IMPACT OF EARLY LIFE NUTRITION ON IMMUNE SYSTEM DEVELOPMENT AND RELATED HEALTH OUTCOMES IN LATER LIFE

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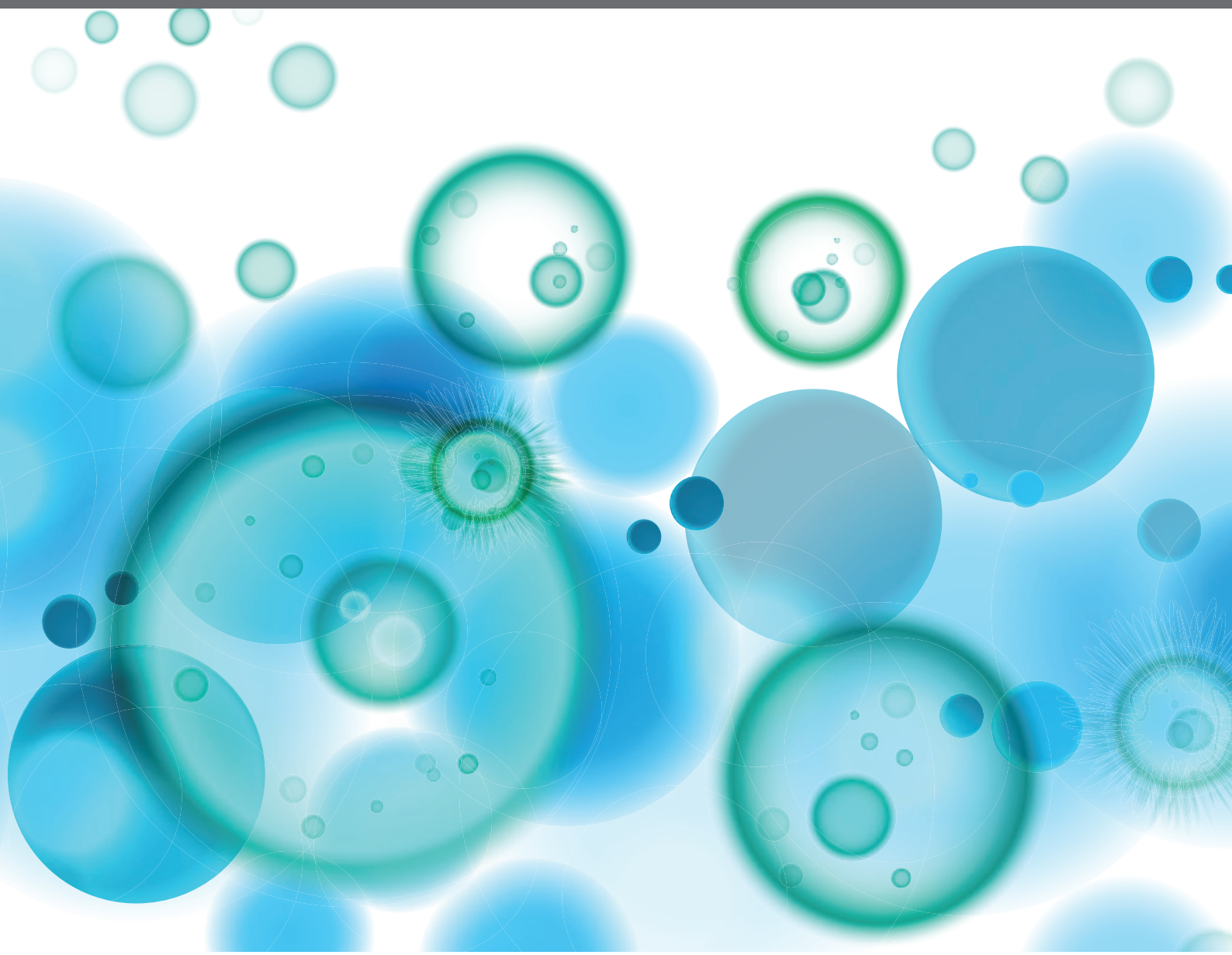

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IMPACT OF EARLY LIFE NUTRITION ON IMMUNE SYSTEM DEVELOPMENT AND RELATED HEALTH OUTCOMES IN LATER LIFE

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EDITORIAL
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Editorial: Impact of Early Life Nutrition on Immune System Development and Related Health Outcomes in Later Life

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Editorial on the Research Topic

Impact of Early Life Nutrition on Immune System Development and Related Health Outcomes in Later Life

EARLY LIFE NUTRITION AND HEALTH OUTCOMES IN LATER LIFE

Human milk (HM) is a complex mixture of macronutrients and bioactive compounds that provide optimal nutrition to infants (1–5). HM has been shown to impact infant's gastro-intestinal tract, immune system, microbiota composition, metabolism and also may have long-term effects on the development of infectious and non-communicable diseases (3, 6–8). The aim of this editorial is to provide a summary of the original research, reviews and opinions regarding key factors affecting human milk composition, and the role of bioactive components of human milk on infants' health.

Maternal obesity and maternal atopy are highly prevalent states that may have an effect on HM composition and infants' health outcomes (9–14). Few studies, however, have attempted to evaluate associations between HM metabolome composition and measures of infants' health and development. For instance, Bardanzellu et al. reviewed different studies for HM metabolite profile from mothers with overweight and obesity in an attempt to determine the milk metabolome composition with respect to obesity. However, the small sample size and large variability of the measures precluded the investigators from drawing conclusions which underscores the necessity of large sample size studies in this area of research. The authors, however, found that the fatty acid profile of human milk was associated with the maternal obesity status. Specifically, higher levels of saturated fatty acids and lower levels of monounsaturated and n-3 long-chain polyunsaturated fatty acids were found in milk of women with obesity compared to milk of women with normal weight. These changes in milk composition may influence long-term weight gain and glucose tolerance, in infants.

Allergic diseases are of a major concern and a significant burden to healthcare. It has been previously shown that HM composition may differ in allergic and non-allergic mothers (15). Recent research from Stinson et al. demonstrated that human milk from atopic mothers had lower levels of short-chain fatty acids (SCFA). Importantly, reduced levels of SCFA during early life may program the gut, microbiota, and obesity in infants. Nutritional interventions during pregnancy and lactation could serve as strategies to mitigate maternal atopy and potentially improve HM composition. For instance, Kao et al. showed that maternal consumption of goat milk during pregnancy and lactation associated with reduced airway inflammation and allergy outcomes in the offspring compared to cow's milk consumption. The goat milk feeding had increased immunoglobulin levels, Th1 cytokine production, and improved NK cell activity in comparison to cow' milk feeding in the offspring. In addition, in an animal study by Adel-Patient et al. showed that altering maternal immune status by sensitizing to different antigens protects offspring by modulating the antibody composition of human milk to specific antigens. In summary, obesity and prenatal antigen exposure of mothers were associated with HM composition and may affect infant health and development, but relationships should be confirmed in methodologically rigorous studies with a large sample size.

Human milk feeding likely protects from pathogens, thereby reducing/preventing negative outcomes associated with infection *via* different bioactives of milk such as human milk oligosaccharides (HMOs) and free amino acids (FAAs) (1, 16–19). Indeed, Carr et al. review highlighted the antipathogenic and immunomodulatory properties of HMOs and Zuurveld et al. reviewed the potential role of HMOs in preventing allergic diseases. In their article Sadelhoff et al., discuss the potential role of amino acids (particularly glutamine and glutamate) in HM to protect against neonatal allergies and infection. Further, using a HM-fed piglet model, Rosa et al. demonstrated the appearance of HM metabolites' in the gut, serum, and urine of HM-fed piglets. Importantly, glutamic acid and glutamate levels were higher in the HM-fed animals relative to the formula fed group suggesting potential benefits of HM FAAs. Also, Rosa et al., study discussed human metabolites such as polyamines and tryptophan impact on immune response.

Human milk has been shown to promote gut microbiota development and function (20–25). In reviewing the literature, Carr et al. comprehensively overviewed the role of HM microbiota on gut microbiota colonization and immune function. This article also discussed the role of human milk components such as HMOs, and IgA impact on gut microbiota. Peroni et al. reviewed the literature regarding microbiome composition and its impact on the development of allergic diseases. Drall et al. demonstrated an association of microbiota composition in exclusively breastfed infants to *C. difficile* colonization. In summary, dietary intake and both pre- and post-natal factors appear to be associated with the gut microbiota composition and its association to pathogens colonization. This may be a focus for the future intervention strategies aiming at improving infants health.

Previous studies suggest antipathogenic effects of HM components and that the addition of these bioactive molecules (i.e., HMOs, lactoferrin, immunoglobulins, and milk fat globule membrane FGM, extracellular vesicles) to infant formulas may benefit child health (20, 26–36), although the studies usually lack methodological rigor and outcomes were based on a small sample size. The studies on recombinant immunoglobulins and bioactivity in the digestive tract are limited. Research from Sah et al. provided some evidence that recombinant antibody towards respiratory syncytial virus (RSV) may impact growth and have neutralization activity against the virus across the GI tract. In another study, Nederend et al. demonstrated that bovine immunoglobulin antiviral activity and T cell response may prevent RSV infection. Interestingly, Adel-Patient et al. found no protection to protein present in cow's milk by feeding the hydrolysates of caseins and *Lactobacillus rhamnosus* GG protobiotic. Thus, future studies are needed to fully understand the protective effects of immunoglobulins, as well as pre and probiotics, before adding these components to infant formula. The combined effect of different bioactive molecules within the formula on infant health and development also requires further investigation.

Human milk may impart benefits through epigenetic programming influencing long-term health by various mechanisms. van Esch et al. provided an overview on the evidence of maternal nutrition, environmental factors impact on milk composition, and how the different components of milk epigenetically program infants' health and dictate allergy and asthma outcomes in later life. Human milk contains extracellular vesicles with microRNAs (miRNAs) as one of the epigenetic molecules (35). Furthermore, Carr et al. provided evidence that miRNAs known to modulate gene expression were associated with immune function in the human milk-fed group compared to formula diet-fed group in the piglet model. Also, the review by Carr and associates highlighted that miRNAs present in human milk may be associated with a beneficial effect for infants' health and immune system.

Finally, Bilsen and colleagues elegantly show how a network-based approach that includes evidence from studies to determine the windows of opportunity to shape lifelong health of infants. This can be used to predict the key candidate markers of early life immune development. Human milk is a complex mixture with several bioactive components providing short and long-term health benefits to infants. We sincerely hope that the article's compilation of the Research Topic on human milk will be useful and interesting to the readers and hope that the knowledge gaps highlighted will be considered for future state-of the art research findings.

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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BRIEF RESEARCH REPORT
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Clostridioides difficile Colonization Is Differentially Associated With Gut Microbiome Profiles by Infant Feeding Modality at 3–4 Months of Age

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Colonization with *Clostridioides difficile* occurs in up to half of infants under the age of 3 months, is strongly influenced by feeding modality and is largely asymptomatic. In spite of this, *C. difficile*'s presence has been associated with susceptibility to chronic disease later in childhood, perhaps by promoting or benefiting from changes in infant gut microbiome development, including colonization with pathogenic bacteria and disrupted production of microbial bioactive metabolites and proteins. In this study, the microbiomes of 1554 infants from the CHILD Cohort Study were described according to *C. difficile* colonization status and feeding mode at 3–4 months of age. *C. difficile* colonization was associated with a different gut microbiome profile in exclusively breastfed (EBF) vs. exclusively formula fed (EFF) infants. EBF infants colonized with *C. difficile* had an increased relative abundance of Firmicutes and Proteobacteria, decreased relative abundance of Bifidobacteriaceae, greater microbiota alpha-diversity, greater detectable fecal short chain fatty acids (SCFA), and lower detectable fecal secretory Immunoglobulin A (sIgA) than those not colonized. Similar but less pronounced differences were seen among partially breastfed infants (PBF) but EFF infants did not possess these differences in the gut microbiome according to colonization status. Thus, breastfed infants colonized with *C. difficile* appear to possess a gut microbiome that differs from non-colonized infants and resembles that of EFF infants, but the driving force and direction of this association remains unknown. Understanding these compositional differences as drivers of *C. difficile* colonization may be important to ensure future childhood health.

Keywords: *Clostridioides difficile*, sIgA, SCFA, infant feeding, microbiome, gut microbiota, metabolites

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INTRODUCTION

Clostridioides (formerly *Clostridium*) *difficile* is a bacterium that is present in the intestine of nearly 40% of infants at 1 month of age, and 30% of infants between the ages of 1 and 6 month (1). *C. difficile* is the main cause of antibiotic-associated diarrhea in adults (2, 3) and although *C. difficile* may not be accompanied by diarrheal illness in infants, it has been associated with atopy and microbial dysbiosis (4–6). Furthermore, despite the lack of immediate risks related to carriage of *C. difficile* in infants, this gram-negative spore-forming bacterium is capable of inducing gut inflammation and disrupting the intestinal epithelial barrier (7, 8). As a result, these less than desirable influences on the intestinal environment may impact the succession and abundance of commensal gut microbiota and overall microbial ecology.

Infancy is a critical period for establishment of the gut microbial ecosystem and immune system priming to confer protection against gut microbial dysbiosis and reduce the risk of negative health outcomes. *C. difficile* is thought to promote colonization of non-commensals and pathogenic bacteria, although this phenomenon has received little attention in infants. In a small group of infants ($n = 53$) (6), one study found that *Ruminococcus gnavus* and *Klebsiella pneumoniae* species were more prevalent in infants colonized with *C. difficile*, while non-carriers were more frequently colonized by *Bifidobacterium longum*. Acquisition of *C. difficile* during infancy has been attributed to several environmental exposures, notably formula feeding (1, 9, 10). Breastmilk bioactive factors, including human milk oligosaccharides and secretory Immunoglobulin A (sIgA), neutralize toxins and bind pathogens, which may account for asymptomatic colonization of the infant gut with *C. difficile* and/or lower colonization rates in breastfed infants vs. infants not fed human milk (11–13). Consequently, infants colonized with *C. difficile* may manifest distinct and persistent changes in their gut ecology, including changes in metabolites, secretory proteins and resident microbiota. Hence, the relationship between *C. difficile* and the infant gut microbiome merits further examination.

In this study, we report the association between *C. difficile* (family Peptostreptococcaceae) and other gut microbiome components, including composition, metabolites and sIgA, to provide insights into ecological factors related to *C. difficile* expansion in infancy. We also explored these differences in exclusively breastfed, partially breastfed, and exclusively formula fed infants to examine the gut microbial community and *C. difficile* colonization infants with distinct diets.

METHODS

Study Design and Population

This study includes a sub-set of 1,562 families enrolled in the CHILD Cohort Study. In this prospective population-based cohort, mothers were recruited and enrolled with informed consent during the second or third trimester of pregnancy between January 2009 and December 2012 from the Vancouver, Edmonton and Manitoba study sites (inclusion and exclusion criteria outlined at www.childstudy.ca) (14). The primary objective of the CHILD Cohort Study was to determine the developmental, environmental, and genetic determinants of later allergy and asthma in childhood (15). All infants included in this subsample provided a fecal sample at 3–4 months of age, which was sequenced by Illumina MiSeq and processed by targeted qPCR to detect *C. difficile*. Within this study, smaller, yet representative, groups of samples were profiled to describe concentrations of fecal metabolites ($n = 467$) and secretory IgA ($n = 731$) (**Supplementary Table 1**). Gut microbiota compositional findings have previously been described for infants in the CHILD Cohort Study (16), but this paper is the first integration and report of 4 characterizations of the infant gut microbiome and gut immunity from the CHILD Cohort Study. The Human Research Ethics Boards at the University of Manitoba, University of Alberta, and University of British Columbia approved this study.

qPCR for *Clostridioides difficile* Detection

Fecal samples of 5–10 grams were collected from infant diapers during home-visits conducted at 3–4 months of age by a research assistant or parents according to an approved protocol (**Supplementary Figure 1**). Samples were aliquoted and stored at −80°C until analyzed. A targeted 16S primer and probe set was used for amplification and quantification of *C. difficile* and followed the methods set by Penders et al. (17). To minimize differential inhibitory effects due to variable concentrations of genomic template DNA in qPCR, all template DNA samples were first normalized by dilution to 1 ng/μL (18). Then, each multiplex assay was prepared to contain 1X QuantiNova Multiplex PCR Kit (QIAGEN), 0.4 μM of each primer, 0.25 μM of each probe and 1 μL [1 ng/μL] of sample DNA in a final volume of 20 μL. qPCR cycling conditions were as follows: initial denaturation for 2 min at 95.0°C, 40 cycles of denaturation for 5 s at 95°C and annealing/extension/reading for 20 s at 60°C. Oligonucleotides were acquired from IDT (Integrated DNA Technologies Inc, Coralville, IA, USA) and reactions were performed on the MiniOpticonTM Real-Time PCR System (Bio-Rad, Hercules, CA, USA). A standard curve was created and employed to determine the efficiency of the *C. difficile* primers and probes by performing five 1:10 serial dilutions of *C. difficile* ATCC 9689D-5 genomic DNA starting at 1 ng/μL. We calculated the lower limit of detection for the multiplex assay to be 1X10-5 ng of DNA or 2 genomes of *C. difficile* based on the amplification data from the serial dilution and the non-template control. Because each template sample represented a different starting mass of stool, the limit of quantification for the analysis was variable from sample-to-sample, and ranged from 514 to 33,333 genomes/g stool. Infants were classified by *C. difficile* colonization status (present in fecal sample, yes/no). Amongst colonized infants, median levels of *C. difficile* (ng/g feces) in infant fecal samples were not different between feeding groups (data not shown).

Fecal Microbiome Analysis

DNA extraction and amplification of bacterial V4 hypervariable region of the bacterial 16S rRNA gene was followed by sequencing and taxonomic classification and was conducted as

previously described (19). To summarize, microbial DNA was extracted from the frozen stool samples mentioned above (80 to 200 mg) using the QIAamp DNA Stool Mini kit according to the manufacturer protocol (Qiagen Inc, Valencia CA). Next, the bacterial 16S rRNA genes were amplified at the hypervariable V4 region using PCR with appropriate primers. PCR products were combined for sequencing, performed using the Illumina MiSeq platform (San Diego, CA). Resultant sequences were taxonomically classified and matched at >97% similarity against the Greengenes reference database in QIIME and filtered/excluded if <60% similarity. Finally, microbiota data were rarefied to 13,000 sequences per sample and relative abundances were calculated. At this time, microbiota diversity within samples (alpha diversity) was calculated using standardized estimators of OTU richness and/or evenness: Chao1 and Shannon diversity indices.

Short-Chain Fatty Acid (SCFA) and Other Fecal Metabolites

In a sub-set of fecal samples ($N = 467$), metabolites were quantified by magnetic resonance spectroscopy (NMR). NMR requires a small quantity of sample for processing and has high reproducibility compared to mass spectrometry (20). Homogenization of 100 mg of sample and subsequent centrifugation were performed as necessary for sample cleaning: Each sample was placed in an Eppendorf tube will 1 mL of ice water, vortexed for 5 min and subjected to sonication for 20 more minutes at 4°C. Samples were then vortexed for another 20 min at 250 rpm. Samples were then centrifuged at 15,000 × g for 1 h at 4°C. The supernatant was removed and placed in a new tube and the process was repeated. The cleaned fecal water was stored at −20°C. After extraction, 280 μL of fecal water was mixed with 70 μL of a standard buffer solution (54% D_2O: 46% 750 mM potassium phosphate (mono- and dibasic) pH 7.0 v/v containing 5 mM DSS-d_6 (2,2-dimethyl-2-silcepentane-5-sulphonate). The sample (350 μL) was then transferred to 3 mm SampleJet NMR tube for subsequent spectral analysis. All ^{1}H-NMR spectra were collected on a 700 MHz Avance III (Bruker) spectrometer equipped with a 5 mm HCN Z-gradient pulsed-field gradient (PFG) cryoprobe. ^{1}H-NMR spectra were acquired at 25°C using the first transient of the NOESY pre-saturation pulse sequence (noesy1dpr), chosen for its high degree of quantitative accuracy.

Prior to spectral analysis, all FIDs (free induction decays) were zero-filled to 250 K data points and line broadened 0.5 Hz. The methyl singlet produced by a known quantity of DSS was used as an internal standard for chemical shift referencing (set to 0 ppm) and for quantification. All ^{1}H-NMR spectra were processed and analyzed using the Chenomx NMR Suite Professional software package version 8.1 (Chenomx Inc., Edmonton, AB) (11). The Chenomx NMR Suite software allows for qualitative and quantitative analysis of an NMR spectrum by manually fitting spectral signatures from an internal database to the spectrum. Typically 90% of visible peaks were assigned to a compound and more than 90% of the spectral area could be routinely fit using the Chenomx spectral analysis software. Most of the visible peaks are annotated with a compound name. We sought to identify all metabolites relevant to microbial production or substrate use. Metabolites were quantified as μmol/gram feces. In this study, we report on a subset of metabolites measured, specifically the SCFAs acetate, butyrate, and propionate, in addition to other metabolites in the metabolic pathways of *C. difficile* including para-cresol, succinate, and glutamate (**Supplementary Figure 2**).

Fecal Secretory IgA

A sub-sample of fecal samples were assayed for sIgA ($N = 731$) using the Secretory IgA ELISA (enzyme-linked immunosorbent assay) kit (ELISA, Immundiagnostik AG assay, Bensheim, Germany). Approximately 14 mg of fecal sample was used for the sIgA analyses. Samples were run in duplicate according to the manufacturer's protocol, as previously described (21), and quantified as the average milligram of sIgA per gram wet weight feces (mg/g). To summarize, a fecal sample aliquot for each infant was thawed and an IDK Extract buffer was used to extract fecal sIgA. Samples were then diluted (1:125) with a wash buffer and placed in a microtiter plate along with controls and standards. Wells were aspirated, washed and 100 μL of conjugate was added and allowed to incubate at room temperature. Samples were then shaken on a horizontal mixer, washed with TMB substrate and incubated in the dark (20 min). An ELISA reader was used to measure the absorption at 450 nm (620 nm reference). The reads were multiplied by 12,500 and compared against a standard curve, created using standards provided with the assay kit, for quantification.

Covariate Data

Breastfeeding status was determined through self-report questionnaires administered to mothers at 3–4 months postpartum ($N = 1{,}554$). A 3-category variable was created for infant breastfeeding status at the time of stool sample collection and questionnaire administration: (1) exclusively breastfed (EBF), (2) partially (i.e., mixed) breastfed (PBF), and (3) exclusively formula fed (EFF). Complete feeding data were missing in 8 infants, leaving a total of 1554 infants (not the full $N = 1{,}562$ with available *C. difficile* and microbiome data) that were stratified by feeding mode.

Statistical Analysis

All statistical analysis was conducted using Stata (version 13), RStudio (version 1.1.456), and the Galaxy platform (MaAslin) between September 2018 and March 2019. Non-parametric (Mann-Whitney U or Kruskal–Wallis test) and parametric (student's *t*-test) tests were used where appropriate (**Supplementary Figure 3**) to compare alpha diversity indices, fecal metabolites, and fecal sIgA according to colonization status. Differences in taxon relative abundance (outcomes) according to *C. difficile* colonization status (predictor) were determined using the multivariate association with linear models method developed by the Huttenhower lab (MaAslin) (22) (available at: https://huttenhower.sph.harvard.edu/galaxy/). Spearman correlations were computed in **Supplementary Table 2**, and heatplots were generated using the gplots package and the heatmap.2() command in R. Scatter bar graphs were generated using the ggplots2 package and the geom_boxplot() and

geom_beeswarm() commands. Statistical significance was defined as a two-sided p or q-value < 0.05, after FDR correction for multiple comparisons.

RESULTS AND DISCUSSION

The prevalence of *C. difficile* colonization among all study infants was 30.9% ($n = 482/1562$), which aligns with previously reported estimates (1). These colonization rates differed between feeding groups: 22.6% for EBF, 36.0% for PBF and 49.6% for EFF infants (χ^2: 76.71, $p < 0.001$, **Figure 1A**, $N = 1{,}554$). The mean Shannon and Chao1 indices for EBF and PBF infants were lower for infants who lacked *C. difficile* compared to infants colonized with *C. difficile,* suggesting that the richness and abundance of the infant gut microbiota are greater and more equally distributed in the presence of *C. difficile* ($p<0.05$, **Figures 1B,C**). No differences in alpha diversity were detected with *C. difficile* colonization in EFF infants. These differences across feeding modality could not be attributed to the normal progression of microbiota development since infant age [median (IQR)] in each of the feeding groups was similar: 3.29 months (1.03) for EBF, 3.33 months (0.94) for PBF, and 3.20 months (1.10) for EFF, $p = 0.27$.

EBF is generally associated with low microbial alpha diversity due to the dominance of *Bifidobacterium* spp. (19, 23). Bifidobacteria thrive on human milk oligosaccharides but their growth is reported to be suppressed with *C. difficile* colonization (6, 24). Accordingly, our regression models revealed that *Bifidobacterium* spp. were less abundant in EBF infants colonized with *C. difficile* than EBF infants who were not colonized (transformed $\beta = -0.06$, $q = 0.021$, **Figure 2**). Bifidobacteria are well-known acetate producers (24, 25) and their presence was positively correlated with this metabolite ($R = 0.56$, $p < 0.01$, **Supplementary Figure 4**). Despite an observed lowered relative abundance of *Bifidobacterium*, we measured higher absolute concentrations of fecal acetate among EBF infants colonized with *C. difficile* ($p = 0.01$, **Supplementary Figure 2**). Many other microbiota produce acetate (26); thus, the greater diversity of microbes we observed in EBF *C. difficile* positive infants likely contributed to higher fecal acetate levels. In our study, acetate concentrations were also positively correlated with the members of the Campylobacteraceae ($R = 0.38$, $p > 0.10$), Peptostreptococcaceae ($R = 0.55$, $p = 0.05$) and Clostridiaceae ($R = 0.58$, $p > 0.10$) families (**Supplementary Figure 4**) which were enriched in EBF infants positive for *C. difficile* ($q < 0.05$, **Figure 2**).

Other microbes that were differentially abundant in the presence of *C. difficile* were members of the Lachnospiraceae and the Ruminococcaceae families, and both were enriched with *C. difficile* colonization among EBF and PBF infants ($q < 0.05$, **Figure 2**). Among EBF infants, we also observed higher absolute concentrations of non-acetate SCFAs (i.e., butyrate and propionate, $p < 0.05$, **Supplementary Figure 2**) when they were colonized with *C. difficile*. The relative abundance of Ruminococcaceae [e.g., *Oscillospira* spp. which are butyrate producers (27)] was positively correlated with butyrate ($R = 0.35$, $p < 0.01$, **Supplementary Figure 4**) and with p-cresol

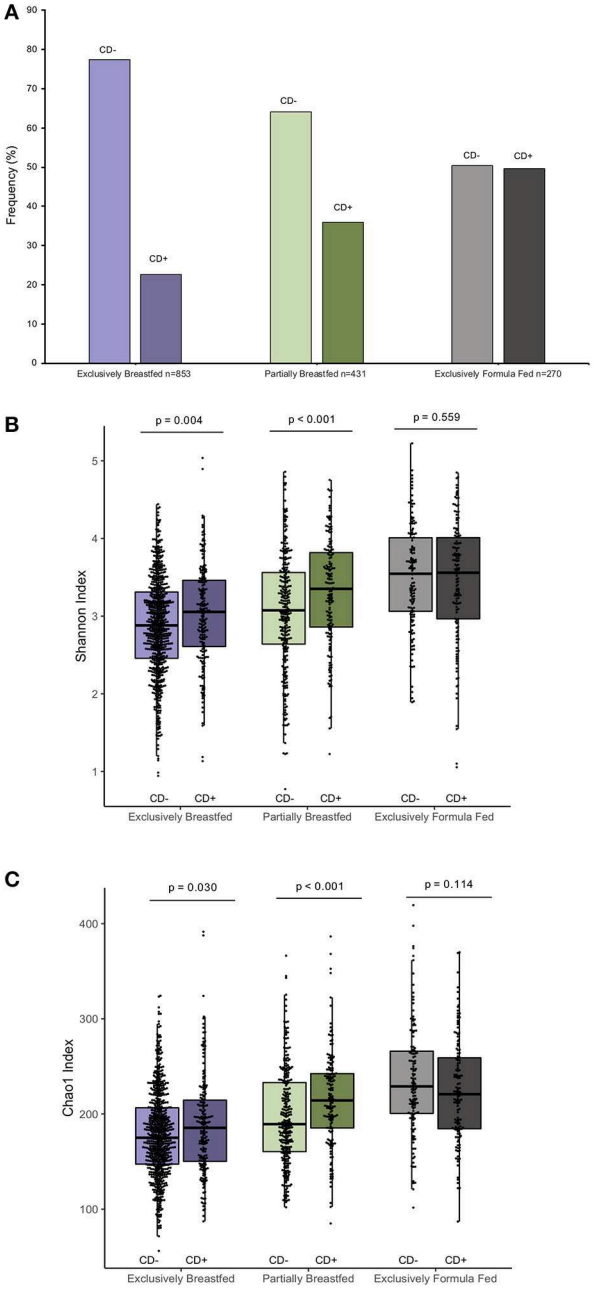

FIGURE 1 | Frequency of *C. difficile* colonization in our study population and infant microbial alpha-diversity according feeding mode ($n = 1{,}554$). Colonization rates differ within feeding groups **(A)** 22.63% of exclusively breastfed infants ($N = 193/853$), 35.96% of partially breastfed infants ($N = 155/431$) and 49.63% of formula fed infants were colonized ($N = 134/270$) (Fishers' exact $p < 0.001$). Scatter box-plots of the median (middle line), Q3 and Q1 quartiles (box limits), IQR (whiskers) and outlying values (dots). Data were normally distributed (**Supplementary Figure 3**) and thus two-sided p-values were calculated with students t-test within infant feeding groups, comparing colonized and non-colonized infants at a significance threshold of $\alpha = 0.05$. Higher α-diversity was observed for infants colonized with *C. difficile* (CD+) and breastfed (either exclusively or partially) than non-carriers (CD–) on the same diet. This was the case for both the Shannon diversity index **(B)** and Chao1 species richness index **(C)**. Purple represents EBF, green for PBF and gray for EFF.

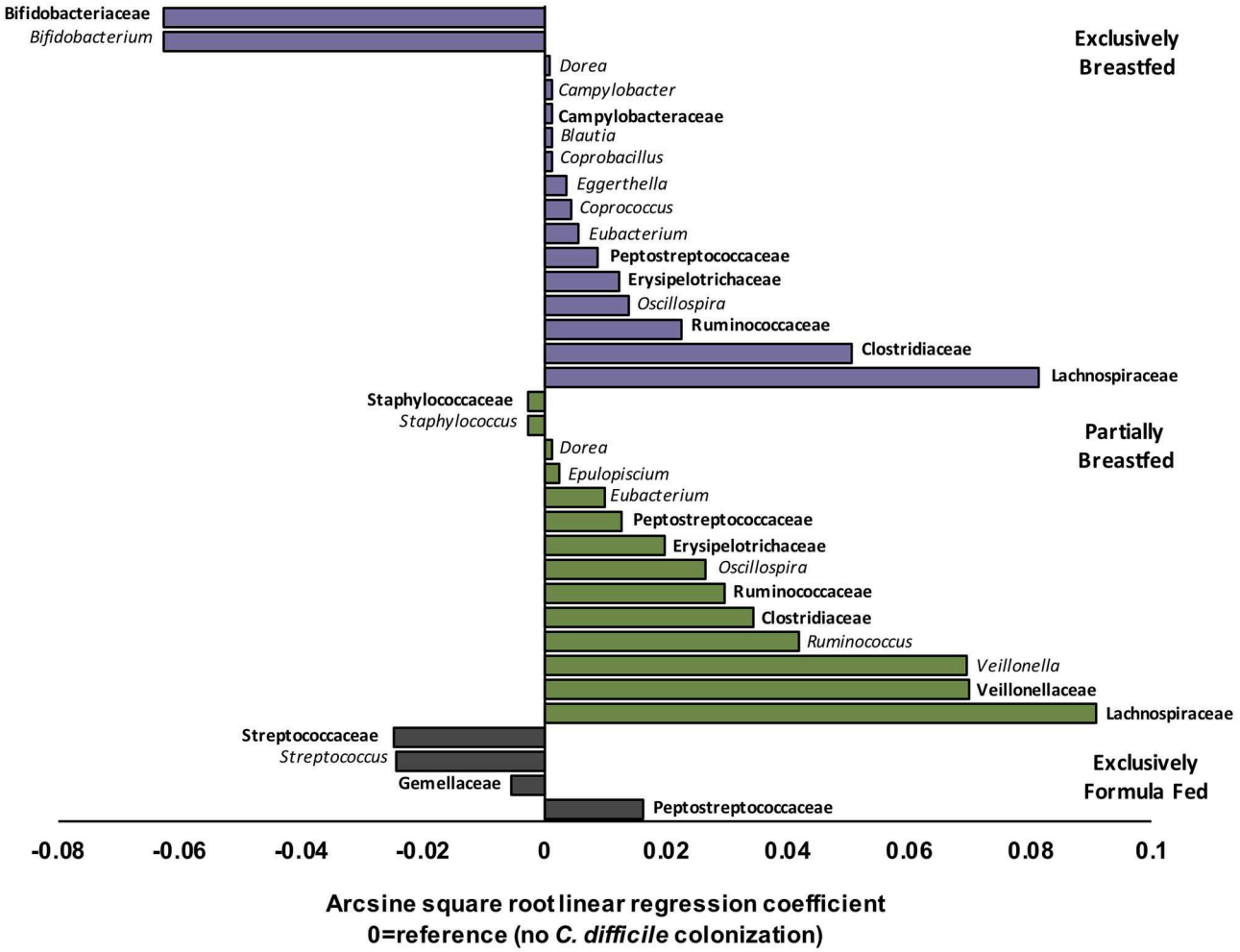

FIGURE 2 | Relative differences in microbiota composition between *C. difficile* carriers and non-carriers across infant feeding groups ($n = 1{,}554$). Multivariate linear regression results (MaAslin) for family (**bolded**) and genus level taxa that are differentially associated with *C. difficile* colonization at 3–4 months of age. Values on the x-axis represent arcsine square root transformed regression coefficients of microbiota relative abundances for each linear model, adjusted for multiple comparisons (FDR correction) to determine which taxa are uniquely associated with *C. difficile* colonization. Each model had a reference of infants without *C. difficile* colonization at 3–4 months. Data shown only for taxa with FDR corrected two-sided q-value < 0.05. Coefficients > 0 (positive values) represent taxa that enriched in *C. difficile* carriers, while coefficients < 0 (negative values) represent taxa that were depleted in *C. difficile* carriers. *P*-values for each regression can be found in **Supplementary Tables 3–5**. Purple represents EBF ($N = 853$, 193 CD+), green for PBF ($N = 431$, 155 CD+) and gray for EFF ($N = 270$, 134 CD+).

($R = 0.27$, $p = 0.08$, **Supplementary Figure 4**), a known product of *C. difficile* amino acid metabolism (28). The fecal concentrations of p-cresol were higher in all infants colonized with *C. difficile,* regardless of infant feeding group ($p < 0.01$, **Supplementary Figure 2**). Lachnospiraceae was weakly correlated with propionate concentrations ($R = 0.18$, $p < 0.01$). Propionate production by Lachnospiraceae is through the 1,2-propanediol and acrylate pathways, which are possessed by *Blautia, Eubacterium,* and *Coprococcus* (29), all genera that were enriched in EBF *C. difficile* carriers ($q < 0.05$ for each, **Figure 2**).

Correlations between microbial relative abundance and butyrate concentrations involved a greater number of gut microbiota in PBF than EBF infants colonized with *C. difficile*. Specifically, Ruminococcaceae (R: 0.25, $p < 0.01$), Lachnospiraceae (R: 0.28, $p < 0.01$) and Clostridiaceae (R: 0.46, $p < 0.01$) were all positively correlated with butyrate and enriched in PBF infants ($q < 0.05$ for each, **Figure 2**). In contrast to EBF infants, Lachnospiraceae taxa in PBF *C. difficile* positive infants were inversely correlated with propionate levels ($R = -0.57$, $p < 0.01$). Since Bacteroidaceae are more abundant with any formula feeding (16), irrespective of *C. difficile* status in the current study, and they predominantly produce propionate (26), these microbiota likely out-competed Lachnospiraceae in the fermentation of substrates in PBF infants to produce propionate via the succinate pathway. Consistently, we observed a positive correlation between propionate concentrations and relative abundance of Bacteroidaceae among PBF and EFF infants, which

was absent in EBF infants and independent of *C. difficile* status (**Supplementary Figure 4**).

Unique to PBF infants colonized with *C. difficile* was a higher relative abundance of *Veillonella* spp. (family Veillonellaceae, $q = 0.002$, **Figure 2**). Also, the relative abundance of *Staphylococcus* spp. (family *Staphylococcaceae*, $q < 0.001$, **Figure 2**) was lower in PBF infants positive for *C. difficile* than non-carriers. Fewer compositional differences were detected with *C. difficile* colonization among EFF infants, relative to breastfed (exclusive and partial) infants and equally no differences were detected in fecal metabolites. The sole family of microbes whose relative abundance was significantly higher in EFF *C. difficile* carriers was its own family, the Peptostreptococcaceae ($q = 4.80E{-}24$, **Figure 2**). As also expected, the Peptostreptococcaceae family were enriched in EBF and PBF infants colonized with *C. difficile* ($q < 0.001$, **Figure 2**).

Other metabolites measured in our study include glutamate and succinate. Glutamate, a metabolite shown to play a role in the establishment of *C. difficile in vivo* (30), was not differentially associated with *C. difficile* colonization in any of the feeding groups (**Supplementary Figure 2**). This metabolite is essential for *C. difficile* pathogenesis but may not be required for asymptomatic colonization in infants. Further, unlike glutamate dehydrogenase, a protein marker of *C. difficile* colonization (30), glutamate is an intermediary metabolite which may be consumed in several microbiota cross-feeding pathways. In fact, fecal levels of glutamate correlated with key microbes that differed by *C. difficile* status in all feeding groups (**Supplementary Figure 4**). Similarly, *C. difficile* utilizes succinate for its expansion and has the ability to ferment succinate to butyrate (31). Consistent with the succinate pathway, succinate concentrations were lower and concentrations of butyrate higher with *C. difficile* colonization in EBF infants and PBF infants ($p = 0.05$, **Supplementary Figure 2**). Since succinate is not easily absorbed by colonic cells (32), as suggested by our findings, levels may be further lowered from cross-feeding by succinate-utilizing members of the "Negativicutes" branch of Firmicutes clade (e.g. *Veillonella* spp.) (32, 33). Indeed, succinate was negatively correlated with Veillonellaceae in PBF infants (**Supplementary Figure 4**).

In addition to examining fecal metabolites, we also measured fecal sIgA levels as a marker of intestinal homeostasis and mucosal immunity (34). As we previously reported, *C. difficile* was associated with lower sIgA concentrations among EBF infants ($p = 0.047$, **Figure 3**) (11). Since infant secretion of sIgA has been positively correlated with breastmilk sIgA levels and breastmilk microbiota, maternal factors may contribute to lower concentrations in the infant (35, 36). Notably, animal models have shown that offspring nursed by mothers who are sIgA-deficient have a different gut microbiota composition than those receiving sIgA through breastmilk (37, 38). Similar to what we observed in EBF *C. difficile* positive infants, reduced fecal sIgA was associated with compositional differences that included an increased relative abundance of Lachnospiraceae and pro-inflammatory microbiota. Previous work from the CHILD Cohort Study has shown that sIgA in breastmilk may be depleted due to factors such as depression (21) or an altered maternal milk

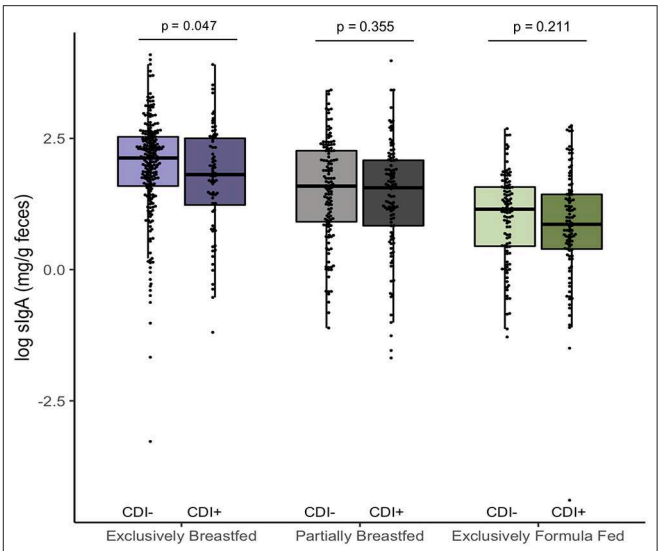

FIGURE 3 | Log transformed measures of fecal secretory IgA, according to infant colonization and feeding mode ($n = 731$). Scatter box-plots of the median (middle line), Q3 and Q1 quartiles (box limits), IQR (whiskers) and outlying values (dots). Two-sided *p*-values were calculated with Mann–Whitney *U*-test of log transformed fecal sIgA (mg/g) comparing colonized and non-colonized infants within the same diet group. Exclusively breastfed infants colonized with *C. difficile* (CD+) had lower median fecal sIgA than non-carriers (CD–) on the same diet. Purple represents EBF ($N = 290$, 72 CD+), green for PBF ($N = 237$, 104 CD+) and gray for EFF ($N = 204$, 101 CD+).

microbiota (36), which may predispose the infant to colonization by *C. difficile* and related dysbiosis. Although sIgA can bind enteric pathogens (34), there is a lack of evidence suggesting that *C. difficile* contributes to the destruction of sIgA or reduce production of this protein.

Finally, some of our findings suggest that the gut microbiota of breastfed (both EBF and PBF) infants colonized with *C. difficile* resembles the gut microbial composition of adults (e.g., increased relative abundance of Firmicutes such as *Eubacterium* spp.) (39). Meta-analytic evidence from cohorts worldwide documents similarity between the gut microbiota of EFF infants and that of adults (23). Extending this evidence, our study suggests that the gut microbiome of breastfed infants colonized with *C. difficile* is compositionally similar to that observed in EFF infants (**Supplementary Figure 5**).

In our large population cohort study, we were not able to categorize infants according to the proportional intake of breastmilk vs. formula, as others have (40). Since our study did not employ culture-based methodology, another study limitation was inability to detect the strains and toxigenic properties of *C. difficile*. Should our study findings continue to align with previous findings, we might expect a prevalence of toxigenic strains to be <10% among infants with *C. difficile* positive samples (12, 41). We are also unable to determine the direction of observed associations: whether *C. difficile* caused gut microbial dysbiosis, or whether gut dysbiosis increased infant susceptibility to *C. difficile* colonization. This could be improved by measuring the *C. difficile* colonization status of infants longitudinally (at more than one time point) to assess if

C. difficile colonization is transient or persistent and whether the microbiome changes precede or follow colonization. However, with enhanced characterization of the gut microbiome beyond taxon composition, our study provides evidence for a putative role of *C. difficile* colonization on the gut microbial ecology of young, full-term infants from a large, general population in North America.

CONCLUSION

We observed a distinct gut microbiome in young infants colonized with *C. difficile* and this distinction depended on the breastfeeding status of the infant. The most noticeable microbiome differences with *C. difficile* colonization, especially depletion of *Bifidobacterium* spp., were among EBF infants. Similar compositional differences among members of the Firmicutes phylum were seen in EBF and PBF infants. However, unique to PBF infants was enrichment of Veillonellaceae. These findings highlight the differential relationship of *C. difficile* colonization on EBF vs. PBF vs. EFF infants, which should be considered in future studies of infants feeding modality and disease risk. In summary, we found differences in the infant gut microbiome with *C. difficile* colonization, but it remains unclear whether *C. difficile* causes these differences or if external factors in early infancy create a niche that is more permissive to colonization. Newer cohorts with available multi-omics data could validate these findings and explore the hypothesized relations between various microbiota and *C. difficile* to further understand colonization of this microbe in infancy and its implications in later childhood health.

DATA AVAILABILITY STATEMENT

The data and analysis code that support the findings of this study can be made available from the corresponding author and CHILD Cohort Study coordinators upon reasonable request. These data, including study participant data, are securely stored in the CHILDdb.ca database.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the University of Alberta, University of British Columbia and University of Manitoba Ethics Boards. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

KD and AK conceived the study. KD performed data analysis, prepared figures, and drafted and edited the manuscript. HT generated gut microbiota operational taxonomic unit profiles using QIIME software. TK conducted DNA extraction and sample preparation for sequencing for microbiome analyses. NM-L performed targeted qPCR for *C. difficile* detection. DW and RM supervised and conducted NMR and fecal metabolite analyses. CF supervised, conducted, and helped interpret the fecal sIgA analyses. MA created the breastfeeding measures. DG, AB, PM, PS, ST, TM, MS, DL, and JS obtained funding and contributed to study design and data collection. AK obtained funding, contributed to data interpretation and critically reviewed the manuscript. All the authors reviewed the manuscript content, provided feedback and approved the final version. AK will serve as guarantor of the manuscript's contents.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.02866/full#supplementary-material

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Goat Milk Consumption Enhances Innate and Adaptive Immunities and Alleviates Allergen-Induced Airway Inflammation in Offspring Mice

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Goat milk (GM), as compared to cow milk (CM), is easier for humans to digest. It also has antioxidant and anti-inflammatory effects and can improve minor digestive disorders and prevent allergic diseases in infants. It is unclear whether GM consumed in pregnant mothers has any protective effects on allergic diseases in infants. In this experimental study with mice, we found GM feeding enhanced immunoglobulin production, antigen-specific (ovalbumin, OVA) immune responses, and phagocytosis activity. The GM-fed mice had an increasing proportion of $CD3^+$ T lymphocytes in the spleen. Splenocytes isolated from these animals also showed significantly increased production of cytokines IFN-γ and IL-10. More importantly, GM feeding during pregnancy and lactation periods can confer protective activity onto offspring by alleviating the airway inflammation of allergic asthma induced by mite allergens. There was a remarkably different composition of gut microbiota between offspring of pregnant mice fed with water or with milk (GM or CM). There was a greater proportion of beneficial bacterial species, such as *Akkermansia muciniphila*, *Bacteroides eggerthii*, and *Parabacteroides goldsteinii* in the gut microbiota of offspring from GM- or CM-fed pregnant mice compared to the offspring of water-fed pregnant mice. These results suggested that improving the nutrition of pregnant mice can promote immunological maturation and colonization of gut microbiota in offspring. This mother-to-child biological action may provide a protective effect on atopy development and alleviate allergen-induced airway inflammation in offspring.

Keywords: goat milk, immune response, pregnancy, allergic asthma, microbiota

INTRODUCTION

An increasing prevalence of allergic diseases, such as atopic dermatitis, allergic rhinitis, and asthma, as well as food allergies, has been noted in western societies (1, 2). Increasing incidences have also been reported in newly developed Asian countries, such as Taiwan (3, 4). These diseases now affect ∼20% of the population worldwide (5, 6); yet the prevalence has increased too rapidly in recent decades to be explained by genetic changes alone (1, 5). This increasing incidence of allergic disease alongside a decreasing incidence of microbial infections in western countries has led to the "hygiene hypothesis" (7). This has been updated to encompass the commensal microbiota in early life (8, 9), which is affected by multiple environmental factors, including the mode of delivery during childbirth (10), breast vs. formula feeding (11), a "Western diet" low in fiber and high in fat content (12), and misuse of antibiotics (13).

Several studies show that children who developed allergies later in life have decreased intestinal microbial diversity, particularly lower levels of *Bifidobacillus* and *Lactobacillus* species in infancy (14). In addition, the pro-inflammatory metabolites produced by dysbiotic microbiota in the neonatal period have been associated with an increasing atopy risk and T-cell differentiation (15). Although breast milk contains numerous allergy-protective bioactive components, such as milk oligosaccharides, polyunsaturated fatty acids, a variety of cytokines of TGF-β and IL-10, and even microbiota (16), there is conflicting evidence on the protective role of breastfeeding in relation to the development of allergic sensitization and allergic diseases (17). A study conducted by Munblit et al. showed that modulation of human breast milk composition may have the potential to prevent allergic disorders in children (18). Human milk composition varies among individuals, which may explain the heterogeneity of these reports. Although, there is evidence that exclusive breastfeeding for 3–4 months reduces the incidence of eczema and is protective against wheezing in the first 2 years of life, there are no short- or long-term advantages for exclusive breastfeeding beyond 3–4 months that have been demonstrated for preventing atopic disease (19).

Previous studies have suggested that goat milk (GM) is easier for humans to digest than cow milk (CM) because its curds are softer (20, 21). The softer curds of GM may be an advantage for adults suffering from gastrointestinal disturbances and ulcers (21). GM contains higher levels of calcium, magnesium, and phosphorous than those of CM and human milk. The higher levels of medium chain triglycerides (MCT) in GM have been recognized as having unique health benefits for infant nutrition (20, 21). Previous studies have demonstrated antioxidant and anti-inflammatory effects of GM (22). For example, Jirillo et al. have shown that GM modulates human peripheral blood mononuclear cells (PBMCs) and polymorphonuclear neutrophils (PMNs) to produce NO, IL-6, IL-10, and TNF-α (22). It is notable that GM is less immunogenic than CM in a murine model of atopy, where the production of IL-4 was lower and IFN-γ was higher from Concanavalin A (ConA)-stimulated splenocytes of GM-fed mice as compared to those of CM-fed mice (23). However, GM is not recommended in CM allergic patients due to the clinically significant cross-allergenicity between CM and GM (24).

Human breast milk contains more than 80 milk oligosaccharides (HMOs). Because of its prebiotic and anti-infective properties, it has been widely recognized as the major source for early life colonization of gut microbiota in infants (25). Recent studies have shown that GM contains the highest level of oligosaccharides among all domestic animals and has significant similarities to human milk oligosaccharides from a structural point of view (26). Though it is clear that a mother's diet influences the health of her fetus in many ways, there is a lack of concrete evidence to link the role of maternal nutrition to the development of allergic diseases in her infants (17, 19). Whether GM consumption by pregnant mothers has atopy protective effects on their newborns is still unclear. This study first evaluates the immune modulation of GM consumption by maternal mice, then it uses pregnant mice and their offspring to verify this hypothesis.

MATERIALS AND METHODS

Animals and Diets

Adult, specific pathogen-free, female BALB/c mice (5–6 weeks old), were purchased from the National Laboratory Animal Breeding and Research Center (Tainan, Taiwan). They were housed in plastic cages with an air filter device and maintained on a standard mouse diet (Lab diet; PMI Feeds, St. Louis, MO, USA) in the Laboratory Animal Center of the College of Medicine, National Cheng Kung University. The composition of the standard diet, which consisted of dry pellets (88%), crude protein (18%), crude fat (3.1%), ash (6.2%), fiber (22%), and carbohydrates (35%). All mice were given *ad libitum* access to deionized water. The GM formula, Mama formulated goat milk (Karihome®), was obtained from Orient EuroPharma Ltd., (Taipei, Taiwan) and manufactured by Dairy Goat Co-operative (NZ) Ltd. (Hamilton, New Zealand). The CM formula was KLIM, powdered milk sold by Nestlé, Switzerland. The GM formula had goat milk protein as the sole protein source, and the CM formula contained cow milk and whey proteins (frequently referred to "whey-enhanced" or "adapted"). In details, the GM formula contained pasteurized goat milk solids (43%), lactose, vegetable oils, minerals, vitamins, acidity regulator (citric acid), choline chloride, L-tryptophan, L-isoleucine, taurine, and L-carnitine. The whey-to-casein ratio was ∼20:80, and 60% of the fat was goat milk fat. The CM formula contained cow milk solids (demineralized whey, lactose, skim milk solids, whey solids, and whey protein concentrate), vegetable oils, soy lecithin, minerals, vitamins, acidity regulator (citric acid and/or calcium hydroxide), choline chloride, L-tryptophan, taurine, and L-tyrosine. The whey to casein ratio was ∼60:40, and cow fat was not included.

Experiment and Study Designs

All animal experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee (IACUC No. 105196 and No. 106244). Groups of 12 mice were first used at 6–8 weeks of age. Milk was administered daily to groups of mice by intra-gastric gavage in 200 μL volume. The

daily milk intake dose for the mice was calculated from the recommended adult human dose of 25 g/200 mL/60 kg (WHO Dietary recommendations/Nutritional requirements) to 8.5 mg for a 20 g mouse. To evaluate the effect of milk consumption on general immune function, mice were fed with either sterile water (W), GM (low dose 1.6 mg, L; medium dose 8.5 mg, M; and high dose 16.6 mg, H), or CM (8.5 mg; C) for 4 weeks before euthanasia. Mice of control group (N) were fed with normal diet without specific treatment.

To assess the effect of milk consumption on antigen-specific immunological response, groups of mice were fed as described above and were sensitized with an intra-peritoneal (i.p.) injection 50 μg ovalbumin (OVA), 2 μL Complete Freunds Adjuvant (CFA) in 200 μL phosphate-buffered saline (PBS) on day 0, and i.p. [50 μg OVA, 6 μL Incomplete Freunds Adjuvant (IFA) in 200 μL PBS] on day 7. They were then euthanized after 3 weeks. OVA-treated mice were fed with either sterile water (WO), GM (low dose 1.6 mg, LO; medium dose 8.5 mg, MO; and high dose 16.6 mg, HO), or CM (8.5 mg; CO) for 4 weeks before euthanasia. Mice of the control group (N) were fed with normal diet without specific treatment.

To evaluate the effects of milk consumption by pregnant mice on their offspring, the grouping and mating design was depicted in **Figure 1**. Female mice were intra-gastrically fed (200 μL) with sterile water (group W), GM (8.5 mg, group G), or CM (8.5 mg, group C) (3 mice/group) after they had been paired with male mice. The total feeding period of female mice began from pairing and continued through pregnancy to the end of a 4-week suckling period. At weaning, the offspring mice were randomly divided into two groups—the control group (WN, GN, and CN) and HDM-stimulating group (WA, GA, and CA)—with 10 mice each. To establish the respiratory allergy model in offspring, they were sensitized with HDM allergen Der p (*Dermatophagoides pteronyssinus*; Allergon, Engelholm, Sweden) on days 0 and 7 by i.p. 200 μL aluminum hydroxide ($Al(OH)_3$) [50 μg Der p/mL $Al(OH)_3$]. Then, mice were intra-nasally (i.n.) delivered by Der p (50 μg/20 μL PBS) daily (5 days). On day 14, mice were challenged with Der p (50 μg/20 μL PBS) by an intra-tracheal (i.t.) route and were sacrificed 2 days later (**Figure 1**). Control mice were sensitized with PBS (i.p. and i.n.) and were challenged with PBS (i.t.). On the weaning day, offspring mice were marked W0, C0, and G0 individually.

Mouse Antibody and Antigen-Specific Antibody Measurements

IgG1, IgG2a, and IgE ELISA kits were purchased from Bethyl Laboratories (Montgomery, TX, USA) and were used according to the manufacturer's recommended protocol. Antigen (OVA)-specific IgA, IgM, IgG, and IgG subclass antibody titers were measured by using an indirect competitive enzyme-linked immunosorbent assay (ELISA) protocol based on previously described methods (27).

Measurement of Total and Der p-Specific IgE in the Serum

Blood was collected from the cheek facial vein of individual offspring on days 0 and 16. The collected samples were left to stand and clot for 1 h at RT, and they were then centrifuged at 10,000 × g for 30 min to obtain the serum. Serum levels of total and Der p-specific IgE were measured by using an ELISA kit (Mouse IgE ELISA Quantitation Set, E90-115, Bethyl Laboratories, Inc., Montgomery, TX, USA) (28).

Splenocyte Culture and Cytokine Measurement

A cellular suspension was produced by mincing individual spleens between two sterile glass slides. The red blood cells were lysed with ACK Lysing Solution (Catalog number: A1049201, Thermal Fisher Scientific Inc., Waltham, MA, USA), and the splenocytes were extensively washed and re-suspended in RPMI 1640 containing 10% fetal calf serum, 0.1% penicillin, 0.1% streptomycin, and 0.1% glutamine. Cells (5×10^6 cells/mL) were cultured in 24-well plates at 37°C in 5% CO_2 and were stimulated with phytohaemagglutinin (1 μg/mL, PHA), ConA (1 μg/mL), or lipopolysaccharide (2 μg/mL, LPS). OVA (10 μg/mL) was used for positive controls and unstimulated cells for background controls. Supernatants were harvested at 48 h and were assayed for the level of IFN-γ, TARC, IL-10, IL-12, and TNF-α concentrations by R&D Systems ELISA (Minneapolis, MN, USA), according to the manufacturer's recommendations. Detection limits were 15 pg/mL for the assays of the abovementioned cytokines.

Passive Cutaneous Anaphylaxis (PCA)

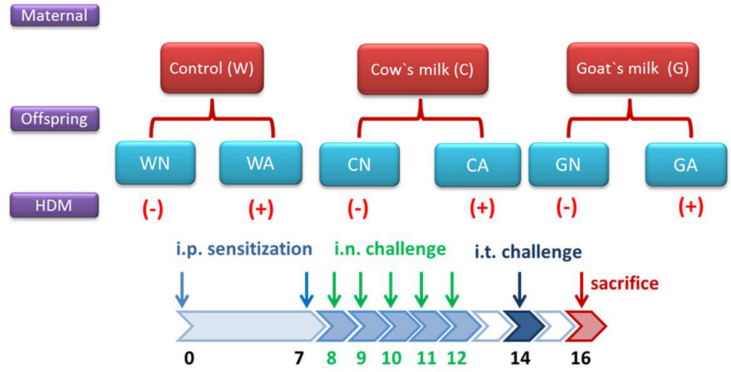
Specific IgE antibody responses to whey proteins were assessed in triplicate by a PCA test in experimental mice. First, 0.1 ml of twofold dilutions of pooled mouse serum samples was intradermally injected into ears of recipient mice. All mice were challenged 48 h later by an intravenous injection of 1 ml of 0.9% saline solution containing 5 mg Evans Blue and 2 mg α-lactalbumin or BSA. The reaction was read 30 min after the challenge. The PCA titer was defined as the highest serum dilution when yielding a positive reaction of at least 5 mm in diameter and expressed as means ± SEM (29).

Airway Hyperresponsiveness Measurement

To measure mechanical properties of mice airways, mice were injected (i.p.) with 100 mg/kg of pentobarbiturate, and tracheotomies were performed on day 16 (48 h after Der p i.t. challenge). Lung function was determined by using the Scireq Flexivent apparatus (SCIREQ, Scientific Respiratory Equipment Inc., Montreal, Canada). Mice were treated with increasing doses of acetyl-β-methylcholine chloride (0–5 mg/mL) (A2251, Sigma-Aldrich, St. Louis, MO, USA). Methylcholine was aerosolized for ventilation by using an ultrasonic nebulizer for 3 min separately. Respiratory system resistance (Rrs) and elastance (Ers) were calculated by using flexiVent software and fitting the equation with airway resistance (Rn), tissue elasticity (H), and tissue damping (resistance) (G). The data from each treatment group was used to calculate the average response.

Broncho-Alveolar Lavage Fluid (BALF) and Lung Tissue Examination

The BALF was collected after two times of instillation and aspiration with 1 mL of cold saline into the trachea. BALF



BALB/c 5 weeks old	i.p.	i.n.	i.t.
Controls (N)	PBS	PBS	PBS
HDM Der p (A)	50 μg Der p / 1 mg Alum / 200 μl	50 μg Der p / 20 μl	50 μg Der p / 50 μl

FIGURE 1 | Scheme of study protocol. The classifications of offspring were based on pregnant mother mice fed with water (W), goat milk (G), or cow milk (C) on weaning period (D0) till 2 days after allergen or PBS sensitization and challenge (D16). The offspring mice were divided into two groups: control groups from pregnant mother mice fed with water (WN), goat milk (GN), and cow milk (CN); and HDM-sensitized and challenged groups from pregnant mother mice fed with water (WA), goat milk (GA), and cow milk (CA).

was centrifuged at 300 × g for 5 min at 4°C to separate cells and supernatants. The total number of cells in two collections was counted with hemocytometer. Differential cell counts of BALF were performed by cytospin. Cells were stained with Liu's stain solution for microscopic examination, and 200 cells were enumerated. Supernatants were stored at −70°C until assay. To examine the bronchial epithelium inflammation in the lung tissue, lobes were fixed by endotracheal perfusion of alcohol-formalin. After perfusion, the trachea was closed with a suture, and the cardiopulmonary tree was then removed and placed in a 10% neutral buffer formalin (pH 7.4) overnight. Lobes were separated and placed in a cassette for automated paraffin embedding. The paraffin blocks were sectioned into 4–5 μm thickness. Sections were stained with hematoxylin and eosin. Photographs were obtained by a Microscope DP70 (Olympus, Shinjuku, Tokyo, Japan) and DP manager system.

Analysis of Gut Microbiome Composition by Axiom Microbiome Array

Stool samples were obtained from groups of offspring after the weaning period and HDM allergen sensitization (day 0), and offspring were sacrificed after allergen intra-tracheal challenge for 2 days (Day 16). Stool samples were frozen then stored at −80°C. A QIAamp DNA Stool Mini Kit was used to purify DNA from frozen stool samples according to protocol. DNA quality was evaluated using MaestroNano spectrophotometry (Maestrogen, Las Vegas, NV, USA) in absorbance ratio A260 nm/A280 nm. The Affymetrix GeneTitan® platform was used to identify the diversity of the microbiome with a Thermo Axiom™ Microbiome array, which can detect more than 12,000 species of viruses, bacteria, fungi, protozoa, and archaea (30). Initially, the 200 ng target probes were prepared to detect each DNA sample, which contained at least 20 μL of good-quality DNA (10 ng/μL). These samples were then amplified, fragmented, and hybridized on a chip followed by a single-base extension through DNA ligation and signal amplification. The array was scanned automatically on a GeneTitan Multi-Channel instrument according to manufacturer's instructions (Thermo Fisher, Waltham, MA, USA).

Microarray Data Analysis

Microarray data were analyzed using MiDAS software (Axiom Microbial Detection Analysis Software), which is based on the Composite Likelihood Maximization Method (CLiMax) algorithm developed at Lawrence Livermore National Laboratory LLNL (31). Probes with signal intensity above the 99th percentile of random control probe intensities and with more than 20% of target-specific probes detected were considered as positives. The microbiome diversity and difference between different samples were calculated by R language. The principal component assay (PCA) was performed by using Python language.

Statistical Analyses

All analyses were conducted in triplicate. Statistical analysis was performed using GraphPad Prism version 5.0a (GraphPad Software, Inc., La Jolla, CA, USA). Data were analyzed using the Student's *t*-test, Kruskal-Wallis one-way ANOVA, and the Dunn's *post hoc* test. If ANOVA assumptions were violated, the Wilcoxon matched-pairs test would be used. Results are expressed as mean ± SEM. Statistical significance was established at the level of $p < 0.05$.

RESULTS

Goat Milk Intake Modulates Immunological Function of Mice

The effects of GM and CM on nutritional immunity were evaluated in mice fed intragastrically with different types of milk for 4 weeks. Control mice were fed with water only. The body weight of the mice increased steadily over the treatment period (weighed once a week) with no difference among groups fed with water, GM (three dosages; L, M, and H), or CM (C) (data not shown). At the end of the treatment period, there was no difference in spleen weight among the six groups (data not shown). Thus, the daily gavage of mice with GM or CM for 4 weeks did not affect weight gain or spleen size.

In contrast, a significant increase in sera immunoglobulin concentration was observed in mice fed with GM or CM. IgA, IgM, and IgG (total) concentrations were significantly higher in cow milk- and goat milk-fed mice compared to control mice (N) ($p < 0.05$) (**Table 1A**). There was a trend of increased IgG2a levels in mice fed with GM, but this was not statistically significant (**Table 1A**). Splenocyte proliferation in response to mitogens PHA, Con A, and LPS was without difference between milk-fed groups and control group (**Table 1B**). Nevertheless, supernatants harvested from 24 h culture of splenocytes in GM treatment groups had increasing concentrations of cytokines (**Table 1C**). Compared to water and CM groups, GM groups (M and H) had a higher level of IFN-γ after LPS stimulation, a higher level of IL-12 after Con A stimulation, and a higher level of TNF-α after PHA or LPS stimulation, particularly with LPS stimulation (**Table 1C**). A flow cytometry analysis of spleen cells demonstrated that 4 weeks' milk treatment had limited effect on the proportion of helper T cells ($CD3^+CD4^+$), cytotoxic T cells ($CD3^+CD8^+$), and B cells ($CD3^-CD45R^+$). Although there appeared to be a trend of an increase in B cells, it was not significant (**Table 1D**). NK-cell activity of splenocytes was increased in mice fed with a low dosage of GM compared to control groups (**Table 1E**). Phagocytic activity was enhanced in mice fed with GM (all dosages) as compared to control and water-fed groups (**Table 1F**).

Goat Milk Intake Increases Antigen-Specific Immunological Response of Mice

The effects of milk consumption on antigen-specific immunological responses were evaluated by extending the above model with OVA immunization protocol. Mice were immunized on Day 14, boosted on Day 21, and sacrificed on Day 28. As described above, there were no significant differences in body weights or spleen size among different treatment groups. A daily milk gavage did not affect food intake compared to the control groups. The immunization protocol induced an antibody response, with the concentrations of total IgM and IgG being increased in sera from all treatment groups compared to non-immunized group (**Table 2A**). OVA-specific IgA, IgM, IgG, and IgG subclass antibodies also significantly increased in immunized groups, and there were higher levels of OVA-specific IgA and IgG in mice treated with GM compared to non-milk-fed immunized mice. OVA-specific IgA levels were the highest when feeding with medium dosage of GM (**Table 2B**). After immunization with an OVA antigen, the proliferation activity of splenocytes increased when cultured with PHA, OVA, and LPS in all immunized groups of mice; neither the milk and non-milk-fed groups nor the GM- and CM-fed groups displayed a significant difference in proliferation activity. The LO group, however, showed significantly decreased cell proliferation at 24 h as compared to the non-milk-fed OVA immunized group (O) (**Table 2C**).

Splenocytes isolated from OVA-immunized mice produced higher levels of IFN-γ and IL-10 after culturing with PHA and OVA antigen than cells from non-immunized mice. When cells were stimulated with LPS, IL-10 production had no difference between these two groups (**Table 2D**). Levels of IFN-γ had no significant difference between CM-fed and GM-fed OVA-immunized mice when splenocytes were cultured with OVA and LPS. But there was higher IFN-γ production in high-dose GM-fed mice compared to CM-fed mice as cells cultured with PHA. Splenocytes of mice fed with milk (CM and high-dosage GM) secreted higher levels of IL-10 than those of control mice after stimulating with OVA (**Table 2D**). After immunization with the OVA antigen, mice fed with GM produced a significantly higher amount of total T cells ($CD3^+$) in their spleens, as compared to non-milk- and CM-fed mice (**Table 2E**). The percentage of other T-cell subpopulations, such as helper T cells($CD3^+CD4^+$) and cytotoxic T cells ($CD3^+CD8^+$), and B cells ($CD3^-CD45R^+$) in the spleens were not significantly different among the six groups (**Table 2E**). To assay the recalled antigen immune response, a delayed hypersensitivity reaction for the swelling of mouse ear skin folds was used as described in method section. **Supplementary Figure 1A** showed that the swelling of the ear skin decreased significantly in GM- and CM-fed mice compared to non-milk-fed mice. A histological examination also showed a significant decrease in epidermis and dermis thicknesses in GM- and CM-fed mice compared to non-milk-fed mice (**Supplementary Figure 1B**).

Goat Milk Feeding in Pregnant Mice Confers Protection of HDM-Induced Allergic Airway Inflammation in Offspring

To explore the protective effect of GM- or CM-fed pregnant mice on allergen-induced airway inflammation in their offspring, we administrated the maternal group with water, CM, or GM daily from mating until offspring were weaned at 4 weeks of age. Offspring mice were divided into six groups (female and male, $n = 6$ in each group) according to the maternal mice feeding models. Sensitized (i.p.), intra-nasal (i.n.), and intra-tracheal (i.t.) challenges with HDM (Der p) or with PBS were carried out on the offspring (**Figure 1**). There was no difference in body weight among the groups of offspring throughout the study (data not shown). The HDM-treated groups (WA, CA, and GA) with exposure to methylcholine induced significantly increasing airway resistance at day 14.

TABLE 1 | Immunological functions of mice fed with water, cow milk, and goat milk.

	Naïve (N)	Water (W)	Cow milk (C)	Goat milk low dose (L)	Goat milk medium dose (M)	Goat milk high dose (H)
(A) IMMUNOGLOBULINS						
IgA (μg/mL)						
Mean ± SEM	128.8 ± 8.6	156.0 ± 11.9	**176.3 ± 19.8**^{*a}	**207.8 ± 15.5**^{**a}	**210.7 ± 23.9**^{**a}	**211.4 ± 10.5**^{**a}
IgM (μg/mL)						
Mean ± SEM	145.9 ± 14.5	125.1 ± 27.6	**271.7 ± 22.2**^{**a}	**244.8 ± 24.6**^{**a}	**309.2 ± 54.6**^{**a}	**28.3 ± 34.0**^{**a}
Total IgG (μg/mL)						
Mean ± SEM	27.9 ± 2.9	30.3 ± 4.1	**34.7 ± 4.14**^{*a}	**38.8 ± 4.1**^{*a}	**48.9 ± 7.3**^{*a}	**56.4 ± 11.9**^{*a}
IgG1 (μg/mL)						
Mean ± SEM	199.8 ± 26.5	167.6 ± 29.1	199.1 ± 28.8	181.9 ± 29.3	165.2 ± 26.1	249.1 ± 27.8
IgG2a (μg/mL)						
Mean ± SEM	9.1 ± 1.7	11.3 ± 3.1	15.5 ± 4.7	24.3 ± 10.6	34.4 ± 19.1	30.3 ± 10.1
(B) SPLEEN CELL PROLIFERATION						
At 24 h (ratio)						
PHA/Medium	1.17 ± 0.08	1.14 ± 0.07	1.21 ± 0.07	1.20 ± 0.09	1.22 ± 0.11	1.20 ± 0.07
Con A/Medium	1.68 ± 0.27	1.32 ± 0.10	1.62 ± 0.31	1.55 ± 0.17	1.61 ± 0.21	1.68 ± 0.22
LPS/Medium	1.09 ± 0.06	1.10 ± 0.02	1.15 ± 0.05	1.12 ± 0.03	1.15 ± 0.05	1.12 ± 0.03
At 48 h (ratio)						
PHA/Medium	1.31 ± 0.13	1.27 ± 0.09	1.27 ± 0.07	1.36 ± 0.11	1.40 ± 0.13	1.41 ± 0.10
Con A/Medium	2.33 ± 0.46	2.10 ± 0.31	2.33 ± 0.32	2.92 ± 0.47	2.93 ± 0.51	3.05 ± 0.45
LPS/Medium	1.17 ± 0.08	1.22 ± 0.06	1.23 ± 0.07	1.19 ± 0.03	1.25 ± 0.04	1.24 ± 0.05
(C) CYTOKINE PRODUCTION						
IFN-γ (pg/mL)						
PHA	117.1 ± 39.6	91.0 ± 22.8	247.2 ± 67.5	227.2 ± 77.4	424.4 ± 157.6	316.0 ± 103.9
Con A	1504 ± 323.8	2121 ± 298.7	1695 ± 284.1	1891 ± 314.0	2192 ± 590.1	1900 ± 331.8
LPS	23.6 ± 5.0	26.9 ± 3.8	25.5 ± 3.5	49.7 ± 14.8	**64.8 ± 20.3**^{*a,*b}	**76.7 ± 19.0**^{*a,*b}
IL-12 (pg/mL)						
PHA	2.88 ± 0.55	2.08 ± 0.67	1.81 ± 0.11	1.85 ± 0.12	1.80 ± 0.19	1.85 ± 0.31
Con A	18.85 ± 6.80	25.68 ± 4.66	27.35 ± 2.40	31.81 ± 5.88	**32.33 ± 6.56**^{*a}	**31.50 ± 5.93**^{*a}
LPS	2.32 ± 0.37	2.31 ± 0.50	2.04 ± 0.16	1.77 ± 0.14	1.80 ± 0.17	1.55 ± 0.12
TNF-α (pg/mL)						
PHA	6.93 ± 1.85	11.27 ± 2.69	10.17 ± 2.67	20.67 ± 8.74	**22.76 ± 5.98**^{*a}	**21.59 ± 6.26**^{*a}
ConA	172.8 ± 22.47	216.7 ± 16.26	201.7 ± 12.54	224.1 ± 21.37	208.3 ± 22.91	205.1 ± 12.56
LPS	55.17 ± 3.53	6.35 ± 3.90	59.00 ± 4.80	**73.48 ± 6.33**^{*a}	**79.04 ± 6.72**^{**a,*b}	**78.97 ± 4.87**^{**a,**b}
(D) FLOW CYTOMETRY						
$CD3^+/CD4^+$	20.40 ± 1.11	21.76 ± 1.74	25.31 ± 2.33	22.80 ± 2.27	19.72 ± 0.45	21.65 ± 2.96
$CD3^+/CD8^+$	10.64 ± 1.46	8.76 ± 1.33	9.95 ± 1.49	9.72 ± 1.11	8.59 ± 1.23	7.90 ± 1.14
$CD3^-/CD45R^+$	30.73 ± 4.88	33.94 ± 3.99	33.91 ± 5.46	36.57 ± 4.18	38.66 ± 3.76	40.75 ± 3.00
(E) NK CELL ACTIVITY (%)						
	36.3 ± 7.3	42.2 ± 8.3	42.2 ± 7.7	**48.9 ± 8.2**^{*a}	43.8 ± 7.7	39.4 ± 5.8
(F) PHAGOCYTOSIS (%)						
	54.8 ± 2.3	56.8 ± 1.8	59.2 ± 2.1	**70.7 ± 6.1**^{*a}	**69.64 ± 7.2**^{*a}	**71.0 ± 14.1**^{*a}

$^{*}p < 0.05$; $^{**}p < 0.01$.

[a]as compared to control group.

[b]as compared to cow milk-fed group.

Bold values as statistically significance.

However, airway resistance was less severe in GA and CA groups (GA: Rrs, 2.358 cm H_2O/mL and Ers, 62.26 cm H_2O/mL, CA: Rrs, 2.527 cm H_2O/mL and Ers, 85.45 cm H_2O/mL) throughout pregnancy and lactation. The decrease in resistance was significant at the concentrations of 2.5 and 5 mg/ml methylcholine inhalation as compared to that of WA group (Rrs, 4.213 cm H_2O/mL and Ers, 137.4 cm H_2O/mL, $p < 0.05$) (**Figure 2A**). In a lung histological examination, non-HDM-sensitized mice (WN, CN, and GN) had minimal inflammatory cell infiltration and lower mucosal thickness (arrow) in the

TABLE 2 | Antigen-specific immune responses in water, cow milk, and goat milk fed mice.

	Naïve (N)	OVA (O)	OVA with cow milk (CO)	OVA with low dose goat milk (LO)	OVA with medium dose goat milk (MO)	OVA with high dose goat milk (HO)
(A) TOTAL IMMUNOGLOBULINS						
IgA (μg/mL)						
Mean ± SEM	189.2 ± 19.1	236.1 ± 42.4	169.7 ± 14.1	195.6 ± 24.0	205.2 ± 20.9	204.7 ± 15.3
IgM (μg/mL)						
Mean ± SEM	169.6 ± 23.6	**455.6 ± 60.7**^{*a}	**708.4 ± 51.3**^{*a,*b}	**770.7 ± 69.6**^{**a,*b}	**798.4 ± 132**^{**a,*b}	**768.5 ± 61**^{**a,*b}
IgG (μg/mL)						
Mean ± SEM	34.3 ± 10.6	63.4 ± 18.1	99.5 ± 13.8	**188.6 ± 35.3**^{**a,*c}	**203.0 ± 40.5**^{**a,*c}	**166.7 ± 15.2**^{**a,*c}
(B) OVA-SPECIFIC IMMUNOGLOBULINS (O.D. 450 nm)						
Spe IgA						
Mean ± SEM	0.02 ± 0.01	0.23 ± 0.06	0.28 ± 0.04	0.28 ± 0.04	**0.38 ± 0.05**^{*a}	0.26 ± 0.03
Spe IgM						
Mean ± SEM	0.04 ± 0.01	**0.91 ± 0.19**^{*a}	**1.02 ± 0.10**^{**a}	**1.20 ± 0.14**^{**a}	**1.17 ± 0.22**^{**a}	**0.99 ± 0.14**^{*a}
Spe IgG						
Mean ± SEM	0.01 ± 0.01	**1.76 ± 0.23**^{*a}	**2.30 ± 0.06**^{*a}	**2.46 ± 0.07**^{**a}	**2.46 ± 0.15**^{*a}	**2.32 ± 0.12**^{*a}
Spe IgG1						
Mean ± SEM	0.01 ± 0.00	**2.18 ± 0.23**^{*a}	**2.71 ± 0.1**^{**a}	**2.60 ± 0.08**^{*a}	**2.66 ± 0.15**^{*a}	**2.75 ± 0.12**^{**a}
Spe IgG2a						
Mean ± SEM	0.01 ± 0.00	0.36 ± 0.10	**0.92 ± 0.29**^{**a,*b}	**0.59 ± 0.17**^{*a}	**0.51 ± 0.11**^{*a}	**0.51 ± 0.09**^{*a}
(C) SPLEEN CELL PROLIFERATION						
At 24 h						
PHA/Medium	1.07 ± 0.04	1.38 ± 0.10	1.22 ± 0.06	1.28 ± 0.09	1.28 ± 0.05	1.27 ± 0.05
OVA/Medium	1.02 ± 0.01	1.21 ± 0.06	1.11 ± 0.04	**1.08 ± 0.02**^{*a}	1.10 ± 0.03	1.13 ± 0.02
LPS/Medium	1.04 ± 0.02	1.27 ± 0.09	1.15 ± 0.07	1.14 ± 0.06	1.17 ± 0.07	1.13 ± 0.04
At 48 h						
PHA/Medium	1.20 ± 0.08	2.46 ± 0.48	2.13 ± 0.36	1.815 ± 0.31	1.98 ± 0.16	1.83 ± 0.15
OVA/Medium	0.95 ± 0.01	1.30 ± 0.12	1.21 ± 0.08	1.20 ± 0.08	1.17 ± 0.05	1.23 ± 0.05
LPS/Medium	1.15 ± 0.09	1.46 ± 0.21	1.30 ± 0.15	1.18 ± 0.10	1.21 ± 0.12	1.17 ± 0.04
(D) CYTOKINE PRODUCTION						
IFN-γ(pg/mL) 48 h						
PHA	543.7 ± 156.4	**905.1 ± 219.5**^{*a}	**838.1 ± 179.3**^{*a}	**1214 ± 266.5**^{*a}	**1463 ± 255.5**^{*a}	**1452 ± 213.2**^{*a*c}
OVA	4.44 ± 0.41	**25.57 ± 3.41**^{*a}	**37.52 ± 5.67**^{*a}	**78.35 ± 19.57**^{*a}	**46.34 ± 7.48**^{*a}	**48.47 ± 11.88**^{*a}
LPS	3.81 ± 0.09	15.04 ± 5.93	**95.40 ± 31.14**^{*a}	**90.87 ± 30.69**^{*a}	**223.9 ± 85.95**^{*a}	**338.7 ± 143.1**^{*a}
IL-10 (pg/mL) 48 h						
PHA	6.68 ± 2.13	17.82 ± 4.16	19.53 ± 3.40	19.13 ± 6.26	23.72 ± 4.11	22.15 ± 2.55
OVA	33.6 ± 8.5	172.3 ± 20.6	**239.3 ± 21.1**^{*a}	167.1 ± 45.6	204.2 ± 25.5	**305.9 ± 59.5**^{*a}
LPS	3.80 ± 0.13	3.81 ± 0.12	3.99 ± 0.14	3.60 ± 0.13	3.88 ± 0.15	3.80 ± 0.13
(E) FLOW CYTOMETRY						
$CD3^+$(MFI)	39.62 ± 1.89	36.96 ± 1.42	34.81 ± 1.47	37.70 ± 1.39	**40.29 ± 1.64**^{*b}	**39.81 ± 1.22**^{*b}
$CD3^+/CD4^+$	17.82 ± 1.34	13.37 ± 1.25	14.71 ± 1.24	15.42 ± 1.77	16.70 ± 1.85	16.22 ± 1.62
$CD3^+/CD8^+$	14.12 ± 1.20	12.43 ± 1.08	12.19 ± 0.77	12.42 ± 0.57	13.16 ± 0.55	12.94 ± 0.88
$CD3^-/CD45R^+$	32.45 ± 2.99	28.64 ± 2.22	29.50 ± 1.34	28.89 ± 1.95	29.65 ± 2.33	29.68 ± 1.68

*$p < 0.05$; **$p < 0.01$.
[a]*as compared to control group (N).*
[b]*as compared to OVA-immunized group (O).*
[c]*as compared to cow milk-fed (CO) group.*
Bold values as statistically significance.

bronchial epithelium than those of HDM-sensitized mice. After being challenged with HDM, GA and CA groups showed significantly decreased inflammatory cell infiltration (12 ± 5 and 7 ± 3 cell/HPF, respectively) and mucosa thickness as compared to those of WA group (35 ± 7 cells/HPF) (**Figure 2B**).

Further analysis of BALF from HDM-sensitized mice showed that there were increasing numbers of eosinophils,

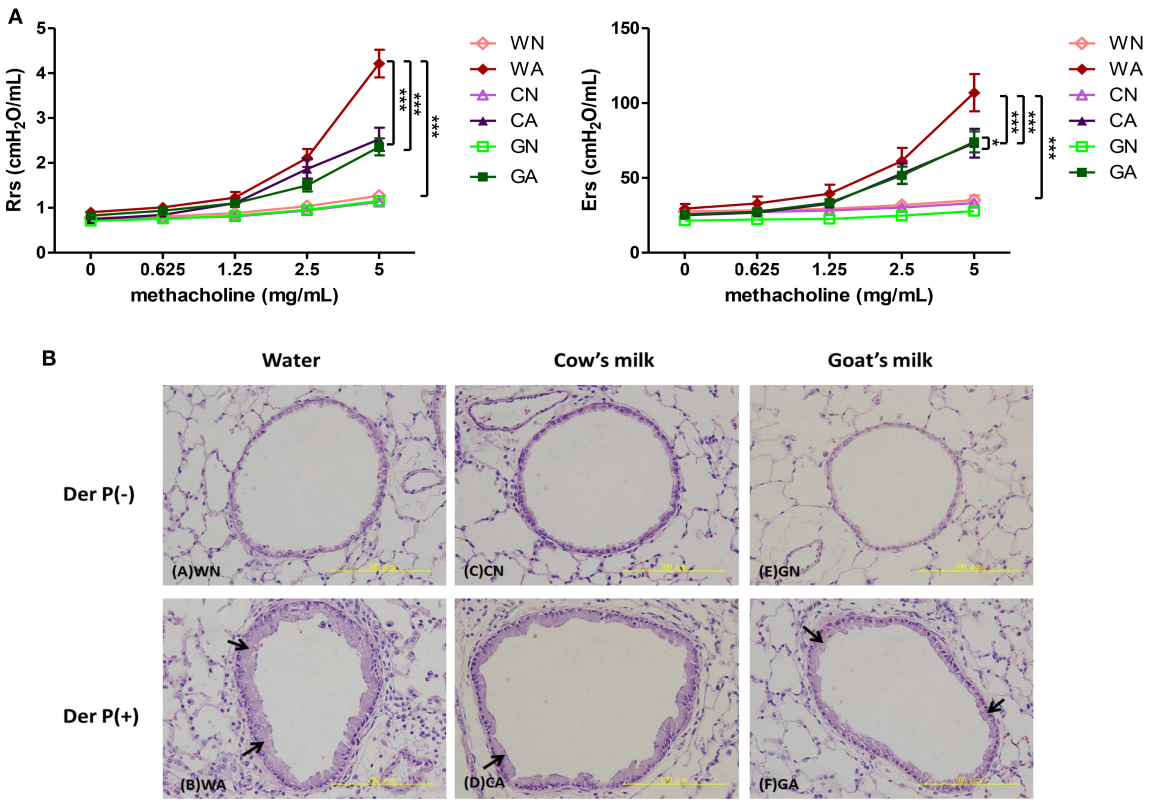


FIGURE 2 | The effects of goat milk feeding in pregnant mother mice on offspring. **(A)** Measurement of airway resistance. **(B)** H&E stains of lung tissues. Each group had 10 mice, and each assay was repeated three times. *P*-value of different groups were compared with those of N groups by Student's *t*-test (***$p < 0.001$). Pregnant mother mice were fed with sterile water (W), GM (G), or CM (C), and offspring were divided into two groups: control groups from pregnant mother mice fed with water (WN), goat milk (GN), and cow milk (CN); and HDM-sensitized and challenged group from pregnant mother mice fed with water (WA), goat milk (GA), and cow milk (CA).

monocytes, and lymphocytes. This confirmed the inflammatory cell infiltration into the lungs. However, BALF from the GA group had lower total cell infiltration levels and fewer numbers of eosinophils compared to those of WA and CA groups (**Figure 3A**). In mice primed with respiratory allergen (HDM), there were significantly higher levels of total IgE and HDM-specific IgE antibodies than those of non-sensitized mice (**Figure 3B**). However, the GA group had significantly lower levels of total IgE compared to WA group ($p < 0.05$). There was a trend of lower levels of Der p-specific IgE antibodies in the GA and CA groups (**Figure 3B**). Assays of cytokine production in BALF showed lower levels of TARC in the GA group compared to the WA and CA groups (**Figure 4A**). The levels of TNF-α in BALF were more reduced in HDM-sensitized mice compared to non-HDM-sensitized mice (**Figure 4B**). There was no significant difference in TNF-α among HDM sensitized and challenged mice. Splenocytes collected from GA group produced the highest levels of IFN-γ following PHA stimulation among the six groups (**Figure 4C**). Furthermore, splenocytes from GA mice produced significantly higher levels of IL-10 after PHA stimulation as compared to cells from the WA and CA groups of mice ($p < 0.05$; **Figure 4D**).

Goat Milk Feeding Induces Gut Microbiota Change in HDM-Sensitized and Challenged Offspring

To analyze gut microbiota among groups of weaned offspring and the effect of gut microbiota on allergen-induced airway inflammation, we collected the stools of the offspring before allergen sensitization (day 0) and 2 days after i.t. allergen challenge (day 16). The detection of the cDNA of stools using Applied Biosystems™ Axiom™ Microbiome Array found the class of *Bacteroidia, Clostridia, Flavobacteriia, Bacilli, Deferribacteres, Verrucomicrobiae*, and *Gammaproteobacteria* as well as some unclassified viruses (**Table 3**). Comparing the ratio of phyla *Firmicutes* to *Bacteroidetes* (F/B ratio), the water-fed (W0) group had a higher F/B ratio (0.79) than the GM-fed (G0) (0.50) and CM-fed (C0) groups (0.54) at Day 0. After HDM allergen sensitization and challenge there was a remarkable increase in the F/B ratio in water-fed mice (0.63 in WN vs. 0.84 in WA), while there was no change of F/B ratio in GM-fed (GN vs. GA) and CM-fed mice (CN vs. CA) (**Figure 5A**). A Weighted Principal Coordinates Analysis (PCoA) for the microbiome of each sample based upon the UniFrac method was performed to compare the overall composition of the bacterial

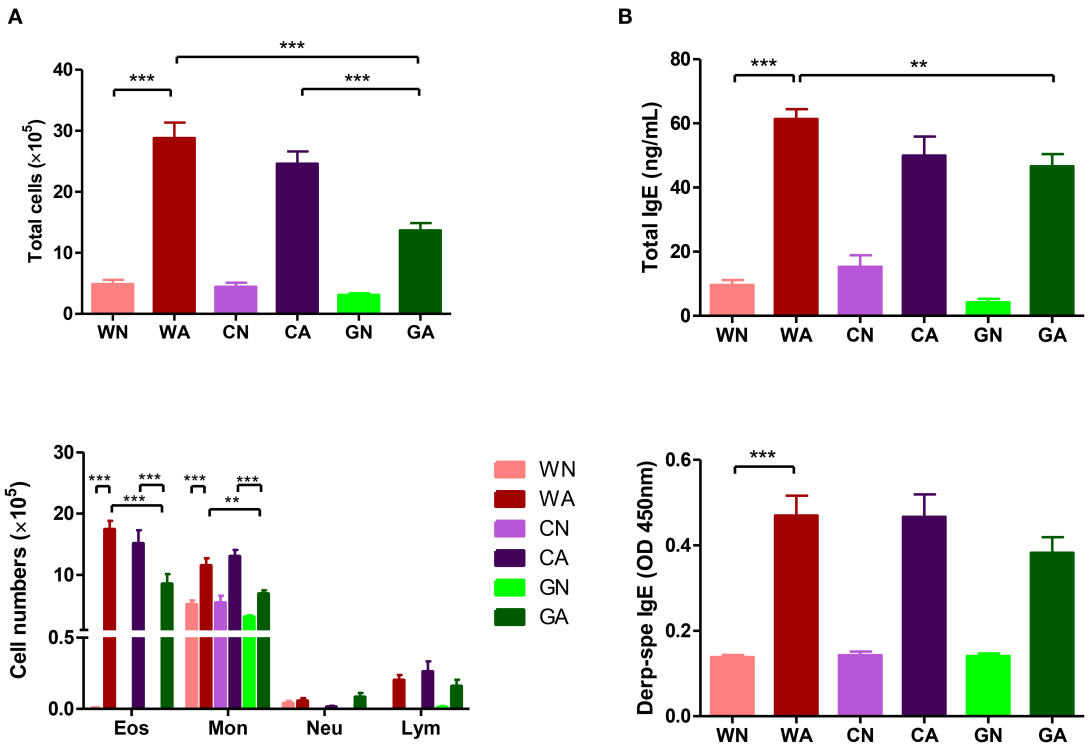


FIGURE 3 | HDM allergen-induced lung inflammation and sera IgE levels in offspring. **(A)** Total infiltrated cells and the number of eosinophils in BALF **(B)** total IgE and Der p-specific IgE levels in sera. Each group had 10 mice, and each assay was repeated three times. *P*-value of different groups were compared with those of N groups by Student's *t*-test (**$p < 0.01$ and ***$p < 0.001$).

community within the samples (**Figure 5B**). Gut microbiota of offspring from water-fed mice had a wider spread in PCoA, while offspring from GM- or CM-fed mice, though not overlapping, clustered in the upper left corner of PCoA, suggesting that these gut microbiotas were more abundant and relating to each other. It was also notable that there was no significant change in the abundance and β-diversity in the gut microbiota between non-sensitized and Der p allergen sensitized/challenged offspring from GM- or CM-fed mice, while gut microbiota of offspring from water-fed mice showed greater change in PCoA between WN and WA. The results from heatmap plots showed there were more dominant strains in the gut microbiota of offspring from GM- and CM-fed mice but less in the offspring of water-fed mice (**Figure 6**). Examples of dominant bacterial strains include *Akkermansia muciniphila*, *Bacteroides eggerthii*, and *Parabacteroides goldsteinii,* which had been reported to be beneficial to human health. In contrast, *Coprococcus catus, Lactobacillus murinus*, *Blautia sp. KLE 1732,* and *Clostridiales bacterium VE202-09* were found to be dominant in the gut microbiota of offspring from water-fed mice but less in the offspring of GM- or CM-fed mice (**Supplementary Figure 2**).

DISCUSSION

Bioactive compounds presenting in food are called nutraceuticals or functional foods. They are beneficial to the human body in many aspects and may go beyond their nutritional roles. Goat milk contains several bioactive compounds that might be useful in relieving cardiovascular disease, metabolic disorders, neurological degeneration, and promoting the establishment of intestinal microbiotas (32). In host immunity, when pathogens invade human body, B cells will generate antibodies to target specific antigens (33). Casein phosphopeptides of GM can increase the level of IgA in stool, which suggests a positive effect on mucosal immunity. Lactoferrin in GM has been demonstrated to play an important role in increasing the activity of NK cells and increasing the phagocytic activity of phagocytes (34). GM can also trigger IL-10, TNF-α, and IL-6 production in blood cells (35).

Our results showed that GM-fed mice could enhance the immune response in antibody production (IgA, IgM, and IgG subclasses) and phagocytosis activity promotion. Compared to CM-fed mice, there were more IFN-γ, IL-12, and TNF-α cytokine production in the culture supernatant of stimulated splenocytes in GM-fed mice. When mice were immunized with a specific antigen (OVA), GM-fed mice, but not CM-fed mice, had more antigen-specific antibodies (IgA, IgM, IgG, and IgG subclasses) than water-fed mice. There was a significant increase in IFN-γ and IL-10 production in the culture supernatant of stimulated splenocytes as well as an increase in the amount of $CD3^+$ T lymphocytes in GM-fed mice. More importantly, we found these enhancements of the immune response in

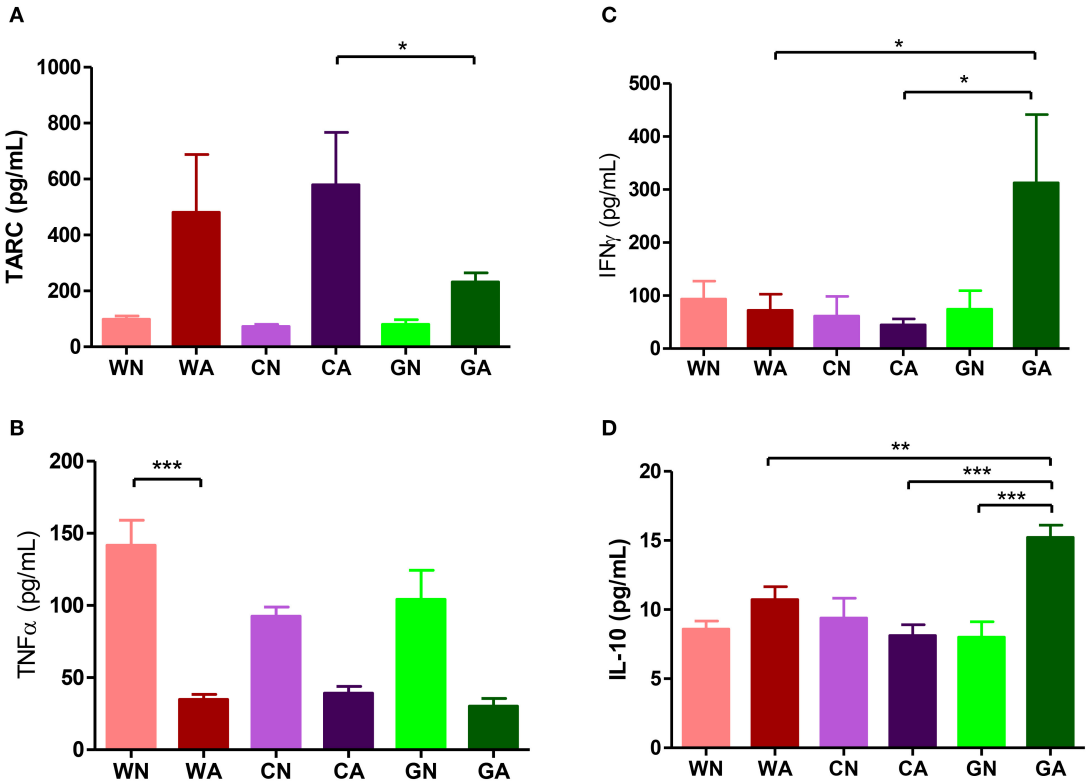


FIGURE 4 | Cytokines production in offspring. **(A,B)** Cytokines levels (TARC and TNF-α) of BALF. **(C,D)** Cytokines production (INF-γ and IL-10) of culture supernatants from PHA-stimulated splenocytes. Each group had 10 mice, and each assay repeated for three times. *P*-value of different groups were compared with those of N groups by Student's *t*-test (*$p < 0.05$; **$p < 0.01$; and ***$p < 0.001$).

TABLE 3 | Goat milk feeding in perinatal period induces gut microbiota change in HDM-sensitization and challenged offspring.

Superkingdom	Phylum	Class	D0W	D0G	D0C	D16WN	D16WN	D16GN	D16GA	D16CN	D16CA
Bacteria	Bacteroidetes	Bacteroidia	45	47	45	45	44	46	45	46	46
Bacteria	Firmicutes	Clostridia	30	25	23	24	33	24	24	26	23
Bacteria	Bacteroidetes	Flavobacteriia	3	3	3	3	1	2	3	3	3
Bacteria	Firmicutes	Bacilli	8	0	3	6	5	2	0	0	0
Bacteria	Deferribacteres	Deferribacteres	1	1	1	1	1	1	1	1	1
Bacteria	Verrucomicrobia	Verrucomicrobiae	1	1	1	1	1	1	1	1	1
Bacteria	Proteobacteria	Gammaproteobacteria	0	1	1	0	0	0	0	0	0
Viruses	Unclassified	Unclassified	1	0	1	0	0	0	1	0	0

innate and adaptive immunities in pregnant mice; mice fed with GM in particular could pass immunity to their offspring to alleviate allergen-induced airway inflammation of allergic asthma. These offspring from pregnant mice fed with GM or CM showed a drastic change of gut microbiota composition after weaning, compared to offspring of water-fed mice. We suspected that GM feeding during pregnancy and lactation might change the composition of breast milk and confer immunological maturation and colonization of gut microbiota on offspring, and this might suppress atopy development and downregulate airway inflammation.

Relationships among a wide spectrum of bioactive factors, such as proteins, polyunsaturated fatty acids, oligosaccharides, microbial content, metabolites, and micronutrients present in breast milk and allergy development in infants have attracted more attention (36–39). Various maternal exposures during pregnancy, such as immunization, dietary patterns, vitamin D, omega-3 fatty acids, and/or probiotics may affect breast milk composition and thereby influence the early colonization of gut microbiota and infant health (16, 40). Early microbial colonization is essential to infants' metabolic and immunological development (41). There is a direct link between microbial colonization and the risk of non-communicable diseases in later life, including allergies (42). After birth, the transfer of microbiota continues during lactation, and it is considered as the cause of differences in gut microbiota between exclusively breast-fed

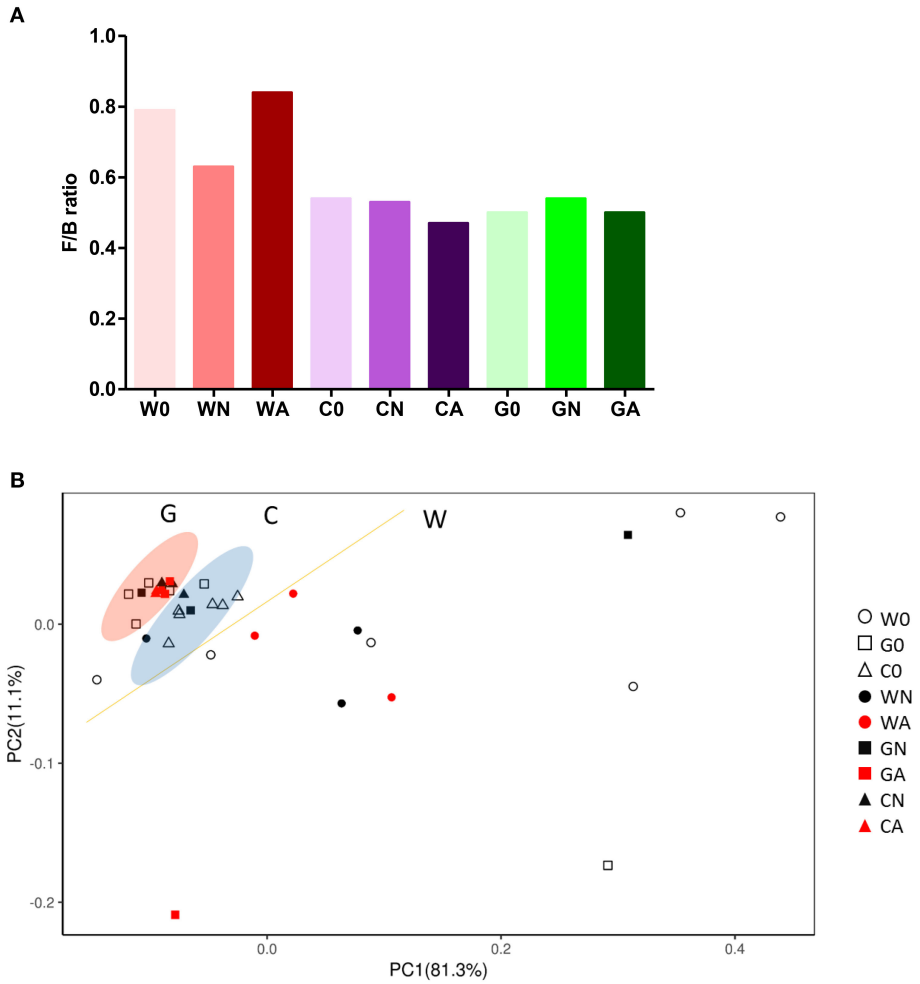


FIGURE 5 | Gut microbiota change in groups of offspring with or without HDM-sensitization and challenge. **(A)** The ratio of *Firmicutes* to *Bacteroidetes* (F/B ratio) in gut microbiota **(B)** Bi-plot representing the weighted Principal Coordinates Analysis (PCoA), pair-wise UniFrac distances showing clustering of bacterial groups from stool samples in groups of offspring.

and formula-fed infants during the first month of life (43). In clinical trials, oral administration of bacterial strains to lactating mothers modulated both human milk composition and infant's gut microbiota. For instance, intake of *Lactobacillus reuteri* led to its detection in the mother's milk and infant stool (44). Another study found that giving *Lactobacillus rhamnosus* to mothers during pregnancy and lactation can reduce the risk of allergy development (45). Probiotic intake during pregnancy and lactation also induced specific changes in infant *Bifidobacterium* colonization and affected breast milk microbiota composition, oligosaccharides, and lactoferrin (46).

While it is clear that mother's diet influences the health of her fetus, there is currently no concrete evidence in the role of maternal nutrition and the development of allergic diseases in children. As compared to formula feeding, there is clear evidence that breastfeeding can increase gut microbial biodiversity in infants. Whether GM consumption during pregnancy and lactation can induce changes of intestinal microbiota in newborns has never been explored. One clinical study (47) was conducted to compare the composition of the stool microbiotas of infants (<2 years old) fed with GM formula, CM-based formula, or breast milk. The results of the beta-diversity analysis showed that gut microbiotas and *Lachnospiraceae* populations were more similar between breast/goat milk comparisons than those between breast/cow milk comparisons. This similarity appeared to be based on the predominance of *Ruminococcus gnavus* among *Lachnospiraceae* in breast/goat milk-fed microbiotas. Our study showed there were significant differences in the intestinal microbiota compositions (PCoA analysis) and decreased *Firmicutes*/*Bacteroidetes* (F/B) ratio in the offspring of GM- or CM-fed pregnant mice compared to those offspring of water-fed mice. Besides, allergen sensitization and challenge induced slight changes in the composition of gut microbiota and F/B ratio in offspring of milk-fed mice, in contrast to the wide swings of change in the offspring of water-fed mice. These results were consistent with previous research that the resilient characteristics and atopy-protective role of colonized gut microbiota could confer from milk-fed

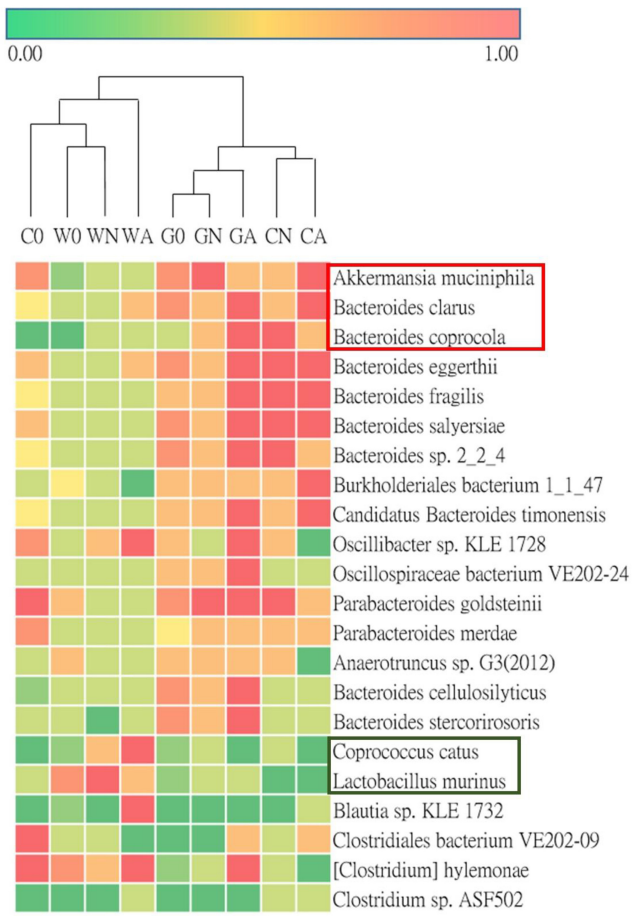


FIGURE 6 | Heatmap for the ratio of detected bacterial strains of microbiota in groups of offspring.

maternal mice to their offspring during pregnancy and lactation periods (48).

The abundance of bacterial species, such as *A. muciniphila* and *P. goldsteinii,* in the offspring's gut microbiota of GM- or CM-fed mice had multiple regulatory functions on glucose metabolism in diabetes and obesity as well as anti-inflammatory action in inflammatory bowel diseases (49–51). *Bacteroides eggerthii* and *Bacteroides fragilis* were reported to be associated with propionate production in human intestine (52). Propionate is a short-chain fatty acid and is suggested to be associated with IL-10-producing regulatory T (Treg)-cell differentiation in gut-associated lymphoid tissues (53). Recently, it had been found that there were reduced *A. muciniphila* and *Faecalibacterium prausnitzii* levels in the intestinal microbiota of children with allergic asthma (54), which might explain the anti-asthma protective role of GM-fed offspring with increasing levels of *A. muciniphila* in their gut microbiota.

In conclusion, this study showed that GM consumption could enhance immune function and antigen-specific immune response in mice. Furthermore, maternal GM consumption during pregnancy and lactation periods could affect the composition of gut microbiota in offspring and protected them against atopy and allergen-induced airway inflammation (**Supplementary Figure 3**). We believe these findings have important clinical implications in the improvement the nutrition of pregnant mothers and components of their breastmilk. Future trials are needed to prove this concept in order to promote maternal health and perinatal nutrition and to reduce allergic diseases in infants.

DATA AVAILABILITY STATEMENT

The microarray data has been uploaded to the GEO—GSE144086. Other raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC No. 105196, and No. 106244), College of Medicine, National Cheng Kung University.

AUTHOR CONTRIBUTIONS

H-FK, Y-CW, and H-YT conducted the experiments of the immunological studies. H-FK, M-HH, and P-CC conducted the experiment of the mouse model of allergic asthma. LW, L-FL, and H-JT for microbiota assays, bio-information, and statistics analysis. W-SK and Z-GL for technical advice. H-FK and J-YW for experimental design and writing up 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/fimmu.2020.00184/full#supplementary-material

Supplementary Figure 1 | (A) Thickness of skin, epidermis, and epidermis of ears and **(B)** H&E stain of ear skins after passive cutaneous anaphylaxis (PCA) test in groups of mice. Each group had 12 mice and each assay was repeated three

times. *P*-value of different groups were compared with those of N groups by Student's t-test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

Supplementary Figure 2 | The ratio of representing bacterial strains in gut microbiota in different groups of offspring, with or without HDM-sensitization & challenge. Female mice were fed with sterile water (W), GM (G), or CM (C) and offspring were divided into two groups: control group (WN, GN, CN) & HDM-stimulating group (WA, GA, and CA). On weaning day, offspring were marked W0, C0, and G0 individually.

Supplementary Figure 3 | Graphic summary of goat milk effects on immune responses and allergy diseases in offspring.

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METHODS
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Characterization of Extracellular Vesicles Isolated From Human Milk Using a Precipitation-Based Method

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Extracellular vesicles (EV) function in intercellular communication, and those in human milk may confer immunologic benefits to infants. Methods of EV isolation such as ultracentrifugation (UC) may not be feasible for the study of EVs in human milk due to the need for large sample volume. A technique to isolate EVs from a small volume of human milk using a precipitation reagent is described herein. Electron microscopy, nanoparticle tracking analysis, and semi-quantitative antibody array were conducted to confirm isolation of human milk EVs. Count, size, protein content, and fatty acid quantification of EVs were determined. This isolation technique yielded 8.9 x 10^9 ($\pm$ 1.1 $\times$ 10^9) EV particles/mL of human milk. The present method meets the Minimal Information for Studies of Extracellular Vesicles (MISEV) guidelines. An established EV isolation method suitable for a low volume of human milk will facilitate further research in this growing area.

Keywords: breastfeeding, dynamic light scattering, exosome isolation, exosome verification, fatty acids, nanovesicles, nanoparticle tracking analysis, scanning electron microscopy

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INTRODUCTION

It is well-known that consumption of human milk is associated with enhanced infant health outcomes in comparison to consumption of infant formula. However, it is not fully known which components of human milk may be responsible for supporting optimal health and development of newborns. Increasing research suggests that EVs from human milk have physiologic function that may impact acute and chronic health outcomes. Human milk EVs promote epithelial cell growth in the intestine (1) and were found to protect intestinal epithelial cells from oxidative stress (2). Additionally, human milk EVs have been implicated in the immune modulating function of human milk, and may play a role in the development of the neonatal immune system (3). These effects may be attributed to the protein, lipid, or microRNA cargo of human milk EVs (3). A reliable method for consistent isolation of EVs from human milk is needed to determine the functional components of EVs to which enhanced infant health outcomes can be attributed.

Although UC is the most commonly used method to isolate EVs from biospecimens (4), the feasibility of this method for human milk research is limited. As a precious biofluid for feeding newborns, acquiring the large volume of human milk needed for EV isolation using UC is not always feasible. Unfortunately, no method of EV isolation has been authenticated for use with a low volume of human milk ($\leq$2 ml); prior studies have isolated human milk EVs from a starting volume of 9 mL (5). As a result, the limitations in conducting research in this area have created a knowledge gap. Additionally, authentication of an EV isolation method from low volumes of

human milk will facilitate research on EVs throughout milk production periods, the course of lactation, over time-of-day variation, and perhaps most importantly in low volume producers, which are not adequately studied.

This limitation has several potential negative consequences. First, analysis of only large volumes may limit research to use of pooled human milk. This measure would result in a greater understanding of average milk composition but not of interindividual variability. Second, analyses may be limited solely to time of lactation when higher volumes of milk are produced. This may then result in a disparate understanding of mature milk relative to early and transitional milks. Finally, research may be limited to studies of mothers with high volume of milk expression instead of low volume producers. Therefore, a strong need exists for a method of isolate EVs from a low volume of human milk.

A novel method for the isolation of EVs requires verification procedures. The International Society for Extracellular Vesicles (ISEV) released MISEV guidelines in 2018 (6) detailing the minimum criteria for confirming isolation of EVs. MISEV guidelines recommend that each EV preparation be (i) defined quantitatively by the source of EVs, (ii) characterized to determine the abundance of EVs by total particle number or protein/lipid content, (iii) tested for components associated with EV subtypes or EVs generically, and (iv) tested for the presence of non-vesicular co-isolated components. This paper describes a precipitation-based method for the isolation of EVs from human milk. The subsequent characterization of EVs suggest successful isolation in compliance with the MISEV guidelines. EVs isolated using the present method are therefore appropriate for downstream characterization and functional analyses to better understand the health and immune-modulating properties of human milk.

METHODS

Mature milk (>6 months after initiation of lactation) was pooled and pasteurized from donors to develop the EV isolation method (Prolacta Bioscience, City of Industry CA). Twelve volunteers also provided samples of expressed milk between 2 and 4 weeks postpartum which were immediately frozen at −80°C. Ethics approval was obtained from the Chapman University Institutional Review Board. The EV isolation method (**Figure 1**) outlined herein is adapted from the instructions for a commercial precipitation reagent (7) and a previously published protocol (8).

EV Isolation Method

Thawing, Defatting, and Removal of Cell Debris

1. Thaw frozen human milk at 4°C. Once thawed, vortex milk for ∼3 s.
2. If milk was not aliquoted into microcentrifuge tubes prior to freezing, aliquot 1.5–2 mL (or desired volume) human milk into microcentrifuge tubes.
3. Centrifuge at 2,000 × g for 10 min to separate and remove the fat layer with a metal spatula. Discard the fat layer and transfer

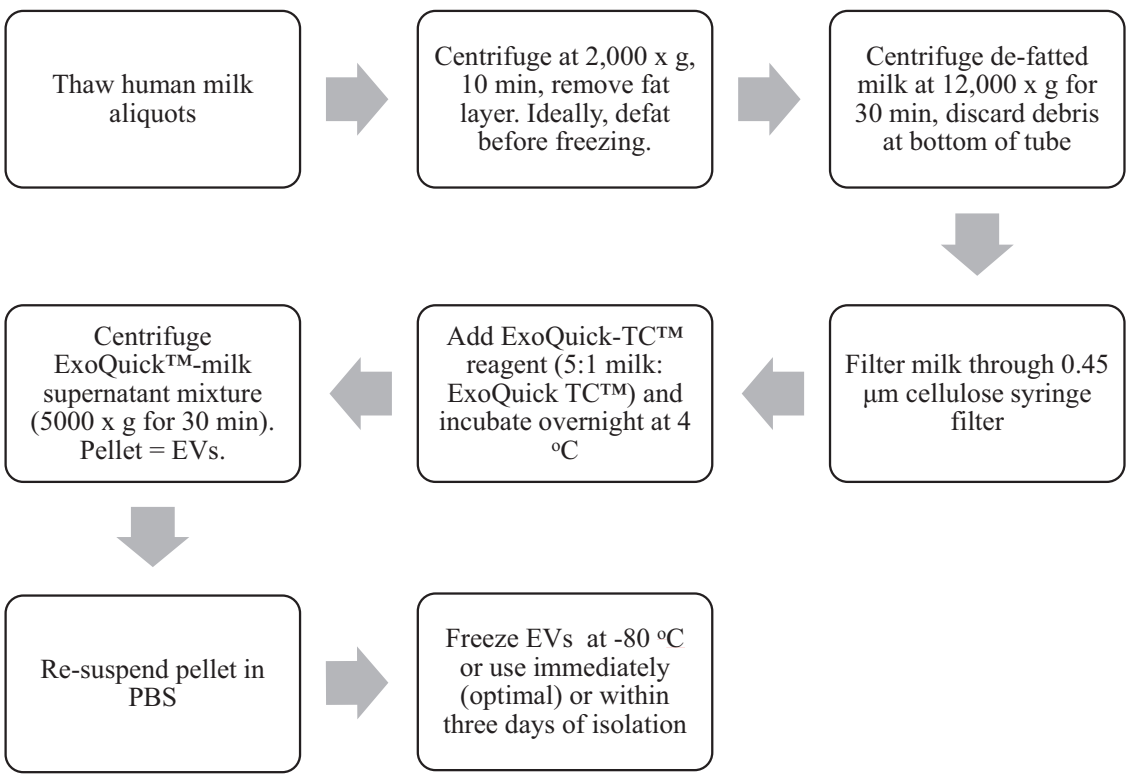

FIGURE 1 | Workflow schematic of EV isolation from human milk using a precipitation-based isolation method.

milk to a new tube. Removing the fat layer also removes milk fat globules (9).
4. Centrifuge the defatted milk at 12,000 × g for 30 min to remove cell debris. Transfer milk supernatant and/or discard pellet.
5. Filter milk supernatant through a 0.45 μm cellulose syringe filter into a new microcentrifuge tube to further eliminate cells and cellular debris.

EV Isolation

6. Using a 5:1 ratio of milk supernatant: ExoQuick-TC™ reagent (System Biosciences, Palo Alto CA), add reagent to the filtered milk and gently invert until mixed.
7. Incubate at 4°C overnight or for at least 12 h.
8. After incubation, centrifuge at 5,000 × g for 30 min (beige pellet will appear at the bottom of the tube).
9. Discard supernatant, and resuspend EV pellet in 100–600 μL PBS (pH 7.4).
10. Depending on downstream application, use resuspended EVs stored at 4°C within 3 days or freeze immediately at −80°C.

Scanning Electron Microscopy

Zeiss Gemini Sigma 300 scanning electron microscope (SEM) was used to visualize EVs isolated from milk expressed at 2 weeks postpartum ($n = 1$ volunteer). EVs were visualized 1 day after they were isolated, resuspended, and stored at 4°C. The original EV resuspension in PBS (500 μL) was further diluted in PBS (1:1,000). SEM slides were prepared with 2 μL of diluted EVs. Argon gas sputter coating of EVs with 3 nm gold-palladium alloy was performed to prevent sample destruction.

Nanoparticle Tracking Analysis

Nanoparticle Tracking Analysis (NTA; Nanosight NS01) was used to determine the concentration and size of EVs isolated from the pooled milk sample. A sample of EVs originally resuspended in PBS (500 μL) and frozen at −80°C was thawed on ice and further diluted in PBS (1:75) prior to injection. Detection threshold was set to four, and three runs each of 30 s in duration were completed and analyzed using NTA 3.1 software. Total yield (EV particles/mL milk) was calculated based on dilution factors and a starting volume of 1.5 mL milk.

Dynamic Light Scattering

The diameter of EVs isolated from the pooled milk sample was measured with a Mobius Dynamic Light Scattering (DLS) instrument (Wyatt Technology) using DLS Firmware Version 1.2.0.0. Laser wavelength was set to 532 nm, and a detector angle of 163.5° was used. DLS acquisition time was set to 5 s and a number acquisition of three was used to perform three technical replications on EVs stored at 4°C over the course of 10 days.

Exocheck Antibody Array

The Exocheck™ Antibody Array (System Biosciences, Palo Alto CA) was used according to the manufacturer's instructions (10) to determine the presence or absence of common EV

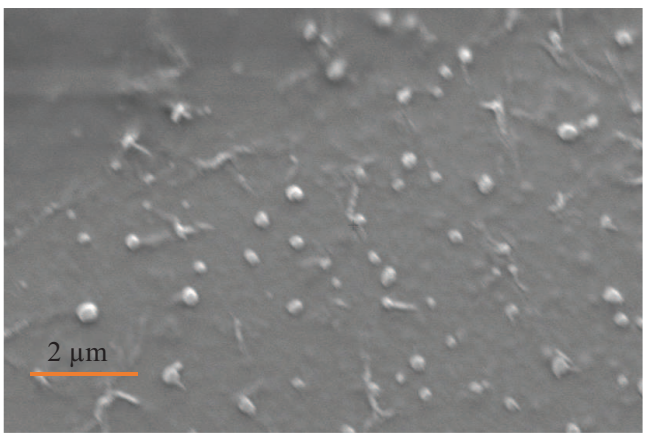

FIGURE 2 | Image of EVs obtained by SEM from participant ($n = 1$), Electron high tension = 5.00 kV, working distance = 20.9 mm, detector = secondary electron, magnification = 8.70 K X, vacuum mode = high vacuum, height = 9.851 μm.

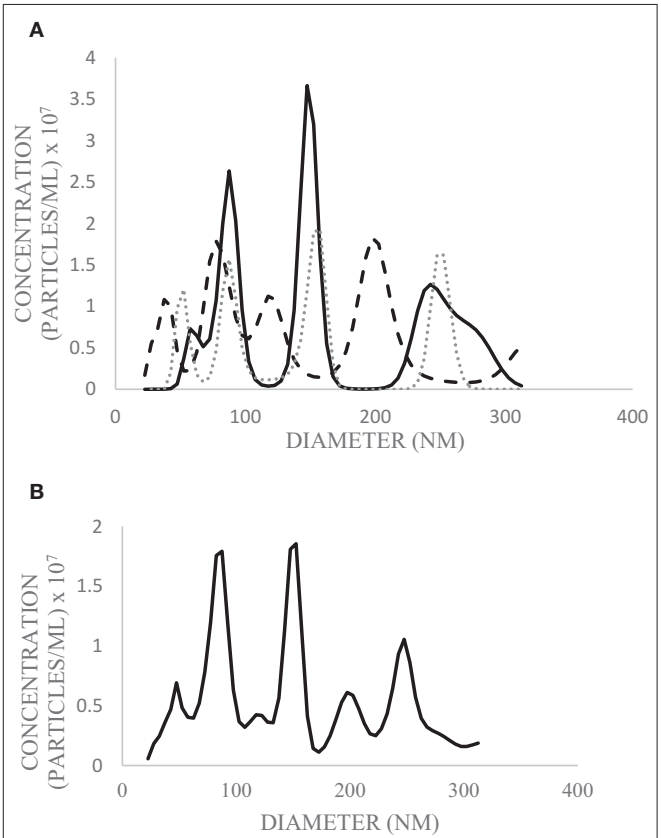

FIGURE 3 | Diameter and concentration of human milk EVs from pooled human donor milk measured by NTA. Technical replicates were performed in triplicate (solid line = trial 1; dashed line = trial 2; dotted line = trial 3) **(A)** and the average of the three runs was calculated **(B)**. The above graphs are plotted from the 10th–90th percentile of EV sizes (22.5–312.5 nm) to exclude particles which do not meet the size criterion for likely EVs.

proteins (CD63, EpCAM, Annexin5, TSG101, Flotilin1, ICAM, ALIX, CD81) in EVs isolated from milk expressed at 4 weeks postpartum ($n = 1$ volunteer). Resuspended EVs were thawed on ice prior to antibody array analysis.

Determination of Total Fatty Acid Concentration

The EVs from which fatty acids were analyzed were isolated using 2 mL aliquots of pooled milk, and with variations in EV isolation steps. A 5:1 and 10:1 ratio of milk supernatant: ExoQuick-TC™ reagent was used with or without (0.45 μm cellulose) filtration or purification using ExoQuick-TC™ ULTRA purification columns according to the manufacturer's instructions (System Biosciences, Palo Alto, CA). Prior to fatty acid analysis, EVs were isolated from the pooled milk sample, resuspended in PBS (500 μL), frozen at −80°C, and thawed on ice. Fatty acid analysis was performed by Creative Biostructure (Shirley, NY USA).

The total fatty acid concentration of EVs was determined by colorimetric analysis in triplicate ($n = 1$ per isolation variation). Standards were prepared with palmitic acid (1 nmol/μL). Samples were diluted and homogenized. Standard dilution (50 μL) or sample (0.5–25 μL) were added to each sample well. The final volume was adjusted to 50 μL with assay buffer. An acyl-coenzyme A synthetase reagent (2 μL) was added to each reaction well, mixed, and incubated (20 min, 37°C). Samples were then incubated (30 min, 37°C) in the dark with reaction mix (2 μL) containing assay buffer (44 μL), fatty acid probe (2 μL), enzyme mix (2 μL), and enhancer (2 μL). Finally, optical density was measured on a microplate reader at 562 nm.

Protein Quantification

A Qubit™ 4 Fluorometer was used to measure the protein concentration in human milk EVs isolated from milk expressed at 2 weeks postpartum ($n = 10$ volunteers). Resuspended EVs were thawed on ice prior to protein quantification. The instrument was calibrated with protein standards according to the manufacturer's instructions (11). EV samples originally resuspended in 600 μL PBS were thawed on ice and diluted in PBS (1:20). Lysis buffer (10 μL) was added and samples were vortexed (Protease Inhibitor Cocktail, RIPA buffer, Thermo Fisher Scientific, Waltham MA). Protein concentration was measured in duplicate after incubating (15 min, room temperature) the lysate (1 μL) with working reagent (199 μL). Protein quantification of EVs was calculated based on dilution factors and a starting volume of 1.5 mL milk.

RESULTS

SEM (**Figure 2**), NTA (**Figure 3**), DLS (**Figure 4**), and an antibody array (**Figure 5**) were used to image, quantify, measure the average diameter, and identify protein markers characteristic of EVs. The image obtained by SEM (**Figure 2**) revealed the size of nanovesicles in the expected range for EVs, approximately 50–350 nm. Results from analysis by NTA (**Figure 3**, **Supplementary Figure 1**, **Video 1**) revealed that the isolation method yielded 8.9×10^9 ($\pm$ 1.1×10^9) particles/mL of human milk. The mean and mode diameter of EVs were 179.3 and 150.3 nm, respectively (**Figure 3**). No standard deviation is reported for the mean since a trimodal distribution of EV populations was observed. Results from DLS (**Figure 4**)

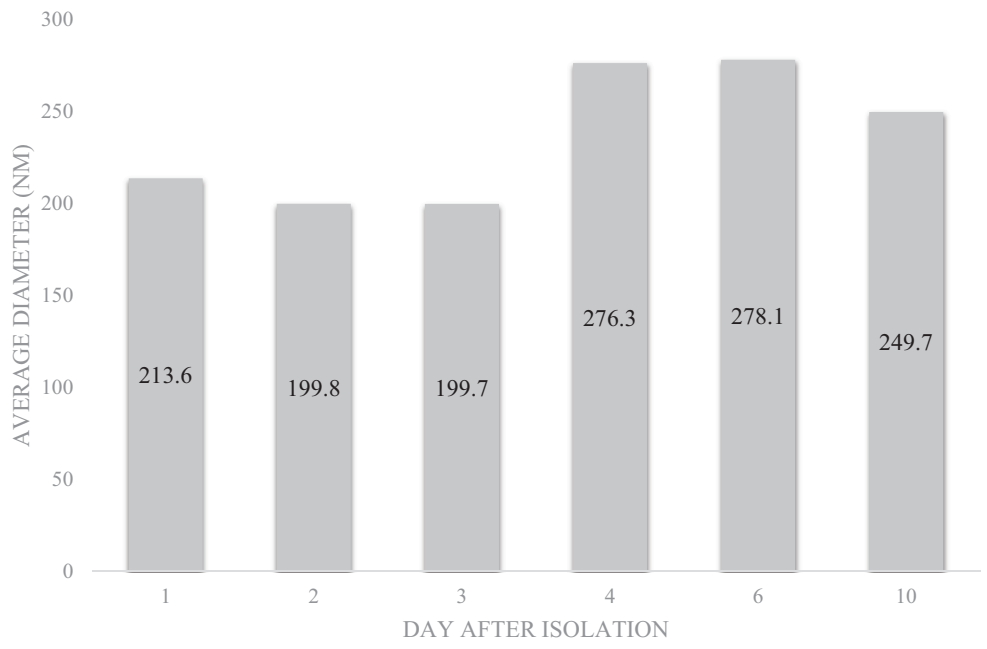

FIGURE 4 | Average diameter of EVs from pooled human donor milk measured for 10 consecutive days after isolation and storage at 4°C. Error bars were excluded because individual standard deviations were <5% of the mean. Pooled SD = 33.45.

showed that the average diameter of EVs 1 day after isolation was 213.6, and 249.7 nm 10 days after isolation. Error bars for individual days were excluded because individual standard deviations for technical replicates were <5% of the mean. Antibody array (**Figure 5**) indicated that the sample was positive for the following known EV markers: cluster of differentiation 81 (CD81), ALG-2-interacting Protein X (ALIX), intracellular adhesion molecule (ICAM), tumor susceptibility gene 101 (TSG101), and Annexin5, and negative for cluster of differentiation 63 (CD63), epithelial cell adhesion molecule (EpCAM), and flotilin1.

After verification of isolation, human milk EVs were characterized by quantifying total fatty acids (**Figure 6**) and protein concentration (**Table 1**). The average total fatty acid concentration of EVs isolated with the recommended method (5:1, filter, no column purification) was 36.94 mg/dL. The mean protein concentration of human milk EVs was 5.08 (±0.15) mg/dL.

DISCUSSION

The method of EV isolation from human milk described herein meets the MISEV criteria (6) for verifying the presence of EVs. EVs isolated with the proposed method were (i) quantified in relation to the source of human milk, (ii) characterized to determine the abundance of EVs by total particle number and lipid & protein content, (iii) tested for the presence of markers associated with EVs, and (iv) tested for the presence of non-vesicular co-isolated components. The method adapted from manufacturer instructions for a precipitation reagent (7) and previous literature (8) was shown to be suitable to adequately characterize EVs isolated from human milk and for downstream applications.

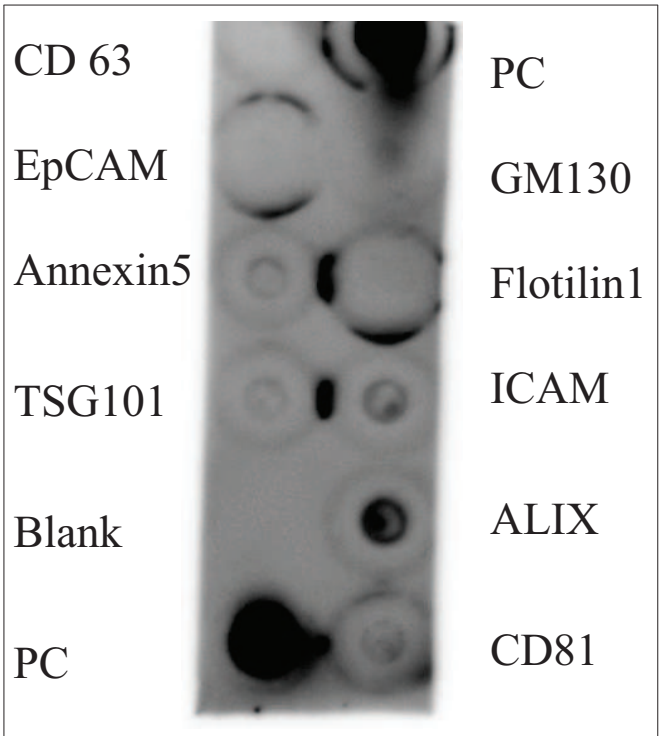

FIGURE 5 | Antibody array of human milk EVs from participant ($n = 1$). PC represents the positive control, and GM130 is a cellular contamination marker. CD 63 = cluster of differentiation 63, EpCAM = epithelial cell adhesion molecule, TSG101 = tumor susceptibility gene 101, ICAM = intracellular adhesion molecule, ALIX = ALG-2-interacting Protein X, CD81 = cluster of differentiation 81.

There is consistency between the average EV diameter measured by SEM, NTA, and DLS (**Figures 2–4**). Unlike NTA which generates size distribution data, DLS measures the average particle diameter. Measurement by DLS then may be skewed by low concentrations of outliers or clustering of particles (12). Therefore, the ∼15% difference in diameter between SEM, NTA, and DLS measurements could be due to overestimation of diameter by DLS. The recommended method presented herein yielded 8.9×10^9 ($\pm$ 1.1×10^9) EV particles/mL of human milk. Another group isolated human milk EVs and reported a yield of 8.0×10^{10} particles/ml of milk using a UC based method (5). The difference in yield could be attributed to the fact that banked, pasteurized milk was used in the present method. Additionally, EVs were frozen and thawed prior to quantification without defatting before initial freezing, which has shown the decreased recovery of EVs (3).

In the MISEV guidelines, it is recommended that operational terminology for extracellular vesicles based on factors such as size be used. EVs <200 nm in diameter would be considered "small," and EVs >200 nm considered medium or large (6). Results from NTA indicated that the greatest concentration of particles is around 153 nm (**Figure 3**), meaning the EV population in highest abundance would be classified as small. The 10th−90th percentile of particle size were graphed (**Figure 3**), as particles outside this range were likely aggregates or fragments.

Because storage conditions may affect EV characterization, MISEV guidelines indicate the importance of describing storage conditions such as storage container, temperature, buffer, freeze-thaw cycles of biofluid and EVs, etc. (6). It was previously found even that storage of EVs for 2 h at 4°C decreased the viability of the exosome population, but the change in size was not measured (3). The timecourse experiment (**Figure 4**) represents storage-induced changes in diameter starting from freshly isolated EVs measured over the course of 10 days. The average diameter of EVs measured by DLS increased over time after isolation and storage. This may indicate swelling and enlargement of EVs, or aggregation of particles. Therefore, when performing studies to determine the relation between structure and function, it may be advantageous to use EVs immediately after isolation.

For protein-based verification of EV isolation, MISEV guidelines stipulate that at least one type of protein in two broad categories should be positively identified and the absence of one negative marker indicated. These categories include transmembrane or GPI-anchored proteins, such as the tetraspanins CD63 and CD81, and EV-recovered cytosolic proteins such as ALIX and flotillins-1 and 2. To verify the absence of non-EV isolated co-structures, markers such as albumin can

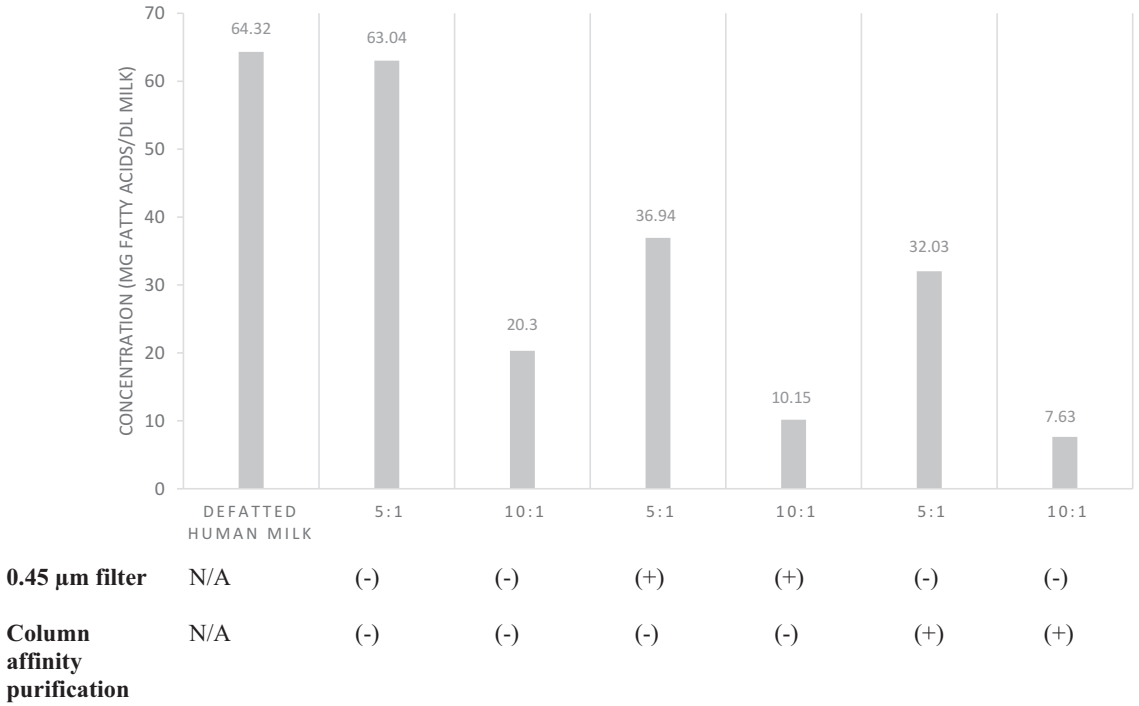

FIGURE 6 | Total fatty acid concentration of EVs isolated from pooled human donor milk. The ratios represent the proportion of milk supernatant: ExoQuick-TC™ reagent used during isolation. Milk was either filtered (0.45 μm) prior to EV isolation or EVs isolated were purified by column affinity after isolation. Error bars were excluded because individual standard deviations for two technical replicates were <5% of the mean. Pooled SD = 18.78.

TABLE 1 | Protein concentration of EVs isolated from human milk ($n = 10$).

Sample	Protein content (mg/dL milk)
1	5.20
2	5.00
3	5.11
4	5.02
5	5.15
6	5.18
7	5.24
8	4.98
9	5.20
10	4.75
Mean	5.08
SD	0.15

For each sample, technical replicates were performed in duplicate.

be used (6). The antibody array (**Figure 5**) verified that human milk EVs isolated were positive for proteins in the tetraspanin and EV-recovered cytosolic proteins category, and also negative for cellular contamination marker.

The amount of exosomal protein has been used as a means of EV quantification (13). Considering that the average protein concentration measured in EVs was 5.08 mg/dL, EV protein comprises ∼0.42% of total protein from mature human milk, assuming the protein concentration of mature human milk is ∼1,200 mg/dL (14). However, it should be noted that protein quantification with biofluids such as human milk may not be a consistent and reliable method of quantification due to the presence of co-isolated molecules. Therefore, we reported the total fatty acid concentration of human milk EVs (**Figure 6**). Based on the assumption that fat content of human milk is primarily in the form of triglyceride, we estimated that EV fatty acids are ∼0.8% of total fatty acids in mature human milk (14).

We compared fatty acid quantification among EVs isolated from human milk with different volumes of reagent, use of size exclusion filter, and with or without column affinity purification. We suggest a supernatant-to-precipitation reagent ratio of 5:1 for optimal yield of EVs to quantify fatty acids. We also suggest filtration of milk by size exclusion after defatting to remove non-EV artifacts such as casein and cellular debris. However, it is unclear whether column affinity purification after EV isolation performs similarly to size exclusion filtration of milk supernatant prior to EV isolation. Although fatty acid quantification was similar after each method, it is unknown if filtration and purification result in differences in the EV populations isolated.

The present method of isolating EVs from human milk fulfills the MISEV criteria by characterizing the EVs with quantitative and qualitative methods, confirming the presence of characteristic EV markers, and confirming (**Figure 5**) the absence of non-EV components. The application of this isolation method

extends beyond the applications detailed in our manuscript. The ability to successfully isolate EVs from small volumes of human milk can be applied to miRNA isolation, proteomics, lipidomics, and functional *in vitro* assays.

CONCLUSIONS

EVs were successfully isolated from human milk using a precipitation reagent. The method yielded $8.9 \times 10^9 \pm 1.1 \times 10^9$ EV particles/mL of human milk. Protein and fatty acid concentration of EVs in human milk were determined and the percentage of fatty acids and protein in EVs relative to the whole milk were ∼0.8% and ∼0.42%, respectively. The method presented is consistent and reliable for isolating, quantifying, and characterizing human milk EVs for research and clinical purposes and in continuing to understand the human milk food matrix. As a dynamic food and biofluid, future study may elucidate how EVs vary over i) early, transitional and mature milk production periods, ii) course of lactation (fore vs. hind milk), and iii) time-of-day variation. This method can be used to elucidate the role of human milk EVs in neonatal health and immune system development, and for applications of formula and human milk fortifier production.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Chapman University IRB. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

DB isolated the EVs from human milk, performed all experiments with the exception of NTA and total fatty acid analysis, and drafted the manuscript. JM oversaw the writing of the manuscript, obtained funding for the study, and collected NTA data.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2020.00022/full#supplementary-material

Video 1 | Human milk EVs from NTA light scatter using Nanosight NS01. Video is a representative segment captured from a 3 × 30 s flux at infusion of 40 μL/min into microfluidics chamber.

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frontiers in Immunology

ORIGINAL RESEARCH
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Seeking Windows of Opportunity to Shape Lifelong Immune Health: A Network-Based Strategy to Predict and Prioritize Markers of Early Life Immune Modulation

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A healthy immune status is strongly conditioned during early life stages. Insights into the molecular drivers of early life immune development and function are prerequisite to identify strategies to enhance immune health. Even though several starting points for targeted immune modulation have been identified and are being developed into prophylactic or therapeutic approaches, there is no regulatory guidance on how to assess the risk and benefit balance of such interventions. Six early life immune causal networks, each compromising a different time period in early life (the 1st, 2nd, 3rd trimester of gestations, birth, newborn, and infant period), were generated. Thereto information was extracted and structured from early life literature using the automated text mining and machine learning tool: Integrated Network and Dynamical Reasoning Assembler (INDRA). The tool identified relevant entities (e.g., genes/proteins/metabolites/processes/diseases), extracted causal relationships among these entities, and assembled them into early life-immune causal networks. These causal early life immune networks were denoised using GeneMania, enriched with data from the gene-disease association database DisGeNET and Gene Ontology resource tools (GO/GO-SLIM), inferred missing relationships and added expert knowledge to generate information-dense early life immune networks. Analysis of the six early life immune networks by PageRank, not only confirmed the central role of the "commonly used immune markers" (e.g., chemokines, interleukins, *IFN, TNF, TGFB*, and other immune activation regulators (e.g., *CD55, FOXP3, GATA3, CD79A, C4BPA*), but also identified less obvious candidates (e.g., *CYP1A2, FOXK2, NELFCD, RENBP*). Comparison of the different early life periods resulted in the prediction of 11 key early life genes overlapping all early life periods (*TNF, IL6, IL10, CD4, FOXP3, IL4, NELFCD, CD79A, IL5, RENBP*, and *IFNG)*, and also genes that were only described in certain early life period(s). Concluding, here we describe a network-based approach that provides a science-based

and systematical method to explore the functional development of the early life immune system through time. This systems approach aids the generation of a testing strategy for the safety and efficacy of early life immune modulation by predicting the key candidate markers during different phases of early life immune development.

Keywords: biomarkers, immune networks, early life, machine learning, text mining

INTRODUCTION

The first 1,000 days of life is a period of growth and development in which the foundations of lifelong immune homeostasis and microbial colonization are established in humans (1). Alterations during this period, due to environmental and host factors, are considered to be potential determinants of health-outcomes later in life (2–4). Therefore, risk reduction measures or immune health interventions during these stages of life may be most effective and efficient for improving health, increasing quality of life, and lowering costs to society due to immune related diseases and disorders.

When developing immune health interventions in early life, the regulatory authorities (EFSA, JECFA) stress the need to address the safety of such interventions. However, currently there is no regulatory guidance about how to assess the risk and benefit balance of such interventions. At the moment final safety confirmation comes from expensive and lengthy clinical follow up studies using a set of guidelines (5–7). Therefore, a need for a science-based system approach to assess the safety and benefit of nutritional immune interventions, with a special focus on early life is clear. With such an approach animal testing can be reduced, refined or replaced.

Key to understanding the potential of early life immunity to shape lifelong immune health is the concept of ontogeny—the immune system development from fetal life through adulthood. Previously, our group made an inventory and compared the maturation of the immune systems of human, mouse, rat, and mini pig, based predominantly on existing (from literature) and newly generated histologic data (8). Critical time windows of immune organ development were identified in human and the above mentioned experimental species. However, less is known about the functional time frames of the developing immune system in humans. This knowledge is crucial to identify factors that need to be considered for assessing the safety and efficacy of early life nutritional interventions and exposure.

As the immune system is an enormously complex system, it is crucial to obtain more understanding about the biological structures and processes to be able to improve human (immune) health. However, due to the enormous wealth of information available, it is extremely difficult to obtain a complete picture of the biological basis of immune related diseases and health. Individual researchers are often restricted to so called "knowledge pockets" (9) covering only a small fraction of all available knowledge, and that fractional information is spread through literature or various databases. This fragmentation of information clearly hampers our understanding of the molecular processes underlying human health and disease. In order to obtain a complete picture, data integration from different sources is required.

Systems immunology combined with bioinformatics can provide sufficient knowledge to identify factors to assess the safety and efficacy of early life nutritional interventions and exposure (10–12). Recent technological advances permit collection and storage of large datasets at molecular and cellular levels (genes, gene products, metabolic intermediates, macromolecules, cells). So far, most studies or research groups collected data sets from several—omics-platforms to understand the larger (systems) picture by putting the pieces together, mostly through association networks (e.g., Protein-Protein Interaction network). Association networks are static and undirected networks. They provide lesser information than a directed causal network. However, creation of system-wide causal networks from omics data is a task that is largely tedious, and not pragmatic. This is because the amount of data spanning the molecular changes in spatio-temporal space is too large to capture the system knowledge within causal network in sufficient detail. Nevertheless, the dynamics of the immune system are better understood and characterized with the use of causal networks. Our intention here is to create causal networks of the early life immune system in a comprehensive and pragmatic manner.

Here, we generated causal immune networks in early life from literature sources that correspond to the 1st, 2nd, 3rd trimester of gestation (resp. EG, LG, MG), birth, newborn and infant period as part of a bioinformatics workflow, which also included subsequent network enrichment steps to generate comprehensive causal early life immune networks. The network-based approach developed here, enabled us to elucidate different phases of early life immune development in a systematical way to predict and prioritize biological functions and genes associated with immune functioning in early life. Moreover, this systems approach aids the development of a science-based testing strategy for assessing the safety and efficacy of early life immune modulation by predicting the key candidate markers during different phases of early life immune development.

MATERIALS AND METHODS

Generation of the Basis of Early Life-Immune Networks Using Text Mining

The entire bioinformatics workflow to generate human early life networks is depicted in **Figure 1**. The first step was to select relevant manuscripts describing immune mechanisms in early life. An inventory of the available literature regarding 6 immune developmental periods [1st/2nd/3rd trimester of

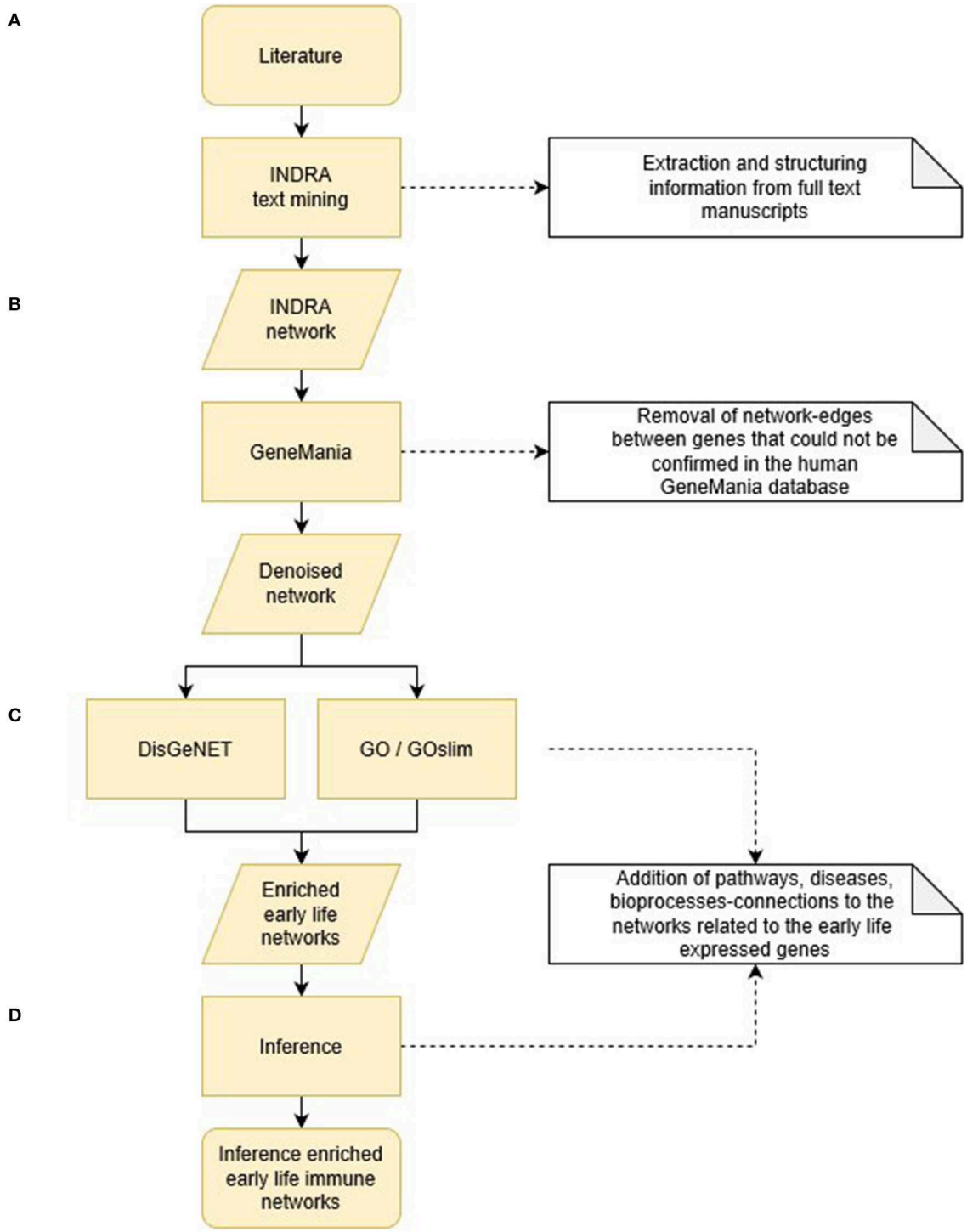

FIGURE 1 | Bioinformatics workflow to generate human early life networks. **(A)** Expert based selection of early life immune manuscripts were divided in 6 early life time periods and subjected to INDRA text mining tool. This resulted in 6 causal INDRA network. **(B)** The gene-gene connections of the INDRA networks were denoised and validated for the human situation by GeneMania. **(C)** DisGeNET and Gene Ontology tools (GO and GOslim) enriched the denoised early life networks by adding gene-disease connections and gene-process/pathway connections. **(D)** Inference calculations enriched the early life networks further by adding process-disease and disease-immune health endpoint connections. All steps together resulted in 6 human early life immune networks. The results of the different programming steps are depicted in **Tables 2**–**4** as indicated.

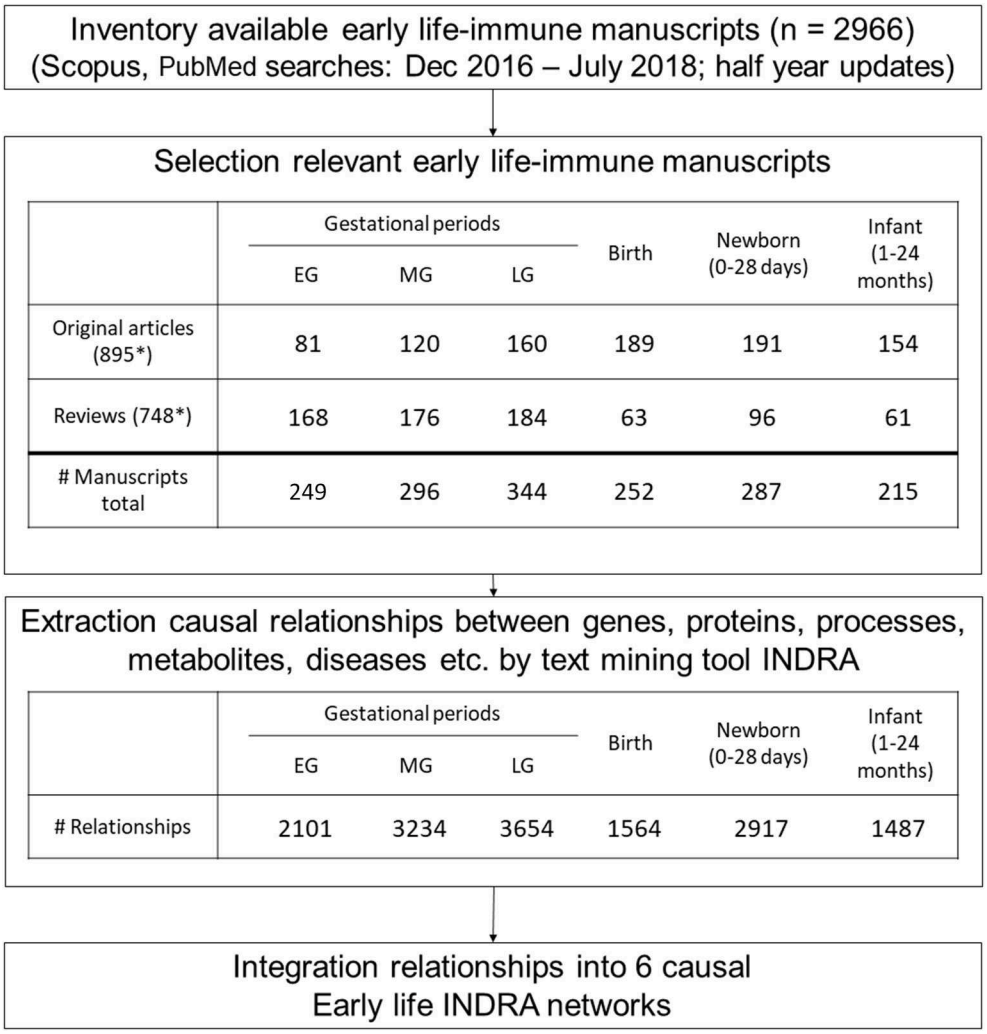

FIGURE 2 | Workflow to generate the basis of early life immune networks by literature. Six causal early life immune networks covering a different early life were generated by selecting appropriate manuscripts from literature after which relationships between biological entities were extracted by the text mining tool INDRA. Next INDRA assembled, de-duplicated and standardized all relationships into causal early life-immune networks each covering a different early life period. These INDRA networks formed the basis of the early life immune networks. *Several unique articles cover multiple early life periods.

gestation, birth, newborn (0–28 days), infant (1–24 months)] in human and experimental animals was made using Scopus and Medline (**Figure 2**). These databases were searched between 1st of December 2016 and 2nd of December 2016 and updated each half year (last update in March 2019). The search strings are depicted in **Table 1**. In total 2,966 articles were selected and manually screened on title, abstract and full text to select appropriate articles. Next, all selected articles were classified into the appropriate early life time period. The lengths of these different time periods in humans and experimental animals have been defined previously by Kuper et al. (8) and reported in **Table 2**.

The text from the manuscripts was moderately preprocessed to correct for obvious noise in text that interfered with the text analyses. Noise correction included deletion of special characters (except numbers, letters, punctuations and hyphens), "Materials and Method" section, d.o.i., terms "fig." and "table," replacement of Greek characters by Roman letters, references containing "et al.," and hyphenation if a word was split into two parts at the end of a line of text. The Python code used to preprocess the manuscripts can be found at https://github.com/TNO/immune_health_textmining/blob/master/PDFminer.py.

After this preprocessing step, INDRA (Integrated Network and Dynamical Reasoning Assembler) text mining platform (www.indra.bio/) was used to extract relationships and structure information on causal mechanisms among biological entities from the selected articles. INDRA is an automated model assembly system interfacing with NLP systems and ontology databases to collect knowledge, and through a process of network assembly, produce causal graph and dynamical models (13–15).

INDRA text mining platform rendered the full texts of the selected articles computationally

TABLE 1 | Search strings used to assess the available literature regarding the immune functional developmental stages in human and experimental animals was performed by searching the databases Scopus and Medline.

Search terms	Combined with species terms	Combined with additional search terms
Thymus OR spleen OR lymph nodes OR Peyer's patches OR bone marrow OR liver	Human OR mini pig OR rat OR mouse	• Functional AND developmental AND stages OR • Immune AND development AND birth OR • Immune AND development AND weaning OR • Immune AND development AND prenatal OR • Immune AND development AND postnatal
Cord blood	Human OR mini pig OR rat OR mouse	• Functional AND developmental AND stages OR • Immune AND development AND birth OR • Immune AND development AND prenatal
*	Human OR mini pig OR rat OR mouse	• Functional AND developmental AND stages AND (amniotic fluid) OR placenta OR (*in utero*) OR intrauterine OR • Immune AND development AND (amniotic fluid) OR placenta OR (*in utero*) OR intrauterine AND birth OR • Immune AND development AND (amniotic fluid) OR placenta OR (*in utero*) OR intrauterine AND prenatal

**No additional organ/tissue-specific term used in this search string which is specifically aimed at the gestational phase.*

TABLE 2 | Developmental early life stages in human, minipig, rat, and mouse [adapted from (8)].

Early life period	EG[a]	MG	LG	Birth	Newborn	Infant
Human	GD0–GW12	GW13–28	GW29–40	–	0–28 days	1–23 months
Minipig	GD0–GD37	GD38–75	GD76–113	–	0–15 days	2–4 weeks
Rat	GD0–6	GD7–13	GD14–21	–	0–7/10 days	1/1.5–3 weeks
Mouse	GD0–6	GD7–13	GD14–21	–	0–7/10 days	1/1.5–3 weeks

[a] *Starts at fertilization/conception.*
EG/MG/LG, early/mid/late gestational period.
GD, gestational day; GW, gestational week.

accessible, identified biologically relevant entities (e.g., genes/proteins/metabolites/bioprocesses/diseases) and extracted relationships among these entities. Next, INDRA assembled, and standardized all relationships among the entities with associated evidence into causal early life-immune networks each covering a different early life period. Neo4J (https://Neo4j.com/) was used as a graph database management system to store, process and visualize the INDRA literature information as two-dimensional networks. This entire workflow is depicted in **Figure 2**.

Code used to generate the INDRA network is part of the INDRA repository and can be found at https://github.com/TNO/immune_health_textmining/blob/master/SRP_Neo4J.py.

Denoising INDRA Literature Networks

In order to eliminate noise from the INDRA literature networks and only depict those relationships for which there is a biological indication that the relationship is valid, all gene-gene relationships in the INDRA literature network were subjected to a denoising step using GeneMania (https://genemania.org/).

Genes coding for proteins described in the INDRA network were entered in the GeneMania Cytoscape plugin (freely available at http://genemania.org/plugin/) to identify human gene-gene associations from its large collection of organism specific functional association data that include protein and genetic interactions, pathways, co-expression, co-localization, and protein domain similarity. These GeneMania-identified human gene-gene associations were compared to the gene-gene associations from the noisy INDRA literature networks, to identify and eliminate non-human specific associations between genes in the INDRA network. In the denoising step the edges (connections) between the genes were eliminated from the network, but not the genes themselves; they remained in the network as disconnected nodes. It must be noted that this step possibly eliminates true early-life gene-gene interactions if they are not represented in the human-specific GeneMania databases, which are mostly based on adult data. However, it is foreseen that this potential loss of information was compensated by the following enrichment steps because

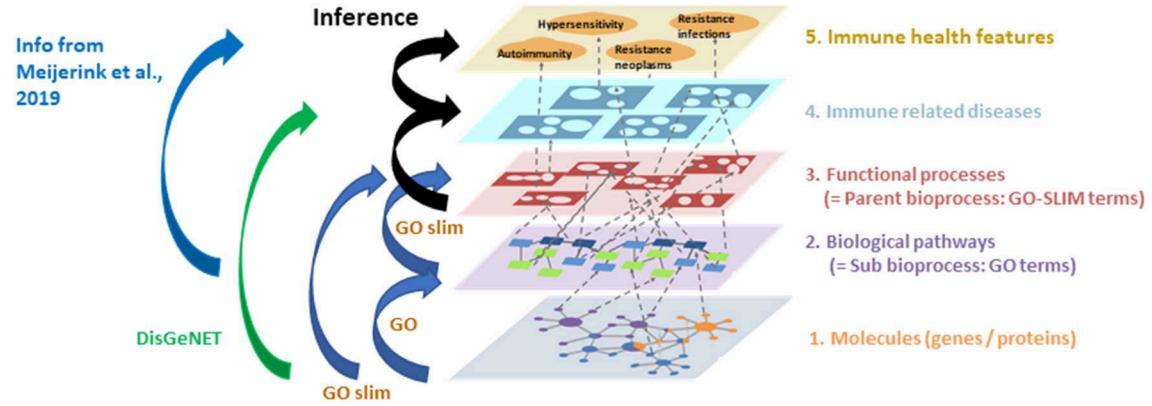

FIGURE 3 | Overview of the steps used to enrich the INDRA networks. The genes described in early life literature (level 1). were entered in (i) DisGeNET to add gene-disease relationships to the network (level 1–4) and (ii) Gene Ontology tools GO/GO-SLIM to add gene-sub bioprocess (level 1–2), sub bioprocess—parent bioprocess (level 2–3) and gene-parent bioprocesses (level 1–3) relationships. Next the GO-terms linking to immune health features described previously in Meijerink et al. (16) were added to the network (level 2–5; blue arrow). The associations between bioprocesses and diseases (level 3–4) and disease–immune health features (level 4–5) were inferred (black arrows) based on the previous enrichment steps (orange arrows).

the disconnected genes remained part of the network. The code used to denoise the INDRA literature networks can be found at https://github.com/TNO/immune_health_textmining/blob/master/SRP_filter_networks.py.

Network Enrichments (Figure 3)

The INDRA network derived from literature reflects only the functionalities of the genes and processes described in literature which provides an incomplete picture of the functionalities of the described genes because the manuscripts usually focus on a specific topic. Therefore, it was important to determine whether the expressed genes are associated with a certain biological process and/or molecular function and/or diseases which were not addressed in the selected manuscripts. This knowledge was retrieved from several databases and added to the networks (enrichment). To enrich the INDRA early life immune literature networks, the genes coding for the proteins in the network were entered into the Gene Disease Association Database (DisGeNET; http://www.disgenet.org/) to retrieve the gene-disease associations using WebGestalt tool (17). The same sets of genes were also entered in the Gene Ontology resource tools (GO enrichment tools GO and GO-SLIM; http://geneontology.org/) to retrieve gene-bioprocess associations (GO/GO-SLIM).

As a final step in the network enrichments, the associations among bioprocesses, immune related diseases and immune health endpoints (16) were inferred based on the enrichment tool specific database knowledge of the number and similarity of the genes related to each of the network entities in different layers in the model (**Figure 3**). As described earlier, Neo4J (https://Neo4j.com/) was used as a graph database management system to store and process all network information, including the literature-derived information by INDRA.

Codes used to generate these enriched networks can be found at https://github.com/TNO/immune_health_textmining/blob/master/SRP_Neo4J.py https://github.com/TNO/immune_health_textmining/blob/master/SRP_add_endpoints_to_disease_nodes.py and https://github.com/TNO/immune_health_textmining/blob/master/SRP_calc_inference.py.

Prioritization Immune Markers in Early Life

In order to identify key early life genes (hub genes), the PageRank centrality score was calculated in the early life networks. The PageRank analysis was launched by Google (the web search engine) to identify significant web pages (18–20) and has been used for the analysis of networks in identifying the important nodes in the network (21). Unlike simply calculating the connections of each gene in the network, the PageRank score measures the importance or popularity of a gene based solely on the interaction (link) structure of the interaction network. It selects the genes that exhibit a high degree, whilst also maintaining the important low-degree genes, which link to other important genes in the network. The underlying assumption is that more important genes are likely to receive more associations from other important genes/bioprocesses/diseases.

The PageRank algorithm code can be found at https://github.com/TNO/immune_health_textmining/blob/master/SRP_calc_pagerank_neo4j.py.

RESULTS

Generation of Early Life-Immune Literature Networks Using Text Mining

The literature covering the information on mechanisms involved in early life immune health is scattered across thousands of scientific papers. Therefore, text mining was applied to enable extracting and structuring information on causal mechanisms to create early life immune networks. In total 2,966 articles were selected using the search strings to explore literature databases. After manual screening 451 original manuscripts and 378 reviews were considered relevant (total number of selected 829 articles). This resulted in a selection of 249 articles for the

1st trimester of gestation, 296 articles for the 2nd trimester of gestation, 344 articles for the 3rd trimester of gestation, 252 articles for birth period, 287 articles for newborn period and 215 articles for the infant period. Please note that some articles covered multiple periods. From these full text articles, INDRA extracted resp. 2,101, 3,234, 3,654, 1,568, 2,917, and 1,487 unique relationships for the 1st, 2nd, 3rd trimester of gestation, birth, newborn and infant period (**Figure 2**). Next INDRA assembled,

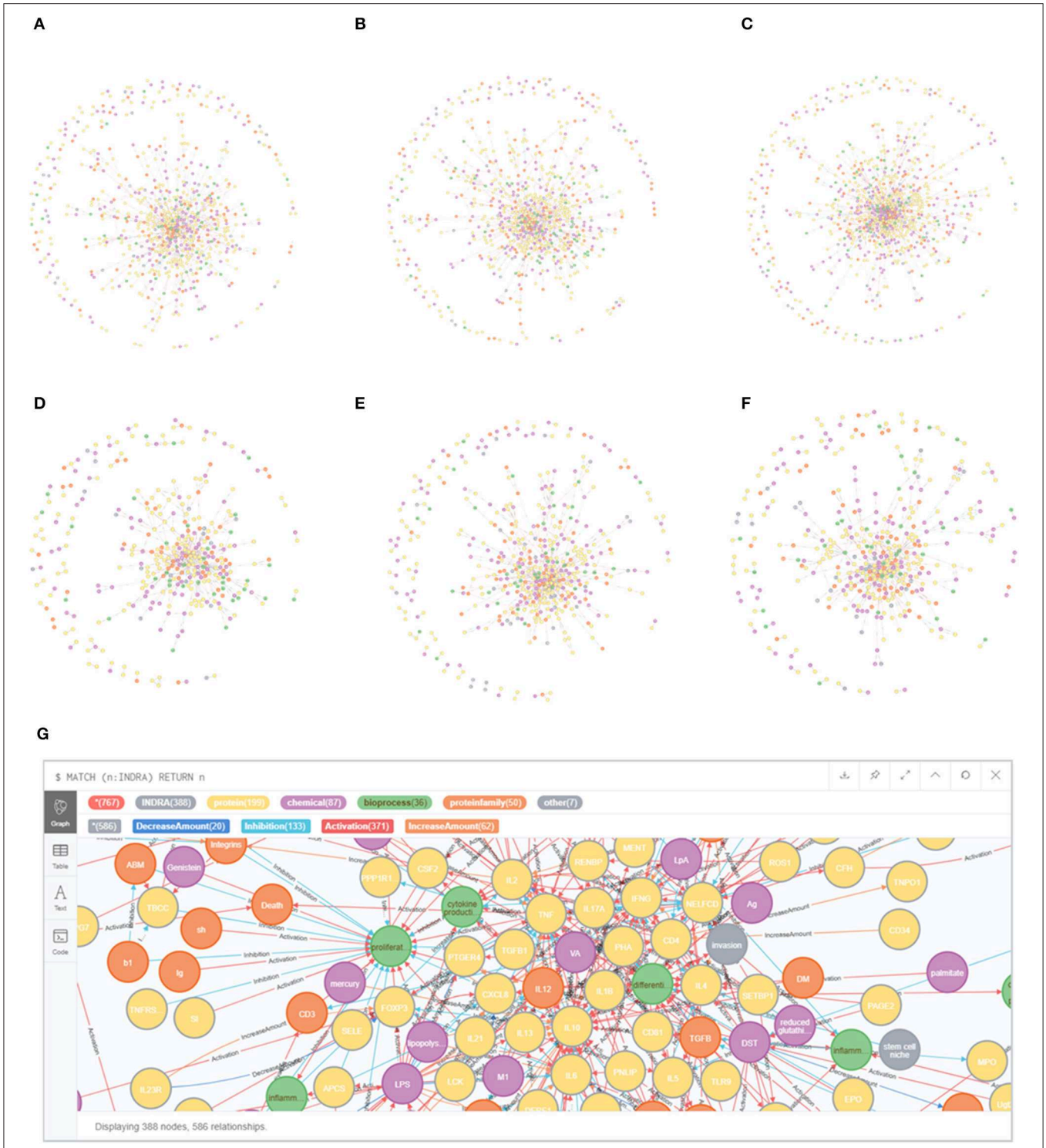

FIGURE 4 | Early life immune networks based on information from early life immune literature and enriched with info from databases and inference steps, each covering a different phase during early life. **(A–C)** EG, MG, and LG; **(D)** birth; **(E)** newborn (0–28 days); **(F)** infant (1–24 months). **(G)** magnification of infant.

de-duplicated and standardized all relationships into 6 large early life-immune networks each covering a different early life period. The Neo4j-based framework enabled the visualization of the early life immune networks as depicted in **Figure 4**. As the networks are very dense in terms of numbers of nodes and edges, it is impossible to extract information directly from these networks without bioinformatical tools. The reason to depict these "unreadable" networks is to illustrate the complexity and density of them. In our methodology we identified 107 genes that have been described in the selected early life literature already during gestation and remained expressed throughout the infant period (**Supplementary Figure 1**).

Denoising Early Life-Immune Literature Networks (Table 3)

Approximately 30% (range 27–32%, depending on early life period) of the connections (edges) between the genes coding for proteins described in the INDRA network were overlapping with the human gene-gene interactions present in the GeneMania consulted databases (**Table 3**), indicating that the denoising step reduced ∼70% (depending on the early life network) of the gene-gene connections in our network. This large reduction may be due to the fact that: (a) The gene-gene connection is solely relevant in early-life situations, which are not reflected in the GeneMania-consulted databases (which contain mainly adult data); (b) The gene-gene connection is non-human specific as the search strings for literature included guinea pig, rat, and mice; (c) Only genes that could be linked to a unique HUGO Gene Nomenclature Committee (HGNC) ID are recognized by GeneMania; and (d) The gene-gene connection is nonsense and should therefore be excluded. It must be noted that only the edges between the genes are removed, but the genes themselves remained part of the network. Although this elimination step possibly also eliminates some of the true early-life gene-gene interactions as suggested above, it is foreseen that this potential loss of information was compensated by the following enrichment steps.

TABLE 3 | Number of edges between genes described in early life (literature info) and their presence in the human GeneMania database.

Network	#Genes/proteins* in early life literature extracted by text mining	#Gene-gene edges in early life literature	#Edges confirmed in GeneMania (%)
EG	440	228	72 (32%)
MG	477	278	84 (30%)
LG	508	319	90 (28%)
Birth	225	162	49 (30%)
Newborn	291	249	68 (27%)
Infant	232	174	51 (29%)

EG/MG/LG, early/mid/late gestation.
**Sometimes it was not possible to distinguish protein names from corresponding gene names in literature. Therefore, all those names were annotated as being both a protein and a gene and regarded as 1 node in the network.*

Network Enrichments

The relationships of genes coding for the proteins that were identified in the early life networks by text mining were enriched by information retrieved from Gene Ontology and DisGeNET databases, respectively, is depicted in **Table 4**. After enrichment, the number of gene—bioprocess relationships were increased 60-fold (approximately). Of note, depending on the early-life time frame, DisGeNET databases introduced numerous gene-disease relationships (ranging from 1,719 to 4,568 relationships) to the early life immune networks. Other than this, the DisGeNET database not being specific to immune-related diseases, numerous non-immune diseases were also added to the early-life immune networks.

Subsequent addition of associations between bioprocesses and immune health endpoints (autoimmunity, hypersensitivity, resistance to neoplasms, resistance to infections) as previously described (16), further enriched the early life immune networks. As a final step in the network enrichments, the connections between bioprocesses and immune related diseases and immune health endpoints were inferred based on the knowledge of the number and the similarity of genes shared among the entities in different layers of the model (**Table 4** and **Figure 3**). The total number of nodes present in the early life immune networks

TABLE 4 | Results of enrichment/inference steps of the early life denoised INDRA immune networks.

	#Gene-bioprocess edges			#Gene-disease edges	#Bioprocess-immune endpoint edges	#Bioprocess-diseases edges	#Disease—immune endpoint edges
Source	Literature	GO-enrichment	GO-SLIM enrichment	DisGeNET enrichment	Meijerink et al. (16)	Inference	Inference
EG	149	9,546	443	3,894	1,121	1,701	1,023
MG	160	10,195	517	4,089	1,132	1,908	1,029
LG	180	10,968	546	4,568	1,246	2,207	1,136
Birth	67	3,929	168	1,719	695	1,073	627
Newborn	102	6,159	231	2,759	832	1,215	752
Infant	86	4,980	296	2,233	770	823	706

Depicted are the number of connections (edges) between biological entities (genes, bioprocesses, diseases, immune endpoints) added to the INDRA immune networks. EG/MG/LG, early/mid/late gestation.

TABLE 5 | Enriched early life immune network nodes.

Type of nodes	EG	MG	LG	Birth	Newborn	Infant
Proteins/genes*	440	477	508	225	291	232
Protein families	101	110	114	62	72	55
Chemicals	175	189	211	93	128	106
Bioprocesses**	51	56	58	36	39	34
GO processes	3,709	3,868	3,988	1,947	2,751	2,289
GOslim processes	55	55	55	59	60	59
Diseases	351	352	400	245	282	257
Immune health endpoint	4	4	4	4	4	4

*Depicted are the number of nodes in the networks after all enrichment/inference steps. These networks formed the basis of the gene prioritization (see **Table 6**). EG/MG/LG: early/mid/late gestation.*

**Using text mining, it was not always possible to distinguish genes from proteins (often same name used).*

***Bioprocesses identified by ontology of INDRA text mining tool.*

after the enrichment and inference steps are depicted in **Table 5**, indicating the complexity of the resulting 6 human early life immune networks.

Gene Prioritization to Identify Key Markers in Early Life

The enriched complex human early life immune networks formed the basis to identify the key markers in early life. The PageRank score of all nodes was calculated in the 6 human early life immune networks which resulted in 6 lists of prioritized immune markers each covering a different early life period (**Table 6**).

In general, the genes coding for the "commonly used immune markers" were highly ranked in all early life periods such as the cytokines including chemokines (e.g., *CXCL8*, *CXCL11*, *CXCL13*), interferons (*IFN*), interleukins (*IL1B*, *IL2, IL4, IL5, IL6, IL7, IL10, IL13, IL15, IL17A),* tumor necrosis factor (*TNF*), transforming growth *factor* (*TGFB*), and other immune activation regulators (e.g., *CD55, FOXP3, GATA3, CD79A, C4BPA*) directly involved in the immune response.

Comparison of the prioritized genes between the different early life periods (**Figures 5A,B**) showed that 36 genes were shown to be central in the network only during the gestational period, whereas others were more prominent in the periods birth, newborn and infant (6 genes: *RBP4*, *IL2*, *HAMP*, *env*, *ALG1*, and *IL1B*) or only in the infant period (14 genes: *TJP1, IL3, PIGS, ANPEP, CXCL11, CLCA3P, JAG1, NTAN1, CYYP1A2, CYP2E1, MADCAM1, VCAM1, GH1*, and *SCB*). Moreover, 11 genes were central in the early life immune networks covering all time periods: *TNF, IL6, IL10, CD4, FOXP3, IL4, NELFCD, CD79A, IL5, RENBP,* and *IFNG*. Most of these genes are immune related, however *RENBP*, renin binding protein, is an important regulator in the renin–angiotensin–aldosterone system. Moreover, *NELFCD*, Negative Elongation Factor Complex Member C/D, is an essential component of the NELF complex, which negatively regulates the elongation of transcription by RNA polymerase II.

Some of the top 50 genes were organ-specific such as *CPA1* (pancreas), *CRH* (neuronal), and *CDX2, MGAM, SI* (intestine). Other genes were specifically involved in pregnancy such as *ERVW-1*, *CSH1, PAEP*, or involved in early life growth, and maturation (e.g., bone/cartilage *CA2,* cell cycle related proteins *CAV1, PRC1*; matrix modulation *FGF4, MMP9, MMP2*) were also identified as central markers.

Interestingly, also a few non-human genes were selected in the top 50 lists (*lectin, cscK, lacZ, rpoD, dop, AtJ1, lanA1, env, ptc*), representing plant, bacterial or viral specific proteins

TABLE 6 | List of prioritized genes per early life time period.

	EG	MG	LG	Birth	Newborn	Infant
CPA1	0,45	0,45	0,45			0,45
CA2	0,41	0,41	0,41			
MMP9	0,41	0,41	0,41			
SMO	0,39	0,39	0,39			
lectin	0,32	0,32	0,32			
PGR	0,30	0,30	0,30			
PSMA7	0,28	0,28	0,28			
TLR2	0,28	0,28	0,28			
PRC1	0,34	0,34	0,34			
DNAJB6	0,28	0,28		0,28		
CASP2	0,29	0,29				
ERVW-1	0,29	0,29				
lanA1	0,28	0,28	0,28			
ATJ1	0,28	0,28	0,28			
IL13	0,51	0,49	0,51	0,26	0,25	
CAV1	0,30	0,30	0,30			
MALL	0,28			0,28		
SPIC	0,28				0,28	
AR	0,34				0,28	
CPM	0,28					
MMP2	0,28					
PTGS2	0,28					
TNIP1	0,28					
CDX2	0,41	0,41	0,41	0,28		
ADCY1	0,52	0,60	0,52			
CXCL8	0,42	0,42	0,41	0,28	0,33	
MRLN	0,31	0,31	0,30			
CXCL13	0,35	0,36	0,29			
DSP	0,29	0,29	0,29			
FGF4	0,31	0,32		0,21		
SIRT1	0,30	0,35				
CD55	0,31	0,31	0,31			
CSH1	0,29	0,29	0,29			
cscK	0,30	0,29	0,30		0,30	
IL7	0,31		0,37			0,23
AHR	0,28			0,28	0,30	
C4BPA	0,29	0,29	0,29			
TNF	0,88	0,89	1,10	0,53	0,41	0,28
CD4	0,45	0,53	0,63	0,42	0,40	0,43
PAEP	0,35	0,35	0,35			
CRH	0,33	0,33	0,33			
MENT	0,28	0,30	0,30			
IFN	0,46	0,42	0,61	0,55		
IL6	0,61	0,67	0,75	0,46	0,70	0,49
IL5	0,29	0,29	0,61	0,27	0,25	0,66
FOXP3	0,33		0,50	0,30	0,65	0,47
IL10	0,53	0,76	0,72	0,98	0,79	0,97
CD79A	0,30	0,31	0,31	0,41	0,52	0,53
NELFCD	0,30	0,41	0,57	0,92	0,76	0,68
IL4	0,31	0,37	0,50	0,66	0,86	0,94
PREP		0,53	0,53			
TXN		0,28				0,28
ptc		0,28				
TEAD1		0,28				
TNFSF11		0,29				
TLR3		0,44	0,61			0,29
HLA-G		0,51	0,66			
LGALS1		0,28	0,34			
GALT		0,30	0,30			
RENBP		0,37	0,34	0,31	0,37	0,38
PTBP1			0,39	0,36	0,30	
DNTT			0,28		0,28	
MTOR			0,28			0,28
KLRK1			0,28			
GATA3			0,30	0,39	0,24	

	EG	MG	LG	Birth	Newborn	Infant
CYP3A4			0,37		0,28	0,37
IFNG			0,33	0,47	0,36	0,41
ALG1				0,25	0,25	0,25
TGFB1				0,28	0,28	
Tnfrsf4				0,28	0,28	
ACKR2				0,25		
BCR				0,22		
CD19				0,22		
CREBBP				0,28		
EGR3				0,25		
GSR				0,21		
HPSE				0,21		
IL15				0,19		
IL17A				0,20		
lacZ				0,22		
NR3C2				0,21		
PTGER4				0,22		
RELB				0,24		
rpoD				0,22		
SLC14A1				0,28		
TBCC				0,27		
THPO				0,21		
RBP4				0,35	0,35	0,35
BTN3A1				0,24	0,25	
SET				0,21	0,24	
HAMP				0,29	0,28	0,40
LILRB4				0,31		0,38
LILRB2				0,21		0,28
env				0,25	0,27	0,26
IL2				0,30	0,34	0,45
IL1B				0,24	0,27	0,34
SGPL1					0,39	0,39
CDH1					0,28	0,28
CP					0,28	0,28
DCT					0,28	0,28
dop					0,28	0,28
FOXK2					0,28	0,28
TECR					0,28	0,28
ACAD8					0,28	
CSF1R					0,28	
GATA6					0,28	
GC					0,28	
HA					0,29	
HPSE					0,24	
IRAK1					0,23	
TLR4					0,29	
MGAM					0,23	0,36
SI					0,23	0,36
IGF1					0,23	0,28
TEC					0,26	0,28
TJP1						0,32
IL3						0,29
ANPEP						0,28
CLCA3P						0,28
CXCL11						0,28
JAG1						0,28
PIGS						0,28
NTAN1						0,25
CYP1A2						0,24
CYP2E1						0,24
MADCAM1						0,23
VCAM1						0,23
GH1						0,23
SCB						0,23

*The PageRank score of all nodes was calculated for each gene in order to identify the most "central" genes in the networks. The top 50 genes (i.e., highest PageRank score) per network are depicted, including their PageRank score. EG/MG/LG, early/mid/late gestation. Descriptions of the genes are described in **Supplementary Table 1**. The light to dark green-gradient reflects the increase in PageRank score.*

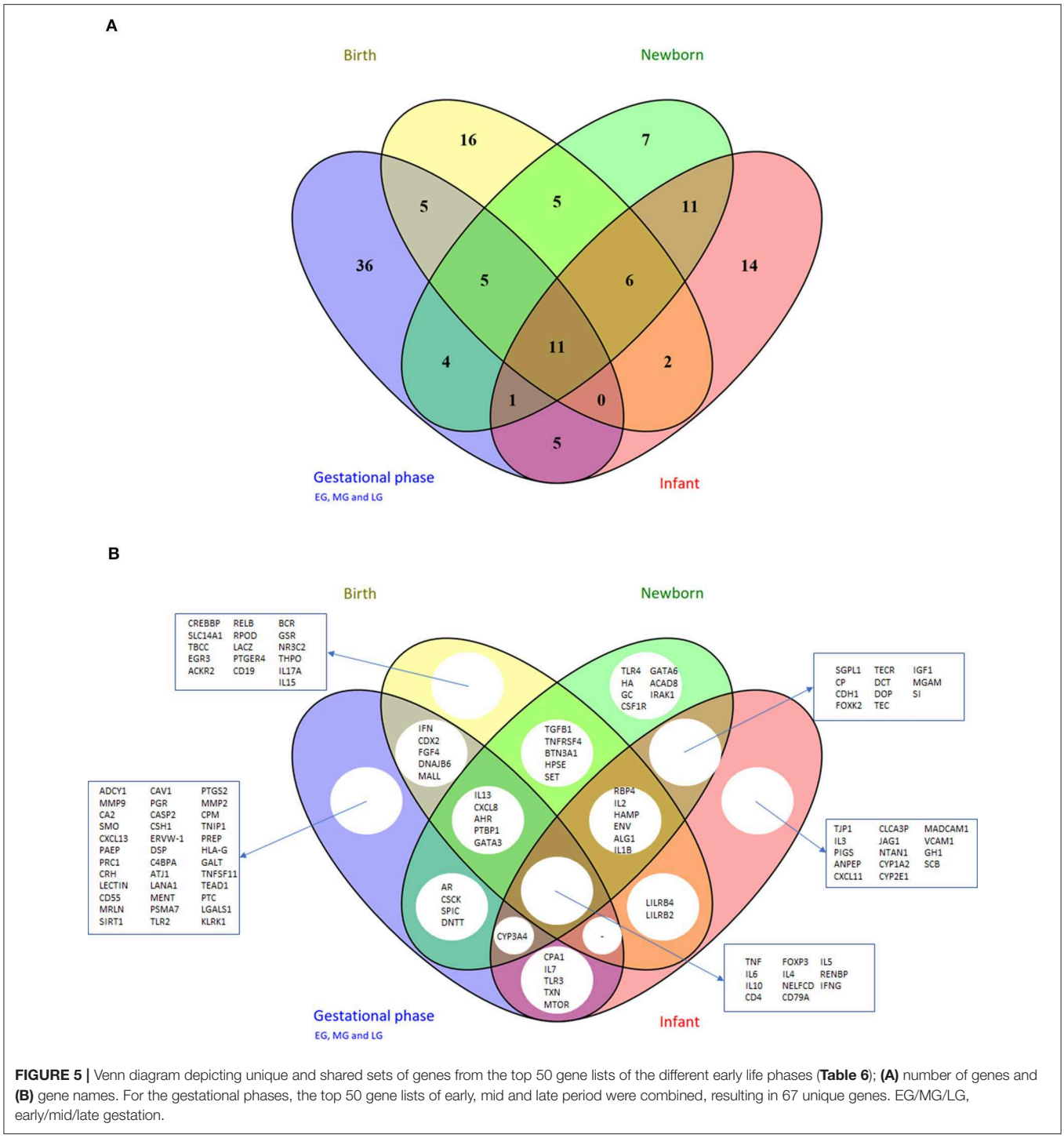

FIGURE 5 | Venn diagram depicting unique and shared sets of genes from the top 50 gene lists of the different early life phases (**Table 6**); **(A)** number of genes and **(B)** gene names. For the gestational phases, the top 50 gene lists of early, mid and late period were combined, resulting in 67 unique genes. EG/MG/LG, early/mid/late gestation.

as key markers. So although the GeneMania denoising step eliminated the gene-gene edges of non-human genes, these non-human genes got central positions in the enriched early life networks.

Concluding, the PageRank analyses resulted in the identification of key early life genes with overlapping genes between the different early life periods, but also genes which were only described in a certain early life period. Moreover, the PageRank analyses confirmed the central role of the "commonly used immune markers" (cytokines, chemokines) in the early life networks, but also identified less obvious key marker candidates.

DISCUSSION

In this paper, we describe an approach to construct early life immune networks to identify and prioritize factors to assess safety and efficacy of early life immune modulation. As an alternative to expensive, hand-built models which can take months to years to construct, a workflow was created to generate causal early life immune networks. Literature-based interactions were used to form the basis of the network. These literature networks were denoised using GeneMania databases and enriched with data from comprehensive databases, such as Gene Ontology and DisGeNET. Thereafter, PageRank algorithm was applied to prioritize candidate genes in the early life networks. The entire pipeline is interpretable and intervenable in a way that domain experts can use our tools to greatly reduce the time required to identify relevant immune markers in early life.

Early life in humans is associated with large developmental milestones in the immune system.

Innate and adaptive immune cells are present early in the fetus during gestation and then expand significantly (8, 22). However, though the innate and adaptive immune cells are already present early during fetal development in the first trimester of gestation, the strength of their effector functions differ considerably from the adult situation. For instance, mature neutrophils are moderately present at the end of the first trimester, and increase steeply in number shortly before birth. Their number then returns to a stable level within days, but they show weak bactericidal functions, poor responses to inflammatory stimuli, reduced adhesion to endothelial cells and diminished chemotaxis (23).

Compared with the adult immune system, which has matured and evolved after years of exposure to antigens and environmental stimuli, the newborn immune system comes from a relatively sterile environment and is then rapidly exposed to microbial challenges (10). It is well-established that these differences in exposure to antigens and environmental stimuli have consequences when examining disease susceptibility. Severe infections remain a leading cause of neonatal morbidity and mortality. The immaturity of the immune system is thought to be an important factor for the increased rate of neonatal infections especially when born preterm but the basis for this is not fully understood (12), although the maturation of the neutrophil and endothelial adhesion function are thought to contribute significantly to the high risk of life-threatening infections in premature infants (23).

Many of our preventive strategies for neonates rely upon our understanding of the adult immune system, because of our limited knowledge of early life immunity. Therefore, there is no consensus regarding which factors should be covered to evaluate the safety and/or efficacy of the early life interventions and how all the available data should be interpreted appropriately. Our bioinformatics approach assumes that the functions of genes and proteins do not change over time. Instead, the biological balances between gene-sets expressed in early life and adult are assumed to change e.g., lower FOXP3 and CTLA-4 expression in activated regulatory T cells from human neonates compared to the adult situation (24). Therefore, the enrichment steps using information from databases (GO and DisGeNET) containing mostly data from adult situations, are assumed to be suitable to enrich the networks with functionalities of the genes/proteins that are described in early life literature. As input for these databases, only genes shown to be expressed in a specific early life period were entered to exclude the possibility that genes/proteins that are not (yet) expressed in that specific time frame would be introduced in the network. As others, we suggest that not the gene function as such, but the context in which the genes are expressed in early life determines the impact of the gene expression on the biological processes, cellular responses and/or cellular phenotype of the immune cell. Especially the microbial context has been suggested to be important: the interactions between the developing immune system and the microbes colonizing the intestine, skin and airways of a newborn child has been suggested by several groups (11, 25, 26). Olin et al. (11) showed that the microbiome diversity increased after birth but children with exceptionally lower diversity indicating bacterial dysbiosis (and high level of activated T cell populations) showed an increased immunological heterogeneity at 3 months of age. Several key immune cell populations (DCs, B cells, NK cells), reach adult-like phenotypes during the first 3 months of life, which suggests that environmental exposures during this period could have influence later in life. For example, differential susceptibility to autoimmunity and asthma may relate to DC exposure to bacterial antigens early in life, which could lead to more tolerogenic DCs later in life (27–29).

Currently only a few biomarkers of inflammation have been developed into biomarker assays approved and recommended by regulatory bodies for use in clinical studies, which includes CRP, TNF-α, serotransferrin and erythrocyte sedimentation rate (30). Although many candidate markers are identified based on preclinical and clinical studies (as listed in the Thompson Reuters Integrity^{SM} Biomarkers Database), only a few are further validated and used for assay development highlighting the classical to clinical biomarkers gap. Moreover, in early life the identification of suitable markers is even more limited due to the fact that immunological studies on newborns tend to be small-scale and focus only on few factors because of limited sample volumes and low-throughput techniques as noted by Schaffert at al. (10). The early life immune networks generated in our approach enabled us to identify and rank genes that have the most central role in the early life immune networks. This is in contrast to earlier identified candidate markers for (pre-) clinical studies which are not specifically aimed at early life and not necessarily prioritized in a biological context.

There are multiple ways to prioritize genes in a biological network (31, 32). In computing network scores, most of the current approaches yield the limitation that the full network topology (systems approach) is not taken into account. Instead, such scoring methods focus on direct links or the most direct paths (shortest paths) within a constrained neighborhood around genes, ignoring potentially informative indirect paths. By applying PageRank algorithm, the full topology of the immune networks is taken into account.

Comparing the top 50 genes of the early life networks of the different time frames shows that many genes are already described in literature early in gestation. In general, the genes coding for the "commonly used immune markers" were highly

ranked in all early life periods such as the cytokines including chemokines and other immune activation regulators directly involved in the immune response. Interestingly, transcription factors GATA-3 and FOXP3 that regulate Th2 and T regulatory cell development are highly ranked in the networks, whereas the gene coding for T-bet (*TBX21*), the transcription factor for Th1 differentiation, was in the lower regions of the priority lists. It has been shown that these 3 transcription factors cross regulate one another: T-bet modulates GATA-3 function and Th2 cytokines block Th1 differentiation (33–36). Additionally, GATA-3 has been shown to inhibit FOXP3 transcription by binding to the FOXP3 gene promoter (37). The low priority ranking of the gene coding for T-bet is in line with the current view of an unbalanced Th1/Th2 neonatal immunity resulting in skewing toward Th2 immunity. Moreover, the genes related to Th17 responses [transcription factor gene coding for RORγT (*RORC*) and *IL17A, IL17F*, and *IL22*], are also of low priority (not in top 50) in the networks. In the context of the neonatal Th2-biased immune response, the inhibitory effect of IL-4 on the development of inflammatory Th17-type responses has been described to represent a major regulation mechanism (38) which may explain the low priority of Th17 related genes and the high priority of IL-4 in the early life networks.

Several non-human genes (lanA1, cscK, dop, rpoD, lacZ, env, ptc, lectin) were ranked in the top 50, which might seem unexpected or perhaps even suggest a flaw in the bioinformatics approach. However, their presence and relevance may well-explained. In our workflow in the denoising step using GeneMania, we removed the connections between genes that were not of human origins, but we did not exclude the non-human genes from the early immune networks: the non-human genes remained in the network as disconnected nodes.

The next step in the generation of early life immune networks was the addition of connections (edges) between the human and non-human genes to human pathways/diseases/bioprocesses (input DisGeNet and GO databases). Genes from rat/mouse/guinea pig will likely not be connected to human processes, so these genes will stay disconnected to the network and therefore have a very low priority in the PageRank scoring. However, some of the non-human genes from mainly viral or microbial origin could be connected in our workflow to multiple human processes/diseases and therefore turned out to be in the top 50 of the PageRank scoring. The relevance of the role of these non-human genes in immune responses could be confirmed by literature: lanA1 (viral protein LanA1; role in host-virus interaction) (39), cscK (bacterial fructokinase; role in TLR4 activation) (40), dop (bacterial pup deamidase; role in resistance to infection) (41), rpoD (bacterial sigma factor for RNA polymerase; role in exponential growth bacteria) (42), lectin (role in activation of innate immune system) (43), lacZ (bacterial beta-galactosidase; Th1-associated) (44), env (viral envelope glycoprotein gp160; role in immune evasion) (45).

Several genes, which are usually not regarded as immune-related, got a prominent position in our early life immune networks such as genes involved in pregnancy, growth, and maturation (e.g., *ERVW-1*, *CSH1, PAEP*, *CA2, CAV1, PRC1*, *FGF4, MMP9, MMP2*). Several intestinal digestion related genes (*MGAM, ANPEP, SI*) were present in the top 50 in the birth-newborn-infant networks, which might be related to start of oral diet after birth. These examples emphasize the role of the immune system on so many other non-immune bioprocesses, which should be taken into account during assessment of possible (side-)effects of immune modulation in early life. Indeed, several chemokines and cytokines selected in our workflow, such as CXCL8, IL-10, TNF, IL1B, TGFb are multifunctional molecules initially described as having a role in endometrial functions and play a role in appropriate embryo implantation or placental functioning (46, 47). Moreover, TNF and TGFb have been identified as core activators of epithelial to mesenchymal transition, which is essential for embryonic development (48, 49). Although our approach to collect and structure and prioritize all available information from literature and databases to identify candidate markers is exhaustive, it also has its limitations due to the natural limitations in the curation process of the usage of enrichment tool-dependent auxiliary databases, and to inaccuracies derived from text mining. Others being annotation issues, such as the incomplete annotation of genes to GO terms and diseases (50, 51). Furthermore, the approach might be subjected to a reporting bias as it can be difficult to distinguish the absence of a gene in early life or a relationship between molecules/pathways from a lack of evaluation. In addition, we do not take the context of the gene expression into account whereas it is known that the context determines greatly the impact of the genes on biological processes, cellular responses and/or cellular phenotypes of the immune cells. Also, the networks are not organ-specific, although organ-specific genes are in the top 50 of prioritized genes, such as *CPA1* (pancreas), *CRH* (brain), and *CDX2, MGAM, SI* (intestines).

The strength/weight of the relationships in the network were not taken into consideration, but merely 6 association networks have been generated of possible biological relationships in early life immunity. The next important step for the applicability of this approach would be to validate these relationships based on gene expression data, which will guide us to validate the networks and moreover enable us to finetune the weighing of the various relationships in the network. This may result in a re-prioritization of the most important genes in a specific period in early life. Moreover, by using gene expression data, it becomes possible to identify critical time frames for specific immune modulation, because depending on the exposure, different pathways/processes may be activated. Even taking into account these current limitations, to the best of our knowledge, this is the first global overview of the early life immune system that can be used as a starting point to select putative markers to monitor the functioning of the early life immune system.

The future step would be to enrich the early life immune networks with early life gene-expression data to generate a quantitative early life immune network for (i) the analysis of mechanisms underlying immune health and disease in early life and (ii) the validation of candidate markers of disease and health.

In conclusion, we describe a network-based approach that provides a science-based and systematic method to explore the functional development of the early life immune system in time. This systems approach aids the generation of a testing strategy for

assessing the safety and efficacy of early life immune modulation by predicting the key candidate markers during different phases of early life immune development.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

JB, MM, LV, and SK contributed to the conception and design of the study. MM, MYM, and TR performed literature database searches and selection. RD, MS, and SK wrote scripts for the preprocessing of the manuscripts, GeneMania denoising, GO/DisGeNET database-searches and inference steps and PageRank algorithm score calculation. JB, MM, and SK wrote the manuscript. JB, RD, MS, MYM, TR, LJ, AK, JG, LK, KK, GH, LV, MM, and SK contributed to manuscript revision, read and approved the 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/fimmu.2020.00644/full#supplementary-material

Supplementary Figure 1 | Venn diagram depicting unique and shared sets of genes of the different early life phases extracted from literature by INDRA text mining (**Table 3**) without PageRank prioritization step. EG/MG/LG, early/mid/late gestation.

Supplementary Table 1 | Descriptions of the prioritized genes listed in **Table 6**.

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Microbiome Composition and Its Impact on the Development of Allergic Diseases

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Allergic diseases, such as food allergy (FA), atopic dermatitis (AD), and asthma, are heterogeneous inflammatory immune-mediated disorders that currently constitute a public health issue in many developed countries worldwide. The significant increase in the prevalence of allergic diseases reported over the last few years has closely paralleled substantial environmental changes both on a macro and micro scale, which have led to reduced microbial exposure in early life and perturbation of the human microbiome composition. Increasing evidence shows that early life interactions between the human microbiome and the immune cells play a pivotal role in the development of the immune system. Therefore, the process of early colonization by a "healthy" microbiome is emerging as a key determinant of life-long health. In stark contrast, the perturbation of such a process, which results in changes in the host-microbiome biodiversity and metabolic activities, has been associated with greater susceptibility to immune-mediated disorders later in life, including allergic diseases. Here, we outline recent findings on the potential contribution of the microbiome in the gastrointestinal tract, skin, and airways to the development of FA, AD, and asthma. Furthermore, we address how the modulation of the microbiome composition in these different body districts could be a potential strategy for the prevention and treatment of allergic diseases.

Keywords: allergy, asthma, atopic dermatitis, food allergy, health outcomes, immune system, children, microbiome

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INTRODUCTION

Over the last few decades, many developed and fast-growing countries worldwide have registered a dramatic increase in the prevalence of allergic diseases, such as asthma, AD, and FA, which currently pose a substantial burden to healthcare systems (1, 2). Thus far, the genetic and environmental drivers of the rapid rise in allergy prevalence remain to be more fully elucidated.

Notably, the evolution of the allergy epidemic has closely paralleled radical environmental and lifestyle changes, such as progressive industrialization and urbanization, widespread sanitation programs and antibiotics use, physical inactivity and highly processed diets. All these changes have led to reduced microbial exposure in early life and loss of microbial biodiversity (3).

Abbreviations: AD, atopic dermatitis; BM, breast milk; CMA, cow's milk allergy; FA, food allergy; SCFA, short-chain fatty acid; TLRs, Toll-like receptors; Treg, regulatory T cell.

Accumulating evidence points to a central role of the human microbiome perturbation in the rising prevalence of allergic diseases. The human microbiome comprises bacteria, viruses, fungi, protozoans, and archaea, which colonize primarily the gastrointestinal tract, but also the airways and the skin surface from the first days of life and gradually develop and diversify concomitantly with the physiological growth of the individual. The resident microbial communities in the human gut and other organs have been shown to modulate both the innate and acquired immune responses. Recent data show that several environmental drivers can affect the microbiome colonization, composition and metabolic activity in infancy, and alter the host functions for nutrition and immunity (4). Indeed, the process of early colonization by a "healthy" microbiome is emerging as a key determinant of life-long health, whereas the perturbation of such a process, has been associated with greater susceptibility to immune-mediated disorders later in life, including allergic diseases (5).

The recent introduction of the next-generation sequencing and genomic analysis to identify different microbial species has led to a greater knowledge of the complex role of the human microbiome in the pathogenesis of FA, AD, and asthma. Here, we review recent findings on the potential role of the human microbiome in the gastrointestinal tract, the skin, and the airways to the development of allergic diseases, and we address how the modulation of the microbiome composition could be a potential therapeutic or even preventive strategy for such disorders.

EARLY LIFE FACTORS MODULATING GUT MICROBIOME COMPOSITION

It is well established that microbiome composition changes dynamically in the first few years of life and can be influenced by several prenatal and postnatal environmental and host-related factors (**Figure 1**) (6). Among these factors, mounting evidence shows that some perinatal factors, such as mode of delivery, breastfeeding, early antibiotic use, and timing and type of complementary feeding, can significantly modulate the gut microbiome composition, which is emerging as a key determinant in developing immune tolerance responses to different antigens (7). The gut microbiome of newborns delivered by cesarean section shows a lower level of commensal bacteria typically found in those born vaginally and high concentrations of opportunistic pathogens typically found in the hospital environment, such as *Enterococcus*, *Enterobacter*, and *Klebsiella* species (8). These differences largely even by the time babies are weaned around 6 to 9 months, except for commensal bacteria *Bacteroides*, which remain absent or at very low levels in most cesarean section infants. Of note, the effect of the cesarean section on the infant microbiome seems to be related to maternal antibiotic exposure before the delivery (8).

Breast milk contributes to the development of healthy gut microbiome. BM contains essential micronutrients and prebiotic compounds, which support the colonization and growth of commensal bacteria, and several immune active factors, oligosaccharides and microbes, which could all modulate host immune responses (9). Term infants born vaginally and breastfed exclusively seem to have the most "beneficial" gut microbiome, with the highest concentration of ***Bifidobacteria*** and lowest numbers of ***Clostridium difficile*** and ***Escherichia coli*** (10).

Shifting from exclusively breastfeeding to complementary feeding at weaning increases the prevalence of *Bacteroides, Bilophila, Roseburia, Clostridium, and Anaerostipes,* and progressively leads to the establishment of an adult-type microbiome (11). In particular, the introduction of solid foods modulates gut microbiome shifting from *Bifidobacterium*-dominant to *Bacteroidetes*- and *Firmicutes*-dominant species, such as the *Clostridium coccoides* and *Clostridium leptum* groups (12). The introduction of solid foods also induces a sustained increase in fecal SCFA levels and expression of genes associated with the adult microbiome's core metabolic functions, such as polysaccharide breakdown, vitamin biosynthesis, and xenobiotic degradation (13).

The first 1000 days of life (i.e., the period from conception to age 2 years) seem to represent the critical window of opportunity for microbiome modulation (**Figure 1**) (6, 14). After this period, the gut microbiome tends to acquire an adult-like configuration with distinct microbial community composition and functions (15). However, several factors can induce significant perturbations to the gut microbiome composition later in life, such as long-term dietary changes, or frequent or prolonged use of antibiotics (13, 16). Notably, a very recent multi-omics integrative analysis showed that antibiotic use in adults induced alterations to the gut microbiome which adversely affected immunogenicity and responses to influenza vaccination (17).

HOW THE GUT MICROBIOME CAN INFLUENCE IMMUNE RESPONSES

Neonatal and infant gut microbiome appear to be involved in gut tolerance modulation and immune system "education" (18, 19). Germ-free animal experiments best describe this mutualistic relationship in animals (20–27). These data may support such a relationship in humans.

Indeed, some recent human studies have addressed the role of the gut microbiome on adaptive and innate immunity in the context of allergic diseases. Christmann et al. (28), reported lower IgG responses to specific clusters of microbiota antigens in infants who then developed allergic disorders in childhood (including skin, respiratory, and food allergies) compared to healthy children. West et al. (29), studied infants at high risk of atopic diseases and showed that depletion of *Proteobacteria* in early infancy is associated with increased Toll-like receptors (TLR)-4 induced innate inflammatory responses, whereas depletion of *Ruminococcaceae* is associated with increased TLR-2 induced innate inflammatory responses. Fujimura et al. (30), reported that infants at risk of asthma have a gut microbial signature with reduced abundance of certain bacteria taxa, such as *Faecalibacterium* and *Bifidobacterium*. Stimulation of adult PBMC with sterile fecal water from these infants then

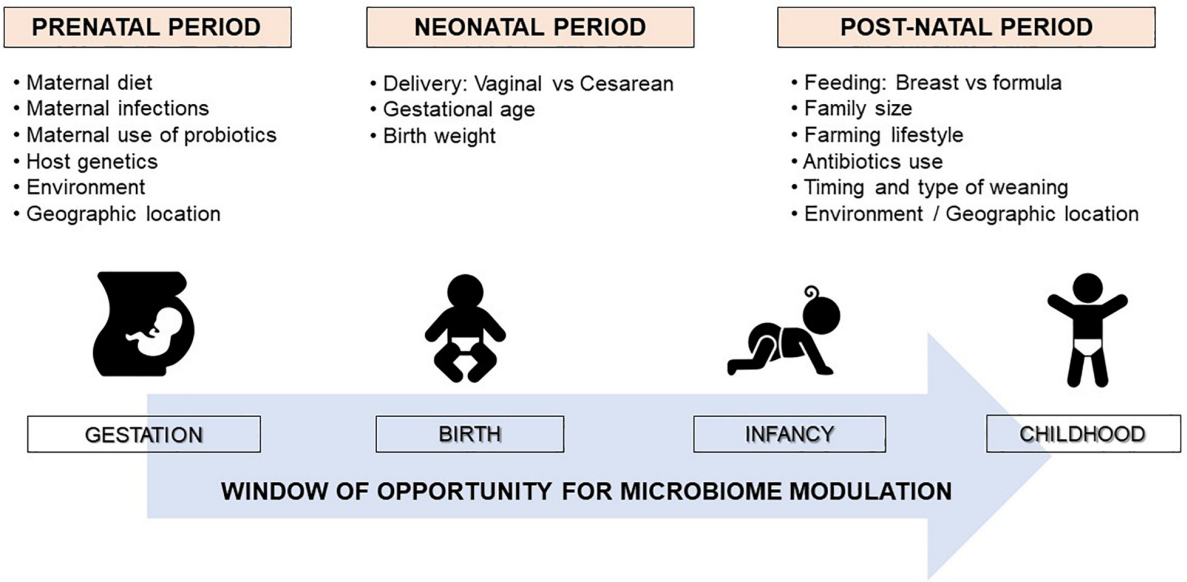

FIGURE 1 | Factors shaping the human microbiome development. The neonatal microbiome is a delicate and highly dynamic ecosystem that undergoes rapid changes in composition in the first few years of life determined by several pre and perinatal factors. The maturation of the gut microbiota toward an adult-like structure largely occurs by the age of 2 or 3 years. Therefore, early infancy could be a critical period for modulating the microbiota to promote healthy growth and development.

led to increases in CD4 + IL-4 producing cells and reduced regulatory Foxp3 cells. Similarly, Sjödin et al. (31), found that the gut symbiont *Faecalibacterium* correlated with the expression levels of regulatory cytokines in children with multiple allergies, suggesting an opportunity to expand such taxa to promote a regulatory tolerogenic immune response.

ROLE OF THE MICROBIOME IN THE DEVELOPMENT OF ALLERGIC DISEASES

The composition of the microbiome varies across different body sites, which constitute unique habitats resulting in varied microbial communities within and between subjects. The greatest concentration and diversity of microorganisms are found in the gastrointestinal tract, which is dominated by facultative and strictly anaerobic bacteria of the phyla *Firmicutes, Bacteroidetes, Actinobacteria, Verrucomicrobia*, and *Proteobacteria* (32).

The mechanisms that mediate host-microbe communications are highly complex; a disrupted dialogue due to altered microbiome seems to negatively impact the immune homeostatic networks and may contribute to the development of hypersensitivity reactions to environmental allergens (33). This connection emerged over the last few decades with the proposed "hygiene hypothesis," based on the epidemiological evidence that environmental drivers increasing early life microbial exposure (such as vaginal delivery, farming life, and furry animals exposure during childhood, large family size, unpasteurized milk consumption and absence of early antibiotic exposure) were associated with a lower risk of developing allergic disorders (34–38). Recent experimental and human investigations have strengthened the mechanistic substance to the hygiene hypothesis, providing evidence for the causal relationship between early life microbial perturbation in the gut, skin, and airways and the development of allergic diseases (**Figure 2**).

Microbiome and Food Allergy

The composition and metabolic activities of the gut microbiome seem to be closely linked with the development of oral tolerance (39). Mortha et al. (40), showed that commensal microorganisms favor the crosstalk between innate myeloid and lymphoid cells that contributes to immune homeostasis in the gut and the development of oral tolerance to dietary antigens.

Infants with CMA have more total bacteria, in particular the anaerobic type, compared to healthy controls after 6 months of milk formula assumption. In addition, higher concentrations of *Lactobacilli* and lower concentrations of *Enterobacteria* and *Bifidobacteria* were observed in infants with CMA (41). Bunyavanich et al. showed that *Clostridia* and *Firmicutes* rates were particularly elevated in the gut microbiota of infants whose CMA resolved by 8 years of age (42). Fazlollahi et al. found that the gut microbiome of children with egg allergy had a greater abundance of the genera from *Lachnospiraceae* and *Ruminococcaceae* than those of healthy controls (43). A prospective study comprising 14 children with FA and 87 children with food allergens sensitization, showed that *Haemophilus, Dialister, Dorea,* and *Clostridium* genera were reduced in participants with food sensitization, whereas, the

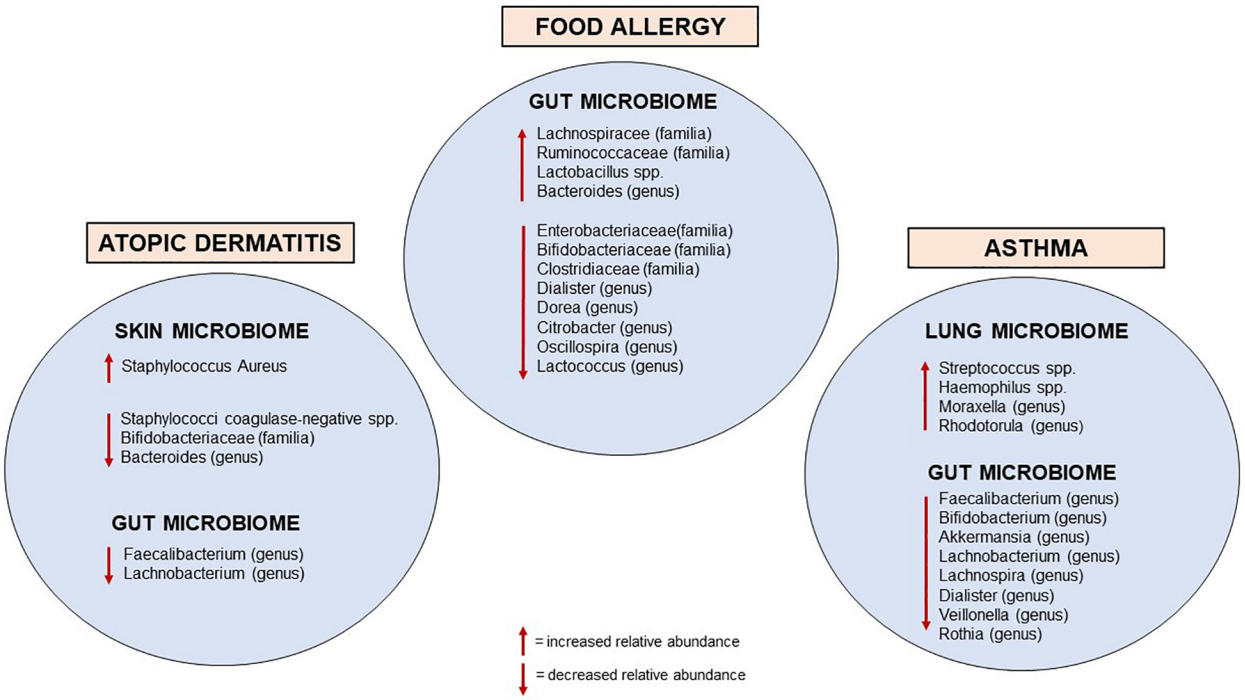

FIGURE 2 | Currently known microbiome perturbations in infancy associated with allergic diseases.

genera *Citrobacter, Oscillospira, Lactococcus,* and *Dorea* were under-represented in participants with FA (44). Furthermore, in subjects with peanut or tree nut allergy, decreased microbial richness and increased concentration of *Bacteroides* species were reported compared to non-allergic controls (45).

Studies in animal models showed that germ-free mice were protected from developing anaphylaxis to cow's milk if colonized with gut microbiome from healthy infants, but not from infants with CMA (46). The transfer of specific bacterial strains, such as *Bifidobacterium* or *Clostridium* species to mice was shown to reducing the risk of food sensitization, by the induction of mucosal Treg (47). *Clostridia* can also stimulate innate lymphoid cells to produce IL-22, which contributes to strengthen the epithelial barrier and decrease the permeability of the gut to dietary proteins (48). Some functional effects of *Clostridia* in FA likely also occur through their fermentation metabolites, such as butyrate, a SCFA with known immunoregulatory and tolerogenic proprieties (49).

Experimental findings showing that the gut microbiome contributes to the development of food tolerance suggest that microbial modulation could be a potential therapeutic strategy for FA. Although the supplementation of an extensively hydrolyzed milk formula with *Lactobacillus casei* and *Bifidobacterium lactis* did not prove to accelerate the resolution of CMA (50), the administration of extensively hydrolyzed casein formula containing the probiotic *Lactobacillus rhamnosus GG* has been shown to promote CMA resolution at 12, 24, and 36 months, compared to non-supplemented hypoallergenic milk formula (51). Of note, the use of such *Lactobacillus rhamnosus GG*-supplemented formula significantly expanded butyrate-producing bacterial strains in the infant gut microbiome compared to non-supplemented formula (49). In another study, the use of an amino-acid based formula containing a specific synbiotics (i.e., a combination of prebiotic blend of fructo-oligosaccharides and the probiotic strain *Bifidobacterium breve* M-16V) has been shown to modulate the gut microbiome and its metabolic activities also in infants with non-IgE mediated CMA (52–54). Recently, an uncontrolled study suggested that oral supplementation with *Lactobacillus rhamnosus GG* could enhance the efficacy of oral immunotherapy in inducing peanut tolerance and immune changes in children with peanut allergy (55). However, further studies including a control group are required to determine whether modulation of the microbiome during immunotherapy will favor the acquisition of sustained unresponsiveness to food allergens.

Microbiome and Atopic Dermatitis

Several factors, such as age, gender, ethnicity, climate, ultraviolet exposure, and lifestyle drivers, can influence the composition of skin microbiome (56). The healthy skin microbiome is represented by *Propionibacterium species*, which are mainly found in sebaceous sites, and *Corynebacterium* and *Staphylococcus* species, which are more abundant in moist microenvironments. *Malassezia* is the predominant fungal flora on human skin (56, 57).

Atopic dermatitis is a complex skin disease characterized by epidermal barrier dysfunction, altered innate/adaptive immune responses and impaired skin microbial biodiversity (58). Loss of

microbial diversity, with the predominance of the *Staphylococcus aureus* over *Staphylococcus epidermidis*, is a characteristic feature at both acute and chronic skin sites of AD (59), which correlates with AD severity and the risk of allergic sensitization to common allergens (60). *Staphylococcus aureus* contributes to the epidermal barrier disruption through different pathways, including the downregulation of terminal differentiation of epidermal proteins, such as filaggrin and loricrin, and the promotion of the skin proteases activities, which directly damage the skin barrier (61, 62).

Coagulase-negative *Staphylococci*, which include *S. epidermidis, S. hominis* and *S. lugdunensis*, can secrete antimicrobial metabolites that limit *S. aureus* overgrowth and biofilm formation (61). In addition, *S. epidermidis* can also activate TLR2 signaling, which can induce the production of keratinocyte-derived antimicrobial peptides and increase the expession of epidermal tight junction proteins (63). Neonatal colonization of the skin by *S. epidermidis* is associated with the induction of specific Tregs that modulate local activation of host immune responses (64). Indeed, it has been recently shown that skin commensal *Staphylococci* species are significantly reduced at 2 months in infants who later developed AD at 1 year, suggesting that targeted topical modulation favoring early colonization with this genus might reduce the risk of later occurrence of AD (65). These findings, together with evidence that regular application of moisturizers repairs the skin barrier and restores commensal bacterial diversity (66–68), constituted the rationale for ongoing research on the application of topical probiotics, such as *Vitreoscilla filiformis* lysate and *Roseomonas mucosa,* as a potential strategy to modulate the skin microbiome and treat AD (69, 70). Preliminary data also showed that the autologous skin transplantation of antimicrobial coagulase-negative *Staphylococci* strains to human subjects with AD could decrease *S. aureus* overgrowth and colonization (71).

Changes in the gut microbiome seem also to contribute to the development of AD. Patients with AD have lower concentrations of *Bifidobacterium* in the gut microbiome than healthy controls, and these counts are inversely related to the severity of the disease (72). Early gut colonization with *Clostridium difficile* was related to the occurrence of AD (73), and lower *Bacteroidetes* diversity at 1 month was associated with AD at 2 years of age (74). There is evidence that pre- and post-natal supplementation with oral *Lactobacillus* and *Bifidobacterium* strains could reduce the risk of AD in infants due to changes in T cell-mediated responses (75). Finally, a recent large prospective study of gut microbiota showed that *Lachnobacterium* and *Faecalibacterium* were significantly less abundant in those children who developed AD by school-age compared to healthy controls. Notably, the differential abundance of these bacterial taxa was documented throughout infancy, which supports the likelihood of their protective role in the development of AD (76).

Microbiome and Asthma in Childhood

Accumulating evidence shows that the composition of the lung microbiome in early life can affect the development of respiratory health or disease (77, 78). Preclinical models support a protective role of bacteria against allergic airway inflammation (79, 80).

The phylum *Bacteroides,* particularly *Prevotella* spp., predominate in the lung microbiome of healthy subjects (81, 82). During the first 2 weeks of life, the lung microbiome promotes the transient expression of programmed death-ligand 1 (PDL1) in dendritic cells, which is necessary for the Treg-mediated attenuation of allergic airway responses (83). Epidemiological evidence shows that children who grow up in a farming environment and are exposed to diverse microbial communities since early life have a lower incidence of allergies (84). Notably, the airway colonization by *Streptococcus, Moraxella,* or *Haemophilus* within the first 2 months of life has been associated with the severity of lower respiratory viral infection in the first year of life, and the risk of asthma development later in life (85). The phylum *Proteobacteria* has also been associated with asthma and neutrophilic exacerbations, whereas *Bacteroidetes* with eosinophilic exacerbations, leading to the consideration that distinct mediators and microbiome profiles may represent different clusters of biological exacerbations (86, 87).

Emerging evidence shows that gut microbial perturbations in early life can also influence the development of allergic airway inflammation. Antibiotic use in neonatal mice favors variations in the microbiome composition, which have been associated with alterations in intestinal Tregs and increased susceptibility to airway hyper-responsiveness (88). Similarly, pre- and post-natal exposures to antibiotics in humans have been associated with an increased risk of developing asthma (89). In a recent longitudinal study, Galazzo et al. (76), showed that the bacterial genera *Lachnobacterium*, *Lachnospira* and *Dialister* were significantly decreased in the gut microbiome of infants who developed asthma by school-age compared to healthy controls. Analysis of the gut microbiome at 3 months of age within the Canadian Healthy Infant Longitudinal Development Study (CHILD) showed a reduction in bacterial taxa of the genera *Lachnospira, Veillonella, Faecalibacterium,* and *Rothia* among infants at risk of childhood asthma (90). In another recent observational cohort study, a reduction of *Lachnospiraceae*, *Faecalibacterium*, and *Dialister* at 1 year of age was associated with an increased risk of asthma at 5 years of age (91).

The protective effect of these bacterial taxa on asthma occurrence could be mediated by their fermentation products (92, 93). *Faecalibacterium prausnitzii* ferments dietary fiber to produce SCFAs, most notably butyric acid (93). Butyrate is the preferred energy source for colonocytes and has anti-inflammatory effects by inducing Tregs and promoting epithelial barrier permeability (94). SCFAs can contribute to the maturation process of dendritic cells in the bone marrow, leading to mature cells with a reduced ability to instigate Th2 responses in the lungs and to induce IgA production by mucosal B cells (94). High levels of gut microbial-derived butyrate in early life reduce the risk of allergen sensitization and asthma occurrence later in life, both in experimental and human studies (94, 95).

Finally, a recent systematic review of studies examining the effect of oral probiotic supplementation on asthma-related outcomes reported no significant differences in children receiving probiotics compared to the control groups regarding asthma control and lung function (96).

CONCLUSION

Early life is a crucial period for microbiome and immune development. The perturbation of the development and maturation of the microbiome during the first few years of life can have a variety of harmful effects on immune health, contributing to determining the development of atopic diseases. Although current understanding of the relationships between early life nutrition, microbiome, and immune system development has significantly increased in recent years, substantial knowledge gaps persist regarding the molecular mechanisms involved. Understanding these mechanisms is of the outermost importance to develop effective prevention strategies for allergic diseases.

AUTHOR CONTRIBUTIONS

DP, GN, IT, PC, and MD made substantial contributions to conception, design, and acquisition of data. GN, IT, PC, and MD drafted the initial manuscript. DP, PC, and MD critically reviewed it for important intellectual content. All authors gave the final approval of the version to be published.

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REVIEW
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Immunomodulation by Human Milk Oligosaccharides: The Potential Role in Prevention of Allergic Diseases

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The prevalence and incidence of allergic diseases is rising and these diseases have become the most common chronic diseases during childhood in Westernized countries. Early life forms a critical window predisposing for health or disease. Therefore, this can also be a window of opportunity for allergy prevention. Postnatally the gut needs to mature, and the microbiome is built which further drives the training of infant's immune system. Immunomodulatory components in breastmilk protect the infant in this crucial period by; providing nutrients that contain substrates for the microbiome, supporting intestinal barrier function, protecting against pathogenic infections, enhancing immune development and facilitating immune tolerance. The presence of a diverse human milk oligosaccharide (HMOS) mixture, containing several types of functional groups, points to engagement in several mechanisms related to immune and microbiome maturation in the infant's gastrointestinal tract. In recent years, several pathways impacted by HMOS have been elucidated, including their capacity to; fortify the microbiome composition, enhance production of short chain fatty acids, bind directly to pathogens and interact directly with the intestinal epithelium and immune cells. The exact mechanisms underlying the immune protective effects have not been fully elucidated yet. We hypothesize that HMOS may be involved in and can be utilized to provide protection from developing allergic diseases at a young age. In this review, we highlight several pathways involved in the immunomodulatory effects of HMOS and the potential role in prevention of allergic diseases. Recent studies have proposed possible mechanisms through which HMOS may contribute, either directly or indirectly, via microbiome modification, to induce oral tolerance. Future research should focus on the identification of specific pathways by which individual HMOS structures exert protective actions and thereby contribute to the capacity of the authentic HMOS mixture in early life allergy prevention.

Keywords: human milk oligosaccharides, mucosal immunity, allergic diseases, early life nutrition, sialyllactose, fucosyllactose, non-digestible oligosaccharides

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INTRODUCTION

Human milk is unique in its composition as it covers all nutritional and physiological infant requirements during the first months of life (1). Therefore, investigating the biological activity of components derived from human breast milk is an area of great interest, in order to identify specific components that support proper immune development in the infant when breastfeeding is not possible. The first indications of a link between breastfeeding and allergy outcome later in life has been published almost a century ago (2). Since then, numerous studies have been conducted to substantiate this suspected link (3–8). Breastmilk is the gold standard in early life nutrition, because of its large range of bio-active protective nutrients essential for healthy development of the microbiome and gastro-intestinal and immune maturation. However, it can also transfer allergens which may cause allergic reactions in atopic or allergic infants. Therefore, the conflicting data presented by these studies demonstrate the importance of studies further evaluating the biological activities of specific constituents found in human milk (9), such as human milk oligosaccharides (HMOS).

HMOS are the third most abundant component of human breast milk after lactose and lipids. The concentration of total HMOS in human breast milk ranges from 5 to 15 g/L, depending on the stage of lactation and genetic background of the mother (10, 11). More than two hundred structurally different forms of HMOS have been identified (12–14). Different structural and functional groups of HMOS have been related to various effects on several aspects of the immune system (15–19), highlighting the need for a diverse mixture of oligosaccharides in neonatal nutrition for optimal immune development.

Maturation of the immune system in the gastrointestinal tract is linked to proper systemic immunity and the establishment of effective oral tolerance for harmless food proteins and commensal bacteria of the host microbiome (20). As microbial colonization coincides with a rapidly maturing immune system in infants, microbial dysbiosis may therefore disturb development of the gastro-intestinal tract and immune system (21). Microbial dysbiosis and immature immune responses are thought to play a crucial role in e.g., necrotic enterocolitis (NEC), a disease characterized by inflammation and necrosis of the intestines affecting especially premature infants (22), whose immune system is not yet fully developed. Pathologies such as NEC and allergic diseases share common ground, as both have been linked to impaired microbial colonization and improper immune maturation.

One of the specific contributions of HMOS in human milk is its prebiotic capacity. Modulation of the infant's microbiome composition into a bifidogenic profile has been shown to have beneficial effects on infant health. Therefore, prebiotics, such as galacto-oligosaccharides (GOS) and fructo-oligosaccharides (FOS), have shown several beneficial immune and microbiome developments in infants (23–25). The specific combination of 90% short-chain (sc)GOS with 10% long-chain (lc)FOS resemble the molecular size distribution of the neutral HMOS fraction found in human milk (26). Prebiotic supplementation with scGOS and lcFOS reduces the incidence of allergy development (26–31). Murine models for both food allergy and house dust mite induced allergic asthma demonstrated the preventive effects of non-digestible oligosaccharides (29, 30). Moreover, scGOS/lcFOS supplemented infant formula in neonates decreased the prevalence of atopic dermatitis and other allergic manifestations (26–28).

Currently, only a small number of *in vivo* studies have investigated immunomodulatory properties and immune development capacities of HMOS. Thus, there are a limited amount of studies that attribute immune development properties to HMOS and individual HMOS structures. Several studies describing immunomodulatory effects of scGOS and lcFOS have been included in this review as they may serve as a framework in which future research could focus on elucidating how immune related mechanisms may be affected by HMOS. In addition, almost no clinical trials have investigated the effects of HMOS supplementation, although the association between the presence of specific HMOS biologically available in human milk and the prevalence of infectious diseases (32–34) or allergic diseases (35–37) has been indicated. The possible biological functions of HMOS gain support from studies that show a potential protective effect of prebiotic administration in *in vitro* models, animal models and human studies against development of asthma or allergy (28, 35, 38, 39). Most of the HMOS are not digested in the upper part of the gastrointestinal tract, but are fermented by local microbiota (40). A large proportion of HMOS will reach the colon intact (40), where they can serve as prebiotics for the colonic microbiota of the infant. Although a large portion of HMOS is metabolized by gut microbiota, some cross the intestinal (sub)mucosa and enter systemic circulation (13, 41, 42), thereby potentially modulating systemic immune functions. This means that HMOS may influence immunity and potentially not only the intestinal microbiome but also the microbiome composition in the lungs, providing a possible explanation for the observation that breastfed infants are less likely to develop asthma during childhood (43). In addition, reduced occurrence (up to 50% reduction) of atopic dermatitis, asthma, recurrent wheeze and food allergy in infants supplemented with prebiotics in early life has been observed (27, 28, 44–46). Despite these observations, little is known regarding the systemic distribution of HMOS in the infant, and how it may influence processes outside the gastrointestinal tract.

The complexity and abundance of oligosaccharides in human milk is unique amongst mammals (47). HMOS play an essential role in the postnatal growth and development of the mucosal immune system. HMOS are made up of monosaccharide units such as glucose (Glc), galactose (Gal), fucose (Fuc), *N*-acetylglucosamine (GlcNAc), and sialic acid with *N*-acetylneuramic acid (Neu5Ac). HMOS synthesis follows a distinct pattern of formation. Each structure has a Gal-Glc unit at the reducing terminus, also known as a lactose unit, containing a β1–4 glycosidic linkage. Elongation of lactose can occur by addition of Gal-GlcNAc units via a β1–3 or β1–6 glycosidic bond to form the linear or branched core structures (see **Figure 1**). The HMOS core structure can be further modified through the addition of Fuc or Neu5Ac residues (48).

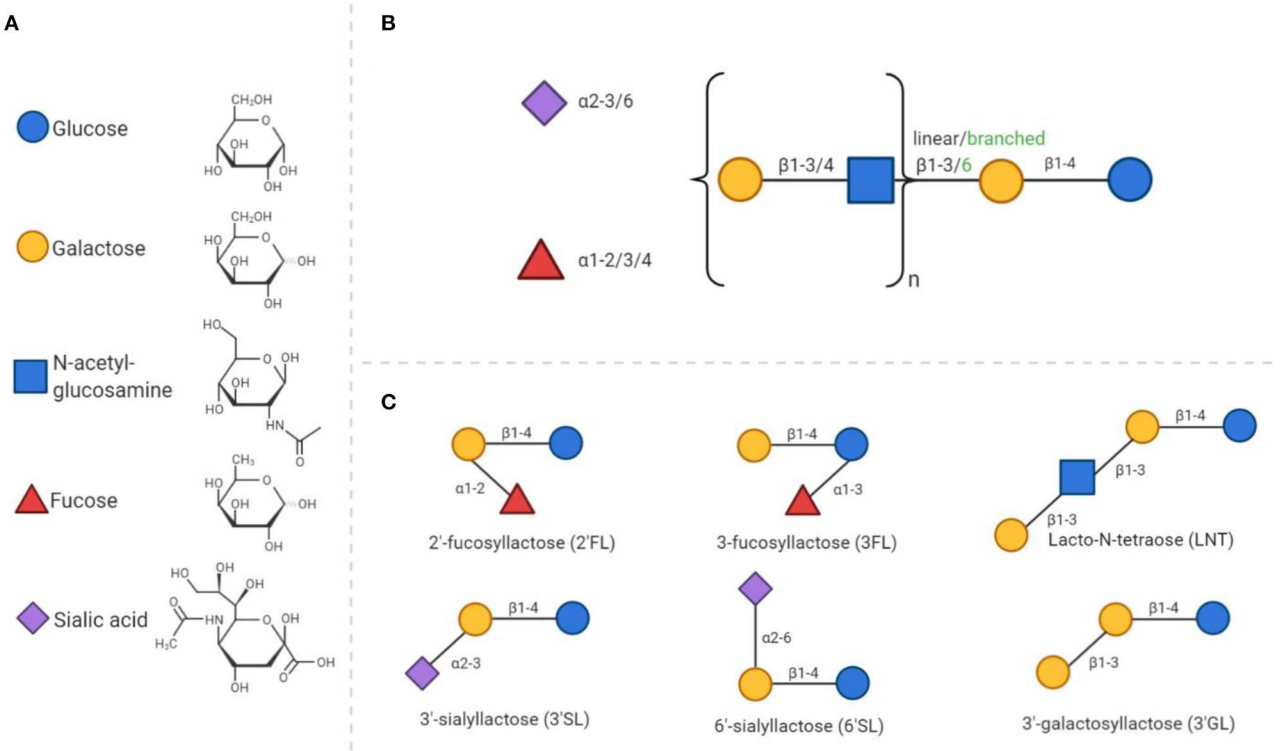

FIGURE 1 | General composition of human milk oligosaccharides and synthetic analogs. **(A)** All HMOS consist of only 5 different monosaccharides. The chemical structures of these monosaccharides are presented in a D- configuration. **(B)** The composition of HMOS follows a distinct structure. Elongation of the core structure and decoration with fucose and/or sialic acid residues leads to the large number of different structures discovered to date. **(C)** As examples, six simple oligosaccharide structures are displayed.

The unique diversity of HMOS also includes galactosyllactoses, with structures based on the elongation of lactose and further galactose residues (49, 50). These types of linkages are indigestible, but fermentable by specific bacteria; leading to the large number of ∼200 distinct structures identified to date. Decoration of the core structure with sialic acid, results in an acidic structure, whereas all other HMOS, including those containing fucose groups, are considered neutral. The composition of HMOS produced by a mother is determined by genetic polymorphisms in genes encoding fucosyltransferases FUT2 [Secretor (Se) gene] and FUT3 [Lewis (Le) gene]. Both genes are polymorphic, the individual expression of these genes are accountable for variable enzyme activity and corresponding variation in HMOS profiles in breast milk (11). Recent data has even indicated that these genetic polymorphisms in mothers, impact immunologic outcome of their children later in life. This effect was demonstrated in children, with a hereditary high risk of developing allergic diseases, who were fed breast milk of FUT2 expressing mothers which decreased the incidence of allergic manifestation of these children at 2 years of age (36). However, from this study it cannot be concluded that solely this genetic polymorphism is related to the allergic outcome of the infant, as many genetic, nutritional and environmental factors contribute to the immune development in neonates.

Synthetically manufactured HMOS or HMOS produced by genetically engineered bacteria, such as 2'-fucosyllactose (2'FL) (51), 3-fucosyllactose (3FL) (52, 53), lacto-N-neotetraose (LNnT) (54), 3'-sialyllactose (3'SL), 6'-sialyllactose (6'SL) (55), and 3'-galactosyllactose (3'GL) (56) have become commercial available just recently. This provides the opportunity to study specific pathways by which individual HMOS structures exert their protective immunologic effects in infants.

ALLERGIC SENSITIZATION AND THE ROLE OF THE EPITHELIAL BARRIER

The prevalence of allergic diseases is rising tremendously, particularly in Westernized regions (57). An allergic disease is an immunological result of complex interactions between genetic, environmental and lifestyle factors mainly triggered by harmless substances (58). Reduced microbial exposure and diversity is one of the many factors that may contribute to the rise in allergic disease prevalence. In allergic sensitization, a harmless, for example food-derived or airborne protein, crosses the mucosal lining and is presented by antigen presenting cells that drive T helper 2 (T_H2) biased immunity contributing to IgE isotype switching of B-cells. Mucosal surfaces with epithelial barriers provide the body with protection from external factors, ensuring that only specific components and nutrients can pass through the epithelium and enter systemic circulation. Allergic sensitization has been linked to dysfunction of the epithelial barrier, both in the

intestine and skin (59, 60). Epithelial barrier integrity depends, among other factors, on the mucus layer covering the single layer of epithelial cells. The mucus layer in the intestines prevents the majority of pathogens and intestinal contents from making direct contact with the epithelial cells (61). In humans, the most abundant protein present in the intestinal mucus layer is mucin 2, which is secreted by goblet cells (62). Several factors, including the microbiota, can influence the composition and therefore the protective effects of the mucus (63). Gut maturation takes place the first couple of weeks after birth rendering a leaky barrier in the first weeks of life (64). This can help to organize oral tolerance induction, but it also provides a risk for allergic sensitization.

Tight junctions strengthen apical connections between epithelial cells that cover the underlying connective tissue, thereby contributing to barrier function. Epithelial tight junction proteins tightly regulate paracellular compartments, preventing transport of large molecules, such as proteins and lipids or microbes and microbial products into the underlying tissue (65). These tight junctions are apically present and are crucial for epithelial barrier integrity. Upon epithelial injury, antigens can cross the epithelium more easily. Cytokines, such as interleukin-8 (IL-8), IL-25, IL-33, and thymic stromal lymphopoietin (TSLP), are produced by the epithelial cells as a response to stress and damage (66). These epithelial cell secreted cytokines influence neighboring dendritic cells (DCs) (67). Generally, DCs in the gastrointestinal tract are hyporesponsive and favor tolerogenic response to prevent unnecessary inflammatory responses to antigens and microbes (68). IL-25, IL-33, and TSLP stimulate the uptake and processing of foreign antigens by DCs and drive these DCs to promote development of T_H2 cells from naïve T cells (69, 70). Consequently, IL-4 and IL-13 produced by the T_H2 cells induces the activation and class-switching of B cells to produce allergen-specific IgE (67). The secreted IgE will bind to the high-affinity Fc receptors on the surface of mast cells. Upon a consequent encounter, the allergen crosslinks the IgE bound to the mast cells, triggering the mast cell to degranulate and release inflammatory mediators, such as histamine, causing the symptoms of allergic disease (71).

Newborns may be particularly susceptible to developing allergic diseases since the immune system after birth is dominated by T_H2 responsiveness (72). Immune maturation involves shifting toward a more T helper 1 (T_H1) prone and regulatory type, which favors the development of adequate immune protection and balanced immune responses (73). The importance of the epithelial barrier and mucosal homeostasis in prevention of allergic sensitization has sparked interest. HMOS may help to support this function by stimulating proper epithelial maturation and microbial colonization (74–76).

HMOS SHAPE THE MICROBIOTA OF NEONATES

The first 1,000 days of life are critical for the development of a diverse, stable gut microbiome (76–78). The initial microbial composition of the gut is determined by host genetics and environmental factors, such as health status, mode of delivery and diet (79). The first bacteria to colonize neonate's intestines are *Enterobacteriaceae* and *Staphylococcus* (80), followed by bifidobacteria and lactic acid bacteria (81). Proper colonization is essential for optimal development and health, as the establishment of a rich and diverse microbiome is related to a decreased prevalence of allergic (82), metabolic and other immunologic diseases later in life (83, 84).

HMOS promote the growth of beneficial bacteria, such as *Bifidobacterium* and *Lactobacillus* species (85, 86). Therefore, HMOS are known for their prebiotic effects and as players in shaping the microbiota of infants as depicted in **Figure 2**. The microbiota supporting effects of HMOS were observed when the gut colonization in breast-fed and formula-fed infants was compared, while addition of scGOS/lcFOS to formula milk was found to bring the microbiome composition closer to that of breastfed infants (87, 88). The microbiota are capable of fermenting oligosaccharides, however the capacity to degrade HMOS is strain-specific and depends on the presence of several genes (89, 90). Several strains of *Bifidobacterium* are well-adapted to digest purified natural HMOS into metabolites such as short chain fatty acids (SCFA) (90–93). Glycosyl hydrolases (GH), expressed by bifidobacteria, cleave monosaccharides from the HMOS and making them available for utilization by the microbe (94). This enzymatic degradation can either occur by membrane-associated extracellular GHs (95) or, as is the case for *Bifidobacterium infantis*, intact HMOS are transported into the cell by Solute Binding Proteins (96) and broken down by GHs inside the cytoplasm (97). The available monosaccharides are assimilated in central metabolic pathways and consequently release large volumes of e.g., SCFAs (98).

Both *B. longum* and *B. bifidum*, the major intestinal bacteria found in breastfed infants, are remarkably well-equipped to metabolize HMOS. In contrast, *B. adolescentis* is often associated with the adult intestinal microbiota, and is a less effective HMOS metabolizer (81, 91, 93). In contrast to *Bifidobacterium* spp., *Bacteroides* spp. are not specifically adapted to metabolize HMOS, but degradation of plant polysaccharides by *Bacteroides spp.* has been indicated (90). As plant-derived oligosaccharides are structurally comparable to human oligosaccharides, the capacity of multiple *Bacteroides* strains to metabolize HMOS is not unexpected (89). Providing a substrate for commensal gut bacteria results in a competitive growth advantage for these bacteria, enhancing proper colonization in the infants intestine and reducing growth conditions for and colonization by pathogenic bacteria (99, 100).

Unlike several species of commensal gut bacteria discussed previously, certain pathogenic species do not use HMOS as carbohydrate source for growth, including *Clostridium difficile, Enterococcus faecalis* and *Escherichia coli* (89). In addition, HMOS can actively bind to several pathogenic microbes and thereby possibly prevent adhesion as first step of infection (101). Infant formula can be supplemented with the prebiotics scGOS and lcFOS in order to promote the growth of various *Bifidobacterium* and *Lactobacillus* strains (102). However, these oligosaccharides do not contain terminal fucose or sialic acid residues, hence missing out biological function of HMOS related to these specific functional groups (103).

Proper colonization of the gut promotes intestinal barrier function and immune maturation (104). The establishment

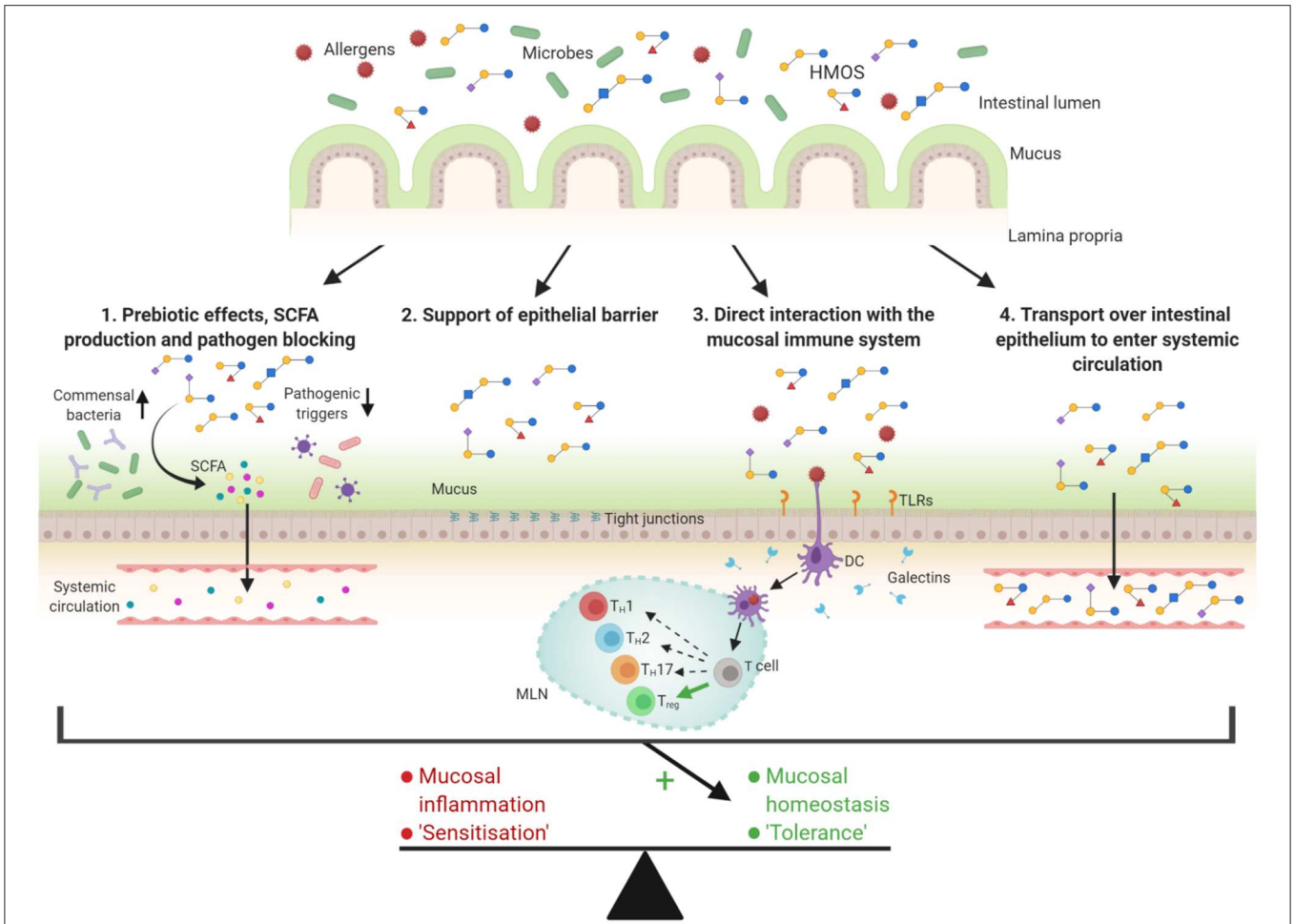

FIGURE 2 | Overview of the possible functions of HMOS related to the prevention of allergic diseases. The diversity in structures suggests engagement in several mechanisms related to maturation of the infant's gastrointestinal tract. (1) HMOS have shown to function as prebiotics and therefore stimulate growth of commensal bacteria. In addition, HMOS have shown to bind pathogens, thereby preventing binding of these pathogens to the intestinal epithelium itself and possible consequent infections. SCFAs produced during HMOS fermentation can enhance epithelial barrier integrity and locally and systemically modify immune responses. (2) HMOS can promote mucus production and epithelial tight junction integrity, thereby supporting the physical barrier between the intestinal epithelium and the gut content. (3) Several mechanisms by which HMOS directly affect the immune function have been described. Modulation of the response of DCs is one of those described mechanisms which may be relevant for the instruction of protective mucosal immune development. (4) Transportation of a small fraction of HMOS over the intestinal epithelium, results in systemic availability of these structures. This suggests an immunomodulatory role for HMOS, also beyond the gastrointestinal tract. All these HMOS related mechanisms can potentially enhance tolerance induction and therefore possibly prevent allergic diseases. Adjusted from Ayechu-Muruzabal et al. (48).

of a rich and diverse microbiome is related to a decreased prevalence of allergic diseases (82). Prebiotics like HMOS can support the growth and function of commensal bacteria and therefore possibly enhance gut microbial diversity. The association between microbial diversity and development of allergic diseases (83, 105) and the role of HMOS in this context, has yet to be elucidated.

METABOLITES OF HMOS INFLUENCE INTESTINAL BARRIER INTEGRITY AND IMMUNE FUNCTION

As described in previous section, HMOS are digested by intestinal bacteria, resulting in various metabolites, among which SCFA are well-known for immunomodulatory properties. The fermentation of major HMOS by bifidobacteria and lactobacilli into SCFA is very efficient (81), hence these bacteria are the dominant suppliers of SCFA in the infant's colon. Butyrate, propionate, and acetate are SCFA metabolites that have gained interest in recent years due to their proposed health benefits. Butyrate is mainly utilized by the epithelial cells, whereas acetate and propionate can be transported across the epithelial barrier to become systemically available in low levels via the bloodstream as depicted in **Figure 2** (106).

Upon absorption by the colonic epithelial cells, SCFA promote several functions of the epithelial barrier. The mucus layer covering the epithelial cells is essential to maintain epithelial barrier integrity. SCFA enhance the mucus secretion by upregulating the expression of mucin 2

(107). Acetate, produced in high levels by *Bifidobacterium* and *Bacteroides* species, increases the expression of genes related to mucus and support goblet cell differentiation (108–110). In addition, SCFA are known to protect against inflammatory insults and fortify the tight junction barrier (111). Promoting and enhancing the epithelial integrity may be of relevance in preventing allergic diseases, as a disrupted intestinal epithelial layer could lead to a compromised local tolerance response in which food allergens are able to reach underlying immune cells intact (112).

In addition, SCFA interact with DC and T cells and therefore modulate inflammatory immune responses. Many of the protective effects of SCFA have been attributed to the interaction with G protein-coupled receptors (GPR) present on intestinal epithelial cells and immune cells (113). Moreover, GPR-independent regulation of the immune response via T cell modulation has been shown in a murine model (114). In this model, SCFA regulate cytokine production via mammalian target of rapamycin (mTOR) by inhibiting histone deacetylase (HDAC) in T cells. In a previous study, butyrate effectively inhibited several HDACs in various cells, among which those that promote the transcription of FoxP3 in T cells, leading to increased expression of this hallmark transcription factor of regulatory T (T_{reg}) cells (115, 116). In addition, inhibition of maturation and differentiation of macrophages and DCs has been demonstrated (117). Suppression of inflammatory responses by butyrate was shown to involve inhibition of the NF-κB pathway in inflammatory cells such as macrophages in the lamina propria (118).

Interestingly, recently it was found that the microbiome of infants who develop allergic diseases during childhood have a reduced genetic potential for butyrate production from complex carbohydrates, supporting the importance of SCFA production in protecting the infant from developing allergic diseases (119). Therefore, supporting the microbial development may be of interest in infants more susceptible to developing allergic diseases (120, 121). All together, as bacterial metabolites of HMOS, SCFA may contribute to the immunomodulatory and protective effects against allergic disease development.

HMOS STRENGTHENING THE INTESTINAL EPITHELIAL INTEGRITY

Beyond their fermentation products, HMOS themselves may directly provide protection from intestinal epithelial barrier dysfunction (122), by promoting epithelial barrier maturation and mucus production (75) (illustrated in **Figure 2**). A mixture of human milk derived HMOS was shown to increase mucus production after 24 h of *in vitro* treatment in two different intestinal epithelial cell lines. The improved mucus production was linked to an upregulation of *Muc2*. In addition, apart from increased mucus production, HMOS could protect against pathogen induced barrier disruption as determined by means of transepithelial electrical resistance (TEER) (123). Furthermore, pollution induced loss of epithelial barrier integrity could be prevented by scGOS and 3'GL as measured in both TEER values and luciferase yellow flux across the intestinal epithelial monolayer in Caco-2 cells (124, 125). It was also demonstrated that supplementation with scGOS resulted in a significant increased rate of tight junction reassembly (124). Interestingly, the galactosyllactose with a β1–3 glycosidic linkage was effective in protecting the intestinal barrier function, whereas the galactosyllactose with an α1–3 glycosidic linkage did not prevent the deoxynivalenol (DON)-induced disrupted intestinal barrier (125). The protective effect of 3'GL on the intestinal epithelial barrier under challenge is structure-specific, which supports the notion that it is critical to understand the function and diversity of the structures within the total pool of HMOS, including the specific benefits of 3'GL within early life nutrition. These studies show that HMOS may directly promote proper development of the intestinal barrier, which strengthens the physical barrier between the intestinal epithelium and the gut content, contributing to lower antigenic load and mucosal homeostasis, which may help to decrease sensitization to food allergens.

In addition to this, the immunologic effects that are mediated through interaction between the intestinal epithelium and the underlying mucosal immune system should be addressed. Administration of synthetic HMOS 6'SL to antigen-antibody complex activated intestinal epithelial cells *in vitro* and resulted in a dose-dependent decrease of IL-8 and CCL20 secretion. Whereas, administration of 2'FL selectively reduced the secretion of CCL20 from the two cell lines used in this study (38). Similarly, a decrease of cytokine and chemokine production was observed upon TNFα stimulation of these cells after 6'SL exposure. Furthermore, comparable outcomes were observed for 3'GL, 4'GL, and 6'GL in an *in vitro* model for the infant intestinal epithelium (50). However, this decrease in cytokine production was not observed when two different intestinal cell lines were exposed to 2'FL (38). Additionally, it was observed that 3'SL, which is an isomer of 6'SL, downregulated the production of pro-inflammatory cytokines in Caco-2 intestinal cells by inhibition of the NF-κB pathway in a PPARγ dependent manner (126). These observations indicate that different functional groups and structures of HMOS exert the anti-inflammatory effects via different mechanisms. Silencing exaggerated or unwanted epithelial cell activation is essential for maintaining mucosal homeostasis.

Data indicated that mice, fed a diet supplemented with GOS for 2 weeks prior to exposure to DON, maintain their normal cellular distribution, as measured by villus height in the proximal small intestine (124). A study in suckling rats investigated the effects of 2'FL on mucosal immunomodulation (19). After treatment with 2'FL for 16 days an overall lower presence of inflammatory cytokines in the intestines compared to a reference group was observed, whereas the ratio of T_H1/T_H2 cytokines remained unchanged. In addition, the height and area under the villi present in the intestines was significantly increased upon supplementation with 2'FL, pointing to a positive effects of this prebiotic on intestinal growth (19). This is linked to the observation that 2'FL and scGOS/lcFOS in early life

TABLE 1 | Overview of HMOS binding receptors, potentially involved in immunomodulation.

HMOS identified as ligands	Receptor	Expression of receptor on	Function of receptor	References
2'FL, 3FL, LNFP-III, LNFP-IV, LNDFH-I	DC-SIGN	Antigen presenting cells	Antigen presentation	(138–140)
3'SL and 6'SL	Siglec 5, 9	Neutrophils, monocytes, dendritic cells	Immune signaling	(138, 141)
LNnT, LNT, LNFP-II, LNFP-III, LNDFH	Galectin 1, 2, 3, 7, 8, 9	Intestinal cells, lymphocytes, antigen presenting cells	Immune signaling	(142–144)
2'FL and 3'SL	TLR4	Most cell types, mainly immune cells	Pathogen detection	(15, 16)

Adapted from Triantis et al. (145).

alter gut microbiome development while supporting vaccination responses (18, 127, 128).

In the light of NEC, especially sialylated oligosaccharides have shown promising outcomes *in vivo* in prevention and development of necrotic intestinal lesions (122). Several studies in neonatal rats have reported reduced pathology scores upon intervention with HMOS mixture (129), or single HMOS alone (130, 131). Although sialylated oligosaccharides have been identified as the protective agents (129), intervention with 2'FL has also resulted in a reduced pathology score in rats (130). Dietary supplementation of 2'FL in preterm pigs had no significant effects on intestinal structure, digestive function and the development of NEC (132). Nonetheless, pooled HMOS, rather than single HMOS, have consistently shown to be most effective in preventing development of NEC (122).

Moreover, it has been shown that HMOS provision early in life can protect against the development of autoimmune diabetes in NOD-mice (133). The number of *in vivo* studies looking into the immunomodulatory effects of single HMOS are rather limited, and currently restricted to only the simple short chain structures. In a murine model for hen's egg allergy, 2'FL or 6'SL were found to reduce allergy symptoms in association with the induction of IL-10$^+$ T_{reg} cells (39). Prebiotic mixtures, such as scGOS and lcFOS, have been studied more extensively for immunomodulatory effects *in vivo*, showing promising results with regards to preventing allergic diseases, such allergic asthma and food allergy and these effects also link to the induction of T_{reg} responses (134–137). This implies a need for additional *in vivo* studies to gain insight in the properties of (single) HMOS to modulate gut maturation and the development of the mucosal immune system. Combining these studies, the direct effects of HMOS on the intestinal epithelial integrity and activation status and possibly the mucosal immune system are only started to be elucidated. The exact mechanisms and pathways involved are not yet fully understood. However, some of the receptors involved in HMOS signaling are identified and will be discussed in the following section.

HMOS BIND TO AND ACT AS RECEPTORS

One potential role of HMOS to modulate the infant's immune system is through receptor binding properties. In fact, multiple classes of human receptors have been described to interact with specific structures of HMOS, as summarized in **Table 1**. These receptors are mainly expressed by innate, adaptive immune cells and epithelial cells, they may therefore play a key role in mucosal immunomodulatory effects of HMOS (145).

Glycan Receptors

Glycan-binding receptors, also known as lectins, are particularly effective in binding HMOS. Many of the receptors belonging to the lectin family are involved in modulation of immune pathways. Lectin receptors consist of several subcategories, such as: membrane bound C-type lectins, sialic acid binding immunoglobulin-like lectins (Siglecs) and soluble type galectins.

The C-type lectin receptor dendritic cell-specific ICAM-grabbing non-integrin (DC-SIGN) is present on the surface of DCs and macrophages. It is usually involved in phagocytosis of pathogens upon recognizing pathogen-related glycoproteins. DC-SIGN has an affinity for HMOS containing α-linked fucose residues (138). A high affinity for 2'FL and 3FL (2 major structures of HMOS) may be of distinct physiological relevance in modulating immune responses in infants. DC-SIGN is expressed by cells in the gastrointestinal tract (139) and this receptor can promote allergen uptake by DCs. This may lead to subsequent T_H2 cell polarization as seen in patients with atopic dermatitis (146). Therefore, even though DC-SIGN can confer protective regulatory immunity in a pre-clinical model for auto-immune disease (147), DC-SIGN signaling may be involved in the sensitization phase of allergic diseases as allergens are capable of DC activation via DC-SIGN binding (148). An HMOS mixture derived from human milk was found to lower the expression of DC-SIGN on DC (140). This indicates that HMOS may be able to reduce DC-SIGN driven allergic sensitization through suppression of DC-SIGN expression on DC and via blocking the DC-SIGN receptor.

Siglecs are expressed by several immune cells that are involved in allergic effector responses, such as eosinophils and mast cells. Siglecs have been associated with binding of sialylated HMOS, although previous results show only affinity of siglec-1, -5, -7, -9, and -10 to 3'SL and 6'SL (141), and more recent data show a more limited binding affinity of Siglecs for HMOS (138). Siglec-9 provides low binding affinity for 3'SL and 6'SL, while siglec-5 has very low affinity for only 3'SL. This study found no other Siglecs to bind sialylated HMOS (138). Hence, the presence of sialic acid alone is not sufficient to ensure functional binding to a Siglec receptor (138). Siglec-7 and siglec-8 have been associated with allergy related immune mechanism (149, 150) making these potential targets for immune modulation by HMOS in relation to allergy prevention.

Galectins are another group of β-galactoside-binding receptors that bind carbohydrate moieties or glycan structures present on proteins. Moreover, galectins are expressed on and/or secreted by several immune cells and intestinal epithelial cells (151). These receptors can directly forward signals into the cell

upon binding to a ligand, but galectins can also be secreted from cells (152). In the secreted form, galectins can act as ligands and bind to receptors, such as TIM-3 and CD44 on other mucosal immune cells (153). Galectins such as galectin-9 have shown to induce T_{reg} cells (154–156). The binding of HMOS to galectins may directly modify galectin release and affect interactions of galectins with other cells, potentially resulting in immune modulation. Of the thirty-two different HMOS structures tested for binding to four galectins (galectin-1, -3, -7, and -9) (142), a total of 25 of these structures were recognized by all four galectins. Significant differences in affinity for each HMOS were observed, i.e., 2'FL, 3'SL, and LNnT were shown to bind galectins, whereas 3FL and 6'SL did not. 2'FL, the most common HMOS in human milk, binds with moderate-to-high affinity to all four galectins, while 3FL a structure very similar to 2'FL, not or weakly binds to any of the four galectins included in the study (142). Similar results were obtained in a different report, including galectin-1, -3, and -7 (143). These findings are supported by a previous study (144), suggesting that all included galectins showed affinity for LNnT, but had no affinity for 6'SL. This study also highlighted the evolutionary conserved binding affinity of galectins for glycans. Galectin-9 is a particularly promising target in allergy prevention strategies, as exposure of intestinal epithelial cells to scGOS/lcFOS together with bacterial CpG DNA or synthetic CpG ODN promoted the secretion of galectin-9 *in vitro*, which resulted in enhanced secretion of IFNγ and IL-10 production by underlying immune cells (154, 157). These cytokines are related to a regulatory type of T_H1 polarization and suppress T_H2 cell activation. Experiments with dietary interventions including scGOS/lcFOS enhanced local and/or systemic galectin-9 levels in murine and human allergy in association with symptom reduction (137). Furthermore, galectins can become systemically available and dampen allergic effector responses as shown in a murine model of food allergy (137).

Pattern Recognition Receptors

Toll-like receptors (TLR) are a family of receptors known to sense common molecules of pathogenic or commensal microorganisms, such as TLR4 ligand lipopolysaccharide (LPS) or TLR9 ligand bacterial CpG DNA. Decreased formation of the three-component complex TLR4, CD14, and LPS, inhibits subsequent pro-inflammatory immune signaling (158). Xiao et al. showed an increase in LPS receptor TLR4 mRNA expression upon stimulation with pooled HMOS isolated from human milk in monocytic derived dendritic cells (moDC) *in vitro*, yet protein levels of this receptor were not increased (140). In addition to affecting TLR4 transcription, HMOS suppress the expression of cluster of differentiation (CD)14, a coreceptor of TLR which is necessary to recognize LPS. 2'FL significantly suppresses CD14 in intestinal epithelial cells (16). In contrast to suppression of inflammation via TLR4 by 2'FL, pro-inflammatory properties related to TLR4 modulation have been described for synthetic 3'SL. In a TLR4-dependent manner, 3'SL was shown to induce intestinal inflammation (15). This pro-inflammatory effect of 3'SL can be explained by mimicking possible structural aspects of pathogenic bacteria, thereby educating and preparing the immune system for possible pathogenic encounters later in life. However, the phenotypical changes of DCs by 3'SL may have been due to LPS contamination of the oligosaccharide during synthesis, since pre-exposure to LPS may contribute to TLR4 silencing (159). However, LPS-containing bacteria are normal components of a healthy intestinal microbiome (160). In this respect, the low level of endotoxins present in purified HMOS used in *in vivo* studies would be minimal compared to the vast amount of endotoxin triggers the infant receives directly after birth. The contradicting results regarding HMOS-induced modulation of TLR4 show that we are only beginning to elucidate the possible immunomodulatory effects of HMOS. In addition, as synthetic (s)HMOS are either derived from enzymatically-processed lactose or produced by *E. coli*. In the latter situation a second possible immune trigger from bacterial byproducts may add to the biological effects of sHMOS structure. The origin of HMOS may influence the immunomodulatory effect, therefore an overview of the source and main outcomes of the studies referred to in this review is provided in **Table 2**.

Pathogen Binding

Besides binding to receptors on the cell membrane, HMOS can act as soluble receptors and bind to several pathogenic bacteria, thereby preventing binding to the intestinal epithelium and subsequent infection (101). Both *in vitro* and *in vivo* studies show that 2'FL attenuated *Campylobacter jejuni* infection (17, 168). However, Coppa et al. did not find inhibition of adhesion of *Escherichia coli, Vibrio cholerae* and *Salmonella fyris* in an *in vitro* intestinal epithelial setting with 2'FL (101). Nonetheless, inhibition of adhesion was observed with 3'SL, 6'SL and 3FL and combinations of these sHMOS. There was a diminished growth of *Streptococcus agalactiae* (group B *Streptococcus*) upon incubation with human pooled natural HMOS, that was attributed to the neutral fraction of the HMOS (169). This effect was supported by other studies, as pooled HMOS inhibited growth of group B *Streptococcus* (GBS) and prevented biofilm formation, although the effects of single HMOS were GBS strain specific (170–172). In this study, the effects of HMOS were compared to scGOS. scGOS did not diminish the growth of group B *Streptococcus* (169), showing that the structures in scGOS in this respect do not exert similar effects as the mentioned HMOS subtypes. These studies indicate that HMOS can also function as decoy receptors, thereby inhibiting growth and adhesion of pathogens in the gastrointestinal tract.

As antibiotic resistance is a growing problem, alternative antibacterial treatments are being investigated (173), including the use of HMOS to potentiate antibiotic functioning (174). It has been recently demonstrated that when exposed to HMOS, GBS becomes sensitive for trimethoprim, an antibiotic to which these bacteria are normally resistant. A significant decrease in metabolic pathways related to membrane construction was observed (175). Furthermore, HMOS were able to sensitize GBS to several antibiotics, such as erythromycin, gentamycin and clindamycin. In addition, an increased sensitivity to gentamycin, when combined with HMOS, in *Staphylococcus aureus* and *Acinetobacter baumanii* was also observed. However, these potentiating effects were obtained for β-lactams and

TABLE 2 | Overview of studies included in this review, which describe effects of non-digestible oligosaccharides (NDO) on immune function.

References	Model	NDO	Main effect of intervention
In vitro			
Gnoth et al. (42)	Caco-2 cells	Isolated HMOS	Neutral HMOS are transported across intestinal epithelia via receptor-mediated transcytosis as well as by paracellular flux, while acidic HMOS are translocated solely via paracellular pathways
Eiwegger et al. (161)	cord blood T cells	Isolated HMOS	Acidic HMOS increased the percentage of IFN? and IL-13 producing T cells as well as CD25+ T cells. IgE and IgG1 production was unaffected
Coppa et al. (101)	Caco-2 cells	Isolated HMOS	Acidic HMOS showed anti-adhesive effects on all 3 intestinal pathogens. Neutral HMOS showed anti-adhesive effects on 2 out of 3 tested pathogens
He et al. (49)	Fetal small intestinal samples	Isolated HMOS	HMOS from colostrum samples were able to attenuate mucosal response to surface inflammatory stimuli, and enhanced maturation of intestinal mucosa
Xiao et al. (140)	human moDCs	Isolated HMOS	HMOs limited LPS maturation of moDCs. HMOS-conditioned moDCs promoted T_{reg} generation
Newburg et al. (50)	T84 cells, H4 cells, NCM-460	Isolated HMOS and GOS	HMOS attenuated surface inflammatory stimuli. HMOS and GOS attenuated NF-κB signaling
Eiwegger et al. (162)	Caco-2 cells	Isolated HMOS and scGOS + lcFOS and AOS	Acidic HMOS increased IFN? and IL-10 secretion and suppressed T_H2 cytokine production in T cells from peanut allergic patients
He et al. (16)	T84 cells, H4 cells	Isolated HMOS, 2'FL^3, LNFP-I^3, 3'SL^3 and 6'SL^3	HMOS and 2'FL inhibited LPS-TLR4 signaling via suppressed CD14 expression. No significant results for any of the other tested NDOs
Holscher et al. (75)	Caco-2Bbe cells, HT-29 cells	Isolated HMOS, 2'FL^1, 3'SL^2 and 6'SL^1	Single HMOS and isolated HMOS decreased proliferation in pre-confluent cells, but increased cell differentiation. isolated HMOS decreased apoptosis and necrosis
Akbari et al. (124)	Caco-2 cells	GOS	GOS improved tight junction assembly and DON induced loss of transepithelial resistance was prevented
De Kivit et al. (154)	T84 cells, HT-29 cells	scGOS + lcFOS	scGOS + lcFOS in combination with *B. breve* M-16V increased epithelial expression and secretion of galectin-9, and enhanced T_H1 and T_{reg} polarization
Hayen et al. (157)	HT-29 cells	scGOS + lcFOS and scFOS + lcFOS	Both mixtures induced enhanced IFN? and IL-10, but suppressed IL-13 and TNFα secretion. scFOS + lcFOS enhanced T_H1 and T_{reg} response in a peanut-specific co-culture (HT-29/PBMC) model
Zenhom et al. (126)	Caco-2 cells	FOS and 3'SL^3	Both decreased levels of inflammation, as IL-12 secretion and mRNA expression of IL-12p35, IL-8, and TNFα was reduced in a dose- and time-dependent manner
Perdijk et al. (163)	human moDCs	GOS, 2'FL^1 and 6'SL^1	None of the oligosaccharides influenced DC differentiation and LPS-induced maturation
Yu et al. (17)	Hep-2 cells, HT-29 cells	2'FL^2	2'FL attenuated *C. jejuni* invasion in both cell lines
Perdijk et al. (159)	human moDCs	3'SL^1	3'SL mediated NF-κB activation via TLR4 induction was explained by LPS contamination
Zehra et al. (38)	T84 cells, HT-29 cells	2'FL^2 and 6'SL^2	2'FL inhibited CCL20 secretion from epithelium upon antigen-antibody complex stimulation. 6'SL inhibited IL-8 and CCL20 secretion from epithelium upon antigen-antibody complex stimulation
Holscher et al. (74)	Caco-2Bbe cells, HT-29 cells	LNnT^3, 2'FL^3 and 6'SL^3	All HMOS inhibited cell proliferation in undifferentiated cell cultures. 2'FL increased alkaline phosphatase and sucrase activity. LNnT increased transepithelial resistance
Varasteh et al. (125)	Caco-2 cells	3'GL^3, 4'GL^3 and 6'GL^3	3'GL prevented loss of transepithelial resistance upon DON exposure, 4'GL and 6'GL had no effect
Pre-clinical			
Xiao et al. (133)	Mice	Isolated HMOS	HMOS intervention delayed and suppressed type 1 diabetes development and reduced development of severe pancreatic insulitis in NOD-mice
Wu et al. (123)	Mice	Isolated HMOS	HMOS increased mucin expression, whereas intestinal permeability was decreased
Jantscher-Krenn et al. (129)	Mice	Isolated HMOS and GOS	HMOS reduced NEC pathology scores, the effects were attributed to DSLNT in the HMOS mixture
Yu et al. (131)	Rats	Isolated HMOS, GOS and synthetic disialylated-GOS	HMOS and sialylated-GOS reduced NEC pathology scores. GOS had no effect on NEC development
Autran et al. (130)	Rats	Isolated HMOS, GOS and synthetic disialylated-GOS	HMOS and sialylated-GOS reduced NEC pathology scores. GOS had no effect on NEC development

(Continued)

TABLE 2 | Continued

References	Model	NDO	Main effect of intervention
Comstock et al. (164)	Pigs	Isolated HMOS, 2'FL^3, 3FL^3, 3'SL^3, 6'SL^3, LNFP-III^3 and LNnT^3	HMOS stimulation IL-10 production by PBMCs. Fucosylated HMOS decreased proliferation of HMOS. Sialylated HMOS increased PBMC proliferation, although less CD4+ cells were observed
Akbari et al. (124)	Mice	GOS	GOS treatment stabilized villus height upon DON exposure
Verheijden et al. (30)	Mice	GOS	GOS prevented induction of airway eosinophilia and T_H2 related cytokine concentrations in lung, similar to budesonide treatment in house-dust mite allergy
Verheijden et al. (135)	Mice	GOS	GOS decreased IL-33 secretion and expression in HDM-induced asthma
Verheijden et al. (165)	Mice	GOS	GOS decreased CCL5 and IL-13 concentration in lung tissue from HDM-induced allergic asthma mice, similar to budesonide treatment
Djouzi and Andlueux (23)	Rats	GOS and FOS	GOS and FOS decreased pH in caecum, increased total SCFA concentration
Verheijden et al. (31)	Mice	scFOS + lcFOS	scFOS + lcFOS in combination with *B. breve* M-16V prevented house-dust mite induced airway inflammation
De Kivit et al. (137)	Mice	scGOS + lcFOS	scGOS + lcFOS in combination with *B. breve* M-16V induced reduced acute allergic skin response, and higher concentrations of galectin-9, which was associated with allergy prevention
De Kivit et al. (166)	Mice	scGOS + lcFOS	scGOS + lcFOS in combination with *B. breve* M-16V in an ovalbumin allergic mouse model, reduced allergic symptoms and increased galectin-9 serum levels. DC activation and T_H2 frequency were normalized in allergic mice
Schouten et al. (134)	Mice	scGOS + lcFOS + AOS	Prebiotic mixtures enhanced percentages of T_H1 cells and decreased Th2 cell percentages were observed. Strong reduction in allergic skin reaction. CD25+ T_{reg} cells were involved in the tolerance induction effect
Kerperien et al. (29)	Mice	scGOS + lcFOS and AOS	Only NDO mixtures reduced allergic skin response, whey-IgG1 levels, T_H2 and T_H17 mRNA expression, and increased Foxp3+ cells
Kerperien et al. (136)	Mice	scGOS + lcFOS + AOS	Prebiotic mixtures increased mRNA expression of IL10, TGFβ and Foxp3, and acute allergic skin response was 50% lower in whey allergic mice when fed the prebiotic mixture. These protective effect were depended on IL10 and TGFβ
Xiao et al. (127)	Mice	scGOS + lcFOS + 2'FL^2	NDOs enhanced influenza vaccine response, higher levels of IgG1, IgG2a, and activated B cells were observed
van den Elsen et al. (128)	Mice	scGOS + lcFOS + 2'FL^2	NDOs improved vaccine-specific antibody response and modulated gut microbiota composition
Yu et al. (17)	Mice	2'FL^2	2'FL attenuated *C. jejuni* colonization, weight loss and inflammatory cytokines
Cilieborg et al. (132)	Pigs	2'FL^3	2'FL intervention did not result in observed differences in bacterial colonization, intestinal function and NEC pathology
Xiao et al. (18)	Mice	2'FL^2	2'FL improved humoral and cellular immune response to influenza vaccination
Azagra-Boronat et al. (19)	Rats	2'FL^3	2'FL increased plasma IgE and IgA levels. Increased intestinal villus height. Higher *Lactobacillus proportion* in cecum
Weiss and Hennet (103)	Mice	3'SL^3	3'SL induced higher degree of resistance to dextran sulfate sodium-induced colitis
Kurakevich et al. (15)	Mice	3'SL^3	3'SL increased colitis, via TLR4 signaling
Castillo-Courtade et al. (39)	Mice	2'FL^2 and 6'SL^2	2'FL and 6'SL attenuated ovalbumin induced allergic symptoms like diarrhea, hypothermia, mast cell number in the intestine, and increased induction of IL-10 producing T_{reg} cells
Clinical			
Newburg et al. (32)	Infants	HMOS in human milk	Higher 2'FL and LNF-I to 3FL and LNF-II ratios in human milk correlated with more protection against diarrhea in infants
Sjögren et al. (35)	Infants	HMOS in human milk	Neutral HMOS concentration in human milk is not related to maternal allergy status nor allergy development in children
Bode et al. (33)	Infants	HMOS in human milk	Higher concentrations of HMOS in human milk were correlated to decreased risk of HIV transmission from mother to child. However, higher concentrations of 3'SL were found in HIV transmitting woman

(Continued)

TABLE 2 | Continued

References	Model	NDO	Main effect of intervention
Wang et al. (88)	Infants	HMOS in human milk	Breastfed infants had relative higher abundances of Bacteroides, and lower proportions of *Clostridium*, *Streptococcus*, *Enterococcus* and *Veillonella* than infants fed formula milk
Kuhn et al. (34)	Infants	HMOS in human milk	Higher concentrations of 2'FL and LNF-I were found in human milk from HIV non-transmitting woman
Sprenger et al. (36)	Infants	HMOS in human milk	FUT-2 associated oligosaccharides in human milk in infants at high risk of allergy development, and born via C-section are associated with lower risk of IgE-associated eczema
Seppo et al. (37)	Infants	HMOS in human milk	Low LNFP-III concentrations in human milk was related to an increased likelihood to develop cow's milk allergy, compared high concentrations of LNFP-III in infants
Grüber et al. (44)	Infants	Neutral oligosaccharides + AOS	Prebiotic supplemented formula resulted in a significant lower rate of atopic dermatitis compared normal formula in infants. Incidence of atopic dermatitis in prebiotic supplemented infants was in a similar range compared to breast fed infants
Moro et al. (27)	Infants	GOS and FOS	GOS and FOS dose-dependently increased in Bifidobacteria and Lactobacilli, in infants receiving prebiotic supplemented formula compared to non-supplemented formula
Arslanoglu et al. (28)	Infants	scGOS + lcFOS	Infants receiving scGOS + lcFOS had a lower incidence of allergic manifestations, in addition, fewer physician-diagnosed respiratory tract infections, fever episodes, and antibiotic prescriptions were recorded
De Kivit et al. (137)	Infants	scGOS + lcFOS	scGOS + lcFOS in combination with *B. breve* M-16V induced higher serum galectin-9 levels, which is associated with allergy prevention
Goehring et al. (167)	Infants	GOS + 2'FL[3]	GOS + 2'FL supplemented formula fed infants had similar plasma inflammatory cytokine concentrations compared to breast fed infants. Infants fed with the GOS diet had significantly increased levels of inflammatory cytokines present in plasma

As HMOS has different origin which may influence the immunological outcome, when possible the origin of the used HMOS was noted. Biological isolated HMOS[1], chemically synthesized[2], bacterial fermentation/synthesis[3] or source unknown. Studies are sorted based on model subgroup (e.g., in vivo), NDO and year of publication.

glycopeptides (176). Next to the above reported antibacterial properties, similarly some viral inhibiting interactions have been described (177). These interactions include binding of 2'FL to conserved epitopes, which are involved in binding to host cells, on norovirus (178, 179). Next to 2'FL, also 3'SL and 6'SL showed to inhibit cell binding in a rotavirus *in vitro* model (180). Some promising results of HMOS intervention have even been observed for influenza and HIV infections (177).

HMOS INTERACT WITH IMMUNE CELLS

HMOS have been detected in the blood, feces and urine of breastfed term and preterm infants (181–184). In breastfed infants, HMOS concentrations in urine appear to be around 10 times higher than in serum (184), which can be explained by clearance of substances from a larger volume of blood and accumulation in a small volume of urine. Direct effects have been demonstrated *in vitro* in bone marrow-derived dendritic cells (BMDC) treated with 2'FL. There was an increase in the percentage of CD40+ and CD86+ BMDCs upon exposure to 2'FL (18). Direct modulation of human moDCs was not found for 2'FL, 6'SL and scGOS (163), but the idea of possible moDC modulation via other HMOS cannot be excluded. BMDC exposed to 2'FL and stimulated by influenza vaccination had a greater capacity to induce CD4+ T cell proliferation in fresh whole splenocytes (18). Low concentrations of a mixture of acidic HMOS, purified from human milk, can alter cytokine production in cord blood mononuclear cells (CBMC) (161). The production of IFNγ and IL-10 in CBMCs was increased upon exposure to acidic HMOS, while IL-13 production remained unaltered, pointing to skewing of the balance toward a regulatory type T_H1 response. Similar effects were observed in a prior study exposing CBMC to acidic HMOS, which resulted in decreased IL-13 production in T cells (162). Mast cell function and direct effects of HMOS on mast cell degranulation were investigated in a murine food allergy model (39). *In vitro* exposure of bone marrow-derived mast cells to 6'SL resulted in significant inhibition of IgE-dependent mast cell degranulation, but only at a relatively high concentration of 1 mg/mL. However, in this same study, 2'FL did not significantly inhibit mast cell activation. Both 6'SL and 2'FL induce $IL10^+$ T_{reg} cells and thereby indirectly stabilize the degranulation of mast cells, in association with reduced food allergy symptoms (39). Hence, HMOS may have the capacity to modulate the immune response via various mechanisms, as indicated by the direct effects of HMOS on several immune cell types.

In the above described murine model for food allergy, 2'FL and 6'SL reduced food allergy symptoms via inducing T_{reg} cells and modulating mast cells (39). After 2'FL and 6'SL treatment during challenge in ovalbumin sensitized mice enhanced the capacity of CD4+CD25+ T_{reg} cells to inhibit mast

cell degranulation *ex vivo* (39), indicating that specific sHMOS support T_{reg} cell function. Similar results were found using scGOS and lcFOS in combination with acidic oligosaccharides or *B. breve* in prevention of food- (29, 166) or asthma-allergy in mice (31, 165). In piglets, either sow-reared or formula fed, peripheral blood mononuclear cells (PBMCs) were isolated (164). PBMCs from formula fed piglets showed more proliferation than sow-reared piglets upon LPS stimulation *ex vivo*, while *ex vivo* addition of sHMOS 2'FL normalized this increased proliferation. The percentage of T helper cells was higher in formula fed piglets compared to sow-reared piglets. *Ex vivo* added synthetic fucosylated and sialylated oligosaccharides downsized the expansion of the T_H cell population in the formula fed piglets, while the cytotoxic T cell population remained unaffected by *ex vivo* sHMOS treatment (164). These results indicate that fucosylated and sialylated oligosaccharides may possess immune regulatory properties, potentially modulating an allergic inflammatory response.

Although clinical trials in this area of research are scarce, data from an initial study indicate that addition of 2'FL to infant formula lowers concentrations of pro-inflammatory cytokines in plasma compared to infants fed a control formula (167). In addition, the decrease of these cytokines in the 2'FL supplemented infants was comparable to the low level of inflammatory cytokines that was measured in plasma of breastfed infants (167). As such, it should be carefully considered whether the effects observed in any of the *in vivo* and clinical studies are caused by a direct effect of the HMOS or indirect immunomodulatory effects as a result of microbiome modulation.

A convincing body of evidence is missing to ascribe clear immune development properties to HMOS and individual HMOS structures, since only a small number of *in vivo* studies describe immunomodulatory properties and immune maturation. In addition, the exact properties of the different groups of HMOS to modulate the immune system are not clear. Therefore, several studies illustrating immunomodulatory effects of scGOS and lcFOS have been described here and summarized in **Table 2**, as they may propose a framework in which future research could focus to elucidate immune related mechanisms affected by HMOS. As synthetically produced HMOS have become available recently, studying these may contribute to acquiring knowledge of the exact properties of HMOS and their specific functional groups in more detail and promote research focussing on allergy prevention. Development of adequate *in vitro* models for allergic sensitization including intestinal epithelial cells and/or dendritic cells, may help understanding the direct immunomodulatory effects of HMOS and their possible role in allergy prevention.

CONCLUSION

The increasing prevalence of allergic diseases has sparked interest in the role of early life nutrition and allergy development. Dietary components drive early life microbiome development as well as gut and immune maturation. HMOS in breast milk exhibit various microbiome modulating as well as mucosal immune maturation properties, which are not yet fully understood. However, in recent years several pathways involved in the effects of HMOS have been elucidated, including their capacities to fortify the microbiome composition and the release of fermentation products including SCFAs, as well as direct binding to pathogens and interactions with the gastrointestinal epithelium and local and systemic immune cells (as illustrated in **Figure 2**). Specific structural groups of HMOS may target several aspects of the immune system and modify immune function, thereby highlighting the need for further research on this topic. In addition, a more diverse mixture of oligosaccharide structures in neonatal formula nutrition may more closely resemble the HMOS composition as available in human breast milk and provide extra benefit for the child. Future research should focus on uncovering the mechanisms and pathways by which HMOS and the specific functional groups present in these HMOS may exert immunomodulatory actions. Ultimately, it would be of utmost value to identify whether specific HMOS structures are capable of contributing to early life allergy prevention.

AUTHOR CONTRIBUTIONS

MZ and NW have written the review. JG, GF, BL, and LW supervised the program. BL and LW have discussed and edited the manuscript. BS made specific contribution to the program with regard to functional oligosaccharides. All authors listed have approved for publication.

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REVIEW
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Free Amino Acids in Human Milk: A Potential Role for Glutamine and Glutamate in the Protection Against Neonatal Allergies and Infections

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Breastfeeding is indicated to support neonatal immune development and to protect against neonatal infections and allergies. Human milk composition is widely studied in relation to these unique abilities, which has led to the identification of various immunomodulating components in human milk, including various bioactive proteins. In addition to proteins, human milk contains free amino acids (FAAs), which have not been well-studied. Of those, the FAAs glutamate and glutamine are by far the most abundant. Levels of these FAAs in human milk sharply increase during the first months of lactation, in contrast to most other FAAs. These unique dynamics are globally consistent, suggesting that their levels in human milk are tightly regulated throughout lactation and, consequently, that they might have specific roles in the developing neonate. Interestingly, free glutamine and glutamate are reported to exhibit immunomodulating capacities, indicating that these FAAs could contribute to neonatal immune development and to the unique protective effects of breastfeeding. This review describes the current understanding of the FAA composition in human milk. Moreover, it provides an overview of the effects of free glutamine and glutamate on immune parameters relevant for allergic sensitization and infections in early life. The data reviewed provide rationale to study the role of free glutamine and glutamate in human milk in the protection against neonatal allergies and infections.

Keywords: human milk, free amino acids, glutamine, glutamate, neonates, immune development, allergies, infections

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INTRODUCTION

Human milk is widely recognized as the best source of infant nutrition. It provides the infant with a highly diverse mix of nutrients that supports optimal development. The health benefits of human milk, however, go beyond that of providing nutrients. An increasing body of evidence suggests that human milk provides the neonate with a protection against a variety of immune-related conditions. For example, it is shown consistently that infants who were exclusively breastfed were less likely to develop respiratory and gastrointestinal infections than infants who fully or partially received an infant milk formula (1–5). This protective effect of breastfeeding against infections may extend well beyond infancy and is indicated to be enhanced upon prolonged breastfeeding (6, 7). Furthermore,

studies have demonstrated that exclusive breastfeeding protects against various allergic diseases, including atopic dermatitis (8, 9), asthma (9–11) and food allergy (12–15), especially if there is a family history of allergic disease (16). For cow's milk allergy, which is one of the most common food allergies in infants, the incidence rate is reported to be up to seven times lower in exclusively breastfed infants, compared to infants fully or partially fed an infant milk formula (17–19). These unique protective capacities of human milk have driven scientific research into the underlying mechanisms in the past decades (15, 20, 21).

At birth, the immune system is immature (22). Compared to adults, the neonatal immune system is characterized by diminished innate effector cell functions, suppressed T-helper 1 (T_H1) immune responses and skewed T cell responses to antigens toward T-helper 2 (T_H2) immunity. These characteristics correlate with an increased susceptibility to infections and allergies in the neonatal period (23, 24). This susceptibility is further enhanced by an immature intestinal barrier function and an incomplete intestinal microbial colonization at birth (23). Various factors in human milk have been identified that could support the development of these immune functions, and thus may contribute to the protection against infections and allergies. For instance, human milk contains immunoglobulin A (IgA) antibodies, which confer protection against pathogens and are reported to induce tolerance to food allergens (25, 26). Moreover, various bioactive oligosaccharides, fatty acids and proteins have been identified in human milk that are capable of modulating immune responses directly, e.g., by regulating immune responses to pathogens (27–29), and indirectly, e.g., by shaping the gut microbiome (29–32). In addition to proteins, human milk also contains protein-unbound, free amino acids (FAAs). Accumulating evidence indicates that certain FAAs are bioactive, and more specifically have immunomodulating capacities (33, 34). Hence, FAAs in human milk may play an active part in an optimal immune development of the infant. However, whereas research on physiological functions of FAAs has made significant progress in recent years, FAAs are typically overlooked in human milk research.

Of the total content of amino acids (AAs) in human milk, 5–10% is present in free form. The FAAs glutamate and glutamine are by far the most abundant, both in absolute sense and relative to their protein-bound form, together comprising almost 70% of all FAAs present in human milk (35). Their levels display unique and consistent patterns over lactation, suggesting that secretion of these FAAs in human milk is a regulated process (35, 36). Interestingly, these structurally related FAAs have been widely associated with immunomodulation, including the modulation of immune mechanisms relevant for the development of allergies and infections. This review aims to describe the current understanding of the FAA composition in human milk, and to provide an overview of the effects of the FAAs glutamine and glutamate on immune parameters relevant for allergic diseases and infections in early life. Ultimately, a better understanding of the composition of FAAs in human milk and their immunomodulating capacities may contribute to the development of new avenues in the prevention of allergies and infectious diseases in infancy.

AMINO ACIDS IN HUMAN MILK: PROTEIN-BOUND AND FREE AMINO ACIDS

It is well known that protein quality and quantity are key aspects of the nutritional value of human milk. The total amino acid (TAA) composition of human milk, including protein-bound AAs and FAAs, is used to evaluate the quantity and the quality of the milk proteins and hence is well characterized (36, 37). However, many studies only report the TAA composition and do not distinguish between protein-bound and FAAs. As a result, data on FAAs in human milk are relatively limited.

FAAs in human milk have been reported to account for ∼5–10% of the TAA content (35, 36). Despite their low abundance relative to protein-bound AA levels, the relevance of FAAs in human milk should not be underestimated. Their levels are approximately 100 times higher than the 0.05% FAA pool in tissues (38) and up to 30 times higher than the FAA levels in plasma of infants (39). Moreover, FAAs in human milk contribute significantly to the initial changes in plasma levels of FAAs following a feed (40, 41) and are indicated to be more readily absorbed (42–44), appear sooner in the circulation and thus might reach peripheral organs and tissues faster than protein-derived AAs. Indeed, differences in plasma FAA levels were observed between infants receiving an infant milk formula containing FAAs and infants receiving an equivalent portion of AAs in the form of intact protein, suggesting differences in absorption kinetics between FAAs and protein-derived AAs (45–47). In contrast to their protein-bound counterpart, FAAs can interact with specific receptors present on a wide variety of cells in various parts of the body, including the intestines, where they can activate specific intracellular pathways and confer physiological effects (34, 48).

While human milk directly supplies infants with FAAs, human milk proteins could also provide the infant with FAAs via proteolysis in the neonatal gastrointestinal tract. However, the contribution of proteolysis of human milk proteins to the FAA supply of infants might be relatively low, as (complete) proteolysis of these proteins in infants is shown to occur to a minimal extent (49–52). Factors contributing to the limited proteolysis of human milk proteins are the relatively low output of pepsin and gastric enzymes observed in infants, the relatively high gastric postprandial pH which leaves proteases largely inactive, as well as the high degree of glycosylation of these proteins (50). Accordingly, it has been argued that the availability of FAAs in the upper region of the gastrointestinal tract, including the upper parts of the small intestine, is almost entirely dependent on the dietary FAA content (48).

The unique abilities of FAAs compared to protein-bound AAs and the relatively inefficient proteolytic capacity of neonates underline the importance of understanding the FAA composition in human milk, separate from the TAA composition.

The FAA Composition in Human Milk is Dynamic and Seemingly Regulated

The composition of human milk is known to be dynamic over the course of lactation. The total protein content has been consistently shown to decrease in the first 3 months of lactation (35, 36). It is argued that this decrease correlates with the infant's protein requirements for growth and that it prevents overfeeding, as milk volume intake increases during this period (53, 54). Not surprisingly, similar dynamics are found for the protein-bound AA content in human milk. For each individual AA the protein-bound form decreases to a highly similar extent during lactation, indicating that the dynamics of protein-bound AAs in human milk during lactation are not AA-specific (35). In contrast, levels of FAAs in human milk display dynamics during lactation that are highly AA-specific: whereas levels of some FAAs decrease in the first 3 months of lactation, others remain stable or sharply increase (35, 36). Remarkably, these FAA dynamics during lactation are consistent in studies across various ethnic groups and geographical locations, indicating that these dynamics are globally consistent and thus seemingly regulated (35, 36, 55, 56).

The underlying mechanisms regulating the dynamics of FAA levels in human milk are poorly understood. Cells of the mammary gland secrete proteases and anti-proteases into human milk that together regulate the cleavage of specific AAs from human milk proteins, generating FAAs and peptides (57). Thus, it can be hypothesized that temporal changes in net proteolytic activity in human milk contribute to the FAA dynamics, although this is unlikely as levels of all major human milk proteases and anti-proteases decrease during lactation, along with levels of their substrates (50, 58). Mammary gland cells can also directly secrete FAAs into human milk via AA transporters present on their cell membranes. Interestingly, animal studies have shown that the expression of certain AA transporters in the mammary gland increases with progressing lactation, whereas that of others remains unchanged (59–62). These expression dynamics throughout lactation appear to be tightly regulated by multiple intracellular signaling pathways (63). Thus, it can be speculated that the dynamic expression of AA transporters on mammary gland cells along lactation contributes to the FAA dynamics in human milk.

To better understand the mechanisms underlying the secretion of FAAs in human milk, several studies examined the influence of maternal characteristics on the FAA composition in human milk. Whereas, FAA levels seem to be independent of the mothers' age (64), maternal body-mass index is reported to slightly influence levels of several FAAs (65, 66). Mechanisms underlying this effect are not known, but may involve the hormone prolactin, as prolactin is involved in regulating FAA transport in the mammary gland and levels of prolactin associate with maternal body-mass index (67–69). Studies investigating the effect of maternal diet on the AA composition in human milk indicate that the TAA composition is largely independent of the AA composition of the diet (70, 71). For FAAs, this relation remains to be examined in humans. However, studies across different geographical locations where different diets are consumed show largely similar levels and ratios of FAAs in human milk, suggesting that maternal diet is not of major influence (35, 36, 55, 56). This is supported by the finding that oral supplementation of a single load of glutamate (6g) in healthy lactating women did not alter levels of any of the FAAs in their breastmilk (72). Moreover, several studies reported that there was no association between maternal plasma levels of FAAs and the FAA levels in human milk (73, 74). In fact, some FAAs were 1- to 15-fold higher in plasma compared to milk, whereas levels of free glutamate were 40-fold higher in milk than in plasma.

All together, these findings indicate that selective FAA transport occurs in mammary tissues during lactation and that levels of FAAs in human milk might be highly regulated throughout lactation.

Correlations of FAAs in Human Milk With Lactation Stage, Gestational Age and Infant Anthropometrics: A Special Role for Free Glutamine and Glutamate?

The FAAs glutamine, glutamate, glycine, serine and alanine in human milk have consistently been shown to increase in the first 3 months of lactation, whereas the levels of most other FAAs remain relatively stable along lactation (35, 36, 55, 56). Of these, glutamate is by far the most abundant, accounting for more than 50% of the total FAA content at any stage of lactation. In addition, glutamate shows the highest absolute increase in concentration along lactation, increasing from ∼1.25 to 1.75 mM from month 1 to 6 of lactation (35). Glutamine, the second-most abundant FAA, shows the highest relative increase in concentration, increasing almost 350% from month 1 to 6 of lactation and reaching a concentration of up to 0.6 mM (35, 64, 75). In addition to the stage of lactation, the gestational age of the infant has also been reported to be a determinant of the free glutamine levels in human milk. A meta-analysis has shown that free glutamine levels in milk for preterm infants are almost three times lower than those observed in milk for term infants in the first month of lactation (36). Levels of all other FAAs were similar in preterm and term human milk samples, indicating that this difference was AA-specific.

Studies investigating associations of FAAs with infant anthropometrics are scarce but do report consistent findings. It was recently reported that free glutamate levels in human milk were significantly higher for term infants that had faster weight gain (76). Moreover, glutamine levels also tended to be higher for fast growing children. Consistent with these findings, another study reported a positive association between free glutamine levels in human milk and infant length at 4 months of age (65). These findings are in line with studies indicating that milk for boys tends to have higher levels of free glutamine and glutamate than milk for girls in the first 3 to 4 months of lactation (35, 76), as boys are known to gain more weight and length than girls in this time period (77).

The finding that levels of free glutamine and glutamate in human milk are relatively high, display unique dynamics along lactation, and are associated with infant anthropometrics urges

the need to understand the functions that these FAAs could have during infant development.

THE DIVERSITY IN PHYSIOLOGICAL FUNCTIONS OF FREE GLUTAMINE AND GLUTAMATE

In the last decade, it has been recognized that glutamine and glutamate are essential AAs at key times in life, including the neonatal period when rapid growth occurs (78, 79). Although these two FAAs are structurally related, they appear to be different in terms of absorption by the infant. Whereas dietary glutamine supplementation in infants leads to higher plasma levels of this AA (80, 81), plasma levels of glutamate are largely unaffected by dietary glutamate (82, 83). This suggests that free glutamate in human milk is almost entirely used by splanchnic tissues, limiting its availability for other tissues, whereas glutamine might also exert direct effects elsewhere in the body. Despite these differences, most of the dietary glutamine and glutamate provided to neonates is consistently shown to be used by the intestines (84, 85). The intestines do not only form a physical barrier to protect against pathogens but are also home to the largest immune organ of the body: the gut-associated lymphoid tissue (GALT). This may explain why glutamine and glutamate are associated with a wide range of physiological functions, ranging from energy provision to cells to more specific immunomodulating functions, many of which could be relevant in the context of the prevention of neonatal allergies and infections. **Figure 1** provides a summary of the demonstrated effects of free glutamine and glutamate in (developing) intestinal tissues, which are described in detail below.

Metabolism of Glutamine and Glutamate in Intestinal Epithelial Cells and Immune Cells: Their Function as Energy Substrate and Protein Precursors

It is well-established that glutamine and glutamate are important energy substrates for intestinal epithelial cells (IECs) and immune cells, especially during periods of rapid growth (86). In fact, studies in young animals and infants have shown that approximately half of the dietary glutamate and glutamine is oxidized by intestinal and immune cells, ultimately leading to the generation of energy for the cells to adequately function and grow (87). Intestinal cells can convert glutamine into glutamate, which is crucial for the usage of glutamine for energy purposes (88). Whereas, human intestinal cells can also convert glutamate into glutamine, this process is limited due to the low glutamine synthetase activity in the small intestine (89, 90). In the neonatal period, this ability may be further limited as studies in young rats demonstrated that glutamine synthetase activity is particularly low in the pre-weaning period (91, 92). Remarkably, IECs as well as immune cells cannot function properly without the availability of exogenous glutamine (93). This, combined with their limited capacity to synthetize glutamine suggests that adequate functioning of these cells in the neonatal period might be partially dependent on dietary-derived glutamine.

Besides serving as energy substrates, free glutamine and glutamate are both specific precursors for glutathione, which is the main antioxidant in IECs and immune cells and critical for the prevention of cellular damage caused by pro-oxidants (94). An imbalance in pro- and antioxidants, known as oxidative stress, stimulates inflammatory responses that can lead to the development and maintenance of allergic disorders (95, 96). Hence, antioxidants like glutathione are considered as preventive or treatment strategy for food allergies (97). It has been reported that both dietary glutamine and dietary glutamate enhance glutathione production and, possibly as a result, reduce oxidative stress in the intestines of weaning piglets (98, 99). In addition, glutamine, but not glutamate, is an important specific precursor for the synthesis of mucins, which are critical for the defense against infections and are suggested to protect against allergic sensitization (100–103). Accordingly, oral glutamine supplementation has been shown to enhance mucin synthesis and to increase the number of mucin-secreting goblet cells in the small intestine of weaned piglets (104).

Effects of Free Glutamine and Glutamate on Intestinal Growth and Barrier Function

In the rapidly growing neonate where the intestines are not yet fully developed, it is crucial to achieve and maintain rapid growth of IECs. Moreover, it is well-established that intestinal barrier function is a crucial factor in the protection against allergies and infections, by preventing allergen and bacterial translocation from the gut lumen into the immune cell-populated lamina propria and mesenteric lymph nodes (105–107). In neonates where intestinal barrier function is immature, proper availability of nutrients that contribute to the growth of IECs and maturation of the intestinal barrier is critical to support this protective effect. Interestingly, free glutamine and glutamate have been shown consistently to influence these processes, by various mechanisms which are further explained in the following sections.

Impact of Glutamine on Intestinal Functions

Glutamine is by far the most widely examined AA in relation to growth and function of IECs. This FAA is known to stimulate IEC proliferation in a variety of ways, as demonstrated in various neonatal IEC lines *in vitro*. For instance, glutamine dose-dependently enhanced cell proliferation and differentiation of neonatal porcine and rat IECs, through activating multiple mitogen-activated protein kinases (MAPKs) (108–110). Moreover, studies in neonatal porcine and adult human IEC lines have indicated that glutamine also promotes growth through augmenting the effects of growth factors, including insulin-like growth factor 1 and epidermal growth factor (108, 111–113). In addition to promoting growth, glutamine has been reported to dose-dependently protect against inflammation-, endotoxin- and oxidant-induced cell death and damage in these IEC lines (114–116). Remarkably, glutamine completely blocked inflammation-induced apoptosis in the adult human epithelial cell line HT-29 when supplied at 0.5 mM, a concentration similar to that of free glutamine in human milk (115).

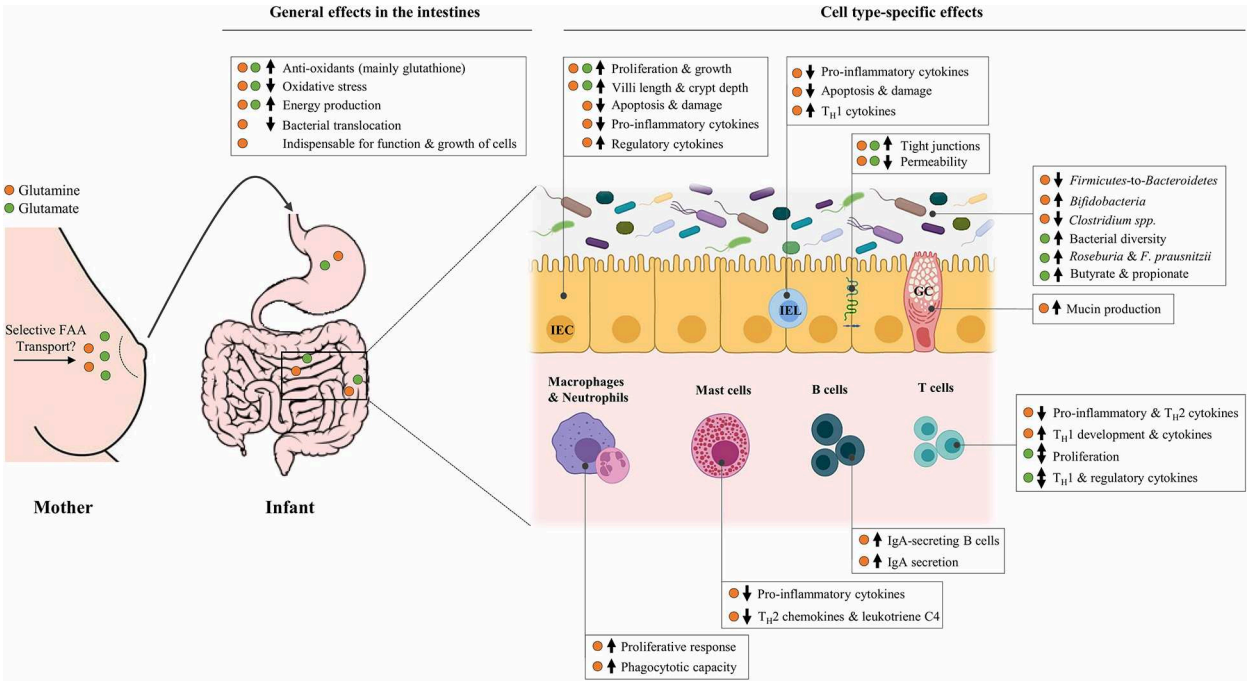

FIGURE 1 | Overview of the potential effects of free glutamine and glutamate, selectively secreted in human milk by mammary gland cells, in the developing infant gut. The ↑ and ↓ indicate an upregulation and downregulation, respectively, of the corresponding target following *in vitro* and/or *in vivo* supplementation with glutamine (•) or glutamate (•). Effects are limited to those that are relevant in the context of allergic sensitization and infections. FAA, Free amino acid; IEC, Intestinal epithelial cell; IEL, Intraepithelial lymphocyte; GC, Goblet cell; T_H1, T-helper 1 cell; T_H2, T-helper 2 cell; IgA, Immunoglobulin A; *F. prausnitzii*, *Faecalibacterium prausnitzii*.

Multiple lines of evidence indicate that glutamine also specifically stimulates intestinal barrier function. For instance, *in vitro* studies with neonatal porcine and human adult IEC lines have revealed that glutamine restriction reduces the expression of the major tight junction proteins, including claudin and occludin proteins, which are vital for intestinal barrier function (110, 117, 118). This was accompanied by a reduced distribution of these proteins at the plasma membrane and an increase in IEC permeability. Remarkably, glutamine supplementation in these *in vitro* models completely reversed this process, suggesting that sufficient availability of free glutamine is crucial for optimal epithelial barrier functions. These effects were mediated through enhanced AMP-activated protein kinase signaling and diminished PI3K/Akt signaling, indicating that glutamine supports intestinal barrier function via modulation of specific intracellular pathways (110, 118).

Consistent with *in vitro* studies in neonatal cells, studies in young animals also suggest a potential role of glutamine in promoting a healthy intestinal development. In rat pups and young piglets, dietary deprivation of glutamine has been reported to diminish intestinal integrity, through breakdown of epithelial junctions and shortening of microvilli (119, 120). Conversely, dietary supplementation of glutamine in young piglets has been consistently reported to increase villus height, inhibit apoptosis and boost proliferation of IECs, increase tight junction protein expression and improve epithelial barrier function (98, 121–123). In addition, glutamine is shown to protect against pathogen-induced intestinal damage *in vivo*. For instance, weaning piglets fed a glutamine-enriched diet prior to challenge with *E.coli* completely maintained villus morphology and tight junction protein expression (124, 125). Moreover, oral supplementation of glutamine prevented endotoxin-induced intestinal damage in suckling piglets (114). Consistent with the ability of glutamine to promote intestinal barrier function, glutamine supplementation is reported to prevent bacterial translocation in various adult animal models of intestinal obstruction (126–131). Whether glutamine can also prevent bacterial translocation in neonatal animals remains to be examined.

Impact of Glutamate on Intestinal Functions

A growing body of evidence suggests that next to glutamine also glutamate has effects on IEC growth and intestinal barrier function. A recent *in vitro* study in neonatal porcine IECs has demonstrated that supplementation of glutamate dose-dependently enhances cell proliferation (132). Moreover, this study showed that glutamate supplementation prevented oxidative stress-induced changes in IEC viability, barrier function and membrane integrity by increasing the abundance of tight junction proteins (132). The ability of glutamate to improve intestinal barrier function is also demonstrated in a study using adult human IEC lines, where glutamate addition significantly reduced phorbol-induced hyperpermeability (133). Remarkably, these effects were observed at a glutamate concentration three

times lower than that present in human milk, highlighting the potency of free glutamate in human milk to exert physiological effects.

In addition to *in vitro* studies, *in vivo* studies in young animals also indicate that free glutamate can promote intestinal development. Supplementation of dietary glutamate to healthy weaning piglets led to an increase in overall intestinal health, as evidenced by higher villus height and enhanced intestinal mucosal thickness and integrity (122, 134). Furthermore, dietary glutamate dose-dependently enhanced the weight of the small intestine, increased the depth of the crypts and the lamina propria, and improved intestinal antioxidative capacities in healthy weaning piglets (99). Finally, dietary glutamate prevented mycotoxin-induced impairments in intestinal barrier function and morphology in young piglets, suggesting that free glutamate may also play a role in the prevention of intestinal damage (135).

As glutamate can be converted into glutamine by IECs, although at limited rates, the effects observed for glutamate may be attributable to the effects of glutamine. However, studies examining effects of both glutamine and glutamate demonstrated differential effects of these FAAs on functions of IECs and intestinal morphology. For instance, weaning piglets supplemented with dietary glutamine alone had higher villi than those piglets supplemented with a combination of glutamate and glutamine, whereas the combination led to the deepest crypts (136). Moreover, glutamine was observed to have protective effects against oxidant- and endotoxin-induced death of porcine neonatal IECs *in vitro*, whereas glutamate had no effect (114). This indicates that the effects of glutamate on intestinal function are not solely exerted through conversion into glutamine.

Effects of Free Glutamine and Glutamate on Immune Cell Functions

In addition to epithelial cells, the immune cells of the GALT also play a crucial role in the prevention of neonatal allergies and infections. The immature neonatal GALT is characterized by the production of higher levels of pro-inflammatory cytokines (137, 138), whereas anti-inflammatory capacities are diminished (139). The pro-inflammatory milieu in the neonatal intestines is indicated to induce T-helper 2 (T_H2) immune activity (140, 141). In contrast, T-helper 1 (T_H1) immunity is highly limited and gradually develops during the postnatal period (142–144). The resulting T_H2-dominant immune milieu is known to increase the susceptibility to allergic sensitization, whereas the minimal T_H1 function correlates with the increased susceptibility of neonates to infections (144, 145). Thus, components in human milk with anti-inflammatory capacities, or components that enhance the development of T_H1 immunity or suppress T_H2 activity might contribute to the prevention of neonatal allergies and infections. Free glutamine and glutamate both have been associated with these immunomodulatory capacities, as described in detail below.

Impact of Glutamine on Immune Cell Functions

The importance of glutamine for the development and function of the immune system is well recognized. Although *in vitro* studies in neonatal cells are lacking, numerous *in vitro* studies in adult cells showed that various immune cells fail to develop and function without adequate glutamine availability (146). For instance, glutamine restriction impaired the growth and differentiation of B and T cells (147) and diminished antigen presentation and phagocytotic capacities of macrophages and neutrophils (148, 149). Conversely, glutamine supplementation dose-dependently enhanced phagocytotic capacities of human neutrophils *in vitro* (150, 151). Consistent with these findings, *in vivo* studies in young animals indicate that glutamine availability modifies intestinal immune cell populations. For example, dietary glutamine dose-dependently increased the number of neutrophils and macrophages in weaning piglets following an LPS-challenge (123, 152), suggestive of enhanced antimicrobial capacities. Moreover, in newly weaned piglets, dietary glutamine decreased the proportion of antigen-naïve T cells in the mesenteric lymph nodes (153), which are reported to be elevated in allergic patients and are proposed as an early life marker for future development of allergies (154, 155). Finally, dietary glutamine increased the number of IgA-secreting B cells in the small intestine of young mice (156) and enhanced intestinal levels of IgA in various weaning animals (157–161). Together, these results indicate that glutamine availability influences immune cell populations in developing intestinal tissues, which in turn may influence antimicrobial and anti-allergic immune processes.

A consistent body of evidence shows that glutamine also exhibits anti-inflammatory capacities. *In vitro* studies demonstrated that glutamine supplementation decreased the production of pro-inflammatory cytokines IL-6, IL-8, and/or TNFα, while increasing the production of anti-inflammatory/regulatory cytokine IL-10 in various activated adult human immune cells, including intra-epithelial lymphocytes (IELs), intestinal mast cells, peripheral mononuclear cells (PBMCs) and monocytes (162–165). Similar findings are reported in healthy young animals. For instance, dietary glutamine reduced levels of pro-inflammatory cytokines (including IL-1 and IL-8) while increasing levels of anti-inflammatory/regulatory cytokines (including IL-10) in the small intestine of healthy weaning piglets (123, 124, 166). Furthermore, in LPS-challenged piglets, dietary glutamine reduced intestinal expression of inflammatory markers, including Toll-like receptor-4 and the nuclear factor NF-κB, suggesting that glutamine might also have potent anti-inflammatory effects in immune-compromised conditions (114).

Glutamine has also been indicated to play a regulating role in the balance between T_H1 and T_H2 immunity, however, *in vitro* studies examining this aspect in neonatal immune cells are lacking. It is reported that adult murine naïve T cells are able to differentiate into T_H2 cells under glutamine-restricted conditions, but not into functional T_H1 cells, indicating that glutamine deprivation may favor T_H2 differentiation (167). Conversely, supplementation of glutamine is reported to enhance T_H1 and/or diminish T_H2 responses of various activated adult immune cells *in vitro*. For instance, glutamine increased the production of T_H1 cytokines IL-2 and IFNy by activated murine IELs and by human lymphocytes and PBMCs, while T_H2 cytokines were unaltered (168–171). In activated human intestinal mast cells, glutamine did not alter the release of T_H1 chemokines, but reduced the release of T_H2 chemokine ligand 2

and leukotriene C4, which are both involved in the pathogenesis of various allergic diseases (164, 172). Although data are limited, *in vivo* studies in young animals also suggest a regulating role of glutamine in the T_H1/T_H2 immune balance. In young mice, dietary glutamine increased the expression of IL-2 and the IL-2 receptor by lymphocytes, indicative of increased activity of and responsiveness to T_H1 stimuli (173). Moreover, dietary glutamine in healthy weaning piglets lowered the production of T_H2 cytokine IL-4 and increased the IFNγ/IL-4 ratio in mesenteric lymph node cells (153). Finally, in weaning rabbits, dietary glutamine upregulated IL-2 and IL-10 expression by IELs, while inhibiting expression of IL-6, an inducer of T_H2 differentiation of naïve T cells (174, 175). Although further confirmation in neonatal animals is critical, these data may indicate that glutamine plays a role in promoting a more balanced T_H1/T_H2 immune system in the neonatal period.

Impact of Glutamate on Immune Cell Functions

Despite dietary glutamate being almost completely used in intestinal tissues, studies investigating the effects of glutamate on intestinal immune cells are lacking. Yet, receptors for glutamate are found on a variety of immune cells, including lymphocytes and dendritic cells, suggesting that glutamate has a role in immune cell functioning (176). Studies using adult human peripheral T cells demonstrated that glutamate at low concentrations ($<100\,\mu M$) dose-dependently increases the proliferative response of T cells to various stimuli (176, 177). At higher concentrations (>1 mM), however, this effect reversed, indicating that glutamate tends to have immunosuppressive properties at higher concentrations (176, 178). Accordingly, it is postulated that the high glutamate concentration in the intestinal microenvironment, which may reach the millimolar range, could prevent inappropriate responses to dietary antigens by exerting immunosuppressive effects on intestinal T cells (178).

Besides regulating T cell proliferation, glutamate availability is also indicated to influence the T_H2 and T_H1 cytokine production by T cells. Glutamate is released by dendritic cells during T cell interaction, where it has dual roles (179). In cases of non-specific antigen presentation, glutamate inhibits T cell activation. However, upon specific antigen presentation glutamate stimulates T cell proliferation and the production of IL-2, IFNγ and IL-10, thereby promoting a T_H1 response (179). This latter process depends on glutamate released from dendritic cells, but also on extracellular glutamate concentrations, suggesting that this process could be influenced by dietary glutamate (179). Accordingly, it is reported that glutamate supplementation of up to 1-2 mM enhanced IFNy and IL-10 secretion by activated adult human peripheral T cells *in vitro*, whereas secretion of T_H2 cytokines IL-4 and IL-5 was unaffected (180). When supplied at even higher concentrations (>5 mM), however, glutamate inhibited IFNy and IL-10 secretion by these cells. Unfortunately, *in vitro* studies in neonatal cells and *in vivo* studies investigating the effects of glutamate on immune cell functions are lacking. Nevertheless, the findings in adult immune cells suggest an immunoregulating role for glutamate, with effects that are highly dependent on the context and the concentration. At concentrations present in human milk, glutamate could be involved in promoting T_H1 immunity and subsequently in reducing the susceptibility to allergic sensitization, although this remains speculative due the lack of evidence in neonatal cells or animals.

Effects of Free Glutamine and Glutamate on the Intestinal Microbiota

Accumulating evidence indicates that the gut microbiota plays a vital role in tolerance induction to dietary antigens (181–183). Accordingly, clinical studies have provided evidence for a link between the microbiota composition in the neonatal period and the development of allergic diseases. It is reported that a higher intestinal bacterial diversity in early life is associated with a lower risk of developing various allergic diseases, including food allergy (184–187). Moreover, infants with an increased colonization of *Firmicutes* and a decreased colonization of *Bacteroidetes* (corresponding to an increased *Firmicutes*-to-*Bacteroidetes* ratio), or a decreased colonization of *Proteobacteria* and *Bifidobacteria* are shown to be at increased risk of developing food allergies (188–191). Mechanisms by which gut microbes modify the susceptibility to allergies are poorly understood but may involve specific modulation of T_H2 and T_H1 immunity (192, 193). The colonization of intestinal microbiota is far from complete at birth and is influenced by various environmental factors, including breastfeeding duration (189). Thus, human milk components that shape the neonatal gut microbiota composition may play an active part in modifying the susceptibility to allergic sensitization. Although data are limited, several studies have shown that glutamine and glutamate can modulate the abundance of gut bacteria that have been associated with the protection against allergic diseases.

Impact of Glutamine on the Gut Microbiota Composition

The ability of dietary glutamine to modify the microbiota composition is shown in various young animals. A study in weaning mice demonstrated that dietary glutamine decreased the content of *Firmicutes* in the jejunum and ileum, and decreased the *Firmicutes*-to-*Bacteroidetes* ratio in the ileum (194). Similar findings are reported in studies in adult pigs and human (195, 196). In weaning rabbits, dietary glutamine specifically reduced the presence of *Clostridium spp.* in the ileum, of which colonization in early life has been associated with increased risk of allergic diseases (197, 198). Finally, a glutamine-enriched diet is also shown to increase the abundance of beneficial *Bifidobacteria* in the jejunum of healthy weaned mice (194), and to decrease potentially harmful microorganisms in adult pigs (196). The mechanisms underlying the effects of glutamine on the gut microbiota composition are poorly understood. It is postulated that glutamine supplementation regulates utilization and metabolism of a variety of AAs in a niche-specific manner, affecting the activity and number of specific microbes (157, 199).

Impact of Glutamate on the Gut Microbiota Composition

To our knowledge, only two animal studies examined the effects of dietary glutamate on the intestinal microbiota composition to

date, both of which used animals in their post-weaning phase. It has been reported that dietary glutamate markedly enhanced the bacterial diversity in the intestinal flora of healthy post-weaning pigs (200). Moreover, the glutamate-enriched diet decreased the *Firmicutes*-to-*Bacteroidetes* ratio in the ileum, although this effect was only seen when given in combination with a high fat diet and was not observed in other intestinal sections. Perhaps more interestingly, dietary glutamate specifically promoted the colonization of *prausnitzii* and *Faecalibacterium prausnitzii* in post-weaning pigs (200, 201). The colonization of *Roseburia* in early life has been positively associated with the acquisition of tolerance to cow's milk (202), and *Faecalibacterium prausnitzii* is indicated to play a role in the prevention of food allergy (203–205). These intestinal microbes are some of the main producers of the short-chain fatty acids butyrate and propionate. Accordingly, a glutamate-enriched diet significantly increased colonic concentrations of these fatty acids in adult pigs (206). Butyrate and propionate both have been associated with the prevention of various allergic diseases and, consequently, high faecal levels of these fatty acids in early life have been associated with a decreased risk of developing atopy (207–209).

CONCLUDING REMARKS

Research indicates that breastfeeding during the first months of life provides protection against immune-related conditions in neonates and later in life. These conditions include gastrointestinal infections and several allergic diseases including food allergy. It is indicated that the transfer of specific immunomodulating components, such as bioactive proteins, from mother to infant through human milk contributes to this protective effect. In addition to proteins, human milk contains FAAs, which have unique characteristics. They are more readily absorbed than protein-derived AAs and can be recognized by specific receptors on various cells. Moreover, whereas protein-bound AAs decrease during the lactation period in a non-AA-specific manner, temporal changes of FAAs in human milk are highly AA-specific. These dynamics in FAA levels are globally consistent and thus seemingly independent of ethnicity, demographics and maternal diet. This suggests that selective FAA transport occurs in the mammary gland, that FAA levels in human milk are strictly regulated and, consequently, that FAAs are likely to be of physiological relevance in the developing infant.

With regards to individual FAAs in human milk, free glutamine and glutamate display particularly remarkable characteristics. They account for almost 70% of the FAA content in human milk, they both drastically increase in the first 3 months of lactation and their levels have been shown to positively correlate with infant growth, suggestive of important functions in the developing neonate. In neonates, dietary glutamine and glutamate are mainly used by the intestines. Remarkably, studies in neonatal immune cells and young animals demonstrate that these FAAs can have a wide range of effects on cells in developing intestines, also at concentrations similar to their levels in human milk. In short, they are reported to increase the growth of intestinal epithelial cells, enhance intestinal barrier function, influence immune cell development and populations in the gut-associated lymphoid tissue, exert anti-inflammatory and potentially T_H1 promoting and/or T_H2 inhibiting effects on various intestinal immune cells, and modify the abundance of gut microbiota that might play a role in allergic sensitization (**Figure 1**). Together, these effects could potentially support neonates in the protection against allergic sensitization and infections.

All together, the findings described in this review warrant further research into the contribution of free glutamine and glutamate in human milk to the protection against neonatal allergies and infections. Levels of free glutamine and glutamate, in addition to that of other bioactive factors that could influence early life immune development, are considerably higher in human milk than in standard infant milk formulas, leading to significant differences in the intake of these FAAs between breastfed and formula-fed children (210–212). As many of the effects of glutamine and glutamate described in this review were concentration-dependent, future studies should address whether this differential intake contributes to the differential occurrence in immune-related conditions between formula-fed and breastfed children.

AUTHOR CONTRIBUTIONS

JS, AH, and SW: conceptualization and literature searches. AH, SW, and JG: supervision. JS: writing original draft. JS, AH, SW, and JG: review and editing. All authors read and approved the manuscript.

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Neonatal Diet Impacts Circulatory miRNA Profile in a Porcine Model

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microRNAs (miRNAs) are conserved non-coding small nucleotide molecules found in nearly all species and breastmilk. miRNAs present in breastmilk are very stable to freeze-thaw, RNase treatment, and low pH as they are protected inside exosomes. They are involved in regulating several physiologic and pathologic processes, including immunologic pathways, and we have demonstrated better immune response to vaccines in piglets fed with human milk (HM) in comparison to dairy-based formula (MF). To understand if neonatal diet impacts circulatory miRNA expression, serum miRNA expression was evaluated in piglets fed HM or MF while on their neonatal diet at postnatal day (PND) 21 and post-weaning to solid diet at PND 35 and 51. MF fed piglets showed increased expression of 14 miRNAs and decreased expression of 10 miRNAs, relative to HM fed piglets at PND 21. At PND 35, 9 miRNAs were downregulated in the MF compared to the HM group. At PND 51, 10 miRNAs were decreased and 17 were increased in the MF relative to HM suggesting the persistent effect of neonatal diet. miR-148 and miR-181 were decreased in MF compared to HM at PND 21. Let-7 was decreased at PND 35 while miR-199a and miR-199b were increased at PND 51 in MF compared to HM. Pathway analysis suggested that many of the miRNAs are involved in immune function. In conclusion, we observed differential expression of blood miRNAs at both PND 21 and PND 51. miRNA found in breastmilk were decreased in the serum of the MF group, suggesting that diet impacts circulating miRNA profiles at PND 21. The miRNAs continue to be altered at PND 51 suggesting a persistent effect of the neonatal diet. The sources of miRNAs in circulation need to be evaluated, as the piglets were fed the same solid diet leading up to PND 51 collections. In conclusion, the HM diet appears to have an immediate and persistent effect on the miRNA profile and likely regulates the pathways that impact the immune system and pose benefits to breastfed infants.

Keywords: breastmilk, infant formula, miRNA, piglet, blood

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INTRODUCTION

The World Health Organization and American Academy of Pediatrics recommend exclusive breastfeeding for the first six months of life, followed by breastfeeding with complimentary foods until 1 year of age (1, 2). It is well-established that breastfed babies have decreased rates of obesity, infections such as otitis media and respiratory tract infections, and decreased asthma and atopic

dermatitis (2). However, the exact mechanisms that make breastfeeding better for infants is still unclear. Multiple components of breastmilk have been shown to impact growth and development as well as immune function including human milk oligosaccharides (3–5), immunoglobulins (6), cytokines (7, 8), and growth factors (9, 10). microRNA (miRNA) are also possible contributors to the benefits of breastfeeding.

miRNAs are conserved non-coding small nucleotide (∼22 nucleotides) molecules (11) that have biological activities in humans (12–15). Breastmilk miRNAs are thought to survive in an acidic environment in the gastrointestinal tract, when exposed to RNase, and be absorbed in the gut (16). miRNAs from bovine milk have been found in the plasma of humans and noted to have a regulatory effect on cell functions (14), such as innate immune, T-cell and B-cell function; several of these miRNAs are also highly abundant in human milk (16, 17). Infant formulas, however, have a decreased amount of miRNA (13, 18). Dietary sources have been shown to contribute an appreciable amount of miRNA to total serum miRNA. For example, when mice are fed a miRNA-depleted cow's milk diet for 4 weeks, they showed a decrease in measured plasma miRNA by ∼ 60% compared to mice fed a miRNA-sufficient diet (19). However, studies are limited in terms of understanding the impact of breastmilk miRNAs and other components on infants' health. Therefore, the purpose of the current study is to determine if neonatal diet influences serum miRNA and if it continues to have an impact after being weaned to a solid diet.

MATERIALS AND METHODS

Animal Study

The piglet study design has been described previously (20). Animal maintenance and experimental protocols followed the ethical guidelines for animal research approved by the Institutional Animal Care and Use Committee (IACUC) and Institutional Biosafety Committee (IBC) at University of Arkansas for Medical Sciences. Briefly, 2 day old male piglets were obtained from a regional commercial farm and transferred to the vivarium at Arkansas Children's Nutrition Center (ACNC). They were then randomized to be fed an isocaloric diet of either dairy-based formula (MF; $n = 26$) or human breastmilk (HM; $n = 26$). Donor human breastmilk was obtained from the Mother's Milk Bank of North Texas, and Similac Advance powder was obtained from Ross Products (Abbot Laboratories). Both HM and MF diets were supplemented to meet the nutritional recommendations of the National Research Council (NRC) for growing piglets. At postnatal day (PND) 14, solid pig starter was introduced until PND 21, at which time all piglets were weaned to an *ad libitum* solid diet until PND 51.

Sample Processing

At 8 h of fasting, blood was collected on the morning of PND 21, 35, and 51 into PAXgene (Qiagen) Blood RNA Tubes. At PND 21 there were 9 MF and 9 HM, 4 MF and 4 HM at PND 35, and 9 MF and 10 HM at PND 51. Tubes were allowed to sit for 2 h at room temperature and then stored at −80°C. Prior to processing, the PAXgene tubes were moved from the −80°C to 4°C overnight and then allowed to sit at room temperature for 2 h. The PAXgene tubes were then centrifuged at 3000 × g using a swing-out rotor (Eppendorf 5810R Centrifuge) for 10 min, and samples were processed with the PAXgene Blood miRNA Kit (PreAnalytiX, Switzerland) to isolate blood RNA according to the commercial protocol. RNA samples were stored at −80°C until needed for small RNA library preparation.

A cDNA sequencing library for miRNA (miRs) was generated using standard methods of the QIAseq miRNA Library Kit (Qiagen, Germany). Small RNA sequencing libraries were constructed using Qiagen's QIAseq® miRNA Library Kit (96) (Qiagen, Germany, cat. 331502) according to the manufacturer's protocol. Briefly, adapter sequences were sequentially ligated to the 3′ and 5′ ends of miRNA in each sample. Adapter ligated miRNAs were then assigned unique molecular indexes (UMI) and simultaneously transcribed into single-stranded cDNA. This was followed by cDNA cleanup per the manufacturer's instruction, and construction of PCR-amplified Illumina compatible sequencing libraries, which involved ligating a 3′ sequencing adapter, and 1 of 48 indexed adapters (QIAseq miRNA NGS 96 index IL) during the amplification process. The sequencing libraries were then subjected to a second library cleanup and validated for fragment size and quantity using an Advanced Analytical Fragment Analyzer (AATI) and Qubit fluorometer (Life Technologies), respectively. Equal amounts of each library were then pooled and sequenced on a NextSeq 500 platform using high output flow cells to generate a ∼5–10 million 75-base single end reads per sample (1 × 75bp SE). All sequencing was performed by the Center for Translational Pediatric Research (CTPR) Genomics Core at Arkansas Children's Research Institute (Little Rock, AR, USA).

Statistical Analysis

Following demultiplexing, miRNA reads were quality checked using FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and MultiQC (21). The fastq files that passed quality control were then adapter trimmed. miRNAs were quantified using Qiagen's primary QIAseq miRNA quantification tool available through GeneGlobe's data analysis center (https://geneglobe.qiagen.com/us/analyze/) against all organisms in miRBase (miRBase v21). miRNA's with low UMI-counts were then removed before downstream analysis. To retain the maximum number of interesting features, miRNA with a minimum of 10 counts-per-million (CPM) in at least 17 libraries were retained for further investigation. The filtered dataset was then normalized for compositional bias using trimmed mean of M values (TMM) (22, 23). edgeR's quasi-likelihood method (*glmQLFTest*) was used to identify differentially expressed miRNA between experimental groups (24–26).

Pathway Analysis

The challenge associated with the piglet model includes finding databases that support miRNA target prediction analysis. As miRNAs are conserved (27–29) and the pig genome is not well-annotated, a human miRNA database was utilized to conduct target prediction analysis using Ingenuity Pathway Analysis software (IPA, Qiagen). The experimentally verified target gene

list of miRNA was generated. The target genes were subjected to canonical pathway analysis that included metabolic pathways, cell cycle regulation, cell growth, proliferation and development, cellular immune response, cellular stress and injury, cytokine signaling, growth factor signaling, humoral immune response, nuclear receptor signaling, organismal growth and development, pathogen-influenced signaling, and transcriptional regulation. The enriched pathways were based on the right-tailed Fisher's exact test (adjusted for False Discover Rate at 5%) that are graphed as negative log *p*-value. These pathways indicate the likelihood of an association of genes to the pathway in MF vs. HM fed piglets at different time points.

TABLE 1 | MF fed piglets have differential miRNA expression at PND 21 relative to HM fed piglets.

miRNA	FC	*p*-value
ssc-miR-708-5p	−39.76665575	0.002
ssc-miR-196b-5p	−3.237684325	0.001
ssc-miR-142-3p	−2.797240418	0.005
ssc-miR-7142-3p	−2.661387334	0.007
ssc-miR-181b	−2.420461905	0.006
ssc-miR-181d-5p	−2.28856399	0.018
ssc-miR-451	−2.276169814	0.012
ssc-miR-181a	−1.857234399	0.038
ssc-miR-1296-5p	−1.49268688	0.019
ssc-miR-148b-3p	−1.359814304	0.045
ssc-miR-28-3p	1.509698075	0.041
ssc-miR-532-5p	1.534975917	0.026
ssc-miR-128	1.559663023	0.019
ssc-miR-574	1.589728656	0.042
ssc-miR-9810-3p	1.613911517	0.030
ssc-miR-335	1.791816536	0.011
ssc-miR-1468	1.796667674	0.048
ssc-miR-7	1.809841726	0.023
ssc-miR-182	1.825348966	0.043
ssc-miR-126-3p	1.928895853	0.022
ssc-miR-99b	1.954175359	0.007
ssc-miR-130a	2.463583469	0.046
ssc-miR-142-5p	2.576233506	0.010
ssc-miR-18b	37.26653433	0.010

Negative fold change (FC) indicates the miRNA is downregulated in MF fed piglets compared to HM fed piglets while positive FC indicates miRNA are upregulated in MF relative to HM group. The data represents values for 9 MF and 9 HM.

TABLE 2 | MF fed piglets have differential miRNA expression at PND 35 relative to HM fed piglets.

miRNA	FC	*p*-value
ssc-miR-18b	−49.72067945	0.048
ssc-miR-135	−42.25878671	0.040
ssc-miR-9	−34.874668	0.049
ssc-miR-32	−7.975548269	0.047
ssc-miR-126-5p	−5.646933269	0.012
ssc-miR-27b-3p	−3.406773728	0.040
ssc-miR-126-3p	−2.826728093	0.051
ssc-miR-628	−2.647555179	0.053
ssc-let-7g	−1.964276243	0.012

Negative fold change (FC) indicates the miRNA is downregulated in MF fed piglets compared to HM fed piglets. The data represents values for 4 MF and 4 HM.

RESULTS

miRNA Expression Profile

miRNA expression analysis was performed on blood samples from MF piglets in comparison to HM piglets at different time points (PND 21, 35, and 51). The reader is referred to **Tables S1–S3** for miRNAs identified using human, mouse, and piglet genome. The data described here are exclusively based on piglet genome. Results demonstrate differential expression of miRNA in the MF group relative to HM fed piglets. At PND 21, 10 miRs were downregulated and 14 were upregulated in MF

TABLE 3 | MF fed piglets have differential miRNA expression at PND 51 relative to HM fed piglets.

miRNA	FC	*p*-value
ssc-miR-708-5p	−181.4471982	0.000
ssc-miR-18b	−24.60941262	0.005
ssc-miR-135	−9.524924918	0.003
ssc-miR-23b	−2.890749819	0.012
ssc-miR-27b-3p	−2.835459396	0.005
ssc-miR-27a	−2.012577166	0.009
ssc-miR-28-5p	−1.795200855	0.040
ssc-miR-24-3p	−1.769462259	0.011
ssc-miR-99a	−1.55286892	0.046
ssc-miR-23a	−1.464557853	0.024
ssc-miR-339	1.393039587	0.040
ssc-miR-339-3p	1.431395735	0.041
ssc-miR-339-5p	1.435181521	0.028
ssc-miR-4334-3p	1.495246365	0.019
ssc-miR-532-3p	1.529235062	0.032
ssc-miR-1307	1.534150221	0.032
ssc-miR-149	1.63922384	0.053
ssc-miR-328	1.745143555	0.044
ssc-miR-320	1.872187384	0.016
ssc-miR-30c-3p	1.901539247	0.029
ssc-miR-199a-3p	2.200123182	0.006
ssc-miR-199b-3p	2.857399975	0.022
ssc-miR-100	3.033459346	0.029
ssc-miR-7139-3p	3.289366861	0.019
ssc-miR-199a-5p	3.552896912	0.000
ssc-miR-204	5.124808887	0.045
ssc-miR-205	8.226824537	0.002

Negative fold change (FC) indicates the miRNA is downregulated in MF fed piglets compared to HM fed piglets while positive FC indicates miRNA are upregulated in MF relative to HM group. The data represents 9 MF and 10 HM.

in comparison to HM fed piglets (**Table 1**). At PND 35, 9 miRs were decreased in MF relative to HM fed piglets (**Table 2**). At PND 51, 10 miRs were downregulated and 17 were upregulated in MF compared to HM fed piglets (**Table 3**). There were several miRNAs that displayed altered directionality depending on PND. For instance, ssc-miR-18b was increased in MF at PND 21 and decreased at PND 35 and PND 51 relative to HM group. ssc-miR-126-3p was elevated in MF compared to HM group at PND 21 and lower at PND 35. Other miRNAs were different only at certain time points. For example, ssc-miR-708-5p was decreased at PND 21 and PND 51 in the MF group relative to the HM group. ssc-miR-135 and ssc-miR-27b-3p were lower at both PND 35 and PND 51 in MF compared to HM group. In addition, miRs found in breastmilk by other research groups (13, 14, 16) such as miR-148 and miR-181 were decreased in MF compared to HM at PND 21. Furthermore, immune system related miRs such as let-7 (30–33) was decreased at PND 35 while miR-199a and miR-199b (34–36) were increased at PND 51 in MF compared to HM.

Target Gene Prediction and Pathway Analysis of miRNAs

IPA identified 17 (out of 24) miRs at PND 21, 7 (out of 9) at PND 35, and 15 (out of 27) at PND 51 with experimentally validated gene targets. miRNAs repress gene translation, therefore, downregulated miRNA is associated with increased gene expression and upregulated miRNA is associated with the decreased gene expression. For downregulated miRNAs in the MF group vs. HM group, the number of unique genes were 37 at PND 21, 159 at PND 35, and 30 at PND 51 (**Figure 1A**). The three common genes between PND 21 and PND 35 are B-cell lymphoma 2 like 1 (*BCL2L1*), Kristen rat sarcoma viral oncogene homolog (*KRAS*), and Vinisin-like 1 (*VSNL1*). The common genes between PND 21 and PND 51 is mitotic arrest deficient 2 like 1 (*MAD2L1*). There are 50 common genes between PND 35 and PND 51. There is one common gene, estrogen receptor 1 (*ESR1*), between PND 21, PND 35, and PND 51. For upregulated miRNA, unique genes were 67 at PND 21 and 60 at PND 51 (**Figure 1B**). There are ten common genes between PND 21 and PND 51. Pathway analysis of the target-predicted genes was performed using IPA in order to further understand the functions possibly regulated in the MF vs. HM group. The top 25 pathways are shown for the different time points in **Figures 2**– **4**. A full list of genes and pathways possibly regulated by miRNA can be found in **Tables S4**–**S8**.

DISCUSSION

Breastfed infants, compared to formula fed, have decreased rates of infections such as otitis media, respiratory tract infections, gastroenteritis, and necrotizing enterocolitis as well as lower rates of obesity and diabetes (2). In previously published work (20), our lab noted that the piglets fed HM had higher serum antibody titers to cholera toxin subunit B and tetanus toxoid than those fed MF. They also had elevated immunoglobulin A producing cells specific to cholera toxin subunit B. The HM fed piglets were noted to have higher T cell proliferation compared to the MF group. There was no difference in body weights or caloric intake between the two groups, thus differences attributed here are likely by diet. Many components of breastmilk contribute to these improved outcomes in infants and new literature suggests miRNA may play a role. While there are studies that describe the different types of miRNA in breastmilk (13, 14, 16), there is no concrete evidence that miRNAs have a direct impact on infant immunity. It is also possible that other breastmilk components alone impact infant circulatory miRNA. To address this, we used a model of formula vs. breastmilk fed piglets collecting circulatory miRNA at different time points of weaning and post-weaning of the neonatal diet.

Kosaka et al. (16) noted expression of multiple miRNA in breastmilk that were predicted to be involved in T- and B-cell function. Specifically, miR-181a and miR-181b were identified in breastmilk. Interestingly, these miRs were decreased in circulation at PND 21 in our MF group compared to the HM

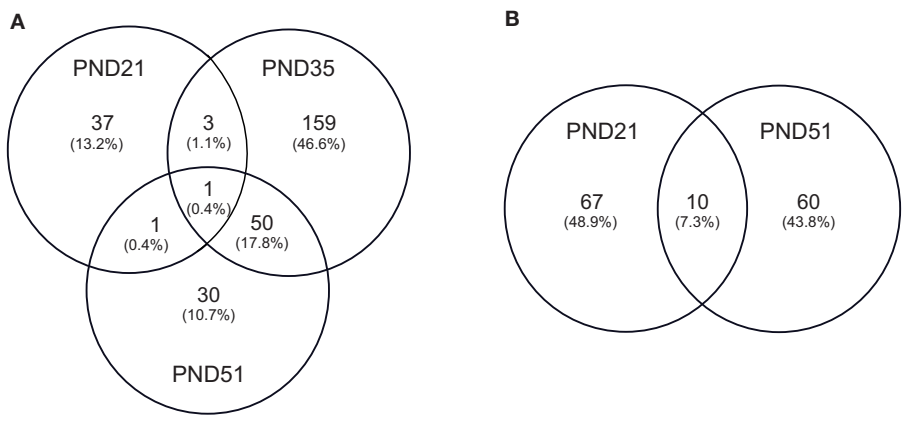

FIGURE 1 | Target predicted genes in MF fed piglets relative to HM fed piglets at PND 21, 35 and 51. **(A)** Venn diagram shows unique and shared genes of downregulated miRNA in MF relative to HM at each time point. **(B)** Venn diagram shows unique and shared genes of upregulated miRNA in MF relative to HM at each time point. The data represents from piglets of 9 MF and 9 HM at PND21, 4 MF and 4 HM at PND 35, and 9 MF and 10 HM at PND 51.

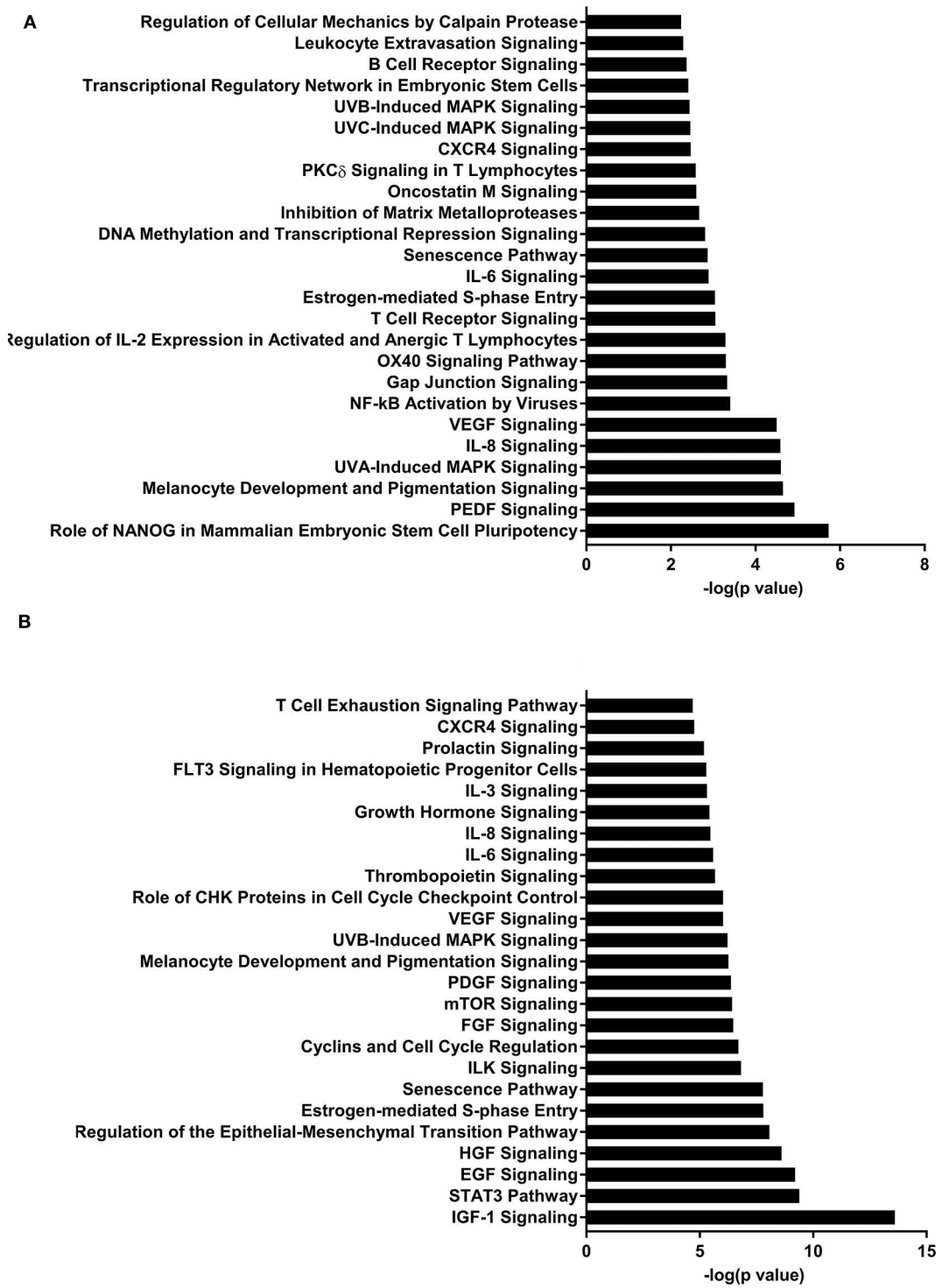

FIGURE 2 | Enriched pathways of MF compared to HM at PND21. **(A)** IPA of target predicted genes for downregulated miRNA in MF relative to HM at PND21. **(B)** IPA of target predicted genes for upregulated miRNA in MF relative to HM at PND21. The data represents from 9 MF and 9 HM fed piglets.

group, suggesting that breastmilk could be the source for these miRNAs. Since these miRNAs are thought to be involved in B- and T-cell differentiation (37), it is plausible that the higher expression in HM fed piglets contributed to the diet-dependent differences in immune cell activity that we previously reported in these animals. The expression pattern of miR-181a during T-cell maturation is dynamic and likely influences development of T-cells (38). miR-181 also plays a role in inflammation. It has

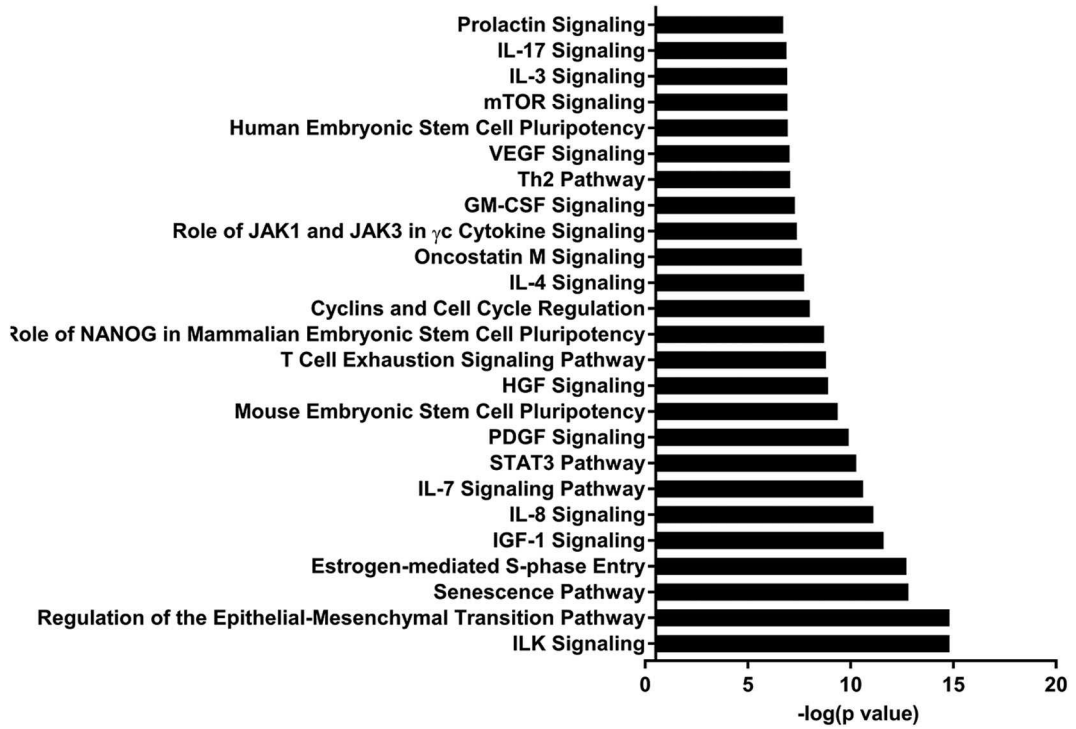

FIGURE 3 | Enriched pathways of MF compared to HM at PND35. IPA of target predicted genes for downregulated miRNA in MF relative to HM at PND35. The data represents from 4 MF and 4 HM fed piglets.

been shown to downregulate production of TNF-α in *Brucella abortus* infections (39). These data, along with ours, suggest that breastmilk miRs are likely involved in protecting infants in modulating the immune system (i.e., to reduce inflammation by infection and to impact T-cell maturation).

Golan-Gerstl et al. and Kahn et al. both showed high levels of miR-148 in pre-term, early term, and term breastmilk (13, 40). Golan-Gerstl et al. also showed significantly reduced amounts of miR-148 in formula compared to breastmilk. The piglets fed MF had a decreased amount of blood miR-148 compared to those fed HM at PND 21. miR-148 family negatively regulates the innate immune response by limiting cytokine production and inhibiting T-cell proliferation initiated by dendritic cell presentation of antigens in a mouse model (41), suggesting a role in reducing inflammatory cytokine production in HM fed piglets.

Let-7 is highly present in both the skim and fat layers of breastmilk (13, 14, 42). It has also been shown in these layers in bovine and goat milk (13). Let-7 regulates the innate and adaptive immune response, plays a role in TLR4 signaling and macrophage activity, and also affects T-cell differentiation and limits B-cell activation (30–33). At PND 35, let-7 had decreased concentration in MF fed group compared to HM group.

At both PND 21 and PND 51, miR-708-5p was significantly decreased in the MF fed piglets compared to the HM fed piglets, ∼40 fold and 180 fold respectively (**Tables 1**, **3**). miR-708 has been shown to target TLR4 (34) suggesting decreased inflammatory pathway activation in HM fed piglets compared to MF fed. miR-708 has also been shown to increase phagocytosis (35) which may allow the HM fed piglets to eliminate pathogens more easily than the MF fed piglets. miR-18b was significantly upregulated at PND 21 but significantly downregulated at PND 35 and 51 in the MF compared to HM fed piglets (**Tables 1–3**). In patients with multiple sclerosis, miR-18b has been associated with relapse (36, 43) so it is possible that it plays a role in inflammation and autoimmune diseases.

The piglets fed MF had higher levels of miR-199a and miR-199b at PND 51 than those fed HM. miR-199b has been found to be significantly increased in nasal mucous extracellular vesicles of adults with allergic rhinitis compared to those that are healthy (44). In asthma patients with a neutrophilic phenotype, plasma miR-199a was significantly increased and correlated negatively with pulmonary function (45). Wang et al. showed in a mouse model infected with *Mycobacterium bovis* that miRNA-199a inhibits autophagy of macrophages and decreases interferon-β production. This allows *M. bovis* to survive and grow in these infected mice (46). miR-199 is associated with allergy and asthma in adults and with bacterial survival in mice. It is possible that this miRNA is involved in increased atopy in formula fed infants (2). These data suggest that the diet could have a persistent effect on miRNA expression and on the immune system.

Several studies have shown that diet alone impacts miRNA levels. In a review by Kura et al. (47), different dietary components such as vitamin D, selenium, and vitamin E impacted blood and cardiac miRNAs that are associated with decreased cardiovascular disease. A high fat diet is associated with decreased miRNA-29b expression in the heart and increases

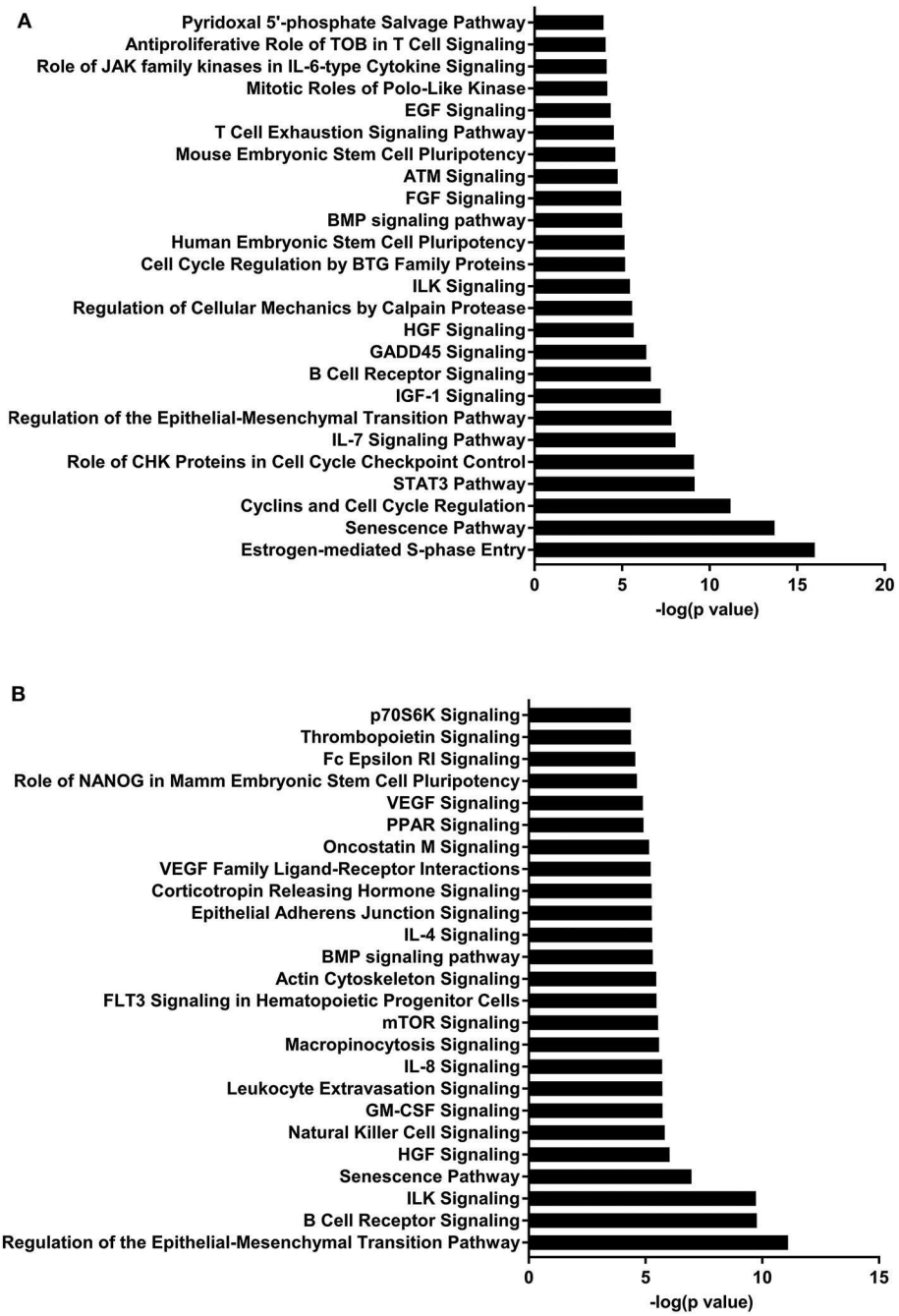

FIGURE 4 | Enriched pathways of MF compared to HM at PND51. **(A)** IPA of target predicted genes for downregulated miRNA in MF relative to HM at PND51. **(B)** IPA of target predicted genes for upregulated miRNA in MF relative to HM at PND51. The data represents from 9 MF and 10 HM fed piglets.

susceptibility to heart injury (48). Dietary compounds have also been shown to change the miRNA expression in skin in patients with psoriasis, helping with treatment of this disease (49). These data suggest that neonatal diet itself can impact miRNA expression. miRNA expression may have an impact in microbiome as well. Zhou et al. showed that mice fed an exosome/RNA depleted diet had different microbiome than mice fed an exosome/RNA sufficient diet (50). In our piglets, miRNA profiles are different in the formula fed vs. breastmilk fed piglets, as are the microbiome profiles [previously published data (51)]. While speculative, it is possible that the miRNA played a part in the neonatal diet-associated differences of the microbiome.

Pathway analysis revealed several pathways involved in immune function. B-cell receptor signaling pathway was likely

upregulated in the MF compared to HM at PND 21 in both the blood and ileal mucosa (Elolimy et al., unpublished results). The B-cell receptor pathway helps with development and differentiation of B-cells after exposure to antigens (52, 53). HM contains immunoglobulins (6, 54) that help in gut mucosa development and likely immune system education. These are not present in formula, which is likely the reason for an upregulation in this signaling pathway in the formula group. In the blood, this pathway is also both increased and decreased at PND 51, which is possibly due to the fact that signaling by miRNA is involved in maintaining homeostasis in the host. DNA methylation and transcriptional repression signaling pathway was also increased in the MF vs. HM group at PND 21 in both the blood and the ileal mucosa (Elolimy et al., unpublished results). DNA methylation involves regulation of gene expression by either inhibiting binding of transcription factor(s) or recruiting gene repression proteins to bind the DNA (55). This implies that MF fed may have a different methylation pattern and therefore gene expression, than HM fed, possibly these impact immune and metabolic realms.

The IL-7 signaling pathway is likely upregulated at PND 35 and 51 in the MF group compared to the HM group (miRNA were decreased). The IL-7 signaling pathway is important for development and differentiation of T-cells and early development of B-cells (56). Multiple cytokines have been found in human milk including IL-7 (57, 58) so it is possible that the HM group did not have an increase in this pathway because they are already exposed to IL-7 from the HM. This also suggests that the neonatal diet has prolonged effects on miRNA and gene expression post-weaning neonatal diet.

The IGF-1 signaling pathway was downregulated at PND 21 (miRNA upregulated) and upregulated at both PND 35 and 51 (miRNA downregulated) in the MF group compared to the HM group. Insulin-like growth factor (IGF)-1 plays an important role in multiple areas of development including cell proliferation and differentiation of tissues (59). Low levels of IGF-1 have been associated with different complications in premature infants including retinopathy of prematurity (ROP) (60) and bronchopulmonary dysplasia (BPD) (61). Interestingly, one study looked at IGF-1 to prevent these complications and decrease rates of ROP (60) which further prompted an ongoing study looking at IGF-1 infusion to prevent BPD. IGF-1 has also been shown in rat models to decrease germinal matrix hemorrhage bleeds (62).

There are several limitations to this study. First, the human breastmilk used was a pool and pasteurized. While miRNA has been shown to survive pasteurization (17), several other components might not survive the pasteurization process. Lactoferrin and secretory IgA are both reduced to some extent by pasteurization (63). We were not able to isolate miRNA from the breast milk samples at the time of this study, therefore, the differences seen in the MF vs. HM fed group are possibly attributable to human milk miRNA, but could also be due to other components in breast milk such as secretory IgA, human milk oligosaccharides, cytokines, etc (3–10). Secondly, the age of the babies of the donor milk mothers varies from about 2 months to 12 months (with an average of 6 months) and breastmilk components change over time (64, 65); thus, differences observed cannot be attributed to specific postpartum milk. Since the source of miRNA in this study was whole blood, future studies are needed to determine the specific cell types involved in miRNA expression profile.

CONCLUSION

Human breastmilk fed piglets were found to have variable amounts of circulatory miRNA compared to formula fed piglets in our pilot study. We proposed that the differential abundances of miRNA impacts immune system in MF vs. HM fed piglets. Further studies should include a human study of serum miRNA in breastmilk vs. formula fed infants as well as miRNA present in their diets. Also, studies looking at specific immune cells and their roles/associations with the miRNA patterns are warranted.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee at the University of Arkansas for Medical Sciences.

AUTHOR CONTRIBUTIONS

LC conducted the RNA isolation, library preparation of study samples, interpreted the data, wrote the manuscript and is responsible for the final content of the manuscript. AB conducted the piglet study. AE performed IPA analysis. SB and CW performed the miRNA sequencing data and statistical analysis. CR and SM performed sequencing. LY acquired the funding, designed the study, edited the manuscript and is responsible for the final content of the manuscript and the principal investigator of this study. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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Prevention of Allergy to a Major Cow's Milk Allergen by Breastfeeding in Mice Depends on Maternal Immune Status and Oral Exposure During Lactation

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Background: The high incidence of food allergy in childhood points to the need of elucidating early life factors dictating allergy susceptibility. Here, we aim to address in a mouse model how the exposure to a major cow's milk allergen through breastmilk of mothers with different immune status influences food allergy outcome in offspring.

Methods: BALB/cJ future dams were either kept naïve, or sensitized through the oral route using cholera toxin ("orally sensitized") or through the i.p. route using alum ("i.p. sensitized"), or rendered fully tolerant (oral gavage without any adjuvant) to bovine β-lactoglobulin (BLG). After mating with naïve males and delivery, mothers were orally exposed or not to BLG during the whole lactation. Then, eight groups of lactating mothers were considered: naïve, i.p. sensitized, orally sensitized, or tolerant, each exposed or not during lactation. In order to specifically address breastmilk effects on their allergy susceptibility, pups from naïve-synchronized mothers were cross-fostered by the different groups of treated dams and lactating mothers at delivery. In some experiments, mothers kept their own pups to address a possible *in utero* effect. BLG antigen, BLG-specific antibodies, and BLG-immune complexes were measured in breastmilk from the different lactating mother groups. Allergic sensitization was monitored in 5-weeks old female offspring (n = 7–8/group of lactating mothers) by determining BLG-specific antibodies in plasma and splenocytes cytokine secretion after i.p. injections of BLG/alum. Allergic reaction to oral BLG challenge was evaluated by measuring mMCP1 in plasma.

Results: Offspring was protected from one allergic i.p. sensitization when nursed by i.p. sensitized mothers, independently of BLG exposure during lactation. Orally sensitized dams conferred protection in offspring solely when exposed to BLG during lactation, while naïve mothers did not provide any protection upon BLG exposure. The levels of protection correlated with the levels of BLG-specific antibodies and BLG-immune complex in breastmilk. There was a trend for decreased sensitization in

offspring breastfed by tolerant and exposed mothers, which was not associated with transfer of specific antibodies through breastmilk. Protection provided by nursing by treated/exposed mothers was not persistent after a boost i.p. injection of the progeny and then did not protect them from an allergic reaction induced at this time point. No additional *in utero* effects were evidenced.

Conclusion: Our study demonstrates the strong potential of breastmilk to modulate immune response to a major cow's milk allergen in the progeny. It highlights the importance of maternal immune status and of her consumption of the allergen during lactation in dictating the outcomes in offspring. This opens perspectives where modulating maternal immune status might increase the chance of cow's milk allergy prevention in breastfed children.

Keywords: breastfeeding, food allergy, prevention, cow's milk, mouse model

INTRODUCTION

Immunoglobulin-E (IgE)-mediated food allergies are hypersensitivity reactions against harmless food proteins occurring in predisposed individuals. Instead of a clinically silent immune regulatory response, food allergic people mount inflammatory immune responses driven by Th2 cells upon ingestion of a food allergen (1). This results from an impaired induction of oral immune tolerance toward food antigens or its breakdown. Because the incidence of allergic disease peaks in infancy and childhood, there is a need to identify which early life factors are dictating allergy susceptibility (1).

The perinatal period is a critical period in which both microbiota implantation and type of feeding impact on the maturation of the gut immune system and the epithelial barrier, and thus on the propensity to develop food allergy later in life. Notably, breastmilk might influence immune system development via the transfer of various immunomodulatory molecules directly acting on the epithelial and immune system, or acting via the microbiota, such as regulatory/pro-inflammatory cytokines, miRNA, immunoglobulins, nutrients, but also metabolic products from the microbiota (2–5). Human breastmilk also contains food antigens, which have been ingested by the mother (6–17). While the factors controlling food antigen shedding in breastmilk are poorly identified, the excretion of food antigens, at low doses and over a long period of time after ingestion (>24 h), appears as a natural process. This might have a role in the education of the immune system to environmental antigens to which the newborn will be naturally exposed: actually, as part of the usual diet of the mother, they might correspond to dietary habits of the family.

Mouse studies evidenced that oral administration of ovalbumin (Ova) to naive mice during lactation led to excretion of Ova in milk, which induced partial protection of the progeny from experimental Ova-induced allergic airway inflammation. The protection was antigen-specific and dependent of transforming growth factor-beta (TGF-β) in breastmilk (18). However, the protective effect provided by Ova-exposure during lactation was far more intense and durable if the mothers were first immunized to the allergen. Breastfeeding-induced tolerance then involves the transfer of IgG-Ova complexes to the neonates, their loading through the neonatal receptor for immunoglobulin constant region (FcR) in the gut and the induction of specific $Foxp3^{+}CD25^{+}$ regulatory cells (19–21). These observations were further extended to mice models of Ova-induced allergic diarrhea (17).

In order to expand the knowledge on how to prevent food allergy by breastmilk, we aimed to address whether observations obtained with an egg allergen could be extended to the major cow's milk allergen, bovine β-lactoglobulin, a frequent cause of food allergy in infancy. Furthermore, in order to better reflect the human setting, we also aimed to assess the role of the immune status of the mother in this protection. We then considered either naïve, tolerant, moderately sensitized, or highly sensitized mothers who were exposed or not to the food allergen during lactation.

MATERIALS AND METHODS

Mice

Female and male BALB/cJ Rj mice, 4 weeks-old, were purchased from CERJ (Centre d'Elevage René Janvier, Le Genest-Saint-Isle, France), and were housed in filtered cages under normal specific pathogen free husbandry conditions, with autoclaved bedding and sterile water. Mice received a diet deprived of animal proteins in which BLG was not detected using specific immunoassays (22).

BLG Purification

Native BLG (BLG) was purified from raw cow's milk (non-heated, Ferme de Viltain, Saclay, France) using selective precipitation and chromatography, and further characterized, as previously described (23).

Sensitization or Tolerization of the Future Dams, and Oral Exposure to BLG During Lactation

A first group of female mice was highly sensitized when 7 weeks-old by i.p. injection of 5 μg of BLG adsorbed on alum (Alhydrogel 3%, Superfos, Danemark, 1 mg/mouse), with a second injection performed 14 days apart, a model known to induce very high levels of IgE and IgG1 specific antibodies and high Th2 cytokine secretion (24–26) ("i.p. sensitized" mothers, $n = 15$). Another group of female mice was moderately sensitized when 4 weeks-old by performing intra-gastric gavage with 2 mg of BLG mixed with 10 μg of Cholera toxin (Sigma-Aldrich, Saint-Louis, US). Gavaged were repeated once a week for 5 weeks. This model allows inducing specific IgE, IgG1, and Th2 cytokine secretion, but that are far lower than induced by the i.p. route using alum (24) ("orally sensitized" mothers, $n = 15$). A third group of female mice was rendered fully tolerant by repeated gavage with 2 mg of BLG alone when 8 weeks old, a model allowing induction of regulatory T cells that prevent any further sensitization to BLG and any induction of BLG-specific antibodies (26, 27) ("tolerant mothers," $n = 15$). An fourth group of female mice was kept untreated (naïve mothers, $n = 15$). When 9 weeks old, and 2 days after the last sensitizing/tolerating treatment, all females were mated with age-matched naïve males. Sixty six percent of the females were pregnant, and at delivery, pups from sensitized/tolerated mothers were replaced by pups from naïve-synchronized mothers in order to exclusively assess breastfeeding effect and not the *in utero* effect (**Figure 1**). Lactating mothers were then exposed or not to 1 mg of BLG by gavage (200 μl/administration, diluted in PBS) every other day starting 48 h after delivery and until weaning. Non-exposed mice received only PBS, so they and their pups had the same handling/stress as in the group of exposed mothers.

In order to assess any additional *in utero* effect, another experiment was conducted in which pups were kept by their respective mother and protocol then performed as before.

Milk Collection

Breastmilk was collected 10 days post-partum from the stomach of 6–10 male pups per mother group. Males were sacrificed 4 h after gavage of the mother with BLG (or PBS) and stomach content was collected and pooled per mother treatment (2–3 mothers per treatment group). Content was weighted and diluted in two volumes of PBS. After vortexing and centrifugation (10,000 × *g*, 10 min, +4°C), supernatants were collected and stored at −20°C until analysis.

BLG, BLG-Specific Ig Antibodies, and BLG-Ig Immune Complexes in Breastmilk

Enzyme immunometric assays were performed in 96-well microtiter plates (Immunoplate Maxisorb, Nunc, Roskilde, Denmark) using AutoPlate Washer, Microfill dispenser and spectrometer equipments from BioTek instruments, Inc (Avantec, Rungis, France).

BLG antigen and BLG-specific IgG1, IgA, and IgE were quantified in serial dilution of breastmilk samples (from 1/5 to 1/625) as previously described (22, 25, 28). As no standard is available for BLG-specific IgA, results are expressed as absorbance measured at 414 nm.

BLG-IgG1, BLG-IgA, and BLG-IgE immune complexes were assayed on plates coated with IgG purified from rabbit hyperimmunized with BLG. Serial dilutions (from 1/5 to 1/625) of breastmilk samples were performed in immunoassay buffer (0.1 M phosphate buffer, 0.1% bovine serum albumin, 0.01% sodium azide) and applied to coated plates for 18 h at 4°C. After extensive wash (0.01 M phosphate buffer pH 7.4, 0.05% Tween 20), acetylcholinesterase (AChE)-labeled anti-mouse IgE, anti-mouse IgG1, or anti-mouse IgA antibodies were applied for 3 h at room temperature, and solid-phase bound AChE activity was determined by addition of 200 μL/well of Ellman's medium. Absorbance was then measured at 414 nm (25, 28). A positive control of IgG1-BLG immune complex was provided by mixing purified anti-BLG IgG1 monoclonal antibodies (10 ng/ml) with purified BLG (1 ng/ml). No specific IgA-BLG or IgE-BLG immune complexes were detected, whatever the group of lactating mothers considered.

Allergy to BLG in Offspring (Figure 1)

Protocol of Induction of Allergy to BLG

When 5 weeks old, the female offspring nursed by the different groups of mothers (e.g., naïve, naïve exposed during lactation, i.p. sensitized, i.p. sensitized exposed during lactation, orally sensitized, orally sensitized exposed during lactation, tolerant, tolerant exposed during lactation) was sensitized to BLG by i.p. immunization with alum (7–8 mice/group of mothers). Plasma samples were collected 20 days later to assess allergic sensitization. To assess the persistency of any effects, a boost injection was performed 21 days after the first i.p. sensitization, and 2 weeks later, all mice were orally challenged with 15 mg of purified BLG. Plasma was collected 60 min later, both to assess allergic sensitization and elicitation of the allergic reaction.

Evaluation of Allergic Sensitization

BLG specific IgG1, IgE, and IgG2a were quantified on plasma samples collected from progeny using BLG-coated microtiter plates (25, 26, 29). Due to high IgG concentrations that might mask epitopes for IgE binding after the i.p. boost (25), a reverse assay using anti-mouse IgE coated plates and AChE-labeled BLG was also performed. Results are then expressed as mAU_{414nm} (no standard available). Non-specific binding was assessed using plasma samples collected from naïve progeny (non-sensitized progeny from naïve and non-exposed mothers).

Evaluation of Allergic Reaction

Mouse Mast Cell Protease-1 (mMCP1), a specific marker of intestinal mast cell activation (Moredun Scientific Limited, Midlothian, UK), was assessed on plasma samples collected 60 min after BLG oral challenge following provider recommendations.

Splenocytes Cytokine Secretion

After oral challenge, spleens were harvested and pooled within each mother treatment group. After lysis of red blood

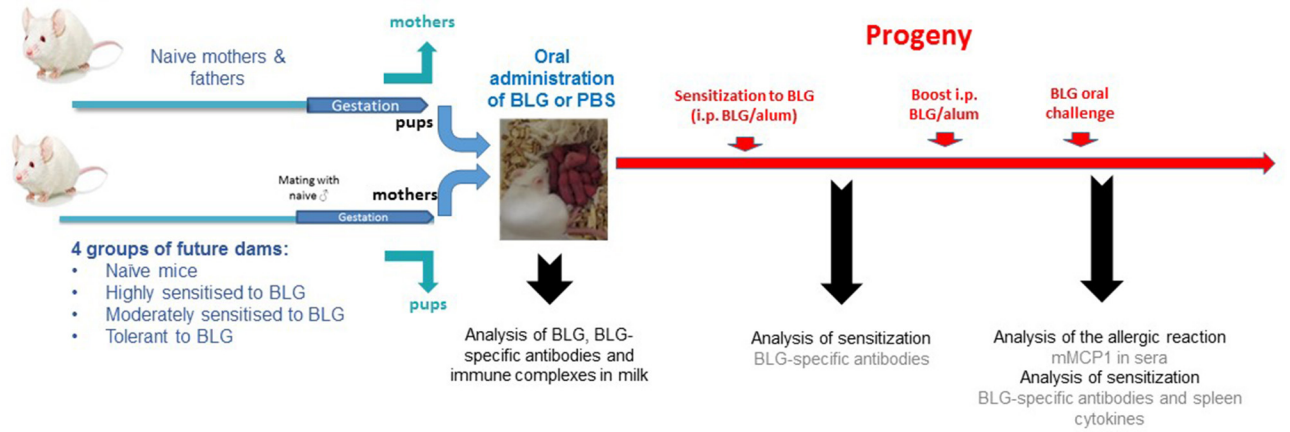

FIGURE 1 | Experimental protocol: BALB/cJ future dams were either kept naïve, moderately (intra-gastric gavage with BLG, and *Cholera toxin*) or highly (intra-peritoneal administration of BLG/alum) sensitized to BLG, or rendered tolerant to BLG (intra-gastric gavage with BLG alone) before mating with naïve males. At delivery, pups of treated mothers were replaced by pups from naïve-synchronized mothers in order to prevent interferences from *in utero* effects. Lactating mothers from each group then received BLG or PBS by i.g. gavage every other day, during the whole period of lactation. Breastmilk was collected 10 days post-partum by pooling stomach contents from 6 to 10 male pups per mother group to assess BLG, BLG specific antibodies, and BLG-immune complexes. The female progeny was then experimentally sensitized by i.p. injection of BLG and Alum when 5 weeks-old and BLG-specific IgE, IgG1, and IgG2a were measured 3 weeks later. A boost injection was performed 21 days after the first i.p. sensitization, and 2 weeks later, all mice were orally challenged with 15 mg of purified BLG. Plasma was collected 60 min later, both to assess allergic sensitization and elicitation of the allergic reaction. Mice were then sacrificed and spleens were pooled to assess *ex vivo* BLG-specific cytokine secretion.

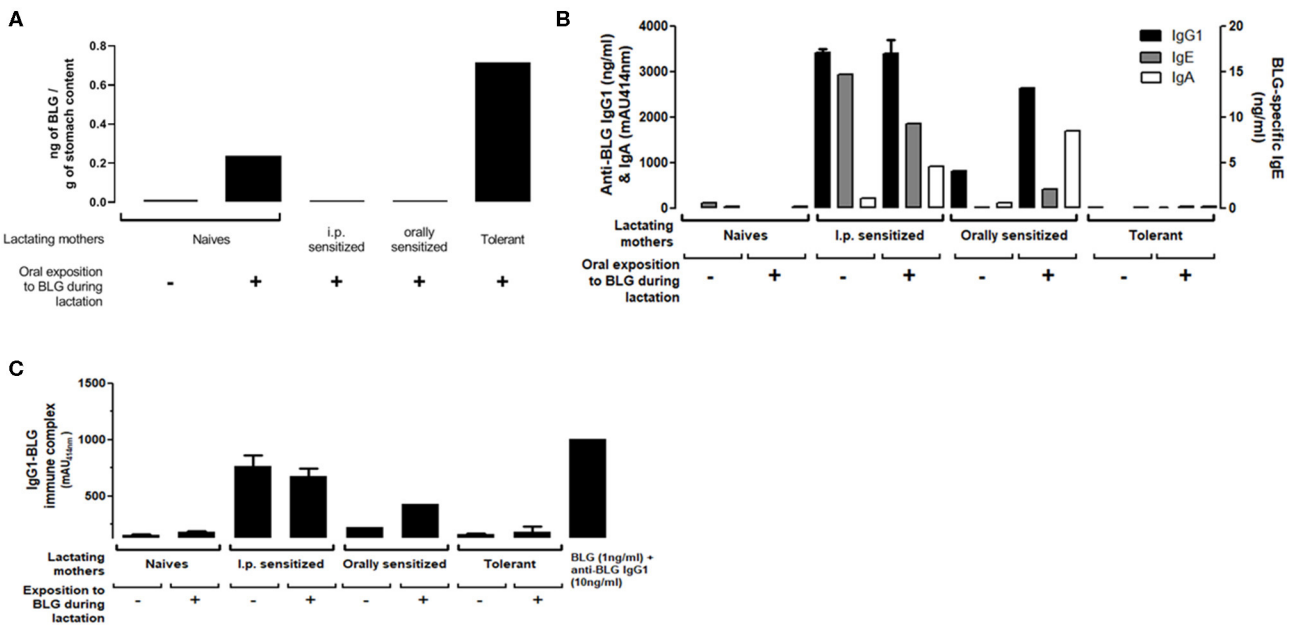

FIGURE 2 | (A) BLG antigen, **(B)** BLG-Specific IgG1 (black bars), IgA (white bars) and IgE (gray bars) and **(C)** IgG1-BLG immune complexes detected in milk collected 10 days post-partum from naïve, sensitized or tolerant mothers exposed (+) or not (–) to 1 mg of BLG every other day during lactation. Assays were performed on stomach contents collected from pups 4 h after the exposition of their fostered mothers. For BLG-specific IgA and IgG1-BLG immune complexes, absorbance signals (mAU_{414nm}) obtained at the 1/25 dilution are reported. A positive control of IgG1-BLG immune complex was provided by mixing purified anti-BLG IgG1 monoclonal antibodies with purified BLG (right bar).

cells (180 mM NH_4Cl, 17 mM Na_2EDTA) and several washes, splenocytes were resuspended in RPMI-10 (RPMI supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U penicillin, 100 µg/mL streptomycin—all from Gibco). Cells were incubated in 96-well culture plates (10^6 cells/well) in the presence of BLG (20 µg/mL), RPMI-10 (negative control), or concanavalin

A (1 μg/mL, positive control) for 60 h at 37°C and 5% CO_2. Each culture conditions were performed in duplicates. Culture were centrifuged (300 × *g*, 10 min) and supernatants were collected and stored at −80°C until further assay for cytokines using multiplexed kits and apparatus from Biorad (Bio-Plex Pro™ Mouse Group I and Bioplex100™ apparatus; Marnes la Coquette, France).

Statistical Analysis

Due to the number of animal included per group ($n < 30$) and as data were not normally distributed, we used non-parametric tests. Presence of differences between groups was first tested using non-parametric Kruskall-Wallis test, and *p*-values calculated using Monte Carlo simulation (10,000 permutations). Pairwise multiple comparison was then performed using Conover-Iman testing, including Bonferroni correction for multiple testing. When specified, Mann Whitney test was additionally performed between two specific groups. All statistical analysis were performed using XLSTAT™ 2019 (version 2.3, Addinsoft, France).

RESULTS

The Transfer of BLG Antigen, BLG-Specific Antibodies, and BLG-Immune Complexes Into Breastmilk Depends on Maternal Immune Status

Using a BLG-specific sandwich immunoassay, we detected BLG in milk collected from naïve or tolerant mothers who had been exposed to BLG during lactation (**Figure 2A**). In contrast, we could not detect BLG in milk collected from orally (moderate sensitization) or i.p. (high sensitization) sensitized mothers orally exposed to BLG during lactation.

BLG-specific IgE, IgG1, and IgA (**Figure 2B**) and BLG-IgG1 immune complexes (**Figure 2C**) were undetectable in milk from naïve or tolerant mothers, whether they had been exposed or not to BLG during lactation. In milk from orally sensitized mothers, BLG-specific Ig and immune complexes were detected and their levels increased with BLG exposure during lactation. I.p. sensitized mothers had the highest levels of BLG-specific Ig and BLG immune complexes. They were not further increased by BLG-exposure during lactation, except for the BLG-specific IgA.

Protection From i.p. Sensitization to BLG in Offspring Depends on Maternal Immune Status and Oral Exposure to BLG During Lactation

The susceptibility of offspring from various mothers' group to be sensitized to BLG was first assessed by measuring BLG-specific antibodies after one i.p. sensitization with BLG in Alum. Mice fostered by naïve mothers exposed or not to BLG during lactation demonstrated comparable sensitization levels, as evidenced by comparable concentrations of BLG-specific IgE and IgG1 (**Figure 3**). A trend in decreased BLG-specific IgE and IgG1 antibodies concentrations were evidenced in progeny fed by tolerant and exposed mothers ($p = 0.06$ and $p = 0.01$, respectively, using Mann-Whitney test and when compared to naïve non-exposed mice), whereas no effect was evidenced in absence of exposure. Progeny fostered by orally sensitized mothers were significantly protected from sensitization only if mothers were exposed to BLG during lactation, although a trend in decreased BLG-specific IgE concentrations was also noticed without this exposure ($p = 0{,}01$ vs. naive non-exposed mother, using Mann-Whitney test). In contrast, progeny fostered by i.p. sensitized mothers were fully protected from sensitization, whether exposed or not during lactation, as evidenced by the nearly absence of specific IgE and the very low concentrations of BLG-specific IgG1.

Protection Is Not Persistent After a Boost i.p. Injection of the Progeny With BLG and Then Does Not Protect the Progeny From Allergic Reaction Elicitation

We further assessed the persistency of the prevention from sensitization observed in the progeny nursed by the different treated/exposed groups of mothers by performing an additional i.p. immunization with BLG and alum and an oral BLG challenge. A significant decrease of BLG-specific IgE concentrations was only observed in the progeny fostered by the i.p. sensitized exposed or non-exposed mothers as compared to naïve non-exposed mothers (**Figures 4A,B**). However, this protection from systemic sensitization was not associated with a reduced allergic reaction as shown by comparable levels of mMCP1 in all the groups (**Figure 4C**). The only group that tended to be protected from the elicitation of an allergic reaction was the progeny fostered by tolerant and exposed mothers ($p = 0.06$ using the Mann-Whitney test). No difference of IgG2a concentrations was observed between the different groups of sensitized progeny (data not shown).

Cellular Response in the Progeny Evidenced Modulated Cytokines Profiles

We then assessed cellular immune responses in the sensitized progeny mice by analyzing cytokine secretion at the end of the experimental protocol. No cytokine secretion was observed after culture with media alone, and Concanavalin A-stimulation led to comparable cytokine secretions in the different groups of mice (not shown). When splenocytes were cultured with BLG, increased secretion of Th1 (IFNγ) and Th17 (IL-17) cytokines was noticed in cells from progeny fostered by naïve mothers exposed during lactation to BLG compared to all the other groups (**Supplementary Figure 1**). A trend in increased BLG-induced Th2 cytokines IL-5 and IL-13 secretion was noticed in splenocytes from progeny fed by non-exposed and sensitized mothers, either i.p. or orally, which was however not associated with higher humoral responses (**Figures 3**, **4**). Conversely, exposure during lactation greatly decreased BLG-induced Th2 cytokine secretion in those groups. Feeding by tolerant mothers also rather led to a decreased Th2 cytokine secretion, whatever the exposure during

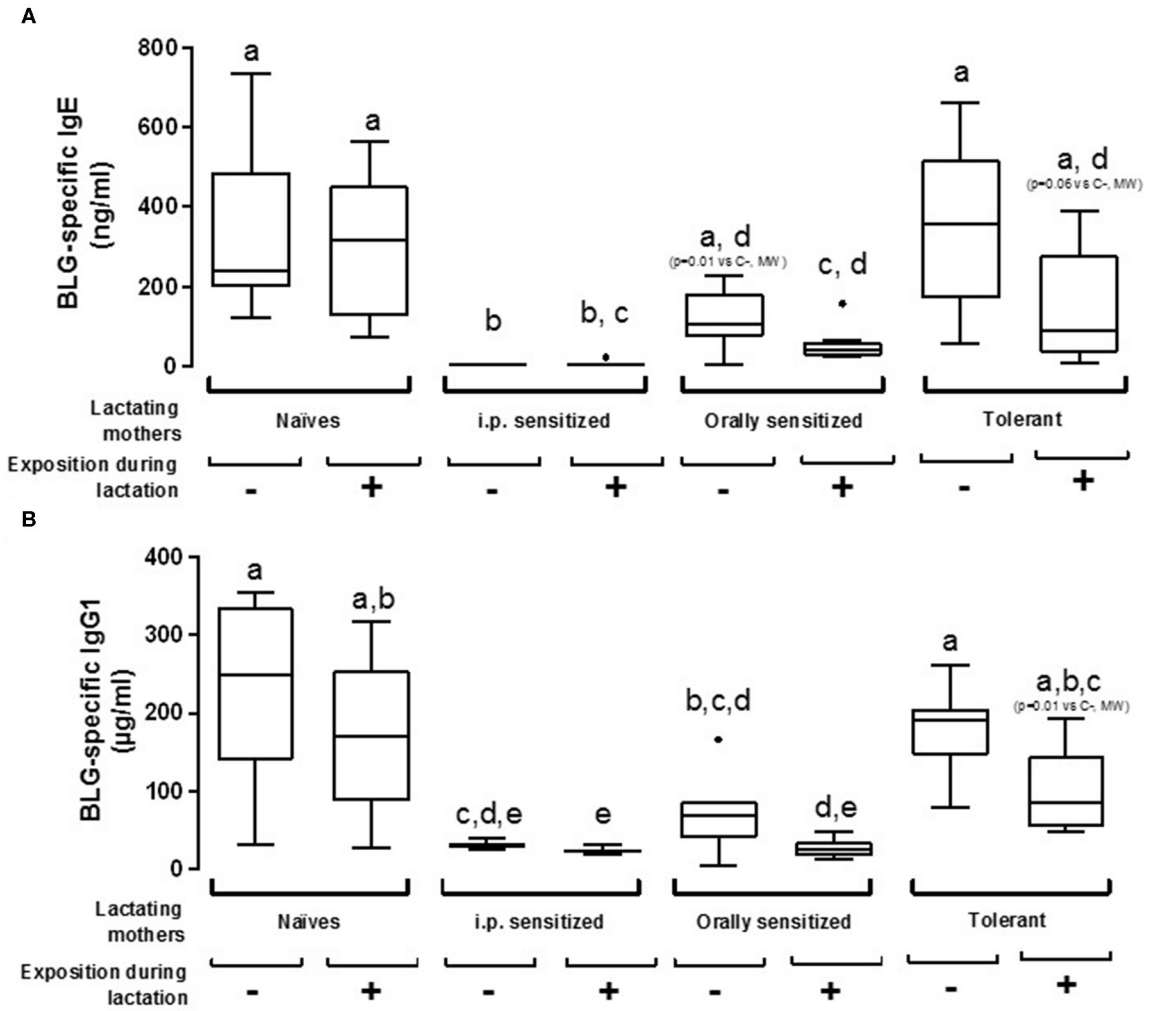

FIGURE 3 | Plasma BLG-Specific IgE **(A)** and IgG1 **(B)** antibodies induced after one i.p. sensitization with BLG/alum in pups fostered by naïve or pre-natally sensitized or tolerated mothers, further orally exposed (+) or not (–) to BLG during lactation. Tukey box and whiskers from 7 to 8 mice/groups are shown. Statistical analysis evidenced differences between groups ($p < 0.0001$, non-parametric Kruskall Wallis test). Pairwise multiple comparisons were then performed using Conover-Iman testing, including Bonferroni correction for multiple testing. Groups indicated with different letters are different from each other's ($p < 0.05$). *p*-values obtained using additional testing against control (naive and non-exposed mothers) by Mann Whitney test are also indicated between brackets.

lactation. Comparable results were obtained for Th2 cytokines and IL-10.

Absence of Additional *in utero* Effects

We finally aimed to assess if *in utero* events might provide additional protective effects in the progeny. Therefore, we compared sensitization in pups nursed by their own mothers vs. pups from naïve-synchronized mothers that were cross-fostered by mothers from the different groups. All mothers were exposed to BLG during the whole breastfeeding period. We found similar IgE and IgG1 responses after the first sensitization (**Figure 5A**) and after the boost injection (**Figure 5B**) in pups form the mothers fostering their own progeny ("*in utero* + lactation") or progeny from naïve-synchronized mothers ("lactation").

DISCUSSION

New epidemiological and interventional studies demonstrate the food allergy preventive effect of early introduction of some allergens in infant diet such as egg and peanut (LEAP and EAT studies, G. Lack and G. du Toit) (30–32). In contrast, the interventional introduction of cow's milk in the diet of breast-fed infant after 3 months is not associated with protection (33). This is in line with the non-interventional large study from Katz and coworkers that evidenced the highest cow's milk prevalence in infants for who regular exposure to cow's milk protein was withheld until the age of 4–6 months (34). In parallel, studies evidenced that regular introduction of cow's milk formula in the first 2 weeks (34) or first 3 months (35) while pursuing

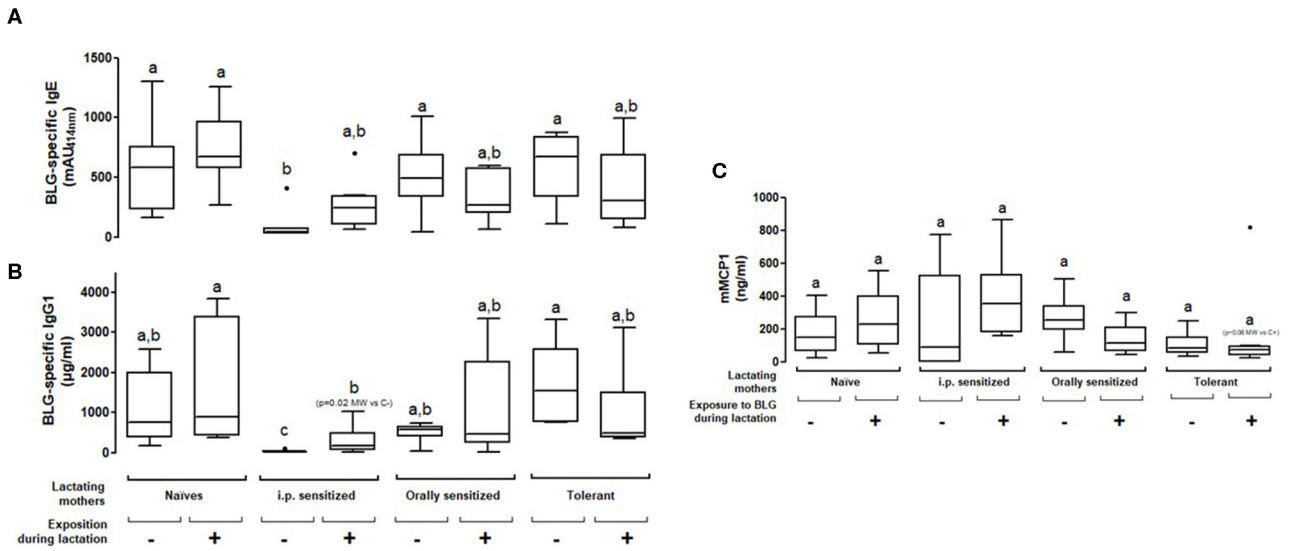

FIGURE 4 | Plasma BLG-Specific IgE **(A)**, IgG1 **(B),** and mMCP1 **(C)** induced after two i.p. sensitizations and then an oral food challenge with BLG in pups fostered by naïve or prenatally sensitized or tolerized mothers, further orally exposed (+) or not (–) to BLG during lactation. Tukey box and whiskers from 7 to 8 mice/groups are shown. Statistical analysis evidenced differences between groups ($p = 0.002$ for IgE and $p = 0.038$ for mMCP1, non-parametric Kruskall Wallis test). Pairwise multiple comparisons were then conducted using Conover-Iman testing, including Bonferroni correction for multiple testing. Groups indicated with different letters are different from each other's ($p < 0.05$). *p*-values obtained using additional testing against controls by Mann Whitney test are also indicated (MW).

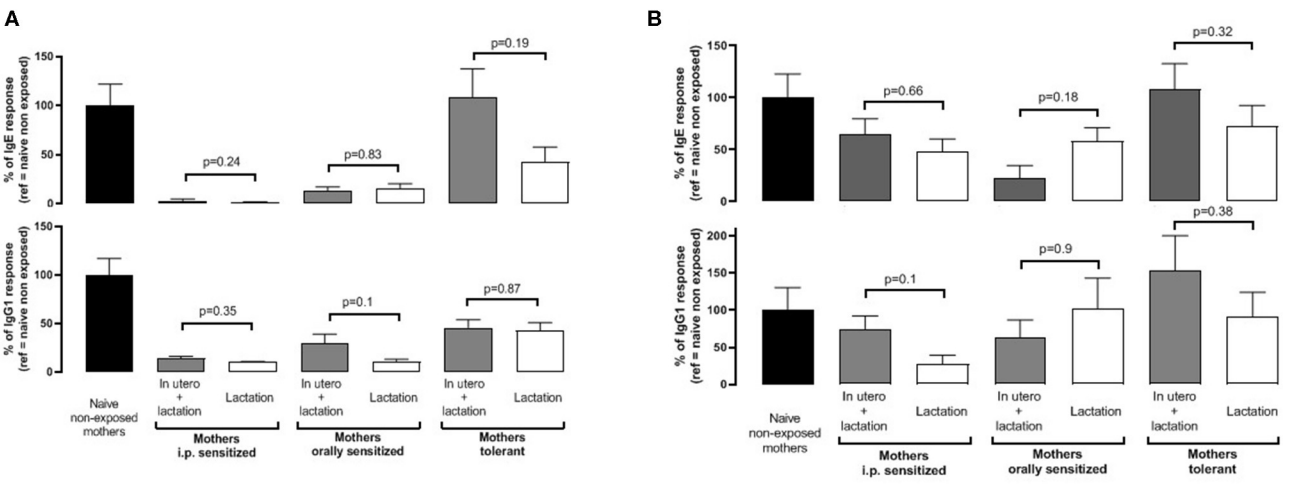

FIGURE 5 | Pups were born and fed by prenatally sensitized (i.p. or orally) or tolerant mothers, further orally exposed during lactation (*in utero* + lactation), or pups from naïve mice were fostered by prenatally sensitized or tolerant mothers further orally exposed during lactation ($n = 5$–12/group). BLG-specific IgE (top panel) and IgG1 (bottom panel) were assayed in plasma from progeny after one **(A)** and two **(B)** i.p. sensitizations with BLG/alum. Specific antibodies are expressed as percentage, with the mean value obtained in naive mothers not exposed during lactation used as a reference (100%). Immune response was compared between "*in utero* + lactation" and "lactation" groups for a same mother pre-treatment, and *p*-values are indicated (Mann Whitney test).

breastfeeding might allow protection. In these studies, no or few information is available on the mother immune status and cow's milk consumption while breastfeeding. Yet, oral exposure in the mother during lactation might already have a significant impact on the breastfed progeny; it has been evidenced recently that early peanut introduction (<12 months) is associated with protection only if the mother consumed peanut while breastfeeding (32). This highlights the need to better understand the way to maximize the chance of food allergy prevention. Here, we then aimed to determine how both the immune status of the mother and her ingestion of a clinically-relevant cow's milk allergen during breastfeeding will impact the allergic outcome in the progeny. Using a mother-child mouse model, we found that these factors do have a major impact on sensitization susceptibility

in offspring. Effect on sensitization ranged between nihil for naïve mothers exposed to BLG to a very potent protection for i.p. sensitized mothers (highly sensitized) ingesting or not BLG during lactation. Tolerant mothers and orally immunized (moderate sensitization) mothers induced some protection from sensitization but only when exposed to BLG during lactation. No additional *in utero* effect was evidenced in our experimental set up.

Actually, when we administered BLG to naïve BALB/cJ lactating mothers, we detected BLG in milk collected on D10 but we could not evidence any significant effect on sensitization of the progeny. Our results are not in line with all those obtained following the same experimental schedule and using Ova as a model allergen (18, 20, 36), although others did not evidence protection and even demonstrated enhanced sensitization in the progeny in similar models (37). Importantly, a mother ingesting a food is most of the time not naïve to this food: she is either tolerant, or sensitized, or allergic and the mothers then produce antibodies (IgGs, IgE, IgA) and have T cells (Treg, T helper) specific to the food antigens. Although a high inter-individual variability was noticed, Ova specific IgG and IgA were detected in more than 95% of transition breastmilk from the French birth cohort EDEN, whereas Ova was detected in only 50% of the samples (17). In the present study, we evidenced that sensitization level of the mothers (naïve, moderately (oral) or highly (i.p.) sensitized) determines the concentrations of IgG, IgE, and IgA specific antibodies in milk, and these increase upon oral exposure in the moderately exposed mothers. The concentrations of antibodies were associated with level of protection in the progeny. This is in line with different studies in human that suggest a protective role of high concentrations of breastmilk specific antibodies on child sensitization, and that exposure of the mother to the food allergens during lactation might increase their concentrations (38, 39).

However, immune complexes might be even more efficient than specific antibodies to protect the progeny, as evidenced in the Ova-model in which breastfeeding-induced tolerance by immunized mothers relies on the transfer of IgG-Ova complexes to the neonates (19, 21). Although immune complexes were not assessed in most of the previous cited studies in humans, IgG and IgA immune complexes with gliadin (9) and peanut allergens (13) were evidenced in human breastmilk. Moreover, oral administration of human breastmilk containing peanut allergens (free and complexed) before weaning induced partial protection from sensitization in a mouse model (13). In the present study, BLG-immune complexes levels are related with level of protection in the progeny. Exposure to the allergen is required to detect immune complex in breastmilk in the orally sensitized mothers, whereas exposure to BLG in the BLG/Alum model is dispensable. This might result from the deposit effect of alum allowing progressive release of the antigen, then available for forming immune complexes independently of oral exposure. Although we did not absolutely prove the direct causal role of Ig and immune complex on protection from sensitization, which might be a limitation of our study, all these results suggest that, in the human condition, oral exposure to the allergen during breastfeeding might be critical to form the Ig-immune complexes necessary to induced efficient protection in the progeny.

Another interesting point is that BLG was not detectable in the BM from sensitized and exposed mothers, whereas we were able to detect BLG in the BM from naïve or tolerant mothers exposed during lactation. As BLG detection relied on the use of an immunometric assay, BLG might not be detectable in the former milks due to a masking effect of specific antibodies present in the breastmilk and/or the presence of BLG mainly as immune complexes. The fact that not all mothers were found to be excretors in various studies might result from the same masking effect (7–12, 14–17). This might then imply that all mothers are actually excretors of allergens in their BM, but as a free and/or complexed form depending on the levels of exposure. This should be taken into account in the association studies relating allergen concentrations in breastmilk and allergic outcome in the progeny.

In our mouse model, tolerant mothers tended to protect offspring from sensitization when exposed to BLG during lactation and this protection was not associated with Ig levels or presence of immune complexes in breastmilk. This suggests that other mechanisms, such as transfer in breastmilk of specific immune cell or immuno-suppressive cytokines, might also be involved in the transfer of protection. The actors and mechanisms involved in the protection provided by tolerant and exposed mothers clearly need additional studies.

It is worth noting that sensitization levels and mMCP1 concentrations after the OFCs were not directly correlated. This may be explained by difference in mast cell density and FceRI expression and will require further investigation.

Finally, another point is that the induction of protection we observed with BLG appears to be less efficient than that observed for Ova, despite the same experimental schedule applied (18–21). Notably, we could not evidence any protective effect of exposure to BLG via breastmilk from naïve mothers, and the protection provided by i.p. sensitized mother did not protect progeny from allergic reaction. These observations suggest that, in addition to the immune status of the mother, the nature of the food allergen itself might be important in dictating the possibility to induce oral tolerance in early life. This is also reflected in epidemiological and interventional studies demonstrating the prevention of food allergy by early introduction of some allergens in the diet such as egg and peanut while this was not observed for other allergens such as cow's milk (33). Future work is needed to elucidate which additional approaches are necessary for successful persistent induction of immune tolerance and prevention of allergic disease to cow's milk allergens in early life. Offspring exposure to BLG after weaning might be required, as suggested for peanut (32). Other strategy might also include supplementation in TGF-β in formula given after weaning (17).

In conclusion, our study demonstrated the strong potential of breastmilk to modulate in the long term the immune response to food allergens in offspring. This protective effect is associated with the excretion of the food allergens and immune factors in breastmilk, some of which are increased by

exposure to the allergen during lactation. Future studies will need to address whether early immune modulation to cow's milk allergen by exposure through breastmilk might lead to a more successful cow's milk allergy prevention by early introduction in child.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article are available from the corresponding author to any qualified researcher on reasonable request.

ETHICS STATEMENT

All animal experiments were performed according to European Community rules of animal care and with authorization N° 91–368 of the French Veterinary Services. All experiments were covered by agreement no. 2009-DDSV-074 from the Veterinary Inspection Department of Essonne (France).

AUTHOR CONTRIBUTIONS

KA-P and VV designed the whole study, analyzed, interpreted the data, and wrote the manuscript. KA-P performed the experiments. HB and SH help to perform some experiments and critically revised the manuscript. FF and CJ critically revised the manuscript. All authors approved the 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/fimmu.2020.01545/full#supplementary-material

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REVIEW
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The Human Breast Milk Metabolome in Overweight and Obese Mothers

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Pre-pregnancy body mass index (BMI) is a major relevance factor, since maternal overweight and obesity can impair the pregnancy outcome and represent risk factors for several neonatal, childhood, and adult conditions, including excessive weight gain, cardiovascular disease, diabetes mellitus, and even behavioral disorders. Currently, breast milk (BM) composition in such category of mothers was not completely defined. In this field, metabolomics represents the ideal technology, able to detect the whole profile of low molecular weight molecules in BM. Limited information is available on human BM metabolites differences in overweight or obese compared to lean mothers. Analyzing all the metabolomics studies published on Medline in English language, this review evaluated the effects that 8 specific types of metabolites found altered by maternal overweight and obesity (nucleotide derivatives, 5-methylthioadenosine, sugar-alcohols, acylcarnitine and amino acids, polyamines, mono-and oligosaccharides, lipids) can exert on the risk of offspring obesity development and other potentially associated health outcomes and complications. However, metabolites variations in samples collected from overweight and obese mothers and the potentially correlated effects highlighted below still need further investigations and should be confirmed in future metabolomics studies on larger samples. Finally, the positive or negative influence of maternal overweight and obesity on the offspring, potentially exerted by breastfeeding, should be analyzed in close correlation with maternal age, genetic and environmental factors, including diet, and taking into account the interactions occurring between BM metabolites and lactobiome. The evaluation of all the factors affecting BM metabolites in overweight and obese mothers can lead to the comprehensive description of such biofluid and the related effects on breastfed subjects, potentially highlighting personalized needs of BM supplementation or short- and long-term prevention strategies to optimize offspring health.

Keywords: breast milk, breastfeeding, obesity, overweight, gestational diabetes mellitus, diabetes, metabolomics

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INTRODUCTION

Obesity is a growing social problem affecting an increasing number of women in reproductive age. It represents a risk factor potentially impairing the pregnancy itself, and even the long-term outcome of the offspring, since it is well known that the overweight condition can be transmitted to the future generations. Several observational studies and some meta-analyses have been carried out to assess whether breastfeeding is positively correlated with a reduction in the incidence of obesity

in later life. Although most of them showed a modest reduction in such risk (1), rigorous reviews (1–3) conclude that there is no clear cause-effect relationship, because of several confusing factors and frequent bias of such studies. Currently, conditions as the socio-economic status of the mother, the level of education, ethnicity, eating habits of the family, and duration of breastfeeding have not been considered and could themselves justify the association. On the other hand, a study reports that the risk of obesity is higher in the non-breastfed children of obese mothers, but remains higher even in those who are breastfed than in the general population (4).

Nutrition in the early stages of life plays a fundamental role in the child's growth and development, and is presumably one of the main players in the "programming" of his future health. In the very early phases of life breast milk (BM) can affect the maturation of organs and systems, influencing the future health.

Human BM is a complex biofluid containing a very large number of components, including macronutrients, hormones, bioactive molecules, stem cells, and microbial communities; each of them is potentially responsible for a certain specific and even synergic influence on the newborn outcome, on its growth and on the development of organs and systems, as deeply reviewed in several paper by ours (5–10).

It could be hypothesized that maternal pathological conditions could influence BM composition, but there are few studies in agreement with these considerations. In a recently published review by our group, to our knowledge the first on this topic, the studies using metabolomics to investigate BM of women with great obstetrical syndromes were discussed; it emerged that some metabolites seem to differentiate BM of healthy women and samples collected from women with preeclampsia, gestational diabetes, and intrauterine growth restriction; these metabolites might be related to the long-term outcomes in the offspring of affected mothers. The results of the analysis seem to highlight the involvement of the mammary gland in the underlying pathological processes and suggest the possibility that BM, while remaining the food of first choice in the early stages of life, could benefit from targeted supplementation to promote a better infant outcome (11).

Up to now, a clear description of BM composition in overweight or obese mothers has not been provided. Metabolomics is an emerging method in the study of BM and has proved useful in differentiating the characteristics of healthy women milk according to gestational age and lactation stage (12–14).

In the present paper, through the review of the metabolomics studies on BM collected from overweight-obese women, for the first time, we aim to investigate the metabolites found altered in the different studies, to assess whether they can have a positive (or negative) effect on the onset of obesity and other long-term complications.

We accurately searched on Medline the whole available literature, in English language, applying metabolomics to characterize BM in overweight and obese mothers; thus, breast milk, overweight, obesity, metabolomics, lipidomics, and oligosaccharides were used as key words.

As result, we found and discussed a single article on untargeted metabolomics (15), a total of four articles on targeted metabolomics (16–19), and 10 articles on lipidomics (20–29) published since 2013–2020. Review articles were excluded.

The main metabolomics differences detected by comparing BM of overweight/obese mothers and lean ones, and the potential short- and long-term effects in offspring have been summarized in **Table 1**. When available, details on maternal age in the different studies have been reported in the footnotes.

NUCLEOTIDE DERIVATIVES

The effects of nucleotides as bioactive substances in the regulation of body functions have been known since long time. *In vitro* and *in vivo* studies showed that nucleotides can promote gut maturation, affect immune modulation enhancing infant antibody response and, in neonatal gut, they can favor the growth of bifidobacteria. In humans, they are considered semi-essential dietary elements, due to the poor capacity of some tissues to synthesize them *de novo*, such as intestinal mucosa and hematopoietic cells. Even if their addition is optional, their pre-constitute mixes are usually present in infant formula milk, to optimize products resembling more accurately mother's samples (31).

Pyrimidine derivatives: in the study by Isganaitis et al., the only one, to our knowledge which performs an untargeted metabolomics analysis, liquid chromatography-gas chromatography-mass spectrometry (UHPLC-GC-MS) was applied to BM analysis at 1 and 6 months post-partum; samples collected from women with BMI > 25 Kg/m^2 were compared with a control group of lean mothers. Pathway analysis indicated that metabolites related to purine and pyrimidine metabolism were the most represented among those found to be significantly different at 1 month post-partum in BM of overweight-obese mothers compared to normal weight controls (15).

Among pyrimidine derivatives, orotate was reduced in the milk of obese-overweight mothers by about 25% (15). Orotate, introduced with food (especially dairy products) or synthesized *de novo* (from glutamine, ATP and CO_2), is an intermediate metabolite of pyrimidine synthesis and a precursor of uridine-mono phosphate (UMP), a nucleotide that plays a central role in different aspects of human metabolism (32). BM contains less orotate compared to milk of other species, and mammary gland is assumed to produce it and to have a high rate of UMP synthetase, an enzyme involved in the transformation of orotate into UMP, readily absorbed in gastro-intestinal tract (33). UMP, in addition to the involvement in nucleic acids synthesis, is the precursor of uridine-di-phosphate (UDP)-sugars, extracellular signaling molecules whose role in inflammatory and immune processes and in obesity-related glucose metabolism has been recently partially clarified.

With regard to the former, UDP-sugars are the major agonist of P2Y14 receptor (P2Y14R), abundantly expressed in leukocytes and other immune/inflammatory cells. They are also involved

TABLE 1 | Main metabolomics differences detected by comparing breast milk of overweight/obese mothers and lean ones in the different studies, and the potential short- and long-term effects in offspring.

Metabolites variation in overweight/obese mothers samples	Potential long term effects in offspring
PYRIMIDINE DERIVATIVES	
Orotate ↓ at 1 month of lactation (15)	Altered glucose homeostasis More weight gain by an inadequate diet Negative effect on the development of immune processes
PURINE DERIVATIVES	
AMP, Adenine ↑ at 1 month of lactation (15)	↑ Overweight risk Protection from obesity associates insulin-resistance Positive effect on the development of immune processes ↑ Neuroprotection ↓ Cardio-vascular risk
Methylthioadenosine ↑ at 1 month of lactation (15)	Protection against cardio-metabolic risk
SUGAR ALCOHOLS	
Erythritol ↑ at 1 month of lactation (15)	↑ Overweight risk
AMINOACIDS (AND ACYLCARNITINES)	
Branched chain aminoacids (BCAAs) ↑ at 3 month of lactation (16)	↑ Cardio-metabolic risk Unfavorable neurological outcomes
3-5Acylcarnitines (ACs) ↑ at 6 month of lactation (15)	
Glutamine ↓ at 6 months of lactation (15)	Altered glucose homeostasis Unfavorable neurological outcomes (as precursor of glutamate)
Asparagine and **Ornithine** ↓ at 6 months of lactation (15)	↑ Cardio-metabolic risk
Aromatic aminoacids and derivatives	
Tyrosine ↑ at 6 months of lactation (16)	↑ Cardio-metabolic risk
Kynurenic acid ↓ at 6 months of lactation (15)	Protection against cardio-metabolic risk from oxidative stress and inflammation
2-Aminobutyrate (2-AB) ↑ at 1 month of lactation (15)	Protection against oxidative stress
Polyamines ↓ at 3 days, 1 month and 6 months of lactation (17)	Less protection against cardio-metabolic risk from oxidative stress and inflammation Less neuroprotection
MONOSACCHARIDES	
1-5 anhydroglucitol (1,5-AG) ↑ at 1 and 6 months of lactation (15)	Emerging hyperglycemia marker **In breast milk** Potential role in describing maternal glycemic control
Arabinose ↑ at 6 months of lactation (15)	Effects on some pathogens, potentially reducing their virulence
Glucose-6-phosphate ↑ at 6 months of lactation (15)	Protection against oxidative stress Providing of energy supply
OLIGOSACCHARIDES	
Lacto-N-fucopentaose I ↓ at 1 month of lactation (15)	↑ Overweight risk ↓ Infant height ↓ Protection against infections Negative influence on neonatal gut microbiota, i.e., reducing *Lactobacillus* spp. (30)
Lacto-N-fucopentaose II ↑ at 1 month of lactation (15)	↑ Overweight risk
Lacto-N-fucopentaose III ↑ at 1 month of lactation (15)	↑ Infant height promotion ↑ Protection against infections ↑ Gut content of *Lactobacillus* spp. (30)
2′-Fucosyllactose ↓ at 1 month of lactation (15) Higher in *Se+* overweight mothers than *Se+* non-overweight ones (observation not confirmed in obese mothers) (18)	No clear associations with infant growth Its reduction could lead to: ↓ Infant weight, height and growth promotion ↓ Protection against infections

(Continued)

TABLE 1 | Continued

Metabolites variation in overweight/obese mothers samples	Potential long term effects in offspring
3′-Fucosyllactose ↑ at 1 month of lactation (15) Lower in *Se+* overweight mothers than *Se+* non-overweight ones. (observation not confirmed in obese mothers) (18)	No clear associations with infant growth
Lacto-N-hexaose ↓ at 3-4 months of lactation (19)	↓ Overweight risk
LIPIDS	
Saturated fatty acids ↑ At 1 and 2 months (23, 24), and at 3 months of lactation (21)	↑ Weight and BMI gain up to 13 months
Palmitic acid (16:0) ↑ at 2 weeks of lactation (25) **Tridecanoic acid (C13:0)** ↑ in colostrum (26)	↑ Overweight risk ↓ Glucose tolerance ↓ Insulin response ↓ Oxidation of fatty acids ↑ Inflammatory and metabolic responses
MUFA/SFA, UFA/SFA ↓ at 3 months of lactation (21)	↑ Weight and BMI gain up to 13 months
Total MUFA ↓ at 1–3 months of lactation (22, 23) **Oleic acid (18:1)** ↓ at 2 weeks of lactation (25)	↑ Overweight risk Worsening of metabolic and lipid profiles
n3 PUFA ↓ at 1–3 months (21, 22, 29) and at 6–7 months of lactation (20) ↓ from 3 days to 2 months of lactation (24) ↑ in colostrum (26)	↑ Overweight risk ↑ Inflammation
ALA, EPA, DHA ↓ at 1–3 months of lactation (22, 23, 29) and from 3 days to 2 months of lactation (24)	↑ Overweight risk Unfavorable sensorineural outcome
n-6 PUFA ↑ at 2 months of lactation (29) and at 6–7 months of lactation (20) **DGLA** ↑ at 2 weeks of lactation (25)	↑ Weight for age z-score ↓ Lengh for age z-score and CC between 2 weeks and 2 months of age ↑ Inflammation
n-6/n-3 ↑ at 1–3 months of lactation (21–23, 29) and from 3 days to 2 months of lactation (24)	↑ Overweight risk ↑ Inflammation
Adrenic acid (22:4 n-6) ↑ in colostrum (26) and at 2 weeks of lactation (25)	Promotion of CNS development
PAHSA levels ↓ at 3 days of lactation (28)	↑ Overweight risk ↑ Inflammation ↓ Glucose tolerance
Conjugated linoleic acid isomers ↑ at 2 weeks of lactation (25) ↓ in colostrum (26)	Conflicting results in the 2 available studies (25, 26)

UFA, unsatured fatty acids; SFA, satured fatty acids; MUFA, mono-unsaturated fatty acids; PUFA, unsaturated fatty acids; ALA, α-linolenic acid; EPA, eicosapentaenoic acic; DHA, docosahexaenoic acid; DGLA, dihomo-gamma-linolenicacid; PAHSA, palmitic acid ester of hydroxystearic acid.
When available, details on maternal age in the different studies have been reported in the footnotes.
Details on maternal age (means ± SDs):
(15): Average age of overweight-obese mothers: 30.5 ± 4.7 years (no statistically significant differences with lean group).
(16): Average age of obese mothers: 30.2 ± 4.7 years (no statistically significant differences with lean group).
(17): Average age of obese mothers: 30.2 ± 5.8 years (no statistically significant differences with lean group).
(18): Average maternal age is reported according to Secretor status, not referring to maternal BMI.
(19): Average age of the mothers in the study: 33.0 ± 4.2 years (considering overweight and lean mothers together).
(20): Average age of overweight mothers: 34.06 ± 3.37 years (no statistically significant differences with lean group).
(21): Average age of overweight mothers: 31.0 ± 5.0 years (no statistically significant differences with lean group).
(22): Maternal age in relation to BMI is not reported.
(23): Average age of overweight-obese mothers: 32.0 ± 4 years (no statistically significant differences with lean group).
(24): Average age of lean mothers: 32.0 ± 4.1 years; Average age of obese mothers: 30.5 ± 5.7 years.
(25): Average age of overweight-obese mothers: 29.9 ± 3.8 years (no statistically significant differences with lean group).
(26): Maternal age in relation to BMI is not reported.
(28): Average age of obese mothers: 35.1 ± 4.3 years (statistically higher than lean group).
(29): Average age of obese mothers: 30.0 ± 5.7 years (no statistically significant differences with lean group).

in the maturation of dendritic cells, in the degranulation of mast cells, and in the promotion of the regenerative processes of hematopoietic cells in the bone marrow (34). These observations suggest that the reduction of orotate in BM of overweight-obese mothers could have an adverse effect on the neonatal immune processes.

With specific reference to obesity, studies in mice models highlighted the abundant presence of P2Y14R mRNA in the pancreas and its implication in the modulation of insulin secretion. Moreover, the agonist action of UDP-sugars on the P2Y14R would seem to blunt the effect of an high fat diet on weight gain (35). In agreement with these reports, the reduction of orotate in BM of obese-overweight mothers could have a long-lasting adverse effect on glucose tolerance and on the protection, exerted by these nucleotide derivatives, against weight gain promoted by an inadequate diet.

Purine derivatives: among the metabolites belonging to purine pathway, adenosine mono phosphate (AMP) and its catabolite adenine were increased in BM of overweight-obese mothers 1 month after birth, while adenosine monophosphate cyclic (cAMP) was reduced (15).

Once introduced with the diet, purine nucleotides no longer necessary for cellular functions are degraded by intestinal enzymes to uric acid. In BM, they can derive from the direct passage from blood to milk or from metabolic processes in the mammary gland (31).

Purines are involved in physiological and pathological processes in all tissues and specifically in adipose tissue. In particular, ATP and adenosine binds to specific receptors of white and brown adipose tissue, both in adipocytes and stromal cells, and their function may be altered in various diseases such as metabolic syndrome (36, 37).

Some of their important functions, exerted mainly through the action of adenosine on A1 receptors, concern the inhibition of lipolysis and the reduction of free fatty acids (whose involvement in the pathogenesis of insulin resistance, diabetes, cardiovascular diseases is recognized), the reduction in insulin resistance is associated with obesity, the increase in leptin production (with an additional beneficial effect on insulin sensitivity), and the increase in the uptake of glucose by adipocytes (with improvement of glucose tolerance but also in triglycerides storage and weigh gain). In agreement, in some studies on obese patients, A1Rs expression was found to be inversely related to the ability to lose weight (38, 39). As well as pyrimidine nucleotides, purine nucleotides positively affect innate immunity and regulates monocytes, macrophages, dendritic cells and mast-cell functions (37, 40). Moreover, their involvement in numerous central nervous system (CNS) functions such as behavior, nociception and locomotion has been highlighted (41).

Finally, the activation of purine pathway found in BM of overweight-obese mothers (15) could promote weight gain long after birth, but also improve glucose tolerance and insulin sensitivity in case of obesity, and reduce cardiovascular risk. It may also have a positive effect on the development of immune and anti-inflammatory processes and on neuroprotection.

5-METHYLTHIOADENOSINE

Methylthioadenosine (MTA) was increased 1 month post-partum in BM of obese-overweight mothers and it was also the only metabolite involved in the overlap between BMI and infant total fat content at 1 month (15). It is a natural nucleoside sulfur containing derived from 5 s-adenosylmethionine (5 SAMe) in polyamine cycle, present in all mammalian tissues including the placenta. 5 SAMe is the substrate of the 5-MTA phosphorylase enzyme, that initiates the salvage pathways leading to the recovery of methionine by one side and adenine (adenosine, AMP) by the other (39). It is an almost exclusively endogenous metabolite (its presence has been described but not quantified in some edible plants) described for the first time in BM by Isganaitis (15). Being a purine nucleoside, we can assume a gastro-intestinal absorption, similar to that of the other purines.

5-MTA has important cellular regulatory functions, including gene expression control, inhibition of cell proliferation, activation of lymphocytes, modulation of tumors invasiveness, regulation of apoptosis, and liver-protection (42). Its potent anti-inflammatory profile has been proven in mice where it prevents lipopolysaccharide-induced death by inhibiting TNFα production (pro-inflammatory cytokine) and iNOS (inducible nitric oxide synthase) gene expression, while enhancing the release of IL-10 (anti-inflammatory cytokine) (42).

In a metabolomics study on an induced rat model of diabetes dating back to 2016, 5 MTA was significantly higher. The study of oxidative stress products showed increased values of superoxide dismutase and hypoxia inducible factor 1 alpha. Both the 5-MAT and the oxidative stress products normalized after treatment with isoflavones, demonstrating their action on the cellular oxidative damage and therefore highlighting related metabolic processes (43). One year later, an untargeted metabolomics study (UPLC-MS) detected an increase in 5-MTA level in the urines of elderly patients with type 2 diabetes (T2D) (44). In a further metabolomics study in Mexican adolescents, conducted with to investigate the metabolites associated with a metabolic disease risk z-score (MetRisk z-score), 5-MTA values showed a significant correlation (45). Finally, 5-MTA levels resulted significantly associated with the BMI in a study conducted on 2,396 unrelated European individuals in the TwinsUK cohort and 724 others of the Health nucleus cohort in three time-points, covering a total interval of 8–18 years (46).

5-MTA should be considered a protective molecule against chronic inflammation and oxidative stress conditions characterizing obesity and metabolic syndrome. In this respect, MTA increase in BM of overweight-obese mothers (15) could be protective for long-term overweight, oxidative stress and cardio-metabolic risk.

SUGAR-ALCOHOLS

Erythritol and ribitol (sugar alcohols) were increased in the milk of obese-overweight mothers 6 months after birth (15). To the best of our knowledge, only another study investigates sugar alcohols in BM (HPLC), even if erythritol and ribitol

were not detectable (47). While ribitol is an endogenous molecule originating from the reduction of ribose in fibroblasts and erythrocytes, erythritol is a low-caloric sugar-substituted sweetener authorized in the USA and it is also present in different foods. It is absorbed from the proximal intestine by passive diffusion. Recently, its serum increase has been observed, in correlation to weight gain, in a cohort of freshmen. At the same time, through *ex vivo* isotopic techniques, endogenous production of erythritol from glucose (48) was detected. Given the wide use of low-caloric sweeteners by obese-overweight individuals, further investigation will be useful to clarify whether its increase in the milk of obese-overweight mothers could be related to this or to other metabolic pathways (48) and the possible long-term effect on glucose metabolism in the infant.

ACYLCARNITINE AND AMINO ACIDS

Acilcarnitine (ACs) and branched amino acids (BCAAs): Three short-chain ACs were increased in the milk of obese-overweight mothers at 6 months after delivery (15). Short-chain ACs derive from the catabolism of BCAAs, rather than from long-chain fatty acids. Although Isganaitis and colleagues did not detect an increase in BCAAs in the milk of overweight and obese mothers (15), De Luca et al. (16), trough UPLC and Tandem Mass (MS/MS), specifically measured free amino acids in the milk of 45 obese women and 45 controls at 1 month post-partum. As result, they found an increase in BCAAs and tyrosine (by 20 and 30%, respectively) in the formers (16). The increase in serum levels of BCAAs and ACs has been found, in several studies, in obese subjects with or without T2D, and has been more correlated with insulin resistance than with obesity (46, 49–52). The same group of Isganaitis (45) carried out an untargeted metabolomic study on 262 children aged 6–10 years divided into two groups (thin and obese non-diabetic): through MS, they found that obese children showed an increase in BCAAs and C3-C5 ACs, significantly correlated with several cardio-metabolic risk indices, including insulin resistance. Interestingly, the pattern was more pronounced in children of mothers with pre-gestational obesity.

An increase in BCAAs may compromise the transport of aromatic amino acids into cells and tissues, reducing the production of serotonin and melatonin (derived from tryptophan) and catecholamines (derived from phenylalanine and tyrosine) in the CNS. Melatonin and serotonin exert their effects both centrally and in the periphery, regulating energy homeostasis through central control of food intake, promoting lipogenesis and glucose metabolism (53).

Finally, literature data on BCAAs and ACs in obese subjects suggest that exposure to these metabolites is associated with increased metabolic risk later in life. It may also lead to a reduced availability of tryptophan and its serotonin and melatonin derivatives, with neurobehavioral impairment and negative effects on lipid metabolism. The abundance of BCAAs and ACs found in BM (15, 16) could in the same way be linked to an increased metabolic and neuropsychiatric risk in the long distance.

Glutamine: glutamine was reduced by about 30% in the milk of obese-overweight mothers at 6 months after delivery (15). It should be noted that an untargeted metabolomics work on the milk of mothers with gestational diabetes (54) showed a reduction of glutamine. Its role in promoting better glucose homeostasis as main precursor of gluconeogenesis in the kidney, representing a main substrate for gut-brain gluconeogenesis system, and an inducer of glucagon-like peptide secretion was highlighted in our recent review (11, 55–58) investigating BM of women with great obstetrical syndromes.

Finally, the reduction of glutamine in the milk of obese-overweight mothers at 6 months after birth (15) may have long-term adverse effects on glucose homeostasis.

Asparagine and ornithine: these aminoacids were reduced by about 50 and 20%, respectively, in the milk of obese-overweight mothers at 6 months after delivery (15). Several studies on the amino acid profile in obese subjects inversely correlate the amino acid asparagine with the cardio-metabolic risk (46, 50, 52, 59, 60); only one recent study, to our knowledge, correlates also ornithine to T2D (61).

Their reduction in the milk of obese women could be a long-term metabolic risk factor in infants.

Aromatic amino acids and derivatives: tyrosine (in addition to BCAAs) was increased by 30% in the milk of obese women 1 month after birth (16). An increase in blood tyrosine is frequently reported in obese subjects (45, 46, 50–52, 62, 63); it is likely to contribute to insulin resistance by glucose production through the pathway of dicarboxilic acid fumarate, the latter also found increased in BM of obese-overweight mothers at 6 months (15).

High levels of tyrosine in the milk of obese women (16) could have a negative impact on BMI and cardio-metabolic risk in later ages.

Kynuretic acid was reduced by about 30% in the milk of overweight-obese women 6 month after birth (15).

In the human body most of kynurenic acid comes from tryptophan catabolism; it is also present in foods and seems to be produced by intestinal microflora in moderate quantities. It can be easily absorbed from the digestive system and transported to the liver and the kidney (64).

In humans, serum kynurenic acid has been positively associated with several cardio-metabolic risk factors as BMI and insulin-resistance (35, 44): however, some reports in humans disagree with experimental studies where kynurenic acid was able to improve energy metabolism and inflammation in mice fed a high-fat diet and to promote weight loss (65, 66). Thus, it is possible that the elevation of this metabolite in individuals with metabolic risk can represent a compensatory mechanism through which kynurenic acid could perform its beneficial action. In this context, the reduction of kynurenic acid in BM of overweight-obese mothers in the Isganaitis study (15) could lead to an increased susceptibility to metabolic risk long after birth, rather than being protective.

2-Aminobutyrate (2-AB): 2-AB significantly increased in samples collected from overweight-obese womenat 1 month of lactation, increased by about 55% ($p = 0.03$) (15). 2-AB metabolismis poorly clarified, especially in BM. It seems involved in defense against oxidative stress; reduced glutathione is a

central metabolite in the intracellular redox state. Glutathione consumption through oxidative stress activates a compensatory glutathione (GSH) synthetic pathway, accompanied by the synthesis of ophthalmic acid, a GSH analog, from 2-AB (67, 68).

We found a single study investigating 2-Ab in BM; in detail, such metabolite was compared between mothers affected by inflammatory bowel disease (IBD) and healthy mothers at 3 and 6 months of lactation. As result, 2-AB was lower in IBD mothers, potentially related to increased pro-inflammatory activity (69).

We strongly believe that 2-AB increase in BM from obese mothers could be a compensative mechanism reflecting an increase in oxidative stress.

POLYAMINES

In the study by Ali et al. (17), 50 mothers (20 lean, 20 obese and 10 obese undergoing dietary treatment), were invited to collect milk at 3 days, 1 month, and 2 months post-partum, to investigate polyamine levels with HPLC. The total polyamine content was reduced in obese mothers at the three time points: the reduction concerned only spermidine and putrescine levels, while spermine was equally represented in the considered groups. The 10 obese mothers undergoing dietary treatment had a higher spermidine and putrescine milk content than the others obese women. The authors assume that the reduced content of polyamines in obese BM could depend on the low quantity of polyamines in the fat- and carbohydrates-rich foods assumed by obese subjects (17). In agreement, higher levels of polyamines were found in subjects who practice the Mediterranean diet (70).

Polyamines are widely present in the human body and are involved in many vital functions. Although they mainly derive from endogenous metabolism, a percentage is produced by food and intestinal flora, especially in case of fiber-rich diet. Once introduced with food, they can be absorbed and distributed to different tissues. Their protective role against cardio-metabolic risk has been highlighted by the study of Eisenberg et al., which finds an association between increased consumption of spermidine and decreased cardiovascular events and mortality (71).

Polyamines also represent oxidative stress and inflammation modulators, often associated with obesity and implicated in the pathogenesis of metabolic syndrome (72). In a study performed on 60 obese children aged 7–14 years, blood levels of polyamines were significantly higher than in controls, and spermine represented a marker of oxidative stress (NO pathway) and inflammation. The authors believe that the increase in these metabolites could be a protective mechanism against obesity-related oxidative stress (73). Recently, an association between serum polyamine and T2D levels has been reported in a cohort of patients with metabolic syndrome (74).

A deregulation of the polyamine system would play a role in neurodegenerative diseases (75, 76) and depression (77) because of their involvement in the modulation of synapsis and in the regulation of the ionic channels that participate in neuronal excitability (72).

Finally, the reduction of polyamines in BM could make newborns of obese mothers in the long distance more susceptible to weight gain, oxidative stress, inflammation and cardio-metabolic risk. Moreover, they may be less predisposed to develop neuropsychiatric disorders.

MONOSACCHARIDES AND OLIGOSACCHARIDES

Below, we review the few available studies regarding human milk monosaccharides and oligosaccharides (HMOs) variations in BM from overweight and obese mothers instead of normal weight mothers' samples, and the consequent effects on neonatal metabolism and infant growth.

It should be underlined that ingested HMOs can reach the distal bowel and colon without undergoing any modification or enzymatic hydrolysis in the stomach and upper GI tract. Thus, HMOs can be metabolized by intestinal microbes and 99% of them is eliminated with the stools. The smallest percentage of them (about 1%) can be absorbed through the intestine and reach the circulation, being transferred to several organs, such as brain, liver, respiratory and urinary tract, where they can exert several functions (7).

- *1,5Anhydroglucitol (1,5-AG) (monosaccharide)* was found significantly increased in BM from overweight and obese mothers at 1 ($p = 0.002$)and 6 months of lactation (increased by 37% at 6 months, $p = 0.003$) (15).

1,5-AG is a very interesting metabolite not previously described in BM. Serum 1,5-AG is a validated short-term marker of glycemic control (78) in patients with type1 and type 2 diabetes, and its role in gestational diabetes is still under evaluation (78–82). During pregnancies affected by diabetes, the mean maternal serum values of 1,5-AG were negatively associated with neonatal birth weight and tended to be lower in infants with hypoglycemia; the magnitude of the difference between hypoglycemic and normoglycemic was greater for gestational diabetes (83).

According to another study, maternal serum level of 1,5-AG at birth was significantly and inversely associated with neonatal complications (such as respiratory distress, hypoglycemia, polycythemia, hyperbilirubinemia, and "large for gestational age" condition), resulting useful in the prediction of complications (84).

In our opinion, 1,5-AG level in BM could reflect maternal glycemic control and help in predicting neonatal outcome in pregnancies complicated by diabetes, even if it was not identified before in BM and its presence requires further clarification.

- *Arabinose (monosaccharide)* was found significantly increased in BM from overweight and obese mothers at 6 months of lactation (increased by 72%, $p = 0.01$) (15).

Arabinose, a five-carbon sugar is a carbon source for many bacteria. In literature, we did not find a metabolic role of such pentose in BM on infant weight gain.

However, in two studies it seems to modulate, in a controversial way, *Pseudomonas aeruginosa* virulence, an

opportunistic human pathogen strongly associated with NEC development (85, 86).

Therefore, arabinose could have a potential role on pathogens virulence, improving our knowledge of BM-related effects and contributing to the optimization of formula milks.

- *Glucose-6-phosphate (monosaccharide)* was significantly higher in BM from overweight and obese mothers at 6 months of lactation (2.07-fold change, $p = 0.01$) (15).

Glucose-6-phosphate (G6P or α-D-glucose-6-phosphate) is involved in protection against oxidative stress, since it guarantees adequate levels of NADPH to modulate the redox state (87).

In literature, we found a single very old study measuring such metabolite in human milk during established times of lactation, including G6P, but the results are not clear (88).

Thus, by the few available evidences, it could be supposed a protective role against oxidative stress and provide energy supply.

- *Lacto-N-fucopentaose I (LNFPI)* was found significantly reduced in BM from overweight and obese mothersat 1 month of lactation, reduced by about 62% compared to samples of normal weight mothers ($p = 0.007$) (15).

LNFPI resulted the most relevant influencer of infant growth, significantly associated with lower infant weight at 1 month and with lower weight and less lean and fat mass at 6 months (89).

LNFPI was also associated with lower infant weight and weight gain at 1 month in another study (18).

Moreover, LNFPI had positive contributions in height-for-age Z scores at 20 weeks (30).

LFNP I was lower in BM of mothers of severely stunted infants vs. healthy controls 6 months after delivery (90).

Therefore, LNFPI in BM seems protective against excessive weight gain in infants and its reduction in overweight and obesity supports the potential pro-adipogenic role of BM in these mothers.

- *Lacto-N-fucopentaose II and III (LNFPII-III)* were found significantly higher in BM from overweight and obese mothers at 1 month of lactation ($p < 0.05$) (15).

At 6 months of lactation, LNFPII was associated with greater fat mass (89).

BM LNFP III positively contributed to infant height-for-age Z scores at 20 weeks (30) and it seems to modulate metabolic pathways in mice, improving glucose tolerance, insulin sensitivity and suppressing liver lipogenesis in experimental model of obesity (91).

In conclusion, little is known on such HMOs and their impact on neonatal growth; LNFP II high level in BM of overweight and obese mothers could suggest a role in infant overweight.

- *2-Fucosyllactose (2′-FL)* was found significantly lower in BM from overweight and obese mothers at 1 month of lactation, reduced by about 38% than normal weight mothers samples (15).

On the contrary, in another study, 2′-FL would be higher in samples from overweight than non overweight mothers (18).

Regarding its relation to infant growth, 2′-FL was found lower in BM of severely stunted infants' mothers vs. healthy controls at 6 months of life (90).

2′-FL seems directly associated to maternal pre-pregnancy BMI, infant weight up to 1 year of life and also child height up to 5 years of life in offspring of *Se+* mothers. According to such data, this HMOs seems to affect child growth up to 5 years of life (92).

Contrarily, in another study 2′-FL did not influence body length, weight, CC or BMI at 4 months of life (93).

In milk from Se+ mothers, 2′-FL resulted the most abundant HMO, positively associated with infant weight velocity from 0 to 5 months of post-natal age and with fat mass index at 5 months. Thus, 2-FL, currently added to some milk formulas, could be involved in excessive weight gain (94).

Maternal BMI at 5 months of lactation has been positively correlated with 2′-FL content (94).

In conclusion, data on 2′-FL seems conflicting; such HMO was associated with a higher maternal BMI and with infant growth, even if its variation in BM from obese mothers should be still defined.

2′-FLwas also hypothesized to increase weight gain when milk formulas are supplemented, effect that results balanced by other HMOs added to such formulations (95, 96).

- *3-Fucosyllactose (3′-FL)* was found significantly increased in BM from overweight and obese mothers at 1 month of lactation ($p = 0.03$) (15).

Contrarily, in another study, 3′-FL was lower in BM from *Se+* overweight mothers than normal weigh ones (18).

Regarding 3′-FL content in BM of overweight and obese mothers, data are controversial and there are not significant associations with infant growth.

- *Lacto-N-hexaose (LNH)* was found significantly lower in BM of obese mothers at 3–4 months of lactation ($p < 0.05$) (19).

At 6 months of lactation, it has been associated with higher infants' body fat mass (89); thus, LNH reduction could protect from weight gain.

Moreover, it could contribute to the lower duration of breastfeeding of obese mothers (18, 19).

LIPIDS

Lipid composition of BM seems influenced by maternal BMI, influencing its inflammatory and oxidative profile (20).

Maternal obesity seems related to the increase of n-6/n-3 fatty acid ratio and to the proportion of long-chain fatty acids (LCFAs) in BM. (96, 21–24). LCFAs are less digestible by the neonatal gut; moreover, long-chain polyunsaturated fatty acids (LCPUFAs), such as eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and arachidonic acid (AA) are involved in several metabolic pathways, including a role as constituents of cells membranes, and in nervous system and retina development (20).

Infant growth up to 6 months of life was correlated with higher levels of dihomo-gamma-linolenic (20:3 n6-DGLA), adrenic (22:4 n-6), palmitic acids, conjugated linoleic isomers and reduced level of oleic acids in BM of overweight-obese mothers (25).

Palmitic acid seems involved in inflammatory and metabolic responses and it could also reduce the oxidation of FAs, alter the insulin response and increase the fat mass (97).

Adrenic acid is abundant in the brain and in myelin lipids, especially in phosphatidylethanolamine. Its important precursor is AA acid, whose conversion into adrenic acid is particularly active in the early stages of life (26). Oleic acid was shown to reduce obesity risk and improve the metabolic and lipid profiles in adults (98). DGLA, other n-6 PUFAs such as AA, and bosseopentaenoic acid (20:5 n-6) are probably involved in the pathogenesis of obesity, promoting adipogenesis and inflammation (20). This group correlates with a greater increase in the weight for age (WFA) z-score, a smaller increase in the lengh for age z-score and of the CC between 2 weeks and 2 months of age. The effects on long distance obesity are clearly to be determined (25).

In another study, samples from overweight mothers showed higher levels of saturated FAs (SFAs), lower amount of n-3 FAs and lower ratio of unsaturated (UFA) to saturated (SFA) FAs, and higher n-6/n-3ratio than normal weight samples. Moreover, total SFAs content in BM was positively correlated while MUFA/SFA ratio and UFA/SFA ratio inversely correlated to infant weight and BMI gain up to 13 months (21).

Successively, the same group investigated the combined effects of maternal pre-pregnancy BMI and food choices on BM triacylglycerols (TAGs) at 3 months of lactation. They evidenced a higher content in 18:3 and a reduced level of 18:0 in normal weight mothers following a recommended food-diet (low fat), than normal weight mothers eating non-recommended foods. Moreover, in samples collected from normal weight mothers eating recommended foods, levels of 50:1 were lower than milk produced by overweight mothers eating recommended food choices. Finally, BM from overweight and obese mothers was characterized by higher levels of saturated FA and lower amount of n-3 FA than non overweight mothers, independently by the diet. Thus, they concluded that maternal BMI and diet can influence the molecular weight distribution of TAGs in BM samples but does not significantly alter their regioisomerism (27).

In BM from obese mothers, an increase in lipid content (10–20%), and higher levels of ALA, n-6/ n-3 ratio and total PUFA were detect, instead of normal weight mothers. In the same study, total MUFAwere significantly reduced in BM from overweight and obese mothers, while 20:1 n-9 were increased (22).

In a similar article,n-3 LCPUFA (including EPA and DHA) were lower while n-6 LCPUFA and n-6/n-3 ratio were higher in overweight mothers at 6–7 months of lactation (20).

A Sweden group demonstrated higher SFAs and n-6/n-3/ratio, and lower n-3LCPUFA (and LA, DHA, EPA) in BM of obese mothers (24).

Total PAHSA levels [the fatty acid esters of hydroxy fatty acids (FAHFAs), namely palmitic acid hydroxystearic acids], endogenous lipid produced by adipocytes in the mammary gland, resulted significantly lower in obese mothers' samples at 3 days postpartum (28).

PAHSA seem to promote gut maturation and secretion of GLP-1, which stimulates insulin secretion and increase glucose tolerance (99).

Interesting findings were also obtained correlating maternal BM lipid content in colostrum and mature milk with infant anthropometry (at 6, 18, and 36 months) and with cognition at 18 months. BM from overweight and obese mothers showed higher SFA levels and n-6/n-3 ratio, and decreased ALA, DHA and MUFA content in mature milk. Infant BMI-z-score at 6 months resulted inversely associated with colostrum levels of n-6 and n-3 LC-PUFAs (e.g., AA and DHA) and positively associated with n-6/n-3 ratio. Cognitive profile evaluated with Bayley scales was positively correlated to colostrum content of n-6 and n-3 PUFAs, DHA, and ALA, and negative correlated to the n-6/n-3 ratio. Thus, according to these data, maternal obesity could increase BMI in the offspring, but n-6/n-3 ratio could impair infant cognition, even if such results should be confirmed (23).

In a further study BM of obese mothers at 2 months post-partum showed a higher n-6/n-3 FA ratio, while total n-3 PUFAs were reduced of 20%, in association to lower levels of DHA, EPA, docosapentaenoic acid and lutein (29).

A unique study of Sinanoglu et al. deeply investigated colostrum lipid content according to maternal BMI. As result, docosadienoic acid (C22:2 n-6), conjugated LA isomers C18:2*c*9*t*11 (rumenic acid) and C18:2*t*11*t*13 were higher in normal weight mothers' BM; total n-3,decanoic (C10:1), tridecanoic (C13:0) and adrenic (C22:4n-6) acids were higher in obese mothers; ALA (C18:3n-3) was higher in overweight mothers' samples (26).

Interest in conjugated LA isomers has grown in recent years due to the increasing number of experimental studies attributing them anti-inflammatory, anti-carcinogenic, antiadipogenic, antidiabetic, and anti-hypertensive properties in animal models. Their increase is probably causedby the excessive dietary intake (25). They may represent afuture nutritional tool to prevent diseases as metabolic syndrome but studies on humans are still necessary (100–102).

The n-6/n-3 ratio in BM increases in proportion with the fat in the diet: this could lead to a higher adipose tissue accumulation in neonates fed with obese mothers' BM. The authors speculate that the increase of PUFA in overweight and obese mothers' BM could determine cardio-protective (probably) compensatory mechanism for their infants, since such mediators are related to a better metabolic profile (26).

CONCLUSIONS

Maternal obesity seems a major risk factor for excessive fetal growth (103), infant overweight and children obesity (104, 105). The negative impact of obesity on children's health can lead to the early development of T2D, the premature onset of cardiovascular complications and, in general, a higher risk of early mortality (106). Recent literature has shown increasing interest in the impact of such disease on the nervous system and in particular,

in the field of neuropsychiatric and behavioral disorders (mainly attention-deficit/hyperactivity disorder, conduct disorders and autism), described only sporadically in the past but now confirmed by large population studies (107, 108). Besides, a reduction in prefrontal cortex thickness and associated executive function deficit was described in a large cohort of obese children aged 9–10 years (109).

In the present review of metabolomics studies on BM from overweight-obese mothers highlighting the differences with samples from the lean ones, some of the metabolites that differentiate the two groups—aminoacids, acyl-carnitines, lipids and oligosaccharides—have been found altered in subjects with the same characteristics and in experimental modes of obesity in several studies and in a relevant number of cases. For these metabolites, which can be considered "major," the results obtained from the aforementioned studies allow for more reliable hypotheses on the meaning of their alteration in BM long after birth. However, it should be noted that it is difficult to make qualitative assessments of the relevance of one group of metabolites in relation to the other.

Between these "major" metabolites, all aminoacids found altered could promote cardio-metabolic risk unlike their derivatives ("minor" metabolites) whose reduction (kinurenic acid) or increase (2-aminobutyrate) might give protection against cardio-metabolic risk from oxidative stress and inflammation.

Among HMOs, those decreased (LNFPI, 2FL, LNH) seem to have a protective effect against excessive weight gain, while NFPII increase could predispose to it.

Our analysis of the papers investigating fatty acids in BM of overweight-obese mothers, in agreement with the unique available meta-analysis (110), highlights an increase in saturated fatty acids, a reduction in monounsaturated fatty acids, a reduction in n-3 LCPUFA, and an increase in n-6/n-3 ratio in most of them. The first two effects could promote excessive long-term weight gain and associated inflammation, as well as reduce glucose tolerance. The increase in the n-6/n-3 ratio, due to the reduction in n-3 or increase in n-6 may impair, as previously mentioned, sensorineural development and promote adipogenesis and inflammation.

Between the "minor" metabolites, the alteration of nucleotides derivatives could promote weight gain after birth and have, except for adenine and MTA, a negative impact on the risk of insulin resistance, T2D, cardio-metabolic disease and on the onset of neurological problems. Increase of adenine, while promoting weight gain, could hinder the insulin resistance associated with obesity (and cardio-vascular risk) and have a positive impact on immune and neurological development. Even MTA increase could be protective against cardio-metabolic risk in offspring.

Lastly, reduction of polyamines could correlate with outcomes related to obesity in the long distance.

Finally, metabolites variations in BM samples collected from overweight-obese mothers and the potentially correlated effects pointed out above, still need further investigations and should be confirmed in future studies on larger sample: metabolomics seems the most suitable technology in this regard. It can lead to the comprehensive description of such biofluid and the related effects on breastfed subjects, potentially highlighting personalized needs of BM supplementation or short and long-term prevention strategies to optimize offspring health.

In our opinion, such topic is of major importance, since pediatric health starts in intrauterine and perinatal life.

AUTHOR CONTRIBUTIONS

VF conceptualized the paper. FB and MP updated the literature and wrote the first version of the review. VF and DP critically revised the text. All authors contributed to the article and approved the submitted version.

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Human Milk From Atopic Mothers Has Lower Levels of Short Chain Fatty Acids

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Short chain fatty acids (SFCAs) are microbial metabolites produced in the gut upon fermentation of dietary fiber. These metabolites interact with the host immune system and can elicit epigenetic effects. There is evidence to suggest that SCFAs may play a role in the developmental programming of immune disorders and obesity, though evidence in humans remains sparse. Here we have quantified human milk (HM) SCFA levels in an international cohort of atopic and non-atopic mothers ($n = 109$). Our results demonstrate that human milk contains detectable levels of the SCFAs acetate, butyrate, and formate. Samples from atopic mothers had significantly lower concentrations of acetate and butyrate than those of non-atopic mothers. HM SCFA levels in atopic and non-atopic women also varied based on maternal country of residence (Australia, Japan, Norway, South Africa, USA). Reduced exposure to HM SCFA in early life may program atopy or overweight risk in breastfed infants.

Keywords: human milk, short chain fatty acids, atopy, allergy, international cohort, breast milk

INTRODUCTION

Human milk (HM) confers numerous benefits to the developing infant, an effect attributed to its many bioactive metabolites. The evidence for some of the long-term health benefits of HM is inconclusive (1). Regarding the prevention of atopic diseases through breastfeeding, this varies across countries and in particular, according to the atopic phenotype of the mother (2). While genetics and epigenetics play a role in the inheritance of atopic disease (3, 4), the role of HM metabolites remains underexplored in this field. Still in its infancy, the study of the HM metabolome has proven valuable in identifying variability by maternal phenotype, diet, and disease state (5, 6). Short chain fatty acids (SCFAs) are key metabolites of microbial fermentation of fiber that have links with host health. Early-life exposure to SCFAs has been shown to protect against atopy (7).

When administered to pregnant mice, the SCFA acetate has prevented offspring from developing atopic airway inflammation (8). These findings are corroborated by human data of associations between high maternal serum acetate levels during pregnancy and decreased risk of respiratory symptoms in young infants (8). Similarly, propionate has been shown to protect against allergic airway disease in mice via its effects on dendritic cell biology (9), while butyrate induces the differentiation of colonic regulatory T cells (10). Further, murine studies have demonstrated that prebiotic fiber supplementation during pregnancy or lactation reduces risk of atopy in offspring (11, 12). Similar trials are currently underway in humans [SYMBA (13) and PREGRALL (14)]. Recently, Lee-Sarwar et al. reported higher fecal acetate levels (relative to total SCFA) in pregnant women of children less likely to develop atopic disease (15).

SCFAs (formate, acetate, propionate, butyrate, and valerate) are intermediate and end products of dietary carbohydrate fermentation by gut bacteria (7). These microbial metabolites are concentrated in the colon and some are distributed systemically after absorption (8, 9, 16). Through their interaction with G-protein-coupled receptors and their inhibition of histone deacetylases, SCFAs are able to elicit a broad range of biological effects, including promotion of regulatory T cell responses and tolerance, mucus secretion and epithelial barrier integrity in the gut, and synthesis of bone marrow dendritic cell precursors (9, 17, 18). A broad range of bacteria are also present in HM (19). HM SCFAs are likely produced by the maternal gut microbiota and distributed to the mammary gland via the circulation. They may also be produced by the resident HM microbiota; however, evidence for this possibility is currently lacking. To date, there has been limited investigation into HM SCFA profiles. Smilowitz et al. were the first to document the presence of acetate and butyrate in HM samples collected 90 days postpartum, finding that these SCFAs were highly variable among women (20). In HM samples from a single woman, acetate, butyrate and formate were detected as early as 24 days postpartum (21). All three of these SCFAs were identified by nuclear magnetic resonance (NMR) at 1-2 months postpartum in a larger study of women (22). Butyrate has also been documented in studies of HM fat or fatty acids (23, 24). Meng et al. reported the presence of acetate and butyrate in HM from women with and without irritable bowel disease, finding higher acetate levels in women treated with aminosalicylates (25). Finally, Gómez-Gallego et al. performed NMR metabolic profiling of 79 HM samples from 4 international cohorts. They identified acetate, butyrate, and formate in these samples and reported differences in acetate and formate levels between countries (26).

Total SCFA levels are elevated in the stool of lactating women at 1 month postpartum compared to non-pregnant women (27), implicating their importance to the nursing infant. HM SCFAs have been shown experimentally to prevent atopic disease, but breastfeeding by atopic mothers does not protect against atopy to the same extent as breastfeeding by non-atopic mothers (2). This discrepancy may be a function of reduced levels of SCFAs in HM among atopic mothers, though this has not been tested. Herein, we profiled SCFA levels in HM samples from atopic and non-atopic mothers from six international sites, including two countries with high rates of atopic disease. We hypothesized that atopic women would exhibit reduced levels of HM SCFAs.

METHODOLOGY

Study Design

In this descriptive study, 109 HM samples from 6 cohort studies from different countries were analyzed (5). The cohorts were from Perth, Australia ($n = 29$ from 2 cohorts); Chiba, Japan ($n = 12$); Detroit, USA ($n = 18$); Oslo, Norway ($n = 40$); Cape Town, South Africa ($n = 10$). These cohorts were sampled across countries to identify women with and without atopic disease. Whenever possible, samples were obtained from women who delivered vaginally and did not receive antibiotics while breastfeeding. To reduce the impact of maternal diet or genetics, an effort was made to obtain samples from women of the same ethnicity within a country. Research ethics approval was obtained from the local ethics committees of participating institutions: Human Research Ethics Committee of The University of Western Australia, Human Research Ethics Committee of the Princess Margaret Hospital, Committee on Human Research of Chiba University, Institutional Review Board at Henry Ford Health System, Norwegian Regional Committees for Medical and Health Research Ethics, and University of Cape Town Human Research Ethical Committee.

Maternal Atopic Status

Maternal atopic status was defined according to maternal report of having asthma, eczema or atopic dermatitis, or a pet, environmental or food allergy (Norwegian, South African women), or atopic sensitization on the basis of at least one blood allergen-specific IgE level $\geq$ 0.35 kU/L (US women) to house dust mite, dog, cat, Timothy grass, ragweed, *Alternaria alternata*, egg, or German cockroach, or at least one blood allergen-specific IgE level $\geq$ 0.7 kU/L (Japanese women) to house dust mite, cat or Japanese cedar, or at least one positive skin prick test (Australian women) to house dust mite, dog, cat, Timothy grass, Japanese cedar where applicable, ragweed, *Alternaria alternata*, egg, or German cockroach. Australian and Japanese atopic women also had a physician-diagnosed history of asthma, eczema or atopic dermatitis concurrent with atopic sensitization.

Human Milk Sample Collection

HM samples were collected 1 month after birth, a time point at which the composition of human milk is thought to stabilize (28). Participants were given written and oral instructions to standardize self-collection of samples. Prior to collection, nipples and mammary areola were cleaned with soap and sterile water, and for the samples from South Africa, additional cleaning was performed with chlorhexidine to reduce contamination by skin microbes. Human milk samples were expressed manually or with an electric breast pump into a sterile tube. Australian samples from non-atopic women (2015) and Norwegian samples (2002) were stored at -20°C, Australian samples from atopic women (2002) and samples from US women (2003) were stored at -80°C. The samples from Japanese women (2010) were initially stored at -80°C before being moved to -30°C. Samples were

shipped on dry ice to The Metabolomics Innovation Center, Edmonton, Canada for processing in 2015.

NMR Analysis

Milk metabolite levels were determined by NMR because of its high reproducibility and coverage of a large range of metabolites. Samples were analyzed as previously reported by Gay et al. (5). Briefly, samples were thawed on ice, mixed thoroughly, and then filtered to remove residual lipids and proteins using a 3-kDa cutoff spin filter at 10,000 × *g* for 15 min at 4°C. Three hundred fifty microliter of filtrate was transferred to a clean tube, and 70 μL of D_2O and 60 μL of standard buffer solution (585 mM $NaHPO_4$ (pH 7.0), 11.667 mM disodium-2,2-dimethyl-2-silapentane-5-sulfonate (DSS), and 0.47% NaN_3 in H_2O) were added. Samples were then transferred to regular NMR tubes for subsequent NMR spectral analysis. All 1H-NMR spectra were collected on a Varian 500 MHz Inova spectrometer equipped with a 5-mm HCN Z-gradient pulsed-field gradient cryogenic probe. 1H-NMR spectra were acquired at 25°C using the first transient of the Varian tnnoesy pulse sequence (chosen for its high degree of selective water suppression and quantitative accuracy of resonances around the solvent). Water suppression pulses were calibrated to achieve a bandwidth of 80 G. Spectra were collected with 128 transient and 8 steady-state scans using a 4-s acquisition time (48,000 complex points) and a 1-s recycle delay. Quality control (QC) mixtures consisting of 4 metabolites at 1 mM were analyzed for every 20 to 25 samples, and a relative standard deviation of <2% was observed. Prior to spectral analysis, all free induction decays were zero-filled to 64,000 data points and line broadened to 0.5 Hz. The methyl singlet produced by a known quantity of DSS was used as an internal standard for chemical shift referencing (set to 0 ppm) and for quantification. All 1H-NMR spectra were processed and analyzed using the Chenomx NMR Suite Professional software package version 8.1. Typically, 90% of visible peaks were assigned to a compound, and more than 90% of the spectral area could be routinely fit using the Chenomx spectral analysis software. Most of the visible peaks were annotated with a compound name and expressed as μmol/L. The limit of detection for these compound was 5-6 μmol/L.

Statistical Analysis

Statistical analyses were carried out using R studio 1.1.414 (Rstudio Inc., Boston, MA, USA) with package nlme for linear mixed models to test statistically significant differences between HM metabolites by atopic status within each country and by country within atopic status. The Tukey–Kramer test was used to adjust for multiple comparisons. Differences were considered to be statistically significant if $p < 0.05$. Partial Least Squared Discriminant Analysis (PLS-DA) plots were created using an Excel add-in Multibase 2015 package (Numerical Dynamics, Japan) to maximize the separation of HM clusters by maternal atopic status. Correlations between SCFAs were determined using Spearman's rank correlation.

RESULTS

Of the 109 participating women, 43% were classified as atopic (**Table 1**). There was generally an even distribution of atopic/non-atopic mothers between the cohorts, except for South Africa, where only non-atopic women were sampled. Overall, 69% of participants were Caucasian. The majority of South African women were of mixed race, 39% of the US cohort were African American, and most of the Australian and Norwegian cohorts were of Caucasian ancestry. Cohorts were comparable with respect to maternal age, parity and pre-pregnancy BMI; Japanese women had the lowest BMI, whereas Australian women were the oldest and had the lowest parity. All but one woman had delivered vaginally. Only nine women reported taking antibiotics and use was during early pregnancy or delivery.

Full metabolomic data from this cohort have previously been reported (5). In brief, HM samples from atopic and non-atopic mothers clustered separately (**Supplementary Figure 1**). For the purposes of this study, we have focused on the SCFAs in HM, which have not been previously reported in this cohort.

Human Milk Contains Short Chain Fatty Acids

All samples contained detectable levels of acetate, butyrate, and formate (**Table 2**). Propionate and valerate were not detected in any of the samples. Butyrate was the most abundant SCFA in these samples (median level of 95.6 μmol/L), followed by acetate (median level of 46.8 μmol/L), and formate (median level of 43.7 μmol/L). There were statistically significant positive correlations between acetate and butyrate (rho = 0.55, $p = 6.66 \times 10^{-10}$) and acetate and formate levels (rho = 0.33, $p = 0.0006$). The SCFA intermediates pyruvate, lactate, and succinate were also detected (**Supplementary Table 1**).

Human Milk Short Chain Fatty Acids Differ Geographically and by Maternal Atopic Status

HM from atopic women had significantly lower levels of the SCFAs acetate ($p = 0.02$) and butyrate ($p = 0.001$) than that of non-atopic women (**Figure 1**). Median levels of these SCFAs in atopic women were approximately half that of their non-atopic counterparts (57% lower for acetate, 62% lower for butyrate). Only for Australian women, of whom 100% were Caucasian, were acetate and butyrate levels significantly lower in those with vs. those without atopy ($p = 0.009$ and $p = 0.002$, respectively). Acetate levels were lower in atopic vs. non-atopic Norwegian (85% Caucasian, $p = 0.009$). The reduction in HM acetate levels with atopy in Japanese women (100% Asian) did not reach statistical significance ($p = 0.2$). Among women from the US (61% Caucasian), HM acetate levels were higher with atopic than non-atopic disease ($p = 0.02$). This difference was driven by samples from atopic Black women as when the comparison was restricted to Caucasian women, differences were no longer statistically significant. HM formate levels were also lower in atopic than non-atopic women (45% lower, $p = 0.056$) (**Figure 1**); this difference was statistically significant within Australian

TABLE 1 | Characteristics of the cohort ($n = 109$).

	Australia ($n = 29$)	Japan ($n = 12$)	Norway ($n = 40$)	South Africa ($n = 10$)	USA ($n = 18$)
Maternal atopy	21 (72%)	6 (50%)	9 (23%)	0 (0%)	11 (61%)
Maternal race					
Caucasian	28 (100%)	0 (0%)	34 (85%)	2 (20%)	11 (61%)
Asian	0 (0%)	12 (100%)	0 (0%)	0 (0%)	0 (0%)
Black	0 (0%)	0 (0%)	0 (0%)	2 (20%)	7 (39%)
Mixed race	0 (0%)	0 (0%)	0 (0%)	6 (60%)	0 (0%)
Other race	0 (0%)	0 (0%)	6 (15%)	0 (0%)	0 (0%)
Maternal age (years)	33.8 ± 5.2	24.6 ± 5.5	29.4 ± 5.2	29.8 ± 4.8	29.6 ± 4.4
Maternal parity	1.3 ± 0.5	1.7 ± 1.0	1.5 ± 0.5	2.0 ± 0.9	2.2 ± 1.2
Maternal pre-pregnancy BMI		20.7 ± 2.5	28.1 ± 6.6	25.0 ± 2.9	27.2 ± 5.6
Maternal antibiotics	4 (14%)*	0 (0%)	5 (13%)^	0 (0%)	0 (0%)
Cesarean delivery	1 (4%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Male infant	13 (46%)	6 (50%)	25 (63%)	5 (50%)	6 (33%)

Values are reported as n (percent) or mean ± SD.
Blank cells represent missing data.
**One case of intrapartum Cefazolin for cesarean delivery, two cases of intrapartum penicillin for Group B Streptococcus, one case of intrapartum antibiotics with no class or reason recorded.*
^All exposures were in early pregnancy. Class of antibiotic was not recorded.

TABLE 2 | Levels of short chain fatty acids detected in 109 human milk samples taken at 1 month postpartum.

	Formate (C1:0)	Acetate (C2:0)	Propionate (C3:0)	Butyrate (C4:0)	Iso-butyrate (C5:0)	Valerate (C5:0)	Iso-valerate (C5:0)
Prevalence	100%	100%	0%	100%	0%	0%	0%
Median	43.7	46.8	-	95.6	-	-	-
Minimum	15.2	13.5	-	4.8	-	-	-
Maximum	4960.3	4307.7	-	409.5	-	-	-

Values are reported as % prevalence or μmol/L.

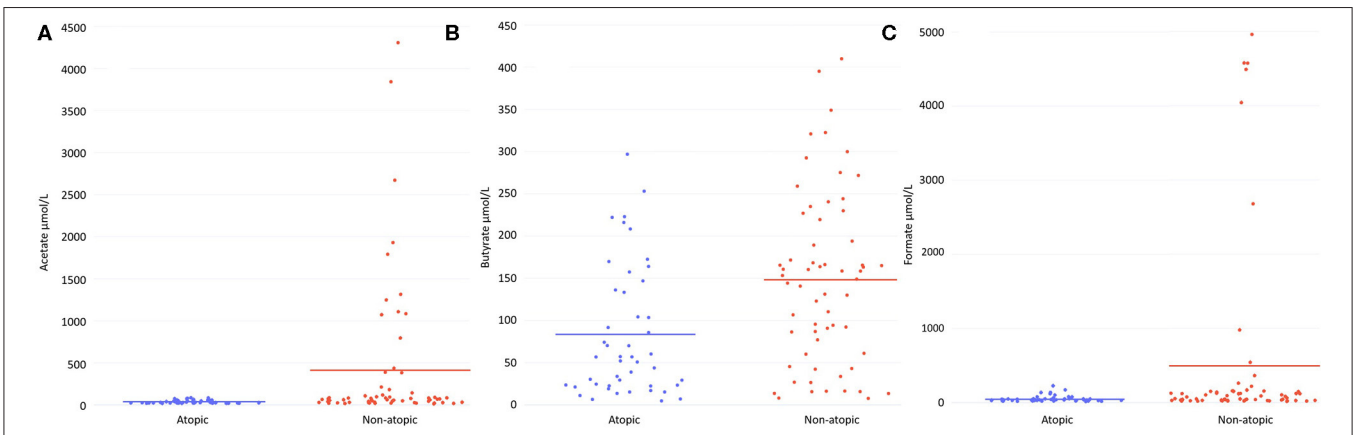

FIGURE 1 | Human milk levels (μmol/L) from non-atopic ($n = 62$) and atopic ($n = 47$) mothers of **(A)** acetate, **(B)** butyrate and **(C)** formate. Lines indicate mean values.

women ($p < 0.0001$) and within Norwegian women ($p = 0.009$). Overall, there were no differences in HM levels of the SCFA intermediates pyruvate, lactate, and succinate between atopic and non-atopic women (**Supplementary Table 1**). However, the HM of Australian women with atopy also had higher levels of lactate ($p = 0.01$) and pyruvate ($p < 0.0001$), and lower levels of succinate ($p = 0.003$) than of women without atopy.

Variations in HM SCFAs levels were also seen between women of the same atopic status living in different countries (**Figure 2**, **Supplementary Table 2**). As tested by mixed linear models, HM

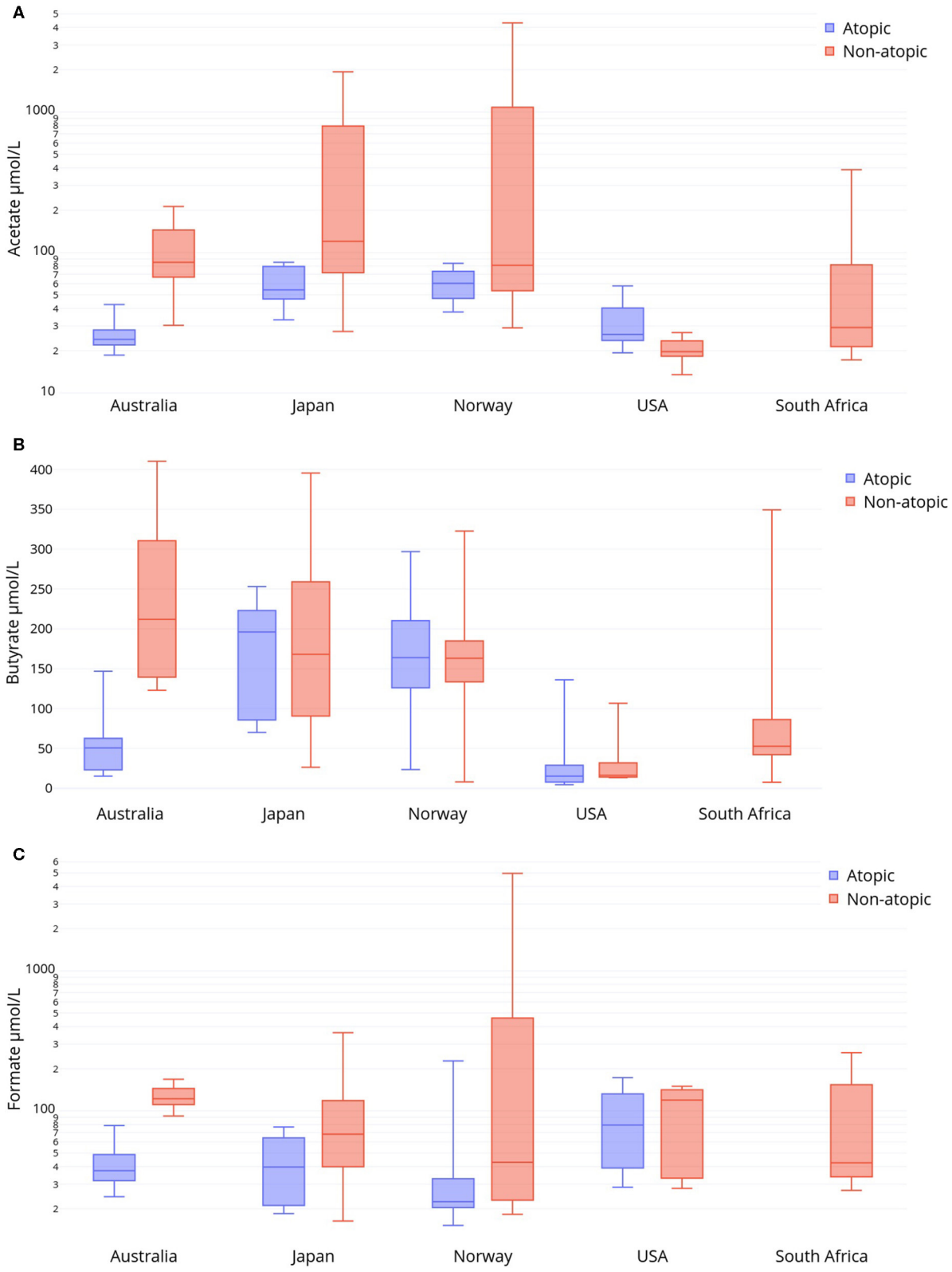

FIGURE 2 | Human milk levels (µmol/L) from non-atopic and atopic mothers in five international sites of **(A)** acetate, **(B)** butyrate and **(C)** formate. Boxes represent median and IQR, whiskers represent range.

levels of butyrate were significantly lower in non-atopic US women compared to those living in Australia, Norway, or Japan ($p = 0.001$, $p = 0.004$, and $p = 0.02$, respectively) but not South Africa. HM butyrate levels were reduced in non-atopic South African women compared to non-atopic Australian women ($p = 0.01$). We conducted a sensitivity analysis that subdivided non-atopic women in the US by race into Black or Caucasian. Only among non-atopic Caucasian women from the US did butyrate levels remain significantly lower than among women in the above comparison.

Atopic women did not differ in their milk acetate or butyrate profiles if they lived in the US vs. Australia, or in Norway vs. Japan. HM from atopic Australian and US women had significantly lower levels of acetate and butyrate that that of atopic Norwegian and Japanese women ($p < 0.001$). HM from atopic Australian women also had lower levels of formate compared to that of US women ($p = 0.02$). No other differences in milk SCFA levels between countries were observed within either non-atopic or atopic women. In a sensitivity analysis that subdivided atopic women in the US by race, atopic Black or Caucasian women from the US continued to differ from Norwegian and Japanese atopic women in terms of lower acetate and butyrate levels in HM. Restricting the US-Australia comparison to atopic Caucasian women did not alter the lack of statistical difference for HM acetate or butyrate.

Levels of SCFA intermediate products also differed by country (**Supplementary Table 2**). Lactate levels were significantly higher in HM from non-atopic Japanese women compared to HM from other non-atopic women (Norway $p = 0.003$, South Africa $p = 0.006$, Australia $p = 0.008$, USA $p = 0.01$). Similarly, lactate was elevated in HM from atopic Japanese mothers compared to atopic Norwegian mothers ($p = 0.01$). HM from non-atopic Japanese and South African mothers had significantly higher levels of pyruvate compared to non-atopic Australian and Norwegian mothers (Japan v. Australia $p = 0.004$; Japan v. Norway $p = 0.008$; South Africa v. Australia $p = 0.02$; South Africa v. Norway $p = 0.04$). Finally, atopic Norwegian women had significantly higher levels of HM succinate compared to mothers from other countries ($p < 0.001$).

DISCUSSION

Here we report that HM contains detectable levels of SCFAs acetate, butyrate, and formate at 1 month postpartum. Collectively, HM levels of acetate and butyrate were significantly reduced in atopic women. This trend was retained for HM acetate in atopic women in Australia, Norway and Japan but not the US. Only among Australian women were HM formate and butyrate levels lower with atopic disease. SCFAs have been shown to be provide protection from allergy and atopy in mice, particularly through their effects on regulatory T cell and dendritic cell biology (8, 9, 16). Higher relative levels of fecal acetate during pregnancy have been associated with reduced risk for hay fever, asthma and wheeze in the offspring of mothers with a history of atopy (15). In their study, fecal acetate levels were higher in mothers of breastfed infants. The ability of SCFAs to inhibit histone deacetylases suggests a role for HM-derived SCFAs in the epigenetic regulation of immune function and postnatal programming of atopy in breastfed offspring. Reduced levels of SCFAs in the HM of atopic women may therefore play a role in the intergenerational transmission of atopic disorders. Indeed, recent data demonstrate that low HM bacterial richness is associated with atopy development in early life (29). Gomez-Gallergo et al. reported country differences in HM SCFA and their correlations with HM microbiota (26). We extend those findings by identifying maternal atopic status as a possible source of variation in HM SCFA.

The reduced levels of acetate in milk from atopic mothers may have other physiological consequences for breastfed infants. In cows, acetate is the major substrate of *de novo* fat synthesis in milk (30). It is unclear whether this is also true for humans (31), but HM acetate levels are found to be weakly correlated with HM fat concentrations (22). In general, breastfeeding is associated with reduced infant adiposity, and gut acetate levels are highest in exclusively breastfed infants (32, 33). SCFAs are involved in several biologic pathways that prevent overweight, including appetite suppression and promotion of fat oxidation over fat synthesis (7). Indeed, HM acetate levels are reported to be negatively associated with infant skinfold thickness (22). Maternal atopic status appears to over-ride the protective actions of prenatal anti-inflammatory cytokines against overweight development in offspring (34). Our study suggests that maternal atopic status may also reduce the availability of HM SCFAs to regulate fat metabolism in the breastfed infant. Acetate and butyrate are also involved in the production of long-chain fatty acids (31). However, contrary to our findings, HM long-chain fatty acid levels do not appear to differ by atopic status (35–37).

HM SCFA levels also varied between our cohorts. This is unsurprising given that the early life gut, adult gut, and HM microbiomes vary geographically (38–41). These metabolites are also likely influenced by regional differences in diet that feed the gut microbiota toward enrichment with Bacteroidetes species in US/European populations. Since we did not collect maternal fecal or HM samples for bacterial profiling, we are unable to link alterations in HM SCFA profiles with specific members of the bacterial community. Gronlund et al. reported reduced bifidobacterial abundance in HM and in the gut microbiota of breastfed infants if mothers had atopic disease (42). Higher bifidobacterial abundance by 3 months of age, followed by an earlier switch to increasing abundance of butyrate-producing bacteria, has been found to be protective against later risk of atopy (43). Recently, *Bifidobacterium*, a key acetate-producing genus, was found to be less abundant in the stool of breastfed infants in the US vs. several African countries (41). Additionally, HM from mothers of US infants exhibited much lower overall bacterial diversity (41). While *Bifidobacterium* spp. chiefly produce acetate, they form symbiotic relationships with butyrate-producers such as *Eubacterium* (44). In HM, acetate and butyrate levels are positively correlated (22). It is thus interesting to note the exceptionally low levels of butyrate in HM from non-atopic US mothers of Caucasian ancestry.

Acetate, butyrate, and formate have been found in HM of women worldwide (20–26). The levels of SCFAs reported here

are in line with those recently reported by Prentice et al. as determined by NMR and GC-MS (at 1-2 months postpartum), and by Wu et al. (across lactation) and Smilowitz et al. (at 3 months) by NMR (20–22). We also confirm the positive correlations of HM acetate with butyrate and formate reported by Prentice et al. The failure by us and others to detect propionate in HM is curious. Presumably, SCFAs, which are produced in the gut, enter HM from the maternal circulation. Thornburn et al. reported that the three most abundantly produced SCFAs in humans (acetate, butyrate, and propionate) were approximately equal in concentration in the sera of pregnant women (median levels 51.4 μmol/L for acetate, 37.1 μmol/L for propionate, and 35.6 μmol/L for butyrate) (8). SCFAs present in HM may be produced by the resident HM microbiota; however, evidence for this possibility is currently lacking. Regardless, the presence of SCFAs in HM likely has important consequences for the developing infant. Endogenous production of SCFAs is low in early infancy (45). Maternally provided SCFAs may, therefore, supplement breastfed infants during the early periods of gut microbiome immaturity.

A major strength of our study is the use of multiple cohorts from around the world. However, this also means that samples were not uniformly collected and stored. Lack of standardized collection by time of day is not an issue for our comparison since there is no evidence for diurnal variation in HM SCFAs (21). On the other hand, some SCFAs are sensitive to storage temperatures higher than −80°C, the temperature at which SCFAs are highly stable for up to 2 months (46). Slight increases to levels of HM butyrate (4 μmol/L) are initially seen after short periods of storage at −20°C compared to storage at −80°C (21), followed by modest declines in butyrate with longer HM storage times at −20°C for up to 16 years (22). Unfortunately, no studies have compared long term storage at −20°C to −80°C. While variation in storage conditions may be an unavoidable limitation of our study, it is unlikely to explain the much lower levels of HM butyrate observed in the Australian atopic samples (stored at −80°C) or to explain within country differences or between country similarities. More importantly, the very large difference in milk butyrate between our Australian cohorts (161 μmol/L lower levels in atopic women) is in the opposite direction to the above stability findings since atopic samples were stored immediately at −80°C, whereas the more recently-collected non-atopic samples were stored at −20°C. Our non-standard definition of "atopy" across cohorts is also a major limitation of this comparison, although similar trends were observed for HM from Norway and Japan despite the absence of serum IgE testing in Norwegian women. Other limitations include not having samples from atopic women from South Africa, and lacking balance in atopy status and number of participants per country. Finally, data were not available for all cohorts on maternal parity, body-mass index or socioeconomic status, but these characteristics have not been found to be correlated with HM SCFA levels (22). On the other hand, this study would have benefited from information on maternal diet, which may have strengthened similar findings by atopic status in two countries with high fermented food intake—Norway and Japan.

CONCLUSION

Our findings suggest that HM SCFA levels may vary by maternal atopic status and country of residence, a finding that could not be attributed to race. Despite sharing Caucasian ancestry, HM SCFA profiles for atopic women differed in Norway vs. the US or Australia. On the other hand, similar HM SCFA profiles by atopic status were seen in Norway and Japan. Lower levels of HM SCFAs have the potential to alter immune programming and fat metabolism in the breastfed offspring of women. This has implications for non-atopic women as well. In our study, this singled out women in the US who had the lowest levels of HM acetate and butyrate compared to non-atopic women in other countries.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Human Research Ethics Committee of The University of Western Australia, Human Research Ethics Committee of the Princess Margaret Hospital, Committee on Human Research of Chiba University, Institutional Review Board at Henry Ford Health System, Norwegian Regional Committees for Medical and Health Research Ethics, and University of Cape Town Human Research Ethical Committee. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

PK performed the NMR analysis. LS drafted the manuscript and contributed to data analysis and visualization. MG contributed to data analysis and visualization. ET, ME, CJ, GW, NS, DC, SP, DM, DG, and AK oversaw recruitment, sample collection, storage, and funding for their respective cohorts. All authors reviewed and critically edited the 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/fimmu.2020.01427/full#supplementary-material

Supplementary Figure 1 | Partial Least Square Discriminant Analysis (PLS-DA) loading plot **(Left)** and scatterplot **(Right)** of human milk metabolites from 109 women in various countries. The score plot shows separation based on maternal atopic status. The loading plot shows the milk metabolites that influence the separation based on maternal atopic status.

Supplementary Table 1 | Levels of short chain fatty acid intermediates detected in human milk samples from atopic ($n = 47$) and non-atopic ($n = 62$) women at one month postpartum. Values are reported as % prevalence or μmol/L.

Supplementary Table 2 | Mean levels of short chain fatty acid and intermediates detected in human milk samples from atopic and non-atopic women at one month postpartum in five international cohorts. Values are reported as μmol/L.

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Bovine IgG Prevents Experimental Infection With RSV and Facilitates Human T Cell Responses to RSV

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Respiratory syncytial virus (RSV) infections represent a major burden of disease in infants and are the second most prevalent cause of death worldwide. Human milk immunoglobulins provide protection against RSV. However, many infants depend on processed bovine milk-based nutrition, which lacks intact immunoglobulins. We investigated the potential of bovine antibodies to neutralize human RSV and facilitate-cell immune activation. We show cow's milk IgG (bIgG) and Intravenous Immunoglobulin (IVIG) have a similar RSV neutralization capacity, even though bIgG has a lower pre-F to post-F binding ratio compared to human IVIG, with the majority of bIgG binding to pre-F. RSV is better neutralized with human IVIG. Consequently, we enriched RSV specific T cells by culturing human PBMC with a mixture of RSV peptides, and used these T cells to study the effect of bIgG and IVIG on the activation of pre-F-pecific T cells. bIgG facilitated *in vitro* T cell activation in a similar manner as IVIG. Moreover, bIgG was able to mediate T cell activation and internalization of pathogens, which are prerequisites for inducing an adaptive viral response. Using *in vivo* mouse experiments, we showed that bIgG is able to bind the murine activating IgG Fc Receptors (FcγR), but not the inhibiting FcγRII. Intranasal administration of the monoclonal antibody palivizumab, but also of bIgG and IVIG prevented RSV infection in mice. The concentration of bIgG needed to prevent infection was ∼5-fold higher compared to IVIG. In conclusion, the data presented here indicate that functionally active bIgG facilitates adaptive antiviral T cell responses and prevents RSV infection *in vitro* and *in vivo*.

Keywords: bovine IgG, RSV, immunoglobulin, prophylaxis, T cell activation

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INTRODUCTION

Respiratory syncytial virus (RSV) infections are a major disease burden in infants and RSV is the second most prevalent cause of death in children, mostly affecting children in low- and middle-income countries (1, 2). It is estimated that 118.200 children died in 2015 because of RSV (1). RSV also is a major seasonal burden to healthcare systems as yearly 3.2 million hospitals admissions are attributed to RSV (1). Efficient protection from RSV will substantially lower

healthcare costs as RSV infections are associated with recurrent wheeze during the first years of life in both healthy preterm and term born children (3, 4). Children are especially vulnerable to RSV during the first 6 months of life, when children are mainly dependent on maternal transferred immunity (5). Specifically infants are unable to produce autologous antibodies and maternal antibody titers decrease quickly within the first months (6, 7). It has been shown that breastfeeding reduces the severity and incidence of RSV infections in children (5). Four months exclusive breastfeeding reduces the risk on respiratory and gastro-intestinal tract infections (8, 9). Yet, most children in developed countries fully rely on bovine milk based infant formulas that do not seem to offer a similar level of protection against these pathogens. The current treatment palivizumab is the only available prophylaxis to protect against RSV (10). Palivizumab binds to the post fusion form of the F protein (11). The F protein undergoes conformational changes after RSV binding facilitating fusion with host cells (11).

Human and bovine milk differ in their composition, e.g., bovine milk has lower molecular weight and less diverse milk oligosaccharides than humans. Even though both human and bovine colostrum and milk contain immunoglobulins, bovine milk has a higher concentration of IgG compared to human milk, in which IgA is the most prevalent antibody (12). The most prevalent immunoglobulin isotype in human milk, IgA, is inversely correlated with respiratory tract infections (13). It is hypothesized that a higher IgG concentration in bovine and other ruminant milk is needed because there is no transfer of maternal immunoglobulins during pregnancy in ruminants, making milk the only source for protective immunoglobulin transfer (14). Despite those differences, it has been demonstrated that consumption of raw bovine milk protects infants against respiratory tract infections and the development of allergies and allergic asthma (15–17). Moreover, immunoglobulins from bovine milk are able to detect several common respiratory tract pathogens like RSV (18). Since raw cow's milk confers the risk of transmitting pathogens to infants, milk is normally heat treated before consumption. Heat treatment of milk reduces the protective effect of bovine milk (15, 19, 20). The amount of intact milk protein thus seems to be correlated to the protective potential of bovine milk, indicating that bovine milk loses its protective potential due to denaturation of milk proteins (19, 20).

Although there is no evidence of gastro-intestinal uptake of bovine immunoglobulins, bIgG is shown to interact with the neonatal Fc receptor (FcRn) (21). Furthermore, bIgG has been shown to bind human FcγRII and is able to form immune-complexes that can mediate activation of monocyte-derived dendritic cells (moDCs) (18, 22, 23). This strongly indicates that supplementation of bIgG to infant formulas could be beneficial for infants.

In the present work, we examined the capacity of purified bIgG to bind RSV, its potential to facilitate RSV-specific T cell responses *in vitro*, and evaluated its prophylactic capacities.

Abbreviations: ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); ADCC, Antibody-dependent cellular cytotoxicity; APC, antigen presenting cells; ATCC, American Type Culture Collection; bIgG, cow's milk IgG; BSA, bovine serum albumin; F protein, RSV fusion protein; FcγR, IgG Fc Receptors; FCS, fetal calf serum FI-RSV, formalin-inactivated respiratory syncytial virus; IC, immune complexes; IMDM, Iscove's Modified Dulbecco's Medium; IVIG, Intravenous Immunoglobulin; KO, knock out; MFI, Mean fluorescence intensity; moDC, monocyte-derived dendritic cells; PBMC, Peripheral blood mononuclear cells; PBS, phosphate buffered saline; PFA, Paraformaldehyde; PFU, Plaque-forming unit; RCF, Relative Centrifugal Force; RPMI, RPMI1640 medium; RSV, Respiratory syncytial virus; SD, standard deviation; WT, wild type.

MATERIALS AND METHODS

Cells and Viruses

HEp-2 cells (ATCC) were maintained in Iscove's Modified Dulbecco's Medium (IMDM, Gibco) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin and 100 ug/ml streptomycin (Life Technologies) at 37°C and 5% CO_2. RSV-A2 and RSV-A2-RL-Line19F were propagated in HEp-2 cells, purified by polyethylene glycol 6,000 precipitation, and resuspended in PBS supplemented with 10% sucrose and stored in liquid nitrogen, as previously described in Jacobino et al. (24).

Bovine IgG

Bovine colostrum was collected from 5 cows within 5 days after calving. The colostrum was cooled and the fat was removed by ultracentrifugation (RCF 100,000*G). The fat free milk serum was stored at −20°C until further purification. After thawing the lipid fraction was removed by centrifugation (RCF 23,500*G), and acidic colostral whey was prepared to remove casein by precipitation with 1 M HCl at pH 4.2. The precipitated casein was removed by centrifugation, adjusted to pH 6.8 with 1 M NaOH, filtered and diluted in 20 mM sodium phosphate, pH 7.0.

bIgG was then isolated from colostral whey by affinity purification using a column consisting of HiTRap Protein G HP (VWR), followed by acid elution with 0.1 M Glycine-HCl, pH 2,7 and dialysis against PBS. Purity was of bovine IgG was checked by SDS-PAGE.

Mice

All experiments were approved by the Animal Ethical Committee of the UMC Utrecht (25). Experiments were performed in C57BL/6 mice purchased from Janviers Lab, or in FcRγ−/− C57BL/6, maintained in the Animal Facility of the UMC Utrecht, or in mFcγR I/II/III/IV−/− C57BL/6 mice, kindly provided by Dr. S.J. Verbeek (LUMC, The Netherlands). Mice were aged 8–20 weeks at the start of the experiments, and littermates were used as controls.

Binding to RSV-Infected Cells

HEp-2 cells were cultured to 70–80% confluency in T75 flasks and infected O/N with 1×10^8 PFU RSV-A2 or RSV-A2-RL-Line19F at 37°C and 5% CO_2. Cells were trypsinized and 1×10^5 cells/well were seeded in 96 well V bottom plates (Greiner bio-one). Serial diluted bIgG, IVIG and palivizumab were allowed to bind for 45 min on ice and detected with αhIgG-RPE or αbIgG-Alexa647 for 45 min on ice. Antibody binding was analyzed by flow cytometry (BD bioscience, Canto II and FACS Diva software). Relative binding was calculated by correcting for the

total infection of the different RSV strains detected by anti-RSV glycoprotein (Merck).

RSV Neutralization Assay

RSV-A2 or RSV-A2-RL-Line19F (MOI 2) was pre-incubated in IMDM supplemented with 1% FCS in the presence or absence of antibodies for 1 h at 37°C. HEp-2 cells (1 × 10e5 cells) were added and incubated for 1 h at 37°C and 5% CO_2. Cells were washed and incubated 24 h in fresh medium at 37°C and 5% CO_2. Cells were trypsinized and infection was stained with 1 ug/ml palivizumab (MedImmune) and 200 times diluted αhIgG-Alexa647 (Southern Biotech). Infection was determined with flow cytometry (BD Bioscience, Canto II and FACS Diva software). The percentage neutralization was calculated by setting the MFI of the uninfected and the infected cells at 0% and 100% neutralization.

Pre- and Post-fusion Protein Binding

96 well maxisorp plates (Nunc) were coated O/N with 100 ng/ml stabilized pre- and post-fusion (F) protein (26–28). In between steps, plates were washed with 0.05% Tween20 in PBS. Plates were blocked with 0.5% gelatin in PBS for 1 h at room temperature (RT). palivizumab, Intravenous Immunoglobulin (IVIG, Nanogam, Sanquin) and bIgG were diluted in PBS and incubated for 2 h at RT. Horseradish peroxidase labeled goat-αhIgG (Jackson) or sheep-αbIgG (Abd Serotec) was used as detection antibody. Plates were developed with ABTS substrate (Roche) and the absorbance was measured at 405 nm with a Multiscan RC (Thermolab Systems).

Human T Cell Activation

PBMC were isolated from blood of healthy donors by ficoll separation and cultured in RPMI1640 supplemented with 5% human AB serum, 100 U/ml penicillin and 100 ug/ml streptomycin (Life Technologies) for 14 days at 37°C and 5% CO_2, 100 ng/ml PepMix RSV (JPT) was added to enrich for the RSV specific T cells. 10 U/ml Interleukin-2 (IL-2) was added to the culture after 7 days. Autologous monocytes were isolated from the PBMC fraction using CD14 magnetic beads (Miltenyi Bioscience) and used as antigen presenting cells. RSV-specific enriched T cells were cell trace violet labeled and incubated with autologous monocytes, pre-F protein and antibodies in Xvivo 15 medium for 5 days at 37°C and 5% CO_2. T cell activation was determined by the number of CD4+ and CD8+ T cells (αhCD3-PerCP / αhCD4-RPE / αhCD8-PE/Cy7) per 10000 sulfate latex beads (Invitrogen) measured with flow cytometry (BD Bioscience, Canto II and FACS Diva software).

Binding of bIgG to Murine FcγReceptors

Bone marrow derived macrophages and dendritic cells were cultured from wild-type (WT), FcRγ−/−, mFcγR I/II/III/IV−/− C57BL/6 mice as described previously (29). 96-well MaxiSorp plates (Nunc) were coated O/N with 10 ug/ml antibody diluted 0.1M NaHPO4, pH 9. Plates were blocked with 1 % gelatin in RPMI1640 (Gibco) for 1 h at room temperature (RT). Cells were labeled with 20 uM calcein AM (Invitrogen) for 30 min at 37°C. 1.5 × 10e5 labeled cells/well were allowed to bind to the coated wells for 45 min at 37°C in 0.1% gelatin in RPMI1640. Binding was defined after several washes with 0.1% gelatin in RPMI1640 and measured (excitation 485 nm, emission 527 nm, ThermoFischer Scientif Fluoroskan Ascent FL) calculated compared to the initial fluorescence.

Internalization Assay Mouse Macrophages

1.5 × 10e8 FITC-labeled *S. aureus* were opsonized with 500 ug/ml IVIG or bIgG or without antibody in 100 μl 1% bovine serum albumin (BSA)-RPMI1640 for 15 min on ice. Washed bacteria were incubated in an effector: target ratio of 1:100 with 1 × 10e5 bone marrow derived WT mouse macrophages in V bottom 96 well plates (Greiner) for 30 min on ice. Cells were washed with 100 ul ice-cold 1% BSA medium and equally divided over 2 wells prior to addition of opsonized bacteria. One part was incubated at 37°C for internalization, while the other part was stained directly. Extracellular immune complexes (IC) were stained with 200x diluted Alexa647 conjugated αhIgG (Jackson) or αbIgG (Jackson) on ice. A decrease in extracellular signal is considered as internalized IC. In addition, cells were washed, fixated with 1% PFA and analyzed by flow cytometry (BD Bioscience, Canto II and FACS Diva software).

RSV Prophylactic Mouse Model

Female FcRγ−/− C57BL/6 mice or wild-type female littermates of the same age were used. Mice were anesthetized (3–4% isoflurane) and administered intranasal with 50 μl antibody diluted in PBS with a varying dosing (0.2–5 mg/kg) of bIgG or IVIG or with a fixed dose of 5 mg/kg bIgG or 1 mg/kg for a similar prophylactic effect on the viral load. Palivizumab was used at 0.05 mg/kg. Mice were intranasally infected with 3 × 10e6 PFU RSV-A2-RL-Line19F in 50 μl PBS after 24 h. Mice were euthanized by intraperitoneal injection of sodium pentobarbital 5 days post infection. A bronchioalveolar lavage was performed, after inflating the lungs, with 1 ml PBS and used to determine the viral load, as described previously (24).

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 6 software. An unpaired Student's *t*-test was used to compare mean values between two groups. Statistical analysis for other multiple comparisons was performed using one-way ANOVA. Statistical significance is indicated as follows: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. All graphs represent mean ± SD of triplicate measurements, unless indicated otherwise.

RESULTS

Bovine IgG Binding and Neutralization of RSV

For this study we made use of RSV-A2 and the more pathogenic strain RSV-A2-RL-Line19F, to evaluate whether the binding of purified bovine colostrum IgG (bIgG) and purified human plasma IgG (IVIG) is equal between both strains. HEp-2 cells were infected with RSV and dose-dependent binding of bIgG and IVIG was analyzed. Binding was compared to the clinically used antibody palivizumab (human IgG1 against RSV F protein). bIgG

bound to the RSV-A2 infected cells, as shown previously, starting from a concentration of 1.2 μg/ml bIgG. Binding of bIgG was equal to cells infected with both RSV strains, similar to what was observed with IVIG and palivizumab (**Figure 1A**).

RSV-specific antibodies, like palivizumab, are known to neutralize RSV and are able to prevent infection in children. To evaluate the *in vivo* protective capacity of bIgG we aimed to use the more pathogenic strain RSV-A2-RL-Line19F, however we first wanted to compare the *in vitro* neutralizing capacity of bIgG between RSV-A2-RL-Line19F and the less pathogenic RSV-A2 strain. Previously, we have shown that bIgG is capable of preventing RSV-A2 to infect HEp-2 cells *in vitro* (18). Therefore, both RSV strains were pre-incubated for 1 h with a serial dilution of palivizumab, IVIG or bIgG. Infection of HEp-2 cells was allowed for 1 h at 37°C and cells were washed three times in fresh IMDM medium after incubation to prevent binding of the anti-RSV antibodies to the infected cells and thereby masking the F protein expression of the cells. Infection was analyzed after 24 h by flow cytometry and the neutralization capacity of the antibodies were calculated. All antibodies were capable of neutralizing RSV and preventing infection, as shown previously. The neutralization capacity of all antibodies was equal between both RSV strains (**Figure 1B**).

Binding of Bovine IgG to Pre- and Post-fusion F Protein

The RSV fusion glycoprotein (F-protein) is a class I viral fusion protein that is involved in the fusion of the virus the host cell. It undergoes a conformational change from the pre-fusion state to the post-fusion state during viral entry. Antibodies directed against pre-fusion F show a higher neutralization capacity than antibodies directed against post-fusion F (27, 30). Specific binding to plate-bound stabilized pre- and post-fusion F was determined. bIgG was found to recognize both the pre- and the post-fusion F (**Figure 1C**). The ratio of pre- vs. post-fusion F specific antibodies was higher for palivizumab and IVIG, but bIgG still recognized the pre-fusion state better than the post-fusion state.

Facilitation of Human RSV-Specific T Cell Activation by Immune Complexes of RSV With hIgG and bIgG *in vitro*

Bovine IgG can engage the human FcγRII on myeloid cells when it is bound simultaneously to RSV. These RSV-bIgG immunecomplexes (IC) can be internalized by FcγRII expressing antigen presenting cells (APC) like monocyte-derived dendritic cells (moDC's). To study whether this uptake can result in antigen presentation and thereby leading to activation of the adaptive immune system, a human T cell activation assay was performed. PBMC from healthy donors were enriched for their RSV specific T cells with a RSV peptide mix. Autologous monocytes were used as APC and co-cultured with the RSV specific T cells and IC, formed by co-incubation of pre-fusion F and palivizumab, IVIG or bIgG in titrated concentrations for 5 days. T cell activation was determined by the proliferation of CD4 and CD8 T cells. IC formed with palivizumab showed optimal activation of both CD4 and CD8 T cells with IC formed with 0.1 μg/ml antibody. The curve of bIgG and IVIG looked highly similar, however with an optimum between 0.2 and 1 μg/ml antibody (**Figure 2**).

FcγR-Dependent Binding and Internalization of Bovine IgG by Murine Macrophages and Dendritic Cells

To investigate whether bIgG can contribute to the prevention and clearance of RSV *in vivo*, we used a murine RSV challenge model. The *in vitro* data with bIgG and human immune cells suggested that there could be a contribution of active clearance by FcγR-expressing immune cells in the elimination or RSV. bIgG is capable to bind the human activating FcγRIIa, but mice do not express the activating FcγRIIa but only the inhibitory FcγRIIb. Therefore, we first examined whether bIgG could bind murine FcγR. Calcein labeled macrophages and dendritic cells, cultured from bone marrow of wild-type (WT) mice, showed binding to plate bound IVIG and bIgG (**Figures 3A,B**). Using cells from the FcγR I/II/III/IV knock out (KO) mouse, lacking expression of all the FcγR, resulted in no binding to IVIG and bIgG equal to the control antibodies. In contrast, the cells of the FcRγ KO mice were still able to bind the control antibody mIgG1 and partly to IVIG via the inhibitory receptor FcγRII, the only FcγR expressed by these mice (**Figures 3A,B**). However, bIgG does not bind to cells of the FcRγ KO mice, demonstrating that binding to bIgG to murine macrophages and dendritic cells is FcγR dependent and only occurs with the activating FcγR. Blocking experiments could not reveal whether one or more of the activating FcγR is responsible for this binding (data not shown).

Next, we examined whether the binding of bIgG to murine FcγR can also induce internalization, as prerequisite for efficient clearance and induction of a memory T cell response. FITC labeled S. aureus were opsonized with or without IVIG or bIgG and incubated with WT mouse bone marrow-derived macrophages. Extracellular IC was determined and compared between 4 and 37°C for internalization (**Figure 3C**). Both IVIG and bIgG showed a decrease in signal on the outside of the cells indicating that the IC were internalized by the macrophages.

In vivo Prophylactic and FcγR Dependent Activity of bIgG

The protective capacity of bIgG was further studied in a prophylactic RSV mouse model. A dilution series of bIgG or IVIG and one dosage of palivizumab was administered intranasally 24 h prior to RSV challenge. IVIG was able to reduce viral load in a concentration dependent manner, while bIgG protected against RSV infection in the airways only at the highest dose (**Figure 4A**). To investigate the underlying mechanism of this protection, we compared the protective effect in WT mice to the effect in FcRγ KO out mice. For optimal comparison between the antibodies, we chose the lowest concentration of antibody that resulted in a protective effect in **Figure 4A**. The level of infection was equal in the PBS treated mice between the WT and the mice lacking the activating FcγR (**Figure 4B**). RSV infection was decreased in all treated WT mice with similar levels between

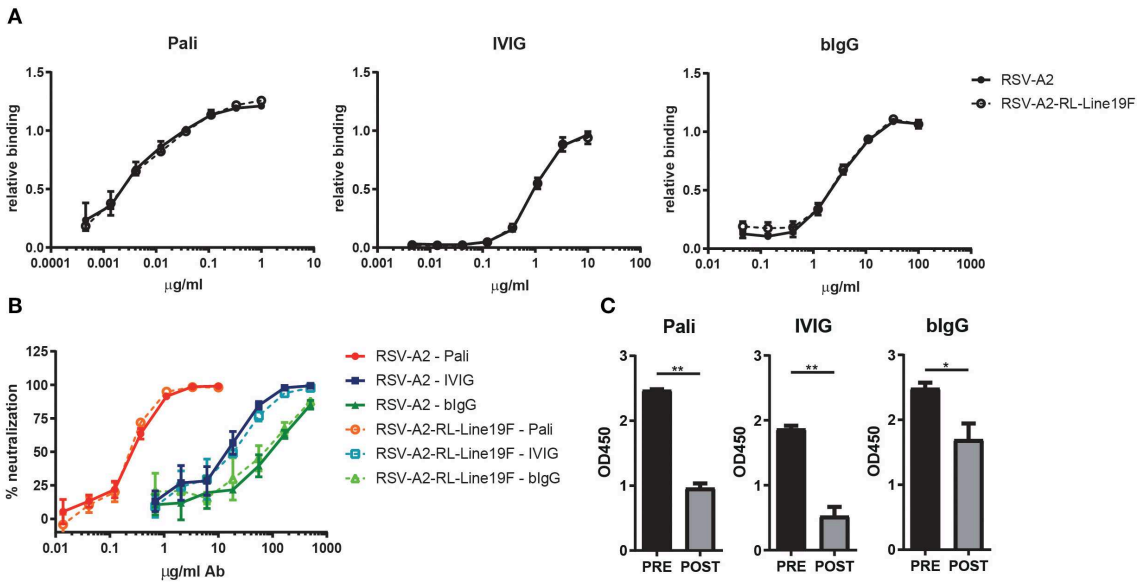

FIGURE 1 | bIgG RSV binding and neutralization. Antibody binding of serial diluted palivizumab (Pali), IVIG or bIgG to RSV-A2 (closed symbols) or RSV-A2-RL-Line19F (open symbols) infected HEp-2 cells. RSV specific binding detected with αhIgG-RPE or αbIgG-Alexa647 and analyzed by flow cytometry. Data corrected for infection rate **(A)**. HEp-2 cells were infected with RSV-A2 or RSV-A2-RL-Line19F which was pre-incubated for 1 h with a serial dilution of palivizumab, IVIG or bIgG. Infection was analyzed by flow cytometry, uninfected cells and no antibody incubation were set as 100 and 0% neutralization, respectively **(B)**. Pre- and post-fusion F glycoprotein specific binding of palivizumab, IVIG and bIgG **(C)**. Median with range of triplicate measurements are shown $^{*}P \leq 0.05$; $^{**}P \leq 0.01$.

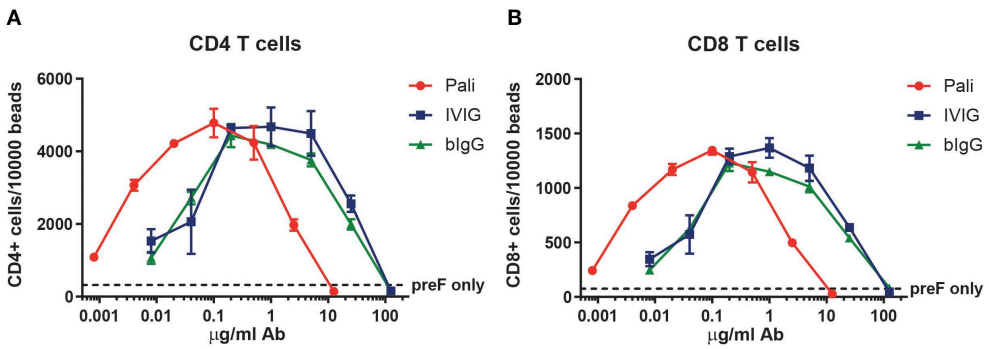

FIGURE 2 | Human T cell activation by RSV prefusion protein-bIgG immunecomplexes. Enriched RSV specific human T cells from healthy donors were incubated with autologous monocytes and immunecomplexes generated by pre-incubating prefusion protein (preF) and a serial dilution of palivizumab, IVIG or bIgG. Activation was determined by the number of CD4 T cells **(A)** or CD8 T cells **(B)** per 10,000 sulfate latex beads with flow cytometry.

the different antibodies. The decrease in viral load was less in the FcRγ KO mice, indicating a role for the activating FcγR, next to the prophylactic neutralizing effect of the antibodies.

DISCUSSION

In this paper, we have demonstrated that bovine IgG binds to two different strains of human RSV, facilitates the activation of RSV-specific T cells, and reduces viral load with RSV in a prophylactic RSV *in vivo* mouse model.

Bovine IgG is able to recognize pre- as well as post-fusion F protein of RSV, although in a lower pre- to post-fusion F protein binding ratio then IVIG and palivizumab. Despite the fact that binding to pre-fusion F protein is associated with a higher neutralization capacity than antibodies that bind to post-fusion F protein, IVIG and bIgG showed a similar neutralization capacity *in vitro*. It has to be noted that for palivizumab lower concentrations are needed to neutralize RSV compared to both IVIG and bIgG. Since IVIG an bIgG are both polyclonal antibodies, it was expected that higher concentrations would have been needed to reach a similar neutralization compared to the monoclonal antibody palivizumab. Here, neutralization was only tested in RSV-A strains and not in B strains. Since the F protein is highly conserved between RSV A and B strains, it is likely that bIgG is able to bind and neutralize RSV-B strains as well (31). Moreover, we observed that bIgG was also able

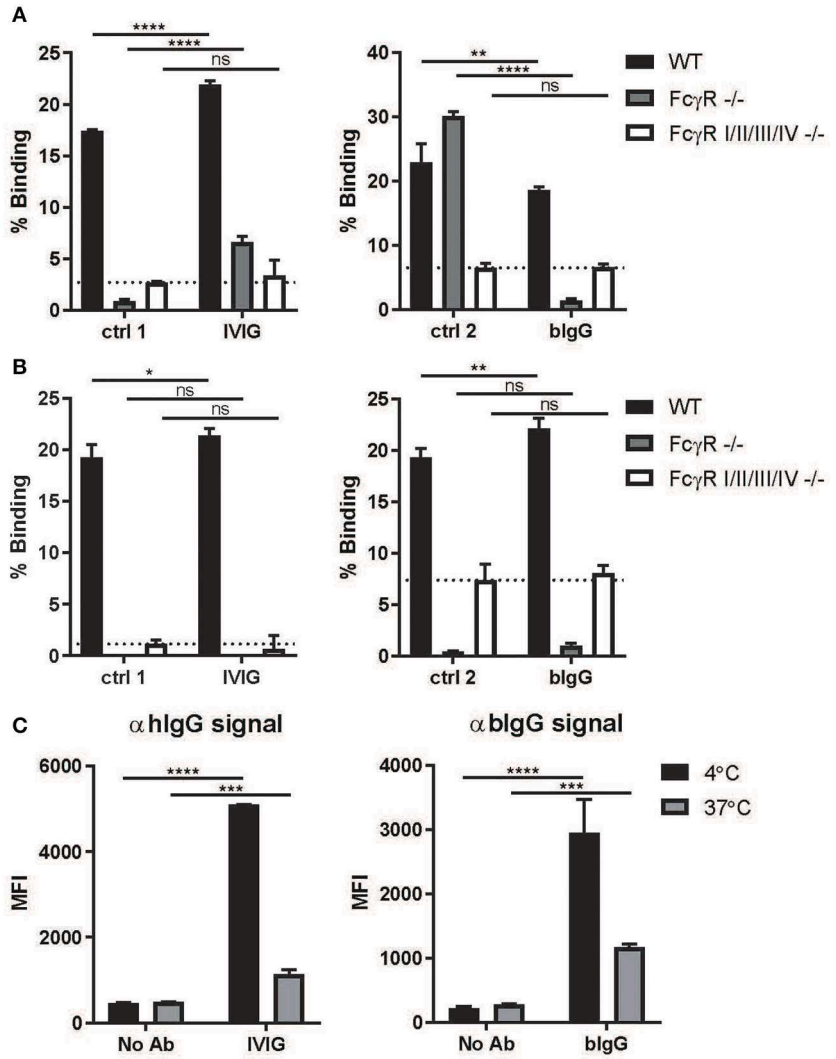

FIGURE 3 | Binding and internalization by activating murine FcγR. Plates were coated with 10 ug/ml IVIG or bIgG and incubated with calcein labeled macrophages **(A)** and dendritic cells **(B)** cultured from bonemarrow of wild-type (WT), FcRγ–/–, mFcγR I/II/III/IV–/– C57BL/6 mice. Binding was compared to human IgG1 (ctrl 1) and mouse IgG1 (ctrl 2) ($N = 3$). FITC labeled S. aureus were opsonized with or without IVIG or bIgG and incubated with WT mouse bonemarrow derived macrophages at 4°C (binding), samples were equally divided and one part was incubated at 37°C for internalization. Extracellular immunecomplexes were determined by Alexa647 conjugated αhIgG or αbIgG and analyzed by flow cytometry. Decrease in signal is considered as internalization **(C)**. Mean with SD of triplicate measurements are shown. $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$; $^{****}P \leq 0.0001$.

to neutralize the more pathogenic strain RSV-A2-RL-Line19. Bovine IgG is able to recognize pre- as well as post-fusion F protein of RSV, although in a lower pre- to post-fusion F protein binding ratio then IVIG and palivizumab. Bovine IgG is directed against the bovine RSV. As the prefusion protein of bovine RSV is not identical to the human RSV, as the homology between human and bovine F protein is about 80% (32), it is expected that bIgG has a lower affinity for human RSV pre fusion protein than IVIG and a monoclonal antibody raised against human pre F protein. In addition, cows are often vaccinated against RSV. These vaccines contain attenuated bovine RSV, for example inactivated with formalin (33). It is known that the pre F protein is not stable, and disappears from the RSV surface upon formalin fixation (34). These observations may explain why bovine IgG binds to a lesser extent to human RSV pre F protein.

When RSV-specific T cells are cultured with autologous PBMC's, bIgG and RSV F protein, bIgG as well as IVIG strongly facilitated T cell proliferation, which indicates activation of the adaptive immune system. A similar effect has been described in mice infected with RSV, oral administration of bovine colostrum led to an increased CD8 T cell activity (35). Particularly in RSV infections, the role of T cells is dubious. T cells are, like in other viral infections, required for viral clearance (36). However, it is hypothesized that T cells are also the cause of the vaccination-enhanced disease during the FI-RSV trial (37, 38). Particularly Th2 cells are suspected to play an important role in RSV bronchiolitis immunopathology

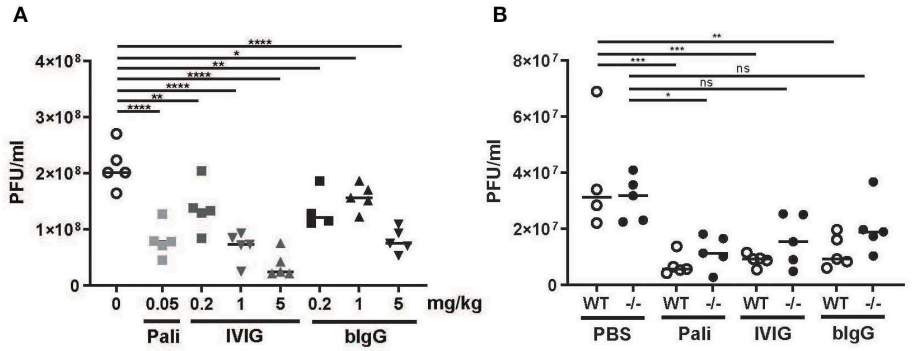

FIGURE 4 | *In vivo* prophylactic activity of bIgG in WT and FcRγ–/– mice. Wild-type (WT) C57BL/6 mice were prophylactically treated with a titration of bIgG or IVIG **(A)** 24 h prior to intranasal infection with 3 × 10e6 PFU RSV-A2-RL-Line19F. RSV load was determined in bronchoalveolar fluid 5 days after infection. The contribution of FcyR was compared in WT (open circles) and FcRγ–/– mice (closed circles) with prophylactic treatment resulting in similar viral load in WT (5 mg/kg bIgG/1 mg/kg IVIG/0.05 mg/kg) **(B)**. *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; ****$P \leq 0.0001$.

due to Th2 cytokine release (37). In literature, the activation of T cells by bovine milk has only been evaluated by Xu et al. (35). In this study, activation of CD8+ T cells was observed after oral ingestion of bovine milk (35). However, this increased CD8+ T cell activation in mice was also associated with a lower burden of disease (35). This indicates that the increased T cell activity against RSV that was observed *in vitro*, is likely to only lead to viral clearance without negatively impacting the infection. Moreover, Den Hartog et al. showed that bIgG is capable of recognizing other common respiratory pathogens like influenza and *Haemophilus influenzae* as well, indicating that bIgG might also activate T cell responses to other pathogens (18).

In order to perform prophylactic RSV studies in mice, we first investigated whether bIgG is capable to engage with murine Fc-receptors. We found that bIgG binds murine macrophages and dendritic cells through one or more activating Fc-receptors. We also showed that opsonization by bIgG enabled murine macrophages and dendritic cells to phagocytose *S. aureus*. It has been shown that bIgG is able to form immune complexes that can lead to opsonization of the pathogen. This opsonization is possible mediated by FcRγII as it has been shown that bIgG is able to bind to this receptor (38–400. Moreover, Inhibition of FcRγIIa lead to inhibition of the opsonization of bIgG-HIV-1 immune complexes (39). The *in vivo* prophylactic studies clearly show that both palivizumab, IVIG and bIgG reduced the RSV load in bronchoalveolar fluid. Interestingly, in the FcRγ-/– mice, less protection from RSV was observed for all three antibody groups: palivizumab, IVIG and bIgG. This indicates that also *in vivo* the activating FcγRs are important for RSV antibodies as was described before for palivizumab by Van Mechelen et al. (40). No statistical relevant difference could be found between the mice that either received bIgG or IVIG, indicating that bIgG is not inferior to IVIG in the protection from RSV in mice. A similar protective effect of bIgG was observed in the study performed by Xu et al., demonstrating that oral intake of bovine IgG protected mice from RSV (35).

Conclusively, our data suggest that addition of bIgG may be a novel strategy to increase the protective potential of infant formulas. As stated before, many children are dependent on bovine milk derived infant formulas as they are not breastfed (41). Previous trials evaluating the effect of raw milk or bovine immunoglobulin rich formulas have already shown their efficacy in the treatment of gastro-intestinal infections with rotavirus and *E. coli*. Another trial performed by Loss et al. remarkably showed that children may benefit from raw cow's milk consumption since the raw cow's milk arm showed fewer respiratory tract infections (among which rhinitis and otitis) and fever episodes compared to the processed milk arms (15, 42). However, consumption of raw cow's milk encompasses risk for young children to transmit several pathogens among which tuberculosis, brucellosis and listeria (15). Adding purified bIgG to infant formulas may thus transfer part of the protective effect of raw bovine milk to microbiologically safe infant formulas.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

All experiments were approved by the Animal Ethical Committee of the UMC Utrecht.

AUTHOR CONTRIBUTIONS

MN designed and conducted experiments and wrote the manuscript. AS wrote the manuscript. JJ and SJ performed experiments. SB, LB, and RN co-supervised the project and critically read the manuscript. CH provided essential materials. JL supervised the project and co-wrote the manuscript.

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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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ORIGINAL RESEARCH
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Partial Degradation of Recombinant Antibody Functional Activity During Infant Gastrointestinal Digestion: Implications for Oral Antibody Supplementation

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Oral administration of engineered immunoglobulins has the potential to prevent enteric pathogen-induced diarrhea in infants. To prevent infection, these antibodies need to survive functionally intact in the proteolytic environment of the gastrointestinal tract. This research examined both *ex vivo* and *in vivo* the functional survival across infant digestion of palivizumab, a model FDA-approved recombinant antibody against respiratory syncytial virus (RSV) F protein. Palivizumab-fortified feed (formula or human milk), infant gastric, and intestinal samples were incubated to simulate *in vivo* digestion (*ex vivo* digestion). Palivizumab-fortified human milk was also fed to infants, followed by collection of gastric and intestinal samples (*in vivo* digestion). Palivizumab was purified from the samples of digestate using protein G spin columns followed by filtration through molecular weight cut-off membranes (30 kDa). Palivizumab functional survival across *ex vivo* and *in vivo* digestion was determined via an anti-idiotype ELISA and an RSV plaque reduction neutralization test. Palivizumab concentration and RSV neutralization capacity both decreased when incubated in intestinal samples (*ex vivo* study). The concentration and neutralization activity of orally-supplemented palivizumab also decreased across infant digestion (*in vivo* study). These results indicate that if recombinant IgGs were selected for oral supplementation to prevent enteric infections, appropriate dosing would need to account for degradation occurring in the digestive system. Other antibody formats, structural changes, or encapsulation could enhance survival in the infant gastrointestinal tract.

Keywords: palivizumab, infant digestion, human milk, antibody functional activity, respiratory syncytial virus

INTRODUCTION

Infectious diarrhea kills more than 2,000 children under 5 years of age every day (1–3). Breastfeeding is associated with lower infection risks in infants (4–6), and human milk enhances passive immunity of breastfed infants by supplying pathogen-specific neutralizing antibodies (7). Following the human milk model of maternal antibodies facilitating immunological protection

for offspring, oral provision of pathogen-specific recombinant immunoglobulins could help prevent diarrheal infections in infants. To prevent infection, however, orally-delivered recombinant antibodies would have to resist degradation from exposure to milk and gastrointestinal proteases and pH changes (from pH 3.5–8) across the gastrointestinal tract (8, 9). Proteolytic enzymes, such as carboxypeptidases, elastase, plasmin, and kallikrein, are present in breast milk (10). These proteolytic enzymes may be active during gastrointestinal digestion, as inactive cathepsin D in breast milk is activated by the acid conditions of the stomach (11). Many digestive enzymes, including pepsin, trypsin, and chymotrypsin, also mix with feed (human milk) during infant digestion. No studies have thus far been reported for the effect of human digestive proteases on viral neutralization; however, these enzymes may degrade antibodies.

The extent to which recombinant antibodies survive across infant digestion remains unknown. Functional survival of recombinant antibodies across digestion needs to be examined to assess their potential as oral supplements to prevent enteric infections.

As a model for examining the functional survival of recombinant antibodies across digestion, we selected palivizumab (a humanized monoclonal recombinant IgG1κ), the only FDA-approved recombinant antibody for use in infants to prevent infections, and which is administered via intramuscular injection. Palivizumab recognizes and binds to the fusion protein (F) of RSV, thereby inhibiting infection of host cells (12, 13). In our previous study (14), palivizumab was not stable across *ex vivo* incubation in infant gastric and intestinal samples, whereas naturally occurring human milk RSV-specific antibodies were stable. Whether the degradation of palivizumab as measured by ELISA across *ex vivo* infant digestion corresponds with a loss of functional capacity of palivizumab to neutralize RSV remained unknown. The aim of this study was to measure the extent to which palivizumab retains functional RSV neutralization capacity across incubation within *ex vivo* infant gastric and intestinal samples (*ex vivo* digestion) and across gastric and intestinal sampling sites after oral supplementation to infants (*in vivo* digestion). This work serves as a model for examining the digestion of recombinant antibodies that can be used to inform future development of oral enteric pathogen-specific recombinant antibodies for the prevention of infectious diarrhea.

MATERIALS AND METHODS

Digestion of Human Milk and Formula (*ex vivo* and *in vivo*)

In vivo digestion samples were collected from infants at the Doernbecher Children's Hospital Neonatal Intensive Care Unit (NICU) located at Oregon Health & Science University in Portland, OR, after obtaining parental informed consent (**Figure 1**). Inclusion criteria for infants in this study were infants already admitted to the NICU, >34 weeks corrected gestational age, with an indwelling nasogastric or orogastric feeding tube and tolerating full enteral feeding volumes (typically 150–160 mL/kg/day). Exclusion criteria were infants with diagnoses that were incompatible with life, infants not being fed enterally, major gastrointestinal system anomalies affecting protein digestion, severe genitourinary anomalies, and significant metabolic or endocrine diseases. Prior to feeding, a nasally-placed tube was placed into the distal duodenum or proximal jejunum. Gastric and intestinal samples were collected from four infant pairs (**Table 1**). Feeds were delivered via nasogastric tubes over 30 min or less. Infants were fed without palivizumab (formula for infant 1, fortified mother's milk for infant 2) or with palivizumab (60 μg/mL in fortified mother's milk for infant 3 and 1,000 μg/mL in unfortified mother's milk for infant 4). This range of feed types represent all common feed types fed to infants in the NICU, allowing us to encompass this potential variability within the analysis of the extent of palivizumab digestion. Two milliliters of feed samples were collected in sterile vials on ice. Each infant's gastric contents (0.5–2 mL) was withdrawn by suction 30 min after completion of feeding into a 3-mL syringe and transferred in sterile vials, and placed on ice. Intestinal samples were collected from the nasojejunal/duodenal tube into sterile vials on ice via gravity flow as the digesta passed the collection tube port. Gastric and intestinal samples collected are, thus, mostly composed of the most proximal feed with the addition of digestive secretions. The sample vials were immediately stored at −80°C. The frozen sample vials were transported on dry ice to Oregon State University and stored at −80°C.

For *ex vivo* digestion of palivizumab (**Figure 1**), the samples [feed (formula for infant 1 or fortified mother's milk for infant 2), gastric, and intestinal samples] were thawed quickly at 37°C with shaking at 300 rpm (∼1 min). Palivizumab was added to samples (feed, gastric, and intestinal) at 11 mg/mL and digested at 37°C with shaking at 300 rpm for 1 h (feed, gastric, and intestinal samples) in an Eppendorf ThermoMixer® C (Eppendorf AG, Hamburg, Germany). The higher concentration of palivizumab used for the *ex vivo* incubation compared with the feeding study was selected to allow the use of lower sample volumes while extracting enough palivizumab for RSV plaque-neutralization assay.

Determination of Palivizumab Content Using Enzyme-Linked Immunosorbent Assay (ELISA)

The Palivizumab content in the samples was determined using an anti-idiotype ELISA with HCA261 (Bio-Rad, Richmond, CA, USA) as a capture antibody and horseradish peroxidase-conjugated goat anti-human IgG gamma chain (STAR 106P, Bio-Rad) as a detection antibody according to the method developed by Bio-Rad, with some modifications. Briefly, 100 μL of HCA261 at 1 μg/mL in phosphate-buffered saline (PBS; pH 7.4) was added in each well of a clear flat-bottom 96-well plate (Nunc MaxiSorp; Thermo Fisher Scientific, Waltham, MA, USA) and incubated overnight at 4°C. Wells of the microplate

Abbreviations: RSV, respiratory syncytial virus; NT, neutralization titer; DMEM, Dulbecco's Modified Eagle Medium; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; PBS, phosphate-buffered saline (pH 7. 4); PBST, PBS containing 0.05% Tween-20; BSA, bovine serum albumin; MWCO, molecular weight cut off.

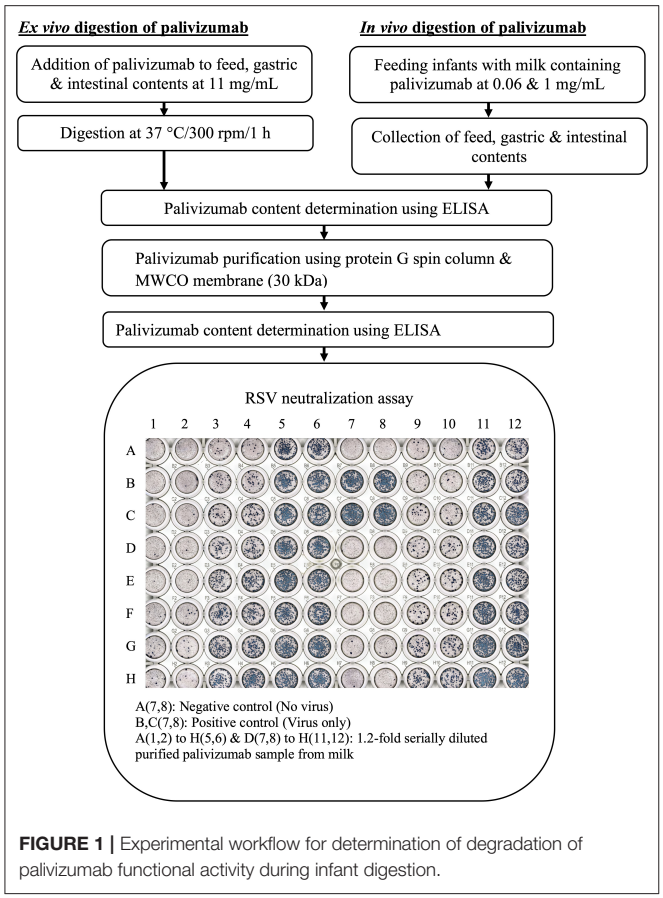

FIGURE 1 | Experimental workflow for determination of degradation of palivizumab functional activity during infant digestion.

were washed three times with 200 μL of PBS containing 0.05% Tween-20 (PBST) (Bio-Rad) and blocked for 1 h with 150 μL of PBST containing 1% bovine serum albumin (BSA) at RT. Samples were diluted 2-fold with PBST containing 1% BSA, and palivizumab standards were prepared in PBST containing 1% BSA in the range of 1–1,000 ng/mL. Palivizumab standards/diluted samples (100 μL) were added to each well after washing three times using PBST and incubated for 1 h at RT. The wells were washed three times with PBST as described above; 100 μL of horseradish peroxidase-conjugated goat anti-human IgG gamma chain detection antibody at 0.13 μg/mL PBST containing 1% BSA were added to each well and incubated at RT for 1 h. After the plates were washed 6 times with PBST as described above, 100 μL of 3,3′,5,5′-tetramethylbenzidine substrate solution (Thermo Fisher Scientific) were added to each well and incubated for 5 min. The reaction was stopped by adding 50 μL of 2 N sulfuric acid and the absorbance was measured at 450 nm using a microplate reader (Spectramax® M2, Molecular Devices, Sunnyvale, CA, USA). The samples from feed, infant gastric, and intestinal contents were tested at least two dilutions with 3 replicates of each dilution. Replicate measurements were averaged. The percentage survival of intact ELISA-detectable palivizumab at each digestion point was determined with respect to the unincubated sample in the *ex vivo* study, whereas it was determined with respect to feed in the *in vivo* study.

TABLE 1 | Demographics of four mother–infant pairs sampled for feed (formula or human milk), gastric, and intestinal contents.

Demographics	Infants[†]
Gestational age at birth, weeks	32.08 ± 4.48 (27.1–38)
Postnatal age at feeding, days	41.50 ± 22.93 (23–75)
Corrected gestational age at feeding, weeks	38.00 ± 2.31 (36.4–41.3)
Body weight at sampling, kg	2.87 ± 0.53 (2.45–3.63)
Length at sampling, cm	46.25 ± 3.77 (42–51)
Head circumference at sampling, cm	35.50 ± 3.54 (31–39.5)
Total kilocalories intake, kcal/kg/day	131.25 ± 25.40 (108–165)
Specific feed volume, mL	43.50 ± 12.23 (30–57)

[†]*Values are mean ± SD (range).*

Percentage survival was determined for each dilution (the average of three replicates) separately and these values were used for statistical analyses.

Purification of Palivizumab Using Protein G Spin Column and 30-kDa Molecular Weight Cut Off (MWCO) Filtration

Milk, gastric and intestinal samples contain substances such as β-casein, milk fat, immunoglobulins (SIgA, IgG, and IgM), lactoferrin, proteases, protease inhibitors, lactoperoxidase, cells, and bacteria that can introduce background effects on the RSV neutralization assay (15–18). Thus, palivizumab was purified from the *ex vivo* and *in vivo* samples using protein G column and 30 kDa-MWCO filtration. Protein G spin column (Thermo Fisher Scientific) and all buffers were equilibrated to RT (30 min). Storage solution of the column was passed through by centrifuging the column at 5,000 × g, 20°C for 30 s. To equilibrate the columns, 400 μL of the Pierce™ protein G IgG binding buffer (proprietary composition, pH 5.0, containing 0.02% sodium azide) were added and the column was centrifuged at 5,000 × g, 20°C for 30 s. The equilibration step was repeated once. The volume of sample added to the protein G column varied based on the infant and sample type, and was selected based on a desired final concentration of 300 μg/mL palivizumab in the purified sample, assuming a standard 50% palivizumab loss after complete extraction (protein G and 30-kDa MWCO filtration). This allowed for a 30-fold dilution to overcome background effects in the neutralization assay while maintaining a target 10 μg/mL palivizumab starting concentration in the neutralization assay. Samples were separately diluted with the binding buffer in the ratio of 1:3 (v/v) to ensure optimal ionic strength and pH for binding. The diluted sample was centrifuged for 10 min at 1,000 × g, 4°C, and the supernatant was collected for palivizumab extraction. The pellet was dissolved in 1 mL binding buffer, centrifuged as described above, and the supernatant was collected and combined with the previous supernatant. An aliquot of this supernatant prepared from sample-buffer mixture (500 μL) was added to a protein G spin column and mixed end-over-end for 10 min and centrifuged at 5,000 × g, 20°C for 30 s. To wash the column, 500 μL

of the binding buffer were added, mixed to resuspend the resin and centrifuged at 5,000 × g, 20°C for 30 s. These wash steps were repeated 9 times. To elute bound palivizumab, 500 μL of the Pierce™ gentle Ag/Ab elution buffer (proprietary composition, high ionic strength, pH 6.6) were added to the column, the column was mixed end-over-end to resuspend the resin and centrifuged at 5,000 × g, 20°C for 60 s. Elution steps were repeated 7 times. To remove remaining interfering substances, the protein G extract was added to a 30-kDa MWCO centrifugal filter unit. Prior to the addition of the sample, 5 mL of Dulbecco's Modified Eagle Medium (DMEM), without serum, were added to the device followed by centrifugation at 3,000 × g, 4°C for 3 min to wash the apparatus. This washing step was repeated once. Four milliliters of each protein G extract were combined with 5 mL of DMEM (no serum, with antibiotic), added to the MWCO device and centrifuged at 1,000 × g, 4°C for 10 min. To allow for additional removal of interfering substances, 5 mL of DMEM (without serum) were added and the MWCO device was centrifuged (repeated 2 times). The retentate (purified palivizumab) was collected, and palivizumab concentration in the purified samples was determined by ELISA. The efficiency of this extraction was not 100% and differed across sample types. To make a fair comparison, the extracted palivizumab concentrations were normalized to a specific dilution of the original concentration prior to the neutralization assay. To do so, a dilution that would bring the original palivizumab concentration close to 10 μg/mL was selected as the target for normalizing the dilution of the purified sample. Purified palivizumab samples were then diluted to reach the concentration of this selected dilution for the respective unpurified sample. This normalized dilution number was used to interpret the results of the plaque assay.

Determination of Plaque Reduction Neutralization Titer

Preparation of RSV Frozen Stock

HEp-2 cells (ATCC® CCL23™) were seeded in a tissue culture flask (75 cm^2) with DMEM containing 10% fetal bovine serum (FBS) and 1% antibacterial-antimycotic solution and allowed to grow until reaching >95% confluency (typically 24–48 h) in a 5% CO_2 incubator at 37°C. The cell monolayer was washed three times with sterile Hank's balanced salt solution and infected with 1 mL of frozen RSV subtype A (Long strain; ATCC® VR-26™; American Type Culture Collection, Manassas, VA, USA) stock (3.74×10^7 plaque-forming units/mL) in 3 mL of virus growth medium (DMEM with antibiotics-antimycotics without serum). The flask was incubated at 37°C in a CO_2 incubator for 2 h. The flask was rocked in the North–South (N–S) and East–West (E–W) direction every 15 min to maintain an even virus distribution and avoid potentially drying the cells. After 2 h of incubation, 10 mL of the virus growth medium were added to stop virus adsorption. The flask was examined every day during post-infection incubation via an inverted microscope for cytopathic effects, namely syncytia formation, rounding and sloughing, to ensure the viral infection had taken place. After 5 days post-infection, the spent media was forcefully mixed 10 times with a pipette to free the infected, weakly attached cell monolayer from the flask and collected in a 50-mL Falcon tube. The pooled cells and supernatants were centrifuged at 280 × g, 4°C for 5 min and the supernatant was collected, leaving ∼200 μL of supernatant in the tube with the pelleted cells. The cell pellet was resuspended with the leftover 200 μL of supernatant and frozen immediately on dry ice, followed by quickly thawing in a 37°C water bath. This freeze-thaw step was repeated 3 times and the tube was agitated with a vortex mixer after each cycle. All the freeze-thawed cell debris was pooled with the saved supernatant, sterile glycerol was added at 15% (v/v) and mixed well with a vortex mixer. The virus suspension was pipetted into cryovials (300 μL/cryovial) and stored at −80°C for long-term storage.

Determination of Neutralization Titer of Samples Against RSV

The plaque reduction neutralization assay was performed with some modifications (19). Briefly, HEp-2 cells were seeded onto a 96-well plate at a density of 3.5×10^5 cells/mL in DMEM containing antibiotic-antimycotic solution (1%) and 10% FBS and grown in a CO_2 incubator until the cells reached >95% confluency. Frozen stock of human RSV (10(6.00)TCID[50]/0.1 mL, HEp2, 2 days; 7×10^6 plaque forming units/mL) was diluted 250-fold in DMEM containing antibiotic-antimycotic solution (1%) without FBS. An aliquot of the diluted virus (40 μL) was mixed with an equal volume of 1.2-fold serially diluted samples in DMEM without FBS (40 μL) in triplicate and pre-incubated for 1 h at 37°C in a CO_2 incubator. After washing the cell monolayer three times with DMEM containing antibiotic-antimycotic solution (1%) without FBS, sample-virus mixtures (25 μL/well) were added to the plate in duplicate wells (for a total of six wells per dilution). The plate was incubated at 37°C in a CO_2 incubator with shaking for 2 h, with intermittent manual rocking each direction (N–S and E–W) every 15 min for 1 min in a biosafety cabinet to enable non-neutralized virus to adsorb onto the cells. The virus-sample inoculum was aspirated, and 0.1 mL of overlay medium (1% methyl cellulose (Spectrum Chemical Manufacturing Corp., New Brunswick, NJ, USA) in DMEM containing antibiotic/antimycotic solution, without FBS) was added to each well and returned to the incubator. Methylcellulose fixed the virus in position to prevent RSV progeny spreading throughout the well and ensure localization of plaques. After 48 h of incubation at 37°C, the overlay was aspirated using a multichannel aspirator. The cells were fixed by adding 100 μL/well of ice-cold acetone:methanol (60:40) for 5 min and air-drying for 30 min. The non-specific sites on the cell monolayer surface were blocked by adding 100 μL/well of 3% skim milk (MilliporeSigma) for 10 min. The cell monolayer was washed three times with PBST. A drop of BLOXALL blocking solution (Vector Laboratories, Inc., Burlingame, CA, USA) was added to each well and incubated for 10 min to inhibit endogenous peroxidase, pseudoperoxidase, and alkaline phosphatase activities. The cell monolayer was washed three times with PBST. The cells were incubated with mouse anti-RSV F protein monoclonal antibodies (MilliporeSigma) at 1:1,500 in PBST (100 μL/well) for 2 h. The cell monolayer was washed three times with PBST and incubated for 1 h in a CO_2 incubator with alkaline phosphatase-conjugated goat anti-mouse IgG antibodies

(MilliporeSigma) at 1:1,500 dilution in PBST (100 μL/well). The cell monolayer was washed three times with PBST. Individual plaques were stained by adding 100 μL per well VECTOR Black alkaline phosphatase substrate (Vector Laboratories, Inc., Burlingame, CA, USA) followed by incubation at RT for 15 min to allow color development. The cell monolayer was washed with PBST. Images of the plate with plaques were recorded using a fluorescence microscope (model: BZ-X710; Keyence Corporation, Osaka, Japan), and plaques were counted manually using Fiji, an open-source image processing package based on ImageJ. Each plate had two wells without RSV (negative control). The sample dilution number for a reduction in 50% plaque neutralization compared with plaque formation in virus-only controls was referred to as 50% neutralization titer (NT_{50}), and it was interpolated from the four-parameter logistic curve drawn from % plaque reduction vs. sample dilution number using GraphPad Prism software (version 8.2.1). A separate NT_{50} value was determined for each of the three experimental replicates based on the average plaque count values of the duplicate wells. The percentage functionality loss of palivizumab at a digestion point was determined with respect to the unincubated sample in the *ex vivo* study, whereas it was determined with respect to feed in the *in vivo* study. Percentage functionality was determined for each of the three experimental replicates separately and these values were used for statistical analyses.

Statistical Analysis

All data passed the Shapiro-Wilk normality test. Unpaired *t*-tests were performed for the *ex vivo* study to evaluate significant differences between the percentage survival of palivizumab relative to time 0 based on ELISA and the RSV neutralization assay at $P < 0.05$ for each infant separately based on measurement replicates (values from at least three dilutions measured in triplicate for ELISA and three independently calculated NT_{50} values based on duplicate wells for the plaque assay). One-way analysis of variance (ANOVA) followed by Tukey Honestly Significant Difference *post-hoc* tests were conducted in the *in vivo* study to evaluate significant differences between the mean percentage survival of palivizumab relative to feed based on ELISA and the RSV neutralization assay at $P < 0.05$ for each infant separately based on measurement replicates (values from at least two dilutions measured in triplicate for ELISA and three independently calculated NT_{50} values based on duplicate wells for the plaque assay). A two-tailed Pearson's correlation test was performed to determine the correlations between percentage palivizumab stability as measured by ELISA and NT_{50} values from the RSV-neutralization assay across gastric and intestinal *ex vivo* and *in vivo* digestion. GraphPad Prism software (version 8.2.1) was used for statistical analyses.

RESULTS

Survival of Palivizumab After *ex vivo* Digestion

To study palivizumab survival across simulated infant digestion, the binding activity of palivizumab was determined via an anti-idiotype ELISA and the functional neutralizing capacity via the RSV plaque-reduction neutralization test after 1 h incubation in human milk, gastric, and intestinal digestates (*ex vivo* digestion).

For Infant 1, palivizumab concentration in formula remained stable after 1 h of incubation as determined by ELISA (**Figure 2A**). Likewise, NT_{50} remained stable (**Figure 2B**). Following 1-h incubation of the infant's gastric sample, palivizumab concentration decreased 72.34% (**Figure 2A**) and NT_{50} decreased 57.87% (**Figure 2B**). After 1 h of incubation of the infant's intestinal sample, palivizumab concentration decreased 51.09% (**Figure 2A**) and NT_{50} decreased 58.47% (**Figure 2B**). The combined data demonstrate that the anti-idiotype binding capacity and neutralization capacity of palivizumab was degraded during *ex vivo* gastric and intestinal digestion in Infant 1 samples. For Infant 2, both palivizumab concentration and NT_{50} were stable after 1-h incubation in fortified mother's milk and the gastric sample (**Figures 2C,D**, respectively). After 1 h of incubation of the intestinal sample, palivizumab concentration decreased 26.74% (**Figure 2C**) and NT_{50} decreased 58.43% (**Figure 2D**).

The combined ELISA and neutralization assay results demonstrated that palivizumab was not digested after *ex vivo* incubation in either the formula or fortified mother's milk, was variably digested in the gastric samples from Infant 1 and Infant 2 and was digested in the intestinal samples from both infants.

Survival of Palivizumab Across *in vivo* Digestion

The extent to which orally-supplemented palivizumab's anti-idiotype binding capacity and RSV neutralization capacity decreased across infant digestion was examined (*in vivo* study). For Infant 3, fed 60 μg/mL of palivizumab in fortified mother's milk, palivizumab concentration was 36.39% lower in the gastric sample than in the feed (**Figure 3A**). The neutralization titer of palivizumab was stable during gastric digestion in Infant 3 (**Figure 3B**). In the intestinal sample from Infant 3, palivizumab concentration was 57.52% lower than in the feed and 21.13% lower than in the gastric sample (**Figure 3A**). Likewise, the NT_{50} in the intestinal sample was 36.13% lower than in the feed and 29.64% lower than in the gastric sample (**Figure 3B**). For Infant 4, fed 1,000 μg/mL palivizumab in unfortified mother's milk, palivizumab concentration and NT_{50} were stable in the gastric sample (**Figures 3C,D**, respectively). Palivizumab concentration in the intestinal sample was 57.49% lower than in the feed and 47.68% lower than in the gastric sample (**Figure 3C**). NT_{50} in the intestinal sample was 63.55% lower than in the feed and 65.00% lower than in the gastric sample (**Figure 3D**). Overall, the neutralization assay results demonstrated that palivizumab was not digested during gastric digestion, whereas it was digested during intestinal digestion of both Infant 3 and Infant 4.

We hypothesized that the functionality of palivizumab could be indicated by ELISA. A two-tailed Pearson's correlation test was performed to determine the correlations between palivizumab percentage stability as measured by ELISA and NT_{50} values from the RSV-neutralization assay across gastric and intestinal *ex vivo*

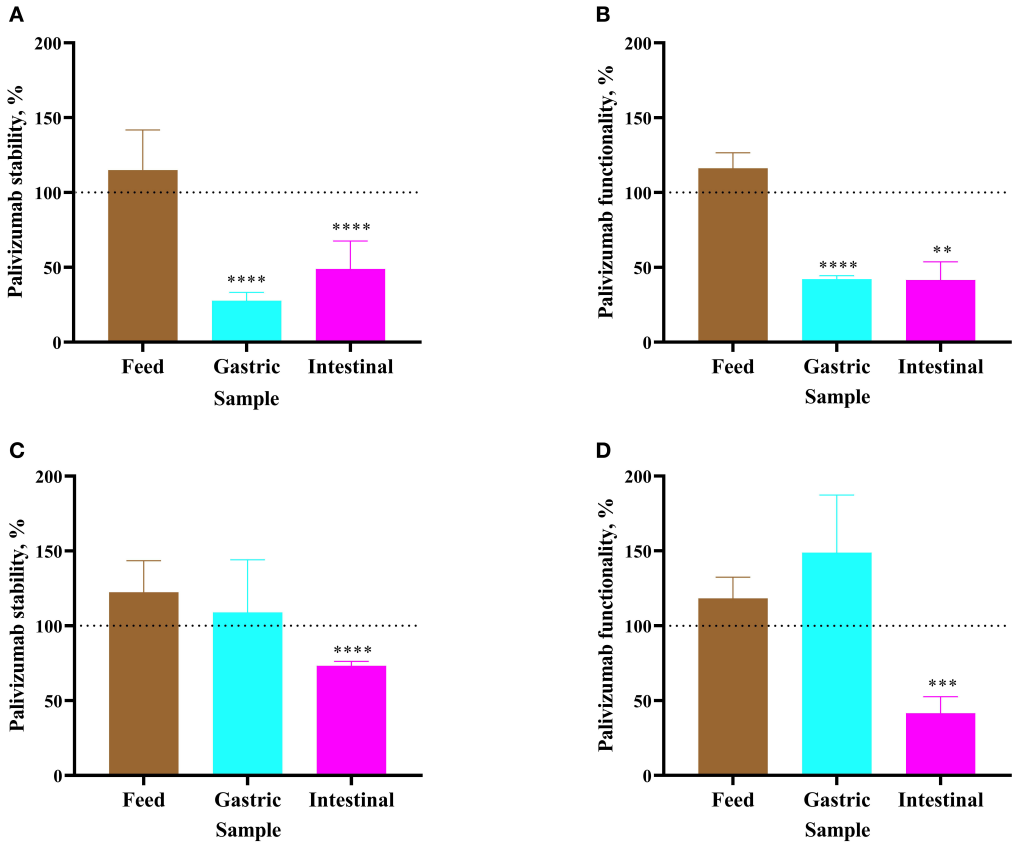

FIGURE 2 | Stability of palivizumab during a 1 h *ex vivo* digestion in feed (brown bars), gastric samples (cyan) and intestinal samples (magenta) in **(A)** Infant 1 and **(C)** Infant 2, respectively, tested by anti-idiotype ELISA and represented as percentage of the original palivizumab content. Stability of palivizumab neutralization capacity across *ex vivo* digestion in the sample from **(B)** Infant 1 and **(D)** Infant 2 based on NT_{50} and represented as a percentage of the original functionality. Values are mean ± SD, $n = 6$ and 3 dilutions for Infants 1 and 2, respectively, measured in triplicate for ELISA and $n = 3$ experimental replicates measured in duplicate for the RSV neutralization assay. Asterisks show statistically significant differences (**$P < 0.01$; ***$P < 0.001$; and ****$P < 0.0001$) between time 0 and 1 h of incubation within each sample type using unpaired *t*-tests. The broken line shows palivizumab stability in the anti-idiotype ELISA and palivizumab functionality in the RSV neutralization assay in feed (0 h), gastric (0 h), and intestinal (0 h) as 100%.

and *in vivo* digestion. These variables were highly correlated ($P < 0.0001$, $r = 0.87$).

DISCUSSION

Diarrhea causes more than half a million deaths each year among children under 5 years old, with most deaths occurring in resource-limited countries (20, 21). Infants are born with naive immune systems, including low levels of intestinal immunoglobulin secretion (22). Feeding infants human milk significantly decreases infectious diarrhea risk, likely in part because milk provides enteric pathogen-specific antibodies (5). Infants can be protected against enteric pathogen-induced diarrhea through fortification of milk or formula with enteric pathogen-specific antibodies. To be effective in preventing enteric pathogen infection, however, oral immunoglobulins need to survive intact after exposure to the digestive system's highly degradative environment, which varies from pH 3 to 8 and contains proteolytic enzymes (8, 23, 24). The extent to which recombinant immunoglobulins remain structurally intact and functional across infant digestion remains unknown. In our previous study (14), we demonstrated that palivizumab was degraded in *ex vivo* infant gastric and intestinal digestion as observed via an RSV F protein-specific ELISA. This result contrasted with the observation that naturally occurring human milk RSV-specific antibodies remained stable across *ex vivo* digestion. As that study did not test the extent to which observed degradation corresponds with loss of RSV neutralizing capacity (i.e., functionality), herein, we examined the survival of palivizumab across *ex vivo* and *in vivo* infant digestion via a plaque reduction neutralization test in addition to an anti-idiotype ELISA.

The anti-idiotype ELISA was selected as a means to determine the extent to which palivizumab remained intact through digestion. To be detected by ELISA, both the Fab and Fc regions of the antibody would have to be sufficiently structurally intact

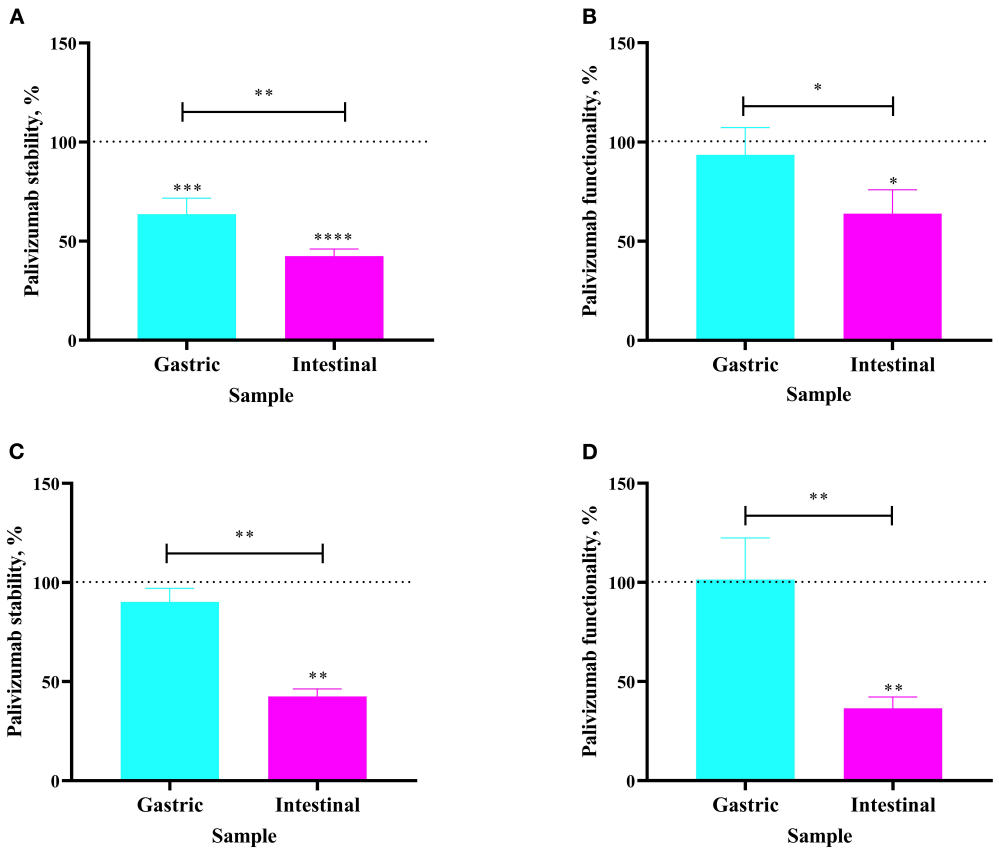

FIGURE 3 | Stability of palivizumab during *in vivo* digestion in **(A)** Infant 3 and **(C)** Infant 4 tested by anti-idiotype ELISA and represented as percentage of the original palivizumab content in feed compared with the gastric (cyan bars) and intestinal (magenta) samples. Stability of palivizumab functionality during digestion in **(B)** Infant 3 and **(D)** Infant 4 tested by plaque neutralization assay and represented as percentage of the palivizumab functionality in the feed sample. Values are mean ± SD, $n = 3$ and 2 dilutions for Infants 3 and 4, respectively, measured in triplicate for ELISA, and $n = 3$ experimental replicates measured in duplicate for the RSV neutralization assay. Asterisks show statistically significant differences (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, and ****$P < 0.0001$) using one-way ANOVA followed by Tukey's multiple comparison tests. The broken line shows palivizumab stability in the anti-idiotype ELISA and palivizumab functionality in the RSV neutralization assay in feed as 100%.

to bind to the anti-idiotype antibody and anti-IgG antibody, respectively. To confirm the extent to which the ELISA could serve as an indicator of palivizumab functionality, we tested the neutralization capacity of palivizumab via the plaque-reduction neutralization test. To be functional in this test, palivizumab must be structurally intact enough to bind to the F protein of RSV to prevent fusion with the host cell, thereby preventing infection.

The digestion of palivizumab was tested with *ex vivo* and *in vivo* approaches. By incubating palivizumab in clinically-collected feedings (formula, fortified mother's milk, and unfortified mother's milk), and gastric and intestinal contents from neonatal intensive care unit patients, we provided conditions highly similar to those of *in vivo* digestion, including the correct concentration of enzymes. This approach more optimally mimics *in vivo* digestion than the typical *in vitro* digestion system (25, 26). Although *ex vivo* digestion overcomes some limitations of *in vitro* methods, it is a static simulation and cannot entirely replicate the dynamic complexity of human digestion. In this study, we therefore also examined *in vivo* digestion of palivizumab in infants.

Palivizumab was degraded across both gastrointestinal digestion *ex vivo* and *in vivo* as determined by ELISA and the plaque-reduction neutralization test. This loss of binding and neutralization capacity indicates that the *ex vivo* and *in vivo* gastrointestinal environments altered palivizumab structure and/or resulted in proteolytic degradation. This observed antibody degradation could result from proteolytic degradation by digestive enzymes encountered during gastrointestinal digestion and/or structural destabilization by the shift from a low gastric pH to a high intestinal pH.

The percentage stability of palivizumab based on the concentrations from the anti-idiotype ELISA and the NT_{50} values from the plaque assay were highly correlated. This correlation indicated that in future experiments, the ELISA method alone can be used as a marker of the functional activity of an antibody across digestion. This finding is an essential discovery on a level of practicality for further implementations of this research in that

the ELISA method has a much higher throughput than does the RSV-neutralization assay.

A limitation of this study is the small number of infants sampled for *ex vivo* and *in vivo* digestive analysis. Though this limitation precludes analysis of the biological variation among infants, the four subjects sampled allows a clear answer to our primary research question: to what extent does a recombinant antibody survive functionally intact in the infant digestive tract. The results from both the *ex vivo* and *in vivo* analysis clearly demonstrate that the infant digestive tract degrades the functional capacity of palivizumab. Likewise, the limited numbers do not allow analysis of the effect of feed type on palivizumab digestion. However, as each infant tested herein was fed a different type of feed, we have encompassed the range of potential variability from this factor within our overall result, that palivizumab is partially functionally degraded across infant digestion.

The partial degradation of functional activities of the recombinant monoclonal antibody palivizumab against RSV suggests that use of recombinant IgG for oral supplementation to prevent enteric pathogens will require either a high degree of antibody dosing to compensate for losses during digestion, antibody encapsulation strategies, or antibody structural changes to enhance antibody stability. Future work should examine the extent to which such approaches can improve the functional survival of recombinant antibodies across infant digestion.

Pathogen-specific recombinant antibodies could have a wide array of applications within the food industry. As an example, enterally-dosed antibodies could be used either to protect against foodborne illness or to modulate the intestinal microbiome. The analytical strategies established herein would be desirable to examine the potential survival and hence, functional capacity of any such antibodies administered with foods.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board of Oregon Health & Sciences University (OHSU IRB #18274). Written informed consent to participate in this study was provided by the participants' legal guardian/ next of kin.

AUTHOR CONTRIBUTIONS

BSa performed ELISA and plaque reduction neutralization assay. BSc led sample feeding and collection. BSa, JL, BK, BH, YW, AO, MA, AO'C, BSc, MP, and DD designed the study and drafted the manuscript. BSa, BSc, and DD had primary responsibility for the final content. All authors contributed to the article and approved the submitted version.

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Administration of Extensive Hydrolysates From Caseins and *Lactobacillus rhamnosus* GG Probiotic Does Not Prevent Cow's Milk Proteins Allergy in a Mouse Model

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Background: Early nutrition may influence the development of food allergies later in life. In the absence of breastfeeding, hydrolysates from cow's milk proteins (CMP) were indicated as a prevention strategy in at risk infants, but their proof of effectiveness in clinical and pre-clinical studies is still insufficient. Thanks to a validated mouse model, we then assessed specific and nonspecific preventive effects of administration of extensive hydrolysates from caseins (eHC) on the development of food allergy to CMP. The additional nonspecific effect of the probiotic *Lactobacillus GG* (LGG), commonly used in infant formula, was also assessed.

Methods: Groups of young BALB/cByJ female mice were pretreated by repeated gavage either with PBS (control mice), or with PBS solution containing non-hydrolyzed milk protein isolate (MPI), eHC or eHC+LGG (eq. of 10 mg of protein/gavage). All mice were then experimentally sensitized to CMP by gavage with whole CM mixed with the Th2 mucosal adjuvant *Cholera toxin*. All mice were further chronically exposed to cow's milk. A group of mice was kept naïve. Sensitization to both caseins and to the non-related whey protein β-lactoglobulin (BLG) was evaluated by measuring specific antibodies in plasma and specific *ex vivo* Th2/Th1/Th17 cytokine secretion. Elicitation of the allergic reaction was assessed by measuring mMCP1 in plasma obtained after oral food challenge (OFC) with CMP. Th/Treg cell frequencies in gut-associated lymphoid tissue and spleen were analyzed by flow cytometry at the end of the protocol. Robust statistical procedure combining non-supervised and supervised multivariate analyses and univariate analyses, was conducted to reveal any effect of the pretreatments.

Results: PBS pretreated mice were efficiently sensitized and demonstrated elicitation of allergic reaction after OFC, whereas mice pretreated with MPI were durably protected from allergy to CMP. eHC+/-LGG pretreatments had no protective effect on sensitization

to casein (specific) or BLG (non-specific), nor on CMP-induced allergic reactions. Surprisingly, eHC+LGG mice demonstrated significantly enhanced humoral and cellular immune responses after sensitization with CMP. Only some subtle changes were evidenced by flow cytometry.

Conclusion: Neither specific nor nonspecific preventive effects of administration of casein-derived peptides on the development of CMP food allergy were evidenced in our experimental setup. Further studies should be conducted to delineate the mechanisms involved in the immunostimulatory potential of LGG and to clarify its significance in clinical use.

Keywords: food allergy, prevention, hydrolyzed formulas, probiotic, cow's milk, mouse model

INTRODUCTION

Type of feeding in early life may determine the propensity to develop a food allergy later in life. One of the main food allergies in infancy is a cow's milk proteins (CMP) allergy, which affects 0.5 to 3% of children in the first year of life (1). It may be severe, persistent and have lifelong implications for health (1, 2). In most allergic children, CMP allergies can be managed using formula based on extensive hydrolysates from whey (eHW) or from caseins (eHC). Those hydrolysates contain CMP-derived small peptides with no more IgE-binding epitopes, thus preventing any elicitation of an allergic reaction in allergic infants. In clinical use, eHC formula allowed for a higher rate of tolerance acquisition to CMP compared to soya or amino acids formula (3). This effect may result from the fact that eHC still contains a large proportion of small peptides derived from caseins that may act as tolerogenic specific T-cell epitopes, or that may display non-specific immunoregulatory properties. Actually, some peptides derived from caseins possess different biological effects, such as anti-inflammatory properties (4), healing of intestinal damages, at least *in vitro* (5), and anti-microbial and immunoregulatory effects [review in (6) and (7)]. Moreover, supplementation of eHC with the probiotic *Lactobacillus rhamnosus* GG (LGG) significantly improved the observed tolerance in clinic (3, 8) and limited other allergic manifestations for up to 3 years when compared to eHC alone (9). The non-specific additional effect of LGG may result from various mechanisms, either direct (e.g., immunoregulation) or indirect (e.g., modification of microbiota composition and function, both important for intestinal barrier integrity) (10).

On the other side, the use of infant formula based on CMP hydrolysates as a diet for allergy primary prevention is a matter of high interest and debate. In the absence of breastfeeding, the use of partial or extensive hydrolysates of CMP was indicated in at-risk infants to prevent allergic sensitization to CMP and to limit the start of the "atopic march." In this selected population, administration in the first 4 months of life of eHC or of partial hydrolysates from whey (pHW) decreased eczema incidence in the first 10 years of life when compared to standard CM formula or eHW. However, no effect on asthma or rhinitis, nor on sensitization to foods or aeroallergens, was observed (11, 12). Other interventional studies (13) or meta-analysis (14) did not support beneficial effects of CMP hydrolysates in at risk infants. A recent population-based study even demonstrated that the use of pHF at 2 months was related to higher risk of food allergy at 2 years of age, both in at risk and non-at risk infants (15). Further research on the impact of early nutrition practices using such formula for food allergy prevention is thus still of major importance in order to provide relevant and scientifically based preventive policies.

Animal models can enable the studying of the impact of postnatal nutrition on the immune responses. Two Th2-biased strains of female mice, namely C3H/HeOuJ [e.g., (16–19)] and BALB/c [e.g., (17, 20–24)], are mainly used to more specifically study food allergy and (early) oral tolerance induction, and their underlying mechanisms. In this context, by using the female BALB/c mouse model, we previously demonstrated that oral administration of the whey protein β-lactoglobulin (BLG) led to a specific tolerance that relies on the induction of regulatory T cells (Treg), and which prevents any further sensitization to this purified cow's milk allergen (23, 25). Large peptides generated from BLG were still efficient to induce tolerance to BLG, whereas products derived from extensive hydrolysis with trypsin, leading to small peptides probably lacking T cell epitopes, were no more tolerogenic. Using an experimental model of allergy to whole CMP, we further evidenced a lower tolerogenic potential of partial hydrolysates from caseins compared to a non-hydrolyzed CMP formula (26). The tolerogenic effect was restricted to the protein source used to produce the hydrolysates, which suggests an antigenic specificity of the induced tolerance. Conversely, others have demonstrated that eHC allowed a partial prevention of allergy in a mouse model of sensitization to BLG (27), which may then rely on non-specific immunomodulatory potency of caseins-derived peptides.

In the present study, we then aimed to assess the effect of administration of eHC on a further experimental sensitization

Abbreviations: BLG, bovine β-lactoglobulin; Cas, caseins; CMP, cow's milk proteins; CT, Cholera toxin; eHC, extensive hydrolysates from caseins; eHW, extensive hydrolysates from whey; HCPC, Principal Component Analysis and Hierarchic Classification on Principal Components; LGG, *Lactobacillus rhamnosus* GG; LP, lamina propria; MLN, mesenteric lymph nodes; mMCP1, mouse mast cell protease 1; MPI, non-hydrolyzed milk proteins isolate; OFC, oral food challenge; PCA, principal component analysis; pHW, partial hydrolysates from whey; PLS-DA, partial least square – discriminant analysis; VIP, variable important in projection.

to CMP, which has never been reported. We evaluated the effect of eHC administration on sensitization to both caseins and whey proteins (BLG) in order to delineate specific from non-specific effects of caseins-derived peptides, respectively, with the nonspecific effect being the mechanism of action suggested by the outcome of clinical CMP allergy studies. We also assessed the additional non-specific effect of the probiotic LGG, a probiotic largely used in infant's formulas.

MATERIALS AND METHODS

Tested Materials

Non-hydrolyzed CMP (Milk protein isolate, MPI; 88% protein, containing both caseins and whey proteins), extensive hydrolysate from caseins (eHC, 85% of equivalent protein); and LGG were provided by Mead Johnson Nutrition (Evansville, IN, United States). eHC corresponds to the one found in Nutramigen formula; eHC peptide length distribution, full MS-based peptidomics description and batch-to-batch variation analysis are described in (28). Commercial whole CM (UHT, Auchan^{TM}, France; 33 mg/ml of proteins) was used for experimental sensitization. For oral food challenge (OFC), commercial ultra-filtrated raw CM (Marguerite^{TM}, Candia, Lyon, France) was defatted (20 min, 400 *g*, +4°C) and freeze dried to increase protein concentration. Dry powder was solubilized in water and CMP concentration adjusted at 80 mg/ml (OFC solution; BCA kit, Pierce, Thermo Scientific, Waltham, United States).

Protocol of Tolerance Induction and CMP Sensitization in Mice

Ethical Considerations

All animal experiments were performed according to the European Community rules of animal care, and with specific Ethical approval from French Minister (authorization #16589 – A17034).

Mice

Females BALB/cByJ mice (3 weeks old, Centre d'Elevage René Janvier, Le Genest Saint-Isle, France) were housed in filtered cages under normal SPF husbandry conditions and received a standard diet (LASQCdiet® Rod16-R, Genobios, Laval, France; 16.9% of proteins) deprived of animal proteins, in which no BLG was detected using specific immunoassays (29). Mice were acclimated for 2 weeks before experimentation. Three days before starting the experiments, mice were randomly allocated to cages corresponding to experimental groups (3–8 mice/cage; see below) and individually identified by ear tattooing. No difference in mean weights was observed between groups (not shown).

Administrations and Samplings

The schedule of the experimental protocol is provided **Figure 1**. Mice received one intra-gastric gavage per day (200 µl/gavage) on days 1, 2, 3, 4 and 8, 9, 10, and 11 with either phosphate buffer saline (PBS, positive control of sensitization), a PBS solution containing eHC, a PBS solution containing eHC plus LGG (10^8 CFU/100 g, similar to ratio in Nutramigen LGG formulation), or a PBS solution containing MPI. Ten mg of CMP were administered by gavage in eHC+/-LGG and MPI groups, corresponding to 1–2% of the total protein intake provided by the standard diet, which was considered as negligible. Administrations were performed following doses and protocol that favor oral tolerance induction (26), using an animal feeding needle (Popper & Sons, New Hyde Park, NY, United States).

After these pretreatments, all mice were submitted to a protocol of experimental sensitization to cow's milk proteins (CMP, i.e., to both caseins and whey proteins), which consisted of repeated administrations of 180 µl of whole CM (eq. to 6 mg proteins/gavage) mixed with 20 µl of the Th2 mucosal adjuvant *Cholera Toxin* (10 µg/mice; Sigma Aldrich, St. Louis, United States) (20). Administrations were performed once a week, for 6 weeks (i.e., on days 15, 22, 29, 36, 43 and 50). On day 56, a first OFC was performed with 20 mg of CMP, and plasma was obtained 3 h later to assess antibodies and mouse mast cell protease-1 (mMCP-1) concentrations (see below). Additional gavages with CM (200 µl) were performed on days 60, 70, 80, and 90 to assess the persistence of any tolerogenic effects upon a chronic exposure. A second OFC was performed on day 95, and plasma collected as previously. One week after, two additional gavages with 200 µl of CM were performed (days 103 and 105). On day 106, mice were finally sacrificed and spleen, mesenteric lymph nodes (MLN) and small intestine were collected in PBS-Glucose (1 g/l) to analyze cellular responses. The group of naïve mice only received the OFCs. All collected samples (plasma, organs) were identified and treated individually.

Experimental Groups

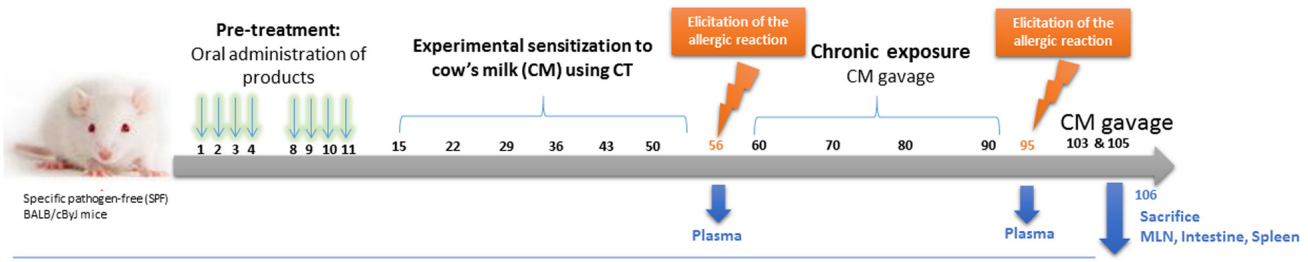
Two separate protocols were conducted (T1: eHC; T2: eHC+LGG) (**Table 1**). For each protocol, two independent experiments (A and B) were performed in parallel, 2 to 3 weeks apart, to assess the reproducibility of any observed effects. In each protocol, 16 mice received PBS (positive control of sensitization), 10 mice received eHC (T1) or eHC+LGG (T2), and 5 mice received MPI as pretreatment. In parallel, six mice were kept naïve (neither pre-treated nor experimentally sensitized to CMP).

Analysis of the Humoral Response

BLG- and caseins (Cas)-specific IgE, IgG1, and IgG2a antibodies were assayed as previously described using allergen-coated microtiter plates (26, 30). For IgG1 and IgG2a, standard curves were performed on each assay plate using mixes of purified and standardized BLG- or Cas-specific monoclonal antibodies produced and characterized in the lab. Results are then provided as ng/mL. For specific IgE, serial dilution of a pool of hyper-immune plasma was used as a standard on each assay plate. Results are then provided as "Arbitrary Units."

Elicitation of the Allergic Reaction

Mouse mast cell protease 1 was assessed as a marker of the elicitation of an immediate intestinal allergic reaction, using commercial kit (Mouse mMCP-1 ELISA Ready-SET-Go!,



Sensitization	BLG & cas-specific IgE, IgG1, IgG2a	X	X
Elicitation of the allergic reaction	mMCP1	X	X
Cellular response	Cytometry : GALT (MLN, lamina propria), Spleen (before & after ex vivo reactivation)		X
	Cytokine assay : Spleen supernatants after specific ex vivo reactivation		X

FIGURE 1 | Experimental schedule.

TABLE 1 | Protocols and subgroups.

Protocol	Sub-groups	Gavage pre-treatment	Experimental sensitization	Number of mice
T1	A	PBS	Cow's milk + CT	8
		eHC	Cow's milk + CT	5
		MPI	Cow's milk + CT	5
		Naive	PBS	3
	B	PBS	Cow's milk + CT	8
		eHC	Cow's milk + CT	5
		Naive	PBS	3
T2	A	PBS	Cow's milk + CT	8
		eHC+LGG	Cow's milk + CT	5
		MPI	Cow's milk + CT	5
		Naive	PBS	3
	B	PBS	Cow's milk + CT	8
		eHC+LGG	Cow's milk + CT	5
		Naive	PBS	3

*Detailed protocol is provided **Figure 1**.*

Affymetrix, eBioscience, San Diego, CA, United States) following the provider's recommendations. No clinical symptoms were evidenced in BALB/c mice when performing sensitization with cholera toxin and an OFC with 20 mg of CMP.

Analysis of Cellular Responses

Extraction and Reactivation of Spleen Cells

After mechanical dilaceration of the spleen (Gentle MACs dissociator, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), red blood cells were lysed (Red Blood cell Lysis Buffer, Sigma). Splenocytes were then washed and finally suspended in RPMI-10 (RPMI supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 U penicillin, 100 µg/ml streptomycin; all from GIBCO®, Thermo Fisher Scientific, Waltham, United States). After numeration and assessment of viability using 7-amino-actinomycin D (7-AAD, Life technologies, Carlsbad, United States), cell concentrations were adjusted. Part of cells were used for T helper (Th) and regulatory T (Treg) cells labeling (see below). Other spleen cells were labeled with CFSE (CFSE Cell Division Tracker Kit, Biolegend, San Diego, United States) following the provider's recommendation. Cells were then dispatched in 96-well culture plates (10^6 cells/well), and purified BLG or Cas [(31); final concentration 20 µg/ml] were added to activate specific memory T cells. Purified proteins were pre-incubated with polymyxin (Sigma-Aldrich, final concentration 50 µg/ml) in order to neutralize any LPS contamination. Efficiency of neutralization was confirmed by the fact that neither cell proliferation nor cytokine secretion was evidenced in spleen cell from naïve mice cultured with BLG or Cas. Concanavalin A (1 µg/ml) was used as a positive control of activation, and RPMI-10 as a negative control (not shown). After incubation for 60 h at 37°C (5% CO2) and centrifugation (300 *g*, 10 min, +4°C), the supernatants were collected and stored at −80°C, and cells were collected for Treg/Th cell staining (see below). IL-5, IL-13, IL-10, IFNγ, and IL-17 cytokines were assayed by multiplexed assays on undiluted supernatants using apparatus and commercial kits from BioRad

(BioPlex200®, BioRad, Marnes-la-Coquette, France), following the provider's recommendations.

Cell Extraction From MLN and Lamina Propria

Cell suspension was obtained from MLN after manual dissociation on a cell strainer (70 μm; BD, Le Pont de Claix, France). Small intestine was collected and flushed with 10 ml of PBS. After Peyer's patches removal, cells were extracted from lamina propria (LP) by successive incubations in HBSS, 2 mM EDTA, 10 mM HEPES, and extracellular matrix digestion (RPMI, 10 mM HEPES, 25 μg/ml Liberase (Roche, Sigma; 0.13 WU), 10 U/ml DNAse I). Numeration and viability were assessed by flow cytometry using 7-AAD, and cell concentrations adjusted in PBS, 1 mM EDTA, 2% FCS for staining.

Cell Staining

5×10^5 cells were stained for Th or Treg using the following anti-mouse antibodies (all from BioLegend, except when specified). *Treg:* PE anti-Foxp3, PerCP/Cy5.5 anti-Helios, PE/Cy7 anti-CCR9, AlexaFluor647 anti-CD39, APC/Fire750 anti-CD45, BV421 anti-LAP, BV510 anti-CD4, BV605 anti-CTLA4, and BV785 anti-CD25. *Th:* PE anti-Foxp3, APC anti-RORγt (eBioscience), PE/Cy7 anti-CCR9, BV421 anti-GATA3, BV605 anti-Tbet, APC/Fire750 anti-CD45, BV510 anti-CD4, and BV785 anti-CD3. All antibodies were first titrated for optimal dilution (0.1–2 μg/ml for 10^6 cells). FcR were blocked using anti-CD16/anti-CD32 (2.4G2, BD Pharmingen, Le Pont de Claix, France), and cells were incubated with antibodies for extracellular labeling for 30 min at +4°C. After washing, cells were fixed and permeabilized (True-Nuclear Transcription factor buffer set kit, Biolegend). After a new incubation with anti-CD16/CD32 antibodies, intracellular staining (Foxp3, Tbet, RORγt, GATA-3, and Helios) was performed for 45 min at +4°C. Compensations were performed using beads (UltraComp eBeads; Life technologies) stained with the same antibodies.

All acquisitions were performed on a Novocyte 13-colors flow cytometer (ACEA Bioscience, Inc., San Diego, CA, United States). Analysis was performed through FlowJo® v10 (FlowJo LLC, Ashland, OR, United States). We first combined analysis of extracellular markers (CD45, CD3, CD4, and CCR9 for intestinal homing) to that of transcription factors (T-bet, GATA-3, RORγt, and Foxp3) to have an overview of Th and Treg cells induced in the intestine. For a more in-depth analysis of Treg cells, we also analyzed Foxp3, Helios, LAP, CTLA-4, CCR9, and/or CD39 expression within $CD4^+CD45^+$ gated cells. $Helios^-Foxp3^+$ cells were defined as "iTreg" (Treg induced in periphery against exogenous antigen) and $Foxp3^-LAP^+$ cells as "Th3" cells (32).

Statistical Analysis

Assessment of Data Homogeneity for a Same Pretreatment Between Subgroups and Protocols

For mice receiving the same pretreatment, homogeneity of data obtained in the two protocols (PBS and MPI) and/or in the different sub-groups (i.e., eHC, eHC+LGG) was checked for each analyzed variable (i.e., all humoral and cellular data, mMCP1 concentrations) [Rcmdr package and "coin" plugin, script for reiteration of oneway_test and adjustment for multiple testing using false discovery rate (fdr), R software]. If no difference was evidenced between subgroups and/or between protocols for a given variable, all data corresponding to this variable were gathered by pretreatment. Conversely, data from protocols or sub-groups were analyzed separately if a significant difference was evidenced.

Thanks to this first analysis, we were able to gather all data obtained for a same pretreatment from the different subgroups and protocols for BLG- and Cas-specific IgE, IgG1, and IgG2a antibodies concentrations and mMCP1 concentrations. Conversely, we observed significant differences for cytokine concentrations for a same pretreatment between protocols and between subgroups. We then expressed each cytokine as a percentage, with PBS pretreated mice taken as an internal reference within each subgroup (100%). Once expressed this way, no statistically significant difference was evidenced for a same pretreatment between protocols and/or subgroups, allowing corresponding data to be gathered. All these gathered data (specific antibodies and cytokines concentrations, mMCP1 concentrations) were then aggregated to perform multivariate analysis (see below), and classical univariate analysis.

For cytometry analysis, a higher heterogeneity was observed between the experiments. We gathered data or had to analyze the data protocol per protocol, or even subgroup per subgroup, depending on the population or organ considered (see section "Results"). Data from cytometry were then analyzed independently from other data using univariate analysis (see below).

Multivariate Analysis

Firstly, we performed a descriptive analysis through a principal component analysis (PCA) of all the aggregated data (antibodies, cytokines and mMCP1 concentrations) obtained from each individual to have an overview of all the individuals, to identify potential outliers (none identified), and to assess the variables which are the most explicative of the whole dataset. Non-supervised clustering was also tested (Hierarchic Classification on Principal Components, HCPC; R software, FactoMineR plugin); HCPC gathers the individuals that are closer when considering all the variables, without any *a priori*: if pretreatments have no effect, individuals will then be homogeneously shared into the different clusters, which is assessed via a chi-square test.

Then, we modeled all the aggregated data (antibodies, cytokines and mMCP1 concentrations) using supervised Partial Least Square-Discriminant Analysis (PLS-DA®, XLSTAT software, Addinsoft, Paris, France), with pretreatment identified as the explicative variable (PBS, eHC; eHC+LGG or MPI). If such a model is successfully constructed, that means that it is possible to classify the mice depending on the pretreatment they received thanks to the analyzed components, and then that each pretreatment may have a specific effect. Such a model will then allow identifying the "discriminant variables", that is to say the set of components that mainly participated in the model construction and then that mainly supported the differences between the groups. Those components are identified thanks to

model-calculated variable important in projection values (VIP), and are selected as showing VIP ± SD > 1.

Univariate Analysis

For a given variable, all groups were compared to all others using pairwise comparison (permutation *t*-test with false discovery rate (fdr) adjustment; R software, RVAideMemoire package). When specified, we also compared all the groups to the PBS group only (non-parametric Kruskal–Wallis and Dunn's post-test, GraphPad Software, San Diego, CA, United States). A $p < 0.05$ value was considered significant. A trend was noticed for *p*-value $0.05 < p < 0.1$.

RESULTS

Sensitization and Elicitation of the Allergic Reaction to CMP in Pretreated Mice

Comparable results were obtained after the sensitization (day 56; **Figure 1**: specific antibodies and mMCP1 concentrations) and after the chronic exposure (specific antibodies and cytokine secretion, mMCP1 concentrations). For clarity, only the later results will be presented in the following.

Multivariate Analysis of the Humoral and Cellular (Cytokines) Parameters

We first performed a descriptive non-supervised analysis (PCA) of the seventeen variables obtained from each individual and that we can gather after the second OFC (BLG and Cas-specific IgE, IgG1 and IgG2a antibodies concentrations, BLG and Cas-specific IL-5, IL-13, IL-10, IL-17, and IFNγ secretions, mMCP1 concentrations; **Supplementary Figure S1**). This analysis highlighted that BLG and Cas-induced IL-5, IL-13, and IFNγ and Cas-induced IL-10 secretions were highly correlated together and are the main contributors of first dimension of PCA, that explained 38.9% of the total variance of the whole dataset. BLG and Cas-specific IgE and IgG1 antibodies, and mMCP1 are the main contributors of the second PCA dimension (16% of total variance). Conversely, BLG- and Cas-specific IgG2a, and BLG-specific IL-10 supported few information, as shown by their low-length vectors in the PCA. Non-supervised HCPC already evidenced a pretreatment effect ($p = 0.0035$), with classification of eHC+LGG mice in a separate cluster (not shown).

Data modeling using supervised analysis (PLS-DA) of the 17 variables led to the construction of a 2-components model with low predictive values (R^2X cum = 0.516, R^2Y cum = 0.171). Actually, only PBS and eHC+LGG pre-treated mice were correctly classified, in two separate groups. This suggests that these mice are not comparable for the global information provided by the 17 variables analyzed. Conversely, eHC mice were classified in the same group as PBS mice, suggesting that PBS and eHC mice are comparable for the global information provided by the 17 variables. BLG and Cas-specific IL-5 and IL-13, anti-BLG IgG1, mMCP-1, and Cas-specific IL-10 were identified as the discriminant variables of the PLS-DA (VIP ± SD > 1; **Supplementary Table S1**), i.e., as the variables that mainly supported the differences identified between the groups.

Univariate Analysis of the Humoral and Cellular (Cytokines) Parameters

In parallel, we performed univariate analysis and graphically represented the data to visualize differences between groups. Anti-BLG and anti-Cas IgE and IgG1 antibodies were significantly induced in PBS-pretreated and CMP-sensitized mice compared to naïve mice (**Figures 2A–D**), which was associated with significant secretion of Th2 cytokines (IL-5 and IL-13) upon BLG and Cas *ex vivo* stimulation, and with significant secretion of Th1 (IFNγ), Th17 (IL-17) and regulatory (IL-10) cytokines, mainly upon Cas re-stimulation (**Figures 3A–I**). In line with this high sensitization status of PBS-pretreated mice, OFC induced a significant increase of mMCP1 concentrations in plasma (**Figure 4**), traducing the elicitation of an allergic reaction in these mice. Conversely, gavage with non-hydrolyzed CMP (MPI pretreatment group) significantly prevented CMP allergy, as evidenced by decrease of specific IgE and IgG1 concentrations (**Figures 2A–D**) and prevention of the elicitation of the allergic reaction (**Figure 4**) compared to PBS-pretreated mice. This was associated with absence of Th2 and IL-10 cytokines secretion, although low but significant secretions of IFNγ and IL-17 were still observed (**Figure 3**).

In line with multivariate analysis, PBS and eHC-pretreated mice were comparable for all the analyzed parameters, i.e., BLG and Cas-specific antibodies (**Figure 2**) and cytokines (**Figure 3**), and mMCP1 release after OFC (**Figure 4**). CMP allergy was also significantly induced in eHC+LGG pretreated mice. However, eHC+LGG pretreated mice had significantly higher BLG-specific IgG1 antibodies concentrations compared to all other groups (**Figure 2C**). A significant/trend increase of anti-BLG ($p = 0.03$) and anti-Cas ($p = 0.1$) IgE antibodies concentrations was also observed in eHC+LGG pretreated mice when comparing all groups to the PBS one. BLG and Cas-induced IL-5, IL-13, IFNγ, and IL-10 secretions were also significantly increased in eHC+LGG compared to PBS and (for some) to eHC pretreated mice (**Figure 3**).

Analysis of Th and Treg Cells in Gut Associated Lymphoid Tissue (GALT) and in Spleen

No significant difference was observed in Th and Treg cell subpopulations frequencies analyzed in the MLN or spleen at sacrifice (not shown).

Lamina Propria

A trend in increased frequency of $ROR\gamma t^+Foxp3^+$ cells was noticed in LP from eHC pretreated mice ($p = 0.09$ versus PBS, MPI and eHC+LGG mice; FDR-adjusted value from pairwise permutation test; not shown). Conversely, a reproducible significant decrease of $CCR9^+CD39^+$ cells within $CD4^+Foxp3^+$ Treg cells in LP from eHC compared to PBS pretreated mice was observed (intra-protocol analysis, not shown). In parallel, a trend in increased frequency of CCR9+Th2 cells was observed

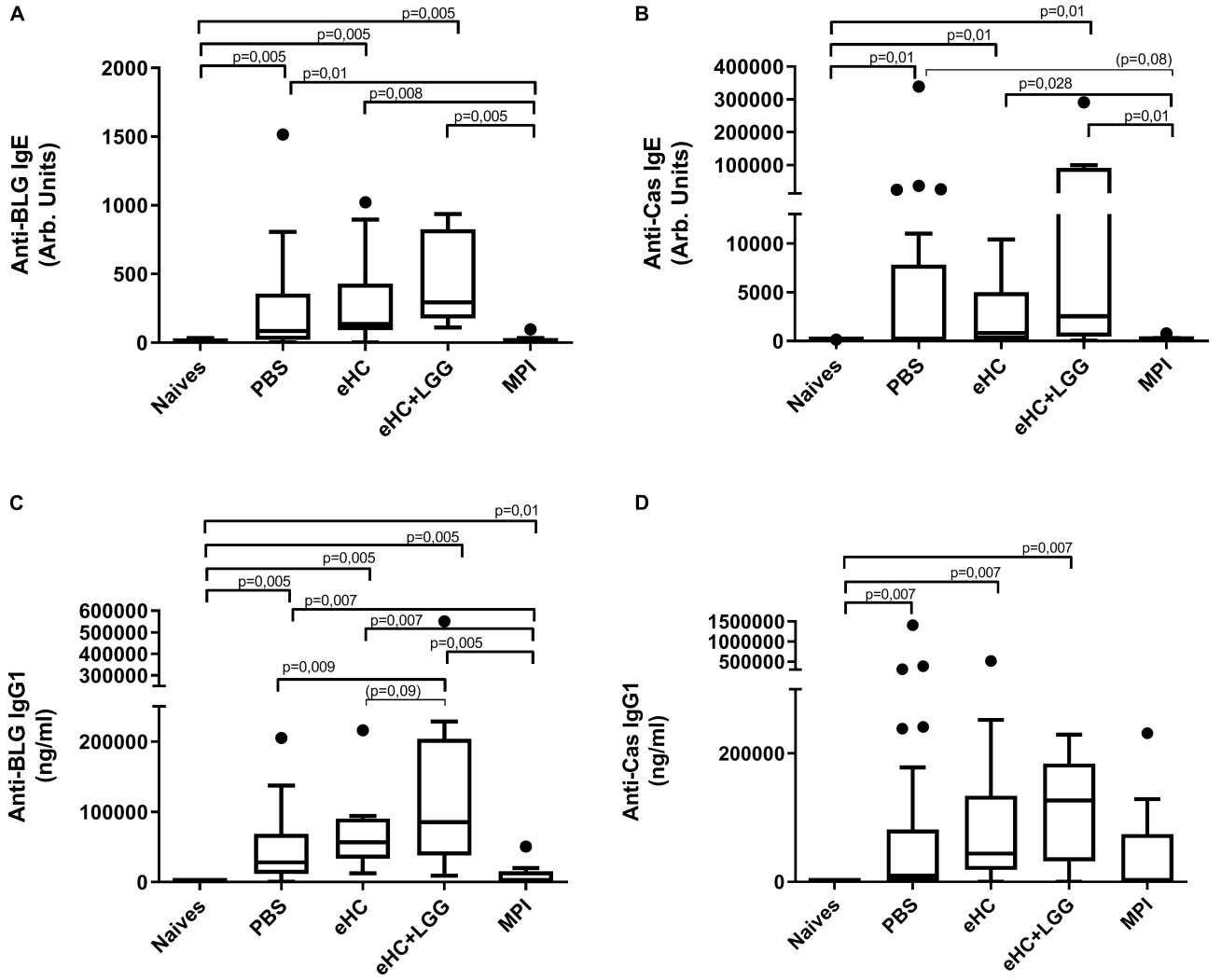


FIGURE 2 | Anti-BLG and anti-Cas IgE [**(A,B)**, respectively] and IgG1 [**(C,D)**, respectively] antibodies concentrations in mice receiving gavage with PBS (n = 32), eHC (n = 10), eHC+LGG (n = 10), or MPI (n = 10) before the oral sensitization to cow's milk proteins. Naïve mice (n = 12) were not treated nor sensitized, but were challenged. Blood samples were obtained after the chronic exposure to CM and 3 h after a second OFC (day 95). Medians (bars) with box and Tukey whiskers are shown for each treatment group. All groups were compared to each other using pairwise comparison and permutation t-test; corresponding fdr-adjusted p-values are indicated. Trend ($0.05 < p < 0.1$) and associated p-value are indicated into brackets.

in LP from eHC+LGG pretreated mice compared to other pretreated groups (**Figure 5**), in line with the higher sensitization status of these mice.

Spleen Cells After *ex vivo* Reactivation

Analysis of splenocytes after specific *ex vivo* stimulation showed a comparable percentage of proliferating cells ($CFSE^{low}$) within $CD45^+CD4^+$ cells in CMP sensitized mice (not shown). The percentage of $CD4^+ROR\gamma t^+$ Th17 cells significantly increased in the eHC group after BLG and/or caseins *ex vivo* stimulation (**Figures 6A,B**). We also observed an increased frequency of $CD4^+GATA3^+$ Th2 cells in eHC mice compared to PBS mice after BLG *ex vivo* stimulation, which was associated with a decrease of $CD4^+Foxp3^+$ frequency (intra-protocol analysis; not shown). No significant change was noticed in eHC+LGG group.

DISCUSSION

The aim of the present study was to assess the effect of administration of an extensive hydrolysate from caseins (eHC), supplemented or not with LGG probiotic, on the further experimental induction of CMP allergy. Thanks to a validated mouse model of CMP allergies, both specific and non-specific effects of casein-derived peptides were assessed.

We evidenced that, as expected, a CMP allergy is efficiently induced in PBS-pretreated and CMP-sensitized mice, as shown by high specific IgE and IgG1 antibody concentrations, high specific Th2 cytokine secretion and high mMCP1 concentrations after OFCs. Conversely, gavage with non-hydrolyzed CMP (here MPI) efficiently prevent further induction of CMP allergy. No protective effect of eHC+/-LGG on the sensitization to casein

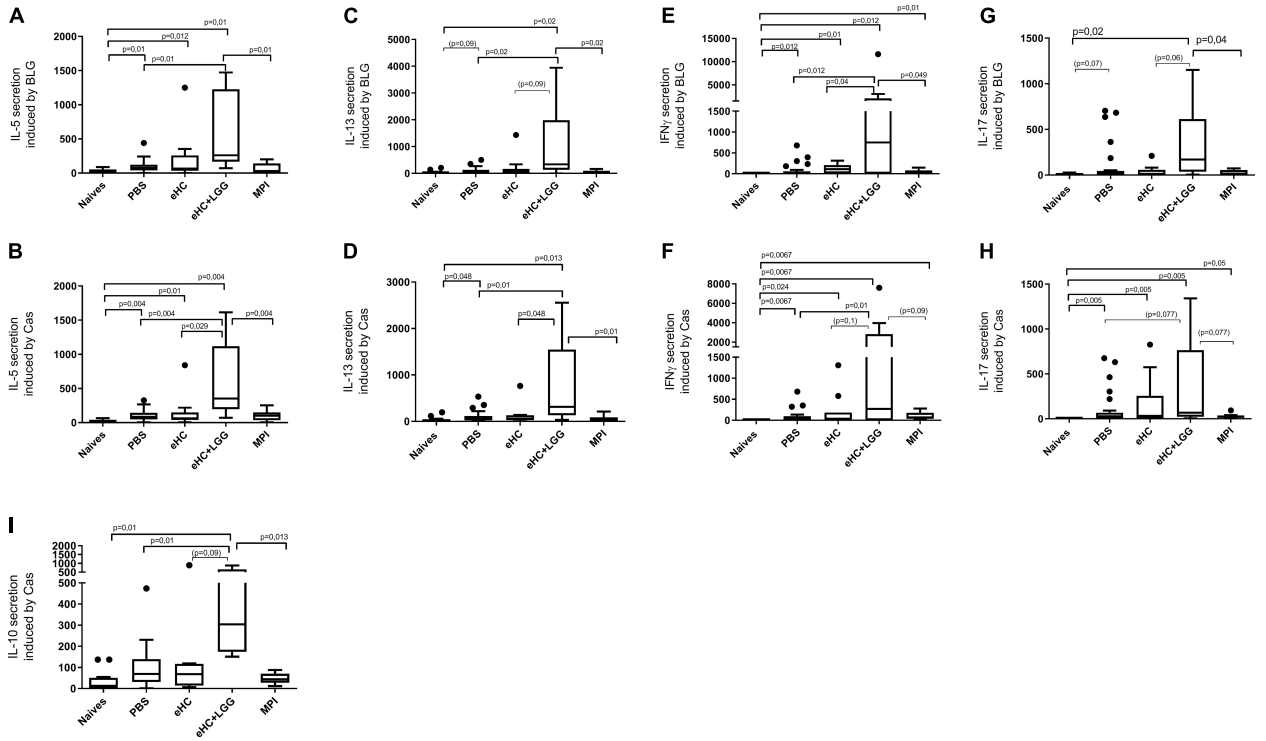


FIGURE 3 | BLG and Cas-induced secretion of IL-5 [**(A,B)**, respectively], IL-13 [**(C,D)**, respectively] IFNγ [**(E,F)**, respectively], and IL-17 [**(G,H)**, respectively], and Cas-induced secretion of IL-10 **(I)** in mice that received gavage with PBS (n = 32), eHC (n = 10), eHC+LGG (n = 10), or MPI (n = 10) before the oral sensitization and then chronic exposure to CM. Naïve mice (n = 12) were not treated nor sensitized. Cytokines were assayed in supernatants obtained from individual spleen cells stimulated *ex vivo* with purified BLG or Caseins. Results are expressed as percentage of secreted cytokines using PBS group as an internal reference within each sub-groups (100%). Medians (bars) with box and Tukey whiskers are shown for each treatment group. All groups were compared using pairwise comparison and permutation t-test; corresponding adjusted p-values are indicated. Trend ($0.05 < p < 0.1$) and associated p-value are indicated into brackets.

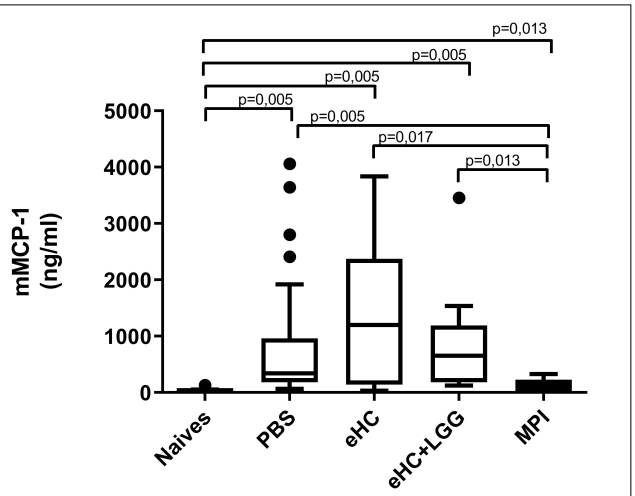


FIGURE 4 | mMCP1 concentrations in plasma from mice pretreated with PBS (n = 32), eHC (n = 10), eHC+LGG (n = 10), or MPI (n = 10) before the oral sensitization, then chronically exposed to CM. mMCP1 was assessed 3 h after an OFC with 20 mg of CMP. Naïve mice (n = 12) were not treated nor sensitized, but were challenged. Medians (bars) with box and Tukey whiskers are shown for each treatment group. All groups were compared to each other using pairwise comparison and permutation t-test; corresponding fdr-adjusted p-values are indicated.

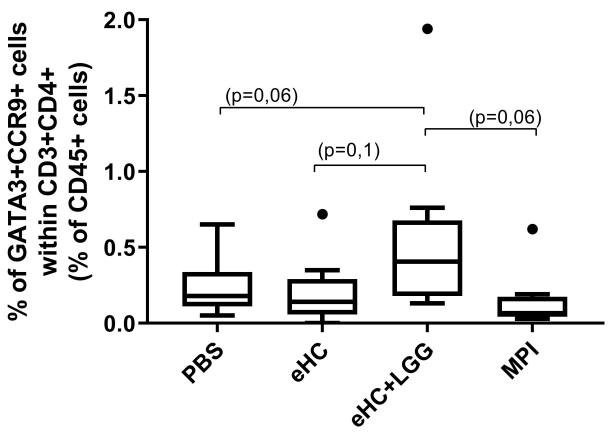


FIGURE 5 | Percentage of Th2 CCR9+ cells in LP from mice of the different treatment groups. Aggregated data from the two protocols are shown. A first gate was designed based on structural parameters (FSC and SSC), and then single cells were selected (FSC-A × FSC-H). Within single cells, $CD45^{+}FSC^{low}$ cells were gated, in which we selected $CD3^{+}CD4^{+}$ T cells. Within these cells, intestinal Th2 cells were identified thanks to the co-expression of transcription factor GATA3 and homing receptor CCR9. Medians (bars) with box and Tukey whiskers are shown for each treatment group. All groups were compared to each other using pairwise comparison and permutation t-test; corresponding adjusted p-values are indicated.

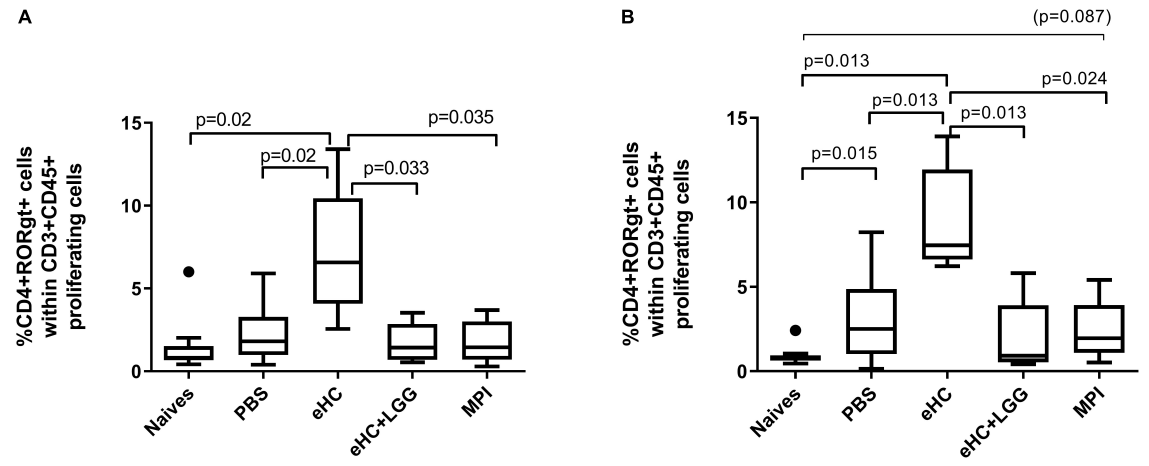


FIGURE 6 | Percentage of CD4+RORγt+ cells within proliferating cells after specific *ex vivo* reactivation with BLG **(A)** or Caseins **(B)**. A first gate was designed based on structural parameters, and then single cells were selected. Within single cells, $CFSE^{low}$ cells (i.e., proliferating cells) were gated and then analyzed for CD3 and CD45 expression. Within $CD3^{+}CD45^{+}$ proliferating cells, co-expression of CD4 and transcription factors RORγt was assessed. Comparable results were obtained when selecting first $CD45^{+}CFSE^{low}$ within single cells, then gating $CD3^{+}CD4^{+}$ cells and analyzing expression of transcription factors within this latter population. Medians (bars) with box and Tukey whiskers are shown for each treatment group. All groups were compared to each other using pairwise comparison and permutation *t*-test; corresponding adjusted *p*-values are indicated. Trend ($0.05 < p < 0.1$) and associated *p*-value are indicated into brackets.

could be evidenced, nor on sensitization to other non-related CMP (here the whey protein BLG), and no protection was provided on elicitation of the allergic reaction to CMP. Although eHC mice could not be distinguished from PBS groups for all analyzed parameters, eHC+LGG mice were characterized by enhanced humoral and cellular immune responses, both to caseins and BLG.

Firstly, we would like to point out that we uniquely analyzed our data through rigorous statistical procedures: (i) assessment of homogeneity of data between protocols and subgroups allowing (or not) to gather data and then to increase statistical power, (ii) descriptive analysis (PCA and HCPC) of gathered data further aggregated, in order to identify potential outliers within the individuals and the most contributive variables in the global response, but also to anticipate differences between groups, and (iii) supervised analysis to identify differences (or their absence) between groups and the variables supporting these differences. Univariate analysis (with correction for multiple testing) allowed comforting these results and visualizing the differences between groups. Such statistical procedure in experimental models may improve the quality, rationalization and robustness of *in vivo* studies that integrate several parameters on the same animal and that aim to compare different (pre)treatments.

Concerning the results obtained with eHC alone, our results are in line with, and extend previous results demonstrating the high and specific prevention potency of non-hydrolyzed CMP (here MPI), and the loss of efficiency of this preventive specific effect while the degree of hydrolysis increases (18, 23, 25, 26). In line with these results, a mix of four 18 amino-acid long synthetic peptides derived from BLG administered orally before oral sensitization to CMP did not prevent a local or systemic CMP allergy (33). In another model, eHW given for 3 weeks through the drinking water (∼180 mg of proteins/day) had no effect on epicutaneous sensitization to BLG, but an attenuation of anaphylaxis and activation of intestinal mast cells was observed after an OFC (34). We then cannot exclude that a longer pretreatment period and higher doses of eHC would have a significant effect on sensitization or elicitation to caseins in our experimental setup. However, 180 mg of whey protein for a 20 g mouse is equivalent to 54 g of protein for an infant of 6 kg. As infant formulas contain 1.3–1.4 g of protein per 100 ml, the quantity of formula ingested by the baby would be 3.8–4.1 L/day.

Alternatively, Aitoro et al. (27) reported prevention from allergy to purified BLG by eHC administration, an effect that then results from non-specific bioactivity of peptides derived from caseins. Discrepancies between this later study and ours should not rely on eHC composition that demonstrated minor batch-to-batch variations (28). In Aitoro's study, eHC was administered through the drinking water as the sole source of food, and was compared to a standard solid diet. However, intervention and standard diets were not comparable for the protein load but also for nutrients such as dietary fibers, fatty acids, vitamin D and folic acids. Those components can critically affect the intestinal barrier, the immune system and the composition and function of the intestinal microbiota, all of which influence a further experimental allergic sensitization. Moreover, they pursued eHC administration during sensitization with BLG and cholera toxin (CT), whereas κ-casein derived glycomacropeptides have been described to inhibit binding of CT to its receptor, at least *in vitro* (35, 36). Glycomacropeptides is hydrolyzed in eHC, but some derived peptides (37) may still interfere with CT and then with the experimental sensitization to BLG. Such non-specific effects could not be evidenced in our experimental setup since eHC administration was not pursued during sensitization.

Considering the cellular responses, we observed a trend in increased frequency of $ROR\gamma t^{+}Foxp3^{+}$ cells in LP, and a significant increase of $ROR\gamma t^{+}$ and $GATA3^{+}$ cells among proliferating splenic cells from eHC pretreated mice. Although

these changes did not affect sensitization and elicitation parameters, further analysis in GALT focusing on these parameters just after the pre-treatment phase would be instructive. RORγt^{+}Foxp3^{+} cells are regulatory cells of importance in the intestine that participate in inflammation control and are induced for example by probiotic strain (38).

Our present study also revealed the strong immunostimulatory potential of LGG. We observed a significant increase of almost all immune parameters in eHC+LGG pretreated mice compared to PBS or eHC pretreated mice. It is worth noting that cellular response differences were mainly revealed through cytokine secretion: small differences were observed through deep cytometry analysis on GALT and spleen cells, even after *ex vivo* restimulation. This thus suggests that the activity (i.e., secretion capacity) rather than the increased frequency or proliferation of specific subpopulations is detectable in our experimental setting. Moreover, despite an increase of specific-antibodies concentrations in eHC+LGG pretreated mice, we did not evidence an increase of mMCP1 concentrations after the OFCs, which would require further investigations (e.g., comparison of mast cell density and FcγRI expression in intestine). Our results are then in contradiction with most of the studies available. For example, more significant preventive (and therapeutic) effects were reported when using eHC+LGG compared to eHC in the BLG-allergy model (27), in line with the clinical results obtained in CMP allergic patients (8). It is clear that the administration of LGG before sensitization (preventive strategy) will not have the same effect than administration of the same compounds in an already sensitized organism (therapeutic strategy). In the therapeutic schedule, Th1/Th17 induced response (as evidenced in our experiments by increased IFNγ and IL-17 secretion in eHC+LGG group) may rather counteract the on-going Th2 immune response, as observed in clinical trials (3, 8, 9). IL-10 induced in the eHC+LGG group may also play a more pronounced regulatory role in this context. But, in the preventive strategy, the time lapse between LGG and sensitizing administration may also be of importance. Actually, transient modification of the gut microbiota composition (unfortunately not assessed in our experiments) and the immune response potentially induced by LGG may amplify the adjuvant effect of CT, or on the contrary repress it, depending on the immune status at the exact moment CT is administered (i.e., "inflammation burst" versus "inflammation resolution"). Further studies combining non-hydrolyzed proteins [e.g., MPI or purified BLG (23, 26)] plus LGG intervention should be conducted to further assess the immunostimulatory effect of LGG and its effect on the induction of oral tolerance. Other probiotics strains should be tested as well in a comparable experimental model.

CONCLUSION

In conclusion, we could not evidence any preventive effect, either specific or non-specific, of administration of extensive hydrolysates from caseins on further experimental CMP allergy. The pre-clinical data we provide are in line with others, and a with recent population-based study that did not observe preventive effect of the use of pHF at 2 months on food allergy, both in at risk and non-at risk infants (15). Altogether, these results then further challenge the use of hydrolysates for allergy prevention. Unexpectedly, we also evidenced that co-administration of LGG with eHC enhanced the immune response induced against CMP. Our results do not challenge the efficiency of eHC supplemented with LGG as a therapeutic strategy for allergic infants evidenced in clinical trials (3, 8). However, and although our findings obtained in a mouse model cannot be translated directly to weaning neonate/infants, further studies in a preventive set up should be conducted to further analyze the effect of early nutritional intervention using LGG on food allergy development, independently of hydrolysates, to understand immune mechanisms involved, and to clarify their significance in clinical applications.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by French Minister (authorization #16589 – A17034).

AUTHOR CONTRIBUTIONS

KA-P designed the whole study, analyzed and interpreted the data, and wrote the manuscript. KA-P, MG, BG, HB, and AC performed the experiments. SH performed some experiments and critically revised the manuscript. CJ critically revised the manuscript. All authors approved the 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/fimmu.2020.01700/full#supplementary-material

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REVIEW
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The Impact of Milk and Its Components on Epigenetic Programming of Immune Function in Early Life and Beyond: Implications for Allergy and Asthma

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Specific and adequate nutrition during pregnancy and early life is an important factor in avoiding non-communicable diseases such as obesity, type 2 diabetes, cardiovascular disease, cancers, and chronic allergic diseases. Although epidemiologic and experimental studies have shown that nutrition is important at all stages of life, it is especially important in prenatal and the first few years of life. During the last decade, there has been a growing interest in the potential role of epigenetic mechanisms in the increasing health problems associated with allergic disease. Epigenetics involves several mechanisms including DNA methylation, histone modifications, and microRNAs which can modify the expression of genes. In this study, we focus on the effects of maternal nutrition during pregnancy, the effects of the bioactive components in human and bovine milk, and the environmental factors that can affect early life (i.e., farming, milk processing, and bacterial exposure), and which contribute to the epigenetic mechanisms underlying the persistent programming of immune functions and allergic diseases. This knowledge will help to improve approaches to nutrition in early life and help prevent allergies in the future.

Keywords: epigenetics, epigenetic imprinting, environmental factors, unprocessed (raw) milk, breastfeeding, allergy, nutritional programming, bioactive milk components

INTRODUCTION

There is increasing evidence to suggest that maternal diet during pregnancy, breastfeeding, early life nutrition, and early life malnutrition can have sustained effects on immunological outcomes, such as respiratory allergies, and metabolic outcomes such as type 2 diabetes and obesity. Nutritional programming during gestation might permanently affect the immunological competence and nutritional status in early life **Figure 1**. This is exemplified by the thrifty phenotype, where the metabolic response to undernutrition during the fetal period is inappropriate during overnutrition later in life, leading to disease manifestations (1). Several studies have since shown that prenatal exposure

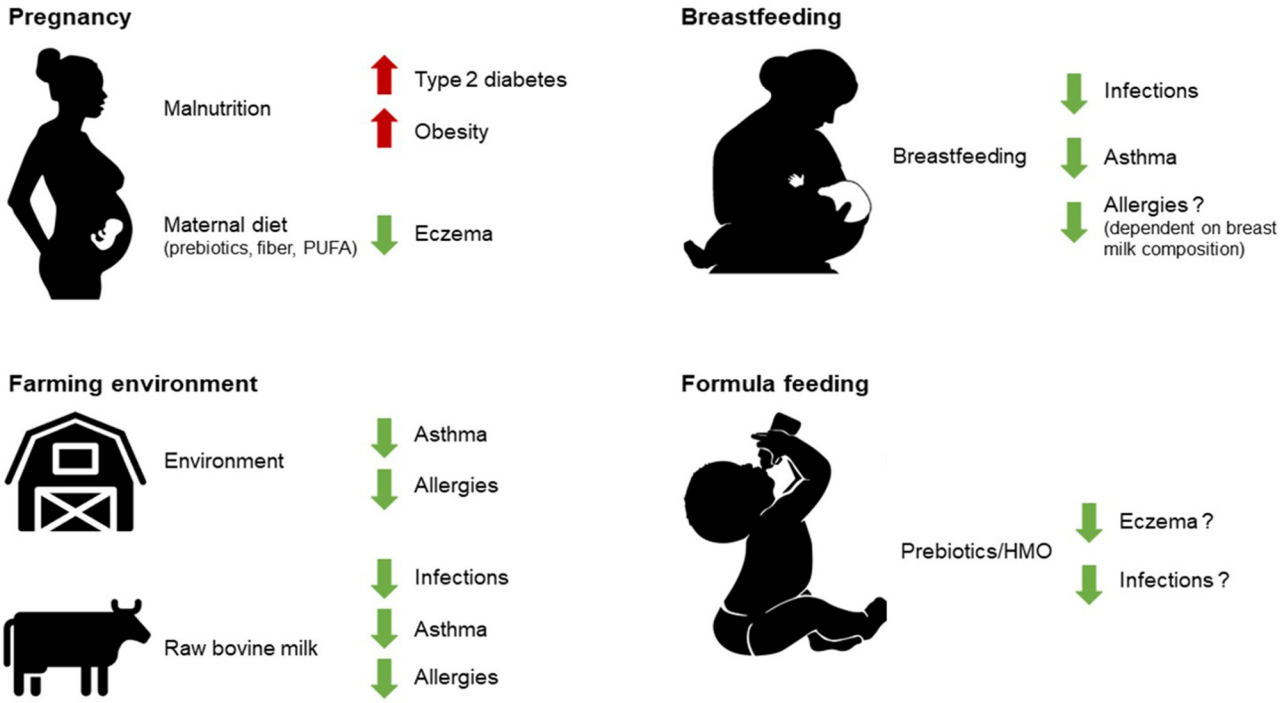


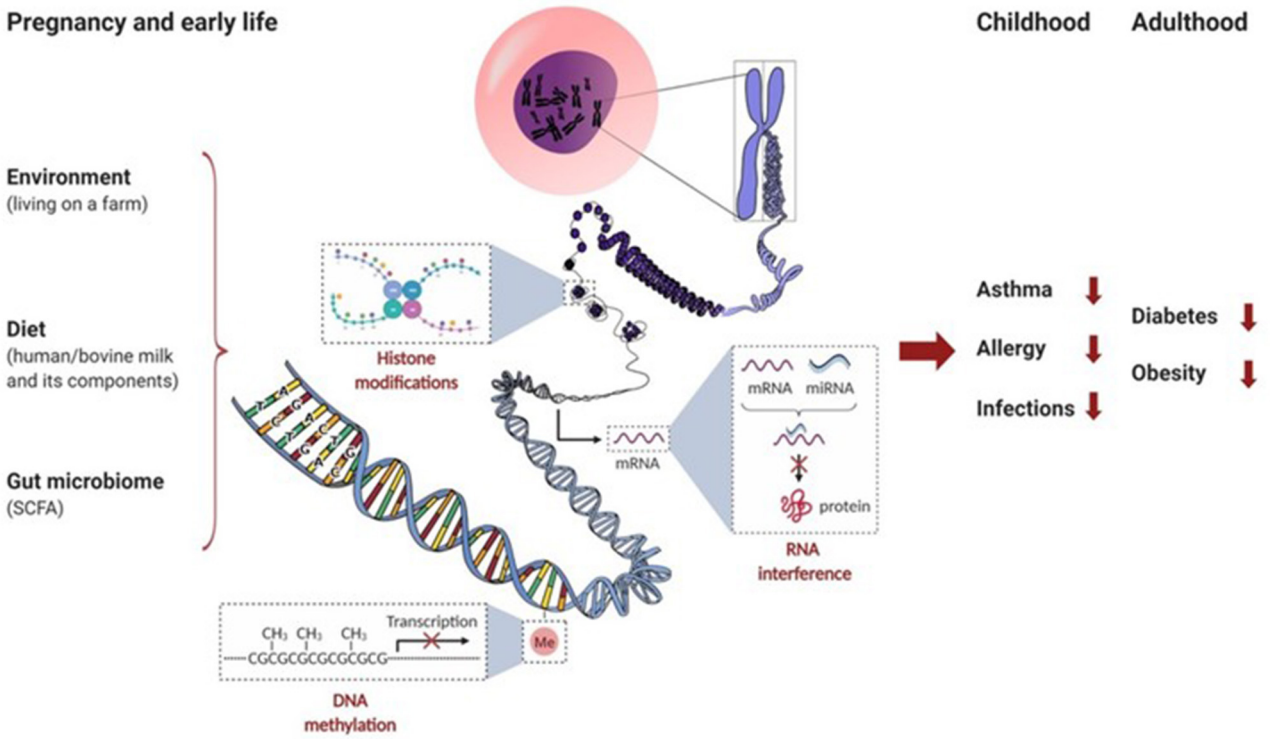
FIGURE 1 | As described in this review, early life nutrition (breastfeeding, raw milk consumption, and some infant formula components), early life environmental exposures (such as farming environment), as well as prenatal development under the influence of maternal diet can all have sustained effects on health outcomes later in life. PUFA, polyunsaturated fatty acids; HMO, human milk oligosaccharides.

to famine is associated with the development of type 2 diabetes later in life (2–4), and an epigenetic link was demonstrated in relation to the Dutch hunger winter where epigenetic modification of the IGF2 gene was shown to be linked to famine during prenatal development (5).

Epigenetic mechanisms may play an important role in these effects. It has even been suggested that early life nutrition forms the basis for susceptibility to a plethora of chronic age-related non-communicable diseases (NCD), like respiratory allergies (6–9). Thus, specific and adequate nutrition during pregnancy and early life are considered important factors that could reduce instances of allergic diseases. Epidemiologic and experimental studies show that nutrition is important for (immunological) health, especially when we are very young and during prenatal development, which may influence health and disease throughout our lives (6, 10). The structures of the mucosal immune system in the gastrointestinal (GI) tract are fully developed *in utero* by gestational week 28 (11). Increasing evidence suggests that maternal diet and other prenatal exposures can influence this development by crossing the placenta (12–14). In the first year of life, the mucosal immune system is further shaped by microbial colonization and oral feeding (15). Breastfeeding is the normal way of providing newborns with nutrients for healthy growth and development and a diet exclusively comprised of breastfeeding has various beneficial outcomes, such as reducing the risk of GI diseases, allergies, colitis, and respiratory infections (16). Besides conferring protection against these short-term outcomes, breastfeeding also reduces the long-term risks of developing diseases like type 2 diabetes and obesity (17). In analogy to breast milk, raw, unprocessed, bovine milk is a rich source of immunomodulatory components (18–20). Studies have indicated that it may protect against common respiratory infections in infants that consume unprocessed bovine milk (21). In addition, epidemiological evidence shows a clear association between the consumption of raw cow's milk and the prevention of allergy development (22–29). Epigenetic mechanisms that are regulated by many immune processes can thereby influence the course of allergic diseases.

Epigenetic mechanisms (**Box 1**) and transcription regulatory factors allow a flexible adaptation in the fetus. They neonate to a fluctuating external environment whereby heritable, non-DNA encoded, alterations in gene expression patterns occur. Especially relevant in early life, several factors drive the epigenetic changes that occur throughout life: environment (e.g., exposure to microbial components in inhaled dust), diet (e.g., components present in breast milk and bovine milk), and the GI microbiota and its metabolites (e.g., through the production of short-chain fatty acids [SCFA] after fermentation of dietary non-digestible oligosaccharides). Thus, environmental, dietary, and microbiota-derived epigenetic modifications during gestation and early life can shape future immunity to the development of diseases like obesity, type 2 diabetes, allergy, asthma, and infections. Most of our

BOX 1 | Epigenetic mechanisms.



Epigenetics refers to systems that control gene expression in a heritable fashion without changing the genomic sequences. The epigenome is much more flexible than the genome and shows different phenotype variations that are influenced by environmental factors and dietary habits. Epigenetic mechanisms include DNA methylation, histone modifications, and RNA interference by microRNAs (miRNAs) (See in this Box figure). Epigenetic mechanisms thus contribute to the regulation of gene expression at the level of transcription by DNA methylation and by modifying chromatin accessibility through posttranslational modifications of histones, and after transcription by mRNA silencing. These epigenetic mechanisms can regulate gene expression by modifying the accessibility of the DNA to transcription enzymes without altering the DNA nucleotide sequence, influencing stability of mRNA or translation efficiency, and others (30–32). The transfer of a methyl group onto DNA, performed by DNA methyltransferases (DNMTs), can directly regulate the rate of gene transcription. DNA demethylation is catalyzed by several enzymes serving as controllers for the equilibrium of DNA methylation (33). For example, methylation of DNA in the promoter regions of cytokines can influence immune responsiveness by steering Th cell differentiation into Th1, Th2, Th17, or Treg (34, 35). For more details see **Box 2**. In addition, histone modifications like acetylation, methylation, phosphorylation and others can also modulate the development and activity of immune cells. Histone acetylation is an important remodeling activity that is catalyzed by a series of enzymes called histone acetyltransferases (HATs). Acetylation is generally considered as a permissive activity that facilitates gene transcription. On the contrary, histone deacetylases (HDACs) reverse HAT activity and tighten up the folding of DNA around the histones and make them less accessible for transcription factors (31, 36). The interplay between HATs and HDACs determines the histone acetylation balance and regulates the gene expression (37, 38) and production of pro-inflammatory (IL-1β, IL-5, IL-6, IL-8, IL-12, and TNFα) and anti-inflammatory mediators (IL-10). Histone methyltransferases (HMTs) and demethylases (HDMs) serve as controller enzymes for the equilibrium of histone methylation (31). Finally, RNA interference can occur by small noncoding RNAs, most notably miRNAs that are found in biological fluids as well as in extracellular vesicles (e.g., in milk). MiRNAs represent short noncoding RNA molecules of 18 to 23 nucleotides that control gene expression by inducing mRNA degradation and/or inhibit post-transcriptional translation. As a result, specific miRNA can silence selective gene expression (32). For example, milk contains extracellular vesicles or exosomes that contain a wide range of microRNAs, including miR-21, miR-29b, miR-148a, and miR-155 that is known to influence Foxp3 expression and Treg development (39).

current knowledge on the environmental and dietary effects on epigenetics and early life immune function comes from epidemiological findings which indicate that children growing up on farms have a decreased risk of developing allergies, especially asthma. For this reason, we will focus this review on the effects of maternal nutrition during pregnancy, the effects of bioactive components in human and bovine milk, and the environmental factors in early life that can contribute to the epigenetic mechanisms involved in the course of allergic diseases.

EPIGENETIC REGULATION OF TH2 DEVELOPMENT IN ALLERGIC DISEASE

Epigenetic changes have been strongly associated with allergies and asthma and might thereby serve as biomarkers. The role of epigenetic mechanisms, particularly DNA methylation, in allergic diseases is at the interface of gene regulation, environmental stimuli, and developmental processes, thereby determining the pathogenesis of asthma and allergy. Alterations of the DNA methylation status in the genes specific for a different subset of T

helper (Th) cells that are considered to be a good example of how epigenetic modulation can influence the development of asthma and other allergic diseases.

The differentiation of naïve CD4+ T cells into Th subpopulations is strictly regulated, with changes in epigenetic marks at main lineage-determining loci encoding transcription factors like GATA3, RORγt, TBX21, and Foxp3 playing a pivotal role. These changes affect the differentiation into mature Th subpopulations, such as Th1, Th2 (and Th9), regulatory T cells (Treg cells), and Th17 (30, 35, 47, 48). In naïve CD4+ T cells, which express a moderate level of GATA3 mRNA after receiving signals via the T cell receptors (TCRs) in the presence of IL-4, activated STAT6 proteins bind to the GATA3 gene locus, driving Th2 differentiation, which is a characteristic in the development of allergy. Differentiation of human CD4+ cells into the Th2 subtype is accompanied by the induction of DNase I hypersensitive (DHS) sites and CpG demethylation around these (DHS) regions within the IL-4 and IL-13 promoters. Extensive studies of the Th2 cytokine locus control region have shown that specific sites undergo rapid demethylation during Th2 differentiation (49).

In addition to DNA methylation, histone modifications are also important in guiding T-cell differentiation. T-bet and GATA3 transcription factors control lineage-specific histone acetylation of IFN-γ and IL-4 loci during Th1/Th2 differentiation. Rapid methylation of H3K9 and H3K27 residues (repressive marks) at the IFN-γ locus was associated with differentiating toward Th1 cells, while demethylation of H3K9 and methylation of H3K27 was associated with Th2 differentiation (49). Epithelial alarmins (IL-25, IL-33, thymic stromal lymphopoietin [TSLP]) induce an inflammatory response in the respiratory mucosal membrane. IL-33 binds to its receptor ST2 on memory Th2 cells and induces epigenetic changes of the IL-5 gene, resulting in the generation of IL-5-producing Th2 cells (47). Thus, Th2 differentiation, which is characteristic of allergy, is triggered by phosphorylation of STAT6 signal transducers and expression of GATA3 and Th2 cytokines, including IL-4 (47).

Demethylation of the IL-4 promoter leads to allergic sensitization (48). Th1 differentiation is in turn triggered by phosphorylation of STAT4 signaling, and expression of the transcription factor T-bet and cytokine. For a more detailed description of epigenetics and T cell development, see **Box 2**. Asthmatic individuals show a lower histone deacetylase (HDAC): histone acetylase (HAT) ratio, i.e., a relative decrease of HDAC enzymes, which is corrected by proper anti-asthma treatment (50). The DNA methylation status of Foxp3 is regulated within a highly conserved region within the CpG-rich Treg-specific demethylated region with a differential Foxp3 demethylation status in children with an active cows milk allergy (CMA) and acquisition of immune tolerance (51).

EFFECTS OF EARLY LIFE NUTRITION ON ALLERGIC DISEASE

The WHO recommends exclusive breastfeeding for infants during the first 6 months of life, and that it should be given alongside complementary feeding up until children are 2 years old (52). If mothers are unable to breastfeed, many children receive early life nutrition alternatives that are based on bovine milk. Therefore, this section of the study is focused on breast milk, bovine milk, and their components.

Effects of Maternal Diet in Pregnancy and Breastfeeding on Allergic Disease

There is increasing evidence to suggest that the maternal diet during pregnancy and breastfeeding can have sustained effects on immunological outcomes in the infant and even have ramifications for their health later in life. The maternal diet can modify some immune supporting micronutrients in breast milk, such as the fat-soluble vitamins A and D, as well as the water-soluble B vitamins, and polyunsaturated fatty acids (PUFA), but maternal diet does not influence other components such as iron and zinc (53). Although there is some conflicting data, supplementation of maternal diet with vitamins and micronutrients during pregnancy and breastfeeding does not seem to prevent infections and allergies in offspring (54, 55).

Supplementation of Maternal Diet With PUFA

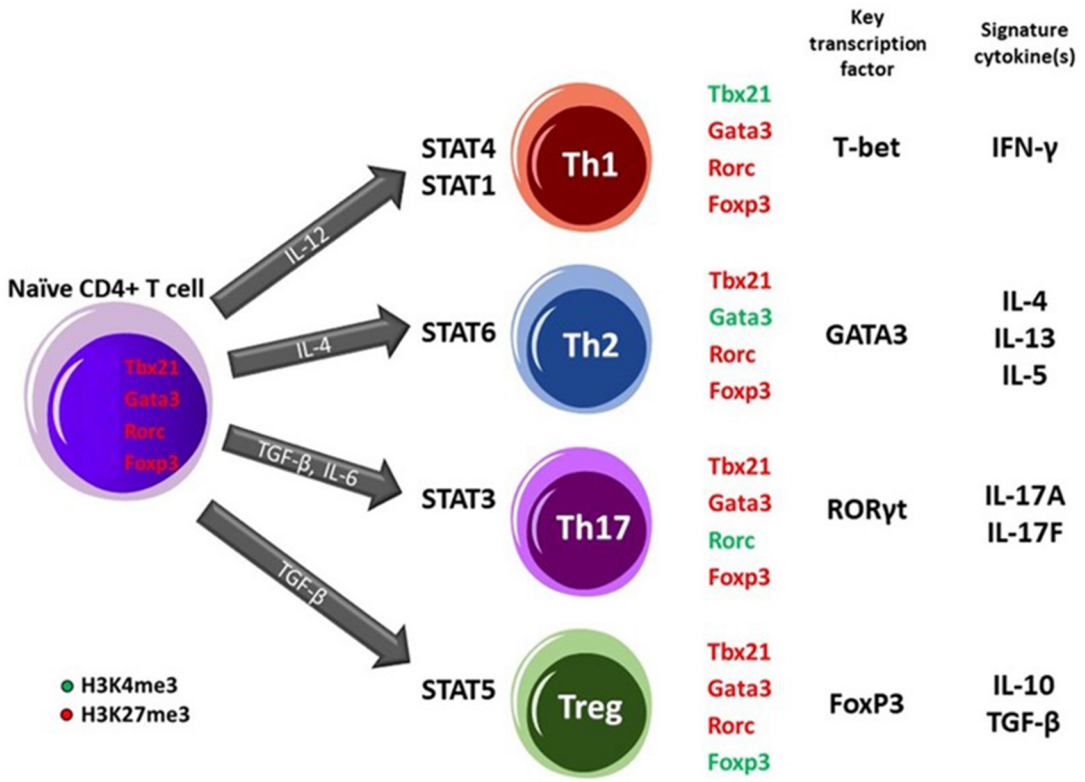
Long-chain PUFA (LCPUFA) induce inflammation by modulating inflammatory mediators like prostaglandins and immunomodulatory factors like IL-10 and TSLP (56). Consumption of omega-3 PUFA correlates with the inhibition of TLR4 signaling and thereby the production of inflammatory cytokines (IL-1, IL-6, and TNFα), which is reflected by a lower risk of allergies, whereas consumption of saturated fats and omega-6 PUFA, a potential trigger for TLR4-induced inflammation, has been associated with a higher risk of allergies. In addition, PUFA supplementation during pregnancy was associated with a reduction in allergic outcomes after birth (57, 58), but not when it was supplemented to infants (8, 59–61), suggesting that pregnancy is an important time that influences the development of the immune system.

Supplementation of Maternal Diet With Pre-/Probiotics

Probiotics are living microorganisms which, when administered in adequate amounts, confer a health benefit to the host. They generally exist of *Lactobacillus, Bifidobacterium,* or *Escherichia* species, which are commonly found in a normal microbiota. Prebiotics are mostly dietary fibers that are non-digestible food ingredients and beneficially affect the host's health by selectively stimulating the growth and/or activity of some genera of microorganisms in the colon, generally lactobacilli and bifidobacteria.

Intestinal microbiota strongly influence the maturation of the immune system (62) and particularly the development of immune tolerance, because they affect the Th1/Th2/Th17/Treg balance. The microbiota composition is modulated by dietary components that help shaping and timing of the composition of the early microbiome (63, 64). In addition, microbiota can be transmitted directly into the uterus during fetal development, passage through the birth canal or during cesarean-section, breastfeeding, and when providing care to the offspring (65, 66).

BOX 2 | Epigenetics and T-cell subset development.



The differentiation of naïve CD4+ T cells upon antigen exposure into effector T helper (Th) subsets (Th1, Th2, and Th17) or induced regulatory T (iTreg) cells relies on epigenetic regulation and the establishment of cell-fate programs (40, 41). DNA methylation and chromatin modifications at pivotal loci in Th cells such as IFN-γ, IL-4 and, Foxp3 contribute to the formation of stable, heritable gene expression patterns. Methylation of CpG dinucleotides specially at promoter or other regulatory regions of genes is generally considered a repressive feature causing silenced genes what mostly seen in (embryonic) stem cells. Targeted loci DNA demethylation is required during early or late hematopoietic cell differentiation (41, 42). For instance, DNA demethylation plays a role in the expression of Th2 cell-related cytokine, IL-4 (43) and, Treg cell-related regulators (44, 45). Besides DNA methylation, histone modifications including acetylation and methylation have a role in the development of Th cell lineage. Histone acetylation, associated with the control of gene expression by condensing or relaxing the chromatin structure to repress or activate transcription, respectively, regulates the expression of several inflammatory mediators of the immune system. In this regard, modifications of histones occur in the enhancer and promoter regions of the STAT4 and STAT1 transcription factor binding sites upstream of the IFN-γ and TBX21 (T-bet) gene to direct Th1 differentiation. In contrast, activation of STAT6 in response to IL-4 occurs leading to the expression of IL-4 and GATA3 transcription factor genes in Th2 differentiating cells. Driving naïve CD4+ T cells toward Th17 phenotype requires STAT3 activation followed by expression of RORC gene encoding RORγt transcription factor and subsequently the production of IL-17 cytokines. Alternatively, upon naïve CD4+ T cells exposure to TGF-β, STAT5 transcription factor engages leading to changes in Foxp3 gene promoter site and commitment of cells into Treg fate. These specific histone modifications lead to engagement of lineage-specific key transcription factors which ensures Th phenotype stabilization and prevents the cells from skewing toward alternative commitments (35, 42, 46).

Food supplements, which are often termed functional foods, have been used to alter, modify, and reinstate pre-existing intestinal microbiota (67). Supplementation of prebiotics, probiotics, and synbiotics (68–74), as well as PUFA (58, 69, 75–77) during pregnancy and breastfeeding, may reduce eczema in infants. This is further supported by preclinical studies, which indicated that supplementing the maternal diet with specific pre- or probiotics affects milk composition (78) and that supplementing non-digestible oligosaccharides diminished allergic disease in offspring (79–81). This may, in part, be linked to the production of SCFA by the intestinal microbiota (82–86). Even though maternal diet during pregnancy and breastfeeding can modulate the prevalence of allergy in the offspring, the potential role of breastfeeding in allergy prevention is still under discussion, as it seems to be linked to variations in breast milk composition rather than to breastfeeding *per se* (53, 87).

Effects of Consumption of Raw Milk and the Farming Environment

Most of our current knowledge on the effects of environment and diet on epigenetics and early life immune function is based on epidemiological findings, which indicate that children who grow up on farms have a decreased risk of developing allergies, especially asthma. Allergies are multifactorial, Th2-driven diseases that are triggered by gene-environment interactions. Environmental factors can interact with genes involved in asthma and allergy development via epigenetic mechanisms, such as DNA methylation and histone modifications. These epigenetic

mechanisms can regulate gene expression by modifying the accessibility of the DNA to transcription enzymes without altering the DNA nucleotide sequence (30, 33). In addition to the consumption of raw cow's milk (22–29), contact with livestock and animal feed along with other farm-related exposures have shown independent protective effects, indicating that a farm/country lifestyle can contributes to a reduced risk of asthma and allergies in children (25, 27, 88–90). Interestingly, the timing of these exposures seems to be crucial, with the strongest effects observed for exposures that occurred *in utero* and during the first year of life (23, 91, 92). Since the protective "farm effect" was demonstrated to sustain into adult life (25), effects might be mediated via epigenetic inheritance/regulation.

Several epigenome wide-association studies concerning allergies have been performed and reviewed (30). These studies showed that allergic disease is accompanied by changing DNA methylation patterns in Th2, Th1, Th17, Th9, and Treg subsets in the affected tissues. DNA methylation changes by demethylation and increased $FoxP3^+$ regulatory T cell numbers in peripheral blood mononuclear cells were shown in 4.5-year-old farm children (93). These regulatory T cell numbers were negatively associated with doctor-diagnosed asthma. It remains to be seen if these changes also precede the onset of allergic disease and can be predictive for allergy development, but questions remain as to how are these epigenetic changes induced. It has been suggested that the epigenome is affected by the farm environment. The first indication for a potential role of epigenetic regulation in the protective "farm effect" was provided by Slaats et al. who demonstrated that DNA methylation of the promoter region of CD14 in placentas of mothers living on farms was lower compared to mothers not living on a farm (94). These lower DNA methylation levels were reflected in higher CD14 mRNA expression levels (95). Interestingly, a higher expression of the CD14 gene was also observed in farmers' children (96). Prenatal farm exposure was also associated with increased gene expression of other innate immune receptors, such as TLR5, TLR7, TLR8, and TLR9, at birth (97, 98) and TLR2 and TLR4 in farm-raised children at school age (95, 96). Maternal exposure to farm environments increases the number of T regulatory (Treg) cells in the cord blood of infants, which is associated with decreased Th2 cytokines and may be linked to demethylation at the FOXp3 promoter (99). Whether epigenetic inheritance is underlying these effects requires further investigation. Further evidence that the farm environment affects the epigenome was provided by a pilot study which showed hypermethylation of genes related to IgE regulation and Th2 differentiation in cord blood from farmers' as compared to non-farmers' children (100). Interestingly, at least part of the protective effect triggered by those factors has been ascribed to the farm bacteria, for instance, *Acinetobacter lwoffii* (101, 102), with a pivotal contribution of downstream epigenetic mechanisms, specifically histone modifications (103).

Milk Components

Human milk contains a unique combination of lipids, proteins, carbohydrates, vitamins, and minerals and thereby provides an ideal source of nutrition for the healthy growth and development of a newborn (104). However, human milk is more than nutrition as it also contains bioactive components that can modulate the immune system, such as immunoglobulins, lactoferrin, human milk oligosaccharides (HMO), long-chain fatty acids, and anti-inflammatory cytokines (18, 105, 106). Most of the immunologically relevant components in breast milk are also found in bovine milk (18). Several key components of breast milk that are not present at high enough levels in bovine milk are added to infant formula to provide the crucial nutrients needed. These include prebiotics or even single HMO like 2'-fucosyllactose (as an alternative to the complex mixture of HMO in breast milk), lactoferrin, PUFA, vitamins, and minerals.

Non-digestible Milk Oligosaccharides

One of the major differences between human breast milk and bovine milk is the amount and diversity of the HMO, i.e., complex, non-digestible oligosaccharides (107, 108). The HMO in breast milk constitutes about 20% of the milk saccharides next to the major carbohydrate in milk, lactose. Human breast milk contains ∼5–15 mg/ml of these non-digestible HMO, consisting of up to 200 or more unique structures. In contrast, bovine milk only contains a few of these oligosaccharides, at much lower levels. One injected, HMO survive passage and digestion through the stomach and small intestine and reach the colon, where they are fermented into SCFA like acetate, butyrate, and propionate (107, 108). In addition, they shape the microbiota by selectively enhancing the growth of bifidobacteria and lactobacilli. These SCFAs serve as an energy source for colonic intestinal tissue and shape the interactions between the host and its gut microbiota. Furthermore, SCFA reduces intestinal pH, limit outgrowth of *Enterobacteriaceae*, and support intestinal barrier function. HMO is the key factor in shaping the development of immunity and early microbiota after birth. HMO have effects on microbiota and infections (107, 108). Of these, 2'-fucosyllactose is the HMO that is most abundantly present in breast milk and has therefore been chosen as the first HMO that was introduced in infant nutrition in 2018.

Prebiotics are non-digestible oligosaccharides like galacto-oligosaccharides (GOS) and fructo-oligosaccharides (FOS), and have widely been used in infant nutrition to mimic the bifidogenic- and SCFA-inducing effect of HMO. There is some evidence that prebiotic oligosaccharides in infant nutrition may prevent eczema in infants (109–112). It is not clear if these effects also extend to the prevention of other allergic diseases, as only one study to date has reported the effects of prebiotics on asthma and food allergy (113). For probiotics, effects are also seen when they are added in infant nutrition (68). As can be seen in detail in Lomax and Calder (114), several studies have reported that infant formula supplemented with prebiotics have a trend toward or even a significant preventive effect on the occurrence of gastrointestinal infections. Trends toward decreased fever episodes, antibiotic use, and upper respiratory tract infections (URTI) have been described. Two studies, by Bruzzese et al. and Arslanoglu et al. and performed with scGOS/lcFOS,

supplemented very young infants from early after birth for 6–12 months (115, 116). Both studies showed a significant reduction in gastroenteritis (115) and a reduction in the total number of infections (116). A study from Westerbeek et al., in which scGOS/lcFOS were combined with acidic oligosaccharides (pAOS) showed a non-significant tendency toward fewer serious infections (117). This study was, however, conducted over a shorter time period, and the infants were preterm. In two other studies infants were older than 6 months (118, 119) were supplemented with oligofructose, one did not show an effect on diarrhea, whilst the latter observed a protective effect against diarrhea. Since these components and their effects have been reviewed in detail previously, we will not address them in detail here, and will instead, only focus on their potential epigenetic and long-lasting immune health effects.

Bioactive Components Besides Non-digestible Oligosaccharides

Both human milk and bovine milk contain many other bioactive components that can modulate immune function [reviewed in (18, 19, 105–107)]. The components in human and in bovine milk that can be isolated in large quantities have largely been studied as separate entities, because they are potential infant nutrition ingredients. Several of these components, such as transforming growth factor-β (TGF-β) (120), bovine lactoferrin (121–124), bovine alkaline phosphatase (19, 125), bovine osteopontin (126, 127), and the milk fat globular membrane (MFGM) (128), as well as milk exosomes (39), have been linked to immunological outcomes with varying levels of evidence (infection, allergy). Another milk component that may have more sustained immunological effects are bovine IgG antibodies. Where IgA is the predominant immunoglobulin isotype in breast milk, bovine milk has a larger amount of IgG (129). Bovine milk IgG (bIgG) has been shown to bind to aeroallergens (130) as well as to respiratory pathogens such as respiratory syncytial virus (RSV), and can inhibit infection of human cells with human RSV (131). Through the formation of immune complexes, bIgG can enhance RSV-specific T cell responses (132). Similarly, bovine colostrum, which is a rich source of IgG can prevent the infection of mice with RSV (133). Different from adaptive immunity, innate immunity was until recently believed to lead to immune memory. However, vaccination studies have shown that after vaccination—that is associated with cross-protection to other pathogens—the innate immune response is increased to the vaccine, but also other pathogens (134, 135). The mechanism of this was elucidated in several mechanistic studies and was shown to be dependent on epigenetic modification of monocytes and macrophages (136–139). Even though epigenetic modification was not directly shown, bovine IgG can induce trained immunity in monocytes (140). In addition to possibly preventing some of the epigenetic modifications induced by infection with respiratory viruses, which would be the result of the lower prevalence of respiratory tract infections (21), bovine IgG may also directly modify subsequent innate immune responses in infants.

(EPIGENETIC) EFFECTS OF HUMAN BREAST MILK AND BOVINE MILK ON ALLERGY OUTCOMES LATER IN LIFE

Several epigenome wide-association studies on allergies have been performed, as reviewed elsewhere (30). These studies have shown that allergic disease is accompanied by changing DNA methylation patterns in Th2, Th1, Th17, Th9, and Treg subsets in affected tissues. The epigenetic mechanism behind T cell subset differentiation is strongly affected by essential micronutrients (folate, vitamins B2, B6, and B12, methionine choline, and betaine) (141), bioactive food components (tea polyphenols, genistein from soybean, isothiocyanates from plant foods, curcumin, and curcumin-derived synthetic analogs) (142), total diet (fiber, protein, fat, and hormones) (143), ethanol, and carbohydrates (144). Dietary compounds, especially vitamin D, folate, and zinc, also have the potency to interfere with DNA methylation and thereby steer the Th1-Th2 balance. In addition to these effects on DNA methylation, prenatal supplementation with PUFA or maternal levels of folate, and microbiota-derived SCFA have been associated with changes in histone acetylation patterns at important T cell differentiation regulating genes (**Box 2**). After birth, these immunomodulatory dietary components are also transferred to the newborn via breast milk.

Epigenetic Effects of Breastfeeding, Raw Milk, and Exposure to the Farming Environment in Early Life

As already mentioned, the mechanisms underlying the anti-allergic effects of human milk are most probably complex, as human milk contains not only nutritional substances but also functional molecules including polysaccharides, cytokines, proteins, and other components forming a real biological system which can modulate and shape the innate and adaptive immune responses of the infant in very early life (104, 145). If and how those components affect the epigenetic status of the growing child and what consequences this has for allergy development need to be addressed in future studies. Considering the observations made about farm milk (see below), as well as indications that breastfeeding may be capable of changing DNA methylation patterns in the offspring (146), such studies are justified.

Epigenetic modulation of the Foxp3 gene by farm milk was demonstrated in an animal model. In this study, exposure to raw, unprocessed, cow's milk for 8 days, increased histone acetylation of Foxp3 in splenocyte-derived CD4+ T cells compared to processed milk exposure (147). In the same study, mice were subjected to an ovalbumin-induced food allergy model after milk exposure and, interestingly, histone acetylation of Th2 genes was lower in raw milk-pretreated mice compared to processed milk-pretreated mice. These mice also showed a reduction in food allergic symptoms (147). As for farm exposure, exposure to raw milk in the first year of life was also associated with changes in gene expression of the innate immune receptors (98). Moreover, it was demonstrated that a polymorphism in the CD14 gene

influenced the protective effect of raw cow milk consumption on allergic diseases (148). DNA demethylation and increased Foxp3+ in the regulatory T cell numbers in the peripheral blood mononuclear cells of 4.5 year-old children were also shown in farm children (93). These regulatory T cell numbers were negatively associated with doctor-diagnosed asthma. It remains to be seen if these changes also precede the onset of allergic disease and can be predictive of allergy development.

There is evidence that the epigenome is affected by the farming environment. The first indication for a potential role of epigenetic regulation in the protective "farm effect" was provided by Slaats et al. who demonstrated that DNA methylation of the promoter region of CD14 in placentas of mothers living on a farm was lower compared to mothers not living on a farm (94). These lower DNA methylation levels were reflected in higher CD14 mRNA expression levels (95). Interestingly, a higher expression of the CD14 gene was also observed in the children of farmers (96). Prenatal farm exposure was also associated with increased gene expression of other innate immune receptors, such as TLR5, TLR7, TLR8, and TLR9, at birth (97, 98) and TLR2 and TLR4 in farm-raised children at school age (91, 96). Maternal exposure to farming environments increased the number of Treg cells in the cord blood of infants, which is associated with decreased Th2 cytokines and may be linked to demethylation at the Foxp3 promoter (50). Whether epigenetic inheritance is the underlying cause of these effects requires further research. Additional evidence that the farm environment affects the epigenome was provided by a pilot study that showed DNA hypermethylation of genes related to IgE regulation and Th2 differentiation in cord blood from the children of farmers as compared to the children of non-farmers (100).

Epigenetic Effects of miRNA Containing Extracellular Vesicles (Exosomes)

Interestingly, both human and cow's milk contain extracellular vesicles, or exosomes, that are resistant to the acidic environment in the stomach and RNAses in the GI tract. These exosomes contain a variety of especially immune function-related microRNAs (miRNAs). miRNAs represent short noncoding RNA molecules that control 40–60% of the total gene expression by inducing mRNA degradation and/or post-transcriptional inhibition of translation. As a result, specific miRNA can silence selective gene expression. The expression of a single gene can be regulated by several miRNAs, and likewise, a single miRNA can regulate over 100 genes (32, 149). This activity thereby constitutes an epigenetic mechanism by which nutritional factors can influence immune activity or the induction of tolerance by affecting the Th1-Th2 balance. Bovine milk exosomes are taken up by human macrophages (150) and epithelial cells (151, 152), exosomes become systemically available in the body of laboratory animals upon oral delivery (153), and bovine miRNA are detectible in the blood after drinking pasteurized milk (154). However, systemic availability could not be demonstrated for breast milk derived exosomes (155) or vegetable derived miRNA (156). Breast milk-derived exosomes were described in 2007 to enhance Treg development *in vitro* (157). Based on miRNA content, bovine milk exosomes contain immunoregulatory miRNAs, like miRNA155, that are involved in the development of Tregs and are thought to play a role in the effect of raw milk consumption on asthma (39). In addition to allergy, orally delivered bovine milk exosomes ameliorated arthritis in a murine model (158), and recent evidence also links milk exosomes to the prevention of necrotizing enterocolitis and intestinal damage in *in vitro* and *in vivo* investigations (159, 160). These studies suggest that miRNAs in human and raw bovine milk exosomes may have epigenetic effects in infants.

Epigenetic Effects of SCFA

Several studies have implicated the SCFA butyrate, propionate, and acetate as epigenetic modifiers of early life immunity, especially in the development of asthma (161). In addition to regulating Treg differentiation and histone acetylation, SCFAs can induce effector T cell differentiation in secondary lymphoid organs by inhibiting endogenous HDAC activity independent of activation of G-protein-coupled receptor (GPCR). In more detail, SCFA can modulate diverse cell processes by two mechanisms, either via interacting with the GPCR (GPR43, GPR41, GPR109A) on the plasma membrane or following a receptor-independent entrance to the cells (162). SCFA entry occurs through passive diffusion or actively by the involvement of two transporters, namely, monocarboxylate transporter 1 (MCT1/SLa16a1) and sodium-coupled monocarboxylate transporter 1 (SMCT1/SLc5a8). These receptors and transporter molecules are widely present in immune and non-immune cells (162, 163). This effect is highly pronounced for butyrate and to a lesser extent for propionate and acetate (164–166). HDAC inhibition allows HATs activity leading to histone hyperacetylation and subsequently an altered gene expression (37) which might, for instance, result in the proliferation of Treg cells (167–169). The significance of this mechanism is illustrated by the fact that bovine, but not human, milk triglycerides contain a relatively high concentration of the SCFA butyrate (18). Altogether, present evidence implies that HDAC inhibitory activity of SCFA might be cell and tissue dependent, and the gene expression pattern is related to the cellular stage and other environmental signals. If bovine milk consumption is associated with decreased allergy prevalence, does this also mean that milk components can affect epigenetic mechanisms? There is no *in vivo* evidence that the induction of SCFA by sialyllactose when ingested in bovine milk, but sialyllactose has been reported to induce SCFA production in *in vitro* fecal microbiota cultures (170) and may thus affect histone acetylation in infants. A high fiber diet (resulting in SCFA production in the colon) or direct feeding of SCFA has been shown to prevent airway inflammation in animal models (84, 85), and SCFA levels in fecal samples of children associated inversely with sensitization to aeroallergens (171, 172).

In addition to allergies, intestinal immunity can also be influenced by microbiota-derived metabolites. For example, tryptophan metabolites can act as aryl hydrocarbon receptor (AhR) ligands, inducing IL-22 and antibacterial peptide production (173), SCFA can directly support the intestinal

epithelial barrier, and bile acids can also be metabolized by the microbiota and influence intestinal barrier function and immunity (174). Two studies reported a decreased risk of wheezing in infants because of high maternal dairy intake (175, 176). Taken together, alterations in the local cellular microenvironment and the microbiome (56) allow milk to induce epigenetic changes in both maternal and neonatal nutrition-mediated genes, which can ultimately affect immune programming in the offspring (177).

CONCLUSIONS

This review summarizes current knowledge on the potential effects of human and bovine milk on neonatal immunity and epigenetic programming and its possible consequences on the development of allergies in early childhood and beyond (see **Figure 1**).

Breast milk is the food of choice for newborns and infants. When breast milk is not sufficiently available, cow's milk based formula is the best alternative, and thus cow's milk has become an integral part of early life diet.

Several epidemiological studies that have shown that exposure to a farm environment as well as to raw/unprocessed cow's milk in the prenatal period and early childhood is associated with protection against the development of asthma and other allergies later in life. Many cow's milk components have been shown to have similar effects on human immune cells as their breast milk counterparts.

Some of the molecular pathways that may explain the association between the consumption of raw milk asthma and allergy may be linked to epigenetics. Epigenetic mechanisms like DNA methylation, but also histone modifications, and non-classical epigenetics represented by miRNA may all contribute to the effects induced by raw cow's milk.

However, milk and dairy products are subject to industrial processing to ensure microbiological safety. As a result, milk proteins can be denatured, and lose their functional activity. In addition, glycation of milk proteins is thought to increase the risk of developing cow's milk allergy, illustrating that preserving milk proteins and preventing glycation may be important innovations to help prevent allergies.

Based on what is currently known on immunological and epigenetic effects that can be exerted by human and different types of bovine milk, future research should focus on enhancing the functional (immunological as well as epigenetic) activity of milk components in early life nutrition, and on establishing epigenetic markers of immunological responses to milk. These could be especially important for diagnostic purposes and assessing the risk of developing CMA. Knowledge gathered during studies on the epigenetic effects of milk can be used in the future to drive the development of preventive or therapeutic anti-allergic strategies based on components that affect epigenetic mechanisms.

Finally, the continuation of epidemiologic and mechanistic studies on the effects of the components of breast and bovine milk on human immune function and health will increase our knowledge and help in finding potential applications that may help prevent allergies in the neonatal period.

AUTHOR CONTRIBUTIONS

All authors contributed to the writing of the manuscript.

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Neonatal Diet Impacts the Large Intestine Luminal Metabolome at Weaning and Post-Weaning in Piglets Fed Formula or Human Milk

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Rosa F, Matazel KS, Bowlin AK, Williams KD, Elolimy AA, Adams SH, Bode L and Yeruva L (2020) Neonatal Diet Impacts the Large Intestine Luminal Metabolome at Weaning and Post-Weaning in Piglets Fed Formula or Human Milk. Front. Immunol. 11:607609. doi: 10.3389/fimmu.2020.607609

The impact of human milk (HM) or dairy milk-based formula (MF) on the large intestine's metabolome was not investigated. Two-day old male piglets were randomly assigned to HM or MF diet (n = 26/group), from postnatal day (PND) 2 through 21 and weaned to a solid diet until PND 51. Piglets were euthanized at PND 21 and PND 51, luminal contents of the cecum, proximal (PC) and distal colons (DC), and rectum were collected and subjected to metabolomics analysis. Data analyses were performed using Metaboanalyst. In comparison to MF, the HM diet resulted in higher levels of fatty acids in the lumen of the cecum, PC, DC, and rectum at PND 21. Glutamic acid was greater in the lumen of cecum, PC, and DC relative to the MF group at PND 21. Also, spermidine was higher in the DC and rectal contents of HM relative to MF at PND 21. MF diet resulted in greater abundances of amino acids in the cecal lumen relative to HM diet at PND 21. Additionally, several sugar metabolites were higher in various regions of the distal gut of MF fed piglets relative to HM group at PND 21. In contrast, at PND 51, in various regions there were higher levels of erythritol, maltotriose, isomaltose in HM versus MF fed piglets. This suggests a post weaning shift in sugar metabolism that is impacted by neonatal diet. The data also suggest that infant diet type and host-microbiota interactions likely influence the lower gut metabolome.

Keywords: human milk, infant formula, neonates, metabolism, host-microbiota

INTRODUCTION

Human milk (HM) contains a diversity of bioactive components including lipids, human milk oligosaccharides (HMOs), a variety of cytokines, and microbiota that can influence the child's development, immune function, and microbiota colonization during early life (1–3). Although studies have indicated the positive impact of HM diet on immune function (4, 5), microbiota

composition (6), and child's growth (7), mechanisms behind these outcomes are poorly understood due to limitations associated with gut sample collection from infants. During early life, cow's milk-based formula (MF) has been chosen as an alternative to human milk (8), but the degree to which MF feeding alters the gastrointestinal tract (GI) milieu relative to HM remains to be fully characterized.

The use of omics technologies such as metagenomics and metabolomics provide platforms to gain new insights about the mechanisms underlying diet-associated differences in the infant's growth and overall health during the neonatal period. For instance, microbiota analysis of infant's stool demonstrated that HM diet shapes microbiota colonization and enriches bacterial species *Bifidobacteria* and *Bacteroides* during exclusive HM feeding relative to formula diet (9, 10). Furthermore, previous studies using metabolomics investigated fecal and serum metabolite profiles of HM versus MF fed infants (11–14). While providing valuable insights, the GI bioregional aspects of HM and MF feeding have remained difficult to study.

We and others reported the use of animal models (primate and piglets) to investigate the impact of MF diet on gut microbiota, immune system, and metabolism (15–22). These models are valuable tools to explore the effects of neonatal regimes on gastrointestinal tract development and maturation (18, 23–25), since they allow the collection of multiple tissues and GI regions for large scale analysis which is limited in human studies (26). Our group developed a piglet model under controlled conditions (i.e., an isocaloric diet of HM or MF, vivarium housing), and have demonstrated that HM-fed piglets had a higher abundance of *Bacteroides* which is similar to the microbiota composition of breast-fed infants (17). Most recently, using the same piglet model our group reported that formula diet could alter the epithelial barrier integrity through disruption of tight junctions in the small intestine of formula-fed piglets compared to the HM-fed (18). These findings are indicative that a piglet model is a promising tool to evaluate the influence of neonatal diet on gut metabolism. Here, we present a comparative metabolomics analysis of the distal gastrointestinal tract of piglets fed HM or MF diet during the first 21 days of life and post-weaning neonatal diet at day 51.

MATERIALS AND METHODS

Experimental Design

The animal study was conducted in accordance with the ethical guidelines for animal research approved by the Institutional Animal Care and Use Committee at the University of Arkansas for Medical Sciences. The detailed experimental design as well as the diet composition were previously published (19). Briefly, White Dutch Landrace Duroc male piglets within 2-d old were randomly assigned to two groups (n = 26/group), fed an isocaloric diet of HM (Mother's Milk Bank of North Texas), or a dairy-based MF (milk formula; Similac Advance powder; Ross products, Abbott Laboratories, Columbus, OH) to meet the nutrient requirements of growing pigs as per the guidelines published by the National Research Council (NRC) (27). At postnatal day (PND) 14 complementary food (i.e., solid pellets) (starter pellets; Teklad, TD 140608; Harlan Laboratories) was introduced to the piglets and weaned to *ad libitum* solid pellets from PND21 to PND51 (19). Piglets were immunized on PND 21 and PND 35 with oral administration of 100 μg of cholera toxin (C8052, Millipore Sigma) and 100 μg of cholera toxin subunit B (CTB; C9903, Millipore Sigma). Piglets also received The DAPTACEL [diphtheria, tetanus, pertussis (DTaP)] vaccine (0.5 mL; Arkansas Children's Hospital pharmacy) by intramuscular injection. Control piglets received vehicle.

Tissue Collection

At PND 21 and 51 piglets were euthanized after anesthetization with isoflurane, followed by exsanguination. Cecum, proximal colon, distal colon, and rectum contents were collected within a scintillation vial by pinching the tissue and sliding the constriction toward the open end. All samples were immediately snap-frozen in liquid nitrogen and stored at −80°C until further analysis.

Metabolite Profiling and Statistical Analyses

Cecum, PC, DC, and rectum contents were subjected to metabolomics analyses using gas chromatography/mass spectrometry (GC/MS) at the West Coast Metabolomics Center at University of California Davis. Approximately 4 mg of contents from experimental samples from each region were used to have a pool for quality control (QC) during the process of the metabolome data. Detailed GC/MS instrument conditions were reported previously (28). Briefly, a total of 0.5 μL of each sample was injected splitless into an Agilent 6890 GC equipped with a Gerstel automatic liner exchange system (ALEX) that includes a multipurpose sample (MPS2) dual rail, and a Gerstel CIS cold injection system (Gerstel, Muehlheim, Germany). The gas chromatograph was controlled using Leco ChromaTOF software. Constituted of helium mobile phase, the gas flow rate through a 30 m long, 0.25 mm i.d. Rtx-5Sil MS column (0.25 μm 95% dimethyl 5% diphenyl polysiloxane film) with additional 10 m integrated guard column (Restek, Bellefonte PA) was 1 mL/min. The transfer line temperature between gas chromatograph and mass spectrometer was set to 280°C. Electron impact was generated by a 70-eV ionization and with an ion source temperature of 250°C. Acquisition rate is 17 spectra/second, with a scan mass range of 85–500 Da. Compounds were identified by comparison with Fiehn lab BinBase database annotations (29), database identifier [i.e., InChI key (30)], the compound annotation metadata (i.e., retention index, quantification mass, BinBase identifier, and mass spectrum), and PubChem annotation (31). A list of peak heights, retention time and mass to charge (m/z) were obtained. 549 metabolites were detected in all samples, including 282 annotated and 267 unknown (non-annotated) metabolites. The unknown metabolites were excluded from the current analysis. The raw data was processed and analyzed in MetaboAnalyst 4.0 (32). On postnatal day 51, diet and immunization interactions

were assessed by Permutational multivariate ANOVA (PERMANOVA) with 999 permutations (**Supplemental Table 1**). No Diet × immunization interaction was observed for cecum ($P > 0.25$), PC and DC ($P \geq 0.42$), and for rectum content metabolites ($P = 0.11$). Therefore, control and immunized data were pooled in the analysis of the PND 51. The QC samples were subjected to multivariate analysis in MetaboAnalyst to check the precision of the metabolomics analysis. The supervised partial least squares discriminant analysis (PLS-DA) score plot for the QC samples (**Supplemental Figure 1**) showed the tight clustering of the QC samples indicating the precise outcome from the metabolites process. Metabolites peak intensities were normalized by the sum of all identified metabolites (33) and log transformed prior to multivariate statistical analysis (34). The PLS-DA score plots were used to see the overall difference between metabolite profiles of HM and MF groups followed by Pattern Hunter analysis in MetaboAnalyst to detect the significant differences in metabolites between groups. A metabolite was considered to be statistically different when *P* - value ≤ 0.05, Benjamini-Hochberg adjusted false discovery rate (FDR) ≤ 0.15, and variable importance in projection (VIP) score > 1.0 (34, 35). Based on the identification of the significantly altered metabolites in HM and MF-fed groups, we calculated the fold change (FC) for each metabolite.

RESULTS

MF Diet-Fed Piglets Have a Distinct Metabolite Profile in the Distal Gastrointestinal Tract Relative to HM Fed Piglets at PND 21

Previously we have demonstrated that microbiota changes were predominant in the large intestine of piglets fed the MF diet relative to the HM group (17). Thus, to evaluate the impact of early diet on the large intestine metabolome, the cecum, proximal colon, distal colon, and rectum contents were examined at PND 21. The PLS-DA model of metabolite showed robust separation of dietary groups at PND 21 in cecal, PC, DC, and rectal regions of the gastrointestinal tract (**Figures 1A–D**).

Metabolite Profile in Different Regions of the Distal Gastrointestinal Tract at PND 21 Is Impacted by Neonatal Diet

At PND 21, within the lumen of large intestine and rectum, a total of 123 cecal, 111 PC, 95 DC, and 62 rectal metabolites from diverse chemical classes including fatty acids, amino acids, lipids, carbohydrates, vitamins, steroids, and co-metabolites were significantly different between HM and MF diet-fed piglets (**Tables 1–7** and **Supplemental Table 2**). The complete list of all detected metabolites (including non-annotated "unknown" metabolites) within each intestinal region is presented in the **Supplementary Table 6**.

Fatty Acids and Polyamines Had Higher Abundances in the Distal Gut of HM Relative to MF Fed Piglets at PND21

The fatty acids myristic, palmitic, linolenic, linoleic, oleic, and palmitoleic were the common metabolites identified throughout the lumen of cecum, PC, DC, and rectum at PND 21, which had greater abundance in the HM than in the MF group. In the lumen of cecum, the saturated fatty acid stearic acid was greater in the HM-fed group relative to the MF group (**Table 1**). In the PC and DC of HM fed piglets, the fatty acids cis-gondoic acid was higher relative to the MF group (**Table 1**). In addition, the fatty acids cis-gondoic had greater abundance in the DC lumen of HM than MF-fed piglets (**Table 1**). Spermidine was another metabolite common to the DC and rectal lumen that was higher in the HM compared to the MF-fed piglets (**Table 2**). However, Putrescine was lower in HM cecal lumen in comparison to MF group.

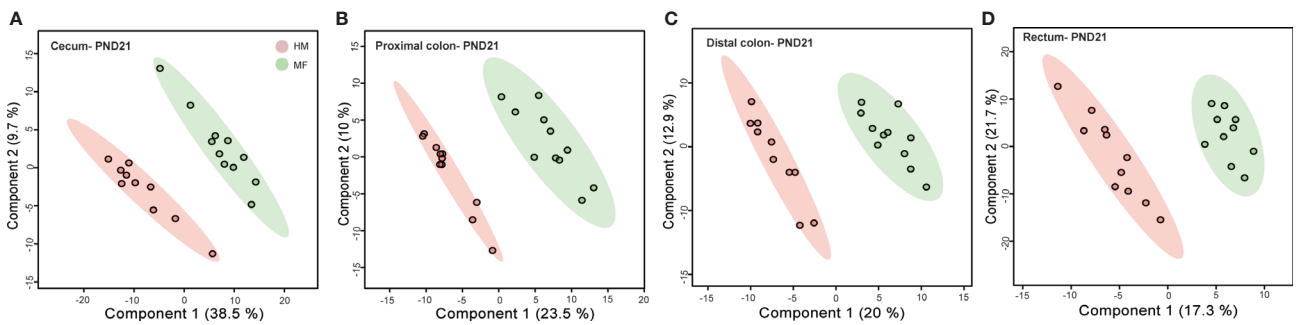

FIGURE 1 | Two-dimensional scores plot of partial least squares discriminant analysis (PLS-DA) model showing how distal gut content abundances of annotated metabolites can discriminate human milk (HM) versus milk formula (MF) feeding groups during the neonatal period in piglets. Panels depict **(A)** cecum **(B)**, proximal colon **(C)**, distal colon, and **(D)** rectal contents at postnatal day (PND) 21. PLS-DA scores (i.e., individual piglet scores) for PLS-DA components (dimensions) 1 and 2 are displayed. Shadows with color are 95% confidence regions. Pink circles indicate individual HM-fed piglets and green circles indicate MF-fed piglets. Sample numbers were n = 8–11 per group.

TABLE 1 | Average abundances [quantifier ion (quantion) intensities] of fatty acids significantly different when comparing human milk (HM) or milk formula (MF) diet groups, in cecum, proximal colon, distal colon, and rectum contents of piglets at postnatal day (PND) 21.

Cecum	HM[1]	SEM[2]	MF[1]	SEM[2]	FC[3]	P[4]	FDR[5]	VIP[6]
Myristic acid	145,457	21,036	73,124	18,704	1.99	0.01	0.03	1.2
Palmitic acid	625,873	39,506	444,733	43,594	1.41	0.01	0.02	1.23
Linolenic acid	16,553	2,620	6,639	1,319	2.49	<0.01	0.02	1.24
Linoleic acid	9,148	1,503	3,055	604	2.99	<0.01	0.01	1.43
Oleic acid	58,553	22,709	7,959	1,415	7.36	<0.01	0.01	1.31
Palmitoleic acid	1,581	214	604	89	2.62	<0.01	<0.01	1.6
Stearic acid	4,829,607	212,505	3,878,986	321,597	1.25	0.03	0.06	1.03
Proximal colon								
Myristic acid	332,535	78,155	121,609	23,150	2.73	<0.01	0.01	1.55
Palmitic acid	1,127,510	94,825	752,618	45,928	1.5	<0.01	0.01	1.55
Linolenic acid	32,957	3,956	17,855	4,297	1.85	0.02	0.06	1.2
Linoleic acid	32,011	5,977	11,235	2,916	2.85	<0.01	0.01	1.51
Oleic acid	159,855	62,469	39,707	21,273	4.03	0.02	0.06	1.18
Palmitoleic acid	4,320	1,317	785	112	5.51	<0.01	<0.01	1.91
Cis-gondoic acid	3,097	327	2,050	225	1.51	0.03	0.09	1.08
Distal colon								
Myristic acid	700,211	64,821	291,343	48,434	2.4	<0.01	0	1.87
Palmitic acid	2,023,370	165,035	1,354,469	78,078	1.49	<0.01	0	1.73
Linolenic acid	75,731	14,902	18,827	4,014	4.02	<0.01	0.01	1.68
Oleic acid	469,449	50,482	73,856	23,936	6.36	<0.01	0	2
Palmitoleic acid	8,349	1,340	615	72	13.57	<0.01	<0.01	2.6
Cis-gondoic acid	5,677	977	2,122	345	2.68	<0.01	0.01	1.65
Stearic acid	8,584,424	666,445	10,187,891	291,167	0.84	0.05	0.15	1.06
Rectum								
Myristic acid	632,851	53,966	401,123	85,521	1.58	0.01	0.07	1.51
Palmitic acid	1,515,125	64,253	1,102,436	96,584	1.37	<0.01	0.02	1.79
Linolenic acid	67,665	12,297	21,882	3,716	3.09	0.01	0.06	1.57
Linoleic acid	54,138	8,348	13,835	3,649	3.91	<0.01	<0.01	2.05
Oleic acid	440,191	80,906	85,398	47,031	5.15	<0.01	0.01	1.89
Palmitoleic acid	8,349	1,340	615	72	13.57	<0.01	<0.01	2.6

[1]*Mean of normalized (mTIC) peak intensities (mz/rt) for human milk (HM) or milk formula (MF) after MetaboAnalyst analyses; n=8–11/group.*
[2]*SEM, Standard error of the mean.*
[3]*Fold change of HM mean to MF mean.*
[4]*P-value ≤ 0.05.*
[5]*FDR, Benjamini-Hochberg adjusted P-value.*
[6]*VIP, Variable importance in projection in PLS-DA models using all annotated metabolites to compare HM and MF within each bio-region. The table only presents metabolites with significant differences between diet groups; all detected metabolites are provided in* ***Supplementary Table 6****.*

TABLE 2 | Average abundances [quantifier ion (quantion) intensities] of polyamines significantly different when comparing human milk (HM) or milk formula (MF) diet groups, in cecum, proximal colon, distal colon, and rectum contents of piglets at postnatal day (PND) 21.

Cecum	HM[1]	SEM[2]	MF[1]	SEM[2]	FC[3]	P[4]	FDR[5]	VIP[6]
Putrescine	4,460	3,457	5,720	1,288	0.78	0.03	0.07	1.01
Distal Colon								
Spermidine	58,259	7,924	14,837	7,484	3.93	<0.01	0.01	1.62
Rectum								
Spermidine	23,474	6,506	4,243	3,592	5.53	<0.01	0.04	1.65

[1]*Mean of normalized (mTIC) peak intensities (mz/rt) for human milk (HM) or milk formula (MF) after MetaboAnalyst analyses; n=8–11/group.*
[2]*SEM, Standard error of the mean.*
[3]*Fold change of HM mean to MF mean.*
[4]*P-value ≤ 0.05.*
[5]*FDR, Benjamini-Hochberg adjusted P-value.*
[6]*VIP, Variable importance in projection in PLS-DA models using all annotated metabolites to compare HM and MF within each bio-region. The table only presents metabolites with significant differences between diet groups; all detected metabolites are provided in* ***Supplementary Table 6****.*

Carbohydrates and Amino Acids Were Higher in MF Fed Piglets Relative to HM Group at PND 21

The carbohydrates 1, 5-anhydroglucitol, galactitol, sorbitol, and fructose were greater in the DC contents of HM-fed relative to MF-fed piglets, while the carbohydrates galactose-6-phosphate and raffinose had greater abundances in the cecal, PC, and DC lumen of MF relative to HM-fed piglets (**Table 3**). Isomaltose, ribitol, and maltotriose were greater in the cecal contents of MF relative to the HM group. In addition, 1, 5-anhydroglucitol,

TABLE 3 | Average abundances [quantifier ion (quantion) intensities] of sugar metabolites significantly different when comparing human milk (HM) or milk formula (MF) diet groups, in cecum, proximal colon, distal colon, and rectum contents of piglets at postnatal day (PND) 21.

Cecum	HM[1]	SEM[2]	MF[1]	SEM[2]	FC[3]	P[4]	FDR[5]	VIP[6]
Galactose-6-phosphate	82	11	216	39	0.38	<0.01	<0.01	1.46
Glucose-1-phosphate	1,059	240	2,373	262	0.45	<0.01	<0.01	1.48
Raffinose	157	34	328	95	0.48	0.03	0.07	1.01
Glycerol	231,576	20,963	340,232	34,945	0.68	0.02	0.05	1.08
Isomaltose	428	59	717	60	0.60	<0.01	0.01	1.37
Maltotriose	356	81	1,456	515	0.24	0.02	0.05	1.08
Ribitol	1,465	195	2,561	325	0.57	0.02	0.05	1.07
Proximal colon								
Galactitol	5,648	2,174	1,427	613	3.96	<0.01	0.02	1.46
Galactose-6-phosphate	153	21	373	80	0.41	0.01	0.03	1.34
Glycerol	400,598	34,375	568,545	43,853	0.7	<0.01	0.02	1.4
Raffinose	180	28	303	42	0.6	0.02	0.08	1.13
Distal colon								
1,5-anhydroglucitol	2,825	495	1,337	156	2.11	<0.01	0.02	1.54
Galactitol	8,608	3,342	882	76	9.76	<0.01	<0.01	1.96
Sorbitol	12,441	4,608	3,973	518	3.13	0.01	0.06	1.29
Fructose	8,678	1,031	5,426	1,139	1.6	0.03	0.1	1.19
Xylulose	7,403	984	3,784	569	1.96	<0.01	0.02	1.49
Ribose	271,496	42,458	143,274	20,425	1.89	0.01	0.03	1.43
Galactose-6-phosphate	136	22	354	80	0.38	<0.01	0.02	1.53
Raffinose	157	17	248	34	0.63	0.01	0.06	1.31
Rectum								
1,5-anhydroglucitol	2,209	130	1,674	224	1.32	0.02	0.12	1.36
Maltotriose	247	33	391	53	0.63	0.02	0.1	1.4
Mannose	5,318	867	9,690	1,390	0.55	0.02	0.1	1.4

[1]*Mean of normalized (mTIC) peak intensities (mz/rt) for human milk (HM) or milk formula (MF) after MetaboAnalyst analyses; n=8–11/group.*
[2]*SEM, Standard error of the mean.*
[3]*Fold change of HM mean to MF mean.*
[4]*P-value ≤ 0.05.*
[5]*FDR, Benjamini-Hochberg adjusted P-value.*
[6]*VIP, Variable importance in projection in PLS-DA models using all annotated metabolites to compare HM and MF within each bio-region. The table only presents metabolites with significant differences between diet groups; all detected metabolites are provided in* ***Supplementary Table 6****.*

mannose and maltotriose were higher in rectal contents in MF group relative to HM group. The essential amino acids histidine, valine, and leucine were greater in the cecal lumen and rectal contents of MF-fed piglets relative to the HM group (**Table 4**). Additionally, threonine, isoleucine, and phenylalanine were greater in the rectal contents of the MF-fed group compared to HM-group. While the non-essential amino acids glycine and proline were greater in the rectal contents, and taurine and cysteine were greater in the cecal contents of MF-fed compared to the HM-fed piglets. In rectal contents, a higher abundance of the amino acids N-acetylornithine, and N-acetylaspartic acid was observed in the HM group (**Table 3**). However, glutamic acid was higher in the HM lumen of cecal, PC, and DC while N-acetyl aspartic acid was higher in PC, DC and rectal contents relative to MF-fed piglets.

Cholesterol and Bile Acids Were Higher in MF Diet-Fed Piglets at PND 21

Cholesterol was significantly higher in the MF group in cecal, PC, and DC lumen (**Table 5**). Interestingly, secondary bile acid deoxycholic acid had greater abundance throughout the 4 regions of the distal gut in comparison to HM-fed piglets. Also, the primary bile acid chenodeoxycholic acid was higher in the luminal contents of PC and DC in the MF group relative to the HM group.

Tryptophan Metabolites Were Impacted by Neonatal Diet in the Large Intestine at PND 21

The metabolites indole-3-propionic acid and 3-hydroxyphenylacetic acid had greater abundance in MF-fed piglets relative to the HM group in the cecal lumen. Within the DC lumen, 5-hydroxy-3-indoleacetic acid and tryptophan were higher in the HM than in the MF group. Additionally, the tryptophan metabolite 5-hydroxy-3-indoleacetic acid was greater in the rectum of the HM relative to the MF group (**Table 6**).

At PND 51 the Metabolite Profile in the Distal Gastrointestinal Tract Is Less Distinct and Showed a Lower Number of Metabolite Differences Between HM and MF

PLS-DA plots demonstrated that the distribution of metabolites had less separation between HM and MF groups at PND 51 (**Figures 2A–D**), except for the rectal contents that had a robust separation of the metabolite profile between HM and MF groups. At PND 51 between HM and MF fed piglets, 15 metabolites were significantly different in cecum and PC, 37 in DC, and 21 in the rectum by using the $P < 0.05$ and a VIP > 1.0 criteria (**Supplemental Table 3**). The lumen of the cecum of HM fed

TABLE 4 | Average abundances [quantifier ion (quantion) intensities] of amino acids significantly different when comparing human milk (HM) or milk formula (MF) diet groups, in cecum, proximal colon, distal colon, and rectum contents of piglets at postnatal day (PND) 21.

Cecum	HM[1]	SEM[2]	MF[1]	SEM[2]	FC[3]	P[4]	FDR[5]	VIP[6]
Histidine	2,041	607	4,831	639	0.42	<0.01	0.01	1.43
Valine	47,321	11,157	121,492	16,114	0.39	<0.01	0.01	1.42
Leucine	68,267	14,347	118,450	16,248	0.58	0.01	0.04	1.12
Isoleucine	39,144	7,645	81,579	12,819	0.48	0.01	0.02	1.22
Methionine	6,886	1,252	11,264	1,274	0.61	0.01	0.04	1.12
Taurine	75	5	152	23	0.49	<0.01	0.01	1.31
Cysteine	832	135	2,285	382	0.36	<0.01	0.01	1.4
Glutamic acid	611,642	67,690	383,277	44,281	1.6	0.03	0.07	1
Proximal colon								
Cysteine	3,074	561	7,987	1,215	0.38	<0.01	<0.01	1.7
N-acetylornithine	1,295	171	2,047	236	0.63	0.03	0.09	1.1
Glutamic acid	1,176,854	153,757	697,884	65,464	1.69	0.01	0.05	1.25
N-acetylaspartic acid	24,555	7,547	12,064	4,117	2.04	0.02	0.07	1.16
Distal colon								
Cysteine	1,494	229	2,757	403	0.54	0.01	0.06	1.3
Glutamic acid	930,473	150,262	306,803	36,781	3.03	<0.01	0	1.86
N-acetylaspartic acid	24,116	10,159	5,426	869	4.44	0.02	0.07	1.27
Rectum								
Histidine	6,240	1,424	14,434	3,220	0.43	0.03	0.14	1.32
Valine	236,629	26,908	517,077	87,043	0.46	<0.01	0.03	1.74
Leucine	262,738	27,431	588,107	113,109	0.45	0.01	0.05	1.61
Threonine	30,098	4,222	70,540	14,278	0.43	<0.01	0.04	1.64
Isoleucine	145,147	17,537	354,847	68,488	0.41	<0.01	0.04	1.66
Glycine	44,615	3,944	89,099	11,985	0.5	<0.01	0.02	1.85
Proline	71,923	9,809	235,145	56,370	0.31	<0.01	0.03	1.75
Methionine	21,104	3,049	53,916	13,521	0.39	0.01	0.08	1.49
Phenylalanine	48,286	6,454	108,093	25,076	0.45	0.03	0.13	1.33
N-acetylornithine	1,798	312	974	238	1.85	0.02	0.11	1.38
Glutamic acid	521,372	106,688	246,722	34,239	2.11	0.01	0.08	1.46
N-acetylaspartic acid	10,420	3,025	3,625	1,075	2.87	0.01	0.06	1.55

[1]*Mean of normalized (mTIC) peak intensities (mz/rt) for human milk (HM) or milk formula (MF) after MetaboAnalyst analyses; n=8–11/group.*
[2]*SEM, Standard error of the mean.*
[3]*Fold change of HM mean to MF mean.*
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[6]*VIP, Variable importance in projection in PLS-DA models using all annotated metabolites to compare HM and MF within each bio-region. The table only presents metabolites with significant differences between diet groups; all detected metabolites are provided in* ***Supplementary Table 6****.*

TABLE 5 | Average abundances [quantifier ion (quantion) intensities] of cholesterol and bile acids significantly different when comparing human milk (HM) or milk formula (MF) diet groups, in cecum, proximal colon, distal colon, and rectum contents of piglets at postnatal day (PND) 21.

Cecum	HM[1]	SEM[2]	MF[1]	SEM[2]	FC[3]	P[4]	FDR[5]	VIP[6]
Cholesterol	8,019	1,200	30,126	3,223	0.27	<0.01	<0.01	1.79
Deoxycholic acid	1,040	193	7,030	1,706	0.15	<0.01	<0.01	1.62
Proximal Colon								
Cholesterol	6,901	883	23,671	2,835	0.29	<0.01	<0.01	1.88
Deoxycholic acid	1,570	393	4,101	852	0.38	0.02	0.06	1.2
Chenodeoxycholic acid	37,595	13,813	89,407	29,531	0.42	0.02	0.08	1.13
Distal Colon								
Cholesterol	18,311	3,627	49,675	4,448	0.37	<0.01	0	1.9
Deoxycholic acid	2,647	713	11,300	1,845	0.23	<0.01	0.01	1.63
Chenodeoxycholic acid	33,830	11,018	82,652	30,280	0.41	0.04	0.13	1.12
Rectum								
Deoxycholic acid	2,805	974	7,852	1,377	0.36	<0.01	0.04	1.68

[1]*Mean of normalized (mTIC) peak intensities (mz/rt) for human milk (HM) or milk formula (MF) after MetaboAnalyst analyses; n=8–11/group.*
[2]*SEM, Standard error of the mean.*
[3]*Fold change of HM mean to MF mean.*
[4]*P-value ≤ 0.05.*
[5]*FDR, Benjamini-Hochberg adjusted P-value.*
[6]*VIP, Variable importance in projection in PLS-DA models using all annotated metabolites to compare HM and MF within each bio-region. The table only presents metabolites with significant differences between diet groups; all detected metabolites are provided in* ***Supplementary Table 6****.*

TABLE 6 | Average abundances [quantifier ion (quantion) intensities] of tryptophan metabolites significantly different when comparing human milk (HM) or milk formula (MF) diet groups, in cecum, proximal colon, distal colon, and rectum contents of piglets at postnatal day (PND) 21.

Cecum	HM[1]	SEM[2]	MF[1]	SEM[2]	FC[3]	P[4]	FDR[5]	VIP[6]
Indole-3-propionic acid	2,155	539	5,569	989	0.39	<0.01	0.01	1.31
3-hydroxyphenylacetic acid	620	67	1,421	159	0.44	<0.01	<0.01	1.65
Proximal Colon								
3-hydroxyphenylacetic acid	884	137	1,806	306	0.49	0.01	0.05	1.24
Distal Colon								
Tryptophan	24,762	4,056	13,072	3,373	1.89	0.01	0.05	1.35
5-hydroxy-3-indoleacetic acid	776	80	344	77	2.25	<0.01	0.01	1.69
Rectum								
5-hydroxy-3-indoleacetic acid	824	87	429	83	1.92	<0.01	0.02	1.79

[1]*Mean of normalized (mTIC) peak intensities (mz/rt) for human milk (HM) or milk formula (MF) after MetaboAnalyst analyses; n=8–11/group.*
[2]*SEM, Standard error of the mean.*
[3]*Fold change of HM mean to MF mean.*
[4]*P-value ≤ 0.05.*
[5]*FDR, Benjamini-Hochberg adjusted P-value.*
[6]*VIP, Variable importance in projection in PLS-DA models using all annotated metabolites to compare HM and MF within each bio-region. The table only presents metabolites with significant differences between diet groups; all detected metabolites are provided in* ***Supplementary Table 6****.*

TABLE 7 | Average abundances [quantifier ion (quantion) intensities] of sugar metabolites (erythritol, lyxose, xylitol, xylose, pentose, xylulose, ribose, maltotriose, isomaltose), tryptophan metabolites (indole-3-propionic acid), and fatty acids (behenic acid) significantly different when comparing human milk (HM) or milk formula (MF) diet groups, in cecum, proximal colon, distal colon, and rectum contents of piglets at postnatal day (PND) 51.

Cecum	HM[1]	SEM[2]	MF[1]	SEM[2]	FC[3]	P[4]	FDR[5]	VIP[6]
Erythritol	1,445	356	761	104	1.9	0.03	0.78	2.02
Indole-3-propionic acid	12,716	2,080	7,397	1,240	1.72	0.03	0.78	2.07
Distal Colon								
Erythritol	1,116	255	652	40	1.71	0.05	0.39	1.57
Lyxose	19,364	3,196	9,660	950	2	<0.01	0.16	2.41
Xylitol	2,899	245	1,950	118	1.49	<0.01	0.16	2.36
Xylose	282,684	50,219	135,380	17,049	2.09	<0.01	0.16	2.25
Pentose	74,638	22,946	27,458	3,058	2.72	<0.01	0.16	2.25
Xylulose	12,922	1,177	8,456	1,028	1.53	0.01	0.22	2.03
Ribose	364,271	36,115	250,569	34,238	1.45	0.03	0.39	1.73
Behenic acid	65,712	3,150	54,919	3,179	1.2	0.02	0.3	1.87
Rectum								
Erythritol	655	32	432	41	1.52	<0.01	0.08	2.96
Maltotriose	586	117	201	14	2.91	<0.01	0.3	2.53
Isomaltose	706	116	445	40	1.59	0.03	0.65	1.9
Behenic acid	40,727	1,532	35,181	1,820	1.16	0.03	0.65	1.92

[1]*Mean of normalized (mTIC) peak intensities (mz/rt) for human milk (HM) or milk formula (MF) after MetaboAnalyst analyses; n=9–15/group.*
[2]*SEM, Standard error of the mean.*
[3]*Fold change of HM mean to MF mean.*
[4]*P-value ≤ 0.05.*
[5]*FDR, Benjamini-Hochberg adjusted P-value.*
[6]*VIP, Variable importance in projection in PLS-DA models using all annotated metabolites to compare HM and MF within each bio-region. The table only presents metabolites with significant differences between diet groups; all detected metabolites are provided in* ***Supplementary Table 6****.*

piglets had higher abundance of indole-3-propionic acid relative to the MF-fed piglets. The sugar alcohol erythritol was a common metabolite in the cecum, DC, and rectum, with higher abundance in the HM group in comparison to the MF group. Additionally, behenic acid was a common fatty acid in the DC and rectal lumen which was higher in the HM-fed relative to the MF-fed piglets at PND 51 (**Table 7**).

Serum Metabolome Impacted by Neonatal Diet at PND 21 and 51

At PND 21, serum metabolome revealed higher abundance of threonic acid and cysteine in the MF relative to the HM fed group. While palmitoleic acid was higher in the HM group. At PND 51, the HM diet resulted in greater abundances of sugar metabolites including maltotriose and xylitol, and greater indole-3-propionic acid relative to MF-fed group. The complete list of serum metabolites impacted by HM and MF diets are presented in the **Supplemental Table 4**.

DISCUSSION

The present study provides metabolite profiles in the cecum, colon, and rectal lumen of HM versus MF feeding regimens in a porcine model at PND 21 and PND 51. We found that diet has a pronounced effect on metabolite profiles in the lumen of the

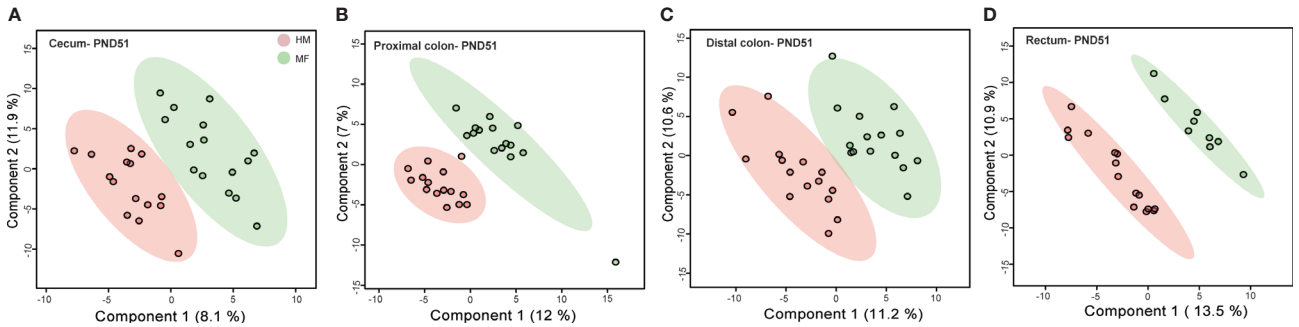

FIGURE 2 | Two-dimensional scores plot of partial least squares discriminant analysis (PLS-DA) model showing how distal gut content abundances of annotated metabolites can discriminate human milk (HM) versus milk formula (MF) feeding groups during the neonatal period in piglets. Panels depict **(A)** cecum **(B)**, proximal colon **(C)**, distal colon, and **(D)** rectal contents at postnatal day (PND) 51. PLS-DA scores (i.e., individual piglet scores) for PLS-DA components (dimensions) 1 and 2 are displayed. Shadows with color are 95% confidence regions. Pink circles indicate individual HM-fed piglets and green circles indicate MF-fed piglets. Sample numbers were n = 9–15 per group.

cecum, PC, DC, and rectum at PND 21 (pre-weaning) but an attenuated effect at PND 51 (~1-month post-weaning). We observed a greater number of metabolite changes in the luminal region of the cecum of HM-fed piglets compared to the MF group at PND 21. A greater abundance of fatty acids and polyamines was observed in HM, while amino acids were higher in MF at PND 21. The persistent effect of the neonatal diet was observed at PND 51 with altered sugar metabolism in HM versus MF fed piglets.

Of particular note was the observation that HM feeding impacted tryptophan metabolism differently than MF feeding, at PND 21. The majority of ingested protein is digested and absorbed by the small intestine (36); however, a significant amount of proteins and amino acids may reach the colon, which is degraded by different microbial species (37). Amino acids in the lower gut may also derive in part from the host (e.g., sloughed tissue, mucous, and epithelial cells from the lining of the intestines) (38–40). In the lumen of DC, tryptophan was higher in the HM-fed group. In addition, a derivative of indole-3-acetic acid (IAA), 5-hydroxy-3-indole acetic acid, was greater in the DC and rectum of HM-fed piglets. Interestingly, we have shown that IAA concentration was also higher in the feces of HM-fed infants at 3 months of age in comparison to formula fed infants (41). *Bacteroides* genera have been shown to convert tryptophan to indole-3-acetic acid. In support of this notion, we have reported a higher abundance of genera *Bacteroides* in infants fed human milk and a higher abundance of genera from class Bacteroidia in the rectal lumen of HM fed piglets (17, 41). These results suggest that tryptophan in the HM group is likely metabolized by distal gut microbiota. In addition, bioactive microbial tryptophan metabolites, indole, indole-3-propionic acid, and IAA have been reported to modulate inflammatory response by promoting IL-22 production in the gastrointestinal tract of mice through the activation of aryl hydrocarbon receptor (AhR) (42, 43). We speculate that the higher tryptophan metabolite levels with human milk feeding promotes the interaction with the host-microbiota which might dampen inflammation.

Neonatal diet also resulted in a divergent fatty acid profile at PND 21 in the large intestine. The human milk lipid profile is variable, and several factors including maternal age, lactation stage, metabolic disorders, maternal diet, among others can modulate the lipid composition (44). HM is composed of more than 200 fatty acids including high levels of oleic and linoleic acids, and these are likely obtained from the mother's diet (45). Essential fatty acids such as linoleic and linolenic cannot be synthesized by the mammalian body from the precursor oleic acid due to the lack of specific enzymes (Δ12 and Δ15-desaturase and hydrogenase), thus adequate intake of these fatty acids through dietary regimens are needed (46). Furthermore, the fatty acid composition of monogastric animals (i.e., piglets) also depends on the dietary intake of fatty acids (47). In our study, throughout the 4 regions evaluated (from cecum to rectum) the linolenic and linoleic essential fatty acids were higher in the HM fed piglets relative to MF at PND 21. Additionally, other fatty acids, myristic, palmitic, oleic, and palmitoleic were common metabolites identified throughout the large intestine of HM-fed relative to the MF-fed group. Studies from our laboratory and others identified higher circulating fatty acids in the HM group. For example, palmitoleic acid was higher in HM-fed serum in comparison to MF-fed piglets (**Supplemental Table 4**), and free fatty acids such as palmitic acid, oleic acid, and stearic acid were higher in the plasma of infants fed HM relative to formula-fed (11). It is suggestive that fatty acids are delivered to infants from HM and in part from the mother's diet. Dietary fatty acids have been shown to exert immunomodulatory effects during inflammatory conditions in humans (48) and in mouse models (49, 50). For example, linolenic acid had an anti-inflammatory effect by decreasing the secretion of the pro-inflammatory IL-6 in an intestinal model using the Caco-2-cell line (51). Additionally, essential fatty acids have been shown to be transferred from sow milk into the piglets' enteric tissues, which might play a role in the immune response and in the epithelial integrity (52). For instance, polyunsaturated fatty acids supplementation to pregnant sows

resulted in lower markers of inflammation in the post weaning period of piglets (53). These data, suggest that fatty acids from mothers' milk exhibit immune protection to infants.

Human milk contains low levels of putrescine compared to spermine and spermidine in term and preterm milk (54). Interestingly, we observed a significantly lower level of putrescine in the lumen of the cecum while spermidine was significantly higher in the lumen of DC and rectum in HM relative to MF. It is possible that HM is the source for these polyamines observed in the distal gut and may provide benefits to infants by various mechanisms. For example, spermine and spermidine play a role in the maintenance of the colonic (55) and intestinal mucosa in mammals (56). Spermidine is considered essential for postnatal intestinal maturation and it has been reported to be higher in human milk than in formulas (57, 58). In addition, spermidine supplementation suppresses inflammatory DC function and systemic inflammation in the psoriasis mouse model (59). Interestingly, human infants fed dairy-based formula had greater levels of the pro-inflammatory molecules (IL8 and IL1β) in the feces compared to HM-fed infants at 1-month (60) and our most recent report suggested higher inflammatory status in MF than HM fed piglets (18). In addition, spermidine has been shown to play a role in autophagy to rejuvenate memory B cell response in older individuals (61). Reduced B cell function causes poor vaccination efficacy and likely a higher incidence of infections. Several studies have demonstrated that HM fed infants have stronger vaccine response and lower respiratory tract infections during the infancy period (1, 2, 62–64). Moreover, in the same piglets we observed stronger vaccine response in HM versus MF fed piglets (19). Also, infant formula supplemented with polyamines increased the number of *Bifidobacterium* species in the large intestine of mice resulting in greater mucin production (65). Thus, the greater level of spermidine upon human milk feeding may benefit the infants by maintaining colon health, microbiota composition, and immune function.

While human milk cholesterol content varies from 90 to 150 mg/L, infant formulas have lower cholesterol content between 20–40 mg/L originated from dairy milk fat (66). Adequate cholesterol dietary intake is essential, especially for growing infants, for the production of steroid hormones, brain development, and lipoprotein metabolism (67, 68). However, a balance between cholesterol absorption and synthesis is required for maintaining whole-body cholesterol homeostasis (69). Formula-fed infants (70, 71) and piglets (24, 72, 73) have been shown to have higher hepatic cholesterol synthesis and fecal bile acid excretion. Fecal sterol excretion followed by intestinal breakdown can be associated with reduced intestinal absorption of cholesterol (68). In the current piglet study, the greater cholesterol detected in the cecum and colon contents of the MF group might be associated with a feedback mechanism (e.g., increased cholesterol synthesis) in response to the low dietary cholesterol uptake. In addition, the cholesterol synthesized in the liver is converted to primary bile acids such as cholic acid (CA), and chenodeoxycholic acid (CDCA) (74). These primary bile acids synthesized from cholesterol in hepatocytes are conjugated to the amino acids taurine or glycine for further biliary secretion (75). In our study, the greater abundance of the bile acids CDCA in the PC and DC lumen was associated with higher levels of amino acids taurine and glycine in the cecal contents of the MF group. In the distal colon, solely gut bacterial bile salt hydrolase (BSH) deconjugates bile acids to form the secondary bile acids deoxycholic acid (DCA) and lithocholic acid (LCA) (76). Importantly, we observed higher DCA in all 4 regions of the distal gut with MF diet suggesting as one of the mechanisms of maintaining cholesterol homeostasis is likely by excretion of secondary bile acids. The implications of a high level of cholesterol and bile acids in the gut can be speculated based on previously published literature (77). For example, bile acids can regulate the epithelial barrier integrity through activation of the farnesoid X receptor (FXR) on intestinal epithelial cells (74). DCA has been shown to induce gut dysbiosis, disrupt bile acid enterohepatic circulation, and promote intestinal inflammation (78). In addition, taurine has been shown to activate *Nlrp6* inflammasome and induce the release of the proinflammatory IL-18 by the intestinal epithelial cells (79). Moreover, the accumulation of DCA in the large intestine has been associated with passive absorption through the colon mucosa (76). Overall, these data suggest that cholesterol and bile acid homeostasis is impacted by the formula diet.

Glutamic acid (glutamate), glutamine, and taurine are the most abundant free amino acids (FAA) in human milk, accounting for approximately 50% of total FAA (80–82) while in dairy-based formulas taurine is the most prevalent FAA (83). In this study, throughout the distal gut regions, higher glutamic acid was detected in HM-fed piglets, likely derived from HM (82, 84). Glutamate intake through the HM diet might benefit the overall neonatal gut health since it has been reported to function as a major energy substrate for intestinal cells (84, 85). Thus, non-essential amino acids intake through human milk might supply infants with readily available nitrogen-compounds. Previous studies demonstrated that standard infant formulas have a lower concentration of free amino acid compared to breastmilk (80, 83) while hydrolysate formulas have a higher amount of amino acids relative to regular formulas (86). In our study, several amino acids (i.e., valine, cysteine, isoleucine, leucine, methionine, cysteine, glycine, histidine, and phenylalanine) were higher in the cecal and rectal contents of MF-fed piglets relative to HM at PND 21, likely due to higher amount of protein in formula. Interestingly, previous studies demonstrated higher levels of circulatory amino acids in formula-fed relative to breastfed infants likely due to higher protein intake with formula diet (11, 12, 87, 88). While we only observed higher cysteine levels in the serum of MF fed piglets (**Supplemental Table 4**), it is possible that in our piglets fasting conditions (8 h) were impacting the circulatory amino acid pool as most of the infant studies measured metabolites after 2–3 h of fasting (11).

Sugar metabolism was impacted by the formula diet relative to the HM diet in piglets. Several metabolites (UDP-glucuronic acid, lyxose, ribonic acid, maltrotriose, UDP-N-acetyl glucosamine, pyruvic acid, threonic acid, raffinose, melibiose, erythrose, xylulose, panose, maltose, mannose) were significantly higher in the MF group relative to the HM group in different regions of distal gut at 8 h of fasting. Interestingly, serum

threonic acid (**Supplemental Table 4**) and urinary threonic acid, ribonic acid, and maltotriose (**Supplemental Table 5**) were also significantly higher in MF relative to HM piglets. Notably, galactose concentration was higher in infant formulas compared to mature human milk (89). In our piglet model MF diet has impacted the carbohydrate metabolism as observed by a higher abundance of galatcose-6-phosphate in the cecum and colon followed by higher glucose-1-phosphate in the cecum of MF-fed piglets at PND 21. Based on previous infant literature and our current data, it is suggestive that formula-fed piglets exhibited a trend to use more of the energy from carbohydrate while HM-fed piglets may use fat as the energy fuel during exclusive neonatal feeding (i.e., PND 21) (11, 13). Additionally, others demonstrated that carbohydrate intake was lower in breastfed infants at 3 and 6 months compared to formula-fed infants (90). Also, metabolites shared between urine and large intestine suggest that these could serve as biomarkers of host health and likely microbial metabolism.

Previous metabolomics studies of infants have shown that the introduction of complementary food minimizes metabolic profile differences in serum while there are clear metabolic changes upon exclusively HM or MF feeding in infants (11). Similarly, we observed less separation of metabolite profile at PND 51 between HM and MF fed piglets. However, sugar metabolites such as erythritol, lyxose, xylitol, xylose, pentose, xylulose, ribose, maltotriose, isomaltose were higher in HM fed relative to MF fed post-weaned piglets. In addition, maltotriose, xylitol followed a similar pattern in the serum of HM fed piglets (**Supplemental Table 4**) suggesting a shift toward carbohydrate metabolism in HM group post-weaning neonatal diet. Persistent effects on microbial metabolism of tryptophan to indole-3-propionic acid was also observed by a higher abundance of this metabolite in cecal lumen and serum of HM fed piglets (**Supplemental Table 4**).

LIMITATIONS

The human milk fed to piglets was a pool from donors at 2 to 12 months of lactation, which is prone to variations on the milk composition including fatty acids. The different stages of

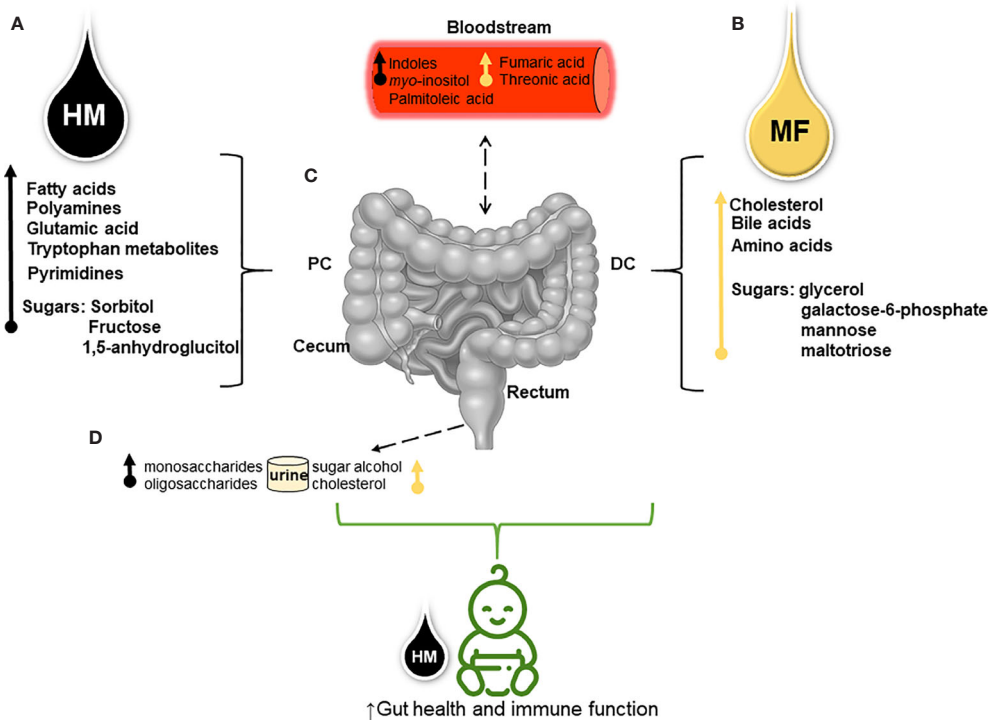

FIGURE 3 | Schematic overview shows the divergent metabolite profile derived from human milk (HM) and dairy-based milk-formula (MF) and their potential effects on neonates' intestinal metabolism **(A)**. Through metabolomics analysis higher fatty acids (myristic, palmitic, linolenic, linoleic, oleic, and palmitoleic acids), spermidine (polyamine), the glutamic amino acid, tryptophan and its derivatives, pyrimidines (thymine, pseudo-uridine, and uracil), and carbohydrates (sugars) were detected in different regions of the distal gastrointestinal tract (gut) [lumen of cecum, proximal colon (PC), distal colon (DC), and rectum] of HM-fed piglets **(B)**. While cholesterol abundance, bile acids (chenodeoxycholic and deoxycholic), essential amino acids (histidine, valine, and leucine), non-essential amino acids (taurine and glycine), and carbohydrates were greater in the luminal distal gut of MF- fed piglets during the first 21 days of life **(C)**. Sugar metabolites and tryptophan derivatives (i.e., indoles) present in the distal gut suggest that neonatal diet interactions with the host-microbiota impact the intestinal metabolism which can be associated with the altered serum metabolites from both diets **(D)**. Diet- microbial interactions reflected in the excretion of mono- and oligosaccharides (i.e., 1,5-anhydroglucitol and raffinose, respectively) in the urine of HM-group compared to sugar alcohols (i.e., threitol) and cholesterol abundance in the urine of MF-group. This model suggests that both HM and MF can impact the host-microbial and the host-intermediate metabolism resulting in a different metabolic profile prior to weaning.

lactation and the variability from the donor mothers might alter the distal tract metabolite profile. The components added to the HM and MF to maintain the requirement of a growing piglet may impact the luminal metabolome.

CONCLUSIONS

Overall, our results showed a distinct metabolome signature between HM and MF-fed during the first 21 days of life. The data presented at PND 21 suggest that human milk feeding may favor the fatty acid metabolism for energy source while MF feeding utilized the sugar breakdown as fuel which is similar with the findings in breastfed vs formula fed infants (11, 13). The greater polyamines and tryptophan pathway metabolites within the distal gut of the HM-fed group may indicate a robust immune response upon human milk than with formula feeding. Also, at PND 21 the higher cholesterol and bile acids in the distal gut of the MF-fed piglets relative to the HM group suggests an impact of formula on cholesterol homeostasis. In contrast, the addition of complementary food (PND 51) resulted in a metabolite profile not as distinguishable and likely shifted to carbohydrate metabolism in HM group. Thus, diet and host-microbiota interactions likely played a role in luminal metabolome (**Figure 3**). Future studies are needed to determine how host physiology (liver and gut tissue) and immune system are impacted at the molecular level by post-weaning neonatal diet.

DATA AVAILABILITY STATEMENT

The raw metabolite data are available online as **Supplementary Table 6**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by University of Arkansas For Medical Sciences.

AUTHOR CONTRIBUTIONS

LY - conceived the study. FR and LY - conducted data anlyses and interpretation, and wrote the manuscript. KM and AB—conducted the study. KW—statistical analysis of the data, AE—input on data analysis, SA and LB—edited the manuscript. All authors contributed to the article and approved the 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/fimmu.2020.607609/full#supplementary-material

SUPPLEMENTARY FIGURE 1 | Two-dimensional scores plot of partial square discriminant analysis (PLS-DA) model showing the distribution of the luminal contents used as quality control pools in the metabolomic analysis. PLS-DA scores (i.e., individual samples) for components 1 and 2 are displayed. Gray circle shadow represents the 95% confidence region. Red circles indicate the individual luminal content samples.

SUPPLEMENTARY TABLE 1 | Prior to metabolome data statistical analysis at PND 51, metabolite abundance in cecum, proximal colon, distal colon, and rectum contents were assessed by permutational multivariate ANOVA (PERMANOVA) including Diet (human milk or milk formula), group (immunization vs control), and their interactions (Diet:group).

SUPPLEMENTARY TABLE 2 | Average abundances (quantifier ion [quantion] intensities) of metabolites significantly altered by diet at postnatal day (PND) 21 (n=8-11/group) across the cecum, proximal colon, distal colon, and rectum contents of piglets fed with human milk (HM) or milk formula (MF) through PND 21.

SUPPLEMENTARY TABLE 3 | Average abundances (quantifier ion [quantion] intensities) of metabolites significantly altered by diet at postnatal day (PND) 51 (n=9–15/group) across the cecum, proximal colon, distal colon, and rectum contents of piglets fed with human milk (HM) or milk formula (MF) through PND 21.

SUPPLEMENTARY TABLE 4 | Average abundances (quantifier ion [quantion] intensities) of serum metabolites significantly altered by diet at postnatal day (PND) 21 (n=25/group) and PND 51 (n=15/group) of piglets fed with human milk (HM) or milk formula (MF) through PND 21.

SUPPLEMENTARY TABLE 5 | Average abundances (quantion peak intensities) of urinary metabolites significantly altered by diet at postnatal day (PND) 21 (n=25/group) and PND 51 (n=15/group), in piglets fed with human milk (HM) or milk formula (MF) through PND 21.

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REVIEW
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Role of Human Milk Bioactives on Infants' Gut and Immune Health

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Exclusive human milk feeding of the newborn is recommended during the first 6 months of life to promote optimal health outcomes during early life and beyond. Human milk contains a variety of bioactive factors such as hormones, cytokines, leukocytes, immunoglobulins, lactoferrin, lysozyme, stem cells, human milk oligosaccharides (HMOs), microbiota, and microRNAs. Recent findings highlighted the potential importance of adding HMOs into infant formula for their roles in enhancing host defense mechanisms in neonates. Therefore, understanding the roles of human milk bioactive factors on immune function is critical to build the scientific evidence base around breastfeeding recommendations, and to enhance positive health outcomes in formula fed infants through modifications to formulas. However, there are still knowledge gaps concerning the roles of different milk components, the interactions between the different components, and the mechanisms behind health outcomes are poorly understood. This review aims to show the current knowledge about HMOs, milk microbiota, immunoglobulins, lactoferrin, and milk microRNAs (miRNAs) and how these could have similar mechanisms of regulating gut and microbiota function. It will also highlight the knowledge gaps for future research.

Keywords: human milk, immunity, infants, neonates, development, breastmilk, immune system, gut

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INTRODUCTION

The immune system is the primary line of defense against environmental exposures such as allergens, bacteria, and viruses. The infant's immune system, often mischaracterized as "immature," is simply naïve to its new extra-uterine environment (1). Normally it undergoes a series of pre-programmed events during early life in response to exposures that occur primarily through the respiratory tract and gastrointestinal tract (GIT) mucosa (2). The infant's immune system at birth has limited anti-oxidant and anti-inflammatory activity in the respiratory and GIT, underdeveloped physical barriers (e.g., tight junctions), limited GIT acidity (chemical barrier), delayed T-cell function and decreased secretion of immunoglobulins [specifically secretory immunoglobulin A (IgA)] (3–5). Early life in humans (from the fetal stage to early months of life) is associated with developmental milestones and human milk provides a medium for inducing both tolerances to antigens and development of a robust immune defense against harmful pathogens. Human milk

feeding has been demonstrated to provide healthy GIT mucosal stimuli, impact gut microbiota composition, and promote the infant's developing immune system likely by human milk bioactives (i.e., HMOs, milk microbiota, miRNA, antibodies, lactoferrin, immunoglobulins, cytokines, and hormones) (6, 7). Careful cultivation of a healthy immune system includes not only protective responses to harmful organisms and antigens (e.g., bacteria, viruses, toxins) but moderating the response to non-harmful antigens in the environment (e.g., food antigens or beneficial commensal organisms) in the form of immune tolerance. The current review focus is on lactoferrin, immunoglobulins, HMOs, milk microbiota, and miRNAs components of human milk and their role in infants' gut microbiota colonization, gut health and immune system modulation.

LACTOFERRIN

Lactoferrin (LF) membrane structure, membrane receptors and transport have been reviewed elsewhere (8). This section will describe the antimicrobial and immune modulatory properties of lactoferrin as well as ongoing clinical studies of formulas supplemented with lactoferrin. Lactoferrin is an iron-binding glycoprotein that exhibits immunomodulatory, anti-inflammatory, antibacterial, antifungal, and antiviral function (**Figure 1A**) (9–11). Human lactoferrin levels change as milk matures with colostrum having higher concentrations in both term and preterm milk (12), however, preterm milk tends to maintain higher levels of lactoferrin over time (12–14). A recent study of Chinese women reported that lactoferrin concentration was 3.16 and 1.73 g/L in colostrum and milk, respectively (15). LF binds free iron which is an essential nutrient for bacterial growth, thus leading to a bacteriostatic effect (16). Also, LF promotes the growth of low iron requiring bacteria thought to be beneficial to humans such as *Lactobacillus* and *Bifidobacterium* (17). Early studies on LF showed a fungistatic effect through iron sequestration (18, 19). Other studies have shown a more direct fungicidal interaction between lactoferrin and the fungal cell surface that is not dependent on iron (20, 21). Furthermore, *in vitro* studies in which skim human milk and bovine milk were incubated with lactoferrin, iron, and fungi (*Candida albicans*) demonstrated that skim human milk inhibits fungal growth while bovine milk did not show a fungistatic effect (22). Additionally, another *in vitro* study showed that human milk LF had higher effect in preventing bacterial growth relative to bovine LF (23) suggesting human milk LF has a superior effect over bovine milk LF. Unfortunately, not all mothers can provide breastmilk for their infants and human milk LF is difficult to obtain for research. Since human and bovine milk LF are highly similar in sequence homology and structure (24, 25), and share similar antimicrobial and immunomodulatory properties (26–29), bovine LF is used more commonly in research.

Lactoferrin has been shown to exhibit immunomodulatory properties in several animal models. For example, mice infected with *Mycobacterium tuberculosis* and supplemented with bovine lactoferrin had decreased levels of *M. tuberculosis* in their lungs as well as decreased inflammation and increased $CD4^+$ and $CD8^+$ cells (30). A porcine model evaluating the impact of lactoferrin on the immune system showed higher levels of natural killer (NK) cells in mesenteric lymph nodes (MLN), peripheral blood monocytes (PBMC), and in the spleen of piglets fed LF supplemented-formula compared to those fed sow milk and standard formula (31). NK cells are part of the innate immune system and provide protection to the neonate against infections as well as release cytokines that activate other immune cells (32, 33). Piglets fed formula supplemented with bovine lactoferrin had increased crypt cell proliferation and serum immunoglobulin G (IgG) compared to piglets fed formula alone (34, 35). Additionally, piglets that received bovine lactoferrin supplemented formula had greater IL-10 and TNF-α production by splenic cells when compared to the control group (35). Collectively, lactoferrin likely plays a key role in the immune response in neonates. Due to these antimicrobial and immunomodulatory properties of lactoferrin, lactoferrin supplementation in preterm infants has been attempted to decrease late-onset sepsis and necrotizing enterocolitis (36). Moreover, the antifungal property of LF is quite important as premature infants are much more susceptible to fungal infections. Thus, several studies of formulas supplemented with bovine LF to support infants' growth and development have occurred. For example, infant formulas supplemented with bovine LF at 0.6 and 1.0 g/L (range of LF concentration found in mature human milk) were compared to a standard cow's milk formula evaluating growth and tolerance in healthy term infants from 12-days old to 12 months of age. This study reported no growth rate difference between formulas, however the bovine LF supplemented formulas had softer stool consistency relative to the infants fed standard formula (37). Several studies have investigated the addition of bovine LF to neonatal diet (breastmilk, donor milk, and/or formula) in premature infants and have not found significant differences in late onset sepsis outcomes (38–40). Future studies are needed to determine the beneficial effect of enteral LF and LF addition to formulas to enhance the anti-pathogenic effects and immune response in term as well as in preterm infants.

IMMUNOGLOBULINS

Immunoglobulins (Igs) are glycoprotein molecules produced by plasma cells. They have been shown to provide passive immunity to infants via transfer across the placenta and during breastfeeding. There are five different types of Igs—IgA, IgG, IgM, IgE, and IgD; however, only IgG, crosses the placenta with the majority being transferred in the 3rd trimester (41, 42). All types of Igs have been found in human milk with the most predominant being secretory IgA (sIgA) followed by sIgG (43). sIgA protects against toxins, bacteria, and viruses by preventing binding to the host or directly neutralizing, and serves as the first line of defense in the intestines (**Figure 1A**) (44–46). sIgA in milk is only partially digested in the stomach of both preterm and term infants while the remainder survives to provide immunity to the lower GI tract (47). Levels in human milk decrease over the

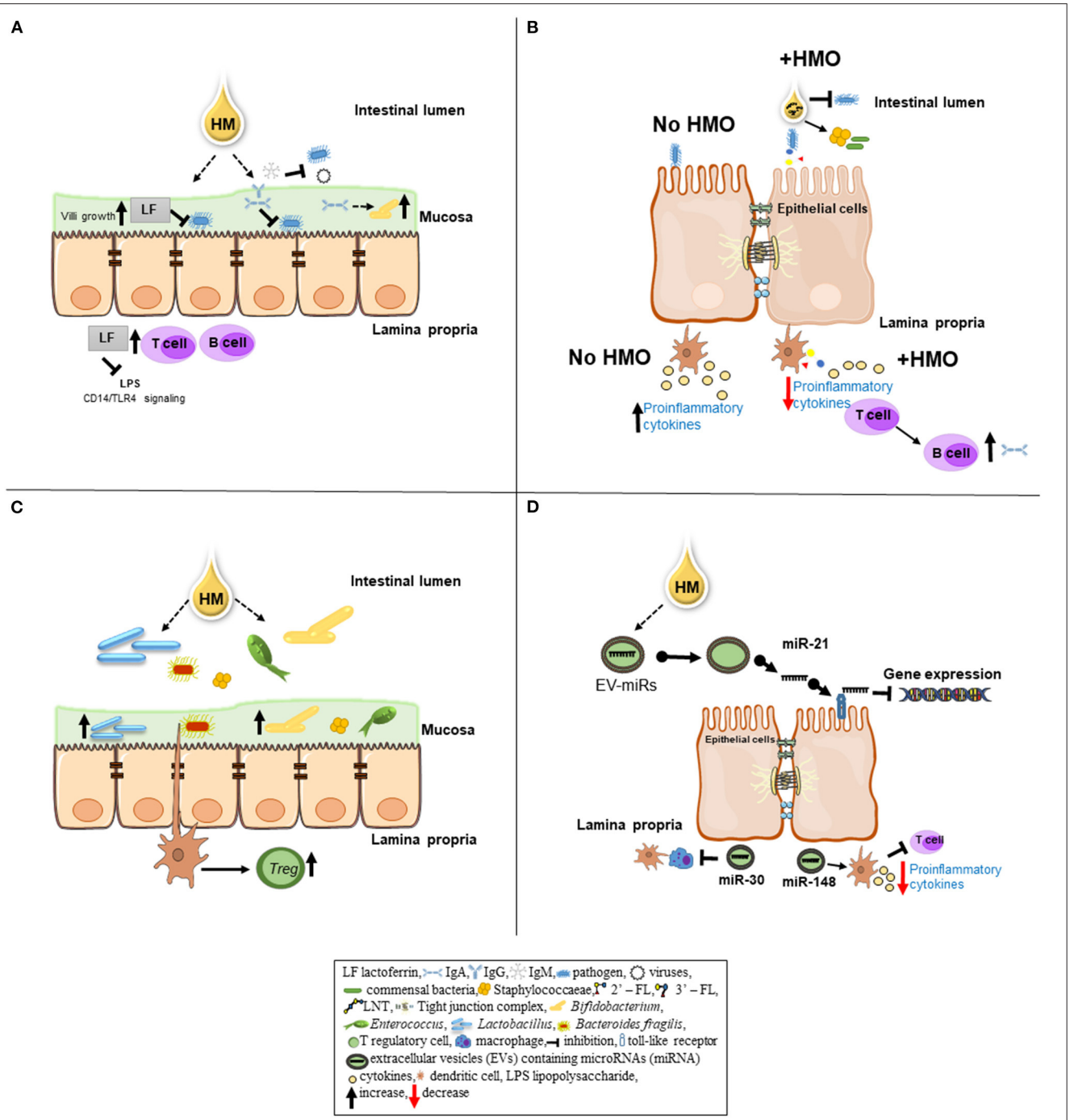

FIGURE 1 | Schematic overview of specific bioactive components of human milk (HM) and their role in immunomodulation. **(A)** An iron-binding antimicrobial protein lactoferrin (LF) inhibits a number of pathogenic bacteria (i.e., *Escherichia coli*) from adhering to epithelial cell. LF can promote the growth of intestinal villi. After pathogenic bacteria invasion into the lamina propria of the epithelial gut cells, LF can inhibit the signal between lipopolysaccharide (LPS) released by gram-negative bacteria and the CD14—TLR complex (macrophage signaling). LF can enhance the maturation of B and T cells to improve the immune response. Immunoglobulins IgA, IgM, and IgG present in HM provide passive immunity to the newborn. IgA and IgG can bind to pathogenic bacteria and prevent them from adhering to the epithelial cells in the gut mucosa. Also, IgA can serve as a substrate to obligate anaerobes (i.e., *Bacteroides*) promoting a healthy microbiota colonization. IgM inhibits enteric bacterial and viral infections by opsonizing the antigen for complement fixation and destruction. **(B)** In the lumen, human milk oligosaccharides (HMO) inhibit bacterial binding to cell receptors by directly binding to the pathogens. HMOs can stimulate the growth of commensal bacteria by serving as substrates. On epithelial cells, HMOs can prevent pathogen binding by acting as binding decoy receptors. Metabolites of HMOs including short-chain fatty acids can influence epithelial cell maturation and intestinal barrier (i.e., tight junctions) function. HMOs can interact with dendritic cells present in the lamina propria leading to T-cell proliferation, subsequently, T/B cell

(Continued)

FIGURE 1 | interaction resulting in increased production of antibodies in order to keep the immune system homeostasis. In the absence of HMOs (no HMO) pathogenic bacteria binding to the epithelial cells increase cytokine production in the lamina propria as a pro-inflammatory response. **(C)** *Bifidobacterium* and *Lactobacillus*, commensal bacteria found in HM, can adhere to intestinal cells, resulting in greater beneficial microbiota colonization. Furthermore, *Bacteroides fragilis* can interact with dendritic cells, resulting in suppression of inflammation by inducing T regulatory cell (Treg) production. **(D)** The extracellular vesicles (EVs) contain cargos such as microRNAs (miRNAs). EV-miRNAs likely have immunological and microbial impact on the gastrointestinal tract of neonates. Human milk miRNAs such as miR-21 can regulate gene expression by binding to toll-like receptors 7 and 8 (TLR7/TLR8). Other milk miRNAs (i.e., miR-148 and miR-30) may play a role in gut immune response by decreasing cytokine production via T-cell inhibition and preventing antigen presentation by dendritic cells and macrophages, respectively.

first 12 weeks post-partum, most significantly over the first week (48, 49). Although it does decrease, infants rely on human milk sIgA initially, as the cells that produce sIgA in the neonatal gut are low at birth and increase by 10–20 times over the first 6 months of life (50). This correlates with a study comparing fecal sIgA levels in breastfed and formula-fed infants which noted that in the first month of life, sIgA levels were much higher in the breastfed group but were more similar between breastfed and formula fed infants at 6 months of age (51). In mothers immunized with the *Neisseria meningococcal* vaccine, IgA antibodies specific for *Neisseria meningitidis* have been shown in human milk for up to 6 months post-partum (52). Interestingly, mother's health status appears to impact sIgA levels in the human milk. sIgA levels have been reported to be lower in the mature milk of mothers with gestational diabetes (53) and in mothers with post-partum stress, anxiety, and depression (54). IgA and IgG levels are lower in the colostrum of mothers with gestational diabetes compared to normo-glycemic women (55, 56). Overall, data suggests that mothers' health condition, vaccination status and lactation period impacts IgA levels in human milk.

IgG is the main immunoglobulin found in serum and is associated with long-term immunity. It not only activates the complement cascade to remove pathogens, but has also been shown to protect against viral infections at the mucosal level through neutralization (57, 58). IgG levels in human milk are low, but increase over time (59). Interestingly, the concentration of IgG is higher in the human milk of exclusively breastfeeding mothers compared to those that are non-exclusive breastfeeding (59). In a mouse model, pathogen-specific IgG was shown to be transferred in milk and protect the pups by coating the pathogen and reducing intestinal colonization (60). Kazimbaya et al. (61) collected human milk samples from mothers prior to their infant receiving the live rotavirus vaccine. For each sample, whole milk, purified IgA, purified IgG, and IgA/IgG depleted milk were isolated. MA104 cells inoculated with the live rotavirus vaccine were exposed to different dilutions of whole milk, purified IgA, purified IgG, and IgA/IgG depleted milk. Interestingly, whole milk and purified IgA and IgG inhibited viral replication suggesting that human milk IgA and IgG can protect against rotavirus infections (61). These studies suggest that human milk IgG plays a role in decreasing infections in infants.

IgM is also transferred to infants via human milk. IgM levels do not vary in human milk in exclusive breastfeeding mothers compared to non-exclusive breastfeeding mothers (59). However, IgM is partially digested by term infants while it is not digested by preterm infants (62). Nevertheless, IgM antibodies protect against bacterial and viral infections by opsonizing the antigen for complement fixation and destruction (63, 64). Serum IgE is associated with a reduction in allergic reactions and parasitic infections. It has also been shown to protect against viruses such as parvovirus B19 (65) and progression of human immunodeficiency virus 1 (HIV-1) (66, 67). Anti-parvovirus B19 IgE antibodies have been found in human milk (68), which might help protect breastfed infants from infection with parvovirus B19. Allergen-specific IgG and IgE antibodies are present in both maternal blood and human milk which may sensitize infants to similar allergens (69). IgD is expressed on mature B cells and it has been shown to bind to certain bacteria resulting in B cell stimulation and activation (70, 71).

Of note, IgA, sIgA, IgM, and IgG concentrations are significantly higher in fresh human milk compared to donor milk (62), which is important to infants in the neonatal intensive care unit due to frequent use of donor milk. This is not unexpected as most donor milk is from mothers of infants that are at least 6 months of age and these samples undergo Holder pasteurization. IgM and IgG are more sensitive to Holder pasteurization than IgA (62, 72, 73). Overall, Igs play a role in reducing pathogenic infections, allergies and likely gut maturation in combination with other components of human milk.

HUMAN MILK OLIGOSACCHARIDES PROMOTE BENEFICIAL MICROBIOTA GROWTH, PROTECT FROM INFLAMMATION, AND PREVENT PATHOGEN INVASION

Human milk oligosaccharides (HMOs) are unconjugated lactose-based carbohydrate structures (74, 75) with concentrations between 7 and 14 g/L in mature milk and 20–24 g/L in colostrum, making HMOs the third most abundant solid component in human milk after lactose and lipids (74, 76). The milk oligosaccharide profile in human milk is more diverse than that of other mammals. For example, the concentration of oligosaccharides in bovine milk is 100 mg/L, and only 50 oligosaccharides structures have been identified in bovine milk (77). However, more than 200 distinct HMO structures have been identified in human milk (74, 75, 78, 79). The structure of HMOs has been reviewed previously (80). The HMOs profile among individual women varies due to differences in the expression of the secretor (Se) and Lewis (Le) genes in the mammary gland. The Se gene encodes for α1,2-fucosyltransferase 2 (FUT2) while the Le gene encodes α1-3/4-fucosyltransferase 3 (81, 82). A systematic review to determine the most abundant HMOs comparing both term and preterm milk reported that for secretor mothers, term milk is most abundant with the neutral

HMOs 2′-fucosyllactose (2′FL), difucosyllacto-N-hexaose II (DF-LNH II), Trifucosyllacto-N-hexaose (TF-LNH), and Lacto-N-Fucopentaose I (LNFP-I) and the acidic HMOs 6′-sialyllactose (6′SL), Disialyllacto-N-Tetraose (DS-LNT), and fucosyllacto-N-neohexaose I (FS-LNnH I). For secretor mothers, preterm milk is most abundant with the neutral HMOs 2′FL, DF-LNH II, LNFP-I, and tetrasaccharides lacto-*N*-tetraose (LNT) and acidic DS-LNT, 6′SL, sialyllacto-N-tetraose c (LST c). Non-secretor milk does not contain α1-2-fucosylated HMOs (83). Additionally, this study revealed that non-secretor term milk is most abundant with neutral DF-LNH II, LNT, and lacto-*N*-neotetraose (LNnT) and acidic 6′SL. Non-secretor pre-term milk is most abundant for neutral DF-LNH II, LNT, and LNFP II and acidic DS-LNT, LSTc, and 6′SL (83). Erney et al. (84) evaluated 435 women from 10 countries and showed a significant variance in expression of HMOs. In particular, European and Latin American mothers had higher 2′FL expression than those in the US or Asia (84). An in-depth evaluation of regional variation in HMO composition evaluating 410 women from 11 different regions in Europe, North and South America, and sub-Saharan Africa showed variation in secretor status based on regions and self-identified ethnicity (85). It also noted variation in total HMO concentration as well as concentrations of all HMO types except LNFP-I. In addition, several HMO concentrations varied based on environment (rural vs. urban Gambia) including higher LNnT and DSLNT in the rural cohort (85). In addition, HMO composition is likely impacted by exercise. For example, recently Harris et al. (86) demonstrated that exercise induces an increase in 3-SL in human and mice during lactation. In conclusion, HMO composition is impacted by geographic location, likely diet, the secretor status of the mother, term vs. preterm milk and exercise. Thus, future studies need to determine how combination of these factors can optimize HMO synthesis and protect neonates during the infancy period.

HMOs Promote Growth of Healthy Gut Microbiota and Exhibit Protection Against Infections

HMOs have been shown to have a prebiotic effect as they are not digested in the gut and reach the large intestine intact where they are utilized by gut microbiota. HMOs have been shown to stimulate gut microbiota growth and composition. *Bifidobacterium,* specifically *Bifidobacterium longum* subsp. *infantis* and its interaction with HMOs has been well-studied. *B. infantis* has greater growth when HMOs, not glucose, are the sole source of carbohydrates (87). Its genome has been shown to contain gene clusters dedicated to HMO metabolism and utilization (88). This ability to grow and metabolize HMOs is not present across all bacteria, but seen in *B. infantis* as well as *Bifidobacterium bifidum*, *Bacteroides fragilis,* and *Bacteroides vulgatus* (89–91). Many bacteria, *Lactobacillus gasseri* and *Enterococcus,* for example, do not grow well, or at all, in just the presence of HMOs (87, 91). In a recent animal study, healthy rats were supplemented daily with 2′-FL from days 2 to 16 of life. At day 8, supplemented animals were noted to have increased villus heights as well as higher *Lactobacillus* proportions in cecal samples. At day 16, animals had higher plasma IgA and IgG as well as more T-cell subsets in their mesenteric lymph nodes (92). This study shows that 2′FL supplementation early in life has a prebiotic effect as well as promotes intestinal growth and immune system maturation.

HMOs not only promote a healthy gut microbiota composition, but also have antimicrobial properties. For instance, α1,2-fucosylated oligosaccharides inhibited *Campylobacter jejuni* infection in mice (93). In addition, 2′FL percentage in milk has been shown to be inversely proportional to rates of *C. jejuni* diarrhea (94). HMOs have also recently been shown to have antimicrobial properties against *Streptococcus agalactiae* [Group B Strep (GBS)], *Staphylococcus aureus,* and *Acinetobacter baumannii* (95, 96) by increasing the sensitivity of such bacteria to several antibiotics, particularly antibiotics to which they are not usually susceptible (97). Overall, HMOs provide some protection to infants against bacterial pathogens.

HMOs protect infants from pathogen invasion by various mechanisms (**Figure 1B**). Several *in vitro* and *in vivo* studies highlighted the antiviral properties against different viruses including rotavirus, norovirus, HIV, and influenza. Rotavirus is the most common cause of severe diarrhea worldwide and accounts for 5% of all deaths among children <5 years of age (98). *In vitro,* 2′FL, 3′SL, 6′SL, and galacto-oligoasccharide reduce infectivity of human rotavirus in MA104 cells, mainly through effects on the virus (99). In experimental settings, 2′FL, LNnT, 3′SL, and 6′SL supplementation in piglets acutely infected with rotavirus downregulated the viral non-structural protein-4 (NSP-4) mRNA expression in the ileum, indicating HMOs inhibit rotavirus replication in the gut (100). Other animal studies in both rats and piglets show that HMOs, in addition to prebiotics, can reduce the length of diarrhea caused by rotavirus (101, 102). HMOs have also been shown to protect against norovirus, the most common cause of acute gastroenteritis outbreaks. Norovirus has been shown to interact with histo-blood group antigens differently with type O having higher susceptibility and B having lower susceptibly to the infection (103, 104). Non-secretors have also been shown to have lower susceptibility to norovirus infections. However, milk from non-secretor mothers does not inhibit attachment of norovirus while milk from secretors does (105). This is likely due to 2′FL binding to the virus and blocking attachment to the gastrointestinal tract (106, 107). 3′FL has also been shown to bind norovirus and block its attachment. Both 2′FL and 3′FL do so by binding to the HBGA pockets on the norovirus capsule, thus, they act as soluble decoy receptors to block pathogens (106). Human milk with higher LDFH-I levels is associated with protection against norovirus as well (94). In both of these gastrointestinal viruses, HMOs have been shown to improve outcomes.

It is estimated that over 38 million people are living with HIV and the rates of transmission from mother to child are as high as 45% (108). In the western world, HIV is considered a contraindication to breastfeeding (109), however, in other countries where access to clean water is unavailable, it is deemed to be the safest option for infant feeding due to lack of nutritional alternatives (110). While breastfeeding is the main post-natal transmission route, many

breastfed infants do not become infected. HMOs have been shown to bind the HIV surface glycoprotein, gp120 and decrease binding to dendritic cells (111). HIV infected mothers, particularly those with higher concentrations of LNnT are less likely to transmit HIV to their infants. Mothers with higher concentrations of 3′SL are noted to have higher transmission rates to their offspring as well as a higher viral load and lower CD4 count (112, 113). Higher concentrations of fucosylated HMOs are also associated with decreased mortality in non-infected infants whose mothers are HIV positive (114). Another viral infection that can be ameliorated with HMOs is influenza. Influenza infects more than 3 million people yearly worldwide and causes over 300,000 deaths (115). An *in vitro* study using pretreated respiratory epithelial cells (Calu-3, 16HBE lines) and PBMCs challenged with either respiratory syncytial virus or influenza and incubated with various concentrations of 6′SL, 3′SL, 2′FL, and LNnT for 24 h showed that 6′SL and LNnT significantly decreased influenza viral load in both airway epithelial cell lines (116). In addition, modified versions of 3′SL and 6′SL have been shown to block hemagglutination and prevent infectivity of influenza viruses (117, 118). HMOs have been shown to improve outcomes in viral gastroenteritis and influenza as well as impact transmission of HIV.

HMOs Improve Gut Barrier Function and Optimize Immune Function

Necrotizing enterocolitis (NEC), a common intestinal disease among premature infants, can cause significant morbidity and mortality [reviewed by Neu and Walker (119)], and is far less common in human milk fed vs. formula fed infants (120). Enteral feeding, including breast- and formula-feeding, impacts the gut maturation of neonates by increasing or decreasing intestinal permeability (121, 122). Decreased intestinal permeability is associated with gut maturation while elevated permeability makes neonates more susceptible to enteric infections and inflammation such as NEC (123, 124). Several studies in animals and humans demonstrated that HMOs may contribute to breastfed infants' lower rates of NEC. In a NEC induction model using neonatal mice, HMO supplemented formula-fed pups had increased mucin expression and decreased intestinal permeability (125). In another rat model of NEC, pups fed HMO supplemented formula had improved survival and the HMO disialyllacto-N-tetraose (DSLNT) was noted to be protective (126). Formulas supplemented with 2′FL have been associated with decreased NEC rates in both mice and rat models (127, 128). However, animal models using preterm pigs have shown only minor effects of HMO supplemented formula on gut microbiota (129) and no effects on gut permeability (130). In addition, several studies have found that milk with lower levels of DSLNT is associated with higher rates of NEC (113, 128). In breastfeeding or pumping mothers, decreased diversity of HMOs, specifically lower concentrations of LNDFH-I during the first month of life is associated with a higher risk for NEC development in preterm infants (131). Clinical trials reported an association of breastfeeding with decreased intestinal permeability at 7 and 14 days of life in preterm infants compared to those that were formula fed (122). In preterm infants, decreased intestinal permeability was associated with increased abundance of *Clostridium* and *Bifidobacterium* during the first 2 weeks of life (132). However, which components of human milk are providing these effects and interactions remains to be determined. Overall, HMOs have been shown to decrease pro-inflammatory cytokine expression, pathogenic bacteria penetration, and intestinal permeability in the gut (125, 133, 134). These findings suggest that not just HMOs alone, but rather HMOs in combination with maternal and/or host microbiota might regulate the intestinal barrier function.

HMOs play an important role in the enhancement of the immune system both locally and systemically. HMOs enhance the functions of human dendritic cells (135), an antigen-presenting cell that plays a pivotal role in the regulation and development of the immature immune system in neonates through the recruitment of functional regulatory T-cells (136). For instance, an *in vitro* approach showed that 0.8, 2 and 5 mg/mL of an HMO mixture upregulated interleukin production (IL-10, IL-27, and IL-6) in dendritic cells (135). Furthermore, HMOs at these concentrations protected dendritic cells against the inflammatory impact of 5 mg/mL lipopolysaccharide (LPS) (135). In a recent mouse model, neutral HMO fractions stimulated the immune response in peritoneal macrophage cells by upregulating the release of nitric oxide (NO), prostaglandin E2 (PGE2), reactive oxygen species (ROS), TNF-α and interleukins such as IL-1β, IL-2, IL-6, and IL-10 (137). Therefore, it is reasonable to hypothesize that certain HMOs can inhibit the pro-inflammatory responses in breastfed infants. In a mouse model, 2′FL supplementation with a dose range of 0.25–5% (w/w) 2 weeks before the primary and booster vaccinations enhanced humoral and cellular immune response to vaccines (138). Mice that received 2′FL had increased levels of vaccine-specific IgG1 and IgG2a in the serum that were 2′FL dose dependent and increased CD27 expression in splenic B-cells. When stimulated *ex vivo*, spleen cells from 2′FL mice had increased interferon-γ production and proliferation of $CD8^+$ and $CD4^+$ T-cells (138). In addition, mice that were fed the 2′FL containing food had increased activation of B-cells, T1-helper cells, and regulatory T-cells in their MLN (135). In a porcine model, piglets that received formula supplemented with HMOs were shown to have increased circulating NK cells and mesenteric lymph node memory T-cells compared to those that only received formula (139). These studies show that HMOs improve immune response to both infections and vaccines.

HMOs have been shown to play a role in toll-like receptors (TLRs) expression. TLRs are a family of pattern recognition receptors that play a key role in the recognition of invading pathogens and initiate host defense (140–142). Studies have reported structure-dependent effects of HMOs on TLR functions. For example, Asakuma et al. (143) showed that 3′SL, 6′SL, and 6′GL increased expression of both TLR2 and TLR4 while LNFP-I upregulated TLR4 in intestinal cell line HT-29 (143). In another *in vitro* study, Cheng et al. (144) reported that 3′-FL activated TLR2 whereas LNT activated

several TLRs in THP1 macrophages. They also found inhibitory effects for HMOs on TLRs *in vitro*. For instance, 6′SL, 2′FL, and LNnT inhibited TLR5 and TLR7 whereas 3′FL inhibited TLR5, TLR7, and TLR8 (144). A recently published study fed mice and premature piglets with 2′FL, 6′SL or lactose supplemented formula. Those fed 2′FL and/or 6′SL were noted to have decreased signs of NEC. 2′FL and 6′FL inhibited TLR4 signaling *in vivo* in cultured IEC-6 enterocytes, in human intestinal explants from NEC patients, and in mouse derived enteroids (145). These studies indicate some role for HMOs in modulating TLRs, however, comparisons are difficult due to differences in studies conducted. The complex effects of different HMOs in modulating TLRs need to be investigated through *in vivo* models. This will enable us to determine the different mechanisms involved in immune modulation by HMOs. Overall, HMOs appear to have a protective effect in reducing inflammation and inducing stronger immune response.

HMOs as Supplements to Boost Immune Function

HMOs and bovine milk oligosaccharides (BMOs) are currently being studied for their ability to improve immune response in infants. Bovine milk serves as a source of simple and complex oligosaccharides that resemble HMOs (146). It is substantially lower in overall total oligosaccharide concentration compared to human milk, however, there are some similarities in the oligosaccharide profile (147). Bovine milk has a much larger proportion of acidic oligosaccharides including 3′SL and 6′SL as well as neutral LNnT, which are identical to the HMOs with the same name (148). Fucosylated structures such as 2′FL have also been isolated from bovine milk, though in far lower concentrations than human milk (146, 148). BMOs have been demonstrated to elicit similar biological functions to those of HMOs including inhibition of pathogen adhesion to intestinal enterocytes, diminished gut permeability, decreased inflammatory markers, and correction of gut dysbiosis (149). Charbonneau et al. (150) investigated breastfed infants' growth parameters and differences in human milk oligosaccharide composition in Malawi (150). This study demonstrated that the human milk of mothers whose infants had poor growth had lower levels of sialylated HMOs and overall lower concentrations of HMOs (150). Based on this data, a germ-free mouse and piglet model was then used to investigate the impact of sialylated HMOs on stunting phenotype. Animals were gavaged with bacterial strains from feces of infants with growth failure and fed a typical Malawian diet. Some of the animals were supplemented with sialylated BMO's (S-BMO) as well. Those that received S-BMO had improved lean body mass gains, improved metabolism, and elevated levels of N-acetylneuraminic acid (150), suggesting sialylated oligosaccharides are involved in infant growth.

Addition of synthesized oligosaccharides to infant formulas is an evolving field. 2′FL is one of the most abundant and well-studied of the human oligosaccharides as previously mentioned. It has been successfully synthesized and shown to be structurally similar to 2′FL found in human milk samples (151). In a neonatal piglet model, enzymatically synthetized 3′SL and 6′SL sodium salt supplemented bovine based formulas were investigated (152, 153). Piglets were fed either a control diet or concentrations of 140, 200 or 500 mg/L 3′SL, and 300, 600, and 1,200 mg/L for 6′SL. These studies showed that the synthesized HMOs are safe and maintain similar growth in supplemented piglets compared to control diet (152, 153). Several clinical studies have evaluated the addition of 2′FL to formula. 2′FL formula fed infants were compared to breastfed infants and all infants had appropriate growth (154). An evaluation of the cytokine profiles in breastfed infants, 2′FL supplemented formula fed infants, and standard dairy-based formula fed infants demonstrated that 2′FL supplemented formula fed infants had lower plasma concentrations of IL-1α, IL-1β, IL-6, TNF-α, and IL-1rα than the standard formula fed infants, and were similar to those that were breastfed (155). 2′FL supplemented formulas have been approved and are being marketed in Europe (156) and the US, however, the supplementation is at much lower concentrations of 2′FL than what is found in human milk. Sialic acid concentrations have also been evaluated in human milk from mothers with term and preterm infants and compared to several infant formulas (157). The highest concentration was noted in colostrum and then decreased over the next 3 months. Milk from mothers with preterm infants had higher levels of sialic acid. Formulas, however, had a much lower sialic acid content, <25% of what was found in human milk (157). Sialic acid is integral to neonatal brain development and childhood malnutrition, specifically decreased sialic acid intake, has been linked to persistent cognitive deficits (158, 159). Thus, future studies of formulas supplemented with sialic acid would need to be tested for the cognitive function in infants and HMO supplementation to formula is an avenue to pursue in the near future.

HUMAN MILK MICROBIOTA IMPACTS COLONIZATION OF GUT MICROBIOTA AND LIKELY IMMUNE SYSTEM DURING NEONATAL PERIOD

Different maternal factors including pathologies of the breast, intrapartum antibiotics, maternal health, body mass index (BMI), parity, gestational age, and geographic location of the mothers can contribute to shaping the milk microbiota (160–166). The early establishment of infant microbiota relies on maternal microbiota and plays a key role in the formation of the gut barrier and the maturation of the immune system (**Figure 1C**) (167). Human milk contains a complex community of bacteria (161, 168) which includes, but is not limited to, multiple genera from *Bifidobacterium and Lactobacillus* spp, *Streptococcus, Staphylococcus, Ralstonia, Bacteroides, Enterobacter,* and *Enterococcus* (161, 167, 169–171). Hunt et al. (172) showed that while there are common genera found in milk, there is variation overtime and between mothers. While most studies have focused on human milk bacterial content, several recent studies have noted fungi present in human milk (173–177). These studies are observational and

further investigation is required to evaluate fungal population variance between mothers, the functions of milk mycobiome in infant gut development, and its interactions with other milk microbiota/bioactives and infant immune system. Due to this constraint, this review will focus on human milk and infant microbiota.

Human milk microbiota likely establishes a healthy profile of intestinal bacteria, leading to the maturation of the innate and adaptive immune systems in infants. For instance, intestinal bacteria promote the development of B-cells in Peyer's Patches and increase the release of mucosal IgA, which acts as the first line of defense (178, 179). Human milk bacteria can also improve the activity against infections through the induction of cytotoxic Th1 cells maturation *in vitro* (180). Interestingly, *Lactobacillus* in the human milk may enhance the release of Th1 cytokines and TNF-α, and activate NK cells, $CD4^+$, and $CD8^+$ T-cells and regulatory T-cells (181). In addition, commensal bacterial in human milk such as *Lactobacillus gasseri* and *Lactobacillus crispatus* have adhesion capacity to the intestinal cells, indicating greater colonization for beneficial bacteria in the gut in breastfed infants (182). In a recent study, Damaceno et al. (182) reported that *Bifidobacterium breve*, *Lactobacillus gasseri* and *Streptococcus salivarius*, limit pathogen adhesion to intestinal epithelial cells *ex vivo* (182). The microbial species identified in human milk have pathogen inhibition and improving immune function properties. Many studies compare human milk bacterial content to stool content of infants. Human milk microbiota composition is also dependent on pumped vs. directly breast fed. Recently, Moossavi et al. (161) noted that providing pumped milk was associated with higher levels of potential pathogens (i.e., *Enterobacteriaceae* and *Enterococcaceae*). Infants fed pumped milk had a lower amount of *Bifidobacterium* in their stool. In addition, Fehr et al. (183) noted that exclusively breastfed infants have a different microbiome than those that are fed pumped milk. The fact that direct breastfeeding vs. pumped milk feeding results in a different gut microbiome in infants needs to be investigated further. It is possible that some of the variations are due to variability in pump hygiene, mothers skin microbiota, and contribution from environment.

Commensal bacteria in human milk may play protective roles against gastrointestinal infections during infancy. Malago et al. (184) found that *Lactobacillus casei*, *Lactococcus lactis* and *Bifidobacterium infantis* suppressed the release of IL-8 in Caco-2 intestinal cell line incubated with pathogenic *Salmonella*, supporting the notion that human milk bacteria could protect the infant intestine against epithelial damage. In a recent study, higher abundance of *Bifidobacterium* at 1 week of life was associated with higher levels of IL-13, IL-5, IL-6, TNF, and IL-1β at 36 months of age compared to children with lower abundance of *Bifidobacterium* at the same time point (185). *Bacteroides* might also play a key role to support the immune system in infants during the early stages of life. In particular, the surface of *Bacteroides fragilis* has polysaccharide A which increases FOXP3 T-cells in the lamina propria resulting in suppression of inflammation (186). In a mouse model, Donaldson et al. (187) showed that *Bacteroides* binds IgA which allows it to colonize the gastrointestinal tract. In conclusion, milk microbiota likely is one of the first things to colonize the infant gut, promote growth of beneficial microbiota, and in turn impact the immune system in infants.

The infant diet also impacts the microbiome of the gastrointestinal tract and immune system in both animal models and clinical studies. In a rhesus macaques model, formula fed infants were noted to have a different gut microbiome including more *Ruminococcus* and less *Lactobacillus*. They also had an increase in pro-inflammatory cytokines TNFα, IFN-γ, IL-1β, and IL-8 (as well as several others) at 1 month of life that decreased overtime (188). Mothers milk fed rhesus macaques are noted to have more memory T-cells as well as T-helper 17 cells compared to formula fed which persists even 6 months after weaning (189). A study of juvenile rhesus macaques noted continued differences, in particular, higher $CD8^+$ T-cell activation (190). These studies show that in rhesus macaques, mothers milk improves immune response while formula changes the microbiome and increases inflammation. There are also several studies carried out with a piglet model that explore diet and its effect on microbiome and the immune system. While many piglet models use sow-fed piglets, this leads to confounding factors due to housing environment, sow milk microbiota, and the maternal environment. Studies from our team housed piglets in the vivarium and fed a regulated diet to eliminate the confounding factors associated with a sow-fed piglet model. Piglets were fed either donor human milk or formula and monitored closely for growth and immune responses. Those fed human milk had a stronger immune response to vaccination in comparison to those fed formula. The piglets who received human milk had lower genera diversity at day 50. At day 21, those fed human milk had higher levels of *Bacteroides* than those fed formula (191, 192). The human milk fed group also had higher levels of T-cell proliferation (191, 192). These results were similar in comparison to infants fed human milk suggesting the strength of the model. For example, in a small comparative study, fecal samples were collected during the first 20 days of life from 6 breastfed and 6 formula fed infants. In breastfed infants, *Bifidobacterium* became the most common gut bacteria while in formula fed infants, *Bacteroides* and *Bifidobacterium* were found in similar amounts (193). Several other studies have found that in early life, stool *Bifidobacterium* amount varies in healthy breastfed infants (194–197). Although the reason is unclear, environment may play a role in this. A recent study found three distinct infant gut microbiota, one low in *Bifidobacterium* but with higher amounts of *Streptococcus*, one with high amounts of both *Bifidobacterium* and *Bacteroides,* and one with higher amounts of *Bifidobacterium.* Overtime, infant stool transitioned from the profile low in *Bifidobacterium* to a profiler higher in *Bifidobacterium* (197). The CHILD cohort has published several studies on infant diet and its impact on microbiome. At 3 months of age, formula fed infants had higher richness and increased *Lachnospiraceae.* Infants who were breastfed but briefly supplemented with formula had lower levels of *Bifidobacteriaceae* and higher levels of *Enterobacteriaceae* at 3 months of age compared to those who did not receive any formula (198). A smaller subset from this cohort noted that formula fed infants had increased richness at 4 months and higher amounts of

Clostridium difficile were noted (195). A 2-year study of infant diet and microbiome revealed that formula feeding in the first 3 months of life is associated with decreased diversity and richness at 12–24 months of life. It is also associated with altered beta diversity (199). Andersson et al. (200) compared infants fed 3 different types of formula to breastfed infants and evaluated immune response through 6 months of age. The breastfed group had an increase in leukocyte count, particularly an increase in neutrophils. Formula fed infants had a decrease in the relative amount of NK cells and an increase in $CD4^+$ αβT-cells. Formula fed infants also had a higher ratio of CD4–CD8 cells (200). Data from these studies indicate that human milk feeding is optimal for microbial colonization, promoting robust immune response and decreasing inflammation in early life.

EXTRACELLULAR VESICLES AND MICRORNA CARGO ROLE IN IMMUNE FUNCTION

Extracellular vesicles is a broad term used to describe vesicles released from many cell types. Readers are referred to O'Reilly et al. (201). for a detailed review of human milk extracellular vesicles (EVs) and their role on infant health. The different methodologies (ultracentrifugation, Exoquick) used to isolate EVs indicate the existence of two subsets such as exosomes (30–100 nm) (202–204) and microvesicles (100–1,000 nm) (205, 206). EVs have been reported to contain various molecules (i.e., proteins, microRNA, metabolites) (207–215). It is yet to be determined whether both exosomes and microvesicles contain miRNAs as most of the methods used so far enrich exosomes. Interestingly, milk seems to contain the highest level of miRNAs compared to its volume. The mechanisms involved in loading the miRNAs to EVs in human milk are still unclear and future research is needed. For a more detailed review of EV biogenesis and cargo composition readers are referred to Spencer and Yeruva (216). The focus of this subsection is to describe EV-microRNA cargo role on infant health.

miRNA are small non-coding RNA (∼22 nucleotides) that regulate post-transcriptional expression of genes and have biological activities in humans (217–219). Human milk contains several miRNAs (218, 220), and these miRNAs survive in the acidic environment in the GI tract and can be absorbed (221). Infant formulas, however, have a significantly lower amount of miRNAs compared with human milk (218, 222). The origin of these miRNAs is still under debate. However, based on the current knowledge on the composition of the EV proteins, breast cell lines, and miRNA profile of mammary gland cells, these miRNAs are likely from immune-related and mammary gland cells (223–225). The literature review of several studies on miRNA profile suggests that miR-148a-3p, miR-22-3p, miR-200a-3p, miR-146b-5p, miR-30d-5p, let-7a-5p, miR-30a-5p, let-7f-5p, let-7b-5p, and miR-21-5p (226–231) were the most abundant in human milk. *In vitro* studies suggest that milk miRNAs are taken up by intestinal, immune, and cancer cell lines (218, 220, 232–236). Future animal models and clinical studies under controlled conditions are needed to determine the bioavailability of these miRNAs.

Few studies have been conducted so far on various factors impacting milk miRNA composition. For example, in mice fed high-fat diet, changes in milk miRNA expression was observed (237). Target prediction analysis of these miRNAs in the high-fat diet group impacted developmental process and transcription. Most recently, Carney et al. demonstrated changes in miRNA profile based on delivery status (preterm vs. term) that appear to influence metabolism and lipid biosynthesis. This suggests gestational age likely plays a role in milk miRNA composition and miRNAs appear to directly influence neonatal health and metabolism. This is an area for future studies to determine the underlying mechanisms involved in milk miRNA composition.

The biological impact of human milk EV-miRNAs on infant health is important to address before supplementing formulas. Previous studies using target prediction analysis of human milk miRNAs provided initial evidence that the majority of these miRNAs are likely impacting the immune system. Also, experimental evidence from *in vitro* and *in vivo* studies using infection and inflammation models suggest that milk miRNAs could impact the immune system. For example, miR-148, present in pre-term and term human milk but significantly lower in formula (218, 226), appears to be the most abundant in human milk. It is shown to regulate the innate immune response in several ways including limiting cytokine production (238). miR-148 also inhibits T-cell proliferation initiated by the presentation of antigens by dendritic cells in a mouse model (238). Let-7 functions to regulate the innate immune system; it limits B-cell activation, affects T-cell differentiation, and regulates TLR4 signaling and macrophage activation (239, 240). miR-30 is important for intestinal epithelial cell homeostasis (241) and the immune response to *Mycobacterium tuberculosis* (242) and influenza infections (243). miR-30 also inhibits antigen processing and presentation by dendritic cells and macrophages (244). Other studies identified miR-181 in human milk (220) which induces B- and T-cell differentiation and development (245, 246) and plays a role in inflammation by downregulating TNF-α production in *Brucella abortus* infections (247). In addition, porcine milk miRNAs were recently shown to reduce LPS-induced apoptosis by preventing TLR4 in intestinal epithelial cells (248). Thus, it is possible that milk miRNAs protect infants from infection, reduces inflammation, and boosts the immune response by various mechanisms (**Figure 1D**).

The potential for human milk miRNAs acting as TLR7 ligand is a novel concept that we put forth in this review. We hypothesize that GU rich motif (GU or GUUG) of human milk miRNAs activates TLR7/TLR8 and could have an adjuvant effect on immune response during vaccination in breastfed infants. For example, milk miR-21, let-7a, and let-7b have a GU rich region and can bind to TLR7/TLR8 receptors (249–252). Thus, milk miRNAs could have dual functions such as TLR7/TLR8 receptors and/or regulatory role by inhibiting gene expression. Mechanistic studies are needed to determine the specific role of milk miRNAs. In addition, whether miRNAs have direct or indirect effects via microbiota on the infant gut and the immune system is not fully understood. However, the evidence so far suggests

that miRNAs could change microbiota composition. Recently, exosome/RNA depleted diet (based on bovine milk exosomes) fed C57Bl6 mice showed changes in the composition of microbiota with relative abundances reported < 1% at family taxonomic level in comparison to exosome/RNA sufficient diet fed mice (253). This study does not show the direct role of miRNAs from bovine milk, nor does it indicate which components of exosomes altered the microbiota composition. However, in a different study it has been demonstrated that bacterial growth is promoted in the presence of certain miRNAs and that endogenous miRNA produced by intestinal epithelial cells alter gut microbial diversity. The increased growth was observed in co-culture of Mission® miRNA mimics and *Fusobacterium nucleatum* (ATCC® 10953) and *E.coli* (ATCC® 47016) (254). Results from this study suggest that miRNAs modulate the gut microbiota; to date, however, no studies investigating the effect of exogenous miRNAs from human milk on neonatal microbiota have been conducted. If miRNAs do indeed promote the survival and growth of gut bacteria, these may serve as a novel component to supplement the infant diet.

PERSPECTIVE AND CONCLUSIONS

Human milk remains the gold standard for infant nutrition. This review summarized several bioactive components of human milk and their impact on infant microbiome and gut/immune function. Human milk oligosaccharides have been shown to have a prebiotic effect, decrease infectivity as pathogen decoys, and enhance the immune system. Milk microbiota appears to help infants' gut and immune system and protect from pathogens. However, several questions remain unanswered that could ultimately improve term and preterm infant outcomes including decreased infection and improved gut and immune function. Mechanistic studies involving animal models in association with clinical trials are needed. While large animal models (piglet and monkey) are advantageous due to the similarities with infant gut physiology (189, 255), they have multiple limitations. These include a low cost-benefit ratio to generate germ-free animal models due to the specialized facilities required, difficulty and expense of knock-out models, issues obtaining species specific reagents and ethical constraints. Animal models have shown differences in offspring gut microbiome and immune response based on diet. Clinical data, while extremely relevant, only allows for association data due to confounding factors. Thus, alternative models such as germ-free mice could be explored to understand the mechanistic questions about milk bioactives. Determining how different human milk bioactives individually and in combination will impact infants' health needs to be pursued.

Future Research

While many questions relating to human milk bioactives have been addressed, there are areas of research that requires future studies. The questions that remain unanswered are: (1) what combination of HMOs or their derivatives should be added to standard formula? (2) should HMOs be added to formula for premature infants? (3) what are the direct and indirect effects of HMOs on infant immune function? (4) how does maternal microbiota transfer into milk and further shape the milk microbiome? (5) does out-of-body bacteria, including skin bacteria, infant oral bacteria, or bacteria from the environment enter the mammary gland and alter milk microbiota? (6) does milk microbiome affect composition of other milk components such as HMOs and miRNAs? (7) how does milk microbiota affect TLRs in the infant gut and does this impact colonization with commensal bacteria and protection from invading pathogens? (8) does the gut milieu (microbiota and mycobiota) interact and how does the interplay impact overall infant health? and (9) how does the addition of different human milk components to formula impact the gut colonization patterns, and in turn, longitudinal infant health? All these questions need further investigation using preclinical and clinical studies. microRNAs are a newer field of study, thus, many questions remain pertaining to how miRNAs interact with the infant gut microbiome and immune system. In conclusion, determining how different human milk bioactives individually and in combination will promote infants' health needs to be pursued.

AUTHOR CONTRIBUTIONS

LC, AE, FR, MV, and LY conceived and wrote the paper. FR made the figures. DM and KM edited the manuscript. All authors contributed to manuscript revision and read and approved the submitted version.

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