of food allergy by way of promoting the tolerogenic effect of $CD103^+$ dendritic cells (143). Initial studies have shown that in term infants, total gut SCFA levels are elevated in formula-fed vs. breastfed infants, however acetate levels in particular are highest with exclusive breastfeeding (141, 144). There are no published studies of SCFA levels in human milk.

HUMAN MILK CELLS

A variety of other factors have yet to be better investigated in terms of the impact on the development of inflammation and immunity. Extremely interesting is recent data suggesting that up to 6% of cells in human milk are stem cells, and mesenchymal stem cells isolated from human milk are potentially reprogrammable to many types of tissue (145, 146). These cells may play a role in development of immune cells, including regulatory T cell, which may suppress antimaternal immunity and lead to microchimerism that induce intestinal tissue repair and immune protection (146). Colostrum is specifically also rich in leukocytes, with breastfed infants being exposed to as much as 10^{10} maternal leukocytes per day, and the role of this exposure in immune development in infants is not yet clear (44). One study of 61 mothers and infants did show that macrophage proportion was significantly smaller in the milk of mothers who had infants with cow's milk allergy compared to mothers who had healthy infants, whereas neutrophil, eosinophil or lymphocyte abundant milk noted significantly more often being received by infants with cow's milk allergy (147). There is still much to learn about the effect of these factors in prevention of allergic disease.

DIETARY ANTIGENS

Maternal dietary antigens, including ovalbumin, β-lactoglobulin, gliadin and peanut, have been detected in human milk generally in quantities varying from undetectable levels to 430 ng/ml (148–155). Although their role in inducing symptoms in already sensitized infants has been shown (150), and the ingestion of egg has been associated with immune markers in infants (155), their role in initial sensitization or tolerance development in humans is still debated.

CONCLUSIONS

The immunomodulatory composition of human milk is surprisingly poorly characterized and varies between mothers. The coarse definition of breastfeeding used in epidemiologic studies does not take into consideration the variability in the numerous immunologically active factors in human milk, which may lead to conflicting data regarding the impact of breastfeeding on immune development and downstream implications on development of prevention of allergic disease. Whereas one mother's milk may be rich is immunoprotective factors, another mother's milk may not; however epidemiologic studies do not differentiate between these two very different infant dietary (and microbial) exposures. In addition, randomized controlled trials, with assignments to either breastfeed or not, are lacking, and definitions of breastfeeding and allergic outcomes vary. Unfortunately, most studies have been underpowered for food allergies or not assessed at all due to methodologic problems of making the firm diagnosis.

The studies above suggest that, upon a closer look, the milieu of biomarkers in human milk varies between mothers and the composition may play a function in progression to or prevention against atopy. The impact of human milk biologically active components can be direct or perhaps due to modulation of intestinal microbial composition and function. Most importantly, the factors do not act in isolation, and the study into the impact of a combination or networks of immune factors in human milk on infant microbiome and immune development is still "in its infancy." Better elucidation of the role of these factors could lead to early targets for treatment and prevention of allergic disease. Further and larger well-characterized studies using prospective cohort data would be extremely helpful in determining the most important factors that likely play a role in development of atopic diseases. The above studies shed a guiding light for future areas of research.

AUTHOR CONTRIBUTIONS

PR wrote sections including introduction, cytokines, soluble as CD14/TLR, fatty acids, human milk cells, and conclusions. AS and KJ mentors and editors, wrote abstract and sections on HMOs, IgA, and microbiome.

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MINI REVIEW
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Human Breast Milk: Exploring the Linking Ring Among Emerging Components

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Maternal breast milk (BM) is a complex and unique fluid that evolution adapted to satisfy neonatal needs; in addition to classical nutrients, it contains several bioactive components. BM characteristically shows inter-individual variability, modifying its composition during different phases of lactation. BM composition, determining important consequences on neonatal gut colonization, influences both short and long-term development. Maternal milk can also shape neonatal microbiota, through its glycobiome rich in *Lactobacilli* spp. and *Bifidobacteria* spp. Therefore, neonatal nourishment during the first months of life seems the most important determinant of individual's outcomes. Our manuscript aims to provide new evidence in the characterization of BM metabolome and microbiome, and its comparison to formula milk, allowing the evaluation of each nutrient's influence on neonatal metabolism. This result very interesting since potentially offers an innovative approach to investigate the complex relationship between BM components and infant's health, also providing the chance to intervene in a sartorial way on diet composition, according to the nutritional requests. Future research, integrating metabolomics, microbiomics and stem cells knowledge, could make significant steps forward in understanding BM extraordinary properties and functions.

Keywords: human milk, metabolomics, microbiota, microbiomics, human milk oligosaccharides, preterm, newborn

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INTRODUCTION

Breast Milk (BM) is a precious fluid which has been considered miraculous since ancient times. Its extraordinary properties have been studied in detail, not resulting fully clarified yet. It can confer protection against a large number of pathologies and exerts a beneficial effect on breastfed newborn's development (1, 2).

BM is the most suitable choice for neonatal nutrition, highly recommended as the exclusive component of the infant's diet for almost 6 months of life (3).

Nutrition in the early neonatal period influences the successive whole life, due to its role in the activation of several metabolic processes, for example, microanatomy development, growth, metabolism, gut microbiological colonization and maturation, immunological system development, brain maturation, and organization (1, 4).

In fact, BM has been associated to many beneficial short-term effects, such as a reduction in necrotizing enterocolitis (NEC) and sepsis (5); a positive influence on long-term outcome (such as neurodevelopment) and a protective effect against infections, overweight, obesity, diabetes and malignant diseases incidence have also been described (6).

BM beneficial effects do not regard exclusively infants' health but could also be exerted on lactating mothers, improving their outcome (6).

BM contains several components, such as lipids, carbohydrates, proteins, vitamins, minerals. Oligosaccharides, the third most abundant constituents of BM, which represent a highly variable fraction of BM, exert several important functions, such as the modulation of neonatal gut microbiota composition, influencing many physiological processes (7).

BM is also defined an "alive" fluid, since it provides to the breastfed newborn maternal soluble bioactive components, growth-factors (GFs), hormones, cytokines, chemokines, immunoglobulins (Ig), and immunological-related cells as leucocytes, cells of both bacterial and maternal origin and finally, as recently demonstrated, also multipotent Stem Cells (SCs) able to integrate *in vivo* in many neonatal tissues and differentiate into mature cells. Finally, great relevance can also be attributed to the presence of maternal milk microbiota (1, 2, 5, 7–9).

BM composition has the extraordinary property to vary according to gestational age (GA) of the neonate and to the lactation phase (5, 6, 10).

Since the degree of prematurity highly influences BM features, the resulting composition is optimal for preterm newborns needs. Macro- and micronutrients levels vary and determine advantages regarding immunity, neurological development, gastrointestinal maturation (9, 11–17).

The presence of several cytokines and chemokines, showing a higher concentration in colostrum, has been widely evidenced and could represent an additional mechanism of protection, especially against NEC and sepsis (17, 18).

It is not fully known how maternal or pregnancy factors could modify their level, although in peripartum infections, in spontaneous preterm delivery and in VLBW neonates lower concentrations of pro-inflammatory cytokines have been measured, potentially protecting against mucosal damage or pathogens (19).

Even the cellular composition of BM varies among samples deriving from mothers of preterm and full-term newborns, meeting the necessities showed by premature neonates during the first phases of life (9) and confirming the extraordinary ability of this liquid to modify itself according to the newborns features and assuming the best qualities for his optimal development (5).

BM related SCs belong to different lineages such as mammary epithelial cells, neuroepitelial-like SCs and mesenchimal SCs (10–15%) (20).

It seems that, among large amount of SCs ingested by the newborns each day, some can pass from BM through neonatal gut and migrate into brain and other organs; there, they can persist and proliferate as in a microchimerism, restoring the involved organs, potentially even after a damage (1, 5, 21). This interaction between the dyad mother-child result very interesting but all the implications should be deeper clarified.

METABOLOMICS

The great relevance of micronutrients in BM is highlighted by the increasing number of metabolomic studies performed to characterize its metabolic profile and inter- and intra- individual variations (8, 10, 16, 22–30).

BM can be analyzed through nuclear magnetic resonance (NMR) and liquid or gas chromatography coupled with mass spectrometry (LC-MS or GC-MS), to evaluate its unique profile (5).

The first metabolomic study investigating BM composition was conducted by Cesare Marincola et al in 2012 (22). This group demonstrated different metabolic features characterizing subsequent lactation stages. Moreover, they found higher levels of lactose and lower levels of maltose in BM samples, compared to formula milk (FM) (1, 17, 22).

Interesting results were also obtained by the numerous successive studies, performed by several groups. The most relevant findings are reported below, and they can be found in a more detailed way and summarized in 2 tables in the recent papers published by Fanos et al. (5) and Bardanzellu et al. (17).

According to the findings of Spevacek and colleagues, (23) the highest variability can be found in preterm samples. They identified and measured variations in 69 metabolites and also demonstrated that lacto-N-tetraose and lysine decreased during the maturation of full-term milk (23).

Another group (10) demonstrated that preterm BM metabolome mostly varies within 5–7 weeks postpartum; after this period it would probably obtain the composition of term milk after this time and BM dependence on GA seems to be reduced (5, 10, 17, 31).

Moreover, in colostrum samples from preterm delivering mothers, an increased level of fucosylated oligosaccharides, fucose, N-acetylneuramic acid and N-acetylglucosamine, citrate and creatinine have been shown (10).

The group of Villasenor et al. also demonstrated a different composition between full term colostrum and mature milk and moreover, our research group detected a higher sample variability in colostrum instead of mature milk belonging to extremely preterms (27).

Summarizing these findings, the highest variability has been evidenced during the first three months of lactation (25), with a high dependence on GA (28); a different metabolitic pattern comparing human colostrum with transition milk and mature was observed (23).

BM from mothers of preterm neonates showed a different composition if compared with full-term newborn mothers' BM. In particular, a higher concentration of proteins and aminoacids, promoting cerebral development and energy production, was observed. This reinforces the concept of BM variability according to the breastfed newborn's peculiarities (17).

Among the genetic factors highly influencing BM composition, four maternal phenotypes, depending on both blood group and expression of two specific genes, were identified.

This influence in particular regards human milk oligosaccharides (HMOs), which constitute the third most abundant solid fraction of BM, following lactose and lipids (1.9–4.5%) (7).

These genes are, firstly, alpha-1-2-fucosyltransferase (secretor gene, FUT2) which is codified by *Se* gene and allows the classification of secretors (Se^+) and non-secretors (Se^-) mothers. Secondly, it is considered alpha-1-3-4-fucosyltransferase gene(Lewis gene, FUT3); it indicates positivity or negativity for Lewis Group (Le^+ or Le^-). According to these considerations, maternal phenotypes can be divided in: Se^+/Le^+, Se^+/Le^-, Se^-/Le^+, and *Se*-/Le$^-$, showing significant differences in BM metabolites (1, 17, 32, 33).

In fact, Se^+/Le^+ mother's BM exhibits all the fucosylated oligosaccharides (2′fucosyl-lactose 2′FL, lactodifucotetraose LDFT, Lacto-N-fucopentaose I LNFPI, Lactodifucoesaose I LNDFHI), while the Se^-/Le^+ mother's phenotype determines the production of samples containing a high concentration of HMOs with (α1-3) and (α1-4)-linked fucose residue, in absence of α1,2-fucosylated structures (32, 34–36).

The great interest concerning BM HMOs composition is also related to their potential influence on microbiota (10, 37, 38).

It has been estimated that FUT2 is expressed in more than 70% of the Caucasian women (34). The absence of α1-2-fucosylated oligosaccharides in BM can lead to several pathophysiological consequences, such as a delayed colonization by *Bifidobacteria* spp., a higher abundance of *Streptococcus* spp. and also functional differences of microbiota metabolic activity. According to some authors, Se^+/Le^+ phenotype results protective against some infections, such as *E. coli* and *Campylobacter* spp. and preventive of NEC (35), while infants fed with BM from Se^-mothers would show a higher risk for diarrheal diseases (11, 39, 40).

In accordance with these data, Bazanella and colleagues analyzed BM samples from Se^+ mothers, demonstrating a higher percentage of fucosylated oligosaccharides instead of Se^- mothers, and the presence of *B. longum* exclusively in the stools of Se^+ breastfed neonates (41).

HMOs are known to decrease with milk maturation (10, 27). According to many studies, total HMOs content, sialic acid, lacto-N-tetraose, LNDFH I, 3′- sialyllactose, 6′-siallylactose, fucose, N-acetylglucosamine, N-acetylneuraminic acid resulted higher in preterm milk (10, 23, 42).

In addition to HMO's, also amino acids and lipids showed a great variability across lactation stages and a great dependence on prematurity. Some amino acids increase, while other reduce their concentration during BM maturation (10, 17, 43).

The studies performed in this field allow to conclude that BM, in particular in the first phases and in the samples obtained by premature delivering mothers, is extremely rich in creatinine and amino acids. These factors are involved in two crucial processes, especially for the vulnerable category of preterm babies: brain development and energetic metabolism (17).

In particular, creatinine, betaine, coline, leucine, isoleucine, and valine take part in cerebral maturation (10, 26, 28, 44, 45), while energy production is closely related to the presence of alanine, glutamate, methionine and creatinine (23, 26, 28, 46, 47).

Coline and betaine could play a role in the reduction of cardiovascular diseases (28, 48). Moreover, acetylcarnitine, betaine, lysine, isoleucine, and taurine levels seem to decrease during milk maturation in samples of mother of full term neonates and not in preterm samples (17).

Other detected metabolites may also take part in several immunity processes, hepatic regeneration, lipid and glucidic metabolism (17, 45, 49–51).

It is also been demonstrated that fatty acids' (FA) composition in BM can be influenced by many factors, not fully understood up to now. Among these, maternal age, nationality, parity, body mass index (BMI), diet, newborn's GA, lactation stage, maternal gestational diabetes mellitus, number and duration of breastfed meals and delivery route can be mentioned, although the entity of their influence is not currently attested. The most represented fractions are tryglicerides, palmitic, oleic, linoleic and alpha-linolenic acids (17, 52–60).

FA's content seems to be higher in colostrum from mothers whose neonates' weight was lower than the 20° centile (52, 61, 62) and this mechanism may probably help to compensate the intrauterine growth restriction occurred in these neonates.

According to the analysis of Collado et al. (63), evaluating BM from preterms and full-term delivering mothers, the content of FAs resulted comparable among colostrum and mature milk samples, although different qualitative profiles were found.

Recently, interesting results confirmed the importance of a metabolomic approach to evaluate the differences occurring in newborns fed with BM or formula milk during the early life. Cesare Marincola et al. (64) detected variable urinary profiles in relation to the kind of diet; several and more numerous trials would be needed to fully understand the clinical implications of these findings, improving our knowledge on BM's effects.

Metabolomics also gave promising results analyzing urine and/or blood samples of breastfed neonates or those from their mothers, to understand biological effects of maternal BM. Two recent studies evidenced as different metabolites can be found in urine or blood of breastfed newborns, instead of those described in samples belonging to FM fed newborns (31, 65).

Moreover, the analysis of urine from breastfeeding mothers revealed different profiles too (5, 66).

In conclusion, BM metabolome varies according to GA and lactation phase, depending on neonatal necessities and especially meeting the peculiar requests of the vulnerable category of premature newborns.

MICROBIOMICS

Even if BM was considered sterile for long time, it has recently been demonstrated, through culture-dependent and -independent techniques, that the microbial community in BM from healthy mothers can contain more than 200 phylotypes, belonging to about 50 different genera (2, 67, 68).

The technological advances, particularly the cultivation independent methods, such as 16S gene sequencing, allowed a deeper analysis of bacterial diversity, giving more detailed information on the populations present in several human fluids, such as BM.

In this sampling, although they permitted to demonstrate a bacterial load between two and three orders of magnitude, which resulted higher than those estimated by cultures, from a critical point of view these technique are not able to discriminate DNA sequences from non-vital bacteria and extracellular DNA that could interfere the amplification by quantitative PCR (qPCR). However, it is also clear that the early stimulations coming from all the microbial products, alive or residual, could influence the newborn immune system (69).

According to the current knowledge, how maternal microbes can reach mammary epithelium and undergo secretion in BM, is still matter of debate. Milk harbored bacteria may derive from the contamination with bacteria from mother's skin (such as *S. epidermidis*) and from infant's oral cavity. On the other side it has been postulated that microorganisms from maternal intestinal tract can reach the mammary gland through a vascular transport via intestinal immune cells, especially dendritic cells (the so-called entero-mammary pathway hypothesis). This entero-mammary pathway allows to consider as maternal gastrointestinal bacteria during pregnancy and lactation could directly influence the infant's immune system. Moreover, a retrograde flow of newborn's microbes could occur during nursing (17, 67, 68, 70, 71). However, due to its peculiar characterization, this community is even more considered as a site-specific microbiota, as demonstrated by several anaerobic species that are identifiable and that are not present both in the skin or in the oral cavity (2, 67, 68).

This community is represented, for about half, by a constant core microbiota with a limited variability, being BM microbiota dominated by *Staphylococcus* spp., *Pseudomonas* spp., *Streptococcus* spp., *Acinetobacter* spp., *Finegoldia* spp., *Anaerococcus* spp., *Actinomyces* spp., and *Enterobacter* spp. (67, 69), with huge differences between colostrum and mature milk (68, 69, 72–77).

On the contrary, the other half seems to be highly dependent on maternal factors, such as ethnicity, diet, drug exposure, environmental factor exposure, mode of delivery (67, 69).

BM is an exceptional source of commensal bacteria for breastfed newborns, representing a dynamic ecosystem for several species, which can modify itself during milk maturation, according to the infant's needs (67, 72). In fact, microbiota shows high variability during the subsequent lactation stages, also highlighted by the possibility to detect different microbial-related metabolites (69, 78). After about one month of lactation, BM microbiota reaches the full maturation and its definitive composition, maintaining therefore a relative stability (67).

Composition and metabolic network of BM microbiota may be considered as an epigenetic determinant of neonatal health (78, 79). Moreover, many microbic-related metabolites represent a linking ring between metabolomics and microbiomics. In particular, BM microbiota is known to shape the newborn intestinal microbiota since the early phases of life. Bacterial genera and species present in colostrum and then in mature milk can positively influence intestinal bacterial network, highly rich in *Bifidobacteria* spp. and *Lactobacilli* spp. Bacterial communities produce metabolites, such as short chain fatty acids (SCFA), mostly butyrate, that may be detected in the fluids by metabolomics and are able to influence several health outcomes of the child.

Moreover, an active role is played by sialylated BM HMOs, which can induce transcriptional responses in the intestinal microbiota (i.e., *B. Fragilis*) potentially influencing even other microbial members, such as *E. coli*. Therefore, through several routes and deep interactions not fully clarified yet, this leads to infant growth promotion, beneficial metabolic pathways and effects on several organs (brain, liver, respiratory, and urinary tract) (36, 80).

The effect of early diet on pigs' intestinal microbiota has been also evaluated by Piccolo et al. (81), demonstrating that neonatal nutrition could characteristically induce different effects according to the different small bowel's region, mostly influencing duodenum microbial composition, since microbial network seems to be functionally defined by the intestinal segment (81).

This bioregional effect of nutrition on the shape of intestinal microbiota also influences the production of different molecules, highlighting the strict dependence of metabolic profile from microbial communities and the influence played by microbiota itself on the host tissue metabolism, as demonstrated through metabolomic analysis (81).

Hunt et al. (68) observed as BM microbial community represents a unique fingerprint characterizing each mother's sample. BM and therefore neonatal intestinal microbiota can be influenced by several factors, as reported in **Table 1**, including genetics (such as secretor status, as previously described), delivery route (in relation to newborn's colonization with maternal vaginal microbiota during spontaneous delivery), maternal weight, diet and lifestyle (in relation to ingested foods and even to maternal diseases or metabolic status), antibiotic therapy administered just before delivery (influencing maternal intestinal microbes), environmental factors, lactation stage or GA (recognized as actors influencing intestinal metabolites and HMOs and thus, indirectly, newborn's microbial community), mastitis or maternal dysbiosis (allowing the newborn to become in contact with potentially dangerous microbial communities) (2, 17, 67, 72, 82, 84, 87).

Due to such reasons, maternal intestinal microbiota influences breastfed newborn, modulating its intestinal microbial community. In fact, several factors leading to maternal dysbiosis (such as an overgrowth of a microbial species instead of the others) and/or breast infection, which has been associated to a reduced variability in microbiota composition, can show direct effects on breastfed neonate's health (2, 73, 74).

Among the other factors, even BM lipid composition, in addition to maternal BMI, seems potentially influence BM microbiota (72, 74).

Finally, even the modality of delivery can modify qualitative bacterial composition of BM. For example, *S. Salivarius*, an oral commensal, was detected only in samples collected from mothers who underwent cesarean section (72, 85).

TABLE 1 | Factors influencing the composition of breast milk and/or neonatal intestinal microbiota and some of proposed mechanisms and exerted effects.

Maternal factors	Effects on BM and/or neonatal gut microbiota
Genetics (1, 16, 26, 27)	Secretor status Se^+ (associated with high presence of *Bifidobacteria* spp. in neonatal stools) and non secretor status Se^- (higher percentage of *Streptococcus* spp.), Lewis gene; ethnicity; other factors not completely known.
Lactation phase (54, 57–59, 63)	Modulation of BM metabolites and microbial community, directly influencing neonatal gut microbiota and metabolic network
Breast milk composition (such as oligosaccharides, lipids) (10, 37, 38, 72)	Modulation of BM metabolites and microbial community, directly influencing neonatal gut microbiota and metabolic network. HMOs influence *B. Fragilis*, *E. coli etc…* HMOs and FAs influence *Bifidobacteria* spp. and *Staphylococci* spp. in neonatal gut
Body mass index (5, 82, 83)	Influence on maternal metabolic status
Diet, lifestyle and habits (2, 5, 17, 67, 82)	Influence played by ingested foods, maternal diseases or metabolic status
Delivery route (vaginal, elective or emergency cesarean section) (5, 72, 82–85)	Induce neonatal colonization with maternal vaginal microbiota during spontaneous delivery. *S. Salivarius* detected only in BM samples from mothers undergone cesarean section. Other factors not completely known
Gestational age at delivery (86)	Modulation of BM metabolites and microbial community, directly influencing neonatal gut microbiota and metabolic network
Administration of antibiotics (17, 33)	Influence on maternal intestinal microbiota
Dysbiosis and/or mastitis (2, 17, 73, 74)	Neonatal contact with potentially dangerous microbial communities

It is clear that BM microbiota plays a central role even in the early colonization of neonatal gastro-enteric tract (67, 88), considering that the breastfed newborn swallows about 1 x 10^{5-8} bacteria/day (67–69, 71, 72, 88, 89). Since BM contains both probiotics (such as *Bifidobacteria* spp. and *Lactobacilli* spp.) and prebiotics (mostly HMOs) it can be considered a natural symbiotic mixture (72, 75).

Thus, neonatal commensal bacteria could be involved in gut tolerance modulation, immune system stimulation and may even inhibit reactions vs. some DNA fragments (78, 90, 91). This would result in a greater protection against several diseases, reducing the rate of enteric and respiratory infections (67, 71, 92).

Moreover, in BM, there are many anaerobic and lactic acid bacteria (69), which could confer further anti-microbial protection and improve nutrients' absorption (67, 68, 71, 72, 88–91, 93).

The recent study of Damaceno et al. (72), investigating BM microbiota in healthy mothers, revealed a bacterial concentration ranging from 1.5 to 4.0 $\log_{10}$ CFU/mL, with the highest concentration in colostrum. In their sample, *S. epidermidis* resulted the predominant species.

Our group (67) evaluated microbiota network in italian mothers, detecting a variable microbial composition duringprogressive lactation stages and even some differences occurring among different populations. In particular, colostrum of Italian mothers mostly contained *Abiotrophia* spp., *Actynomicetospora* spp., *Aerococcus* spp., *Alloicoccus* spp., *Amaricoccus* spp., *Bergeyella* spp., *Citrobacter* spp., *Desulfovibrio* spp., *Dolosigranulum* spp., *Faecalibacterium* spp., *Parasutterella* spp., *Rhodanobacter* spp., *Rubellimicrobium* spp. (67, 88, 94). Other authors also demonstrated a high prevalence of *Weisella* spp., *Leuconostoc* spp., *Staphylococci* spp., *Streptococci* spp., and *Lactobacilli* spp. (82).

In mature BM from italian mothers, *Abiotrophia* spp. and *Aerococcus* spp. were also present, in addition to *Acetanaerobacterium* spp., *Aciditerrimonas* spp., *Acidocella* spp., *Aminobacter* spp., *Bacillus* spp., *Caryophanon* spp., *Delftia* spp., *Microvirga* spp., *Parabacteroides* spp., *Phascolarctobacterium* spp., and *Alistipes* spp. (67). Other authors also reported the presence of *Veillonella* spp., *Leptotrichia* spp., *Prevotella* spp. (82)., *Enterococcus* spp., *Lactococcus* spp., *Actinomyces* spp., *Corynebacterium* spp., *Kecuria* spp., *Escherichia coli* spp., *Klebsiella* spp., and *Raistonia* spp. species (88).

In the same study evaluations were conducted in colostrum and mature milk from mothers living in Burundi, where so many genetic but also environmental factors can be taken into account to explain huge differences in microbial composition. Several differences in the microbiota network have been observed also in different lactation stages of the same population and it is clear that the differences between these two populations may influence the findings (67).

Analyzing the gut microbiota of breastfed neonates, and comparing it to FM fed infants, different levels of *Proteobacteria* spp., *Bacteroides* spp., *Actinobacteria* spp., and *Firmicutes* spp. were detected (95); moreover, *Bifidobacteria* spp. resulted one of the most represented species, especially *Bifidobacterium longum* subsp. *longum* and *infantis*, and *B. breve* (96), which also showed a high concentration in breastfed neonate's stools (2, 86).

Bifidobacteria spp., *Lactobacilli* spp., *and Bacteroides* spp. proliferation is useful to face intestinal aggressive pathogens' invasion (such as *Salmonella* spp., *Lysteria* spp., and *Campilobacter* spp. (97, 98). Moreover, BM has a buffering capacity, that allows acidifying the intestinal content in order to make it more fermentable by the bacteria of the proximal colon. BM shows an inhibitory effect on the growth of *Clostridi* spp., *Bacteriodes* spp. and other anaerobic bacteria.

The great influence exerted by BM on neonatal intestinal microbial composition allow to indicate this community with the expression milk-oriented microbiota (MOM) (1).

This effect mainly occurs through the action of the glycans, constituted by free HMOs, glycolipids and glycoproteins and highly contained in BM. In fact, as previously reported, they act as prebiotics, since they do not undergo absorption in proximal gut (38, 99) and represent growth substrates for specific hugs (67, 68, 71, 72, 88, 96, 100–103).

Therefore HMOs, and even FAs, influence the growth of some beneficial species in neonatal gut, such as *Bifidobacteria* spp. and *Staphylococci* spp., related to several positive effects (38, 96, 104–106). For example, a better response to vaccines, an improved function of the intestinal barrier and a protection against intestinal infections (38, 107–109).

Moreover, bacterial species like *Bacteroides* spp., *Bifidobacteria* spp. and *Lactobacilli* spp. are very important for HMOs' metabolism (72), promoting their degradation into sugars available for energy production. In addition, *Bifidobacteria* spp., *Lactobacilli* spp., *and Bacteroides* spp. can induce short chain fatty acids (SCFAs) production, playing a role in gut mucosa homeostasis and in lipid metabolism (97, 98).

A recent study of Karav et al. (96) demonstrated that a endo-β-N-acetylglucosaminidase (EndoBI-1) found in several *Bifidobacteria* spp. promotes the cleavage of N-linked glycans fragments. These can influence bacterial selective growth, especially allowing *Bifidobacterium longum* subsp. *Infantis* (*B. infantis*) proliferation and even interfering with other subspecies' metabolism. On the contrary, in the same study, *B. infantis* did not result able to grow exclusively in presence of the de-glycosylated protein fraction, confirming the role played by glycans in bacterial growth.

In this perspective, *Bifidobacteria* spp. and other species able to perform the initial de-glycosylation seem advantaged, since this represents a key passage. In fact, many studies demonstrated that the shape of gut microbiota, through the influence of microbial growth, is better promoted by HMOs and deconjugated glycans instead of the whole glycoprotein or glycolipid (96, 110–120).

This topic represents an interesting field of research, being not full clarified up to now. It would be very promising to find a link among the exact and inter-individual BM composition, the exerted influence on intestinal microbiota of the newborn and its resulting clinical phenotype. In fact it could be a suitable substrate for therapeutic beneficial applications modifying the final outcomes.

For example, BM content in HMOs and even neonatal gut microbiota showed some differences in under nutrition models, leading to an impaired infantile development. Therefore, it could be very useful to perform some dietetic strategies, adapted to these needs, which could treat or prevents several disorders, including under nutrition (38, 121).

CONCLUSIONS

BM exceptional features make it a very precious fluid, whose extraordinary properties and functions have not been fully clarified yet. It would be very interesting to understand all maternal factors influencing BM composition, also regarding SCs, in terms of quality and quantity (21, 122).

In the last years the importance of metabolomics has been highlighted, especially due to its role in characterizing metabolites related to microbial network. This integrated approach to the triad nutrients-microbes-metabolites can allow the identification of the effective bacterial taxa in BM and therefore transferred to the newborn (78, 123, 124), since we know that BM is the best modulator of neonatal microbiota (125).These findings would help to clarify, and even predict, BM influence on neonatal short- and long-term outcomes. Moreover, such observations may result useful to perform a sartorial approach through targeted strategies which potentially could, improve neonatal or even maternal health through the modulation of BM microbiota (2).

Finally, these evidences suggest the possible importance of bacterial supplementation of FM. The detailed knowledge of BM composition could allow to produce the best artificial products to provide to the nourished newborn a FM resembling, in the most accurate way, BM characteristics (78, 126).

AUTHOR'S NOTE

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AUTHOR CONTRIBUTIONS

FB made the selection of the papers from the literature. FB wrote the paper. VF and DP conceived the paper and FS and PA revisioned the manuscript.

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The Prebiotic and Probiotic Properties of Human Milk: Implications for Infant Immune Development and Pediatric Asthma

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The incidence of pediatric asthma has increased substantially in recent decades, reaching a worldwide prevalence of 14%. This rapid increase may be attributed to the loss of "Old Friend" microbes from the human microbiota resulting in a less diverse and "dysbiotic" gut microbiota, which fails to optimally stimulate immune development during infancy. This hypothesis is supported by observations that the gut microbiota is different in infants who develop asthma later in life compared to those who remain healthy. Thus, early life exposures that influence gut microbiota play a crucial role in asthma development. Breastfeeding is one such exposure; it is generally considered protective against pediatric asthma, although conflicting results have been reported, potentially due to variations in milk composition between individuals and across populations. Human milk oligosaccharides (HMOs) and milk microbiota are two major milk components that influence the infant gut microbiota and hence, development of the immune system. Among their many immunomodulatory functions, HMOs exert a selective pressure within the infant gut microbial niche, preferentially promoting the proliferation of specific bacteria including *Bifidobacteria.* Milk is also a source of viable bacteria originating from the maternal gut and infant oral cavity. As such, breastmilk has prebiotic and probiotic properties that can modulate two of the main forces controlling the gut microbial community assembly, i.e., dispersal and selection. Here, we review the latest evidence, mechanisms and hypotheses for the synergistic and/or additive effects of milk microbiota and HMOs in protecting against pediatric asthma.

Keywords: human milk, microbiota, human milk oligosaccharides, immune development, asthma, pediatrics, probiotic, prebiotic

BREASTFEEDING, ASTHMA, AND THE MICROBIOTA

Breastfeeding has many established benefits for maternal and child health (1), including a potentially protective effect against pediatric asthma development. In a meta-analysis of 117 studies, Dogaru et al. found that breastfeeding was associated with a 22% reduced risk of asthma, with the strongest effects observed during early childhood (2). This association was not seen among adults in the population-based UK Biobank study (3); however, breastfeeding data were self-reported and did not account for duration or exclusivity. Several plausible mechanisms have been proposed to explain how breastfeeding might protect against asthma (4). For example, breastfeeding appears to mitigate the harmful effects of asthmogenic exposures including air pollution (5) and psychosocial stress (6). In addition, breastfeeding has been shown to support lung growth (7) and enhance lung function (8). Recently in the Canadian Healthy Infant Longitudinal Development (CHILD) Study, we reported a dose-dependent reduced risk of wheezing (9) and asthma (10) among breastfed children. These associations were stronger among infants fed at the breast compared to those receiving pumped breast milk, although both were superior to infant formula (10). This suggests that the act of suckling and/or skin-to-skin contact may contribute to the protective effect of breastfeeding. Alternatively or in addition, this finding could reflect a role for bioactive components in human milk, which may be altered during the pumping and storage process.

Breastfeeding also profoundly influences development of the infant oral and gut microbiota (11), which have been independently linked with asthma development (12, 13). Recent increases in asthma prevalence (14) have been attributed to a loss of diversity within the human microbiota due to the dramatic lifestyle changes in the last century (15). "Old Friend" and "missing microbe" hypotheses speculate that our increasingly hygienic lifestyle and overuse of antibiotics have resulted in the loss of specific bacteria from the modern day human microbiota (15, 16). Given the co-adaptation and co-evolution of the "ancient" microbiota with the human immune system, it is plausible that their loss could result in aberrant immune responses leading to allergic, autoimmune, and inflammatory diseases, including asthma (16). This hypothesis is supported by evidence that skin and nasal microbiota differ between adjacent but socioeconomically contrasting regions of Finnish and Russian Karelia with discordant prevalence of asthma and allergy (17). Consistent with this line of evidence, gut microbiota profiles have also been shown to differ between infants who do or do not develop asthma in the CHILD cohort and other longitudinal studies (12, 13). In addition, early life exposures that alter the microbiota (such as antibiotic use, cesarean delivery, and formula feeding) have been linked to asthma development (18).

Human milk oligosaccharides (HMOs) and microbiota are two major milk components that influence infant gut microbiota and hence, development of the immune system. As such, breastmilk has prebiotic and probiotic properties that can modulate two of the main forces controlling the gut microbial community assembly, i.e., dispersal (acquiring new bacterial species) and selection (achieving a permissive environment to facilitate sustainable colonization). If these processes are disrupted, the infant gut microbiota developmental trajectory will be altered, potentially leading to a suboptimal final composition. This could be one of the underlying mechanisms of predisposition to a range of chronic diseases including allergy and asthma (19) (**Figure 1**). Here, we review the latest evidence and hypothesized mechanisms for the synergistic and/or additive effects of HMOs and milk microbiota in protecting against pediatric asthma.

PREBIOTIC AND PROBIOTIC PROPERTIES OF HUMAN MILK

Breastfeeding affects both gut microbiota and immune system development (20, 21). Human milk functions as a bioactive food consisting of all essential nutrients plus immune components, hormones, HMOs, and microbiota, which serve crucial roles in early life metabolic and immune system homeostasis and development (22). HMOs and the microbiota are of particular interest because of their influence on the infant gut microbiota and potential long-term health importance (22) (**Table 1**).

Human Milk Oligosaccharides

HMOs constitute the third largest component of human milk (31). These structurally diverse carbohydrates are synthesized by sequential addition of monosaccharides to lactose, and various α-glycosidic linkages of fucose and/or sialic acid to the core molecules (23, 36). More than 100 different HMOs have been identified, with the amount and composition varying substantially between women and over the course of lactation (25, 28, 37, 38). HMO fucosylation is regulated by enzymes encoded by the fucosyltransferase 2 (FUT2) and FUT3 genes, which determine secretor status and Lewis blood group status, respectively (23, 24). However, geographical variation in HMO composition suggests that non-genetic factors such as sociocultural and environmental factors may also play a role (24, 25). In the CHILD cohort we have observed that, beyond genetic FUT2 secretor status, HMO composition is associated with ethnicity, lactation stage, parity, geographic location, season of collection, and breastfeeding exclusivity (26).

Although still an emerging field of research, many biological functions have been attributed to HMOs (24, 31). The majority of ingested HMOs reach the lower intestinal tract where they can function as prebiotics, providing selective substrates for gut bacteria (30), as discussed below. Preliminary evidence also suggests that specific HMOs could directly modulate the immune response, with studies in pigs (39) and mice (40, 41) demonstrating direct effects on viral pathogens as well as host immune cells. In breastfed infants, approximately 1% of HMOs are absorbed into the peripheral circulation, potentially reaching all organs including the lungs (31), thus it is plausible that HMOs could affect lung mucosal immunity by interacting with airway

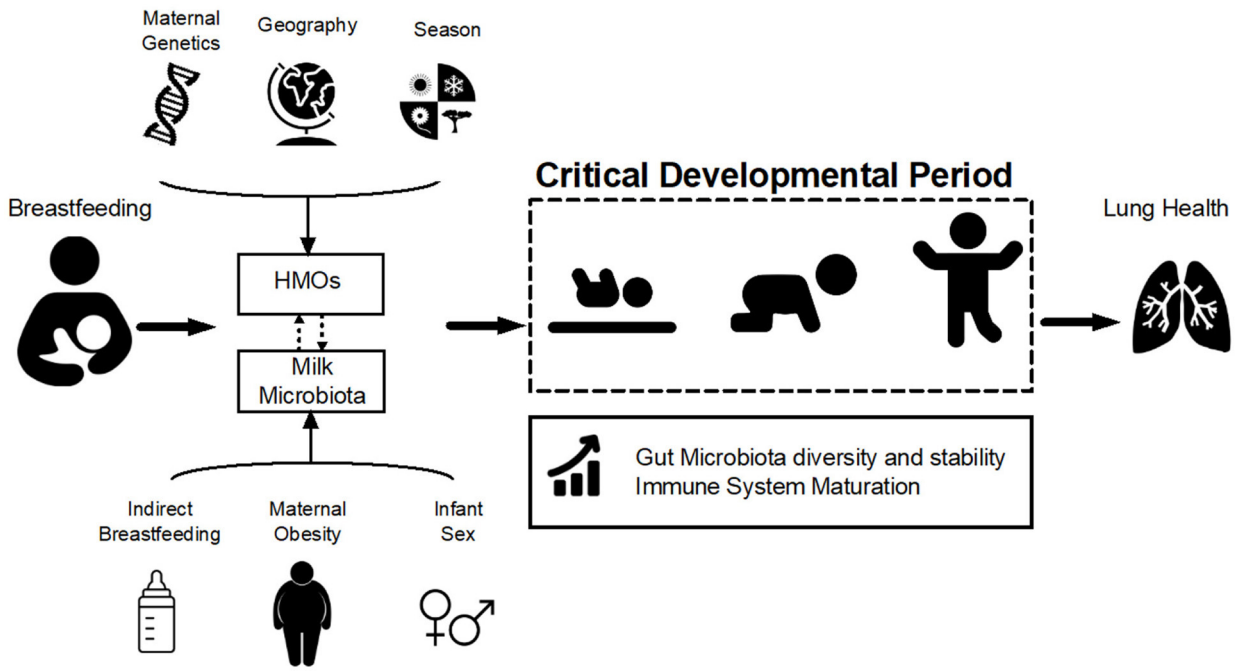

FIGURE 1 | Hypothesized pathways of association between breastfeeding and lung health. Breastfeeding influences infant gut microbiota development and stability during the critical developmental period in early life via two of its main components: human milk oligosaccharides (HMOs) and milk microbiota. HMOs exert a selective pressure within the infant gut microbial niche, preferentially promoting the proliferation of specific bacteria including *Bifidobacteria*. Milk is also a source of viable bacteria originating from the maternal gut and infant oral cavity. HMO composition is influenced by maternal genetics, geography, and season while microbiota is affected by maternal weight status, mode of breastfeeding and infant sex (**Table 1**). Variations in HMOs and milk microbiota could modulate the effect of breastfeeding on the infant gut microbiota, which in turn shapes the infant immune system and could ultimately influence lung health and asthma development.

TABLE 1 | Evidence on factors influencing the composition of human milk oligosaccharides (HMOs) and milk microbiota and their association with pediatric asthma.

Milk components	Influenced by	Impact on gut microbiota	Association with asthma
Human milk oligosaccharides	Host genetics (23, 24) Geography (24, 25) Season (26, 27) Parity (26) Lactation stage (28) Birth mode (29)	Influence nutrient availability for gut bacteria (30) Enrich *Bacteroides* and *Bifidobacterium* spp. (30) Prevent pathogen colonization (31) Modify host-microbe interaction (32)	No published evidence, but associations found for atopic sensitization and food allergy (26, 33)
Milk microbiota	Birth mode (22) Mode of breastfeeding (22) Maternal BMI (34) Infant sex (34)	Provide pioneering species (11) Enrich "beneficial" bacteria (35) Increase colonization efficiency	No published evidence

epithelia, immune cells, potential pathogens or resident microbes, providing another mechanism for protecting against asthma.

While the mechanisms are not fully elucidated, there is some evidence that supplementation with prebiotics (42, 43) or HMOs (44) may be protective against allergy and asthma in animal models and human studies. A recent systematic review reported a reduction in asthma among high-risk infants given prebiotics, such as galacto-oligosaccharide (GOS) and fructo-oligosaccharide (FOS) (45). However, GOS and FOS are structurally distinct from HMOs, which have not been widely studied in relation to asthma, although a few studies have examined their association with allergic disease. One study found that infants receiving milk with low Lacto-N-fucopentaose III concentrations were more likely to develop cow's milk allergy (33), an effect that might be modulated by birth mode (29). In the CHILD cohort, we have observed that HMO composition (but not any individual HMO) is associated with the development of allergic sensitization during infancy (26). Associations with asthma during early childhood are currently being explored. Altogether, epidemiological and experimental studies support the prebiotic effects of HMOs

and suggest a potential role in asthma development (**Table 1**), but further research is needed to confirm and characterize this relationship.

Milk Microbiota

Culture-dependent and independent studies have confirmed the presence of bacteria in human milk (46). It is estimated that breastfed infants receive 10^4-10^6 bacteria per day (based on an average daily consumption of 800 mL of milk) with most isolated species belonging to the genera *Staphylococcus*, *Streptococcus*, *Lactobacillus*, and *Bifidobacterium* (47). Culture-independent (DNA sequencing-based) approaches in the CHILD cohort (34) and others (48) have recovered a higher diversity of bacteria in breast milk including lactic acid bacteria (*Enterococcus* and *Lactococcus*), oral-derived (*Veillonella* and *Gemella*), skin-associated (*Cutibacterium* and *Staphylococcus*), and environmental bacteria (*Pseudomonas* and *Sphingomonas*) with a high degree of inter-individual variability. While bacterial load appears to remain constant during milk maturation (49), it gradually decreases during one feed (50).

The milk microbiota is suggested to originate from the maternal gut, breast tissue, or infant oral cavity (51). Depending on the source of bacteria, different factors may contribute to shaping the milk microbiota (**Table 1**). For example, while maternal factors could influence the mother's gut bacteria, early life factors such as mode of delivery and mode of breastfeeding (directly at the breast vs. expressed and bottled breast milk) could potentially alter the exogenous bacteria derived from the infant (22). In the CHILD cohort, we have found that mode of breastfeeding was significantly associated with milk microbiota composition, with expressed milk feeding favoring depletion of *Bifidobacteria* and enrichment with potential pathogens and environmental bacteria (34). We also observed some sex-specific associations (e.g., maternal BMI associated with milk microbiota only if the infant is female) while other factors demonstrated a phylum-specific effect (e.g., maternal atopy associated with Actinobacteria richness) (34). As discussed below, the milk microbiota is suggested to provide a mechanism of vertical microbial transmission from the mother to the infant.

To the best of our knowledge, no study to date has directly linked milk microbiota with pediatric asthma and allergy. However, we have previously reported that indirect breastfeeding was associated with higher risk of pediatric asthma in the CHILD study (10), and we have also observed that mode of breastfeeding is consistently associated with milk microbiota composition in this cohort (34). It is plausible that the milk microbiota could play a role in asthma development, conceivably via the modulation of gut microbiota. Studies to date have been inconclusive regarding the use of commercial probiotics for preventing asthma (52); however, the natural milk microbiota is a complex community, and hence, may be more effective in this regard, as it has presumably evolved to optimally support infant gut microbiota and immune development.

CONTRIBUTION OF HMOS AND MILK MICROBIOTA TO INFANT GUT MICROBIOTA DEVELOPMENT

Dietary exposures are among the most influential factors shaping the infant gut microbiota. Breastmilk not only provides nutrients to the infant, but is also a source of probiotics (milk microbiota) and prebiotics (HMOs) contributing to the establishment of the infant gut microbiota (11). The human gut microbiota develops through a complex process of stepwise successions beginning at birth (53). First colonizers of the infant gut are facultative anaerobes, including the *Staphylococcus*, *Streptococcus*, and *Enterococcus* genera, and the Enterobacteriaceae family. These pioneering bacteria reduce the redox potential and hence, create a favorable condition for the growth of obligate anaerobes such as the *Bifidobacterium*, *Bacteroides*, *Clostridium,* and *Eubacterium* genera (53). In contrast to the mature gut microbiota, the infant gut is dominated by *Bifidobacterium* species prior to weaning, including *B. longum*, *B. breve*, and *B. bifidum* (35, 54). HMOs are specifically utilized by *Bacteroides* and *Bifidobacterium* spp. (30) leading to dominance of these taxa in breastfed infants (55), with different *Bifidobacterium* species and strains demonstrating different preferences for specific HMOs (56). Furthermore, rates of absorption and excretion are different for different categories of HMOs (57), resulting in different amounts reaching the colon; further highlighting the nuanced impact of the HMO composition on the infant gut microbiota. HMOs could also indirectly affect the gut microbiota composition through their decoy activity that prevents pathogen colonization (31). Additionally, HMOs can modify host-microbe interactions by affecting epithelial cell turnover (32) and glycocalyx mucus formation (58).

In addition to the prebiotic role of HMOs, the viability of milk bacteria suggests that human milk could function as a probiotic shaping the infant gut microbiota by facilitating dispersal (22). Milk microbiota may provide pioneering species and impact the final gut microbiota composition (59). Human microbiota studies demonstrating strain similarities between maternal gut, milk, and infant gut support this hypothesis (54), and find that *Bifidobacterium* spp. constitute the majority of shared taxa between maternal milk and infant stool (35). It is plausible that human milk specifically enriches and protects "beneficial" bacteria as they are transported through the infant's acidic stomach environment to the lower intestinal tract. Milk could also function as an incubator of bacteria to increase their "dose," thereby increasing the likelihood of successful colonization in the infant gut (60).

INFANT GUT MICROBIOTA, IMMUNE SYSTEM DEVELOPMENT, AND ASTHMA

The precise role of milk microbiota and HMOs in shaping the infant gut microbiota and immune development is an active area of research with potential implications for asthma prevention. The gastrointestinal tract is the largest immune organ in the human body and it plays a crucial role in the

education and subsequent maturation of immune system by (i) maintaining immunological tolerance to food components as well as the commensal microbiota, and (ii) acquiring the capacity to appropriately respond to pathogenic microbes (61, 62). Moreover, the immune system is essential in keeping the balance of bacterial communities and preventing dysbiosis (61, 63). This host-microbiota crosstalk is shaped early in life (63, 64). It has been hypothesized and experimentally demonstrated that alteration of the early gut microbiota can disrupt the microbially-mediated mechanisms of immunological tolerance, resulting in predisposition to allergic disorders including asthma (61, 65).

Development of the immune system begins in utero, and continues during infancy (18). At birth, the adaptive immune system is dominated by the T-helper cell type 2 (Th2) which will later shift to Th1 and Th17 phenotype (66, 67). Delayed or impaired Th2/Th1 transition during early infancy is associated with increased risk of atopic disorders, including asthma (18). It has been shown that the gut microbiota stimulates regulatory T cells that help monitor the Th1/Th2 balance (18, 61, 62). Furthermore, exposure to microbes or microbial products such as microbe-associated molecular patterns (MAMPs) or short chain fatty acids (SCFA) during the early infancy is essential for stimulating the infant's naïve and immature immune system (53, 67), gut-associated lymphoid tissue development (64, 66), and B cell maturation (68). Thus, early life microbiota disruption could lead to alterations in several immune parameters including regulatory T cells proliferation, Th17 response, and IgE response (53), all of which contribute to asthma development.

A growing body of evidence supports the role of gut microbiota in the development of allergic disorders (64, 69, 70). However, it is not clear whether the risk of asthma development is modulated by presence of specific bacteria, temporal succession patterns, or the overall taxonomic and functional diversity of the microbial community (53). Observational human studies have indicated that lower gut microbiota diversity early in life could be associated with higher risk of asthma at later time points (12). In contrast, gut microbiota diversity and overall community composition were not associated with atopic wheezing in the CHILD cohort, although lower relative abundances of specific bacterial genera including *Faecalibacterium*, *Lachnospira*, *Veillonella*, and *Rothia* were associated with this phenotype (13). These genera were further shown to reduce clinical features of asthma including respiratory tract neutrophil and lymphocyte infiltrations and histopathological alterations in a mouse model of ovalbumin-induced asthma (13). Similarly, other studies have found that higher relative abundances of specific taxa including *Clostridium difficile* and *Clostridium neonatale* in the infant gut microbiota are associated with an increased risk of asthma (18, 71–74). Despite the discrepancies between studies on the important microbial features potentially associated with asthma, they have consistently shown that gut microbiota status in infancy, more so than early childhood, is associated with an altered risk of asthma. Collectively, this evidence supports the existence of a "critical period" during which the gut microbiota shapes the infant immune landscape and thus susceptibility to asthma later in life (70, 75).

CONCLUSIONS AND FUTURE DIRECTIONS

Breastfeeding profoundly influences the infant gut microbiota, and emerging evidence indicates that divergence from this evolutionarily conserved process can alter immune system maturation and influence asthma development. However, much remains to be discovered about the underlying mechanisms, including the prebiotic and probiotic properties of human milk. For example, we do not fully understand how HMOs and milk microbiota modulate the overall gut microbial community and whether this involves many or just a few key taxa. Moreover, there is tremendous variation in HMO utilization by different bacterial strains, and some HMOs are not utilized by the gut microbiota. The source and fate of bacteria in human milk is also uncertain. HMOs and milk microbiota may influence infant microbial communities at body sites other than the gut, including the airways and nasopharyngeal cavity, which may also play a role in asthma development. In addition to HMOs and microbiota, other milk components likely also impact the gut microbiota and/or the immune system, including cytokines, antimicrobial compounds, and immunoglobulins. These diverse components have largely been studied in isolation, yet they co-exist and interact in the mammary gland and the infant gut. New methods and integrated approaches will be required to disentangle the complex and dynamic interactions between HMOs, milk microbiota, and other milk components and their collective impact on the infant gut microbiota, immune function, and pediatric asthma. Understanding these processes will help define the role of breastfeeding and human milk in normal development, and could ultimately inform new microbiota-based strategies for asthma prevention.

AUTHOR CONTRIBUTIONS

All authors contributed to the conception and design of this review and wrote sections of the manuscript. SM and MA compiled and integrated the individual sections. All authors contributed to manuscript revision, read and approved the submitted version.

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REVIEW
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Immunological Effects of Human Milk Oligosaccharides

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Human milk oligosaccharides (HMOs) comprise a group of structurally complex, unconjugated glycans that are highly abundant in human milk. HMOs are minimally digested in the gastrointestinal tract and reach the colon intact, where they shape the microbiota. A small fraction of HMOs is absorbed, reaches the systemic circulation, and is excreted in urine. HMOs can bind to cell surface receptors expressed on epithelial cells and cells of the immune system and thus modulate neonatal immunity in the infant gut, and possibly also sites throughout the body. In addition, they have been shown to act as soluble decoy receptors to block the attachment of various microbial pathogens to cells. This review summarizes the current knowledge of the effects HMOs can have on infections, allergies, auto-immune diseases and inflammation, and will focus on the role of HMOs in altering immune responses through binding to immune-related receptors.

Keywords: human milk oligosaccharides, HMO, infection, immunity, infant, allergy, benefit, health

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INTRODUCTION

Based on its richness in immune-related components like human milk oligosaccharides (HMOs), milk proteins and lipids, breastmilk can be seen as the first functional food humans encounter during their life (1). HMOs comprise a group of structurally complex, unconjugated glycans found in human breastmilk (see **Figure 1**). Although the amount and precise composition of HMOs varies depending on time of lactation and the genetic makeup of each woman as well as potential environmental exposures, human breast milk contains an average of 5–15 g of oligosaccharides per liter, making HMOs the third most abundant solid component of breast milk after lactose and lipids (2). Each oligosaccharide is built on a lactose backbone expanded by the addition of galactose, N-acetylglucosamine, fucose or sialic acid, branched and elongated in different ways, generating approximately 200 different structures identified to-date (3). As they are only minimally digested in the gastrointestinal tract, HMOs reach the colon intact or are absorbed in small quantities, reach the systemic circulation and are excreted in urine (4). In this way, they may exert a plethora of functions at multiple sites throughout the body and beyond the intestinal lumen and intestinal mucosal surfaces, including the urinary tract or the immune system. HMOs were first described as prebiotic substrates for the infant gut microbiota, promoting the establishment of bifidobacteria and lactobacilli, based on striking differences in microbiota composition between breastfed and bottle fed infants (5).

However, HMOs are now recognized to have various additional benefits for the developing neonate. HMOs may modulate neonatal immunity by altering host epithelial and immune cell responses in the infant gut (6), modify immune responses systemically or act as soluble decoy receptors to block the attachment of various microbial pathogens to cell surface receptors (7), not only in the intestine but also in other sites such as the urinary tract (8). The benefits of HMOs can

extend to health outcomes beyond infancy such as allergies (9) or cognitive functions (10), making HMOs the focus of intense current scientific research with increasing number of studies unraveling their role in human physiology.

This review summarizes recent findings, discusses the proposed modes of action, and identifies future prospects and scientific challenges, with a focus on immunity and infection.

HMO ABSORPTION

HMOs are resistant to digestion in the infant GI tract (11). Both neutral and acidic HMOs can cross the epithelial barrier, but active transport over intestinal epithelial monolayers has only been demonstrated for neutral HMOs (12). These findings suggest that HMOs may be taken up into the human body. Indeed, HMOs have been detected in feces and urine of breastfed infants (13–17), but also directly in the peripheral blood (18–21). However, lower concentrations of HMOs are detected in blood compared to urine, which may be a reflection of accumulation in urine from a larger volume of blood. For example, concentrations of 2′-fucosyllactose (2′FL), were around 1.5 mg/l in peripheral blood and 100 mg/l in urine (20).

Absorption of orally administrated single HMOs was also shown in an adult rat model showing indeed that the intestinal epithelium is permeable to HMOs although to a different extent in infancy and adulthood (22). Therefore, these publications indicate that HMOs may, in addition to effects in the GI tract, have effects throughout the human body. Such effects can be conveyed directly through binding to receptors for HMOs, or indirectly via induction of short chain fatty acids and other metabolites produced by the microbiota.

POTENTIAL HMO RECEPTORS, THEIR EXPRESSION AND FUNCTION

Potential HMO Receptors

Several classes of lectins (glycan-binding proteins) have been described in the literature that have different functions and ligand specificities, namely galectins, siglecs, c- type lectins, and selectins. Different HMOs can bind to these different types of receptors on human cells, primarily expressed on cells of the immune system.

Galectins are lectins that bind N-acetyllactosamine or lactose containing sugars (23–25). Galectins can also bind sulfated, sialylated or fucosylated galactose moieties (25). The work of Hirabayashi et al elegantly shows the oligosaccharide specificity of galectins for several HMO structures (23, 25, 26). More recently, Prudden et al. confirmed binding of HMOs with a terminal type 1 and 2 LacNAc to galectin 9 with a preference for type 1 structures on a solid surface (27). Similar findings were reported for HMO binding specificity for galectins in solution, corroborating these initial studies (28, 29).

Another family of lectins involved in HMO binding are the sialic acid binding immunoglobulin- like lectins (Siglecs). Siglecs have been shown to bind sialylated HMOs (30). Sialyllactose has been shown to bind to sialoadhesin (Siglec-1) (31), but also to Siglec-5 and Siglec-10 (32), Siglec-7 (33), and Siglec-9 (34). However, the affinity of sialyllactoses for Siglecs are relatively low.

In addition to galectins and siglecs, HMOs also interfere with another family of lectins involved in cell adhesion, the selectins (2, 35). Selectins bind to glycans that carry sialylated Le bloodgroup epitopes (36), which are sialylated and fucosylated lacto-N-bioses (Galβ1-3GlcNAc) or N-acetyllactosamines (Galβ1-4GlcNAc)—very similar to HMOs. In fact, HMOs contain Le blood group antigens (37) and are able to reduce selectin-mediated cell–cell interactions (38, 39). In addition, HMOs have been shown to interact with selectins (40), and integrins (39).

Finally, HMOs can bind to C-type lectins like DC-SIGN and Dectin-1. C-type lectins containing an EPN-motif (Glu-Pro-Asn) have high specificity for mannose- and fucose terminating glycans, whereas the presence of a QPD-motif (GlnPro-Asp) is important for galactose-or N-acetylgalactosamine(GalNAc) terminating glycans (41). HMOs were shown to bind specifically to DC-SIGN expressed by DCs (42). Although HMO binding to DC-SIGN seems to be weaker than binding to galectins, it was shown that structures containing α-linked fucose could bind to DC-SIGN (34). The results were also confirmed by binding of DC-SIGN to beads derivatized with 2′-FL or 3-FL, but not with LNT.

A limited number of reports have also discussed the possibility of binding of HMOs to other receptors belonging to the Toll like receptor (TLR) family that typically bind to pathogen-related molecules. TLR-4 dependent effects of HMOs have been described in two papers in which HMOs tested *in vivo* required the expression of TLR-4 for their effect (43, 44). However, formal demonstration of the binding of the HMOs (3′SL and LNFPIII) to TLR-4 in direct binding assays was not provided. In addition, in relation to TLR-signaling of HMOs, a recent paper highlighted the effect that low level LPS contamination of the commercially available HMO 3′SL can have in these studies, indicating that caution is warranted when studying TLR-mediated effects (45).

An overview of putative receptors for HMO is shown in **Table 1.**

Expression Profiles and Functions of Potential HMO Receptors

Galectins are mainly expressed on T cells, and can regulate T cell function (46), but are also present on intestinal epithelial cells (47–49), and on antigen presenting cells and granulocytes (25). Galectins can convert signals into the cell after binding to their ligands directly, but galectins can also be secreted, after which they bind to glycoproteins or receptors at cell surfaces and hence can regulate cell functions (50–52). Binding of HMOs or lactose can thus have direct effects or inhibit the interaction of galectins with their ligands on other cells.

Siglecs are involved in the immune system in multiple ways (53). Siglecs 1–16 are expressed on a variety of blood cells, including monocytes, macrophages, dendritic cells, neutrophils, eosinophils, basophils, and NK cells (53, 54). In contrast to galectins and Dectin-1, Siglecs are not expressed by intestinal epithelial cells. Many of the Siglecs have an intracellular

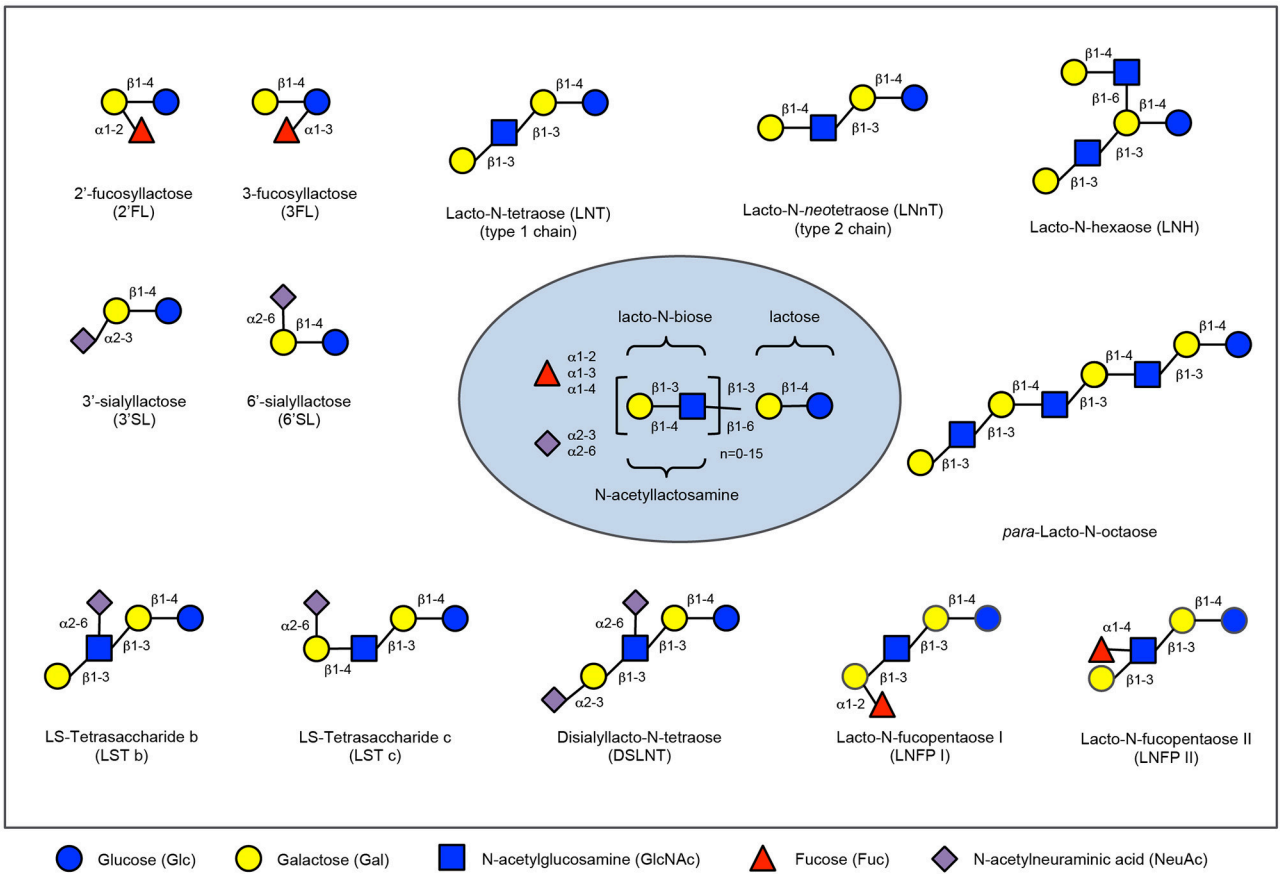

FIGURE 1 | Human milk oligosaccharide composition blueprint. HMO composition follows a basic blueprint shown in the center. HMOs can contain 5 different monosaccharides in different number and linkages, namely glucose (blue circle), galactose (yellow circle), N-acetylactosamine (blue square), fucose (red triangle), and sialic acid (purple diamond). All HMOs carry lactose at the reducing end. Lactose can be fucosylated or sialylated to generate the small HMOs 2′-fucosyllactose and 3-fucosyllactose or 3′-sialyllactose and 6′-sialyllactose, respectively (upper left corner). Alternatively, lactose can be elongated with type 1 or type 2 disaccharide units to form linear or branched HMOs (upper right corner). Elongated HMOs then can be sialylated (lower left corner) or fucosylated (lower right corner) or both sialylated and fucosylated (not shown). The HMOs in this figure are only a few relatively simple examples. So far, more than 150 different HMO structures have been identified.

immunoreceptor tyrosine-based inhibitory motif (ITIM), and are thus known as regulators of immune responses.

Selectins are cell adhesion molecules that mediate the earliest stages of leukocyte trafficking. At sites of inflammation, leukocytes need to migrate from the blood stream through the endothelium into sub endothelial regions of inflammation (55, 56). Induced by pro-inflammatory cytokines, endothelial cells express P- and E-selectin, which bind to glyco-conjugates on leukocytes passing by with the blood stream. This initial contact decelerates the leukocytes and makes them roll over the endothelial cell layer. Subsequently, additional adhesion molecules bring leukocytes to a complete stop and facilitate their transmigration into sub endothelial regions. Initial selectin-mediated rolling is essential for leukocyte extravasation and mucosal infiltration.

Sialylated HMOs have been shown to interact with selectins (40), and integrins (39), and affect leukocyte-endothelial cell and leukocyte-platelet interactions (39, 57–59). Similarly, sialylated HMOs reduce PNC formation and subsequent neutrophil activation in an *ex vivo* model with whole human blood (38). In both cases, non-sialylated HMOs are ineffective and pooled HMOs are more effective than monovalent sialyl-Le X, indicating the importance of Sia and suggesting potential multivalent interactions with higher molecular HMOs that carry more than one sialylated blood group epitope.

C-type lectins are primarily expressed by antigen presenting cells (monocytes, macrophages, dendritic cells) and are of crucial importance for regulating immune responses to pathogens. The can be divided into four subgroups, the sialo-glycoprotein receptor family (e.g., DC-SIGN), the dectin-1 subfamily of asialo glycoprotein receptors (e.g., Dectin-1), the DCIR subfamily (e.g., DCIR), and the Mannose receptor family (e.g., CD206) for a review see Geijtenbeek and Gringhuis (60).

In general, c- type lectins are primarily expressed on dendritic cells and macrophages, and play a role in the internalization of saccharide-containing antigens, resulting in antigen presentation (41). However, dectin-1 can also be detected on intestinal epithelial and on M cells, and play a role in IgA transcytosis

TABLE 1 | Putative receptors for HMOs on the immune system.

Type of lectin	Name	Function of receptor	Ligand specificity	Known HMO ligands	Expression	Reference
C-type lectins	DC-SIGN	Immune (Antigen presentation)	α-fucosylated structures, mannose	2′-FL, 3FL, LNFP-III, LNFP-IV, LNDFH-I	Antigen presenting cells	(34)
Siglecs	Siglec-5, Siglec-9	Immune	sialylated HMO, α2,3- and α2,6-linked sialic acids	3′ and 6′ Sialyllactose	Neutrophils, monocytes, dendritic cells	(34)
Galectins	Galectins 1, 2, 3, 7, 8. 9	Immune	N-acetyllactosamine or lactose	LNnT LNT NFP-I LNFP-II LNFP-III LNDFH FucLac a-GalLac but not 6SL	Intestinal cells, lymphocytes, antigen presenting cells	(23, 25, 26)
Selectins		Leucocyte adhesion	Sialylated and fucosylated lacto-N-bioses (Galβ1-3GlcNAc) or N-acetyllactosamines	sialyl-Lewis x	Leucocytes, endothelium	(37, 39)

(61–63). Apart from promoting antigen presentation, some c- type lectins like DCIR may—just like Siglecs—contain an immunoreceptor tyrosine-based inhibitory motif (ITIM) -motif in their intracellular domains, that inhibit immune activation.

DC-SIGN interacts with a variety of pathogens, including HIV-1, and binding of HMOs inhibited the transfer of HIV-1 to CD4+ T lymphocytes. These data may suggest that oligosaccharides act systemically and are thereby modulating the immune response in a microbiota-independent manner. In addition, recent publications have also demonstrated that the c-type lectin Dectin-1 can modulate innate immune function, possibly explaining the cross-protection against other pathogens seen after vaccination (64, 65).

The functions of these receptors thus indicates that binding of HMOs to these structures may result in regulation of adaptive and innate immune protection against infection and inflammation.

EFFECTS OF HMOS ON INFECTION, ALLERGY AND IMMUNE PARAMETERS IN HUMAN STUDIES

As can be seen in **Table 2**, there are currently only a few infant studies on effects of HMOs on infection and immune function. Most of these studies are observational studies on breastfeeding infants, correlating HMOs in breastmilk with these outcomes. Placebo controlled studies with HMOs have been performed but have to date focused on safety rather than on anti-infective and immunomodulatory effects (18, 77).

Four of these studies showed an effect of HMOs on prevention of diarrhea (67, 69), respiratory tract infections (69), and severe outcomes like sepsis and death (75). Morrow et al. showed in another study that HIV exposed, non-infected children receiving breastmilk of secretor+ mothers have a reduced risk of early mortality compared to secretor—breastfeeding (66).

Also, in relation to cow's milk allergy, the level of Lacto-N-fucopentaose (LNFP) III in breast milk correlated with the prevalence of cow's milk allergy (9). Similarly, Sprenger et al. reported that FUT2-dependent breast milk oligosaccharides, with the levels of 2′FL as proxy for secretor status, were associated with lower levels of IgE-mediated allergies and eczema (70).

Finally, Biesbroek et al. reported recently that 6 week old breastfed children have a different nasopharyngeal microbiota, suggesting that milk components like HMOs may influence the nasopharyngeal microbiota composition—which may contribute to the protective effect of breastfeeding on decreased respiratory infections (76).

It should be stressed that none of these studies have formally demonstrated direct effects of HMOs, and that other breastfeeding components may be associated with the effects described. Only in a recent study (71) the administration of 2′-FL in combination with LNnT could reversely correlate with parentally reported episodes of bronchitis, lower respiratory tract infections, and use of antipyretics or antibiotics at different ages.

However, as reviewed in detail in section Effects of HMOs on Infection, Allergy and Immune Parameters in Human Studies and in several recent reviews (2, 35, 78–88), quite some information is available of effects of effects of HMOs on microbiota composition, pathogens, and pathogen adhesion *in vitro*, as well as on infection *in vivo*. In addition, effects on intestinal epithelium and barrier function, as well as immune function have been described in these reviews and the underlying literature.

In infants another study showed that the secretor or non-secretor genotype of mothers of infants that were breastfed correlated with enterocolitis (low secretor) and sepsis (non-secretor) (66).

It has been shown as well that the amount of 2-linked fucosylated oligosaccharides in breast milk inversely correlates with the incidence of diarrhea in infants (89), and similarly the amount of fucosyl oligosaccharides in breast milk inversely correlates with the severity of infection with E coli that has a stable (68). Similarly, the amount of 2FL inversely correlated with Campylobacter diarrhea (89).

Bode et al demonstrated that the risk of HIV transmission in breastfeeding children of HIV infected mothers inversely correlates with HMO concentration (73). In another study the amount of LDFH-1 in breast milk inversely correlated with norovirus diarrhea (67). In addition to effects of HMOs on

TABLE 2 | Human studies with HMOs and measured outcomes.

Title of study	Health-related effects	HMO used	Target group	Study setup	Outcome of effect HMO (Short)	Reference
Fucosyltransferase 2 non-secretor and low secretor status predicts severe outcomes in premature infants.	Mortality, necrotizing enterocolitis (NEC), sepsis	Breastmilk	Infants ($n = 410$)	Observational study	Mortality, NEC and gram - sepsis increased in infants receiving low secretoe status breast milk	(66)
Human milk oligosaccharides are associated with protection against diarrhea in breast-fed infants.	Diarrhea	Breastmilk	Infants ($n = 93$)	Observational study	(1) High levels of 2-FL in breastmilk protective against Campylobacter diarrhea (2) High levels of lacto-N-difucohexaose (LDFH-I), also a 2-linked fucosyloligaosaccharide, protective against calicivirus diarrhea	(67)
Innate protection conferred by fucosylated oligosaccharides of human milk against diarrhea in breastfed infants	Diarrhea	Breastmilk	Infants ($n = 93$)	Observational study	Breast milk with higher 2-linked to non-2-linked fucosyloligosaccharide ratios affords greater protection against infant diarrhea	(68)
Early consumption of human milk oligosaccharides is inversely related to subsequent risk of respiratory and enteric disease in infants.	Diarrhea and respiratory infection	Breastmilk	Infants ($n = 49$)	Observational pilot study	LNF-II levels in breast milk and in infant feces at 2 weeks of age (as representative of total HMO) associated with fewer infant respiratory problems and gastropintestinal problems by week 6 and week 12	(69)
FUT2-dependent breast milk oligosaccharides and allergy at 2 and 5 years of age in infants with high hereditary allergy risk	Eczema	Breastmilk	Infants at risk for allergy ($n = 266$)	Observational study (in placebo arm of controlled study)	At 2 years, but not at 5 years, FUT2-dependent oligosaccharides associated with lower IgE-associated eczema manifestations. Only in C-section-born infants with high allergy risk	(70)
Human milk oligosaccharides and development of cow's milk allergy in infants	CMA	Breastmilk	Infants with ($n = 35$) and children without CMA ($n = 39$)	Observational study	Infants receiving breast milk with low LNFP III levels more likely to become affected with CMA than infants receiving higher levels of LNFP III	(9)
Effects of infant formula with human milk oligosaccharides on growth and morbidity: A randomized multicenter trial	Respiratory infection (bronccitis) and antibiotic use	Formula containing 2′fucosyllactose (2′FL) + lacto-N-neotetraose (LNnT)	Infants receiving cow's milk-based infant formula ($n = 87$) vs. the same formula with 2′FL and LNnT ($n = 88$)	Multicenter, randomized, double-blind trial	Infant formula supplemented with 2′FL and LNnT associated with lower parent-reported morbidity (particularly bronchitis) and medication use (antipyretics and antibiotics)	(71)
Infants fed a lower calorie formula with 2′-fucosyllactose (2′FL) Show Growth and 2′FL Uptake Like Breast-Fed Infants	Growth	Formula supplemented with 2-Fucosyllactose (2′FL) and galactooligosaccharides (GOS)	Infants exclusively formula-fed in 3 groups: (1; $n = 101$ control formula GOS 2; $n = 104$ formula high GOS and low 2′FL 3; $n = 109$ medium GOS and medium 2′FL) or breastfed ($n = 106$) from enrollment to 4 mo of age	A prospective, randomized, controlled, multicenter growth and tolerance study	Growth and 2′FL uptake similar to breast milk	(18)

(Continued)

TABLE 2 | Continued

Title of study	Health-related effects	HMO used	Target group	Study setup	Outcome of effect HMO (Short)	Reference
Similar to those who are breastfed, infants fed a formula containing 2′-fucosyllactose have lower inflammatory cytokines in a randomized controlled trial	Immune parameters	Formula supplemented with 2-FL and GOS	Infants exclusively formula-fed in 3 groups: (1; $n = 75$ control formula GOS 2; $n = 76$ formula high GOS and low 2′FL 3; $n = 78$ medium GOS and medium 2′FL) or breastfed ($n = 86$) from enrollment to 4 mo of age	Observational substudy nested within a randomized, double-blind, controlled study	Infants fed formula supplemented with 2′-FL exhibit lower plasma and *ex vivo* inflammatory cytokine profiles, similar to those of a breastfed reference group	(72)
Human milk oligosaccharide concentration and risk of postnatal transmission of HIV through breastfeeding.	HIV transmission	Breastmilk	Breast milk of HIV-infected women who did not transmit HIV despite breastfeeding ($n = 86$), and uninfected women ($n = 36$)	Nested case-control study was conducted within a larger cohort study	(1) Higher concentrations of non-3′-SL HMOs were associated with protection against postnatal HIV transmission (2) A trend toward higher concentrations of lacto-N-neotetraose (LNnT) being associated with reduced transmission	(73)
Human milk oligosaccharides differ between hiv-infected and hiv-uninfected mothers and are related to necrotizing enterocolitis incidence in their preterm very-low-birth-weight infants	NEC, HIV infection	Breastmilk (secretor/nonsecretor)	HIV infected mothers ($n = 41$ of which 22 secretor, 19 non-secretor) and non-infected mothers ($n = 41$ of which 20 secretor, 21 non-secretor)	Substudy of a larger clinical trial on HIV-infected and HIV-uninfected mothers and their preterm infants	(1) HIV-infected mothers have higher relative abundances of 3′-SL in breastmilk (2) Low concentrations of DSLNT in brestmilk increased infant's risk of NEC	(74)
Growth and Morbidity of Gambian Infants are Influenced by Maternal Milk Oligosaccharides and Infant Gut Microbiota.	Morbidity	Breastmilk	Mother/infant pairs ($n = 33$, of which 21 secretors and 22 non-secretors)	Sub-study embedded within a randomized trial	(1) Higher breast milk levels of lacto-N-fucopentaose I (secretor) associated with decreased infant morbidity (2) Higher breast milk levels of LNT (non-secretor) associated with higher infant morbidity 3) Breast milk levels of 3′-sialyllactose indicator of infant weight-for-age	(5)
Oligosaccharide composition of breast milk influences survival of uninfected children born to hiv-infected mothers in Lusaka, Zambia	HIV infection, mortality	Breastmilk	HIV-infected children ($n = 103$) and HIV exposed uninfected children ($n = 143$).	Nested case-cohort study	High levels of fucosylated HMOs in breastmilk of mothers of HEU children protective against mortality	(75)
The impact of breastfeeding on nasopharyngeal microbial communities in infants.	Respiratory infection	Breastmilk	Infants receiving exclusive breastfeeding ($n = 101$) vs. and exclusive formula feeding ($n = 101$)	Case-cohort analysis	(1) Association between breastfeeding and microbial community composition in the upper respiratory tract (2) Possible link to protective effect of breastfeeding on respiratory infections and wheezing in early infancy	(76)

intestinal infections, HMOs have been linked to other infections such as infections of the urogenital tract (8, 90), and airway infection (69, 91, 92).

Such results were expanded lately with measuring inflammatory cytokines in systemic circulation if infants receiving infant formula supplemented with 2′-FL (72).

Much more is known about the direct effects of HMOs on pathogens, on adhesion and infection in *in vitro* models, and in animal models.

EFFECTS OF HMOS ON IMMUNE FUNCTION AND INFECTION IN *IN VITRO* AND ANIMAL STUDIES

Effects on Bacterial Adhesion and Infection

HMOs have been shown to prevent adhesion of several potential pathogens to epithelial surfaces in the intestine and other organs by acting as decoy receptors for bacterial pathogens like Campylobacter or *E. coli* (86, 93–95).

Several recent manuscripts report specific effects of isolated HMOs. For example, Weichert et al. showed that 2′FL, and to a lesser extent 3FL, reduce the adhesion of Campylobacter, EPEC, Salmonella and Pseudomonas, although the inhibitory effects were very small (96). Sialylated oligosaccharides were shown to reduce adhesion of EPEC (97, 98). In addition to reducing the adhesion of entire bacteria, HMOs may also compete with binding of bacterial toxins and mitigate their diarrheal activity (99, 100).

However, not always is the beneficial effect of HMOs to bacterial infection arising from preventing association or invasion of the pathogen. For example, HMOs can alter gene expression in intestinal cells that can block infection of Listeria monocytogenes (101), or have a direct effect on the growth of pathogens as was shown for neutral HMOs and especially LNT and LNFP I against group B Streptococcus (102). Similarly, HMOs were shown to modulate hyphal induction in Candida albicans, which is necessary for invasion of the intestinal epithelium (103). Another mechanism could be attenuation of pathogenic virulence through metabolites from fermentation of HMOs from the intestinal microflora. That seems to be at least partially the case for *Escherichia coli* O157:H7 and Salmonella typhimurium (104). When bifidobacteria of human and bovine origin were grown on medium containing 3′-SL, they could produce metabolites that could block expression of virulence genes in both pathogens. In addition, HMOs may have an indirect effect on bacterial infection by reducing epithelial inflammatory responses as it has been shown for 2′-FL and Campylobacter-induced inflammation (105).

While 2′-FL protected against adherent-invasive *E. coli*-induced pathology in mice (106), it failed to improve *E. coli*-induced diarrhea in piglets (107). These contradicting results could be explained by the difference in model, virulence of different *E. coli* strains, dosage and timing of administration of pathogens and HMOs, etc.

Effects on Intestinal Viruses

Shang et al. demonstrated that different HMOs can bind to norovirus (LNFPII and 2′FL) and Norwalk virus (LNFP I and LNDFHI), indicating that several potential Noro- and Norwalk virus-binding glycans are present in HMOs that can play a role in viral infection (108). Notably, they also showed that LNFP III-HSA and 2′-FL-BSA – but not heir monovalent forms (LNFP III-Gly and 2′-FL-Gly) bound to VA287 capsids. This suggests that polyvalent oligosaccharides on a carrier protein may be more potent in anti-adhesion effects than their monovalent sugars themselves. However, recently it was also shown that 2′-FL can block both the GI.1 and GII.17 noroviruses from binding to HBGAs (109).

In addition to effects on gut bacteria, HMOs can also have effects on viral pathogens as rotavirus, norovirus, and HIV [reviewed in (85)].

In Rotavirus infected piglets, HMO-supplemented piglets had a shorter duration of diarrhea compared to the control Group (110, 111). There have been several HMO structures identified that bind the glycan rotaviral receptor VP8*. The sialic acid containing HMOs inhibited rotavirus infection *in vitro*, but *in vivo* both neutral HMOs and sialic acid containing HMOs decreased replication during acute RV infection *in situ*. These data are confirmed by recent *in vitro* findings where 2′-FL, 3′-SL, and 6′-SL could block infectivity of human rotaviral strains in cells (7). Apparently simple HMO structures can act as decoy receptors for viruses. However, since there are differences in the infectious mechanism of porcine and human rotaviral strains extrapolation from porcine to human models can be treacherous and more research is needed to clarify the role of HMOs in rotaviral infections.

Effects on Respiratory Viruses

In addition to effects on intestinal pathogens, HMOs have been suggested to also play a role in infections from respiratory viruses. For example, 2′FL was shown to decrease RSV viral load, whereas LNnT and 6′SL decreased influenza viral load. Also effects were observed on innate cytokines in response to both viruses (92) suggesting an effect of HMOs on respiratory virus infection. This is supported by an early study by Stepans-Flanders on the fact that HMO consumption is inversely linked to respiratory infection (69). In this study higher LNFPII levels in breastmilk correlated with decreased respiratory and gastrointestinal infections in early infancy.

Immobilized 3′SL and 6′SL haven been shown to prevent infectivity of influenza viruses as a result of blocking the haemagglutins of influenza viruses (112, 113), and Yu et al. identified a number of additional sialic acid containing HMOs that bind to influenza virus (114). The effects of these HMOs was confirmed in a functional infection assay *in vitro*, where 6′SL and LNnT were shown to reduce the viral load of influenza in airway epithelial cells, and 2′FL did the same for respiratory syncytial virus (RSV) (92). In one recent *in vivo* study 2′FL enhanced responses to vaccination in mice (115). The mechanism was postulated to involve also a direct effect of 2′FL on dendritic cells as shown *in vitro*. However, concentrations used in their experiments were more than 1000-fold higher than what has

been described to be found in circulation (20), warranting further clarification and research on the mechanism of action and relevance to human breast-fed infants.

Enterocolitis

In relation to necrotizing enterocolitis, Jantscher-Krenn et al. noted in a rat model that disialylated LNT (DSLNT) increased survival rates and improved pathology scores (116), while low amounts of DSLNT in mother's milk could be a predicting risk factor for the development of NEC in premature infants (117) corroborating the previous findings. More HMOs could have a beneficial effect in NEC as it was shown also in a rat study where 2′-FL ameliorated the pathology of NEC, however there was no association between 2′FL and NEC risk in the corresponding human cohort (115). Similar observations were made for rats fed sialylated galacto-oligosaccharides (Sia-GOS) (118). Studies in mice have also shown a beneficial effect of 2′-FL in an induced NEC model (119). However such effect could not be seen in a piglet model where piglets born with caesarian section were fed control formula or formula supplemented with 2′-FL and were let to develop NEC spontaneously (120). Differences between induction vs. natural progression to NEC or species differences could account for such outcomes. In infants another study showed that the secretor or non-secretor genotype of mothers of infants that were breastfed correlated with enterocolitis (low secretor) and sepsis (non-secretor) (66).

Effects of HMOs on Intestinal Epithelium

In another study immunomodulation by 2′FL *in vivo* was shown to be dependent on the downregulation of CD14 on intestinal epithelial cells (106). As CD14 is a co-receptor for LPS and is involved in TLR-4 signaling, this may lead to decreased inflammatory responses in the intestine after exposure to LPS.

In a recent study by He et al. the effect of colostrum oligosaccharides on gene expression in fetal immature intestinal mucosa was tested (121). They identified several immune related pathways were induced by HMOs, such as Immune cell communication, homeostasis, and intestinal immune differentiation. HMOs could reduce the response to TLR stimuli, and induced cytokines that are involved in tissue repair. 3′, 4', and 6′ galatosyllactoses were the most potent oligosaccharides.

Sialyllactose has been described to be able to promote the differentiation and growth of human intestinal epithelial cells as measured by upregulation of expression of alkaline phosphatase (122). Alkaline phosphatase is a molecule that is important in maintaining gut barrier function, possibly through the inactivation of LPS, by cleaving of a phosphate group from LPS. This suggests that on the one hand epithelial cells may yet have a receptor that recognizes Sialyllactose, and that Sialyllactose may be beneficial for promoting a good epithelial barrier in the gut.

In addition, two recent papers suggest that SL or goat milk oligosaccharides containing SL may have an effect on epithelial cells via activating through TLR4 (43, 123).

In contrast, another paper showed that 3′SL had anti-inflammatory activity by reducing the expression of IL-12 and IL-8 in Caco-2 cells, mediated via NFkB, and stimulates the anti-inflammatory nuclear receptor PPARg (124). Especially neutral HMOs have been shown to have an anti-inflammatory effects on the intestinal epithelium in *in vitro* inflammatory models (106, 121).

Lane compared effects of HMOs and BMOs on gene expression in HT-29 cells, noting that "both treatments including a response to stimulus, signaling, locomotion, and multicellular, developmental and immune system processes" (125).

Combined, these studies suggests that milk oligosaccharides contribute to the development and maturation of the intestinal immune response.

Effects of HMOs on Immune Function

Acidic HMOs (but not acidic cow's milk oligosaccharides) were shown to induce IFN-g and IL-10 in human cord blood T cells, and could decrease IL-4 production in allergen-specific T cells (126). These data suggest that acidic HMOs may downregulate Th2 responses in infants as well.

Such results were expanded lately with measuring inflammatory cytokines in systemic circulation if infants receiving infant formula supplemented with 2′-FL (72). In this study, supplementation of 2′-FL alone could lower levels of TNFα, IL-1α, IL-1β, and IL-6 resembling those found in breast fed infants. In early studies LNFP III and LNnT were shown to have immunosuppressive effects (127, 128), and LNFPIII can induce IL-10 in macrophages (129, 130). Unexpectedly, a link with helminth infections exists. It is now known that upon infection with helminths confers a protective effect on allergy development (131, 132). Schistosoma eggs, but not the helminth itself, induce potent IL-10 responses that inhibit Th2 responses (133, 134). These effects are at least in part mediated by the oligosaccharides LNFPIII GalNAcβ1-4(Fucα1-2Fucα1-3) GlcNAc (LDN-DF) and Lewis-X that is present in the egg shells. These oligosaccharides are also found in breast milk, suggesting a functional anti-allergic/anti-inflammatory role of these HMOs. Likewise, Comstock et al. demonstrated a similar effect of HMOs *in vivo* in piglets, where HMOs induced IL-10 levels and inhibited T cell proliferation (135). The same was noted by Hester et al that showed enhanced T helper type 1 (interferon-gamma) and anti-inflammatory (interleukin-10) cytokines in the ileum in response to HMO supplementation of piglets in a rotavirus infection model (110).

Interestingly, LNFPIII and Lewis X glycoconjugates can also inhibit TLR signaling in innate immune cells through possible involvement of c-type lectins (132). LNFPIII is a very well-studied HMO that has been linked to many different effects including hepatosteatosis and insulin resistance (136), autoimmunity (137), and transplantation (138). Especially in the case of insulin resistance and autoimmunity, HMOs have been shown to elicit a protective effect in a murine model of Type 1 Diabetes (T1D) (139). The paper shows that supplementation of HMOs can alter microbiota composition and SCFA production in a NOD-mouse model that can prevent spontaneous progression to diabetes. The protective effect of SCFA-producing diets on T1D has been documented beforehand (140, 141). On the other hand, by increasing barrier integrity

HMOs may also reduce gut permeability, which has been argued to contribute to the onset of T1D (142).

Indirect Effects of HMOs on Intestinal Epithelium and Immune Function via SCFA

Another important role of HMOs is establishing and maintaining the intestinal microbiota. Breastfed infants have higher numbers of beneficial bifidobacteria and lactobaccilli than bottle fed infants. This is the result of preferential fermentation of HMOs by the microbiota by bifidobacterial and lactobacilli (143–145). Upon fermentation of HMOs these bacteria produce, in addition to lactic acid, the short chain fatty acids (SCFA) butyrate, actetate, and propionate. These SCFA improve intestinal barrier function (146) lower the pH in the colon, and have well established anti-inflammatory properties (147).

The notion that composition and metabolic activity of the intestinal microbiota affects the development of allergies has become clearer over the last years (148–151). Exactly how the microbiota composition influences allergy development is not clear at this point, but data from animal models strongly suggest a protective role for SCFA (152–155). A similar role was shown for the SCFA receptors GPR43 in asthma, arthritis, and colitis models (156), and for GPR41 in allergic airway inflammation.

Taken together, these data suggests that HMOs may also have an indirect effect on allergy.

HMOs AND ALLERGY

Several, but not all, studies on the association between breastfeeding and allergy have shown effects on allergic outcomes (157–159). One of the factors that may explain the conflicting findings described above may be the result of differences in breastmilk composition (in relation to milk proteins) (160). None of these studies have correlated their findings with HMO composition.

However, two studies have done just this. In a cohort study of cow's milk allergy (CMA) (9) it was observed that the concentration of 6SL, DSLNT, LNFPI, and LNFPIII was lower in the breast milk of mothers having infants with CMA. After further corrections, only breast milk levels of LNFPIII associated reversely and significantly with development of CMA. In the same study it was observed that FUT2 status of mothers correlated with a delayed onset of CMA while CMA infants born to non-secretor mothers (FUT2 negative) were prone to acute CMA (IgE-mediated). FUT2 status seemed to play a role also in IgE-mediated eczema developed in infants born with C-section (70). In a study of 266 infants followed for 5 years, they observed that infants born to secretor mothers had lower incidence of IgE-mediated eczema. That effect was evident at 2 years but not at 5 years of age though. 2′-FL (one of the main HMOs produced by secretor mothers) was shown to have a significant association with any allergic disease, acute or delayed in infants born with C-section in the same study. 2′-FL and 6′SL had also a beneficial

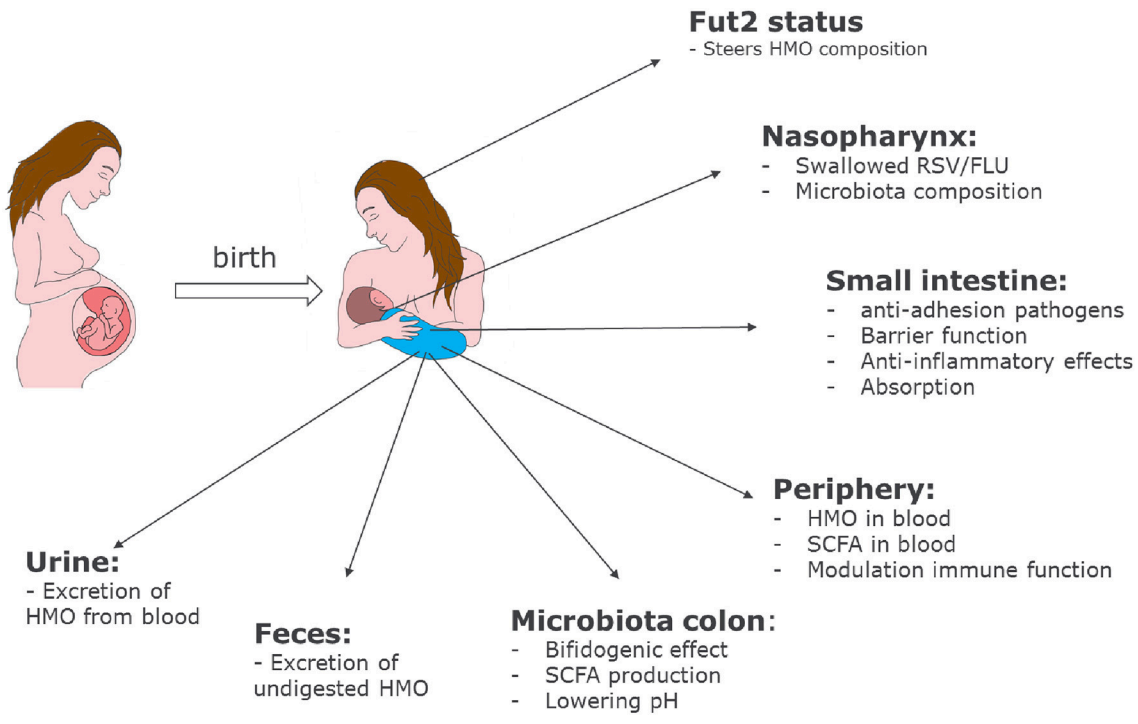

FIGURE 2 | Role of HMOs in developmental physiology of infants: Depending on the genetic background of the mother, HMO composition may differ. Depending on the HMO composition in breast milk, benefits of HMOs span a broad range from shaping the infant microbiome to preventing infections and having systemic effects in the infant after absorption in the intestine.

effect in a mouse model of OVA-induced allergy (161). Both HMOs could increase numbers of IL10 producing Treg cells and alleviate allergic symptoms but through different mechanisms.

CONCLUSIONS

HMOs contribute to the development of the microbiota and the immune system of newborn infants. The mechanisms by which HMOs contribute have become clearer over the past few years, and our current knowledge is summarized in **Figure 2**.

However, despite many *in vitro*- and animal experiments, HMOs have not been tested extensively in placebo controlled infant studies. It is clear that several HMOs will be introduced in the near future into infant nutrition to supplement or replace non-human prebiotics like galactooligosaccharides and/or fructooligosaccharides. Prebiotics have been added to infant nutrition in the early 2000's as non-digestible oligosaccharides in an attempt to mimic some of the function of HMOs. With these prebiotics a large number of studies have shown effects on intestinal infection, respiratory infection and allergy (162–167). As the selection of prebiotics is based on functional similarities with HMOs, and extrapolating from *in vitro* and animal experiments with HMOs, it is to be expected that inclusion of HMOs to infant formula will have additional benefits to infant health, and may supplement the functionality of the prebiotics that are already used. Still more research is needed to clarify whether HMOs may also have a therapeutic rather than a protective effect in human immune disorders. Our emerging evidence for the beneficial effects of HMOs once again provide a powerful rationale to encourage women to breastfeed their infants to provide the full scope of benefits that stem from a diverse composition of HMOs that is provided through mother's milk and could potentially be personalized to match the genetic context and environmental exposures of the mother-infant dyad.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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TGF-β Concentration in Breast Milk is Associated With the Development of Eczema in Infants

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Background: Transforming growth factor (TGF)-β in breast milk is crucial for mucosal immune system in the neonatal period. We hypothesized that the level of exposure to TGF-β from breast milk in the first month of life is related to the development of eczema later in life. Thus, the present study investigated whether changes in TGF-β levels between colostrum and mature milk are associated with such occurrence in a birth cohort study.

Methods: Colostrum and 1-month breast milk samples were collected from mothers who participated in our birth cohort study. TGF-β1 and TGF-β2 levels in breast milk were measured using a commercial ELISA kit. The development of eczema in the first 6 months after birth was assessed based on parent's response to a questionnaire. Levels of TGF-β1 and TGF-β2 were compared in breast milk from mothers of infants with and without eczema.

Results: In children with eczema, TGF-β1 levels were higher in colostrum, but lower in 1-month milk. A lower TGF-β1 ratio (1-month milk/colostrum) was related to the development of eczema during the first 6 months of life. There was no difference in TGF-β2 ratio (1-month milk/colostrum) between eczema group and control group.

Conclusions: Concentration of TGF-β1 but not TGF-β2 in breast milk during the first month after birth may be associated with eczema later in life. Factors that increase TGF-β1 levels in breast milk may play a role in preventing allergic disease.

Keywords: TGF-β, breast milk, infants, allergy, eczema

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INTRODUCTION

Breast milk contains a wide variety of immune mediators, such as secretory immunoglobulin A, soluble CD14, and transforming growth factor (TGF)-β (1). These mediators are crucial for mucosal immune system in the neonatal period.

TGF-β may play a role in preventing allergic diseases. In animal models, the oral administration of TGF-β1 maintained the biological activity in the intestinal mucosa and enhanced the induction of oral tolerance (2). It has also been reported to inhibit inflammation in the intestinal epithelium and systemic production of interleukin-6 and interferon-γ, reducing the incidence of

necrotizing enterocolitis (NEC) (3). In contrast, developmental defects rather than immunological dysregulation were observed in TGF-β2- and TGF-β3-deficient mice (4, 5).

Several studies have been conducted to address whether the levels of TGF-β in breast milk are related to the development of allergic diseases. Although a review article reported TGF-β in breast milk is an effective cytokine in preventing allergic disease (6), the results of several other studies were controversial (7–9). Hence, we hypothesized that the amount of TGF-β received in the first month of life is important in the prevention of allergy, and in the present study we investigated whether changes in the TGF-β cytokine level in breast milk from colostrum to mature milk were related to the future development of eczema in infants.

MATERIALS AND METHODS

Study Design

This study is part of a birth cohort study of 500 newborn infants, which was conducted in Kawatetsu-Chiba Hospital from January 2007 to May 2008. The study design has been described in detail elsewhere (10). Briefly, all participants received a questionnaire when their infants were born. Data on parental allergic disease and various exposures were obtained. The parents also answered questionnaires on symptoms related to eczema in their infant at 1, 4, and 6 months of age. Eczema was defined as itchy skin rashes lasting >2 months in 6-month-old children.

Infants who presented with eczema at 6 months were included in the eczema group. As for the control group, we randomly selected infants who did not have eczema at 6 months. Both groups were matched in terms of feeding methods and history of atopic dermatitis or other allergic diseases in the mother (asthma, allergic rhinitis, food allergy, and pollen hypersensitivity). Levels of TGF-β1 and TGF-β2 in breast milk were measured in both groups.

The study protocol was approved by the Ethics Committee of Chiba University Graduate School of Medicine (No. 570). All parents provided written informed consent.

Breast Milk Collection and Processing of Breast Milk

Colostrum samples were collected within 5 days post-partum and mature milk samples were collected 1 month after delivery. Each sample was frozen within 12 h of collection and kept at −80°C. After thawing, the samples were centrifuged at 10,000 g for 10 min, the cellular debris and fat layer were discarded, and the clear middle layer was used for analyses.

Measurements of TGF-β1 and TGF-β2 in Breast Milk

TGF-β1 and TGF-β2 levels in breast milk were measured with the Quantikine Human TGF-β1 and TGF-β2 Immunoassays (R&D Systems Inc, MN, USA). Activation of TGF-β in breast milk was performed using 1N HCl and neutralized with 1N NaOH. The sensitivity threshold for both the TGF-β1 and TGF-β2 assays were 31.25 pg/mL. TGF-β1 levels below 31.25 pg/mL were also measured since we could confirm linearity with sequential dilution down to 1.95 pg/mL. We therefore defined 3.9 pg/mL as the detection limit of the TGF-β1 assay in this study. Since the clear middle layer was diluted 40 times in the neutralization step for TGF-β1 analysis, 156 pg/mL was defined as the detection limit. Undetectable levels of TGF-β1 were set as half the value of the minimum detectable level at 78 pg/mL. The clear middle layer was diluted 7.8 times in the neutralization step for TGF-β2 analysis, 243.75 pg/mL was defined as the detection limit. Undetectable levels of TGF-β2 were set as half the value of the minimum detectable level at 121.9 pg/mL.

Statistical Analysis

Differences in the characteristics of the participants were assessed by using chi-squared test or Fisher's exact test and expressed as p values. Mann-Whitney *U*-test was used to compare TGF-β levels in both colostrum and mature milk and TGF-β ratio (1-month milk/colostrum) between eczema and control group. Changes in TGF-β levels from colostrum to mature milk was assessed with the Wilcoxon signed rank test. Adjusted logistic regression analyses were conducted to evaluate the association between eczema and TGF-β ratio. The TGF-β ratio was categorized into tertile as low, medium, or high levels.

For all analyses, $p < 0.05$ was considered statistically significant. Statistical analysis was performed with SPSS software ver. 11.0 (SPSS INC., Chicago, IL, USA).

RESULTS

Study Subjects

Fifty-one infants developed eczema at 6 months of age. Because of the lack of mother's milk, 8 infants with eczema were excluded from the study, leaving 43 subjects for final analysis. As controls, 53 children without eczema were randomly selected. **Table 1** shows the characteristics of the participants. The only difference between the two groups was gender proportion, with a higher number of girls in the control group.

TABLE 1 | Characteristic of the participants.

	Eczema group (*n* = 43)	Control group (*n* = 53)	*p*-value
Sex (boy: girl)	30:13	21:32	<0.05
Father allergy	26	40	0.12
Mother allergy	28	38	0.49
Mother atopic dermatitis	11	12	0.74
Mother smoking	2	5	0.37
Pet	11	16	0.62
Feeding methods			
Mixed	22	30	0.59
Breast feeding			
0–1 months	6	3	0.15
1–5 months	4	4	0.52
≥ 6 months	11	16	0.62

TGF-β Levels in Colostrum and Infant Eczema

In colostrum, TGF-β1 was undetectable in 29 samples of the control group, but only in 6 samples from the eczema group. TGF-β2 was detectable in all 96 samples. Undetectable TGF-β1 level in colostrum was set at 78 pg/mL.

Figure 1 shows the comparison of TGF-β1 and TGF-β2 levels in colostrum in both groups. TGF-β1 levels were significantly higher in the eczema group with a median 878.8 pg/mL (range 78–2,921 pg/mL) than that of control group (median: 78 pg/ml, range 78–5,538 pg/ml, $p < 0.01$, **Figure 1A**), while the levels of TGF-β2 was not significantly different between eczema group and control group (median: 4,129 pg/mL, range 1,175–31,900 pg/mL; and median: 4,977 pg/mL, range 721.2–26,260 pg/mL, respectively, $p = 0.92$, **Figure 1B**).

TGF-β Levels in 1-Month Milk and Infant Eczema

In 1-month milk, TGF-β1 were undetectable in 19 samples from the control group and 24 in the eczema group. TGF-β2 were undetectable only in 4 samples of the eczema group. Undetectable levels of either TGF-β1 in 1-month milk was also set at 78 pg/mL. Undetectable levels of TGF-β2 in 1-month milk was also set at 121.9 pg/mL.

Figure 2 shows the comparison of TGF-β1 and TGF-β2 levels in 1-month milk in both groups. In contrast to colostrum, TGF-β1 levels in the eczema group was lower than those in the control group (median: 78 pg/mL, range 78–1,768 pg/mL; and median: 311.2 pg/mL, range 78–3,903 pg/mL, respectively, $p < 0.01$, **Figure 2A**), while there was no significant difference in the TGF-β2 levels between eczema group and control group (median:1,692 pg/mL, range 121.9–12,370 pg/mL; and median 1,550 pg/mL, range 255.2–8,273 pg/mL, respectively, $p = 0.65$, **Figure 2B**).

TGF-β Levels Ratio and Eczema in Infants

Figure 3 shows the change in TGF-β levels in colostrum vs. 1-month milk in both groups. TGF-β1 concentration did not decrease in the control group ($p = 0.21$); however, there was a significant reduction in the eczema group ($p < 0.01$, **Figure 3A**). TGF-β2 concentration significantly decreased in both groups ($p < 0.01$, **Figure 3B**).

TGF-β1 ratio (1-month milk/colostrum) was lower in the eczema group than in the control group ($p < 0.01$, **Figure 4A**).

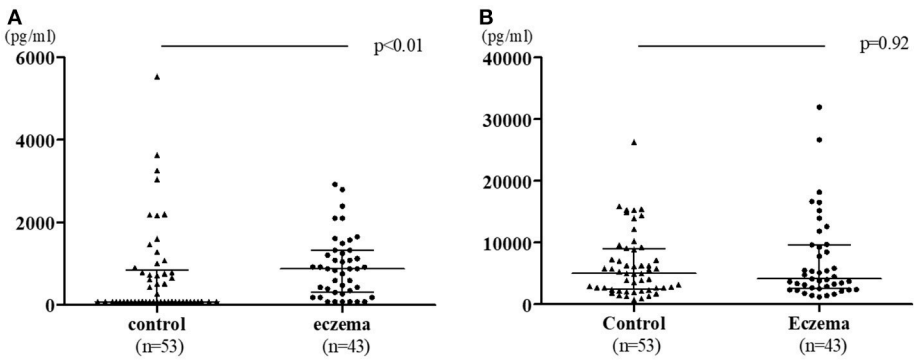

FIGURE 1 | TGF-β1 **(A)** and TGF-β2 **(B)** levels in colostrum in eczema group (43 subjects) vs. control group (53 subjects). Data is shown with medians and interquartile ranges.

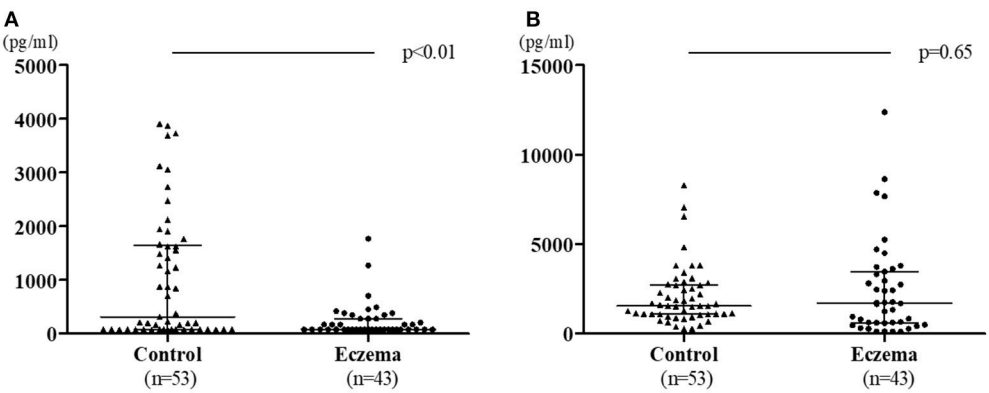

FIGURE 2 | TGF-β1 levels **(A)** and TGF-β2 levels **(B)** in 1-month milk in eczema group (43 subjects) and control group (53 subjects). Data is shown with medians and interquartile ranges.

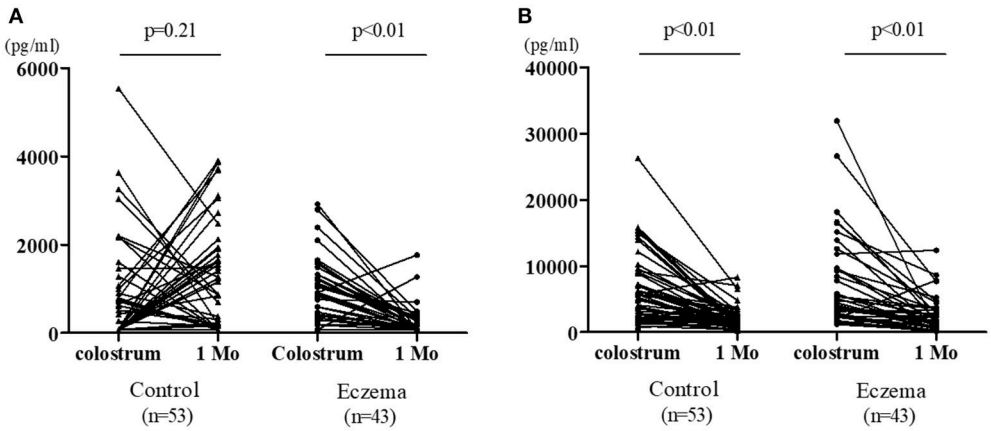

FIGURE 3 | Individual values of TGF-β1 **(A)** and TGF-β2 **(B)** in colostrum and 1-month milk in eczema group (43 subjects) and control group (53 subjects).

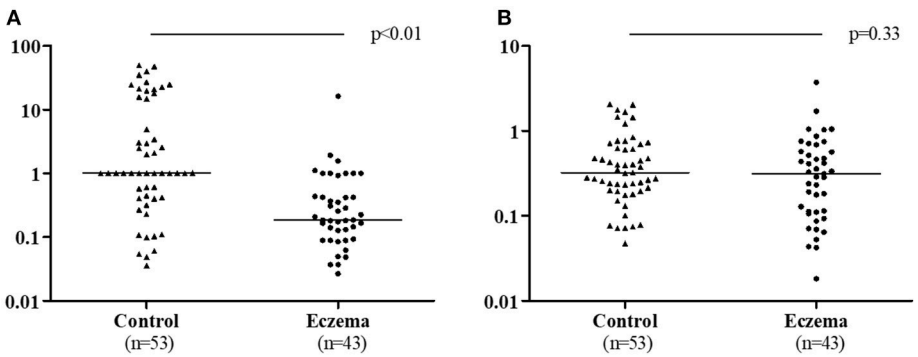

FIGURE 4 | TGF-β1 ratio (1-month milk/colostrum) **(A)** and TGF-β2 ratio (1-month milk/colostrum) **(B)** in eczema group (43 subjects) and control group (53 subjects).

The TGF-β2 ratio was not statistically different between the two groups ($p = 0.33$, **Figure 4B**).

Multivariable Analysis

Table 2 describes the results of multivariate analysis with logistic regression. The risk of eczema was inversely related to TGF-β1 ratio.

Effect of Maternal Allergic History on TGF-β Levels in Breast Milk

We also compared TGF-β1 and TGF-β2 levels in both colostrum and 1-month milk in allergic mothers. The levels of TGF-β1 and TGF-β2 in colostrum were not significantly different between allergic mothers (median: 511.1 pg/mL, range 78–5,538 pg/mL; and median: 4,826 pg/mL, range 721.2–31,900 pg/mL, respectively, $p = 0.56$), and in non-allergic mothers (median: 511.1 pg/mL, range 78–2,799 pg/mL; and median: 4,553 pg/mL, range 1,402–16,650 pg/mL, respectively, $p = 0.55$), neither were their concentrations in 1-month milk in allergic (median: 169.1 pg/mL, range 78–3,903 pg/mL; and median: 1,678 pg/mL, range 121.9–12,370 pg/mL, respectively, $p = 0.4$) and non-allergic mothers (median: 183.4 pg/mL, range 78–3,119 pg/mL; and median: 1,129 pg/mL, range 121.9–7,852, respectively, $p = 0.28$).

TABLE 2 | Multiple regression analysis for the effect of TGF-β ratio on eczema.

Factor		Adjusted odds ratio	95% CI	*p*-value
Sex (Male)		2.084	0.774 ~ 5.610	0.146
Mother allergy		0.560	0.185 ~ 1.699	0.306
Breast feeding by 1 month		1.203	0.444 ~ 3.288	0.711
TGF-β1 RATIO (1-MONTH/COLOSTRUM)				
Low	0.0267–0.224	1		
Middle	0.229–1	0.161	0.049 ~ 0.534	<0.01
High	1.013–49.585	0.035	0.006 ~ 0.188	<0.01
TGF-β2 RATIO (1-MONTH/COLOSTRUM)				
Low	0.0182–0.214	1		
Middle	0.231–0.464	0.848	0.250 ~ 2.878	0.792
High	0.473–3.731	2.959	0.702 ~ 12.467	0.139

Logistic regression models adjusted for sex, maternal history of allergies, breast feeding by 1 month and TGF-β ratio.

DISCUSSION

We demonstrated that principally a lower TGF-β1 ratio (1-month milk/colostrum) was related to later eczema in infants.

Reports on the relationship between TGF-β in breast milk and onset of allergic diseases are controversial. Some studies have reported that neither TGF-β1 nor TGF-β2 was associated with the onset of allergic disease (7–9, 11), while others have demonstrated such an association (12–14). In the present study, we found a significant relationship between TGF-β1 levels in breast milk and the development of eczema. In the eczema group, the TGF-β1 concentration was higher in colostrum, but lower in 1-month milk. In contrast with previous reports, we measured TGF-β at multiple time points and assessed the TGF-β ratio.

The higher dose of TGF-β1 (long breast feeding and medium-high TGF-β1) in breast milk were shown to have a protective effect against wheezing (12); although the outcome was different, we saw a similar pattern with eczema in our study. Riggoti et al. reported that TGF-β1 was significantly higher in the mature milk of non-allergic mothers, and infants fed with this TGF-β1-rich breast milk had less risk of developing atopic diseases (15), further emphasizing the protective effects of this cytokine against eczema. Their results might be due to genetic causes as they had grouped their cohort based on the mother's allergic background. Levels of TGF-β1 reportedly decreases from colostrum to mature milk in urban mothers but not in rural mothers (16). These findings and our results suggest that a significant decrease in TGF-β1 in colostrum to mature milk is associated with later allergic disease.

Lower levels of TGF-β2 in colostrum were observed with *Lactobacillus reuteri* supplementation during pregnancy, leading to less sensitization in children of those mothers during the first 2 years of life (13). Nonetheless, higher levels of TGF-β2 in breast milk were reportedly related to higher risk of eczema (14). In our study, both TGF-β2 levels and 1-month/colostrum ratio for TGF-β2 were not different between eczema group and control group.

The factors influencing the levels of TGF-β in breast milk are not well known. TGF-β levels in breast milk differed between countries (17, 18), and even races in the same country (19, 20). Similar to our results, no difference was observed in the allergic history of mothers (21). There have been conflicting results between the supplementation of probiotics during pregnancy and TGF-β1 levels, with a previous study reporting a significant increase in TGF-β1 levels (22); although, other investigations did not support such a result (23–27). Further studies are needed to clarify the factors influencing the levels of TGF-β in breast milk.

As for the limitations of this study; first, in our recruitment, we had more boys in the eczema group. And second, we could not evaluate long-term outcomes. Nonetheless, the present study suggests that TGF-β1 levels in breast milk are related to the occurrence of eczema later in life.

In conclusion, the present investigation suggested that a higher 1-month/colostrum ratio for TGF-β1 is associated with a more consistent concentration or an increase of TGF-β1 across the first month, which has a protective effect in reducing the risk of eczema. Additional studies are needed to evaluate the relationship between TGF-β levels in breast milk and the development of allergic disease. Identifying factors that increase TGF-β1 levels in breast milk may contribute to preventing allergic diseases.

AUTHOR CONTRIBUTIONS

YM wrote this manuscript and did statistical analysis. EC-A wrote this manuscript. FY, TN and NoK provided critical advice. YK and NaK conducted study. HO, MK and EM measured TGF-β. NS conducted study and wrote this manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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SYSTEMATIC REVIEW
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Human Milk Oligosaccharides and Associations With Immune-Mediated Disease and Infection in Childhood: A Systematic Review

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Complex sugars found in breastmilk, human milk oligosaccharides (HMOs), may assist in early-life immune programming and prevention against infectious diseases. This study aimed to systematically review the associations between maternal levels of HMOs and development of immune-mediated or infectious diseases in the offspring. PubMed and EMBASE databases were searched (last search on 22 February 2018) according to a predetermined search strategy. Original studies published in English examining the effect of HMOs on immune-mediated and infectious disease were eligible for inclusion. Of 847 identified records, 10 articles from 6 original studies were included, with study quality ranging from low to high. Of three studies to examine allergic disease outcomes, one reported a protective effect against cow's milk allergy (CMA) by 18 months of age associated with lower lacto-*N*-fucopentaose (LNFP) III concentrations (OR: 6.7, 95% CI 2.0–22). Another study found higher relative abundance of fucosyloligosaccharides was associated with reduced diarrhea incidence by 2 years, due to (i) stable toxin-*E. coli* infection ($p = 0.04$) and (ii) "all causes" ($p = 0.042$). Higher LNFP-II concentrations were associated with (i) reduced cases of gastroenteritis and respiratory tract infections at 6 weeks ($p = 0.004$, $p = 0.010$) and 12 weeks ($p = 0.038$, $p = 0.038$) and (ii) reduced HIV transmission (OR: 0.45; 95% CI: 0.21–0.97) and mortality risk among HIV-exposed, uninfected infants (HR: 0.33; 95% CI: 0.14–0.74) by 24 months. Due to heterogeneity of the outcomes reported, pooling of results was not possible. There was limited evidence that low concentrations of LNFP-III are associated with CMA and that higher fucosyloligosaccharide levels protect infants against infectious disease. Further research is needed.

Keywords: oligosaccharides, human milk, breastfeeding, infants, allergy and immunology, respiratory tract infections, diarrhea, HIV

INTRODUCTION

Human milk contains a wide range of immunologically active components with the potential to protect against disease (1, 2). Research has emphasized the importance of human milk as an influential early-life exposure for the development of a healthy immune system; however, the mechanisms for this are still not clearly understood. Some studies have shown positive immunological effects and

anti-infectious properties of human milk, particularly in the prevention of respiratory and gastrointestinal infections (3, 4). Other research suggests that breastfeeding influences the intestinal microbiome, which may in turn influence autoimmune and allergic disease development (4). The association between human milk and allergic disease is controversial, with numerous studies reporting inconsistent results (5). A possible explanation for the contradictory findings may lie in the diverse composition of bioactive factors present in human milk (6) and even when specific milk components are addressed, there may be significant differences in both quantity and variety between human milk from different mothers.

Human milk oligosaccharides (HMOs) are a key constituent of human milk. They are a structurally and biologically diverse group of complex indigestible sugars (7, 8). To date, more than 200 different oligosaccharides have been identified, varying in size from 3 to 22 monosaccharide units (9).

The most common HMOs are the neutral fucosylated and non-fucosylated oligosaccharides (10–13). The quantity and structure of these HMOs differs significantly among women and is dependent upon Secretor and Lewis blood group status (14, 15). Mutations in the fucosyltransferase 2 (FUT2) secretor gene results in human milk that is deficient in α1,2-linked fucosylated oligosaccharides (12).

Human milk oligosaccharides provide no direct nutritional value to the infant, and there is only minor absorption across the intestinal wall with approximately 1–5% detectable in serum and urine (8). It is proposed, instead, that HMOs have many different roles to play for the infant. They are preferred substrates for several species of gut bacteria and act as prebiotics, promoting the growth of beneficial intestinal flora and shaping the gut microbiome, thereby affecting immune responses (8, 16, 17). Short-chain fatty acids generated by the gut microbiome breaking down HMOs are critical for intestinal health. They further favor the growth of benign gut commensals along with providing nourishment for epithelial cells lining the intestine (18). HMOs also directly modulate host-epithelial responses, favoring reduced binding of pathogenic microbiota to the gut epithelium. Gut microbiota composition differs between formula-fed and breastfed infants, possibly due to the absence of HMOs in infant formula milk (19). There is also evidence that HMOs act as decoy receptors, inhibiting the binding of enteric pathogens to prevent infection and subsequent illness (20). Furthermore, HMOs provide a selective advantage for colonization by favorable bacteria, thereby inhibiting the growth of pathogenic species.

Despite substantial interest in this area, to date no systematic review has been undertaken to assess the effects of HMOs on disease prevention. This systematic review aims to identify and summarize the current evidence of the associations between HMOs and immune-mediated or infectious diseases in early childhood. Establishing a clear link between HMOs and disease outcomes may lead to intervention strategies.

METHODS

Search Strategy

PubMed and EMBASE electronic databases were systematically searched (last search date 22 February 2018) for original studies examining the effect of HMOs on childhood immune-mediated and infectious disease outcomes. The search strategy included MeSH and free text terms for HMOs, allergic disease, immune-mediated disorders and clinical infections (see Table E1 in Supplementary Material). All original studies published in English were included. Papers that did not report original results, or outcome data of interest, were excluded.

Titles and abstracts of papers were screened by two authors (Alice M. Doherty and Xin Dai) for inclusion. Reference lists of primary articles and related reviews were checked to identify any other studies appropriate for inclusion. Studies assessed as eligible, potentially eligible or unclear, were retrieved in full text where available. Any uncertainty concerning inclusion of specific studies was resolved by discussion with a third author (Adrian J. Lowe). Outcomes of interest were the development of any immune-mediated diseases (allergic or autoimmune disorders) or clinical infections in childhood.

Data Extraction

Study characteristics were extracted and tabulated from each of the included studies. The data extracted included the following: author's name, date of publication, study design, location, population, exposure classification, outcome definitions, effect sizes, confounders and tests for potential effect modification, and potential sources of bias.

Quality Assessment

The Newcastle-Ottawa scale was used to assess the quality of individual studies (21). The quality assessment was performed independently by two authors (AD, XD) to meet PRISMA guidelines. Each study was scored using a star (*) method to report the quality based on selection of sample, comparability, and the ascertainment of the exposure or outcome measures for case–control or cohort studies, respectively. Included studies were graded on total score: unsatisfactory = 0–3; low = 4–5; moderate = 6–7; and high = 8–9 (see Table E2 in Supplementary Material). Studies were not excluded based on quality assessment.

Statistical Analysis

Where two or more papers reported the association between the same HMO and outcome, we pooled results using meta-analysis. The I^2 statistic was used to document heterogeneity of study results, and random effect models were used where there was wide spread differences between studies ($I^2 > 80\%$).

RESULTS

The search identified 847 articles (see **Figure 1**). After title and abstract screening, 48 articles were selected for full-text assessment. In total, 10 records were included (see Table E3 in Supplementary Material for reasons for exclusion), from 6 original studies. Three articles reported on allergic disease outcomes (22–24), four articles on diarrheal disease outcomes, all from a single study (11, 25–27), one article on respiratory and gastrointestinal tract infections (28), and two articles on HIV outcomes from a single study (29, 30).

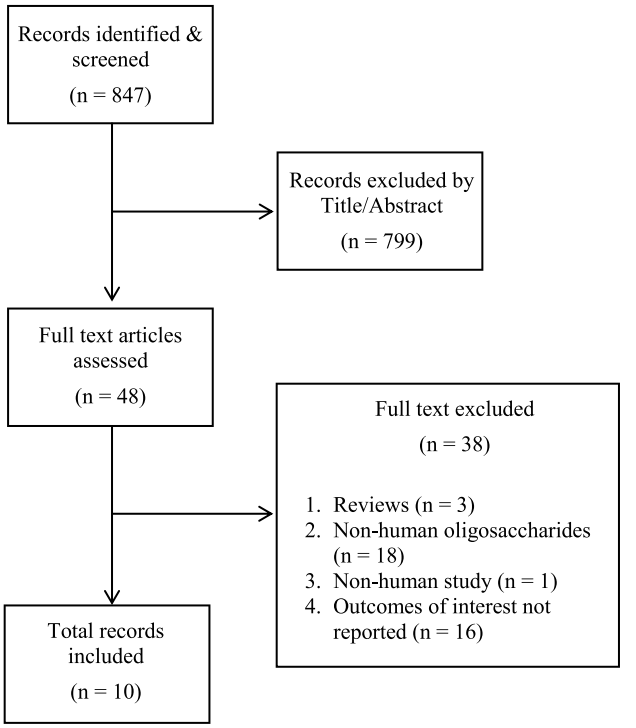

FIGURE 1 | PRISMA flow diagram. Human milk oligosaccharides on immune-mediated and infectious disease outcomes.

Study Characteristics

Three prospective cohort studies assessed associations with allergic disease outcomes, all from Scandinavia, with sample sizes ranging from 20 (22) to 266 (23) mother–infant pairs (see **Table 1**). One study was population based (22), while the other two cohort studies sampled participants with parental allergic disease (23, 24). A prospective cohort of 93 mother–infant pairs conducted in Mexico City investigated associations between HMOs and infectious diarrhea (25). One prospective cohort study, of 73 participants, examined associations between HMOs and respiratory tract infections and gastroenteritis (28). The two publications examining associations with HIV outcomes were from one nested case–control study conducted in Lusaka, Zambia (29). Bode et al. reported HIV transmission from HIV-infected mothers to their exposed infants (29), and Kuhn et al. recorded mortality rates among HIV-exposed infants (30). Participants comprise 103 HIV infected and 143 HIV exposed but uninfected (HEU) children were randomly selected from an early weaning trial comprising of 958 HIV-infected mother–infant pairs (31).

HMO Assessment

Human milk oligosaccharides were quantified by high-performance liquid chromatography in all included studies. Sjögren and colleagues collected milk samples 2–4 days postpartum and reported exposure as median concentrations (nmol/mL) of nine common neutral oligosaccharides in colostrum (see Table E4 in Supplementary Material) (22). Sprenger et al. measured the

TABLE 1 | Characteristics of studies included examining the association between maternal levels of human milk oligosaccharides (HMOs) and allergic and infectious diseases.

Reference	Design	Location	Sample size	HMO exposure	Outcome
Allergic disease					
Sjögren et al. (22)	Prospective cohort	Sweden	20 mother–infant pairs	Concentration of 9 HMOs[a] at 2–4 days of age	Any allergic disease[b] at 18 months of age
Sprenger et al. (23)	Prospective cohort	Finland	266 mother–infant pairs	Levels of FUT2-dependent HMOs measured at mean 2.6 days of age	• Any allergic disease[c] and/or sensitization[d] • Eczema and/or sensitization At 2 and 5 years of age
Seppo et al. (24)	Prospective cohort	Finland	145 mother–infant pairs	Concentration of 19 HMOs[a] at median 1.0–1.4 months of age	Cow's milk allergy by 18 months of age
Infectious disease					
Newburg et al. (25)	Prospective cohort	Mexico	93 mother–infant pairs	Mean fucosyloligosaccharide ratios at 1–5 weeks postpartum	• Diarrheal symptoms • Clinical diarrhea At 2 years of age
Stepans et al. (28)	Prospective cohort	USA	73 mother–infant pairs	Levels of HMO *LNFP-II* at 2 weeks postpartum	• Respiratory problems • Gastrointestinal tract problems • Ear infections At 2, 6, and 12 weeks of age
Bode et al. (29) and Kuhn et al. (30)	Nested case–control	Zambia	203 mother–infant pairs	Fucosyloligosaccharide concentration at 1 month postpartum	HIV infection (29) and mortality (30) at 24 months of age in children born to HIV-infected mothers

HMO, human milk oligosaccharide; FUT2, fucosyltransferase 2; 2'FL, 2-linked fucosylated oligosaccharides; LNFP-II, lacto-N-fucopentaose II.

[a]*Refer to Table E2 in Supplementary Material.*

[b]*Allergic disease defined as bronchial asthma, allergic rhinoconjunctivitis, atopic eczema, and food allergy.*

[c]*Allergic disease defined as asthma, allergic rhinitis, and eczema.*

[d]*Sensitization defined as any positive skin prick test.*

presence or absence of FUT2-dependent oligosaccharides in mothers' milk, collected at a mean postpartum day of 2.6; infants were classified as either FUT2-positive (had consumed levels of any FUT2-dependent HMOs) or as FUT2-negative (no FUT2-dependent HMOs) (23). Seppo et al. assessed concentration of 19 HMOs in milk samples at a median 1.0 month in mothers of non-cow's milk allergy (CMA) infants and at median 1.4 months in mothers of CMA infants (24). An internal standard (Raffinose) was added to milk samples to allow for absolute quantification. Newburg et al. examined variations in fucosyloligosaccharides in human milk collected at 1–5 weeks postpartum (25). Exposure to HMOs was measured as the mean fucosylated oligosaccharide ratio: the concentrations of α1,2-linked fucosyloligosaccharides compared with oligosaccharides that contain only α1,3- and α1,4-linked fucose. Stepans et al. measured HMO exposure as levels of a major oligosaccharide, lacto-*N*-fucopentaose II (LNFP-II), at 2 weeks postpartum as a representative of HMO consumption (28). The HIV study defined the exposure of interest as HMO concentration, measured first as a continuous variable and second as a dichotomous variable with infants categorized as above or below the median HMO concentration (1.87 g/L) (29). Several different HMO groups were assessed. Human milk samples were collected at 1-month postpartum and were analyzed for HMO composition.

Outcome Assessment

Allergic Disease

Sjögren et al. characterized children as "allergic" if clinical symptoms of allergic disease at 18 months of age were present and "non-allergic" if no clinical symptoms of allergic disease were apparent, along with a negative skin prick test (SPT) (22). Allergic disease was defined as bronchial asthma, allergic rhinoconjunctivitis, atopic eczema, and food allergy, although it was not clear if this was based on parent report or clinical examination. Sprenger measured the associations with any physician diagnosed allergic disease (food allergy, eczema, asthma, and allergic rhinitis) and/or IgE-associated disease (any allergic disease and sensitization as assessed by SPT) at 2 and 5 years of age (23). Seppo et al. measured outcomes of CMA by 18 months of age (24). Cases of CMA were confirmed by a positive oral food challenge at median 6 months of age.

Diarrhea

Outcomes reported were all diarrhea episodes due to stable toxin (ST)-*E. coli* infection and diarrhea as a result of all causes (25). Diarrheal episodes were determined by the study physician. All diarrheal episodes were assessed using a standardized scoring system (32, 33). ST-*E. coli* related diarrhea was tested in a laboratory according to previously published methods (34).

Respiratory and Gastrointestinal Tract Infections

Outcomes were cumulative occurrences of either (a) respiratory problems, consisting of upper respiratory infections (runny nose or cold), cough, or pneumonia; (b) gastrointestinal tract problems, which included vomiting, diarrhea, or colic; and (c) ear infections; by 2, 6, 12, and 24 weeks (28). Associations with ear infection outcomes were reported as not significant.

HIV

Bode et al. (29) reported the outcome measure as HIV transmission postpartum. The same study population was used to measure mortality in infants exposed to HIV infection during and after breastfeeding (30). HIV infection was established by heel-stick blood samples collected first at birth, then at 1 week of age, then monthly to 6 months of age, and subsequently every 3–24 months of age (29). HIV DNA was tested by polymerase chain reaction. Causes of deaths were ascertained *via* verbal autopsy and a review of medical records. Death after weaning was defined if breastfeeding had ceased independently of events before death (30).

Study Quality

The included studies ranged from low to high quality (see Table E5 in Supplementary Material). Selection of participants was adequately reported for all included papers. The ascertainment of HMO exposure was based on laboratory assays for all included studies. Allergic disease outcomes were determined *via* parental reports in one study (22) and medical diagnosis for two studies (23, 24). While Newburg et al. reported physician confirmed diarrhea episodes (25), Stepans et al. used respiratory and gastrointestinal infections as reported by mothers (28). HIV status was confirmed *via* blood samples collected at several age intervals (29). Loss to follow-up was only reported in one study (28). Four of the six original studies considered possible bias as a result of confounding or effect modification (23, 24, 28, 29). One of the papers investigating allergic disease outcomes adjusted for potential confounders (siblings, delivery mode, gender, allergic parents, and gestational age) and tested for interactions (FUT2 status and delivery mode; and FUT2 status and siblings) (23), while another study adjusted for the age of the infant, maternal atopy, duration of lactation and Secretor status (24). Stepans et al. adjusted for breastfeeding behavior (28). Measures of association between HMOs and HIV transmission were adjusted for two potential confounders identified in the study, white blood cell count and human milk HIV RNA viral load at 1 month (29). Potential confounders and interaction terms for the association between HMOs and diarrheal diseases were reported as not significant; however, the authors did not discuss what covariates were tested (25). Sjögren et al. did not discuss confounding (22).

Study Findings

Allergic Disease

One of the three allergic disease studies found evidence of an association between HMOs and allergic disease (22, 24). Sjögren reported a weak trend for higher total concentrations of neutral oligosaccharides in the breastmilk consumed by infants who developed allergic disease by 18 months ($p = 0.12$) (22) (see **Table 2**). Seppo observed that infants who consumed breastmilk with low lacto-*N*-fucopentaose (LNFP) III concentrations (<60 nM) had an increased likelihood of CMA compared with higher concentrations of LNFP-III (OR 6.7, 95% CI 2.0–22) (24). Seppo also noted that infants who received human milk with lower levels of LS-tetrasaccharide c, disialyllacto-*N*-tetraose (DSLNT) and 6′-sialyllactose were more likely to develop atopic dermatitis. Although Sprenger reported a significant interaction with mode of delivery (C-section or vaginal birth) ($p = 0.016$)

TABLE 2 | Associations between HMO concentration and allergic disease outcomes.

Reference	Age (months)	Allergy status at 18 months		*n*	Conc. (g/L)[a]	*p*-Value
Sjögren et al. (22)	18	No allergy		11	7.53 (5.94–11.01)	0.12
		Allergy		9	9.88 (4.90–13.90)	
Reference	**Age (years)**	**Delivery mode**	**FUT2-positive (%)**	**FUT2-negative (%)**	**OR (95% CI)[b]**	***p*-Value**
Sprenger et al. (23)	2	Vaginal	32	27	1.3 (0.52–3.24)	0.658
		C-section	30	57	0.32 (0.06–1.6)	0.203
	5	Vaginal	52	42	1.47 (0.64–3.37)	0.406
		C-section	57	57	0.98 (0.20–4.94)	1.0
Reference	**Median (IQR) age in months**			***n***	**Median conc. (nM) LNFPII[a]**	***p*-Value**
Seppo et al. (24)	CMA	1.4 (0.7–2.8)		39	29	0.007
	No CMA	1.0 (0.12–1.9)		40	57	
	Atopic dermatitis	LSTc				0.019
		DSLNT				0.028
		6′SL				0.044

FUT2, fucosyltransferase 2; CMA, cow's milk allergy; LSTc, LS-tetrasaccharide c.
[a]Median concentration of HMOs.
[b]Unadjusted odds ratio.

TABLE 3 | Associations between human milk oligosaccharide concentration and diarrhea infection.

Reference	Cause of diarrhea infection		Mean fucosyloligosaccharide ratio[a]	SE	*p*-Value
Newburg et al. (25)	Stable toxin-*E. coli* infection	Infected, symptomatic	4.4	±0.8	
		Infected, asymptomatic	8.4	±1.0	0.04
		Uninfected	8.6	±1.1	0.04
	All causes	Moderate/severe symptoms	6.1	±0.9	
		No symptoms	10.5	±1.9	0.042

[a]Mean fucosyloligosaccharide ratio refers to the concentrations of α1,2-linked fucosyloligosaccharides compared with oligosaccharides that contain only α1,3- and α1,4-linked fucose.

(23), an adjusted regression model found no statistically significant association between oligosaccharide status and allergic disease, regardless of mode of delivery.

Diarrhea

It was found that infants with diarrhea due to ST-*E. coli* infection had a significantly lower mean fucosyloligosaccharide ratio than asymptomatically infected infants or uninfected infants (25) (see **Table 3**). In addition, lower fucosyloligosaccharide ratios were associated with more severe diarrheal disease due to any cause. Infants who developed moderate to severe diarrhea of any cause were fed with human milk that had lower fucosyloligosaccharides ratios than infants with no symptoms (25).

Respiratory and Gastrointestinal Tract Infections

Higher levels of LNFP-II in colostrum were associated with reduced respiratory infections by 6 and 12 weeks, after controlling for breastfeeding behavior (see **Table 4**).

Increasing LNFP-II concentration was also associated with reduced gastrointestinal illness in infants at 6 and 12 weeks (26). No significant results were reported at 24 weeks postpartum.

TABLE 4 | The effect of HMO concentration on respiratory and gastrointestinal tract infections.

Reference	HMO	Time (weeks)	*N*	Odds ratio[a]	95% CI	*p*-Value
Stepans et al. (28)	*Respiratory tract infection*					
	LNFP-II	6	45	0.672	0.457, 0.989	0.01
		12	42	0.797	0.620, 1.026	0.04
		24	33	0.882	0.697, 1.115	0.28
	Gastrointestinal tract infections					
	LNFP-II	6	44	0.662	0.468, 0.935	0.004
		12	42	0.806	0.632, 1.029	0.04
		24	34	1.048	0.928, 1.182	0.41

HMO, human milk oligosaccharide; LNFP-II, lacto-N-fucopentaose II.
[a]Associated change of odds due to a 1 μm LNFP-II change/mL milk.

HIV

There was a non-significant trend toward a reduction in HIV transmission risk postpartum (29). An association was found between higher total HMO concentration and reduced risk of HIV transmission after adjustment for maternal CD4 cell count and human milk HIV RNA viral load at 1 month (see **Table 5**). Assessment of individual oligosaccharides found non-3′-sialyllactose HMOs to reduce HIV transmission at concentrations above the median (OR 0.38; 95% CI: 0.17–0.82). No other significant reductions in HIV transmission were reported for the other oligosaccharides, instead higher concentrations of 3′-sialyllactose oligosaccharides were associated with an approximately 2-fold increased risk of transmission (adjusted OR: 2.21; 95% CI:

TABLE 5 | Associations between HMO concentration and HIV transmission and mortality in children born to HIV-infected mothers.

HIV transmission			**TR**	**NTR**	**OR (95% CI)**	
Reference			***n*(%)**	***n*(%)**	**Unadjusted**	**Adjusted[a]**
Bode et al. (29)	Total log_{10} HMO conc.				0.43 (0.14–1.32)	0.31 (0.08–1.21)
	Median HMO conc. (g/L)	≥1.87	31 (41.9)	43 (58.1)	0.62 (0.34–1.15)	0.45 (0.21–0.97)
		<1.87	50 (53.8)	43 (46.2)		
Mortality					**Adjusted HR (95% CI)[b]**	
Reference	**Oligosaccharide**				**HIV infected**	**HEU**
Kuhn et al. (30)	Non-2′FL HMOs (LNFP-II/III + 3FL) > 200 mg/L				0.89 (0.38–2.08)	0.28 (0.13–0.67)
	2′FL + LNFP I > 550 mg/L				1.44 (0.64–3.21)	0.33 (0.14–0.74)
	LNT > 585 mg/L				1.43 (0.77–2.67)	0.58 (0.34–0.98)

TR, HIV transmitting; NTR, HIV-non-transmitting; HEU, HIV-exposed uninfected; 2′FL, 2-linked fucosylated oligosaccharides; LNFP, lacto-N-fucopentaose; LNT, lacto-N-tetraose; 3FL, 3-fucosyllactose; HMO, human milk oligosaccharide.

[a]*ORs adjusted for maternal CD4 cell count and log_{10} breast-milk HIV RNA viral load at 1 month.*

[b]*HRs adjusted for maternal CD4 cell count, mother symptomatic, maternal death, more than 2 other children aged <5 years in household, sex, and breastfeeding status.*

1.04–4.73) (29). For HEU infants, higher HMO concentrations were found to reduce mortality during, not after, breastfeeding for both 2-linked fucosylated as well as non-2-linked fucosylated oligosaccharides, following control for maternal CD4 cell count and human milk HIV RNA viral load at 1 month (30). No significant associations between HMOs and mortality were observed for HIV-infected children.

DISCUSSION

The six studies included in this systematic review have published 10 articles but provide only limited evidence that HMOs are associated with allergic and infectious diseases in early life. No studies were published on the associations between HMOs and other immune-mediated conditions. In terms of allergic disease, only one study showed that LNFP III was associated with increased risk of CMA. For infectious disease, the evidence was stronger although still limited with one study reporting that increased maternal levels of fucosyloligosaccharides were associated with reduced risk of diarrhea up to 2 years of age, as well as respiratory and gastrointestinal tract infections at 6 and 12 weeks of age. Evidence for an association between high HMO concentration and HIV outcomes was reported in two studies. In infants with a HIV positive mother, HMOs above the median concentration (≥1.87 g/L) had reduced risk of HIV infection. In addition, high concentrations of HMOs during breastfeeding were associated with a lower mortality rate for HIV-exposed, uninfected (HEU) infants but not for HIV-exposed infected infants.

Despite the recent interest and discussion surrounding breastmilk oligosaccharides and their potential impact on disease outcomes, very little original research has been conducted in this field. Furthermore, there are a number of important limitations with the available evidence, which prohibit strong conclusions being made at this time. Where two or more studies examined associations with similar outcomes, different measures of association were used (odds ratios versus mean differences in maternal HMO between affected and unaffected infants), preventing any pooling of the results in a meta-analysis. Most of the included studies measured disease outcomes from very small sample sizes (between 20 and 266 mother–infant pairs) both affecting the precision of the effect estimates and limiting the statistical power to detect important associations.

There were disparate HMO exposure classifications between the studies, making it difficult to compare the results of each study. Sprenger et al. measured exposure to HMOs as presence or absence of FUT2-dependent oligosaccharides (23). Using this dichotomy assumes that infants who consume high concentrations of FUT2-dependent oligosaccharides are just as likely to develop allergic disease as those exposed to low concentrations. Newburg et al. defined exposure to HMOs in terms of fucosylated oligosaccharide ratios (concentrations of α1,2-linked fucosyloligosaccharides compared with oligosaccharides that contain only α1,3- and α1,4-linked fucose), thus grouping a range of HMOs (25), whereas the remaining four studies measured associations with several specific common HMOs (22, 24, 28, 29). Furthermore, most of the techniques used to measure levels of HMO are unable to quantify absolute levels of HMO. Only three papers discussed using an internal standard in the HMO quantification process (23, 24, 29).

With over 200 structurally unique HMOs, it is possible that other important oligosaccharides not considered in the six studies may have important biological effects. It remains possible that the included studies have not measured the important HMO for each of the assessed outcomes. However, with so many different forms of HMOs, testing associations for each HMO will result in multiple comparisons, leading to spurious associations. As this area is novel, such exploratory "hypothesis generating" studies are still needed, but, care should be taken not to over-interpret any one finding in the absence of replication across cohorts.

Potential confounding was accounted for differently in each study. Four of the six original studies adjusted for potential confounders (see Figures E1–E3 in Supplementary Material) (23, 24, 28, 29). The remaining articles failed to acknowledge possible confounding (22) or stated confounding was not significant, with no description of how this was confirmed (25). Two of the allergic disease studies adjusted for a range of potential confounders in their analysis including siblings, gender, allergic parents/maternal atopy, and gestational age, which largely

appears appropriate (23, 24). Bode et al. noted that associations between advanced maternal HIV infection, including low maternal CD4 counts and viral load, and higher HEU mortality were confined to breastfed children, thus CD4 counts and viral load were controlled for in the regression model (29). Stepans et al. controlled for breastfeeding behavior in the analysis; defined as the proportion of days breastfed (28), but it is unlikely that the duration of breastfeeding would confound the relationship between HMO consumption and disease outcomes as maternal levels of HMOs were measured for all women at one fixed time window—2 weeks after recruitment. As all of the available evidence is derived from observational studies (cohort and nested case–control), it is subject to potential unmeasured confounding factors.

It is possible that a range of factors modify the effects of HMOs, and this has been examined in some of the included studies. Delivery mode was reported to be a significant modifier of the association between the presence of FUT2-dependent oligosaccharides and allergic disease development, although the strata-specific effects were quite weak (23). It is possible that breastfeeding duration is an effect modifier; longer breastfeeding duration may result in a larger amount of HMOs consumed by the infant, thereby affecting outcomes. Similarly, total volume of breastmilk consumed may also vary between infants and could modify these associations. This possibility has not been examined previously.

Follow-up was complete for five of the six studies (22–25, 29). However, it is not clear whether the authors only reported results on participants with outcome data. Stepans reported that only 34 participants from the original sample of 73 (46%) remained after 24 weeks, leading to a high risk of attrition bias.

The generalizability of results may be affected by population homogeneity in the regions in which participants were recruited. As the proportion of women with FUT2 gene mutations varies in different ethnic populations, lack of genetic diversity among study participants may result in associations that are unique to that specific population. For example, in the study by Newburg et al., all participants secreted fucosylated HMOs (no mutations in the FUT2 gene in the Mexican population). This meant that there was less variation in the levels of FUT2-dependent oligosaccharides and hence higher fucosyloligosaccharide ratios were reported than would potentially be found in other ethnic populations. Therefore, these results cannot be directly translated to European populations where there is a higher proportion of non-secretors (35, 36). An additional limitation in these studies is that while the Secretor status of the mother is often known, based on expressed breastmilk HMOs, the status of the infant has not been measured. Secretor status of the child may also modify the associations between HMO and clinical outcomes. However, infant Secretor status is difficult to measure.

This review has a number of strengths and limitations. We prospectively registered the review, searched multiple databases, had duplicate study selection, and listed reasons for excluding studies and quality assessed the included papers. While only published works were included in the predetermined search strategy, additional sources in the form of conversations with colleagues were used to identify possible unpublished manuscripts. Publication bias may have influenced the results of this review, although this seems unlikely given the preponderance of negative associations that have been reported to date. While our search strategy allowed for their inclusion, none of the published papers reported outcomes beyond infancy or reported autoimmune disease outcomes, which are areas for future research.

CONCLUSION

We identified limited evidence to support a possible role for HMOs to influence cows' milk allergy, diarrheal diseases, respiratory, gastrointestinal tract infections, and HIV infection in the infant in early life. Despite these positive findings, the evidence base is very limited and has numerous issues, with varying quality of the included studies from low to high. Further research into this area is needed, using larger observational studies with appropriate measures of outcomes and exposures and better control for confounding. Future research would benefit from considering multiple HMO exposures, which could be grouped appropriately, either by similar biological effects or by patterns of associations. Improved understanding of the complex chemical structures of oligosaccharides in milk may potentially allow for the design of intervention studies, to increase exposure to specific HMOs, which may reduce the burden of these conditions.

AUTHOR CONTRIBUTIONS

AL and LB conceived the work. AD developed the search strategy and AD, AL, and XD systematically assessed studies for inclusion. AD developed the first draft manuscript, with input from AL, and all authors drafted and revised the manuscript and approved the final submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fped.2018.00091/full#supplementary-material.

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GENERAL COMMENTARY
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Commentary: Association of Breast Milk Fatty Acids With Allergic Disease Outcomes—A Systematic Review

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We were pleased to read the systematic review of studies investigating associations between breast milk fatty acids and allergic disease outcomes by Waidyatillake et al. (1). A comprehensive overview of systematic reviews in allergy epidemiology (2) up to 2014 has identified several systematic reviews on the association of fatty acid intake with allergic disease risk (3–8). We are aware of two further systematic reviews on this topic published in 2015 (9, 10). While these previous reviews have largely synthesized evidence on fatty acid supplementation, also during lactation, only one of them has explicitly synthesized studies ascertaining evidence on breast milk fatty acid levels—but only for asthma as an outcome (8). We thus hope that this systematic review by Waidyatillake et al. will help to spur interest on this potentially important topic.

We have published an original study (11) after the search period of the most recent systematic review which offers additional insight and may thus provide further guidance for future investigations. In our study (11), we investigated associations between breast milk fatty acid composition and several wheeze phenotypes as well as asthma diagnosis up to age 13 years. Here, we addressed several limitations implicated by Waidyatillake et al. (1) as cause for further study (i.e., large sample size, analysis of diverse outcomes, comprehensive statistical analysis, and adjustment for multiple potential covariates or confounders but not for potential mediators). We also improved upon previous studies by employing statistical methodology to account for constant-sum constraint (11), a potentially serious issue when analyzing concentration data (12, 13).

As in the majority of the studies reviewed by Waidyatillake et al. (1), we identified no convincing evidence of association between fatty acid composition and childhood respiratory outcomes (11). Furthermore, we showed that previously reported significant associations for omega-3 and omega-6 fatty acids may have been overstated due to spurious correlation between fatty acids which was not accounted for (11). Yet despite our null findings, we also do not believe that this is the end of the story. Further study of breast milk fatty acids may shed light on potentially more complex relationships between breastfeeding and disease.

Since our results diverge from previous studies likely due to different statistical methodology, we believe a good first step could be to re-examine existing data using simple statistical methodology similar to our own (11). As the sum concentration of all fatty acids within any breast milk sample is bound at 100% of total fat weight, known as the constant-sum constraint, analysis of raw data

may often result in spurious correlations (12, 13). Interestingly, though these analytical methods for compositional data analysis have been used extensively in investigations of bovine milk (14), few previous studies of human breast milk constituents have truly accounted for compositionality (15). Results from appropriate re-analyses of existing data would serve to test the validity of previous findings. Moreover, they could be used to identify correlations between fatty acid constituents which may be more meaningful to infant and childhood health and disease outcomes than total fat proportions of single fatty acids or fatty acids grouped based on chemical similarities (like grouping all omega-3 fatty acids). Importantly, we show that associations may be attenuated toward the null if negatively correlated (or non-correlated) fatty acids are grouped and analyzed as such (11).

In addition, breastfed children often constitute their own specific subgroup who may be more likely to have healthier and more educated mothers than children who were never breastfed or were breastfed for only a short period of time (16). To overcome this potential selection bias, it may be important to investigate those children not receiving breast milk alone or at all. In early infancy, before introduction of solids, infant formula will be their source of nutrition. Thus, future studies could embark on the strategy of investigating the association of fatty acid profiles of both breast milk and infant formulas forming the diet of an unselected study population with allergic outcomes.

Furthermore, fatty acid concentrations in breast milk have been shown to vary over the course of lactation (17). Infant formula may provide a more stable fatty acid profile which may allow researchers to identify associations which are difficult to observe in breast milk. Following the idea that the source of fatty acids could be extended from pure breast milk to formula, future investigations should also pay attention to the evidence of intervention studies on fatty acid supplementation in pregnancy, during lactation, or during infancy. Obviously, timing of exposure is an important issue. Moreover, for maternal supplementation, maternal uptake and secretion into breast milk can lead to variation across mothers. Also, child uptake of fatty acids may differ by a various factors associated with infant feeding such as timing, frequency, and maternal diet. Therefore, another approach to exposure assessment may be to directly measure fatty acid levels in infant serum. However, this may be limited due to practical and ethical reasons in small infants but could be easier and potentially informative in animal model settings.

Finally, early infancy is a particularly important period for development of the immune system and of the gut microbiome which may potentially be associated with childhood atopic outcomes. Therefore, we agree with the current review and believe future studies should add to ours by analyzing fatty acids and other constituents in colostrum which may be differentially associated with disease outcomes.

To move forward, we suggest (i) building on the existing systematic reviews, (ii) employing the centered log ratio transformation to overcome spurious correlation, (iii) considering alternative ways of grouping fatty acids, (iv) reducing selection bias by sampling infant formula, (v) further investigating other forms of exposure assessment (upstream as maternal fatty acid supplementation, downstream, or as part of animal models), and (vi) bearing in mind timing of exposure.

AUTHOR CONTRIBUTIONS

Both CL and JG have conceived and written this commentary.

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