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Gut Microbiome and Breast-feeding: Implications for Early Immune Regulation

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Abstract

Establishment of the gut microbiome during early life is a complex process with lasting implications on an individual's health. Several factors influence microbial assembly; however, breastfeeding is recognized as one of the most influential drivers of gut microbiome composition

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during infancy, with potential implications for function. Differences in gut microbial communities between breastfed and formula-fed infants have been consistently observed and are hypothesized to partially mediate the relationships between breastfeeding and decreased risk for numerous communicable and non-communicable diseases in early life. Despite decades of research on the gut microbiome of breastfed infants, there are large scientific gaps in understanding how human milk has evolved to support microbial and immune development. This review will summarize the evidence on how breastfeeding broadly impacts early life gut microbiome composition and function and discuss mechanisms by which specific human milk components shape intestinal bacterial colonization, succession, and function.

Keywords

Human milk; breastfeeding; microbiome; infant immunity; IgA; human milk oligosaccharides

Introduction

Infancy is a critical period for establishment of the gut microbiome, a complex process that is known to have a long-term impact on health and risk for disease. Microbial colonization of the gastrointestinal (GI) tract is fundamentally linked to metabolic programming, immunologic maturation, and proper gastrointestinal development.[1,2] Perturbations in colonization in infancy have been associated with an increased risk for multiple conditions, including asthma, atopic dermatitis, food allergy, diabetes, inflammatory bowel disease, and obesity, highlighting early life as a critical window to shape the gut microbiome.[3] Multiple perinatal factors, such as gestational age, mode of delivery, and antibiotic usage, impact microbial assembly in the GI tract, but infant diet is recognized as one of the most significant factors associated with early microbial structure.[2,4,5] Human milk has evolved over time to not only provide nutrients necessary for growth but also a myriad of bioactive molecules that profoundly influence microbial colonization and succession.[6,7] Breastfeeding has been shown to be protective against diarrheal disease and respiratory infections and associated with decreased risk for non-communicable, inflammatory diseases[8], benefits that are hypothesized to be at least partially conferred through the gut microbiome.[9] This review will explore how breastfeeding impacts the early life gut microbiome with an emphasis on mechanisms by which different human milk components shape bacterial colonization and fitness.

Gut Microbiome Composition in the Breastfed Infant

Much of the understanding about the impact of breastfeeding on the infant gut microbiome has been generated by decades of research highlighting differences in fecal bacterial composition between breastfed (BF) and formula-fed (FF) infants, specifics of which have been reviewed extensively by us and others.[5,9–11] In a simplified, albeit generalizable model of succession, the GI tract is initially colonized by facultative anaerobes, such as *Staphylococcus, Streptococcus, Enterobacteriaceae,* and *Lactobacillus.* These pioneering taxa create an environment suitable for succession of obligate anaerobes, such as *Bifidobacterium, Clostridium,* and *Bacteroides*.[5,10] Broadly speaking, formula feeding is

associated with a more diverse fecal microbiota as well as a more mature microbiome as assessed by microbiota age and microbiota-for-age Z-score[4,9], metrics originally developed by Subramanian and colleagues to compare assembly of the microbiome in malnourished relative to healthy infants of a similar chronological age.[4,9,12] These features are often a result of high abundances of certain *Bifidobacterium* species that predominate during infancy in breastfed infants, in part, due to their unique capacity to metabolize human milk oligosaccharides (HMOs).[2,4,9] Consumption of human milk has also been associated with higher abundances of *Lactobacillus* and lower abundances of *Proteobacteria, Veillonella, Clostridium,* and *Bacteroides,* though results vary among studies.[2,4,5,9,13] Breastfeeding exclusivity (i.e. exclusive vs partial)[13] and duration[14] also impact the infant fecal microbiome, and dose-dependent effects of breastfeeding on gut microbiota composition have been demonstrated.[13] A meta-analysis by Ho and colleagues also identified higher abundances of *Eubacterium, Veillonella,* and *Bacteroides* in the fecal microbiome of partially breastfed compared to exclusively BF infants.[9] Cessation of breastfeeding, even more so than solid food introduction, dramatically shifts the infant microbiota towards a more mature and diverse, adult-like configuration marked by increased levels of Firmicutes.[2,4,15,16]

Though assessed in fewer studies, the demonstrated impact of human milk is not limited to the taxonomic profile (species abundance) but also extends to both metabolic capacity (functional genes) of resident microbiota and their output (metabolites). Studies employing shotgun metagenomic sequencing, which sequences all genetic material in a microbial community, have shown breastfeeding to be associated with enrichment of microbial genes related to carbohydrate and lipid metabolism, including fatty acid biosynthesis,[4] as well as oxidative phosphorylation and vitamin B synthesis.[2] On the other hand, the microbiome of infants not receiving any human milk has been characterized by greater abundance of genes related to bile acid synthesis and methanogenesis[2] as well as nucleotide and amino acid metabolism[4], processes that are characteristic of a more mature microbiome seen in adults.

Metabolomic studies have also demonstrated that the fecal metabolite pool discriminates BF and FF infants.[17,18] BF infants have been shown to exhibit lower levels of conjugated and secondary bile acids but higher levels of sulfated bile acids[18], the latter of which may represent an attempt at detoxification by decreasing buildup of bile acids.[19] Furthermore, breastfeeding is associated with higher levels of several aromatic amino acid metabolites, including anti-inflammatory, indole-3-lactic acid (ILA)[17,18], which will be discussed in more detail later in this review. Findings on the effects of breastfeeding on fecal organic acid and short chain fatty acid (SCFA) concentrations are mixed. BF infants from 3-5 months of age have been shown to exhibit increased levels of lactate and relative proportion of acetate compared to FF infants.[20] However, in another study, breastfeeding was associated with increased fecal concentrations of butyrate and isovalerate at both 3 and 6 months relative to infants receiving cow's milk or soy formula.[17] SCFA are rapidly absorbed by colonocytes[21] and SCFA and organic acids can be utilized by other resident microbiota, thus fecal concentrations may not accurately represent production, which is a consistent limitation in fecal metabolomic studies. Still, how these diet-related differences in functional capacity of and metabolic output by the microbiome influence infant health and development has not been fully resolved.

The Role of Specific Human Milk Components in Shaping the Infant Gut Microbiome

Human milk has several factors that may impact the composition and function of the infant gut microbiome. We discuss some of the most prominent factors below.

Human milk is a rich source of oligosaccharides, which select for bifidobacteria that mitigate intestinal inflammation and promote intestinal immune development

Human milk has evolved to supply essential nutrients as well as other non-essential components to support infant health and survival.[7] The third largest fraction of molecules secreted in human milk are glycans referred to as human milk oligosaccharides (HMOs), composed of up to five monosaccharide building blocks varying in length and composition.[22] HMO biosynthesis takes place in the mammary gland and is achieved by successive action of specific glycosyltransferases.[23] Approximately 200 HMO structures have been identified to date[24], where fucose and sialic acid moieties contribute to diversity and complexity relative to non-human mammalian milk.[25] HMO concentrations vary among individuals and across lactational stages[26], but median concentrations are approximately 8 g/L.[27] These molecules are non-nutritive to the infant and arrive intact to the infant colon similar to soluble dietary fibers.[27–29] Thus, HMOs are available exclusively for utilization by microbiota capable of doing so.[30]

Several taxa within the genus *Bifidobacterium* have evolved mechanisms to bind, import, and catabolize HMOs intra- and extra-cellularly.[31,32] Bifidobacteria ferment HMOs, and as a result, decrease the pH of the gut lumen through the secretion of organic acids, i.e. acetate, lactate, and formate (Fig 1). These organic acids promote the production of additional short-chain fatty acids (SCFA) by heterologous microbiota, collectively decreasing the pH of the gut lumen. SCFA are absorbed in the gut and are used as a source of energy by colon and liver cells, as well as peripheral tissues.[33,34] However, the capacity of various bifidobacteria to ferment HMOs differs according to their makeup of HMO utilization genes within their genome. *Bifidobacterium longum* subsp. *infantis* (*B. infantis*) possesses all five of the HMO utilization gene clusters and is therefore a “superuser” of HMOs. Clusters H1 and H4 are rather unique to *B. infantis,* and *B. breve* has some genes of H2 and H5 and even H3. Species that harbor these gene clusters have a significant competitive advantage over other bacteria, allowing them to predominate the infant gut microbiome. In addition to SCFA related to energy metabolism, bifidobacteria produce indole-3-lactic acid (ILA) as a catabolite of tryptophan.[35] ILA is hypothesized to be partly responsible for the anti-inflammatory effects associated with high abundances of *B. infantis* colonization in infancy as it interferes with transcription of the inflammatory cytokine IL-8 in enterocytes.[36] In addition, tryptophan metabolites were found to regulate the expression of the ligand-binding subunit of the IL-10 receptor, implicating their role in the maintenance and repair of the intestinal epithelial barrier.[37,38] HMOs may also shape the infant gut microbiome by acting as a decoy receptor for pathogens, preventing attachment to the intestinal epithelium and subsequent infection.[39]

HMOs select members of the genus *Bifidobacterium*, which often represent 50-70% of the infant gut microbiome early in life.[40,41] However, it is likely that HMOs alone do not determine the presence or abundance of bifidobacteria. Several pre- and postnatal exposures such as cesarean sections, presence of older siblings, antibiotic use, short duration or non-exclusive breastfeeding, and urban lifestyles have been demonstrated to impact the succession of bifidobacteria early in life.[42–45] The effect of *Bifidobacterium* on development of infant immunity was examined recently by Henrick et al.[46] Infants with expanded bifidobacteria had contracted populations of neutrophils, basophils, plasmablasts, and memory CD8+ cells as well as invariant T cells (MAIT) indicating effects on both the innate and adaptive immune system. They also had higher frequency of non-classical monocytes, which have been considered anti-inflammatory, and antigen-experienced CD39R+ regulatory T cells (Tregs) as well as decreased levels of inflammatory cytokines TNFα, IL-17A, and IL-1α as well as Th2 cytokine IL-13. Furthermore, infants harboring high abundances of bifidobacteria also had elevated levels of Treg-associated cytokines IL-27 and IL-10 and IL-1 inhibitor, IL1RA, but also unexpectedly elevated IL-6. To our knowledge, this is the only information available on the impact of *Bifidobacterium* succession on early immune phenotypes, warranting further investigation and replication in future cohort studies.

The prevalence of bifidobacteria, especially *B. infiantis,* in western populations is reduced compared to global populations, in particular relative to developing countries where breastfeeding rates and durations are historically higher.[47] It is notable that infant formulas enriched with soluble fructo- and galacto-oligosaccharides enrich the infant gut with infant-type bifidobacterial taxa, although they may do so by non-specifically promoting other bacterial groups.[48]

B. infantis is a model taxon to elucidate mechanisms of attenuating intestinal inflammation and dysregulation. *B. infantis* decreases flagellin- and pathogen-induced IL-8 secretion in epithelial cells.[49] High relative abundance (>50%) of *B. infantis* in early infancy is associated with improved CD4 T-cell responses and vaccine memory[50], and high bifidobacterial taxonomic diversity is positively correlated to salivary secretory IgA levels, a marker for protection against development of allergic symptoms.[51] The absence of HMO-utilization genes from the microbial metagenome, rather than the absence of specific taxa, is associated with dysregulated immunity.[46] This finding was supported through a probiotic intervention with *B. infantis* that fully utilizes HMOs, preventing intestinal T helper 2 and T helper 17 cytokine response in favor of T helper 1 and Tregs. This is hypothesized to be due to upregulation of galectin-1 in T cells by *B. infantis*-derived ILA. Additionally, supplementation with *B. infantis* has been shown to reduce necrotizing enterocolitis in pre-clinical and clinical trials.[52,53] However, a randomized controlled trial deploying *B. breve*[54] did not demonstrate similar effects, highlighting further both the potential utility of *B. infantis* as well as important functional differences among species or strains of *Bifidobacterium*.

SIgA drives establishment and maintenance of the infant gut microbiome through specific and non-specific mechanisms

Human colostrum and mature milk contain high amounts of immunoglobulin A (IgA) antibodies and relatively low levels of IgG, which contrasts with plasma where IgG outnumbers IgA antibodies. This difference in the relative proportions of IgA to IgG in mucosal secretions is the result of active transport of IgA across the mammary epithelium, resulting in the formation of secretory IgA (SIgA).[55–57] SIgA consists of two or more IgA monomers linked together by joining (J) chain and secretory component (SC). Milk SIgA is produced from mucosal-homing α4β7, CCR10+ plasma cells (PCs)[58] that are induced within gut-associated lymphoid tissues in response to food antigens, enteric pathogens and commensal biota[59], which then home to the mammary gland through an entero-mammary pathway.[60] Gut- and other mucosa-derived PCs preferentially secrete IgA dimeric and polymeric forms of IgA (not monomeric) into the mammary gland parenchyma.[61] Dimeric and polymeric IgA is recognized by the pIgR on the basolateral membrane of mammary epithelial cells and is then transcytosed through the cell and secreted apically after proteolytic cleavage of the pIgR secretory component (SC).[62] SC, which is acquired during IgA transport across the mammary gland epithelium, renders SIgA resistant to intestinal proteases and plays a role in antibody localization within mucus[63] (Fig 2). SIgA is highly glycosylated with over 16 N-linked glycan sites located on the Ig, SC, and J chain.[64]

IgA in Immune Exclusion, Neutralization, and Inhibition of Bacterial Colonization.

SIgA serves to protect the newborn from enteric and respiratory infections, although we are only just beginning to understand the mechanisms by which this occurs.[65–67] Cryo-electron microscopy indicates that SIgA assumes a V-shape, with the Fc elements tethered by their tails and the variable regions (Fab) projecting outwards.[68,69] This molecular architecture explains how SIgA is so efficient at cross-linking and agglutinating viruses and bacterial cells *in vitro* and *in vivo.*[70,71] SIgA-mediated agglutination results in mucus entrapment and clearance from the intestinal lumen via peristalsis, a phenomenon referred to as immune exclusion.[72] Other mechanisms of immune protection by SIgA in the newborn likely include direct neutralization of toxins and microbial virulence factors, as well as inhibition of bacterial colonization and invasion.[70] Maternally-derived SIgA can also suppress innate inflammatory responses by sequestering microbial pathogen-associated molecular patterns (PAMPs) and limiting their uptake into the neonatal intestinal mucosa.[73,74]

Some examples of human milk (HM) antibody associated protection against infectious diseases include studies showing that higher colostrum and mature milk antibody levels against enteric pathogens have been associated with protection against diarrheal diseases caused by rotavirus[75,76], *Shigella*77, *Giardia lamblia*[78,79], *Entamoeba histolytica* and *Cryptosporidium* spp[80] and *Campylobacter*[81]. Studies of HM antibodies to respiratory pathogens have focused on the associations between maternal vaccination during pregnancy and HM IgG and IgA levels to pertussis, pneumococcus, influenza, and meningococcus.[82] However, few studies have attempted to assess the degree of clinical benefit from HM antibodies on rates of disease in breastfed children. A report in Bangladeshi mothers described significantly higher vaccine-specific, viral neutralizing IgA levels to influenza

A/New Caledonia in HM collected from women that were vaccinated compared to those not vaccinated.[83] Furthermore, exclusive breastfeeding in the first 6 months of life significantly decreased the expected number of infant febrile respiratory illnesses, suggesting protection against influenza by these milk antibodies. Lastly, capsular antibodies to Group B *Streptococcus* (GBS) in HM have been associated with reduced risk of developing late-onset disease.[84] More recently, neutralizing antibodies against SARS-CoV2 have been also found in HM from women that were infected with and/or vaccinated against SARS-CoV2[85–89], although their role in protection against COVID-19 disease in breastfed infants has yet to be demonstrated. Other examples of virus-specific IgA activity playing a role in gut protection are from rotavirus and HIV-1.[90 91,92]

While direct evidence for a role of HM antibodies in protecting the newborn gut against enteric infections is lacking, the association of antigen-specific antibodies in HM and reduced disease incidence in infants is difficult to refute. In the case of HIV-1 infection, HM SIgA against viral envelope glycoproteins was associated with reduced postnatal transmission risk.[93] Indeed, there are ongoing vaccination efforts to stimulate HIV-1 specific SIgA in HM as a means to reduce vertical transmission of the virus.[94] In the case of rotavirus (RV), maternally derived RV-specific SIgA (and IgG) proved able to neutralize homologous virus *in vitro*.[90] However, in the context of oral RV vaccination, RV-specific SIgA in HM reduced overall seroconversion rates, presumably due to “neutralization” of the vaccine itself.

Human Milk SIgA, Microbial Colonization and Immune Regulation.

SIgA in HM has profound long-term effects on the infant microbiota and immune regulation. Both poly-reactive[95] and somatically hypermutated commensally-induced specific IgA[96] bind commensal biota within the intestine. Mechanisms of SIgA interactions may explain the discrepancies related to binding specificity. Many IgA-commensal interactions are glycan-mediated, either from bacterial surface glycan structures like LPS on Gram negative species[97], teichoic acid on Gram positive organisms[98], and exopolysaccharides[99], or from the SIgA glycoprotein itself, rich with N-linked glycans.[100] Huus et al. offers a deep review of the many diverse mechanisms of SIgA-microbial interactions.[101] In the intestine, SIgA directly impacts microbial colonization by promoting biofilm formations[102] of slow-growing bacteria through enchained growth[103], which enhances mucosal adhesion by glycan-glycan interactions and improves microbial cross-talk[102]. SIgA can also alter exopolysaccharide expression in commensal biota[104] and promote niche colonization.[105] In the colon, SIgA promotes colonization in the luminal but not epithelial mucosa, reducing aberrant host immune response and promoting tolerance.[106] SIgA can also have a prebiotic effect, promoting growth of certain bacterial species. Some commensal microbes, like *B. infantis,* secrete endo-β-N-acetylglucosaminidase enzymes that can cleave the chitobiose core of N-linked glycans on SIgA, or other glycoproteins, which frees oligosaccharides that the bacteria can then use as an energy source.[107,108]

In mice, breeding schemes with pIgR (polymeric Ig receptor) knock-out ($pigr^{-/-}$ or KO) and heterozygote mating pairs provide unique opportunities to understand the impact of maternal SIgA (wt) vs lack of maternal SIgA (KO) on the developing neonate, as mouse

pups do not begin endogenous SIgA production until weaning.[57] Maternal SIgA was found to improve barrier protection, shape the gut microbiota, and program immune tolerance in pups, the effects of which remained through adulthood.[106] In humans, maternal IgA does not cross the placenta, but infants begin producing their own SIgA sometime between 1-8 weeks of age.[109,110] Longitudinal salivary IgA levels in infants have been shown to peak around 4-6 weeks with a later decline between 3-6 months of age,[111–113] though this pattern may differ at other mucosal sites, such as the intestine. Breastfed infants have been shown to have increased fecal IgA concentrations compared to formula-fed infants in the first 3 months of life.[114,115] SIgA levels measured in infants are still considerably lower than in adults; therefore, SIgA in HM can supplement infant production during this window when infants are particularly vulnerable to infection. Additionally, Ramanan et al. found milk IgA led to coating of inflammatory commensal biota in the infant gut, which led to reduced induction of pro-inflammatory cytokines and thus induction of RORγ Tregs. This maternal IgA:infant microbiota interaction created a multigenerational transmission of immune phenotype programming.[116] Collectively, information from observational studies in humans, animal studies, and *in vitro* investigations show maternal milk-derived SIgA as a strong driving force in the establishment and maintenance of the infant gut microbiome, although studies in human are scarce.

In the PASTURE study (Protection Against Allergy: Study in Rural Environments)[117], regression analysis demonstrated an inverse relationship between the levels of IgA intake from HM feeding and the development of atopic dermatitis.[118] Similar findings of higher total and cow's milk-specific IgA levels in HM being associated with protection against cow's milk allergy have also been reported by us.[119,120] These associations do not necessarily indicate causation, although we showed HM IgA antibodies to prevent milk antigen transcytosis in a Caco2 monolayer consistent with immune exclusion. Other mechanisms could include IgA effect mediated by the gut microbiome and direct immune effects.[120] Recently, we also identified differential glycosylation of IgA1 and pIgR, in addition to other glycoproteins, in HM of mothers whose infants did and did not develop atopic disease.[121] These different glycan compositions could elicit direct effects on the infant immune system by controlling access to antigens or indirectly through effects on microbial composition. Indeed, SIgA is proposed to sculpt the newborns commensal microbiota though direct coating of beneficial bacterial species, while excluding harmful species, possibly in conjunction with maternally-derived IgG.[104,122–124] Gopalakrishna et al. found a significant inverse correlation between maternally-derived IgA coating of infant enteric bacteria and the development of necrotizing enterocolitis, a disease afflicting premature infants and a lead cause of infant mortality, indicating the essential role of milk IgA in regulating the infant gut microbial community.[66]

Human milk as a potential microbial reservoir for the infant gut

Findings over the last two decades support the presence of a diverse microbial ecosystem within HM.[125] *Staphylococcus* and *Streptococcus* are the most prevalent and generally most dominant bacterial taxa in HM, but *Gemella, Pseudomonas, Corynebacterium, Bacteroides, Veillonella, Rothia, Lactobacillus,* and *Actinomyces*, among others, have also been consistently identified at notable abundances using 16s rRNA sequencing.[126–129]

Origins of the HM microbiome are not entirely clear but remain an area of active investigation.[125,130] Findings from studies employing 16S rRNA sequencing suggest that a large proportion of bacteria in HM arise from breast skin and the infant oral cavity,[131–133] the latter of which is believed to colonize HM during retrograde backflow of infant saliva into the mammary gland during suckling.[134] Though these studies have not identify a large proportion of shared bacterial taxa between HM and maternal fecal bacterial communities, gut-associated, obligate anaerobes have been detected in HM, identifying the maternal gut as another possible origin site.[130,135,136] Although debated, maternal gut bacteria are proposed to reach the mammary gland via immune-cell mediated translocation through an enteromammary pathway, the presence of which has been most strongly supported by evidence from rodent studies[137–139] as well as the detection of orally-administered probiotic strains in HM.[140,141] Regardless of origin, HM remains a small, but consistent source of microorganisms for the infant gut, with an estimate that infants ingest roughly $1 \times 10^7 - 1 \times 10^8$ bacterial cells per 800 mL of HM.[142] While the bacterial composition of HM is fairly consistent within an individual, it is highly unique among mothers[128,143,144] and may be one of the factors driving differences in gut microbiota composition, not only between BF and FF infants but also among infants who are exclusively BF.

Though not predominating, *Bifidobacterium* has also been identified in HM in appreciable numbers using both culture-dependent and independent techniques.[135,144,145] Infant-associated bifidobacterial species, such as *Bifidobacterium longum, Bifidobacterium bifidum, and Bifidobacterium breve*, have all been detected in both HM and infant stool of breastfeeding dyads, but counts were not associated between the two sites.[145] High co-occurrences of *Actinomyces, Atopobium, Rothia,* and *Streptococcus* have been demonstrated between HM and infant fecal microbial communities,[13] and other studies have further demonstrated shared taxa between sites.[133] In an in-depth analysis of microbial relationships between breastfeeding dyads, it was estimated that HM contributed on average 5% of the bacteria present in the infant gut at 2 days of age but decreased to less than 1% beyond 1 month, similar to results from our own studies.[44,131,132] Though estimated contributions are low, the HM microbiota may play a role in early seeding of the infant gut, which could influence later microbial succession based on the principle of priority effects.[146] Additionally, it is possible that the HM microbiota may contribute to the microbiome of the proximal gastrointestinal tract thereby indirectly shaping composition of the distal colon and feces[123], relationships that may have gone undetected due to the use of fecal samples as a proxy of intestinal microbial communities.

Part of the ongoing investigation of how and if the HM microbiome impacts that of the infant gut is the question of bacterial viability in HM, as much of the recent work has been done using culture-independent sequencing techniques. Recent data from Stinson et. al showed that roughly half of the DNA isolated from HM arose from non-viable cells, findings which may help to explain the lack of robust associations between taxa abundance in HM and infant feces and certainly warrant further exploration.[147] Further, other components in HM may mediate the impact of the HM microbiota on the infant gut microbiome. For example, SIgA could potentially neutralize and/or inhibit colonization of HM microbiota within the infant gut[148] through previously discussed mechanisms. At the same time, one study has shown that up to 40% of the bacteria in HM are IgA-coated,[149]

which may promote their colonization within the infant gut.[148,150] There still remains a paucity of information on the functional capabilities of the bacteria or other microorganisms in HM, such as bacteriophage.[151,152] This is largely due to limitations presented by the low biomass nature of HM but represent critical areas for future research.

Gut dysbiosis is associated with atopic disease

As discussed above, HM has the capacity to impact the infant gut microbiome, which plays a significant role in development of many atopic diseases. Increasing evidence indicates that gut dysbiosis in early life negatively impacts the development of the immune system and precedes development of multi-sensitized atopic disease,[153] atopic dermatitis,[154] food allergy,[155,156] and asthma (Fig 3).[157] This may be in part due to induction of Tregs. Animal models of food allergy highlight a protective role of a consortia of clostridia in the induction of Tregs[158] and the MyD88/RORγt pathway in Tregs,[159] important for development of oral tolerance, as well reduction in intestinal permeability and subsequent sensitization to dietary antigens.[160] Similarly in human studies, clostridia were enriched in stool of infants who outgrew milk allergy by age 8 years[161] and were underrepresented at 3-6 months in stool of those who later developed food sensitization.[156] Bifidobacteria also appear to play a role in anti-allergic immunity as lower levels in the first 6 months of life have been associated with a higher incidence of atopic disease,[162] multi-sensitized atopy,[153] and cow's milk allergy.[163] Our own studies demonstrating high abundance of *B. infantis* in farming lifestyle populations with low rate of allergic diseases compared to urban lifestyle high-risk infants further highlight the potential protective effect of *Bifidobacterium*.[44] This protective effect may be in part due to production of ILA as well as organic and SCFA as previously discussed.

In a recent study by Depner and colleagues, the fecal microbiome at 2 months of age was associated with breastfeeding and protection against asthma; however, this protective effect was mostly associated with abundance of *Bacteroides* rather than *Bifidobacterium*.[164] In the same study, a more mature microbiome at 12 months of age, characterized by higher abundances of *Coprococcus* and *Roseburia* and abundance of genes related to butyrate synthesis, were associated with protection against later asthma development. Similarly, findings from the CHILD cohort in Canada suggest differential abundance of multiple taxa in the first year of life between infants who were and were not sensitized to food and aeroallergens at 12 months of age, including a number of genera in the Clostridia class such as *Coprococcus, Roseburia,* and *Blautia* among others.[165] Metagenomic data from the GUSTO cohort also recently demonstrated aberrant longitudinal development in microbial composition and function in atopic infants.[166] At just 3 weeks of age, infants who developed eczema exhibited decreased abundance of *Bacteroides fragilis (B. fragilis)* and increased abundance of *Escherichia coli* and *Klebsiella pneumoniae*, along with increased expression of virulence genes coding for adhesion, invasion, and flagellin. All three of these species were also associated with lipopolysaccharide (LPS) biosynthesis, suggesting that differences in LPS immunogenicity among early bacterial colonizers may influence allergy development similar to in autoimmunity.[167] *B. fragilis* also harbors some capacity to metabolize HMOs[168] and produce SCFA.[169]Additionally, *B. fragilis* produces multiple surface polysaccharides, the most immunodominant of which is Polysaccharide A (PSA).

A study in germ-free animals demonstrated a critical role of PSA in immune homeostasis, specifically regulation of Th1/Th2 balance[170], highlighting a potential protective role of early colonization of *B. fragilis* against atopic disease. Subsequently at 3, 6, and 12 months, atopic infants harbored a lower abundance of multiple bacterial species known to produce short-chain fatty acids (SCFA), butyrate and propionate.

To date, multiple cohorts have demonstrated associations between low levels of fecal SCFA, propionate and butyrate, and/or genes related to biosynthesis in the first two years of life and increased risk for eczema, food allergy, atopic sensitization, and asthma.[166,171–173] SCFA are prominent mediators of host-microbe interactions[174,175] and likely influence atopic disease risk through their roles in epithelial tight junction assembly and barrier maintenance,[174,176] direct modulation of immune cell function[175], and their influence on host gene expression via histone deacetylase (HDAC) inhibition.[179–183] Through HDAC inhibition, acetate and butyrate have been shown to upregulate *FOXP3* transcription, thereby inducing Treg differentiation and subsequent prevention of allergic and airway disease[177,178] as well as amelioration of colitis[183] in murine models.

Does breastfeeding mediate the relationship between the gut microbiome and allergic disease?

Despite the potential for HM to impact the infant gut microbiome, the association between breastfeeding and development atopic diseases is less clear with most consistent studies showing a protective effect with exclusive breastfeeding on atopic dermatitis and recurrent wheeze/asthma.[184] The conflicting findings may be due to methodological limitations including reverse causality, varying definitions of breastfeeding, diagnostic methods for atopic disease, and/or sample size with most being underpowered for food allergy. Importantly, the term ‘breastfeeding’ does not account for the complexity of and/or interactions between the many HM factors, levels of which vary between mothers with different lifestyles[185] and geographic locations.[186] This may lead investigators to potentially miss the many nuances of the effect of breastfeeding on the gut microbiome. Finally, randomized controlled trials in this area, which are necessary to assess the true relationships of breastfeeding on the gut microbiome, on allergic diseases and on overall immune development, are lacking for obvious ethical reasons and are difficult due to confounding effects among these and other demographic variables. However, given that allergic outcomes such as atopic dermatitis and childhood asthma could be repressed in cohorts that are exclusively breastfed early in life[8,44,187] and are colonized predominantly with infant-type bifidobacteria[41], it is possible that breastfeeding creates a specific “niche” in the gut microbiome or in the progression of gut colonization that is supporting anti-allergic immune development. However, due to obvious ethical issues relating to randomization of breastfeeding, this relationship is difficult to establish.

Conclusions

The connection between HM, the infant gut microbiome, physiology and health is an active area of scientific inquiry. In addition to providing essential nutrients for growth, HM has evolved to contribute to guiding the assembly of the bacterial communities that colonize the

gut during early life. As an example, the complexity of HMO biosynthesis in the mammary gland [23] matched with similarly complex utilization of HMO by bifidobacteria[108], especially *B. infantis*, suggests a remarkable evolutionary adaptation to select bacterial species for early gut microbiome. This alone suggests that there is a specific benefit from these bacterial species, and that HM has also evolved to indirectly support the infant also indirectly by supporting a specific microbial composition. While the relationships between HMOs and members of the infant gut microbiota are fairly well understood, the potential mechanisms by which other components, those discussed in this review as well as others, shape bacterial communities in early life are only now emerging.

Similarly, while studies have identified relationships between bacterial taxa and metabolites with allergic disease, it is unclear how breastfeeding may mediate this relationship. In addition to deeper sequencing of the infant gut microbiome to gain better taxonomic resolution and insight into functional capacity, there is a need to characterize how HM components act synergistically to influence microbial development and metabolic output. Importantly, there it will be critical to conduct these studies longitudinally as the microbiome and infant diet rapidly change over the first year of life. Though not discussed in this review, assembly of other microorganisms, such as viruses, in the infant gut microbiome “niche” are shaped by breastfeeding and hold the potential to influence infant immune development, either independently or through interkingdom interactions with bacteria.[188] Furthering our knowledge of the dynamic relationships between HM, the infant microbiome, and immune development will open the door for the development of microbiota-tailored interventions to improve human health.

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Conflict of Interest:

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Abbreviations

BF	breastfed
FF	formula-fed
GI	gastrointestinal
HM	human milk
HMO	human milk oligosaccharide
ILA	Indol-3-lactic acid
PAMPS	pathogen-associated molecular patterns

PC	plasma cells
pIgR	polymeric immunoglobulin receptor
SCFA	short-chain fatty acids
SIgA	secretory immunoglobulin A
SC	secretory component

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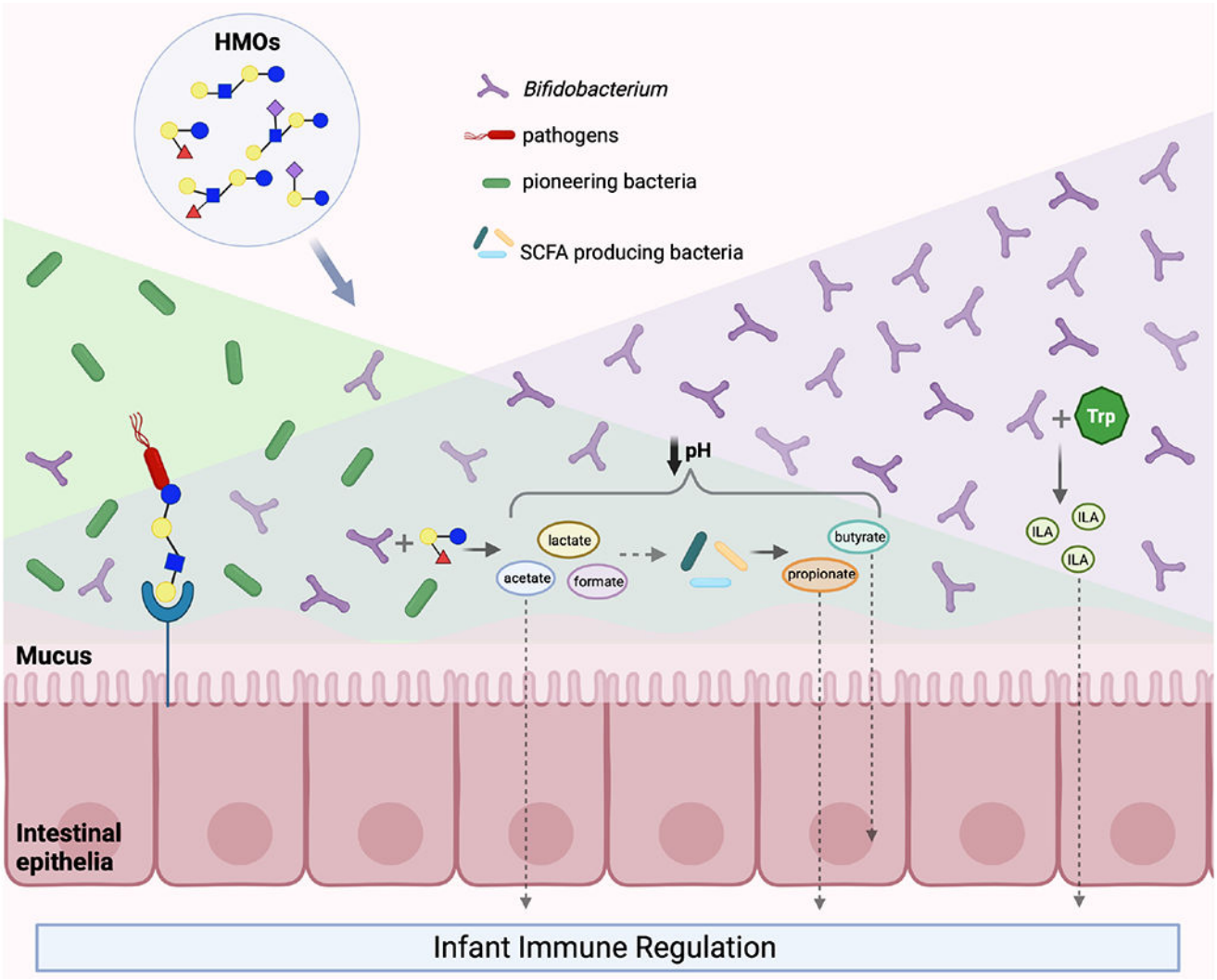

Fig 1. Human Milk Oligosaccharides (HMO) drive *Bifidobacterium* colonization and shape the infant metabolome.

HMOs select for and drive colonization of specific *Bifidobacterium* species within the infant gut microbiome. *Bifidobacterium* produce acetate, formate, lactate, which can be further metabolized into SCFA by other resident microorganisms. Certain species of *Bifidobacterium*, such as *B. infantis*, can metabolize tryptophan into indole-3-lactic acid (ILA), among others. SCFA and ILA can be reabsorbed into the gut epithelium and redistributed to peripheral tissues, influencing the infant immune system through a variety of proposed mechanisms. Figure created with BioRender.com

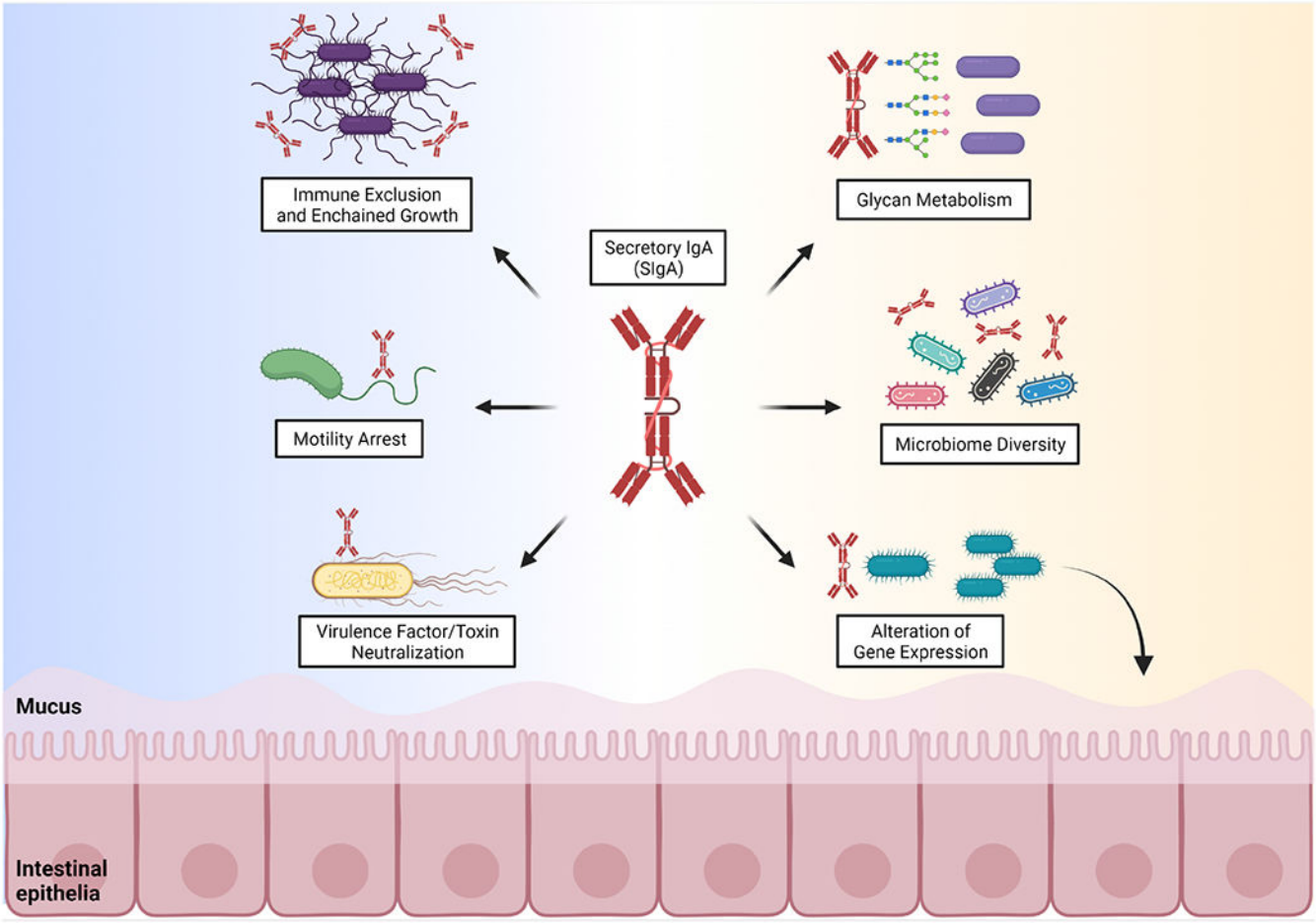

Figure 2: The biological activities of SIgA.

SIgA antibodies exert unique effector functions depending on the species being interacted with. SIgA mediates immune exclusion and enchained growth, restricts motility, and inhibits expression of toxins and virulence factors to defend against pathogenic organisms. Alternatively, SIgA supports commensal species by altering bacterial gene expression that in turn enhances growth and mucus colonization, supplying glycan residues as nutrients, and promoting overall diversity of the microbiome. Figure adapted from Yang and Palm, 2020[189]. Figure created with BioRender.com

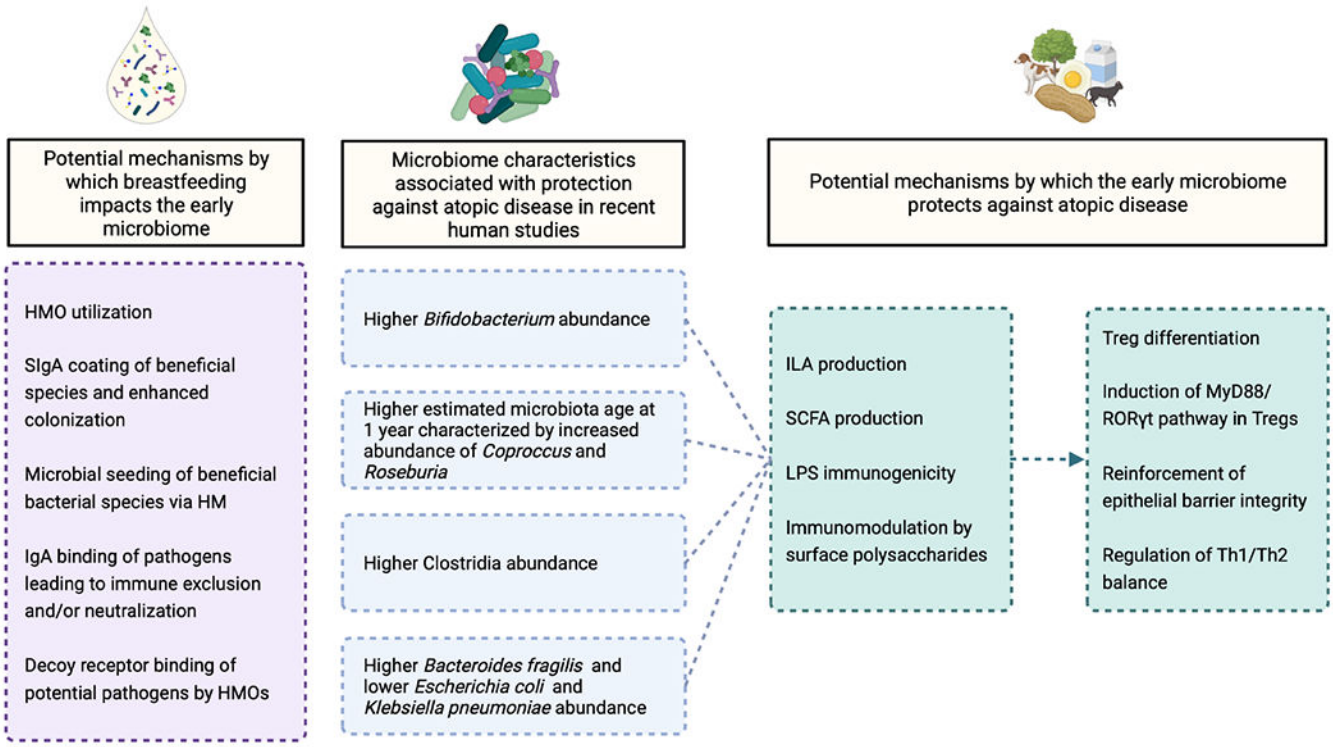

Figure 3: Potential relationships between breastfeeding, microbiome composition, and protection against atopic disease in early life.

There are multiple potential mechanisms by which human milk components, specifically HMOs, SIgA, and microbiota, may shape the infant microbiome, metabolite production, and early immune development. Recent studies have highlighted differential abundance of several bacterial taxa that discriminate infants who do and do not develop atopic disease, though how breastfeeding specifically mediates these relationships remains unclear. The proposed mechanisms by which the early microbiome protects against atopic disease include metabolite production, such as short-chain fatty acids (SCFA) and indole-3-lactic acid (ILA), LPS immunogenicity and immunomodulation by surface polysaccharides, which can result in downstream Treg differentiation and corresponding induction of pathways associated with oral tolerance, reinforcement of the epithelial barrier, and regulation of Th1/Th2 balance. Figure created with BioRender.com